

Timing and targeting of Type III secretion translocation of virulence effectors in *Yersinia*

Sofie Ekestubbe



Department of Molecular Biology

Umeå Centre for Microbial Research (UCMR)

Laboratory for Molecular Infection Medicine Sweden (MIMS)

Umeå University

Umeå 2017

Copyright © Sofie Ekestubbe
ISBN: 978-91-7601-639-8
Cover design: Sofie Ekestubbe
Elektronisk version tillgänglig på <http://umu.diva-portal.org/>
Printed by: UmU Print Service, Umeå University
Umeå, Sweden 2017

Till min familj

Most bacteria are good guys

that enable us to live happily ever after.

But this is not that kind of story...

TABLE OF CONTENTS

Table of Contents	i
Abstract	iii
Papers Included in this Thesis	iv
List of Abbreviations	v
Sammanfattning på Svenska	vii
1 Introduction	1
1.1 Virulence	3
1.2 Secretion systems in Gram-negative bacteria	4
1.2.1 <i>Secretion across the bacterial envelope</i>	5
1.2.2 <i>Secretion across host cell membranes</i>	7
1.3 The Type III Secretion System	8
1.3.1 <i>T3SS, a secretion system that translocates</i>	9
1.3.2 <i>Origin and acquisition of the T3SS</i>	9
1.3.3 <i>The structure of the T3SS</i>	11
1.3.3.1 <i>Assembly of the T3SS</i>	12
1.3.4 <i>The function of the T3SS</i>	13
1.3.5 <i>Regulation of the T3SS</i>	13
1.3.5.1 <i>Temperature regulation</i>	14
1.3.5.2 <i>Cell contact</i>	14
1.4 Secretion through the T3S organelle	15
1.4.1 <i>The sorting platform</i>	15
1.5 Translocation by the T3SS	16
1.5.1 <i>The translocator proteins</i>	16
1.5.2 <i>Pore formation</i>	17
1.5.3 <i>The one-step model of translocation</i>	18
1.5.4 <i>The two-step model of translocation</i>	18
1.6 <i>Yersinia</i>	20
1.6.1 <i>The route of infection</i>	20
1.6.2 <i>Phagocytosis</i>	20
1.6.2.1 <i>β1-integrin triggered phagocytosis</i>	21
1.6.3 <i>The virulence plasmid</i>	23
1.6.4 <i>Regulation of the T3SS in Yersinia</i>	23
1.6.4.1 <i>The low calcium response</i>	23
1.6.4.2 <i>Copy number</i>	24
1.6.4.3 <i>Regulated expression and secretion</i>	25

1.6.4.4	<i>Regulated translocation</i>	27
1.6.5	<i>The Effectors in Yersinia</i>	28
1.6.5.1	<i>YopH</i>	29
1.6.5.2	<i>YopE</i>	30
1.6.5.3	<i>YopK</i>	31
1.7	<i>YopN</i>	33
1.7.1	<i>Secretion and translocation of YopN</i>	34
1.8	<i>LcrV</i>	35
1.8.1	<i>The V-antigen</i>	35
1.8.2	<i>LcrV – a regulator of Yop synthesis and secretion</i>	35
1.8.3	<i>The structure and localization of LcrV</i>	36
1.8.4	<i>LcrV is one of the translocator proteins</i>	37
2	Objectives of this Thesis	39
3	Results and Discussion	40
3.1	The LcrV-LcrG heterodimer interacts with YopD	40
3.2	Indications that YopN is an effector	42
3.3	No evidence for a sorting platform in <i>Yersinia</i>	44
3.4	Characterization of the central region in YopN	45
3.5	YopN has a role in translocation of effectors	46
3.6	Using red blood cells as a model for T3SS pore forming activity	48
3.7	LcrV N-terminal mutants as a tool to study translocation	49
3.8	LcrV is involved in intracellular targeting of YopH	50
3.9	Timing and targeting of effector translocation	51
4	Main Findings in this Thesis	56
5	Future Perspectives	57
6	Acknowledgements	59
7	References	61

ABSTRACT

The Type III secretion system (T3SS) is an important virulence mechanism that allows pathogenic bacteria to translocate virulence effectors directly into the cytoplasm of eukaryotic host cells to manipulate the host cells in favor of the pathogen. Enteropathogenic *Yersinia pseudotuberculosis* use a T3SS to translocate effectors, Yops, that prevent phagocytosis by immune cells, and is largely dependent on it to establish and sustain an infection in the lymphoid tissues of a mammalian host.

Translocation into a host cell requires specific translocator proteins, and is tightly controlled from both the bacterial and host cell cytoplasm. We aimed to investigate two of the regulatory elements, YopN and LcrV, to gain more insight into the translocation mechanism.

Two separate regulatory complexes regulate expression and secretion of Yops, however, the processes are linked so that expression is induced when secretion is activated. A complex, including YopD, prevents expression of Yops, while YopN-TyeA and LcrG block secretion. LcrV is required to relieve the secretion block, by sequestering LcrG. We verified that LcrG binds to the C-terminal part of LcrV, which is consistent with what has been shown in *Y. pestis*. In addition to their regulatory roles, both LcrV and YopD are translocators and are assumed to interact at the bacterial surface, where LcrV promotes insertion of YopB and YopD into the host cell membrane. However, here we show that purified YopD failed to interact with LcrV, instead YopD solely interacted with a complex of LcrV-LcrG. This indicates that LcrV and YopD interact in the bacterial cytosol, which may be important for regulation of Yop expression and secretion.

The established role of YopN is to block secretion prior to host cell contact. We found that deleting the central region (amino acids 76-181) had no effect on the regulatory role of YopN in expression and secretion of Yops. Interestingly, we found that, even though the YopN $_{\Delta 76-181}$ mutant secreted the translocators with similar kinetics as the wild type strain, translocation of the effector YopH, into HeLa cells, was significantly reduced. Consequently, the YopN $_{\Delta 76-181}$ mutant was unable to block phagocytosis, almost to the same level as the Δ lcrV mutant which is completely unable to translocate YopH. Our results indicate that YopN is involved in the translocation step in addition to its role in regulating secretion.

Further, we show that the amino terminal of LcrV, in the context of translocation, is involved in the early intracellular targeting of YopH in order to block phagocytosis efficiently and sustain an *in vivo* infection. LcrV mutants that failed to efficiently target YopH intracellularly were severely attenuated also for *in vivo* virulence.

All together, we show that LcrV and YopN are involved in more steps in the regulation of translocation, than what was known before. Our studies also highlight that early translocation is essential for *Yersinia* to block phagocytosis, which in the end is essential for *in vivo* virulence.

PAPERS INCLUDED IN THIS THESIS

Rogne P, Ekestubbe S, Nordfelth R, Forsberg Å and Wolf-Watz M (2017). **Type III secretion regulatory proteins LcrV, LcrG and YopD, in *Yersinia*, form a tripartite complex** (Manuscript)

Bamyaci S*, Ekestubbe S*, Nordfelth R, Ertmann S, Edgren T, Forsberg Å. (2017). **YopN is required for efficient translocation and virulence in *Yersinia pseudotuberculosis***. (Manuscript) *These authors contributed equally to this work.

Ekestubbe S, Bröms JE, Edgren T, Fällman M, Francis MS and Forsberg Å. (2016). **The amino-terminal part of the needle-tip translocator LcrV of *Yersinia pseudotuberculosis* is required for early targeting of YopH and *in vivo* virulence**. *Front. Cell. Infect. Microbiol.* 6:175.

LIST OF ABBREVIATIONS

5' -UTR	5' -untranslated region
aa	Amino acid
ATP	Adenosine triphosphate
Bla	Beta-lactamase
CBD	Chaperone binding domain
CD	Calcium dependent
CI	Calcium independent
Cop	<i>Chlamydia</i> outer protein
FAC	Focal adhesion complex
FAK	Focal adhesion kinase
GAP	GTPase activating protein
GTP	Guanosine triphosphate
HA	Hemagglutinin
IM	Inner membrane
Inv	Invasion
Ipa	Invasion plasmid antigen
IVIS	<i>In vivo</i> imaging system
kDa	Kilo Dalton
Lcr	Low calcium response
MAPK	Mitogen activated protein kinase
MLN	Mesenteric lymph node
mRNA	Messenger RNA
Mxi	Membrane expression of Ipa
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B-cells
OM	Outer membrane
Org	Oxygen-regulated gene
Pcr	<i>Pseudomonas</i> calcium response
PMF	Proton motive force
Pop	<i>Pseudomonas</i> outer proteins
PP	Peyer's patch

PTPase	Protein tyrosine phosphatase
RACK1	Receptor for activated C-kinase 1
RBC	Red blood cell
RBS	Ribosome binding site
Sep	Secretion of <i>E. coli</i> proteins
Spa	Surface presentation of antigen
SPI	<i>Salmonella</i> pathogenicity island
T3SA	Type III secretion apparatus
T3SS	Type III secretion system
Tat	Twin-arginine translocation
TM	Transmembrane
TS	Temperature sensitive
TyeA	Translocation of Yops into eukaryotic cells A
Yop	<i>Yersinia</i> outer proteins
Ysc	<i>Yersinia</i> secretion

SAMMANFATTNING PÅ SVENSKA

Bakterier finns överallt omkring oss, på oss och i oss. De allra flesta är harmlösa och många är livsviktiga för oss. Men det finns även bakterier som gör oss sjuka. Forskning på de virulensmekanismer som sjukdomsframkallande (patogena) bakterier använder ger oss större möjligheter att motverka dessa patogener utan att påverka den normala bakteriefloran i vår kropp.

Många patogena bakterier (t.ex. *Salmonella*, *E. coli* och *Yersinia*) som infekterar däggdjur använder sig av en speciell virulensmekanism, kallad typ III sekretionssystem (T3SS). T3SS kan beskrivas i tre delar; 1) en sekretionsapparat (T3SA) som sitter förankrad i bakteriens cellmembran, med en nålliknande struktur som sträcker sig ut från bakteriens yta, 2) regulatoriska proteiner som säkerställer att T3SA endast är aktiv vid rätt plats och tidpunkt, samt 3) toxiska proteiner (effektorer) som levereras in i våra celler (värdceller) via T3SA, i en process som kallas translokering.

För att effektorerna ska kunna translokeras in i värdcellen krävs att bakterien har bundit till ytan av värdcellen. Nålen kommer då i kontakt med värdcellmembranet och en signal skickas till bakteriens insida att effektorerna ska translokeras. Effektorerna har oftast en enzymatisk funktion men exakt vad de gör inne i värdcellen är unikt för varje bakterieart.

Yersinia pseudotuberculosis är en enteropatogen bakterie som infekterar oss i magtarmkanalen via kontaminerad mat och vatten. När *Y. pseudotuberculosis* når tarmen passerar den över tarmväggen och når den underliggande lymfatiske vävnaden som är målvävnaden för *Y. pseudotuberculosis*. I den lymfatiske vävnaden finns delar av vårt immunförsvar som består av olika typer av celler (lymfocyter) som är specialiserade på att upptäcka och eliminera främmande material, såsom patogena bakterier. En av lymfocyternas viktigaste uppgifter är att aktivt ta upp och förstöra eller avdöda dessa bakterier genom en process som kallas fagocytos. För att överleva i denna fientliga miljö använder *Y. pseudotuberculosis* sitt T3SS för att translokera effektorer som paralyserar lymfocyterna och blockerar därigenom fagocytos. Fagocytos är en snabb process och signaleringen som leder fram till det startar direkt när bakterien fäst på cellytan. För att hinna blockera fagocytos måste därför *Y. pseudotuberculosis* translokera effektorerna omedelbart vid cellkontakt. Flera regulatoriska proteiner samarbetar för att säkerställa att effektorerna translokeras omedelbart samt att de dirigeras till sina målproteiner inuti värdcellen. Dessa två aspekter är otroligt viktiga för T3SS, och är det som åsyftas i titeln som ”timing och targeting”.

I den första studien undersökte vi hur tre av de regulatoriska proteinerna (LcrG, LcrV och YopD) interagerar med varandra. LcrG behövs inuti bakterien för att blockera T3SA så att inga effektorer translokeras innan bakterien har bundit till en värdcell. YopD i sin tur förhindrar produktionen av effektorer, till dess T3SS är aktiverat. Vid cellkontakt, binder LcrV till LcrG och upphäver då blockeringen. Translokeringen kan starta varpå YopD transporteras ut från bakterien vilket resulterar i ökad produktion av effektorer som translokeras efter hand. Tidigare forskning har visat att LcrV och YopD binder till varandra och det förutsattes att detta skedde utanför bakterien i samband med att LcrV placerade YopB och YopD i värdcellmembranet. Våra undersökningar visar däremot att YopD inte binder till LcrV i ren form, utan endast binder ett komplex av LcrV-LcrG. Det är möjligt att LcrV-LcrG och YopD interagerar med varandra inuti bakterien, möjligtvis som ett steg i regleringen av produktion och export av effektorer.

I den andra studien undersökte vi YopN, som tillsammans med LcrG blockerar T3SA. Tidigare forskning har visat att YopN kan ha flera funktioner och våra resultat visar att YopN dessutom reglerar translokering. För att kunna påvisa detta konstruerade vi en YopN variant (mutant) med bibehållen regulatorisk funktion. Det som skiljde YopN mutanten från vildtypen var att translokeringen försenades när celler infekterades med YopN mutanten. Således verkar YopN ha en viktig funktion i timingen av translokering. Till följd av att effektorerna inte translokerades fort nog kunde YopN mutanten inte blockera fagocytos, vilket är förödande för *Y. pseudotuberculosis*' överlevnad vid en infektion.

I den tredje studien undersökte vi LcrV i sin roll som translokator. Tidigare forskning har visat att LcrV behövs för att sätta in YopB och YopD i värdcellmembranet där de främjar translokering av effektorerna, och det antogs att detta var LcrV's huvuduppgift under translokeringen. När effektorerna translokerats, dirigeras de till sina målproteiner och våra resultat visar att LcrV kan medverka även i detta steg. Två LcrV mutanter som translokerade effektorer lika snabbt och lika mycket som vildtypen, visade sig ändå inte kunna blockera fagocytos. När vi undersökte hur en effektor, YopH, dirigerades till sitt mål fann vi att LcrV mutanterna inte lyckades dirigera YopH lika effektivt som vildtypen. Detta indikerar att LcrV har en funktion i targeting av effektorerna.

Sammantaget visar vi att både YopN och LcrV är involverade i flera steg i translokering av effektorer, samt att deras funktioner är viktiga för *Y. pseudotuberculosis* förmåga att etablera en infektion och överleva i ett värdjur.

Life on Earth began roughly four billion years ago when organic material that could replicate itself came into existence. This was the base for the genetic material that is harbored by all living organisms. Over time life evolved to create the biodiversity of organisms that inhabit the world today. Based on the genetic material, life is divided in three domains; Archaea, Bacteria and Eukarya (Figure 1). Archaea and bacteria are prokaryotes, which are single-cell organisms encased by one or two lipid membranes and a cell wall, that separates the cell interior (cytoplasm) from the environment. Prokaryotes lack intracellular organelles, such as a separated nucleus, and the genetic material resides in the cytoplasm of the cell. Eukaryotes, on the other hand, are more complex organisms with several intracellular membrane-encased organelles and a nucleus separating the genetic material from the cell cytoplasm. Eukaryotic life forms range from unicellular organism such as amoeba and plankton to complex multicellular organism such as fungi, plants and animals.

Prokaryotes were among the first organisms to colonize earth and arose at least 3.5 billion years ago. Their amazing ability to quickly evolve and adapt to changes in the environment allow them to survive and thrive, even in the most uninhabitable of places.

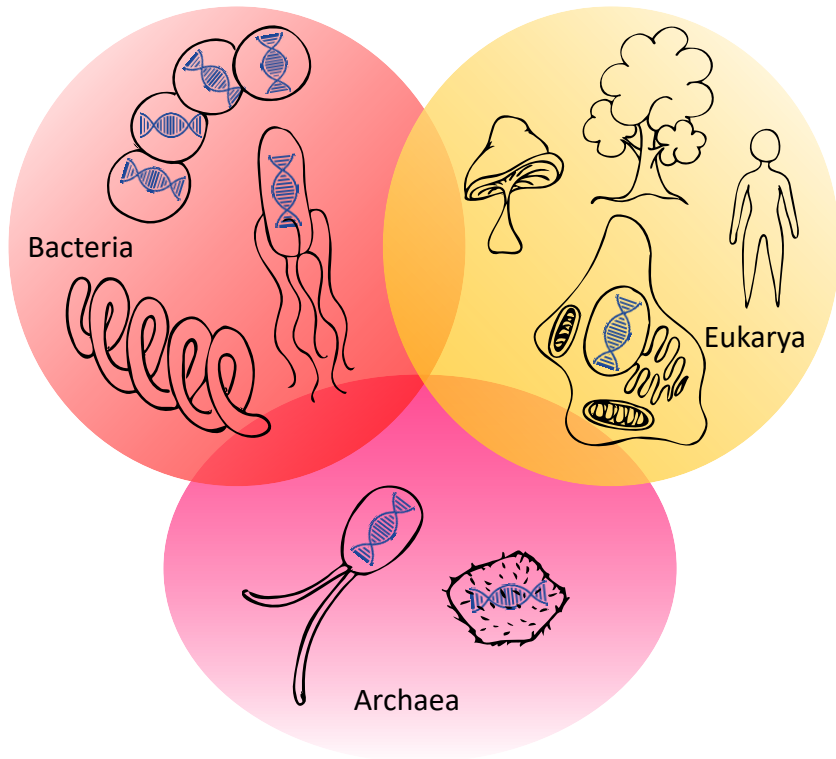


Figure 1. The three domains of life. Based on the genetic content, organic organisms are separated into three domains; Bacteria, Archaea and Eukarya. Eukarya is, morphologically, the most diverse domain with organism ranging from unicellular, such as amoeba, to complex multicellular organisms like fungi, plants and animals.

Archaeal species are generally more difficult to isolate and cultivate, compared to bacteria, and therefore our knowledge about them is limited. Much more is known about bacteria, and especially about bacteria that interact with us. Animals, including humans, provide residence for several prokaryotic species, both archaea and bacteria, and we rely on them for our survival and well-being. Parts of our skin and our intestines are covered with bacteria and it was estimated that a human body contains ten times more bacterial cells than human cells, which puts a perspective on our dependence on these organisms.

Even though we are completely dependent on bacteria to survive, there are parts of our body, such as most of our internal organs, where bacterial growth could be devastating for us. Most bacteria have a considerably shorter generation time than eukaryotic cells, and if left to roam free, they could easily outmaneuver us.

1.1 Virulence

The co-evolution of bacteria and eukaryotes has pushed the development of strategies that allow us to co-exist. Higher eukaryotic organisms have evolved immune systems to keep colonizing bacteria in check and prevent harmful infections. Humans have an intricate immune system, which includes various types of cells that scavenge our bodies continuously and quickly remove any foreign and harmful material, including invading bacteria. Naturally, bacteria have evolved strategies of their own to evade our immune system and survive in the host. These survival strategies are often displayed as virulence.

The word *virulence* is derived from the Latin word *virulentus* meaning “full of poison”, and is a measure of the ability of bacteria to cause disease in a host, and a parameter to distinguish between pathogenic and non-pathogenic bacteria.

As the saying goes; it takes two to tango, virulence is a phenomenon arising from the interaction between pathogen and host and can only be displayed in a susceptible host. It is difficult to draw a clear line between pathogenic and non-pathogenic bacteria. Pathogenic bacteria can fail to cause disease in individuals that are immune. At the same time, non-pathogenic bacteria may suddenly cause disease in individuals that for one reason or another has become susceptible to infection.

One feature that pathogenic bacteria have in common is the expression of virulence factors that support their lifestyle and promote survival of the pathogen. There is a great deal of variation in virulence factors displayed by different pathogens, depending on the lifestyle niche of the pathogen. Pathogens that replicate in the tissues of the host generally express virulence factors that help them evade the host immune system. Some pathogens

replicate intracellularly and express virulence factors that aid attachment to and invasion of host cells. Other pathogens replicate extracellularly and thus express virulence factors that block uptake. Some pathogens also express toxins and other harmful compounds that block or damage cellular processes in the host.

1.2 Secretion systems in Gram-negative bacteria

To cause the desired effect the virulence factors must be secreted from the bacterial cytosol by a secretion system. Depending on the composition of the cellular envelope, bacteria are divided into Gram-positive and Gram-negative (Figure 2). The cell envelope of Gram-positive bacteria consists of an inner membrane and a thick peptidoglycan cell wall. Gram-negative bacteria, on the other hand, have an inner- and an outer membrane, separated by a periplasmic space containing a thin peptidoglycan layer.

Once secreted, the virulence factor can either be deployed on the bacterial surface or secreted out from the bacteria altogether. The process of protein secretion requires energy, which is generally provided through hydrolysis of ATP by an ATPase associated with the secretion system. The energy needed can also be provided through the proton motive force (PMF) and in some cases by a combination of ATP and PMF [1].

Transport across the inner membrane is accomplished by the Sec or twin-arginine translocation (Tat) pathways. The Sec and Tat pathways are highly conserved and are present in both prokaryotes and eukaryotes. In bacteria these pathways are commonly employed to insert proteins into the cytoplasmic membrane or transport proteins to the periplasm. Some of the proteins are further secreted by other secretion systems (section 1.2.1).

The Sec system transports unfolded proteins through a membrane embedded channel, formed by SecY, SecE and SecG. The proteins are targeted to the SecYEG by SecB or the signal-recognition particle and the transport is driven by the ATPase SecA [2].

The Tat system consists of three proteins; TatA, TatB and TatC, and transports folded proteins. The substrates are recognized by a TatB/TatC complex and are targeted to TatA, which forms a channel in the inner

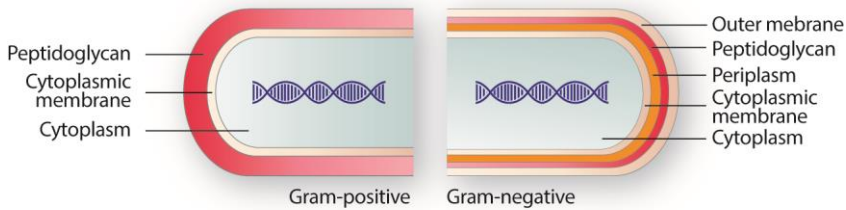


Figure 2. Composition of the cell envelope of Gram-positive and Gram-negative bacteria. Reprinted with permission from Ummehan Avican ISBN 978-91-7601-607-7.

membrane, through which the substrate is transported. Transport via the Tat pathway is driven by the PMF [3].

Since Gram-negative bacteria have an outer membrane in addition to the inner membrane, protein transport by the Sec and Tat pathways is not sufficient to secrete the proteins out of the bacteria. Consequently, they have acquired several secretion systems to accomplish this. To date, six secretion systems have been identified in Gram-negative bacteria, named type I secretion system, (T1SS), T2SS, T3SS, T4SS, T5SS and T6SS. The contributions to virulence by the different secretion systems vary. Many of them are involved in metabolism and are important for survival and fitness of the bacteria, and are thus present also in non-pathogenic bacteria [1].

1.2.1 Secretion across the bacterial envelope

T1SS, T2SS and T5SS secrete their protein substrates from the bacteria out to the surrounding environment (Figure 3). Some of the substrates associated with virulence are for example, hemolysin A in *Escherichia coli* (T1SS), Cholera toxin in *Vibrio cholera* (T2SS) and IgA protease in *Neisseria gonorrhoeae* (T5SS) [1], [4].

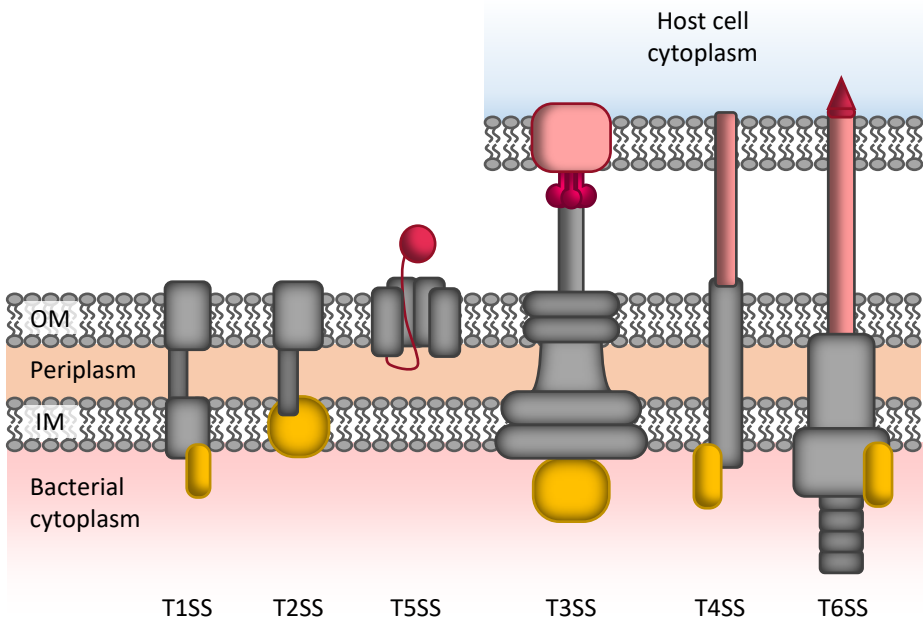


Figure 3. Illustration of secretion systems in Gram-negative bacteria. T1SS, T2SS and T5SS secrete their substrates to the extracellular space. T3SS, T4SS and T6SS target their substrates to the cytoplasm of a host cell.

T1SS forms a structure that spans both bacterial membranes and is thus independent of the Sec and Tat pathways. An inner membrane ABC transporter is linked via a membrane fusion protein to a pore in the outer membrane. The substrates are secreted in an unfolded state and the secretion is driven by the ABC transporter ATPase [5].

Secretion by T2SS and T5SS occur in two steps where the proteins are transported across the inner membrane by either Sec or Tat. These secretion systems are therefore said to be Sec- or Tat-dependent.

T2SS forms an outer membrane channel, which consists of a protein called secretin. The secretin contacts an inner membrane platform that is linked to a cytoplasmic ATPase. The ATPase drives the secretion of folded proteins through the secretin channel [6].

T₅SS differs from the other secretion systems in that it does not form a stable secretion apparatus, instead the protein substrates themselves promote secretion. A β -barrel domain forms a channel in the outer membrane through which the substrate domain is secreted in an unfolded state. The β -barrel and the substrate domain can either be transcribed as one protein or as two separate proteins [7].

1.2.2 Secretion across host cell membranes

T₃SS, T₄SS and T₆SS are independent of the Sec and Tat pathways, as they form secretory channels that span both bacterial membranes. In that sense they resemble the T₁SS, however these systems are unique in that they transport their substrates (called effectors) across three membranes – the bacterial envelope and into an adjacent cell (Figure 3). As such, these secretion systems are designed to interact directly with other cells and are perhaps more typically associated with virulence compared to the previously mentioned systems.

T₃SS specifically targets eukaryotic cells and the system requires close contact with the host cell. The system is related to the flagella system and forms a basal body that spans the bacterial envelope followed by a needle filament on the surface of the bacteria [8]. The secreted proteins are delivered directly into the host cell and *in vivo* there is little or no secretion into the extracellular space [9]. T₃SSs are important for virulence in many pathogenic bacteria, and as it is the focus of this thesis it will be discussed in detail in the following sections.

T₄SSs are versatile and can secrete both DNA and protein, either to the surrounding environment or directly into an adjacent cell. The target cells can be both other bacteria and eukaryotic cells. T₄SS comprise three subfamilies; conjugation systems, effector translocation systems, and DNA uptake/release systems [10]. DNA conjugation is an important bacterial trait and T₄SS are widely spread in both bacteria and archaea. The secretion apparatus consists of an inner membrane complex and an outer membrane complex that are linked by a stalk. A pilus extends from the outer membrane complex and may be involved in sensing target cell contact [11], [12]. T₄SSs

are associated with virulence in some pathogenic bacteria, e.g. *Helicobacter pylori* and *Agrobacterium tumefaciens*. Most commonly, virulence-associated T4SSs belong to the effector translocator subfamily.

T6SS is structurally similar to bacteriophage tails and may have evolved from these. A membrane complex anchors a contractile sheath containing an inner tube. In the inactive state the contractile sheath is extended in the bacterial cytosol. Upon activation the sheath contracts, pushing the inner tube towards the target cell and deliver the proteins by puncturing the target cell envelope [13]. Interestingly the T6SS effectors are encoded together with genes providing immunity to the effector, and studies of e.g. *V. cholera* and *Pseudomonas aeruginosa*, suggest that bacteria utilize the T6SS to target other bacteria in the competition for the same environment [14].

1.3 The Type III Secretion System

The T3SS is an important virulence mechanism found in many Gram-negative pathogens, including *Salmonella*, *Shigella*, *Chlamydia*, *E. coli*, *Pseudomonas* and *Yersinia*. The T3SS consists of a complex machinery that actively delivers effector proteins into the cytoplasm of eukaryotic host cells. Thereby, allowing the bacteria to communicate directly with the host cell. Pathogens use their T3SS to translocate toxic effector proteins in order to hijack the host cell and promote its own survival [15].

The T3SS was first discovered in *Yersinia*, when it was found that proteins encoded on a virulence plasmid, had to be delivered into the host cells through a bacteria-host cell contact-dependent mechanism [16]. The name T3SS was coined in 1993 [17] and a few years later, the structure of the type III secretion apparatus (T3SA) (Figure 4) was revealed by electron microscopy of isolated T3SAs from *Salmonella* [18].

Activation of the T3SS require tight contact between bacteria and host cell, and translocation is polarized, meaning that translocation of effectors only occur at the site of interaction [9]. The bacteria display several T3SAs on the surface, however, only those in close contact with the host cell will be active [19].

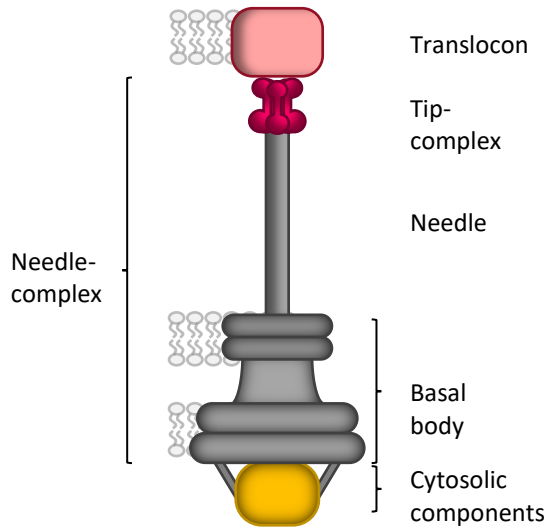


Figure 4. Schematic illustration of the T3SS. The basal body spans the bacterial membranes and a needle-like filament extends from the surface and is capped by a tip complex. The whole needle complex is traversed by a narrow channel. Upon host cell contact a translocon is inserted in the host cell membrane and proposedly form a pore. The ATPase is associated with the cytosolic complex at the base of the T3SA.

1.3.1 T3SS, a secretion system that translocates

As indicated by the name the T3SS is a secretion system, and the objective is to deliver proteins directly into another cell. This process is, however, termed translocation. For clarification, in this thesis ‘secretion’ will refer to transport of proteins through the T3SA out into the extracellular space, which is mostly an *in vitro* phenomenon when the T3SS is induced artificially, whereas, ‘translocation’ will refer to transport of proteins into host cells (Figure 5).

1.3.2 Origin and acquisition of the T3SS

The T3SS is evolutionary related to the flagella and similarities between the systems can be seen both in structure and in function [20], [21]. There are different theories of how these systems evolved, i.e. which came first

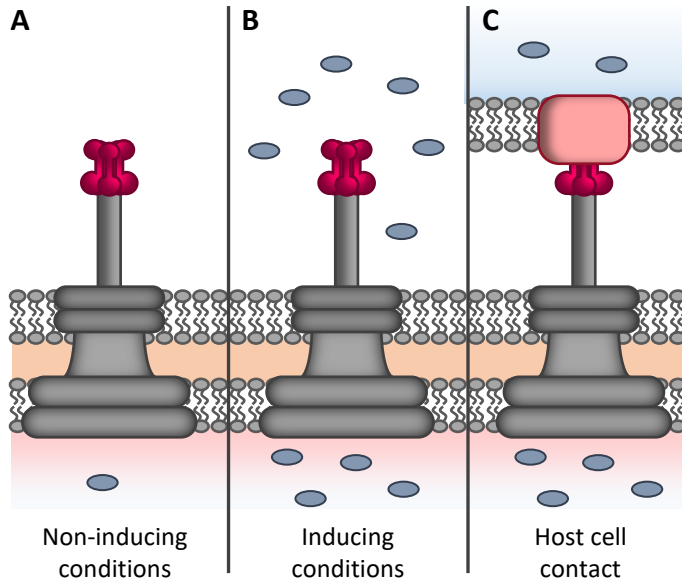


Figure 5. The concepts of secretion and translocation. (A) When bacteria is growing in non-inducing conditions, i.e. prior to host cell contact, the T3SS is in stand-by, secretion is blocked and expression remains low. **(B)** If the T3SS is artificially induced in vitro, expression of effectors is upregulated and there is secretion to the medium. **(C)** When the T3SS is induced by host cell contact, the expression is upregulated, however the effectors are translocated directly into the host cell without leakage to the surroundings.

[20], [22]–[25]. Considering that bacteria evolved roughly two billion years before the multicellular eukaryotes that make out the targets for T3SS (plants, animals), and also the ancient need for bacteria to be able to move towards more beneficial environments, it seems more likely that T3SS evolved from the flagella and not the other way around [20], [25].

Phylogenetic studies of T3SS ATPase and 16S RNA show that bacteria and T3SS have evolved independent of each other, which strongly indicate that T3SSs are transferred horizontally [22]. This is also supported by the fact that T3SSs most often are encoded by gene clusters on pathogenicity islands or plasmids, which can be easily transferred from one bacteria to another [26].

Based on phylogenetic studies, T3SSs can be grouped into seven different families, where the most well-studied include the Ysc-system in *Yersinia* and *Pseudomonas*, the Inv-Mxi-Spa-system (SPI-1) in *Shigella* and *Salmonella*, and the Ssa-Esc-system (SPI-2) in *E.coli*. Regardless of any differences at the genetic levels and in individual T3SS components, the overall T3SS mechanism is highly conserved. The experimental support for that comes from studies of T3SS in *Yersinia*, *Pseudomonas*, *Salmonella* and *Shigella* which allowed secretion as well as translocation of heterologous effectors into the target cells [27]–[29].

While the overall structure and function of the T3SS is highly conserved, there is great variability in the effector proteins. They are unique for each species, and as a result, the bacteria-host interaction is unique for each pathogen.

Many T3SS effectors are modular and can contain several different domains, enzymatic or protein interaction domains. Often these domains are functionally similar to eukaryotic proteins, and it is possible that they were acquired from eukaryotic cells by horizontal gene transfer and then rearranged to modular domains [30].

One thing that effectors from different pathogens have in common is that they often mimic eukaryotic enzymes. The effectors also work in concert with each other and may target the same eukaryotic process from several different angles [31]. Many pathogenic bacteria carry effectors that modulate the dynamics of the actin cytoskeleton and intervene with phosphorylation signaling cascades within the host cell.

1.3.3 The structure of the T3SS

The proteins that make up the T3SS can be divided in four parts 1) a structured unit, the T3S apparatus (T3SA), through which proteins are secreted, 2) regulatory proteins that ensures the timing and precision of T3SS activity, 3) translocator proteins which facilitate the translocation across the host cell membrane, and 4) the secreted effector proteins which are delivered into the host cell [32].

The structural unit (Figure 4) is built up by ~20 different proteins and contains a basal body anchored in the bacterial envelope that is traversed by a hollow rod. An ATPase is associated with the cytosolic side of the basal body through interaction with cytoplasmic T3SS components. On the surface of the bacteria a needle-like filament extends from the basal body out in the extracellular space and is capped by a needle-tip complex. Upon host cell contact, two additional proteins are inserted in the host cell membrane where they form a translocon. Altogether this creates a hollow structure, proposed to link the bacterial cytoplasm with the host cell cytoplasm and this structure is believed to be similar for all T3SSs [32]–[37]. The energy needed for secretion is supplied by the ATPase and the PMF [38]–[40].

1.3.3.1 Assembly of the T3SS

The T3SA was first visualized in *S. typhimurium* by Kubori et al., through the use of electron microscopy [18]. Since then, both electron and fluorescence microscopy techniques have developed to the point where the individual components of the T3SA could be visualized and based on this, the order of assembly was proposed [35]. The structure and assembly of the T3SA have been studied extensively in *Salmonella*, *Shigella* and *Yersinia* and the names of the components below, and in following sections, are derived from the *Yersinia* Ysc T3SS, unless otherwise stated.

In *Yersinia*, assembly of the T3SA starts as two separate events. The outer membrane secretin (YscC) is formed and reaches down towards the inner membrane where YscD assemble the outer MS-ring. Meanwhile the export apparatus (YscRSTUV) is formed separately in the inner membrane and associates with the inner MS-ring (YscJ), and is then incorporated into the outer MS-ring [41], [42].

Following the assembly of the outer and inner membrane components into one complex, the ATPase (YscN) bound to the C-ring complex (YscQLK) is recruited to the base of the T3SA [41]. At this point the T3SA is able to secrete and to complete its structure the inner rod component (YscI) and needle subunits (YscF) are secreted through the T3SA and assembled [43].

Following completion of the needle filament, controlled by YscP [44]–[46], the needle tip protein (LcrV) is secreted and localizes at the distal end of the needle [47], [48]. At this point the T₃SA will be in stand-by mode until the bacteria has made contact with a eukaryotic host cell.

1.3.4 The function of the T₃SS

The T₃SS is an important virulence mechanism in many pathogens, which rely on it to translocate effectors that are toxic to the host. There are however, examples of bacteria that use their T₃SS to establish a symbiotic interaction with their host, e.g. *Rhizobium* spp [49]–[51]. This is an example of how T₃SSs can trigger completely opposite outcomes, depending on the activity of the translocated effectors.

This is also illustrated among pathogenic bacteria; some pathogens, such as *Salmonella* and *Shigella*, use their T₃SS to trigger uptake into host cells, while others, such as *Pseudomonas* and *Yersinia*, use it to block uptake [52]–[55].

1.3.5 Regulation of the T₃SS

As for any type of cell it is important for bacteria to conserve energy and therefore metabolic processes are often regulated in response to environmental cues to ensure that the right function is served at the right time. This is also true for virulence mechanisms such as the T₃SS. Besides being energetically costly for the bacteria to express the T₃SS when it is not needed, it could also be devastating for the bacteria to have an unregulated T₃SS, e.g. the *Yersinia* $\Delta yopK$ mutant which hyper-translocates effectors *in vitro* cell infection models, is attenuated for virulence *in vivo* (section 1.6.5.3). It is possible that uncontrolled activity of the T₃SS might cause a stronger immune response, giving the host an upper hand and ability to clear the infection.

The environmental triggers of T₃SS expression vary depending on the niche of the particular pathogen. For example *Salmonella* and *Yersinia* replicate in different organs in human (mammalian) hosts and they also have different pathogenic lifestyles. *Salmonella* replicates intracellularly,

while *Yersinia* replicates extracellularly and therefore the environmental signals they respond to are different. One thing that is common for many T3SS of human pathogens is that expression of T3SS is induced when the temperature is elevated to 37°C which is the body temperature of mammals [56]. Another induction signal is linked to the establishment of bacteria-host cell contact [57].

1.3.5.1 Temperature regulation

When bacteria are ingested by a mammalian host the temperature is elevated as the body temperature is generally higher compared to the surrounding environment. The increased temperature triggers expression of the T3SS master activator LcrF [58]–[60]. Extensive studies in *Yersinia* have shown how this thermoregulation is achieved. LcrF is a member of the AraC-like transcriptional activators and once expressed it binds to the promoter regions of T3SS genes and activates their transcription [59], [61]–[63]. Expression of LcrF is tightly thermoregulated, both at the transcriptional and the translational level. At low temperatures, YmoA blocks transcription of *lcrF* by binding to the 5′-UTR of the *yscW-lcrF* operon [60], [64]. At 37°C, YmoA is degraded by proteases and as a result, *lcrF* is transcribed [64], [65]. If transcription of *lcrF* is forced at low temperatures under experimental conditions, translation is still blocked through the presence of a stem-loop structure upstream of *lcrF*, making the ribosome site inaccessible for the ribosome. At 37°C the stem-loop structure is destabilized allowing access of the ribosome and translation of LcrF [64].

As the T3SS gene products are being expressed, the basal body, needle and tip complex of the T3SA are assembled, after which, the T3SA is ready to be activated upon host cell contact [35], [66].

1.3.5.2 Cell contact

Activation of the T3SS, i.e. translocation of effector proteins, requires close contact with the host target cell [9]. Prior to host cell contact, the expression of effectors remains low, through the action of anti-activators [67]–[72]. When the bacteria have attached to a host cell, secretion/translocation is

induced and the anti-activators are secreted resulting in an upregulation of expression and secretion of effectors and translocators [9], [73]–[76].

It has been suggested that the tip complex senses host cell contact and propagate the signal down the needle, to relieve the secretion block inside the bacteria [77]–[79].

1.4 Secretion through the T3S organelle

There are two classes of secreted substrates; the effector proteins that are translocated into the host cell where they elicit a biological response, and the translocator proteins which facilitate the delivery of effectors across the host cell membrane [80], [81].

A hallmark of T3SS is that the effectors are delivered directly into the host cell cytoplasm without significant leakage to the extracellular space [9]. Components of the T3SS needed for secretion of virulence effectors were identified early, but the secretion mechanism remained a mystery until the structure of the T3SA was visualized by electron microscopy [18]. The structure revealed a channel spanning the length of the T3SA and it was suggested that the effectors were transported through the T3SA [32].

The narrow channel, only 2-3 nm in diameter, suggested that the effectors had to be secreted in an unfolded or partially folded state, and the energy required for unfolding of the effectors would be supplied by the T3SA-associated ATPase [38]. Thanks to advanced microscopy techniques, two independent groups were able to show the secretion of unfolded substrates through the T3SA [82], [83].

1.4.1 The sorting platform

In *Salmonella*, the C-ring complex (SpaO, OrgA, OrgB) has been proposed to function as a sorting platform loaded with translocator proteins. In the absence of translocators the C-ring complex was instead loaded with effectors. This indicated that the C-ring complex preferably interacts with translocators ensuring their secretion before the effectors [84]. It was found that the secretion regulator InvE, which belongs to a family of proteins sometimes referred to as ‘gate-keepers’, was required to load the

translocators onto the sorting platform. When *invE* was deleted the translocators could not be secreted [84]. Similar mechanisms seem to occur in *Shigella* and *E. coli*, resulting in a secretion hierarchy for translocators and effectors. The consequence of deleting the gate-keepers in *E. coli* (*sepL*) and *Shigella* (Δ *mxiC*) is similar to that of Δ *invE* [36], [78], [85].

The C-ring complex is part of the general structure of the T3SA and is formed also in *Yersinia* (YscQ,K,L) [41], [86], however, it is questionable if it functions as a sorting platform also in *Yersinia*. The *Yersinia* homolog to InvE, YopN-TyeA, regulate secretion, however, there does not appear to be a secretion hierarchy for translocators and effectors. A Δ *yopN* or Δ *tyeA* mutant hyper-secretes both translocators and effectors [87]–[89](Paper II).

1.5 Translocation by the T3SS

The intricate assembly and extensive regulation of the T3SS, all boils down to one thing – translocation. The fate of every T3SS effectors is to efficiently reach into the eukaryotic cell and like a ninja strike its molecular target, leaving no traces behind.

1.5.1 The translocator proteins

Three translocator proteins aid the delivery of effectors over the host cell membranes. A hydrophilic translocator forms the tip complex on the needle [47], [48], [90]–[92], and two hydrophobic translocators are inserted into the host cell membrane [93]–[97]. There is no redundancy between the translocators, all three are required for translocation to occur [9], [98]–[100]

Homologs of the hydrophilic translocator include LcrV (*Yersinia*), PcrV (*Pseudomonas*), SipD (*Salmonella*), IpaD (*Shigella*) and EspA (*E. coli*). It has been shown that the hydrophilic translocators are required for insertion of the hydrophobic translocators into host cell membranes, and without the hydrophilic translocator the translocon cannot be formed [93]–[96]. Based on this, it was suggested that the tip complex functions as a platform for insertion of the hydrophobic translocators and formation of the translocon

[101]. In line with this, it was shown that LcrV needs to be secreted to function as a translocator [102].

The hydrophobic translocators harbor one or two transmembrane domains (TMs), and homologs include YopB/YopD (*Yersinia*), PopB/PopD (*Pseudomonas*), SipB/SipC (*Salmonella*), IpaB/IpaC (*Shigella*) and EspD/EspB (*E. coli*).

In some cases the translocators also have a regulatory role. Studies from *Shigella* and *P. aeruginosa* indicated that the tip complex can function as a sensor for host cell contact [77]–[79], [103]. This seems logical since the tip-complex is positioned in close proximity to the host cell membrane when the bacteria has attached to a host cell. The tip complex in *Shigella* consists of 4 molecules IpaD and 1 IpaB molecule. As part of the tip complex both IpaD and IpaB have regulatory roles and deletion of either of the corresponding genes results in deregulated secretion [104]–[106]. The *Yersinia* translocator YopD regulates expression of effectors by blocking access of the ribosome to the *yop* mRNA, as a result, deletion of *yopD* leads to constitutive expression of Yops [67]–[69] (section 1.6.4.3).

1.5.2 Pore formation

The hydrophobic translocators share some homology with pore-forming toxins [107] and numerous studies using both artificial membranes and eukaryotic cell models have shown that the hydrophobic translocators form pores in the host cell membrane (*Yersinia*, *Shigella*, *Salmonella*, *E.coli*, and *Pseudomonas*). The methods used to study pore formation measures leakage of small substances over the cell membrane and gives an indirect measurement of pore formation. The methods include leakage of hemoglobin from red blood cells (RBC) [99], uptake and release of impermeable dyes like Ethidium bromide and Calcein [108], or leakage of LDH [109].

By infecting cells in the presence of different sugar molecules the size of the pore has been estimated to be 2-3 nm wide [99], [110]–[116] which corresponded nicely to the inner diameter of the needle [117].

Through the isolation of host cell membranes, it has been established that the hydrophobic translocators are inserted into the membrane and isolation of a native translocon, by Montagner et al., estimated the size of the translocation pore to be between 500-700 kDa corresponding to 15-20 YopB/YopD monomers [118]. Actual visualization of the translocation pore has proven far more difficult, however, by adding purified PopB and PopD, Schoehn et al. could show the presence of ring-like structures in liposomes [119].

In the case of *Yersinia*, pore formation assays require the use of $\Delta yopK$, $\Delta yopE$ or multi-effector mutants, due to the low pore forming ability of wild type strain *Yersinia*. This has led to speculations that the effectors plug the pore [120] and this may or may not be part of the feed-back regulation that occurs *in vivo* (section 1.6.4.4). It may also mean that the pore is not a stable entity that once inserted into the membrane will stay in the membrane.

1.5.3 The one-step model of translocation

The widely accepted mode of translocation by the T3SS is the one-step model where the effectors are secreted and translocated directly from the bacterial cytoplasm into the host cell cytoplasm through the T3SA.

This model is based on the observations that 1) translocation is polarized and no effectors leak out to the infection media, 2) the hollow structure of the T3SA in combination with the translocation pore in the host cell membrane forms a hollow conduit, connecting the bacteria and host cell, and 3) the overall resemblance to the flagella system where the flagella substrates are secreted through the basal body.

The injection model is indeed quite logical, there are however, studies that instead argues for a two-step model of translocation.

1.5.4 The two-step model of translocation

Persson et al. showed that a truncated version of the *Yersinia* effector YopH lacking amino acids (aa) 49-154 was not translocated into HeLa cells although it was secreted from the bacteria, thereby showing that YopH has a translocation domain separated from the secretion signal [121]. Fusion of

YopE and YopH secretion signals to Cya triggered efficient secretion of the hybrid proteins to the bacterial surface, yet the hybrid proteins could not be translocated into host cells [122], [123]. The secretion signals of YopE and YopH were mapped to aa 1-15 and 1-17 respectively, while translocation required the first 50 aa of YopE and the first 71 aa of YopH [122]. If the injection model is true that would suggest that once the effectors have been targeted to the T3SA for secretion, translocation into host cells would follow automatically. The presence of a separate translocation domain argues for a two-step process where secretion and translocation are separate events [123].

Results contradicting the injection model also comes from the *Shigella*-field where the translocators IpaB, IpaC and IpaD were present on the bacterial surface prior to host cell contact [71], [124]. Upon host cell contact these proteins were released from the surface [124]. Interestingly, latex-beads coated with Ipa proteins triggered uptake into HeLa cells in a similar way as the *Shigella* T3SS [125].

Surface localization of effectors and translocators has been observed in *Yersinia* as well and, more importantly, Akopyan et al. showed that surface-localized effectors could be translocated into host cells [29]. Purified YopH coated on bacteria was translocated into neutrophils and HeLa cells by both *Yersinia* and *Salmonella*. These results strongly argue for a two-step translocation mechanism perhaps similar to the AB-toxin where the B-moiety binds to the host cell membrane and deliver the toxic A-moiety into the cell. In this model the translocators would represent the B-moiety whereas the effectors are the A-moiety [126].

One model does not exclude the other and it is possible that at least *Yersinia* use both ways of translocation *in vivo*. Release of translocator-effector complexes from the bacterial surface upon host cell contact may ensure instant delivery of effectors, while awaiting translocation to establish through the T3SA.

1.6 *Yersinia*

There are three human pathogenic species of *Yersinia*; *Y. pseudotuberculosis*, *Y. pestis* and *Y. enterocolitica*, and they all rely on their T3SS to promote infection and survive in a host. The T3SS in *Yersinia* belongs to the Ysc-family of T3SSs (section 1.3.2).

Y. pseudotuberculosis and *Y. enterocolitica* cause gastrointestinal disease in humans and infections are often self-limiting. *Y. pestis* on the other hand is far more virulent and infection with *Y. pestis* is fatal unless treated with antibiotics very early after infection.

The pathogenic *Yersinia* species share a tropism for lymphoid tissues which can seem paradoxical to the extracellular lifestyle of these pathogens, as the lymphoid tissues are the reservoir of the host's immune cells.

1.6.1 The route of infection

Y. pseudotuberculosis and *Y. enterocolitica* infects via the fecal-oral route upon ingestion of contaminated food or water. The ingested bacteria travel down the gastro-intestinal tract and reach the intestines where *Yersinia* passes through the intestinal epithelium. The intestinal epithelium forms a barrier that most bacteria cannot pass through, however specialized cells (M-cells), which are sampling content of the intestines, are targeted by *Yersinia*. *Yersinia* attaches to the M-cells via invasin- β 1-integrin interaction and thereby promotes its own uptake. The bacteria are then released on the other side in the lymphoid tissue (Peyer's patches, PPs) below [127]–[131].

Yersinia colonizes the PPs and can then spread further through the lymphatic system to the mesenteric lymph nodes (MLNs), spleen and liver. *Y. pseudotuberculosis* and *Y. enterocolitica* can spread to the blood stream (sepsis). It is, however, very rare that the infection reaches that far in humans.

1.6.2 Phagocytosis

Phagocytosis is a process by which a eukaryotic cell internalizes large particles. In mammals, and other higher vertebrates, phagocytosis is

associated with the host immune response, whereby foreign and potentially harmful particles, such as microbes, are engulfed by phagocytic cells and cleared from the host [132].

Essentially all host cells are capable of phagocytosing particles, however, innate immune cells such as macrophages, dendritic cells and neutrophils are professional phagocytes. They are equipped with multiple types of receptors that collectively recognize a wide range of foreign particles. Some receptors bind the particle indirectly, through antibodies or other host-derived proteins coated on the surface of the particle, while other receptors bind directly to ligands expressed by the particle itself [133], [134].

Regardless of which type of receptor is involved, the phagocytic process is initiated by binding of a ligand to the receptor, thereby activating the receptor. This is followed by kinase activation and phosphorylation signaling in the cytosol. The actin cytoskeleton is rearranged to encapsulate the particle in a phagosome that buds off from the membrane. Through fusion to other cytosolic vesicles the phagosome eventually matures into a phagolysosome where the particle is degraded [55] (Figure 6).

1.6.2.1 β 1-integrin triggered phagocytosis

The integrin receptors are a large group of receptors that are expressed on professional phagocytes as well as non-professional phagocytes. These receptors bind a variety of ligands in e.g. the extracellular matrix or on other cells. The cytosolic domain of the receptor is coupled to the actin cytoskeleton and is involved in processes such as migration and proliferation. Integrin receptors can also recognize ligands expressed by microbes [135].

Pathogenic species of *Yersinia* express the adhesion molecule invasin on the surface, which binds to β 1-subunit of the integrin receptor. The interaction between invasin and β 1-integrin leads to activation and clustering of the integrin receptors at the site of binding and formation of focal adhesion complexes (FAC) [136], [137]. FACs are distinct structures associated with the cytoplasmic domain of the activated receptor and are made up of multiple proteins that are recruited to the receptor upon

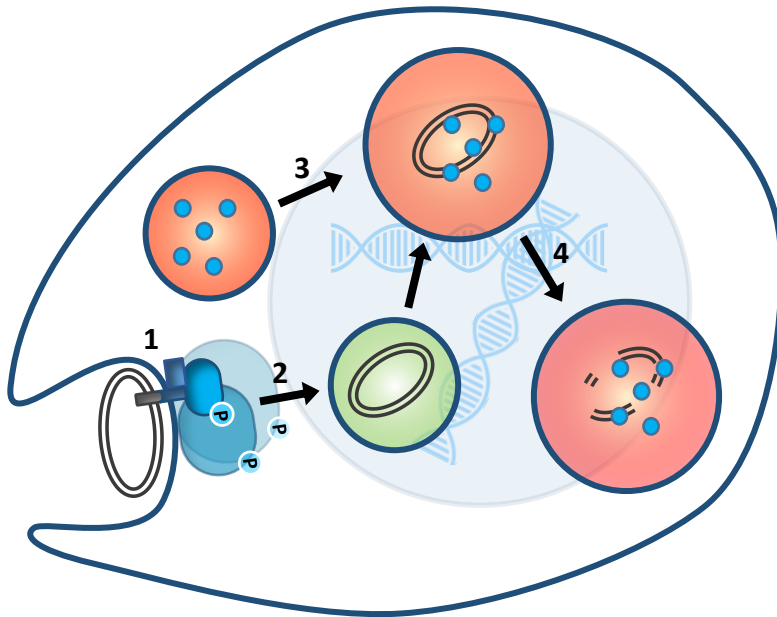


Figure 6. Outline of the phagocytic process. (1) Bacteria bind to receptors in the cell membrane (e.g. β 1-integrin) which initiates a phosphorylation-signalling cascade in the cell, and actin rearrangement to form pseudopods that wrap around the bacteria. **(2)** The bacteria is taken up into a phagosome. **(3)** The phagosome fuses with a lysosome forming the phagolysosome, which is an acidic compartment containing a lot of different digestive enzymes. **(4)** The bacteria is eventually degraded in the phagolysosome.

activation. First structural proteins, such as α -actinin and paxillin are recruited to the cytoplasmic domain of the receptor and form a structural link between the receptor and the actin filaments. Subsequently, signaling proteins, typically tyrosine kinases such as Focal adhesion kinase (FAK), are recruited and launch a rapid phosphorylation signaling cascade, which ultimately leads to rearrangement of the actin filaments into pseudopods that surround and engulf the bacteria [138]–[140] (Figure 6).

Yersinia depend on its T3SS to survive in a host, and the main mission of the T3SS is to block phagocytosis [55]. Several of the effectors in *Yersinia* act in concert to block phagocytosis [141], [142], and they will be discussed in section 1.6.5.

1.6.3 The virulence plasmid

The pathogenic species of *Yersinia* all carry a 70 kb virulence plasmid which encodes all the components of their respective T3SS [143], [144]. These include 1) *Yersinia* secretion (Ysc) proteins that build the T3SA, 2) *Yersinia* outer proteins (Yop) which includes the effector- and translocator proteins, 3) Low calcium response (Lcr) proteins that regulate expression and secretion, and 4) Specific Yop chaperones (Syc) that chaperone members of group 3.

1.6.4 Regulation of the T3SS in *Yersinia*

The *Yersinia* T3SS is regulated by temperature and host cell contact as described in section 1.3.5. The regulatory features that apply to, or are unique for, *Yersinia* will be described in more detail in this section.

1.6.4.1 The low calcium response

Yersinia has a unique requirement for calcium when grown *in vitro* at 37°C [145]–[147]. This calcium requirement was found to be associated with the presence of the virulence plasmid. When *Yersinia* is grown at 37°C in presence of calcium (non-inducing conditions) the genes encoding the T3SS are expressed at a basal level and the system is assembled. If calcium is removed from the culture medium at this point (inducing conditions), it results in massive expression and secretion of Yops [75], [148], [149], and the bacteria stop growing. This phenomenon is referred to as the low calcium response (LCR) [150]. The growth restriction is most likely a result of the energy cost of the T3SS rather than a metabolic need for calcium [151]. The LCR has been very useful to decipher the functions of the T3SS gene products, and T3SS mutants in *Yersinia* can be categorized as calcium dependent (CD), calcium independent (CI) or temperature sensitive (TS). Calcium dependent strains are the wild type strain and mutants that are not affected in the overall T3SS *in vitro* [148], [149], which also include mutations in the Yop effectors, meaning that an *in vitro* CD phenotype can still be avirulent *in vivo*. CI strains are generally downregulated in T3SS activity and grow equally well in the presence or absence of calcium [148],

[149]. Mutants that fall within this category are those that fail to produce a functional T3SA (*ysc* mutants) or lack the positive regulators like *lcrF* and *lcrV* (section 1.6.4.3). In contrast to CI, TS strains cannot grow at 37°C regardless of the calcium concentration and they constitutively express (and in some cases secrete) Yops [152]. Mutants in this category typically lack negative regulators, such as *lcrG*, *yopN*, *tyeA*, *lcrQ* or *yopD* (section 1.6.4.3).

It is not known how the calcium dependent Yop expression and secretion correlate to the *in vivo* infection. The cytoplasmic concentration of calcium inside eukaryotic cells is very low (micromolar), compared to the extracellular concentration of calcium (millimolar), which correlates to the calcium concentrations used during *in vitro* growth. One theory is that docking of the T3SA to the host cell allows the bacteria to sense the intracellular environment, i.e. the low calcium concentration, and that would trigger the expression and subsequent secretion and translocation of effectors into the cell [32]. The translocation-dependent feed-back mechanism fine-tune the expression and translocation to accommodate the particular bacteria-cell interaction, so the growth restriction that occurs when the T3SS is induced *in vitro* likely does not occur *in vivo*.

1.6.4.2 Copy number

T3SS activity requires intimate bacteria-cell contact, or in the case of *Yersinia*, *in vitro* induction can be achieved by removing calcium from the growth medium. Upon induction of the T3SS, expression of Yops increases dramatically [75], [148], [149] and in a recent publication Wang et al. showed that *Y. pseudotuberculosis* can regulate the copy number of the virulence plasmid [153]. The virulence plasmid copy number was slightly higher when *Y. pseudotuberculosis* was grown at 37°C, compared to 26°C, under non-inducing conditions. However, when the bacteria were shifted to inducing conditions the copy number increased almost 3-fold. Interestingly a $\Delta yopD$ mutant which display a TS phenotype, with constitutive Yop expression at 37°C, also had a higher copy number compared to the wild type strain in non-inducing conditions. The importance of regulating virulence plasmid copy number was verified *in vivo* as a strain with only one copy of

the virulence plasmid was less virulent than the isogenic wild type strain and failed to cause systemic spread in mice. These results show that the copy number strongly contribute to T3SS gene expression [153].

1.6.4.3 Regulated expression and secretion

Expression and secretion of Yops are tightly linked through a feed-back mechanism requiring secretion of anti-activators before Yops can be translated (Figure 7). When host cell-contact is established the anti-activators are secreted and expression of Yops is upregulated and vice versa, when secretion is blocked the Yop expression remains low.

In absence of host cell-contact (or in presence of calcium *in vitro*) secretion of Yops is blocked by YopN-TyeA and LcrG, if either one of the corresponding genes are deleted it results in constitutive expression and secretion of Yops [87]–[89], [154]–[157]. YopN-TyeA needs to be targeted to the T3SA by the chaperone complex SycN/YscB in order to regulate secretion [158]–[160], and it was shown that YopN interacted with the inner rod component YscI in the T3SA [161]. A study in *P. aeruginosa* showed that both LcrG and TyeA homologs (PcrG and Pcr1 respectively) interacted with the YscV (PcrD) in the export apparatus, and with each other [162]. These results argue for a model where LcrG, YopN and TyeA blocks secretion by forming a complex at the base of the T3SA.

LcrV is a positive regulator of secretion and the interaction between LcrV and LcrG is required for secretion of Yops. A model was suggested where the intracellular levels of LcrV increases quickly upon host cell contact, allowing LcrV to titrate LcrG away from the T3SA, thereby relieving the secretion block. In support of this, an *lcrG* mutant, which failed to interact with LcrV, constitutively blocked secretion [163]–[165]. It is not known how YopN-TyeA are affected by the removal of LcrG, however, considering that LcrG and YopN-TyeA associate to YscV, it is possible that removal of LcrG, by LcrV, leads to dissociation of YopN and TyeA from the T3SA, and subsequent secretion of YopN, thereby relieving the secretion block. Curiously, low levels of Yops are secreted and dispersed on the bacterial surface before host cell

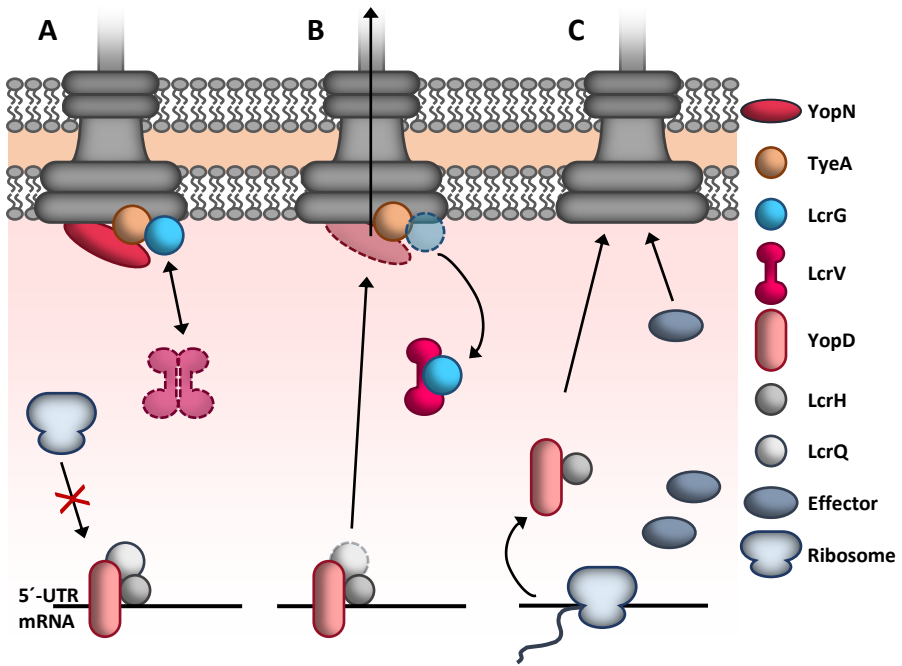


Figure 7. Simplified model of the regulation of expression and secretion. (A) YopN-TyeA and LcrG block secretion by binding to the base of the T3SA. Low levels of LcrV are present in dimerized form, *yop* expression is blocked by YopD-LcrH and LcrQ. (B) Upon target cell contact, LcrV sequesters LcrG from the T3SA and YopN is secreted, this allows secretion of LcrQ, which possibly destabilizes the YopD-LcrH-mRNA complex. (C) YopD-LcrH detaches from the mRNA and the ribosome gain access and translation starts. The Yop effectors and translocators are secreted.

contact [29], [98], and it is currently unknown how they pass the secretion block.

Before secretion is induced, Yop expression remains low by the actions of YopD, LcrH and LcrQ. YopD forms a complex with LcrH which binds to the 5'-UTR of *yop* gene mRNA, and in doing so, likely block the access of the ribosome to the ribosome binding site and promote degradation of the *yop* gene mRNA [69], [166]. LcrQ is also required for the negative regulation of Yop expression although the regulatory mechanism of LcrQ is not known. It has been suggested that LcrQ, YopD and LcrH form a tripartite complex at

the mRNA. When *Yersinia* has attached to a host cell, LcrQ is secreted which in turn may destabilize the YopD-LcrH complex, causing its release from the mRNA. The ribosome can then access the RBS on the mRNA and translation is initiated resulting in a quick up regulation of Yop expression [68], [75], [167], [168]. A recent study showed that LcrQ counteracted the effect of LcrF, and it was suggested that LcrQ may regulate transcription of T3SS encoding genes. As LcrQ is secreted (upon host cell contact) the level of LcrF exceed that of LcrQ and transcription is activated [169].

1.6.4.4 Regulated translocation

Translocation of effectors into host cells is the be-all and end-all of T3SS induced pathogenicity, nevertheless, translocation follows the minimalistic principle that less is more. The effectors have specific targets within the cells and once those targets have been hit, there is no point in continuing the translocation. On the contrary it is actually detrimental for the bacteria to translocate too much, as is exemplified in the *Yersinia* $\Delta yopK$ mutant which hyper-translocates effectors yet is attenuated for *in vivo* virulence [170], [171].

It has been shown for both *Pseudomonas* and *Yersinia* that translocation of effectors inhibit subsequent translocation of effectors through a feed-back loop [109], [172]. It is largely unknown how the feed-back mechanism work, but in *Yersinia* it is dependent on the enzymatic activity of the effector YopE, which is similar to eukaryotic GTPase activating proteins (GAP) (section 1.6.5.2). In short, the GAP activity of YopE disrupts the actin cytoskeleton of the host cells and inactivation of the GAP-activity results in loss of the feed-back regulation and over-translocation of effectors (including YopE). This suggests that the feed-back loop is coupled to the activity of the effectors and relayed back to the translocation machinery through the actin filaments [109]. YopK (section 1.6.5.3), which is specific to *Yersinia* spp., is also important for regulation of translocation. As mentioned above a $\Delta yopK$ mutant hyper-translocate effectors into cells, without affecting either expression or secretion of Yops, which means that YopK acts specifically at the level of translocation [173]. YopK bridges the translocon to the β 1-

integrin, which in turn connects to the actin cytoskeleton, suggesting that YopK may transmit the signal from YopE back to the T3SA [109], [171], [174] (Figure 8).

1.6.5 The Effectors in *Yersinia*

Yersinia spp. encode six effectors; YopE, YopH, YopJ, YopM, YpkA and YopT. YopE and YopH will be discussed in more detail in the sections below.

YopJ, YopP in *Y. enterocolitica*, is a serine/threonine acetyltransferase which inactivates kinases in the MAPK- and NF- κ B signaling pathways [175]–[177]. By blocking these pathways, YopJ blocks cytokine production and promotes apoptosis of the infected cell [178]–[181].

YopM is the only effector that lacks enzymatic activity. It has a leucine rich repeat domain and a nucleus localization signal and localizes in the nucleus [182], [183]. YopM has been shown to inhibit pro-inflammatory responses, possibly by upregulating the anti-inflammatory IL-10, through binding to ribosomal S6 protein kinase and protein kinase C-related kinase [184], [185]. Further it has been suggested that YopM induces apoptosis by activating caspase-3 [186].

YpkA (YopO in *Y. enterocolitica*) has two enzymatic domains, both of which target actin filaments and contribute to anti-phagocytosis. A serine/threonine kinase domain becomes activated upon binding to globular actin in the host cell, and phosphorylates proteins involved in the regulation of actin polymerization [187]–[189]. A guanine nucleotide dissociation-like inhibitor (GDI) domain binds to RhoA and Rac1 and lock them in their inactive state, thereby inhibiting actin polymerization [190]

YopT also contributes to anti-phagocytosis by disrupting the actin filaments. YopT is a cysteine protease which targets and cleaves GTPases of the Rho family, causing them to mis-localize in the cell [191]–[194]. Due to a deletion of the *yopT* gene, this effector is not present in all serotypes of *Y. pseudotuberculosis*, however the function of YopT can be complemented by YopE [191], [195].

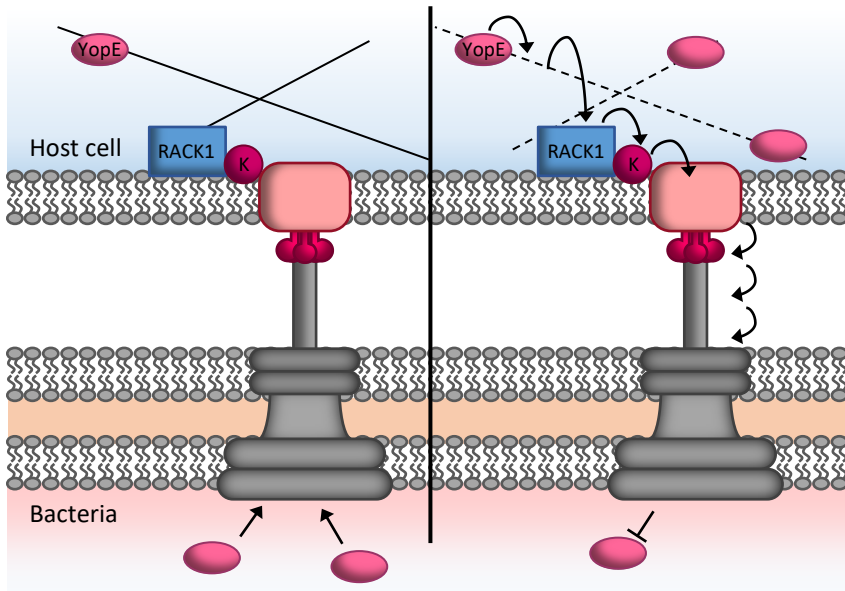


Figure 8. Translocation dependent feed-back signalling in *Yersinia*. YopE and YopK regulate the level of translocation. The GAP activity of YopE results in disruption of the actin filaments in the host cell, which prevents the cell from phagocytosing the bacteria. Successful disruption of the actin filaments sends a signal back to the bacteria, possibly via RACK1 and YopK, to stop translocating.

1.6.5.1 YopH

YopH was discovered almost 30 years ago as a crucial virulence factor of *Y. pseudotuberculosis*, when it was found that YopH protected *Yersinia* from phagocytosis [196], [197]. YopH, together with YopE, is the most abundant effector in *Y. pseudotuberculosis* and since its discovery a lot of research has been done on this effector.

YopH is a very powerful protein tyrosine phosphatase (PTPase) [198], [199]. The PTPase domain is located in the C-terminal, aa 206-468, and a cysteine residue at position 403 is critical for YopH activity [198].

YopH targets cytosolic proteins that are associated with the FAC in epithelial cells and macrophages. Among the proteins identified as targets of YopH are FAK, p130cas, paxillin and Fyb [200]–[202]. When *Yersinia*

attach to a host cell, through the invasin- β 1-integrin interaction, these proteins are rapidly phosphorylated, however translocation of YopH reverse the process, by specific dephosphorylation (Figure 9). The YopH dependent dephosphorylation of cytosolic host cell proteins can be seen as early as 30 seconds after cell infection [136].

Activation of β 1-integrin receptors also results in a massive elevation of the intracellular calcium concentration and this influx of calcium is seen immediately after *Yersinia* attaches to the host cell. Translocation of YopH blocks the calcium spiking, showing that YopH is translocated within seconds after attachment [203].

The importance of YopH to *Yersinia* virulence *in vivo* is illustrated by that a $\Delta yopH$ mutant fails to disseminate beyond the PPs, compared to e.g. a $\Delta yopE$ mutant, which can spread to the MLN before it is cleared [204], [205].

1.6.5.2 YopE

Translocation of YopE into eukaryotic cells is followed by a characteristic rounding of the cells as YopE elicits a cytotoxic response. This morphological change is caused by a collapse of actin filaments [16], which is in turn caused by the intrinsic GAP-activity of YopE [206]–[208].

The GAP domain of YopE contains a conserved arginine finger, which is also found in eukaryotic GAPs, and a conserved arginine residue at position 144 is crucial for the GAP activity of YopE [206]–[208]. YopE targets RhoA, Rac1 and CDC42 *in vitro* and RhoA and Rac1 have been verified to be *in vivo* targets as well [206], [207], [209].

Through its effect on the actin cytoskeleton, YopE plays an important part in the anti-phagocytic action of *Yersinia*. Interestingly, it was shown that YopE is the sole determinant in blocking phagocytosis by dendritic cells [210], due to the specific mechanism by which dendritic cells engulf bacteria. Macrophages engulf bacteria by a different mechanism, where both YopH and YopE are required for anti-phagocytosis [210].

Another important function of YopE is to regulate translocation levels, through a feed-back mechanism. A $\Delta yopE$ mutant hyper-translocates the

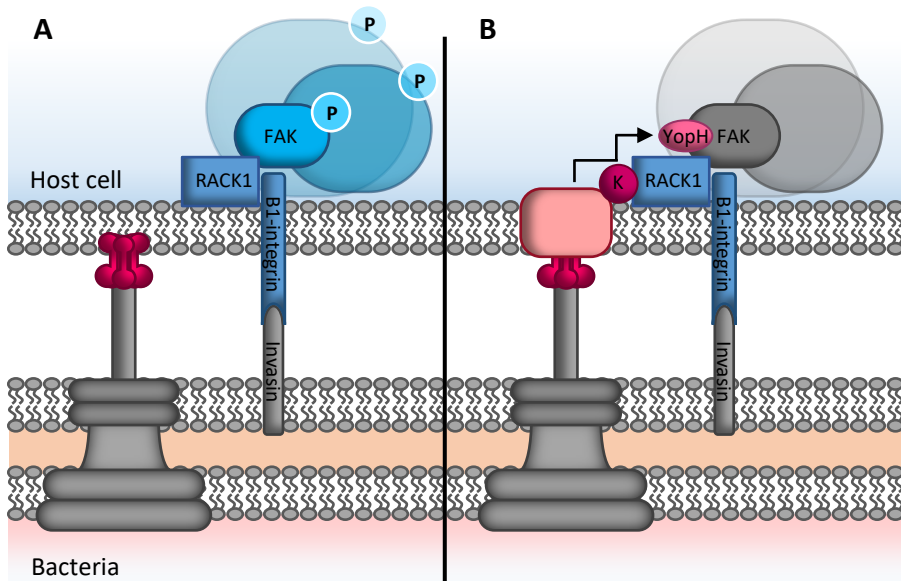


Figure 9. YopH-dependent phagocytosis inhibition. (A) *Yersinia* attaches to the host cell through invasins binding to β 1-integrin. This binding initiates a phosphorylation signalling cascade that ultimately results in phagocytosis of the bacteria. **(B)** *Yersinia* translocates YopH which is guided by YopK to FAK and other intracellular target proteins. YopH quickly dephosphorylates its target proteins thereby terminating the signalling cascade.

other effectors [109], [211], similarly to a $\Delta yopK$ mutant [171], [173], [212], [213]. The precise mechanism behind the feed-back regulation is not known, however, it is coupled to the GAP activity of YopE, as the GAP mutant YopER144A hyper-translocates itself and YopH [209]. Further, it was shown that inhibition of actin polymerization resulted in lowered translocation levels [120], [214]. It is therefore tempting to think that the feed-back regulation stems from YopE activity, resulting in rearrangement of the actin cytoskeleton which, by extension, connects to YopK and the translocon (Figure 8).

1.6.5.3 YopK

Though not an effector *per se*, YopK is translocated and serves its function from within the infected host cells.

YopK is specific for *Yersinia*, and it is found in all three pathogenic strains of *Yersinia* (YopQ in *Y. enterocolitica*). There are no genetic homologs identified in T3SSs of other species, however, the EspZ protein in *E. coli* may be a functional homolog to YopK, in the sense of regulating translocation levels [215].

YopK was, early on, identified as an important virulence factor as a $\Delta yopK$ mutant was attenuated for virulence in mice, despite that it induced cytotoxicity and blocked phagocytosis *in vitro* [170]. It was later found that the $\Delta yopK$ mutant translocated more YopE and YopH into HeLa cells compared to the wild type strain while on the other hand, overexpression of YopK lowered the translocation levels. YopK did not affect either expression or secretion levels of the effectors, which meant that YopK specifically regulated translocation [173].

The hyper-translocation phenotype of the $\Delta yopK$ mutant was coupled to the increased hemolytic activity, and osmo-protection assay using sugars of different sizes suggested that the $\Delta yopK$ mutant form larger pores in the host cell membrane and therefore translocates more [173]. Isolation of RBC membranes verified that the $\Delta yopK$ mutant insert more YopB and YopD into the membrane [171] (Paper III), which agrees with the increased hemolytic activity in this mutant.

By use of a glycogen synthase kinase tag, Garcia et al., showed that YopK is translocated into host cells [216] which was also verified by Thorslund et al., using the beta-lactamase reporter system [171]. Further it was shown that YopK needs to be translocated into the host cell to regulate translocation [212]. Interactions between YopK and YopD suggests that YopK is associated with the translocon once it has been translocated [171], [212].

Interestingly, RACK1 has been identified as a target for YopK. RACK1 is a scaffold protein, which binds to the cytoplasmic domain of β 1-integrins. *Yersinia* is dependent on the interaction between YopK and RACK1 to block phagocytosis and Thorslund et al. proposed a model where YopK bridges the translocon to the focal adhesion complexes, through its interaction with RACK1, and guide the effectors to their targets, thereby ensuring an immediate block in the intracellular signaling [171].

The localization of YopK between the translocon and RACK1 also places YopK in an optimal position to receive and transmit the feed-back signal from YopE to cease translocation [109], [171], [174]. If YopK is absent, transmission of the feed-back signal is lost and as a result translocation continues (Figure 8).

1.7 YopN

YopN is a 32 kDa secreted protein which regulates secretion. The C-terminal end of YopN interacts with the small cytoplasmic protein TyeA (aa 212-222 and 243-293) and an N-terminally located chaperone-binding domain (CBD, aa 32-76) binds the cognate chaperone complex SycN/YscB [158], [159] (Figure 10). All these four proteins are required, along with LcrG, to regulate the timing and precision of secretion during infection, and deletion of either of the corresponding genes results in a TS phenotype [88], [154], [155], [158], [159].

YopN-TyeA is the *Yersinia* homolog of a family of proteins (gate-keepers) that regulate secretion. Homologs from other species include PopN-Pcr1 (*P. aeruginosa*), InvE (*Salmonella* SPI-1), MxiC (*Shigella*) and SepL (*E. coli*). The sequence homology between these proteins is quite low, yet the overall function as secretion regulators is conserved.

Unique for the Ysc T3SS is that the gate-keeper is encoded as two separate proteins, whereas homologs from the other T3SS families are encoded as a single protein. Interestingly though, TyeA binds to the C-terminal of YopN and the structure of the YopN-TyeA complex is strikingly similar to that of MxiC [34], [217], [218].

The interaction between TyeA and YopN is central to secretion regulation. The TyeA binding site has been mapped to aa 248-293 of YopN [88], [219], however the crystal structure of the complex indicated that there is a second interaction site at aa 212-222 [217]. If TyeA is deleted or the YopN-TyeA interaction disrupted, secretion regulation is lost [88], [156], [220].

YopN-TyeA function depend on the YopN chaperone complex SycN/YscB and most likely the function of the chaperone is to pilot the YopN-TyeA complex to the T3SA [160].

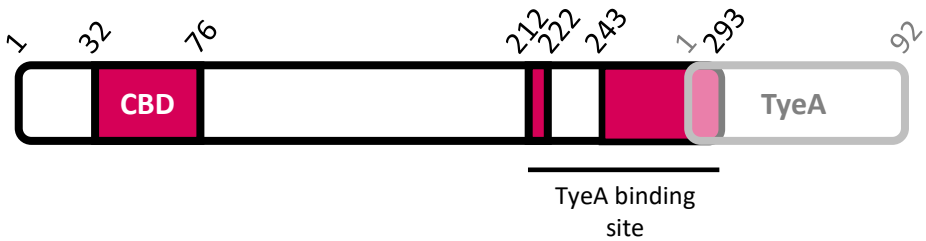


Figure 10. Schematic diagram of YopN. The coding sequence of *yopN* and *tyeA* overlap, however they are expressed as two separate proteins. TyeA bind post-translationally to the C-terminal end of YopN. The SycN/YscB chaperone complex bind to the chaperone binding domain (CBD).

1.7.1 Secretion and translocation of YopN

The established role of YopN is to regulate secretion, yet YopN is also translocated into host cells [221].

Under secretion permissive conditions YopN, but not TyeA, is secreted along with the other Yops and it has been suggested that secretion of YopN is the trigger that induces secretion of translocators and effectors [156]. Intriguingly, Day and co-workers also showed that YopN is translocated into the eukaryotic cell in a YopB-dependent manner [221]. However, it is not clear if YopN is translocated as a step in relieving the secretion block, or if YopN has a specific function or target inside the host cell.

The YopN homolog from *Chlamydia*, CopN, was shown to sequester tubulin, and when expressed intracellularly in yeast, CopN caused cell cycle arrest, possibly by preventing microtubule assembly [222], [223]. Intracellular expression of YopN did not cause cell cycle arrest [222], however that does not exclude that YopN could have a different intracellular target.

1.8 LcrV

LcrV is a 37 kDa multifunctional protein. It serves three different roles in three different locations, inside the bacteria, at the tip of the YscF needle and secreted from the bacteria.

1.8.1 The V-antigen

The name LcrV derives from Low calcium response Virulence antigen. LcrV was the first identified virulence factor in *Yersinia*, discovered in *Y. pestis* and named V antigen as it was only produced by virulent strains of *Y. pestis* [224]. Later it was also discovered in *Y. pseudotuberculosis* [225] and *Y. enterocolitica* [226].

LcrV is a secreted soluble protein and in 1963 Lawton et al., purified LcrV and showed that passive immunization with antibodies raised against LcrV protected mice against challenge with *Y. pestis* [227]. They also showed the first indications that active immunization with purified LcrV protected against *Y. pestis* infection. This was later confirmed when it was shown that active immunization with LcrV protected mice against up to 10^6 lethal doses of *Y. pestis* [228], [229].

Further studies revealed that LcrV contains a minor (aa 2-135) and a major (aa 132-275) protective epitope and monoclonal antibodies (mAb7.3) directed against the major epitope gave passive immunity against infection with *Y. pestis* and *Y. pseudotuberculosis* [230]–[232]. Due to the antigenic property of LcrV, it is pursued as a vaccine candidate [233], [234].

1.8.2 LcrV – a regulator of Yop synthesis and secretion

LcrV functions as a positive regulator therefore deletion of *lcrV* leads to downregulated Yop expression and secretion [98], [235].

The gene encoding LcrV is transcribed as part of the *lcrGVH-yopBD* operon and LcrV interacts with LcrG as well as YopB and YopD [163], [236].

1.8.3 The structure and localization of LcrV

From the early studies of LcrV it was known that LcrV was secreted from the bacteria, and in 1999 Pettersson et al. showed that LcrV localizes at discrete foci on the bacterial surface [98]. A few years later it was shown that LcrV localizes to the distal end of the needle where it forms a distinct tip structure, containing a 'head', 'neck' and 'base' (Figure 11) [47]. The surface localization of LcrV and positioning on top of the YscF needle fits with the antigenic property of LcrV and suggests that the protective antibodies block T₃SS function rather than LcrV alone.

LcrV is shaped like a dumbbell with an N-terminal- and a central globular domain separated by a coiled-coil motif (Figure 11). LcrV majorly consists of α -helical content as revealed by the crystal structure [237], [238]. LcrV contains 12 α -helices of which two (α_7 and α_{12}) form the coiled-coil motif. The N-terminal globular domain consists mainly of α -helices, while the central globular domain consists of a mixture of short α -helices and β -strands that are connected by unstructured coils and turns. Further studies showed that LcrV homologs from *Pseudomonas*, *Shigella*, *Salmonella*, *Burkholderia* and *E. coli* share the dumbbell shaped structure. Even though the sequence homology between the homologs is rather low, the structural conservation of the proteins is very high, especially in the coiled-coil motif. The coiled-coil motif is also the most sensitive to mutations as disruption of the coiled-coil most likely disrupts the structural integrity of the whole protein [237].

Computer modelling of LcrV on top of the YscF needle indicated that LcrV tip-complex consists of a pentamer [96]. Broz et al. also showed that the N-terminal and C-terminal of LcrV is facing down towards the needle and that the N-terminal globular domain points out from the needle and forms the 'base'. The central coiled-coil forms the 'neck' and the central globular domain forms the 'head' [96]. Overall, the structure of LcrV and the pentamer fits very well with electron micrographs of the tip complex [101], [239].

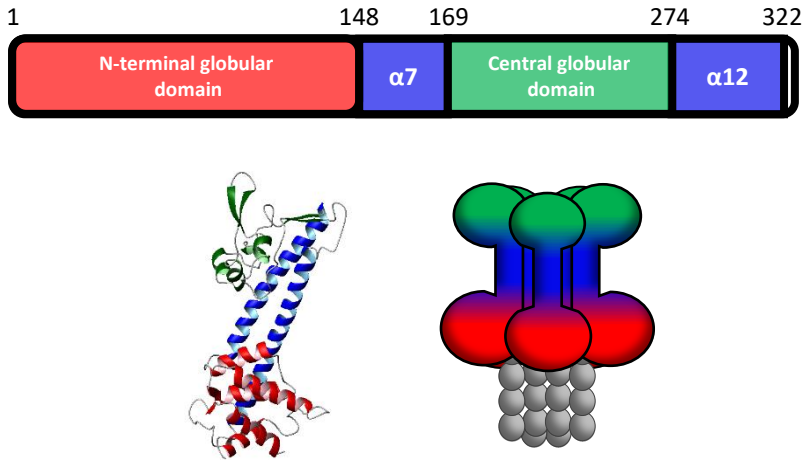


Figure 11. Illustration of LcrV. (A) Diagram of the domains in LcrV, α -helices 7 and 12 form the coiled-coil that separates the two globular domains. (B) X-ray structure of LcrV (PdB: 1R6F, reference 237). (C) Illustration of the LcrV pentamer which forms the tip complex, with the characteristic 'head' (central globular domain), 'neck' (coiled-coil) and 'base' (N-terminal globular domain) configuration.

1.8.4 LcrV is one of the translocator proteins

Beside its role in regulation, LcrV is also one of three translocator proteins required for translocation of effectors across the host cell membrane. The two other translocators, YopB and YopD, were shown to be directly involved in translocation [9], [99], and both YopB and YopD were shown to interact with LcrV [236] which suggested that LcrV might also be involved in translocation. The regulatory phenotype of *lcrV* mutants impeded studies of LcrV's role in translocation, however, by use of polyclonal antibodies directed against LcrV, Pettersson et al. showed that LcrV was a true translocator protein, and in-frame deletions identified a central and C-terminal regions of LcrV, that were specifically required for translocation [98]. Studies followed to show that LcrV was required for pore formation by YopB and YopD in RBC membranes [112], [240] and the localization of LcrV at the tip of the

needle agreed nicely with this and suggested that LcrV constitutes a platform for pore formation [101]. In further support of this it was shown that LcrV secretion was a requirement for translocation and *in vivo* virulence [102]. By producing hybrid proteins of LcrV homologs of different sizes it was shown that the N-terminal globular domain of LcrV was required for membrane insertion of YopB [96].

2 OBJECTIVES OF THIS THESIS

The molecular mechanism of effector translocation and how it is regulated is largely unknown, therefore the studies in this thesis were undertaken to analyze different aspects of translocation.

The specific aims were:

- To characterize the central region of YopN
- To analyze if YopN has an additional role besides regulating secretion
- To perform an in-depth analysis of the role of LcrV during translocation

T3SS assembles into a complex machinery dedicated to translocate a specific set of substrates into eukaryotic cells. The system is vital in many bacteria-eukaryote interactions, especially as a virulence mechanism in pathogenic bacteria. Expression of the T3SS inflicts a metabolic burden on the bacteria and is therefore only expressed and active when needed. Several regulatory proteins coordinate the expression and activity of the T3SS with high precision (sections 1.6.4.3 and 1.6.4.4).

Past studies have revealed that expression and secretion (*in vitro*) or translocation (*in vivo*) are linked through feed-back mechanisms. In this thesis I show that two of these regulatory proteins, YopN and LcrV, are involved in additional events, feeding in to the regulation of the T3SS.

3.1 The LcrV-LcrG heterodimer interacts with YopD

Before secretion is induced, *yop* gene translation is blocked by YopD, LcrH and LcrQ [67]–[69]. When secretion is induced, LcrQ is secreted [75]. Presumably YopD and LcrH detach from the mRNA at this point, allowing translation to start.

LcrG regulates secretion together with YopN and TyeA. Studies in *Pseudomonas* showed that TyeA (Pcr1) and LcrG (PcrG) interacted with YscV (PcrD) and with each other, and the results suggested that LcrG and YopN-TyeA form a complex which blocks access of T3SS substrates to the secretion channel through the interaction with YscV [162]. LcrV, which is a positive regulator, is believed to sequester LcrG from the T3SA when host cell contact is established [163], [164]. This possibly leads to secretion of YopN and opening of the secretion channel.

In addition to their regulatory functions inside the bacteria, both LcrV and YopD are translocator proteins and the interaction has been mapped to the amphipathic domain in YopD [241]. Further it was shown that the N-terminal globular domain of LcrV was required to insert YopB and YopD in host cell membranes suggesting that this was the site of interaction [96],

[103]. Considering that LcrV is required for membrane insertion of YopB and YopD, the interactions between them would logically occur on the surface of the bacteria.

In Paper I we set out to map the LcrV-YopD interaction in LcrV and investigate if YopD competes with LcrG for the interaction site. Considering that interaction between LcrV and YopD has been shown before [241], we were somewhat surprised to find that YopD failed to interact with LcrV under the conditions used (Paper I). Previous studies of purified proteins in *Pseudomonas*, showed that PcrV and PopD did not interact with each other *in vitro* [119], [242], and now we show that the same is true for *Yersinia* LcrV and YopD. It is assumed that LcrV and YopD interact directly with each other *in vivo*, during translocation, yet since LcrV forms a pentamer on the needle tip [96] it is probable that LcrV needs to oligomerize before the interaction with YopD can occur. Purified LcrV appears in dimeric form which may be unsuitable for YopD interaction.

All the more fascinating was that YopD readily bound to the LcrV-LcrG heterodimer (Paper I, Figure 5). Since LcrG is not secreted, any interaction involving LcrG ought to reflect an event that occurs in the bacterial cytoplasm. We also assume that the LcrV-LcrG heterodimer is the natural cytoplasmic form of LcrV. This raises the question if the interaction is relevant for the regulation of Yop expression and/or secretion?

Costa et al. successfully pulled down YopD with GST-LcrV from the cytoplasm of *Yersinia* and our results suggest that LcrG was present in that complex. Further, in line with the theory that YopD-LcrV interaction is involved in the regulation, is that residues in the amphipathic domain of YopD, that were required for interaction with LcrV, were also required for YopD-dependent regulatory processes in the bacterial cytoplasm [241].

YopD was also pulled-down using TyeA-GST [88], suggesting that there may be a link between the YopD/LcrH/LcrQ complex which blocks *yop* gene translation and the LcrG/YopN-TyeA complex that blocks secretion. It is possible that an interaction between the two complexes (or parts of them) is a step in the secretion of the anti-activators to de-repress *yop* gene translation.

3.2 Indications that YopN is an effector

Under secretion permissive conditions YopN, but not TyeA, is secreted along with the other Yops and it has been suggested that secretion of YopN is the trigger that induces secretion of translocators and effectors [156], [243]. Intriguingly, Day et al. also showed that *Y. pestis* translocated YopN into the eukaryotic cell in a YopB-dependent manner [221]. The question remains, if YopN is passively translocated as a step in relieving the secretion block or if YopN also has an intracellular function?

YopN is translocated at very low levels and to detect YopN intracellularly, Day et al. employed a GSK tag which becomes phosphorylated by eukaryotic kinases [221]. We could confirm that, similarly to *Y. pestis*, YopN is also translocated by *Y. pseudotuberculosis*. As expected, the wild type strain translocated very low levels of YopN, however by combining the proteinase K protection assay with TCA precipitation of the cytosol we were able to concentrate the cytosolic proteins and detect YopN. To optimize detection of YopN, we constructed a hemagglutinin (HA) tag in the C-terminal of YopN. Since there is an overlap of the *yopN* and *tyeA* sequences, we wanted to ensure proper expression of *tyeA* by expressing the YopN-HA construct *in-trans*, from an arabinose inducible promoter in a non-polar $\Delta yopN$ mutant (Paper II). To analyze if translocation of YopN was regulated by YopK we constructed a $\Delta yopN/\Delta yopK$ double mutant and expressed the YopN-HA in this background (Paper II). Interestingly, we found that YopN translocation was indeed upregulated in the absence of YopK (Figure 12, Ekestubbe unpublished), which shows that YopN translocation is regulated in the same way as the other effectors. These findings indicate that YopN is not passively translocated as a step in relieving the secretion block, but rather that YopN is actively translocated. As absence of YopE also results in upregulated Yop translocation it remains to be analyzed if the YopE-dependent feed-back regulation applies to YopN.

While no known function for translocated YopN inside the host cell has been assigned yet, expression of CopN (*Chlamydia*) in yeast cells lead to cell cycle arrest [222]. Further, it was shown that CopN prevented microtubule

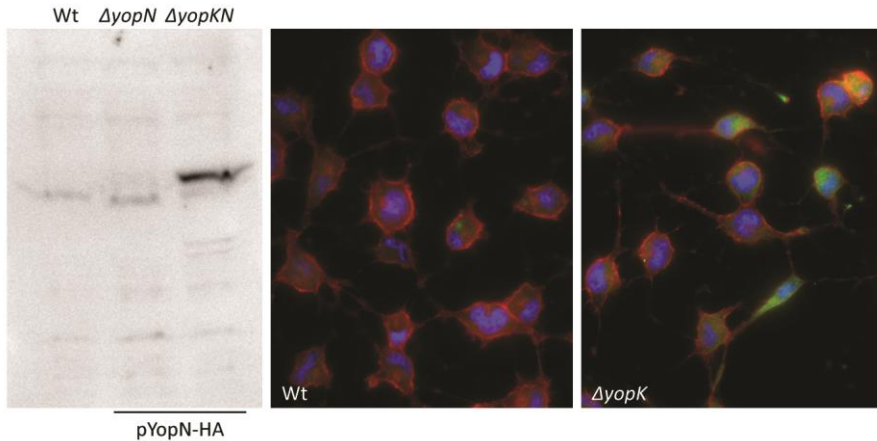


Figure 12. YopN translocation is regulated by YopK. HeLa cells were infected with the indicated strains for 2 hours. For Western blot analysis (left), the cells were fractionated and the cytosolic proteins were precipitated. For immunostaining (right) the cells were fixed and membranes were stained with Texas Red-conjugated WGA (red) and YopN-HA was stained using α -HA antibody followed by Alexa-488-conjugated secondary antibody (green). Nuclei were stained with DAPI (blue).

assembly by sequestering tubulin [223]. Intracellular expression of YopN did not cause cell cycle arrest [222]. However, considering the very different lifestyles of *Yersinia*, an extracellular pathogen, and *Chlamydia*, an intracellular pathogen, it would not be very surprising if YopN has a different eukaryotic target than CopN.

To our knowledge, translocation of gate-keepers from other T3SS subfamilies, has not been shown, and considering the low level of homology between the gate-keepers, translocation is not necessarily a common trait to all of them. In contrast to YopN, InvE (*Salmonella*) and SepL (*E. coli*) remain in the bacterial cytosol also after secretion has been induced [244], [245], which argues against translocation of these proteins into host cells. It has been shown though, that like YopN, MxiC (*Shigella*) and PopN (*P. aeruginosa*) are secreted during inducing conditions [243], [246], so it is possible that they are also translocated. Future studies will have to reveal if

translocation of gate-keepers is a common trait and, if so, what their intracellular targets and functions are.

3.3 No evidence for a sorting platform in *Yersinia*

In *Salmonella*, *E. coli* and *Shigella* there is evidence for a secretion hierarchy where translocators are secreted before the effectors, which is regulated by the gate-keeper [84], [85], [243], [247].

In Paper II, we show that *Yersinia* lacks this YopN-dependent secretion hierarchy and this suggests that the C-ring complex in *Yersinia* does not act as a sorting platform. Deletion of *yopN* results in constitutive expression and secretion of both translocators and effectors (Paper II, Figure 2). Our results highlight that gate-keepers regulate secretion differently in the different subfamilies. In *Yersinia* both effectors and translocators are surface localized before host cell contact, which also argues against the presence of a sorting platform or secretion hierarchy in *Yersinia*. The question is why some T3SS subfamilies employ a sorting platform while others do not. This is likely an adaptation to fit the particular life style niche of the pathogen.

Yersinia replicates in lymphoid tissues and must continuously battle professional phagocytes. To prevent uptake by phagocytes, translocation must occur immediately. In that sense it may be advantageous for *Yersinia* to keep both effectors and translocators surface localized prior to host cell contact. In support of this argument, it was shown by Akopyan and Edgren et al., that surface localized YopH was translocated, and translocation was immediate and efficiently blocked the intracellular calcium signaling that precedes phagocytosis. Further, they showed that YopH coated on the surface of *S. typhimurium* was translocated as well [29], however it has not been shown if *Salmonella* naturally positions its effectors on the surface. It is possible that the one-step translocation is the main mode of translocation in *Salmonella*, while *Yersinia* employ both one-step- and two-step translocation to ensure quick translocation of effectors to block uptake.

In vitro studies of *Salmonella* showed that this pathogen translocated the effector SipA quickly after host cell contact [248], however it is not known if this is as crucial *in vivo* as it is for *Yersinia* [55].

3.4 Characterization of the central region in YopN

It is fairly common that T3SS substrates have more than one function, e.g. YopD and LcrV are required for regulation of the T3SS and for translocation of effectors. The indication that YopN is actively translocated into host cells support the notion that also YopN may be a multifunctional protein. In absence of YopN both expression and secretion are highly upregulated and this phenotype could mask any other possible function of YopN. In Paper II we therefore aimed to construct YopN mutants that retained full regulatory control.

The interaction between YopN and its cognate chaperone complex and TyeA has been characterized previously [88], [158], [159], [217], yet no known function had been ascribed to the central part of YopN. We decided to target this region of YopN with the aim to construct mutants that were unaffected in regulation of Yop expression and secretion, to gain further insights into the function of this part of YopN.

Based on domain predictions of YopN, we constructed three in-frame deletions generating YopN $_{\Delta 131-167}$ HA, YopN $_{\Delta 170-207}$ HA and YopN $_{\Delta 76-181}$ HA, all containing a C-terminal HA-tag (Paper II, Figure S1). The constructs were cloned under an arabinose inducible promoter and expressed in *trans* in the non-polar $\Delta yopN$ mutant.

Trans-complementation of the $\Delta yopN$ mutant with the three YopN variants showed a variation in the regulation phenotype. The YopN $_{\Delta 170-207}$ HA variant could partially rescue the $\Delta yopN$ phenotype, as complementation with this variant reduced, yet did not completely block, the expression and secretion of Yops in non-inducing conditions. Expression of YopN $_{\Delta 131-167}$ HA resulted in a slightly deregulated phenotype, where low levels of Yops were expressed and secreted also during growth in non-inducing conditions. Interestingly, the largest deletion YopN $_{\Delta 76-181}$ HA rescued the $\Delta yopN$ phenotype, and brought back the regulation of expression and secretion to wild type strain levels (Paper II, Figure 2-4).

It is interesting that the largest deletion (aa 76-181) retained full regulatory control of secretion. Considering that the smaller deletion (aa 131-

167) falls within the large deletion, one would expect that this part is redundant for the regulation. However, this was apparently not the case, since the YopN $_{\Delta 131-167}$ HA variant did not bring back full regulatory control.

Our results indicate that aa 182-207 are important for the regulatory function of YopN, and this agrees with the study performed by Ferracci et al., where several point mutations that downregulated secretion, localized to this region [156]. It is not known what this part of YopN does, however one can speculate that the deregulated phenotype of YopN $_{\Delta 170-207}$ HA results from failure to interact with TyeA or YscI. Studies in *Shigella* showed that the interaction between MxiC (YopN) and MxiI (YscI) was an important step in the secretion regulation. Point mutation in *mxiC* identified residue 206 (corresponding to residue 223 in YopN) as important for the interaction [161]. The interaction site of YscI has not been mapped in YopN and it is possible that YscI interact upstream of TyeA which would position it in the proximity of the deleted region in YopN $_{\Delta 170-207}$ HA. Studying the interactions between the YopN variants and TyeA and YscI could shed light on the different regulatory phenotypes of the YopN variants.

3.5 YopN has a role in translocation of effectors

The gate-keepers have been implicated in translocation of effectors in *Yersinia*, *Salmonella* and *E.coli*. The studies of *Yersinia yopN* and *tyeA* mutants have provided conflicting results as to the requirement of the gate-keeper for translocation [9], [88], [89], [221]. Results from *Salmonella* and *E. coli* showed that deletion of *invE* or *sepL*, respectively, blocked translocation. However, considering that deletion of *invE* and *sepL* abrogated secretion of the translocators in *Salmonella* and *E. coli*, the translocation defect might very well be an indirect effect caused by the lack of translocator secretion [244], [245].

Rosqvist et al. reported that a $\Delta yopN$ mutant caused delayed translocation of YopE [9], however, when we infected HeLa cells with the new non-polar $\Delta yopN$ mutant we saw a quick cytotoxic response, similarly to that caused by the wild type strain. Interestingly, quantification of translocated YopH showed that the $\Delta yopN$ mutant actually translocated somewhat higher levels

of YopH compared to the wild type strain. Complementation with YopN-HA brought the translocation back to wild type strain levels (Paper II, Figure 5).

When we complemented the $\Delta yopN$ mutant with the YopN $_{\Delta 76-181}$ HA variant we found that the translocation was significantly downregulated. This is particularly interesting since this YopN variant retained full regulatory control. Quantification of translocation levels was done by introducing a YopH-beta-lactamase fusion protein (YopH-Bla), and we could verify that deletion of this part of YopN resulted in delayed translocation. After 30 minutes of infection the amount of YopH in cells infected with the YopN $_{\Delta 76-181}$ HA strain was 65% lower than that in cells infected with the wild type strain (Paper II, Figure 5). However, after 60 minutes the translocation levels were similar between the YopN $_{\Delta 76-181}$ HA and the wild type strain. Considering that the YopN $_{\Delta 76-181}$ HA mutant displayed normal secretion of the translocators, our results indicated a novel and specific role of YopN during translocation. To reveal a possible delay in secretion of translocators we performed a kinetic assay and found that there was no difference in secretion kinetics between the YopN $_{\Delta 76-181}$ HA mutant and the wild type strain, showing that indeed YopN is directly involved in translocation. The fact that prolonged infection allowed the YopN $_{\Delta 76-181}$ HA mutant to catch up to the wild type strain indicates that the requirement of YopN is in the early stages of translocation.

The YopN $_{\Delta 131-167}$ HA mutant translocated YopH at similar levels as the wild type strain, while the YopN $_{\Delta 170-207}$ HA translocated at similar levels as the $\Delta yopN$ mutant. This is interesting because it shows an indirect role of YopN in translocation as well. If YopN is deleted or dysfunctional, translocation is upregulated which suggests that YopN may be part of the feed-back regulation.

The significance of the translocation defect was further strengthened when the YopN $_{\Delta 76-181}$ HA strain was analyzed for phagocytosis inhibition. All and all, the ability to block phagocytosis reflected the translocation ability of all the YopN variants. Strikingly, while the wild type strain efficiently blocked phagocytosis (20% uptake) the YopN $_{\Delta 76-181}$ HA was almost as inefficient as the $\Delta lcrV$ mutant, with an uptake of 75% and 95% respectively

(Paper II, Figure 6). It should be noted here that the $\Delta lcrV$ mutant suffers a complete downregulation in the T3SS, while the YopN $_{\Delta 170-207}$ HA mutant, in that sense, only suffers from delayed translocation. This stresses the importance of efficient, early translocation, to survive the infection.

3.6 Using red blood cells as a model for T3SS pore forming activity

For translocation to work, all three translocators must be secreted. Further, YopB and YopD need to be inserted into the host cell membrane, where they are proposed to form a pore through which the effectors are translocated.

For the most part membrane insertion of YopB and YopD and pore formation have been studied in RBCs, and in past studies, there has been a strong correlation between ability to form pores in RBCs and ability to translocate [99], [102], [112], [171].

The use of RBCs as the model for studies of T3SS induced pore formation and insertion of translocators in plasma membranes is widely spread. A lot of knowledge also comes from using these methods in terms of T3SS function. However the research field has progressed to a point where the use of RBCs needs to be questioned. We are no longer dealing with knock-out mutants with a black or white phenotype, instead we are down to the tortuous point mutations' impact on structure and function of the protein. When studying T3SS at this high resolution the methods needs to be adjusted to illustrate a more *in vivo*-like situation.

In Paper III we saw a striking phenotype of two LcrV mutants (section 3.7), which were able to translocate effectors without forming pores in RBCs. This initially sidetracked us to investigate a possible mechanism of how translocation occurred without membrane localization of YopB and YopD. When we then analyzed membrane insertion in HeLa cells, which is the cell system we use for our translocation studies, we found that YopD was indeed inserted into the membrane. YopB could not be detected in our assay, and although we cannot say for certain that YopB is inserted, we find it most

likely that YopB along with YopD localizes in the membrane during infection with the LcrV mutants, just as they are during infection with the isogenic wild type strain.

The discrepancy between RBCs and HeLa cells instead highlights that pore formation in RBCs can be an artefact and not a true reflection of translocation ability, and this type of inconsistencies has actually been shown before. For example, *Yersinia* YopD mutants with strongly reduced hemolytic activity, which still provoked a strong cytotoxic response in HeLa cells, have been identified [241]. Also *Shigella* IpaB mutants have been identified, that were invasive yet unable to insert translocators in RBC membranes. Similar to our observation, the translocators could be recovered from HeLa cell membranes [104].

Ideally, one would analyze all aspects of T3SS in the same cell-based system, however, due to various reasons this is not accomplishable at all times. It should be prioritized when dealing with phenotypes in the gray-zone, such as the LcrV mutants, where there is not an all-or-nothing response.

3.7 LcrV N-terminal mutants as a tool to study translocation

In contrast to the novel finding that YopN is involved in translocation, it is well established that the needle tip proteins (LcrV) are required for translocation, possible by forming a platform for insertion of the hydrophobic translocators [101]. Like YopN, LcrV is also involved in regulation, so in-depth functional analysis of LcrV requires mutants that separate one function from another. On account of the central- and C-terminal α -helices that form the coiled-coil, LcrV is very sensitive to genetic manipulations. This makes it challenging to construct mutants that affect translocation alone without affecting regulation.

It was previously shown that frame shifting the extreme N-terminal of LcrV did not affect Yop expression or secretion. The resulting LcrV+1 and LcrV-1 mutants also induced cytotoxicity in HeLa cells [102]. When we

analyzed these LcrV^{+/-1} mutants using the RBC-model, we were quite surprised to find that they were completely devoid of hemolytic activity (section 3.6). In Paper III we investigated the translocation ability of these mutants in more detail.

3.8 LcrV is involved in intracellular targeting of YopH

Efficient translocation is central in preventing uptake by phagocytes and, by extension, it is central to survival of *Yersinia* in a host. We knew from our earlier study that the LcrV⁺¹ and LcrV⁻¹ strains induced cytotoxicity in HeLa cells [102], which is an indirect measure of translocation ability. When we analyzed the intracellular levels of YopE and YopH we found that the LcrV mutants translocated the effectors at levels similar to the wild type strain (Paper III, Fig. 3 and 4). However, despite that the effectors were successfully translocated, the LcrV mutants were impaired in their ability to block phagocytosis. Roughly 45% of the LcrV mutants were phagocytosed in comparison to 20% of the wild type strain (Paper III, Fig. 6).

Phagocytosis is a rapid process and to block it *Yersinia* must translocate effectors immediately at cell contact. The fact that the LcrV^{+/-1} mutants were unable to fully block phagocytosis suggested that they were affected in the very early events of translocation. Unlike the YopN76-181HA mutant, where reduced translocation was displayed after 30 minutes of infection, kinetics experiment with the LcrV^{+/-1} mutants could not detect any differences from the wild type strain (discussed further in section 3.9).

Due to the powerful enzymatic activity of YopH, translocation can be monitored during the first minutes of infection. Fällman and co-workers identified intracellular targets of YopH in both HeLa cells and macrophages. Further, they showed that YopH was translocated and reached these targets within a minute after cell contact [136], [137], [200]. We therefore decided to pursue YopH targeting, to analyze early translocation in the LcrV^{+/-1} mutants.

The interaction between *Yersinia* and the host cell, through invasin and β 1-integrins, initiates rapid phosphorylation of several intracellular proteins. YopH targets several of these and quickly dephosphorylate them [136], [137],

[200], [202]. By measuring YopH dependent dephosphorylation of one of these targets, FAK, we got an indirect measure of the targeting efficiency. Using this approach we found that the LcrV+/-1 mutants inefficiently targeted YopH to FAK. Interestingly, the phosphorylation level of FAK was intermediary in cells infected with the LcrV+/-1 mutants, compared to cells infected with the wild type strain and a strain expressing inactivated YopH (Paper III Fig. 7). This argues that the LcrV+/-1 mutants' impaired ability to block phagocytosis is a reflection of their inefficient intracellular targeting of YopH.

Given that the LcrV+/-1 mutants failed to fully block phagocytosis, one could expect them to be affected in *in vivo* virulence as well. Strikingly, we found that the LcrV+/-1 mutants were severely attenuated for virulence almost to the point of the $\Delta lcrV$ mutant. Mice survived intraperitoneal challenge with a high dose (10^5 CFUs) of LcrV+1 or LcrV-1, while mice infected with the wild type strain succumbed to the infection within seven days (Paper III Fig. 8). This is in line with a previous study, showing that targeting of YopH to the FAKs is required for *in vivo* virulence [137]. Using the *in vivo* imaging system (IVIS) we found that when mice were infected orally the LcrV+/-1 mutants colonized PP and in a few cases they reached the MLNs, where they were eventually cleared (Ekestubbe, unpublished data). These results also highlight the requirement of efficient targeting of YopH, a $\Delta yopH$ mutant fails to disseminate from the PP during *in vivo* infection, while a $\Delta yopE$ or a $\Delta yopK$ mutant reaches the MLN where they are eventually cleared [204], [205], [249].

3.9 Timing and targeting of effector translocation

It is well established that LcrV is required for translocation, and it was initially thought that the requirement of LcrV was to promote pore formation. During the last decade, compiling evidence suggest that the tip protein and translocon have a more dynamic role during translocation. Numerous studies suggest that the tip protein and translocon senses host cell contact. Through structural rearrangement they can transmit signals,

through the needle, between the cell and bacteria to initiate, regulate and terminate translocation [77]–[79], [103], [250], [251].

In Paper III we show that LcrV is involved in the early intracellular targeting of effectors, which is in line with a dynamic tip and translocon. In addition we show that YopN is involved in the regulation of translocation, which has not been shown before. Genetic manipulations to LcrV or YopN resulted, in both cases, in a translocation defect that consequently affected *Yersinia*'s ability to block phagocytosis (Paper II, Figure 6 and Paper III, Fig. 6). The question is at what level YopN and LcrV affect translocation. Is it timing or targeting?

Our data suggests that YopN ensure the timing of translocation. The YopN $_{\Delta 76-181}$ HA strain translocated low amounts of YopH-Bla, compared to the wild type strain, after 30 minutes infection. However, after 60 minutes infection the YopN $_{\Delta 76-181}$ HA had caught up with the wild type strain, and there was no difference in the amounts of intracellular YopH (Bamyaci & Ekestubbe unpublished). This suggests that even though the YopN $_{\Delta 76-181}$ HA strain secrete the effectors and translocators with similar kinetics as the wild type strain, there is a glitch during the translocation step that results in delayed translocation (Figure 13). The delayed translocation seem to be general as YopE dependent cytotoxicity was also delayed in this strain, and quantification of translocated YopE-Bla showed the same results a for YopH-Bla (Bamyaci & Ekestubbe unpublished).

Regarding the LcrV $_{+/-1}$ mutants, they cause a delayed effect of YopH, however, it is difficult to deduce if they affect timing or targeting. That is, is the immediate translocation of YopH delayed, or is YopH, once translocated, not targeted efficiently to the FACs? Our attempts to analyze translocation kinetics, by using the cytotoxicity assay, could not reveal any translocation defect in LcrV $_{+/-1}$ mutants (Paper III). In a further attempt to analyze early translocation levels, we took advantage of the LcrV $_{+/-1}$ mutants expressing YopH_{FL}-Bla and quantified translocation levels in the Tecan plate reader (as described in Paper II). Using this approach we were able to detect translocation as early as after ten minutes of infection and we found that the LcrV $_{+/-1}$ mutants translocated YopH-Bla at the same levels as the wild type

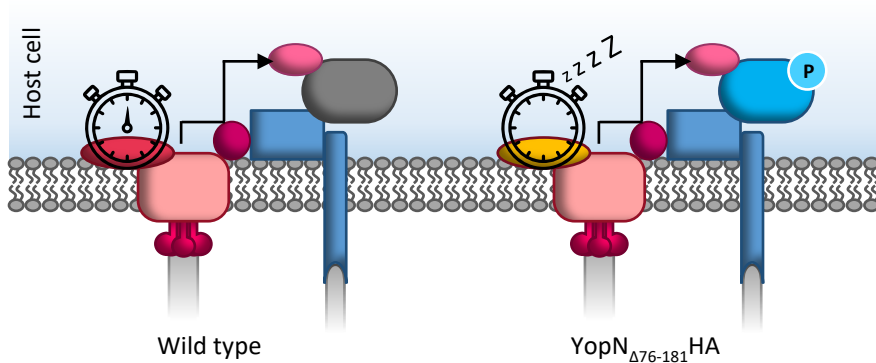


Figure 13. Hypothetical model of YopH targeting in the *YopN*_{Δ76-181}HA strain. Translocation of YopH was significantly lower in cells infected with the *YopN*_{Δ76-181}HA strain compared to the wildtype after 30 minutes infection, however after 60 minutes infection there was no difference in translocation levels, suggesting that translocation is delayed in the *YopN*_{Δ76-181}HA strain.

strain (Figure 14 A, Ekestubbe unpublished). As we have shown that YopH targeting to FAK was reduced in the *LcrV*^{+/-} mutants, compared to the wild type strain, after ten minutes infection, this indicates that *LcrV* is involved in intracellular targeting and not timing (Figure 14 C).

The intracellular targeting of YopH involves YopK. YopK interacts with both YopD and the eukaryotic protein RACK1, and by extension, may therefore be a link between the translocon and the FAC, which guides YopH to the correct place after translocation. In that context a *ΔyopK* mutant fails to target YopH efficiently to the FAC, however in the phagocytosis inhibition assay this is compensated by the massive amount of YopH that is translocated [171]. Interestingly, when we analyzed our *ΔyopK/LcrV*^{+/-} double mutants for phagocytosis inhibition we found that they, like the *LcrV*^{+/-} mutants, suffered impaired ability to block phagocytosis (Figure 14 B, Ekestubbe unpublished). This suggests that the hypertranslocation phenotype cannot compensate the loss of YopK when the *LcrV*^{+/-} variants are expressed instead of wild type strain *LcrV*. These results indicate that *LcrV* manage the intracellular targeting of effectors in cooperation with YopK (Figure 14 C).

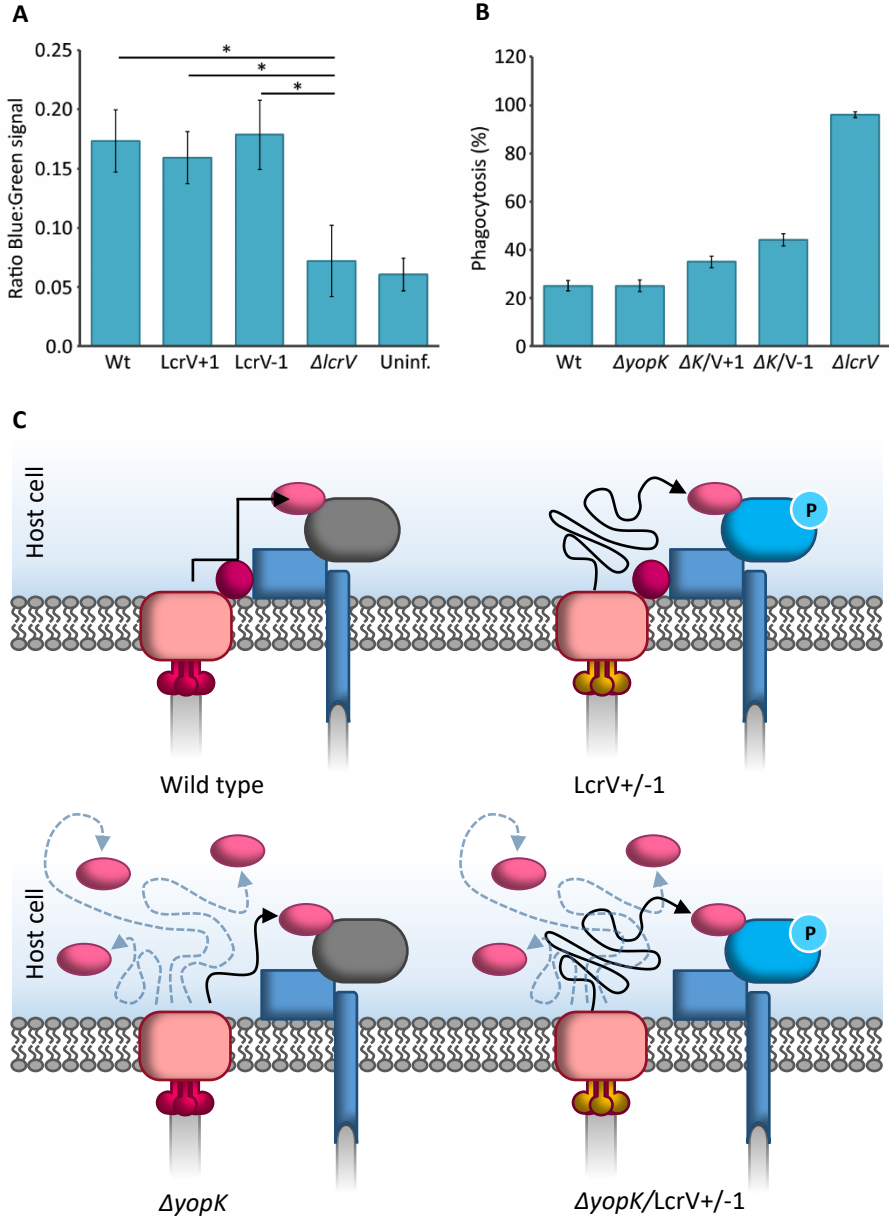


Figure 14. YopH targeting in the LcrV+/-1 mutants. (A) Translocation of YopH_{FL}-Bla into HeLa cells after 10 minutes infection. **(B)** Phagocytosis inhibition of $\Delta yopK/LcrV+/-1$ strains. **(C)** Hypothetical model of YopH targeting. The wild type translocates and targets YopH directly to its eukaryotic target. The LcrV+/-1 mutants seem to translocate YopH as quickly as the wild type but fail to target YopH correctly. The $\Delta yopK$ mutant cannot target YopH, however this defect is compensated by the massive translocation in this mutant. In the $\Delta yopK/LcrV+/-1$ mutants, the hyper-translocating phenotype does not seem to compensate the mistargeting of YopH.

- YopD interacts solely with an LcrV-LcrG complex, which suggest that these proteins interact in the bacterial cytoplasm and that the interaction between them may be important for regulation of the T₃SS.
- Similar to Yop effectors, YopN translocation is also regulated by YopK.
- Deletion of aa 76-181 of YopN generated a YopN variant which retained the regulatory phenotype of the wild type strain.
- YopN is directly involved in translocation of effectors into host cells.
- Impaired early translocation of YopH, seen in the YopN Δ ₇₆₋₁₈₁HA mutant and the LcrV $^{+/-1}$ mutants resulted in significantly reduced ability to block phagocytosis.
- Methods to study the early events of translocation are important to detect subtle phenotypes that may be important for *in vivo* virulence. Further, methods to study pore formation and translocator insertion in host cell membranes is best performed in nucleated cells, as opposed to RBCs.
- Frame-shift mutations in the extreme N-terminal of LcrV did not affect the regulatory role of LcrV.
- LcrV is required for early efficient targeting of YopH to the intracellular target FAK.
- Slight defects in the early intracellular targeting of YopH, by the LcrV $^{+1/-1}$ mutants, had a major impact on the *in vivo* virulence of *Yersinia*.

To deduce how YopN is implicated in translocation, it would be very interesting to investigate the membrane insertion of YopB and YopD in host cell membranes in the YopN $_{\Delta 76-181}$ HA mutant. Further it remains to be elucidated if YopN regulates translocation from within the host cell or not. It is possible that YopN is translocated and localizes to the translocon, somehow gating the translocation from this position. To elucidate this it should first of all be investigated if YopN $_{\Delta 76-181}$ HA is also translocated. Further, interaction studies between the translocators and YopN or YopN $_{\Delta 76-181}$ HA could give valuable information of how YopN affect translocation.

Another approach would be to combine the YopN $_{\Delta 76-181}$ mutation with $\Delta yopK$ and $\Delta yopE$ mutations. Since both $\Delta yopK$ and $\Delta yopE$ mutants hyper-translocate effectors and the YopN $_{\Delta 76-181}$ HA seem to delay translocation, the combination between them can give meaningful information on if/how these protein interact during translocation.

The fact that YopN is translocated, in what seems to be a regulated manner, indicates that YopN has a function in the host cell cytoplasm and it would be most interesting to do a screening against eukaryotic proteins to investigate if YopN has a eukaryotic target protein. If that was the case, that would open up for a whole new line of assays to investigate the role of the interaction during translocation and virulence.

The interaction between YopD and the LcrV-LcrG complex raises many questions. First of all, the interaction between them should be verified in *Yersinia*, which can be done using pull-down assays of the individual proteins. It was shown previously that LcrV and YopD co-purified [241], and it should be investigated if LcrG is present in that complex. An LcrG mutant which does not interact with LcrV has been identified earlier [165], and could be incorporated as a control. The next thing to elucidate is if the interaction between LcrV-LcrG-YopD is biologically relevant. Both YopD and LcrG have several other identified interaction partners in the bacterial cytosol, which opens up for an extensive study on how these proteins interact. Delineation

of the genes in combination with pull-down assays and *in vitro* Yop expression and secretion assays can go a long way in answering these questions.

6

ACKNOWLEDGEMENTS

There are so many people that has helped making this dissertation a reality. This section is for YOU!

To my supervisor, **Åke**, I want to thank you for taking me on as a PhD-student and giving me such an interesting project! Your scientific thinking and ability to see the bigger picture has been both inspiring and helpful. Thank you for seeing the potential in me, especially at times when I failed to see it myself. To **Maria Fällman**, I want to thank you for being very positive and supporting during our meetings, for which I am truly grateful! Your input into my project has been very valuable. To **Roland Rosqvist**, who supervised me when I first started in the lab. His enthusiasm for science, regardless of what the results were, has inspired me throughout my whole project.

I had the great joy of sharing the lab and office with some wonderful people. Finaste **Ümmühan**, I consider myself incredibly lucky to have met you. We became instant friends and over the years we have shared so many deep, philosophic discussions, and just as many gibberish conversations in different accents, and countless laughs! You have supported and encouraged me at all times, and especially through the hardest times of my life, without you I think I wouldn't have finished this! Sötaste **Lisa**, you are like a vitamin injection wrapped in sunshine and craziness! You inspire me so much in how you are able to make conversation with anyone at any time, and surprise people with your attack-hugs. Thank you for all the hugs, te och äggmackor, spontaneous dancing in the hallways, laughter and friendship! **Kemal**, you are such a great guy, funny and always very helpful, thank you for the help with the cover and figure. Also, thank you for all the nice trips and the hospitality when I attended yours and **Ümmühans** wedding! **Sarp**, I was so happy when you started in the lab, it felt really nice to have another type3 person in the lab to ventilate ideas and questions with. Your laid-back personality and passion for science has helped during days when I've been not-so-in love with science. I wish you nothing but good-friday-results and good luck with your dissertation! **Roland N**, thank you for help in the lab and interesting discussions that have made me run and search the literature and my data to find out what LcrV really does. **Francesco**, thank you for letting me borrow your pipettes, I wish you good luck with your project! **Tomas**, your enthusiasm for science is inspiring. To both you and **Helen**, thank you for helping me time and again! **Margareta**, thank you for many nice talks and help during my first time as a PhD student! Thank you,

Frederic, for all the help with protein purifications. Also a heartfelt thank you for letting me borrow your flat at a very crucial time-point in my life! **Stefan, Ann-Catrin, Elin**, for always taking the time to help me! **Nelson**, for many interesting discussions about science and life! **Sveta, Christopher, John, Christer**, for all the chitchats during lunch, fika, in the hallways, and during teaching. **Niko, Akbar** and **Salah**, for friendly smiles and small talk whenever we pass each other by. The past and present members of **grpMFä, grpMFr, grpHWW, grpVS, grpAFa** for nice discussions in the journal clubs and Thursday meetings, and a special thank you to **Matthew** for all the help with the project and Paper III, and to **Anna** for taking the time to help me when I needed and for donating J774 cells again and again.

Till mina kursare **Sofie J., Sofie G. & Jenny**, tack för alla trevliga middagar och fikor vi haft.

Till mina vänner utanför Molbi, **Jonny & Marlene** för alla mysiga träffar, middagar och danser. **Kamilla**, för alla träffar som snart blir fler och tätare ☺ Extra tack till både **Kamilla** och **Sofie J.**, för alla trevliga barn-snack!! Tack till alla goa glada dansvänner som jag träffat häruppe, vi ses på danser framöver!

Till **Mamma, Pappa, Jocke, Anita & Evelina, Jonas, Caroline, Emelie & William**, jag vill tacka er för allt stöd och all kärlek som ni ger mig! Jag är så otroligt stolt över att vara en del av vår familj. Mamma och Pappa, ni är min klippa! Ni ställer upp förbehållslöst vad det än gäller och när jag blir stor vill jag bli som er!

Till Rikards familj, **Hans, Kerstin, Erika, Jonas, Pontus, Elias & Lilly**, tack för att ni fått mig att känna mig som en del av er familj från första stund! Men hä ju skovvalottom! I sko lära mig bonschka, i nöges ba djära klart häina avhandlingen ☺

Rikard, min kärlek! Du och **Melvin** är mitt allt! Jag är otroligt glad att det blev vi två. Du har varit en fantastisk vän men en ännu bättre livspartner. Tack för att du har stått ut med mina stressutbrott och överdimensionerade diskussioner om saker som egentligen kanske inte är så viktiga. Du har varit förstående och hänsynsfull men framförallt kärleksfull och utan det stödet hade den här boken inte blivit skriven! Du är underbar, jag älskar dig!

Vår underbara son, **Melvin**, Du är tveklöst det bästa som hänt oss och att se dig växa och lära dig saker är så fascinerande. Längtan att se vad du ska lära dig näst och insikten av att njuta här och nu gör varje dag lite bättre än den som var. Jag älskar dig!

- [1] E. R. Green and J. Mecsas, "Bacterial Secretion Systems: An Overview.," *Microbiol. Spectr.*, vol. 4, no. 1, Feb. 2016.
- [2] J. A. Lycklama A Nijeholt and A. J. M. Driessen, "The bacterial Sec-translocase: structure and mechanism.," *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, vol. 367, no. 1592, pp. 1016–28, Apr. 2012.
- [3] B. C. Berks, "The Twin-Arginine Protein Translocation Pathway," *Annu. Rev. Biochem.*, vol. 84, no. 1, pp. 843–864, Jun. 2015.
- [4] T. R. D. Costa, C. Felisberto-Rodrigues, A. Meir, M. S. Prevost, A. Redzej, M. Trokter, and G. Waksman, "Secretion systems in Gram-negative bacteria: structural and mechanistic insights," *Nat. Rev. Microbiol.*, vol. 13, no. 6, pp. 343–359, May 2015.
- [5] M. H. H. Lenders, S. Reimann, S. H. J. Smits, and L. Schmitt, "Molecular insights into type I secretion systems," *Biol. Chem.*, vol. 394, no. 11, pp. 1371–1384, Jan. 2013.
- [6] K. V Korotkov, M. Sandkvist, and W. G. J. Hol, "The type II secretion system: biogenesis, molecular architecture and mechanism.," *Nat. Rev. Microbiol.*, vol. 10, no. 5, pp. 336–51, Apr. 2012.
- [7] I. Gawarzewski, S. H. J. Smits, L. Schmitt, and J. Jose, "Structural comparison of the transport units of type V secretion systems," *Biol. Chem.*, vol. 394, no. 11, pp. 1385–1398, Jan. 2013.
- [8] A. Diepold and J. P. Armitage, "Type III secretion systems: the bacterial flagellum and the injectisome," *Philos. Trans. R. Soc. London B Biol. Sci.*, vol. 370, no. 1679, 2015.
- [9] R. Rosqvist, K. E. Magnusson, and H. Wolf-Watz, "Target cell contact triggers expression and polarized transfer of Yersinia YopE cytotoxin into mammalian cells.," *EMBO J.*, vol. 13, no. 4, pp. 964–72, Feb. 1994.
- [10] C. E. Alvarez-Martinez and P. J. Christie, "Biological diversity of prokaryotic type IV secretion systems.," *Microbiol. Mol. Biol. Rev.*, vol. 73, no. 4, pp. 775–808, Dec. 2009.
- [11] H. H. Low, F. Gubellini, A. Rivera-Calzada, N. Braun, S. Connery, A. Dujancourt, F. Lu, A. Redzej, R. Fronzes, E. V Orlova, and G. Waksman, "Structure of a type IV secretion system.," *Nature*, vol. 508, no. 7497, pp. 550–3, Apr. 2014.
- [12] M. Trokter, C. Felisberto-Rodrigues, P. J. Christie, and G. Waksman, "Recent advances in the structural and molecular biology of type IV secretion systems.," *Curr. Opin. Struct. Biol.*, vol. 27, pp. 16–23, Aug. 2014.
- [13] F. R. Cianfanelli, L. Monlezun, and S. J. Coulthurst, "Aim, Load, Fire: The Type VI Secretion System, a Bacterial Nanoweapon," *Trends Microbiol.*, vol. 24, no. 1, pp. 51–62, 2016.
- [14] A. B. Russell, S. B. Peterson, and J. D. Mougous, "Type VI secretion system effectors: poisons with a purpose.," *Nat. Rev. Microbiol.*, vol. 12, no. 2, pp. 137–48, Feb. 2014.
- [15] D. Büttner, "Protein export according to schedule: architecture, assembly, and regulation of type III secretion systems from plant- and animal-pathogenic bacteria.," *Microbiol. Mol. Biol. Rev.*, vol. 76, no. 2, pp. 262–310, Jun. 2012.
- [16] R. Rosqvist, a Forsberg, and H. Wolf-Watz, "Intracellular targeting of the Yersinia YopE cytotoxin in mammalian cells induces actin microfilament disruption.," *Infect. Immun.*, vol. 59, no. 12, pp. 4562–9, Dec. 1991.
- [17] G. P. Salmond and P. J. Reeves, "Membrane traffic wardens and protein

- secretion in gram-negative bacteria.," *Trends Biochem. Sci.*, vol. 18, no. 1, pp. 7–12, Jan. 1993.
- [18] T. Kubori, "Supramolecular Structure of the Salmonella typhimurium Type III Protein Secretion System," *Science (80-.)*, vol. 280, no. 5363, pp. 602–605, Apr. 1998.
- [19] M. Kudryashev, A. Diepold, M. Amstutz, J. P. Armitage, H. Stahlberg, and G. R. Cornelis, "Yersinia enterocolitica type III secretion injectisomes form regularly spaced clusters, which incorporate new machines upon activation," *Mol. Microbiol.*, vol. 95, no. 5, pp. 875–884, Mar. 2015.
- [20] R. M. Macnab, "The bacterial flagellum: reversible rotary propellor and type III export apparatus.," *J. Bacteriol.*, vol. 181, no. 23, pp. 7149–53, Dec. 1999.
- [21] J. E. Galán and A. Collmer, "Type III Secretion Machines: Bacterial Devices for Protein Delivery into Host Cells," *Science (80-.)*, vol. 284, no. 5418, 1999.
- [22] U. Gophna, E. Z. Ron, and D. Graur, "Bacterial type III secretion systems are ancient and evolved by multiple horizontal-transfer events," *Gene*, vol. 312, pp. 151–163, 2003.
- [23] L. Nguyen, I. T. Paulsen, J. Tchieu, C. J. Hueck, and M. H. Saier, "Phylogenetic analyses of the constituents of Type III protein secretion systems.," *J. Mol. Microbiol. Biotechnol.*, vol. 2, no. 2, pp. 125–44, Apr. 2000.
- [24] M. H. Saier, "Evolution of bacterial type III protein secretion systems," *Trends Microbiol.*, vol. 12, no. 3, pp. 113–115, 2004.
- [25] S. S. Abby and E. P. C. Rocha, "The Non-Flagellar Type III Secretion System Evolved from the Bacterial Flagellum and Diversified into Host-Cell Adapted Systems," *PLoS Genet.*, vol. 8, no. 9, p. e1002983, Sep. 2012.
- [26] C. WINSTANLEY and C. A. HART, "Type III secretion systems and pathogenicity islands," *J. Med. Microbiol.*, vol. 50, no. 2, pp. 116–126, Feb. 2001.
- [27] R. Rosqvist, S. Håkansson, A. Forsberg, and H. Wolf-Watz, "Functional conservation of the secretion and translocation machinery for virulence proteins of yersiniae, salmonellae and shigellae.," *EMBO J.*, vol. 14, no. 17, pp. 4187–95, Sep. 1995.
- [28] E. Frithz-Lindsten, A. Holmstrom, L. Jacobsson, M. Soltani, J. Olsson, R. Rosqvist, and A. Forsberg, "Functional conservation of the effector protein translocators PopB/YopB and PopD/YopD of Pseudomonas aeruginosa and Yersinia pseudotuberculosis," *Mol. Microbiol.*, vol. 29, no. 5, pp. 1155–1165, Sep. 1998.
- [29] K. Akopyan, T. Edgren, H. Wang-Edgren, R. Rosqvist, A. Fahlgren, H. Wolf-Watz, and M. Fallman, "Translocation of surface-localized effectors in type III secretion.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 108, no. 4, pp. 1639–44, Jan. 2011.
- [30] J. Stavrinides, W. Ma, and D. S. Guttman, "Terminal Reassortment Drives the Quantum Evolution of Type III Effectors in Bacterial Pathogens," *PLoS Pathog.*, vol. 2, no. 10, p. e104, 2006.
- [31] J. E. Galán, "Common Themes in the Design and Function of Bacterial Effectors," *Cell Host Microbe*, vol. 5, no. 6, pp. 571–579, 2009.
- [32] J. E. Galán and H. Wolf-Watz, "Protein delivery into eukaryotic cells by type III secretion machines.," *Nature*, vol. 444, no. 7119, pp. 567–73, Nov. 2006.
- [33] J. Enninga and I. Rosenshine, "Imaging the assembly, structure and activity of type III secretion systems," *Cell. Microbiol.*, vol. 11, no. 10, pp. 1462–1470, Oct. 2009.
- [34] J. E. Deane, P. Abrusci, S. Johnson, and S. M. Lea, "Timing is everything: the regulation of type III secretion," *Cell. Mol. Life Sci.*, vol. 67, no. 7, pp. 1065–

- 1075, Apr. 2010.
- [35] A. Diepold and S. Wagner, "Assembly of the bacterial type III secretion machinery.," *FEMS Microbiol. Rev.*, vol. 38, no. 4, pp. 802–822, Jul. 2014.
- [36] B. Hu, D. R. Morado, W. Margolin, J. R. Rohde, O. Arizmendi, W. L. Picking, W. D. Picking, and J. Liu, "Visualization of the type III secretion sorting platform of *Shigella flexneri*.,," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 112, no. 4, pp. 1047–52, Jan. 2015.
- [37] A. Nans, M. Kudryashev, H. R. Saibil, and R. D. Hayward, "Structure of a bacterial type III secretion system in contact with a host membrane in situ.," *Nat. Commun.*, vol. 6, p. 10114, 2015.
- [38] Y. Akeda and J. E. Galán, "Chaperone release and unfolding of substrates in type III secretion," *Nature*, vol. 437, no. 7060, pp. 911–915, Oct. 2005.
- [39] T. Minamino and K. Namba, "Distinct roles of the FlII ATPase and proton motive force in bacterial flagellar protein export," *Nature*, vol. 451, no. 7177, pp. 485–488, Jan. 2008.
- [40] P.-C. Lee and A. Rietsch, "Fueling type III secretion.," *Trends Microbiol.*, vol. 23, no. 5, pp. 296–300, May 2015.
- [41] A. Diepold, M. Amstutz, S. Abel, I. Sorg, U. Jenal, and G. R. Cornelis, "Deciphering the assembly of the *Yersinia* type III secretion injectisome.," *EMBO J.*, vol. 29, no. 11, pp. 1928–40, Jun. 2010.
- [42] A. Diepold, U. Wiesand, and G. R. Cornelis, "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.*, vol. 82, no. 2, pp. 502–14, Oct. 2011.
- [43] A. Diepold, U. Wiesand, M. Amstutz, and G. R. Cornelis, "Assembly of the *Yersinia* injectisome: the missing pieces.," *Mol. Microbiol.*, vol. 85, no. 5, pp. 878–92, Sep. 2012.
- [44] T. Kubori, A. Sukhan, S. I. Aizawa, and J. E. Galán, "Molecular characterization and assembly of the needle complex of the *Salmonella typhimurium* type III protein secretion system.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 97, no. 18, pp. 10225–30, Aug. 2000.
- [45] K. Tamano, E. Katayama, T. Toyotome, and C. Sasakawa, "Shigella Spa32 is an essential secretory protein for functional type III secretion machinery and uniformity of its needle length.," *J. Bacteriol.*, vol. 184, no. 5, pp. 1244–52, Mar. 2002.
- [46] L. Journet, C. Agrain, P. Broz, and G. R. Cornelis, "The Needle Length of Bacterial Injectisomes Is Determined by a Molecular Ruler," *Science (80-.)*, vol. 302, no. 5651, 2003.
- [47] C. a Mueller, P. Broz, S. a Müller, P. Ringler, F. Erne-Brand, I. Sorg, M. Kuhn, A. Engel, and G. R. Cornelis, "The V-antigen of *Yersinia* forms a distinct structure at the tip of injectisome needles.," *Science*, vol. 310, no. 5748, pp. 674–6, Oct. 2005.
- [48] M. Espina, A. J. Olive, R. Kenjale, D. S. Moore, S. F. Ausar, R. W. Kaminski, E. V Oaks, C. R. Middaugh, W. D. Picking, and W. L. Picking, "IpaD localizes to the tip of the type III secretion system needle of *Shigella flexneri*.,," *Infect. Immun.*, vol. 74, no. 8, pp. 4391–400, Aug. 2006.
- [49] C. Marie, W. J. Broughton, and W. J. Deakin, "Rhizobium type III secretion systems: legume charmers or alarmers?," *Curr. Opin. Plant Biol.*, vol. 4, no. 4, pp. 336–342, 2001.
- [50] V. Viprey, A. Del Greco, W. Golinowski, W. J. Broughton, and X. Perret, "Symbiotic implications of type III protein secretion machinery in *Rhizobium*," *Mol. Microbiol.*, vol. 28, no. 6, pp. 1381–1389, Jun. 1998.
- [51] M. Fauvart and J. Michiels, "Rhizobial secreted proteins as determinants of host specificity in the rhizobium–legume symbiosis," *FEMS Microbiol. Lett.*,

- vol. 285, no. 1, 2008.
- [52] E. J. McGhie, L. C. Brawn, P. J. Hume, D. Humphreys, and V. Koronakis, "Salmonella takes control: effector-driven manipulation of the host.," *Curr. Opin. Microbiol.*, vol. 12, no. 1, pp. 117–24, Feb. 2009.
- [53] N. Carayol and G. Tran Van Nhieu, "The inside story of Shigella invasion of intestinal epithelial cells.," *Cold Spring Harb. Perspect. Med.*, vol. 3, no. 10, p. a016717, Oct. 2013.
- [54] M. Galle, I. Carpentier, and R. Beyaert, "Structure and function of the Type III secretion system of *Pseudomonas aeruginosa*.," *Curr. Protein Pept. Sci.*, vol. 13, no. 8, pp. 831–42, Dec. 2012.
- [55] M. Fällman and A. Gustavsson, "Cellular mechanisms of bacterial internalization counteracted by *Yersinia*.," *Int. Rev. Cytol.*, vol. 246, no. 5, pp. 135–88, Jan. 2005.
- [56] L. Schwiesow, H. Lam, P. Dersch, and V. Auerbuch, "Yersinia Type III Secretion System Master Regulator LcrF.," *J. Bacteriol.*, vol. 198, no. 4, pp. 604–14, Feb. 2016.
- [57] R. S. Dewoody, P. M. Merritt, and M. M. Marketon, "Regulation of the Yersinia type III secretion system: traffic control.," *Front. Cell. Infect. Microbiol.*, vol. 3, no. February, p. 4, Jan. 2013.
- [58] J. Yother, T. W. Chamness, and J. D. Goguen, "Temperature-controlled plasmid regulon associated with low calcium response in *Yersinia pestis*.," *J. Bacteriol.*, vol. 165, no. 2, pp. 443–7, Feb. 1986.
- [59] G. Cornelis, C. Sluifers, C. L. de Rouvoit, and T. Michiels, "Homology between virF, the transcriptional activator of the Yersinia virulence regulon, and AraC, the Escherichia coli arabinose operon regulator.," *J. Bacteriol.*, vol. 171, no. 1, pp. 254–62, Jan. 1989.
- [60] G. R. Cornelis, C. Sluifers, I. Delor, D. Geib, K. Kaniga, C. L. Rouvoit, M.-P. Sory, J.-C. Vanooteghem, and T. Michiels, "ymoA, a Yersinia enterocolitica chromosomal gene modulating the expression of virulence functions," *Mol. Microbiol.*, vol. 5, no. 5, pp. 1023–1034, May 1991.
- [61] P. Wattiau and G. R. Cornelis, "Identification of DNA sequences recognized by VirF, the transcriptional activator of the Yersinia yop regulon.," *J. Bacteriol.*, vol. 176, no. 13, pp. 3878–84, Jul. 1994.
- [62] C. L. Rouvoit, C. Sluifers, and G. R. Cornelis, "Role of the transcriptional activator, VirF, and temperature in the expression of the pYV plasmid genes of Yersinia enterocolitica," *Mol. Microbiol.*, vol. 6, no. 3, pp. 395–409, Feb. 1992.
- [63] N. P. Hoe, F. C. Minion, and J. D. Goguen, "Temperature sensing in Yersinia pestis: regulation of yopE transcription by lcrF.," *J. Bacteriol.*, vol. 174, no. 13, pp. 4275–86, Jul. 1992.
- [64] K. Böhme, R. Steinmann, J. Kortmann, S. Seekircher, A. K. Heroven, E. Berger, F. Pisano, T. Thiermann, H. Wolf-Watz, F. Narberhaus, and P. Dersch, "Concerted actions of a thermo-labile regulator and a unique intergenic RNA thermosensor control Yersinia virulence.," *PLoS Pathog.*, vol. 8, no. 2, p. e1002518, Feb. 2012.
- [65] M. W. Jackson, E. Silva-Herzog, and G. V. Plano, "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.*, vol. 54, no. 5, pp. 1364–1378, Oct. 2004.
- [66] A. G. Portalio, K. C. Tsolis, M. S. Loos, V. Zorzini, and A. Economou, "Type III Secretion: Building and Operating a Remarkable Nanomachine," *Trends Biochem. Sci.*, vol. 41, no. 2, pp. 175–189, 2016.
- [67] M. S. Francis, S. A. Lloyd, and H. Wolf-Watz, "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.*, vol. 42, no. 4, pp. 1075–1093, Nov. 2001.
- [68] E. D. Cambronne and O. Schneewind, “*Yersinia enterocolitica* type III secretion: yscM1 and yscM2 regulate yop gene expression by a posttranscriptional mechanism that targets the 5’ untranslated region of yop mRNA,” *J. Bacteriol.*, vol. 184, no. 21, pp. 5880–93, Nov. 2002.
- [69] Y. Chen and D. M. Anderson, “Expression hierarchy in the *Yersinia* type III secretion system established through YopD recognition of RNA,” *Mol. Microbiol.*, vol. 80, no. 4, pp. 966–980, May 2011.
- [70] C. Parsot, E. Ageron, C. Penno, M. Mavris, K. Jamoussi, H. d’Hauteville, P. Sansonetti, and B. Demers, “A secreted anti-activator, OspD1, and its chaperone, Spa15, are involved in the control of transcription by the type III secretion apparatus activity in *Shigella flexneri*,” *Mol. Microbiol.*, vol. 56, no. 6, pp. 1627–1635, 2005.
- [71] R. Ménard, P. Sansonetti, C. Parsot, and T. Vasselon, “Extracellular association and cytoplasmic partitioning of the IpaB and IpaC invasins of *S. flexneri*,” *Cell*, vol. 79, no. 3, pp. 515–525, 1994.
- [72] M. Mavris, A.-L. Page, R. Tournebize, B. Demers, P. Sansonetti, and C. Parsot, “Regulation of transcription by the activity of the *Shigella flexneri* type III secretion apparatus,” *Mol. Microbiol.*, vol. 43, no. 6, pp. 1543–1553, Mar. 2002.
- [73] R. Ménard, P. Sansonetti, and C. Parsot, “The secretion of the *Shigella flexneri* Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaD,” *EMBO J.*, vol. 13, no. 22, pp. 5293–302, Nov. 1994.
- [74] M. K. Zierler and J. E. Galán, “Contact with cultured epithelial cells stimulates secretion of *Salmonella typhimurium* invasion protein InvJ,” *Infect. Immun.*, vol. 63, no. 10, pp. 4024–8, Oct. 1995.
- [75] J. Pettersson, R. Nordfelth, E. Dubinina, T. Bergman, M. Gustafsson, K. E. Magnusson, and H. Wolf-Watz, “Modulation of Virulence Factor Expression by Pathogen Target Cell Contact,” *Science (80-.)*, vol. 273, no. 5279, 1996.
- [76] A. J. Vallis, T. L. Yahr, J. T. Barbieri, and D. W. Frank, “Regulation of ExoS production and secretion by *Pseudomonas aeruginosa* in response to tissue culture conditions,” *Infect. Immun.*, vol. 67, no. 2, pp. 914–20, Feb. 1999.
- [77] A. K. J. Veenendaal, J. L. Hodgkinson, L. Schwarzer, D. Stabat, S. F. Zenk, and A. J. Blocker, “The type III secretion system needle tip complex mediates host cell sensing and translocon insertion,” *Mol. Microbiol.*, vol. 63, no. 6, pp. 1719–1730, Jan. 2007.
- [78] I. Martinez-Argudo and A. J. Blocker, “The *Shigella* T3SS needle transmits a signal for MxiC release, which controls secretion of effectors,” *Mol. Microbiol.*, vol. 78, no. 6, pp. 1365–78, Dec. 2010.
- [79] a D. Roehrich, E. Guillosoy, A. J. Blocker, and I. Martinez-Argudo, “*Shigella* IpaD has a dual role: signal transduction from the type III secretion system needle tip and intracellular secretion regulation,” *Mol. Microbiol.*, vol. 87, no. 3, pp. 690–706, Feb. 2013.
- [80] G. R. Cornelis, a Boland, a P. Boyd, C. Geuijen, M. Iriarte, C. Neyt, M. P. Sory, and I. Stainier, “The virulence plasmid of *Yersinia*, an antihost genome,” *Microbiol. Mol. Biol. Rev.*, vol. 62, no. 4, pp. 1315–52, Dec. 1998.
- [81] G. R. Cornelis, “Molecular and cell biology aspects of plague,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 97, no. 16, pp. 8778–83, Aug. 2000.
- [82] J. Radics, L. Königsmayer, and T. C. Marlovits, “Structure of a pathogenic type 3 secretion system in action,” *Nat. Struct. Mol. Biol.*, vol. 21, no. 1, pp. 82–7, Jan. 2014.
- [83] K. Dohlich, A. B. Zumsteg, C. Goosmann, and M. Kolbe, “A substrate-fusion protein is trapped inside the Type III Secretion System channel in *Shigella*

- flexneri.," *PLoS Pathog.*, vol. 10, no. 1, p. e1003881, Jan. 2014.
- [84] M. Lara-Tejero, J. Kato, S. Wagner, X. Liu, and J. E. Galán, "A sorting platform determines the order of protein secretion in bacterial type III systems.," *Science*, vol. 331, no. 6021, pp. 1188–91, Mar. 2011.
- [85] W. Deng, Y. Li, P. R. Hardwidge, E. A. Frey, R. A. Pfuetzner, S. Lee, S. Gruenheid, N. C. J. Strynadka, J. L. Puente, and B. B. Finlay, "Regulation of type III secretion hierarchy of translocators and effectors in attaching and effacing bacterial pathogens.," *Infect. Immun.*, vol. 73, no. 4, pp. 2135–46, Apr. 2005.
- [86] M. W. Jackson and G. V. Plano, "Interactions between type III secretion apparatus components from *Yersinia pestis* detected using the yeast two-hybrid system.," *FEMS Microbiol. Lett.*, vol. 186, no. 1, 2000.
- [87] A. Forsberg, A. M. Viitanen, M. Skurnik, and H. Wolf-Watz, "The surface-located YopN protein is involved in calcium signal transduction in *Yersinia pseudotuberculosis*.," *Mol. Microbiol.*, vol. 5, no. 4, pp. 977–86, Apr. 1991.
- [88] M. Iriarte, M. P. Sory, A. Boland, A. P. Boyd, S. D. Mills, I. Lambermont, and G. R. Cornelis, "TyeA, a protein involved in control of Yop release and in translocation of *Yersinia* Yop effectors.," *EMBO J.*, vol. 17, no. 7, pp. 1907–18, Apr. 1998.
- [89] L. Sundberg and A. Forsberg, "TyeA of *Yersinia pseudotuberculosis* is involved in regulation of Yop expression and is required for polarized translocation of Yop effectors.," *Cell. Microbiol.*, vol. 5, no. 3, pp. 187–202, Mar. 2003.
- [90] M. Sani, A. Botteaux, C. Parsot, P. Sansonetti, E. J. Boekema, and A. Allaoui, "IpaD is localized at the tip of the *Shigella flexneri* type III secretion apparatus," *Biochim. Biophys. Acta - Gen. Subj.*, vol. 1770, no. 2, pp. 307–311, 2007.
- [91] S. Knutton, I. Rosenshine, M. J. Pallen, I. Nisan, B. C. Neves, C. Bain, C. Wolff, G. Dougan, and G. Frankel, "A novel EspA-associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells.," *EMBO J.*, vol. 17, no. 8, pp. 2166–76, Apr. 1998.
- [92] C. K. Yip, B. B. Finlay, and N. C. J. Strynadka, "Structural characterization of a type III secretion system filament protein in complex with its chaperone," *Nat. Struct. Mol. Biol. Publ. online 26 December 2004*; | [doi:10.1038/nsmb879](https://doi.org/10.1038/nsmb879), vol. 12, no. 1, p. 75, 2004.
- [93] J. Goure, A. Pastor, E. Faudry, J. Chabert, and I. Attree, "The V Antigen of *Pseudomonas aeruginosa* Is Required for Assembly of the Functional PopB / PopD Translocation Pore in Host Cell Membranes," vol. 72, no. 8, pp. 4741–4750, 2004.
- [94] J. Goure, P. Broz, O. Attree, G. R. Cornelis, and I. Attree, "Protective anti-V antibodies inhibit *Pseudomonas* and *Yersinia* translocon assembly within host membranes.," *J. Infect. Dis.*, vol. 192, no. 2, pp. 218–25, Jul. 2005.
- [95] W. L. Picking, H. Nishioka, P. D. Hearn, A. Baxter, A. T. Harrington, A. Blocker, W. D. Picking, and M. A. Baxter, "IpaD of *Shigella flexneri* Is Independently Required for Regulation of Ipa Protein Secretion and Efficient Insertion of IpaB and IpaC into Host Membranes IpaD of *Shigella flexneri* Is Independently Required for Regulation of Ipa Protein Secretion and Effici," 2005.
- [96] P. Broz, C. a Mueller, S. a Müller, A. Philippsen, I. Sorg, A. Engel, and G. R. Cornelis, "Function and molecular architecture of the *Yersinia* injectisome tip complex.," *Mol. Microbiol.*, vol. 65, no. 5, pp. 1311–20, Sep. 2007.
- [97] P.-J. Mattei, E. Faudry, V. Job, T. Izoré, I. Attree, and A. Dessen, "Membrane targeting and pore formation by the type III secretion system translocon.,"

- FEBS J.*, vol. 278, no. 3, pp. 414–26, Feb. 2011.
- [98] J. Pettersson, A. Holmstrom, J. Hill, S. Leary, E. Frithz-Lindsten, A. von Euler-Matell, E. Carlsson, R. Titball, A. Forsberg, and H. Wolf-Watz, “The V-antigen of *Yersinia* is surface exposed before target cell contact and involved in virulence protein translocation,” *Mol. Microbiol.*, vol. 32, no. 5, pp. 961–976, Jun. 1999.
- [99] S. Håkansson, K. Schesser, C. Persson, E. E. Galyov, R. Rosqvist, F. Homblé, and H. Wolf-Watz, “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.*, vol. 15, no. 21, pp. 5812–23, Nov. 1996.
- [100] A. Boland, M. P. Sory, M. Iriarte, C. Kerbouch, P. Wattiau, and G. R. Cornelis, “Status of YopM and YopN in the *Yersinia* Yop virulon: YopM of *Y. enterocolitica* is internalized inside the cytosol of PU5-1.8 macrophages by the YopB, D, N delivery apparatus.,” *EMBO J.*, vol. 15, no. 19, pp. 5191–201, Oct. 1996.
- [101] C. a Mueller, P. Broz, and G. R. Cornelis, “The type III secretion system tip complex and translocon.,” *Mol. Microbiol.*, vol. 68, no. 5, pp. 1085–95, Jun. 2008.
- [102] J. E. Bröms, M. S. Francis, and A. Forsberg, “Diminished LcrV secretion attenuates *Yersinia pseudotuberculosis* virulence.,” *J. Bacteriol.*, vol. 189, no. 23, pp. 8417–29, Dec. 2007.
- [103] E. I. Armentrout and A. Rietsch, “The Type III Secretion Translocation Pore Senses Host Cell Contact.,” *PLoS Pathog.*, vol. 12, no. 3, p. e1005530, Mar. 2016.
- [104] a D. Roehrich, I. Martinez-Argudo, S. Johnson, A. J. Blocker, and A. K. J. Veenendaal, “The extreme C terminus of *Shigella flexneri* IpaB is required for regulation of type III secretion, needle tip composition, and binding.,” *Infect. Immun.*, vol. 78, no. 4, pp. 1682–91, Apr. 2010.
- [105] A. J. Olive, R. Kenjale, M. Espina, D. S. Moore, W. L. Picking, and W. D. Picking, “Bile salts stimulate recruitment of IpaB to the *Shigella flexneri* surface, where it colocalizes with IpaD at the tip of the type III secretion needle.,” *Infect. Immun.*, vol. 75, no. 5, pp. 2626–9, May 2007.
- [106] S. Johnson, P. Roversi, M. Espina, A. Olive, J. E. Deane, S. Birket, T. Field, W. D. Picking, A. J. Blocker, E. E. Galyov, W. L. Picking, and S. M. Lea, “Self-chaperoning of the type III secretion system needle tip proteins IpaD and BipD.,” *J. Biol. Chem.*, vol. 282, no. 6, pp. 4035–44, Feb. 2007.
- [107] M. L. Barta, N. E. Dickenson, M. Patil, A. Keightley, G. J. Wyckoff, W. D. Picking, W. L. Picking, and B. V Geisbrecht, “The structures of coiled-coil domains from type III secretion system translocators reveal homology to pore-forming toxins.,” *J. Mol. Biol.*, vol. 417, no. 5, pp. 395–405, Apr. 2012.
- [108] C. Neyt and G. R. Cornelis, “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.*, vol. 33, no. 5, pp. 971–981, Sep. 1999.
- [109] M. Aili, E. L. Isaksson, S. E. Carlsson, H. Wolf-Watz, R. Rosqvist, and M. S. Francis, “Regulation of *Yersinia* Yop-effector delivery by translocated YopE.,” *Int. J. Med. Microbiol.*, vol. 298, no. 3–4, pp. 183–92, Apr. 2008.
- [110] A. Blocker, P. Gounon, E. Larquet, K. Niebuhr, V. Cabiaux, C. Parsot, and P. Sansonetti, “The tripartite type III secretion system of *Shigella flexneri* inserts IpaB and IpaC into host membranes.,” *J. Cell Biol.*, vol. 147, no. 3, pp. 683–93, Nov. 1999.
- [111] D. Dacheux, J. Goure, J. Chabert, Y. Usson, and I. Attree, “Pore-forming activity of type III system-secreted proteins leads to oncosis of *Pseudomonas*

- aeruginosa-infected macrophages,” *Mol. Microbiol.*, vol. 40, no. 1, pp. 76–85, Dec. 2001.
- [112] A. Holmstrom, J. Olsson, P. Cherepanov, E. Maier, R. Nordfelth, J. Pettersson, R. Benz, H. Wolf-Watz, and A. Forsberg, “LcrV is a channel size-determining component of the Yop effector translocon of *Yersinia*,” *Mol. Microbiol.*, vol. 39, no. 3, pp. 620–632, Feb. 2001.
- [113] T. Ide, S. Laarmann, L. Greune, H. Schillers, H. Oberleithner, and M. A. Schmidt, “Characterization of translocation pores inserted into plasma membranes by type III-secreted Esp proteins of enteropathogenic *Escherichia coli*,” *Cell. Microbiol.*, vol. 3, no. 10, pp. 669–679, Oct. 2001.
- [114] S. J. Daniell, N. Takahashi, R. Wilson, D. Friedberg, I. Rosenshine, F. P. Booy, R. K. Shaw, S. Knutton, G. Frankel, and S.-I. Aizawa, “The filamentous type III secretion translocon of enteropathogenic *Escherichia coli*,” *Cell. Microbiol.*, vol. 3, no. 12, pp. 865–871, Dec. 2001.
- [115] T. Miki, N. Okada, Y. Shimada, and H. Danbara, “Characterization of *Salmonella* pathogenicity island 1 type III secretion-dependent hemolytic activity in *Salmonella enterica* serovar Typhimurium,” *Microb. Pathog.*, vol. 37, no. 2, pp. 65–72, Aug. 2004.
- [116] A. Chatterjee, C. Caballero-Franco, D. Bakker, S. Totten, and A. Jardim, “Pore-forming Activity of the *Escherichia coli* Type III Secretion System Protein EspD,” *J. Biol. Chem.*, vol. 290, no. 42, pp. 25579–94, Oct. 2015.
- [117] A. Blocker, N. Jouihri, E. Larquet, P. Gounon, F. Ebel, C. Parsot, P. Sansonetti, and A. Allaoui, “Structure and composition of the *Shigella flexneri* ‘needle complex’, a part of its type III secretion,” *Mol. Microbiol.*, vol. 39, no. 3, pp. 652–663, Feb. 2001.
- [118] C. Montagner, C. Arquint, and G. R. Cornelis, “Translocators YopB and YopD from *Yersinia enterocolitica* form a multimeric integral membrane complex in eukaryotic cell membranes,” *J. Bacteriol.*, vol. 193, no. 24, pp. 6923–8, Dec. 2011.
- [119] G. Schoehn, A. M. Di Guilmi, D. Lemaire, I. Attree, W. Weissenhorn, and A. Dessen, “Oligomerization of type III secretion proteins PopB and PopD precedes pore formation in *Pseudomonas*,” *EMBO J.*, vol. 22, no. 19, pp. 4957–67, Oct. 2003.
- [120] G. I. Viboud and J. B. Bliska, “A bacterial type III secretion system inhibits actin polymerization to prevent pore formation in host cell membranes,” *EMBO J.*, vol. 20, no. 19, pp. 5373–82, Oct. 2001.
- [121] C. Persson, R. Nordfelth, A. Holmstrom, S. H>>kansson, R. Rosqvist, and H. Wolf-Watz, “Cell-surface-bound *Yersinia* translocate the protein tyrosine phosphatase YopH by a polarized mechanism into the target cell,” *Mol. Microbiol.*, vol. 18, no. 1, pp. 135–150, Oct. 1995.
- [122] M. P. Sory, a Boland, I. Lambermont, and G. R. Cornelis, “Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the *cyxA* gene fusion approach,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 92, no. 26, pp. 11998–2002, Dec. 1995.
- [123] K. Schesser, E. Frithz-Lindsten, and H. Wolf-Watz, “Delineation and mutational analysis of the *Yersinia pseudotuberculosis* YopE domains which mediate translocation across bacterial and eukaryotic cellular membranes,” *J. Bacteriol.*, vol. 178, no. 24, pp. 7227–33, Dec. 1996.
- [124] M. Watarai, T. Tobe, M. Yoshikawa, and C. Sasakawa, “Disulfide oxidoreductase activity of *Shigella flexneri* is required for release of Ipa proteins and invasion of epithelial cells,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 92, no. 11, pp. 4927–31, May 1995.
- [125] R. Ménard, M. C. Prévost, P. Gounon, P. Sansonetti, and C. Dehio, “The secreted Ipa complex of *Shigella flexneri* promotes entry into mammalian

- cells.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 93, no. 3, pp. 1254–8, Feb. 1996.
- [126] T. Edgren, A. Forsberg, R. Rosqvist, and H. Wolf-Watz, "Type III secretion in *Yersinia*: injectisome or not?," *PLoS Pathog.*, vol. 8, no. 5, p. e1002669, May 2012.
- [127] C. Hanski, U. Kutschka, H. P. Schmoranzler, M. Naumann, A. Stallmach, H. Hahn, H. Menge, and E. O. Riecken, "Immunohistochemical and electron microscopic study of interaction of *Yersinia enterocolitica* serotype O8 with intestinal mucosa during experimental enteritis.," *Infect. Immun.*, vol. 57, no. 3, pp. 673–8, Mar. 1989.
- [128] R. R. Isberg and J. M. Leong, "Multiple β 1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells," *Cell*, vol. 60, no. 5, pp. 861–871, 1990.
- [129] A. Grützkau, C. Hanski, H. Hahn, and E. O. Riecken, "Involvement of M cells in the bacterial invasion of Peyer's patches: a common mechanism shared by *Yersinia enterocolitica* and other enteroinvasive bacteria.," *Gut*, vol. 31, no. 9, pp. 1011–5, Sep. 1990.
- [130] I. B. Autenrieth and R. Firsching, "Penetration of M cells and destruction of Peyer's patches by *Yersinia enterocolitica*: an ultrastructural and histological study," *J. Med. Microbiol.*, vol. 44, no. 4, pp. 285–294, Apr. 1996.
- [131] M. A. N. N. Clark, B. H. Hirst, and M. A. Jepson, "M-Cell Surface β 1 Integrin Expression and Invasin-Mediated Targeting of *Yersinia pseudotuberculosis* to Mouse Peyer's Patch M Cells," vol. 66, no. 3, pp. 1237–1243, 1998.
- [132] P. Chavrier, "Molecular basis of phagocytosis," *Semin. Immunol.*, vol. 13, no. 6, pp. 337–338, 2001.
- [133] T. E. Tjelle, T. Løvdal, and T. Berg, "Phagosome dynamics and function," *BioEssays*, vol. 22, no. 3, pp. 255–263, Feb. 2000.
- [134] O. V. Vieira, R. J. Botelho, and S. Grinstein, "Phagosome maturation: aging gracefully.," *Biochem. J.*, vol. 366, no. Pt 3, pp. 689–704, Sep. 2002.
- [135] A. van der Flier and A. Sonnenberg, "Function and interactions of integrins.," *Cell Tissue Res.*, vol. 305, no. 3, pp. 285–98, Sep. 2001.
- [136] K. Andersson, N. Carballeira, K.-E. Magnusson, C. Persson, O. Stendahl, H. Wolf-Watz, and M. Fällman, "YopH of *Yersinia pseudotuberculosis* interrupts early phosphotyrosine signalling associated with phagocytosis," *Mol. Microbiol.*, vol. 20, no. 5, pp. 1057–1069, Jun. 1996.
- [137] C. Persson, R. Nordfelth, K. Andersson, A. Forsberg, H. Wolf-Watz, and M. Fallman, "Localization of the *Yersinia* PTPase to focal complexes is an important virulence mechanism," *Mol. Microbiol.*, vol. 33, no. 4, pp. 828–838, Aug. 1999.
- [138] J. Liliental and D. D. Chang, "Rack1, a Receptor for Activated Protein Kinase C, Interacts with Integrin Subunit," *J. Biol. Chem.*, vol. 273, no. 4, pp. 2379–2383, Jan. 1998.
- [139] M. D. Schaller, "Biochemical signals and biological responses elicited by the focal adhesion kinase," *Biochim. Biophys. Acta - Mol. Cell Res.*, vol. 1540, no. 1, pp. 1–21, 2001.
- [140] E. Zamir and B. Geiger, "Components of cell-matrix adhesions," *J. Cell Sci.*, vol. 114, no. 20, pp. 3577–3579, 2001.
- [141] B. Raymond, J. C. Young, M. Pallett, R. G. Endres, A. Clements, and G. Frankel, "Subversion of trafficking, apoptosis, and innate immunity by type III secretion system effectors," *Trends Microbiol.*, vol. 21, no. 8, pp. 430–441, 2013.
- [142] K. Pha and L. Navarro, "Yersinia type III effectors perturb host innate immune responses.," *World J. Biol. Chem.*, vol. 7, no. 1, pp. 1–13, Feb. 2016.
- [143] I. Bölin, L. Norlander, and H. Wolf-Watz, "Temperature-inducible outer membrane protein of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*

- is associated with the virulence plasmid.," *Infect. Immun.*, vol. 37, no. 2, pp. 506–12, Aug. 1982.
- [144] T. Michiels, P. Wattiau, R. Brasseur, J. M. Ruyschaert, and G. Cornelis, "Secretion of Yop proteins by Yersinia," *Infect. Immun.*, vol. 58, no. 9, pp. 2840–9, Sep. 1990.
- [145] L. L. KUPFERBERG and K. HIGUCHI, "Role of calcium ions in the stimulation of growth of virulent strains of Pasteurella pestis.," *J. Bacteriol.*, vol. 76, no. 1, pp. 120–1, Jul. 1958.
- [146] R. R. Brubaker, "Growth of Pasteurella pseudotuberculosis in simulated intracellular and extracellular environments.," *J. Infect. Dis.*, vol. 117, no. 5, pp. 403–17, Dec. 1967.
- [147] P. Gemski, J. R. Lazere, and T. Casey, "Plasmid associated with pathogenicity and calcium dependency of Yersinia enterocolitica.," *Infect. Immun.*, vol. 27, no. 2, pp. 682–5, Feb. 1980.
- [148] I. Bölin and H. Wolf-Watz, "Molecular cloning of the temperature-inducible outer membrane protein 1 of Yersinia pseudotuberculosis.," *Infect. Immun.*, vol. 43, no. 1, pp. 72–8, Jan. 1984.
- [149] I. Bölin, D. A. Portnoy, and H. Wolf-Watz, "Expression of the temperature-inducible outer membrane proteins of yersinia.," *Infect. Immun.*, vol. 48, no. 1, pp. 234–40, Apr. 1985.
- [150] J. D. Goguen, J. Yother, and S. C. Straley, "Genetic analysis of the low calcium response in Yersinia pestis mu d1(Ap lac) insertion mutants.," *J. Bacteriol.*, vol. 160, no. 3, pp. 842–8, Dec. 1984.
- [151] Å. Forsberg, I. Bölin, L. Norlander, and H. Wolf-Watz, "Molecular cloning and expression of calcium-regulated, plasmid-coded proteins of Y. pseudotuberculosis," *Microb. Pathog.*, vol. 2, no. 2, pp. 123–137, 1987.
- [152] J. Yother and J. D. Goguen, "Isolation and characterization of Ca²⁺-blind mutants of Yersinia pestis.," *J. Bacteriol.*, vol. 164, no. 2, pp. 704–11, Nov. 1985.
- [153] H. Wang, K. Avican, A. Fahlgren, S. F. Erttmann, A. M. Nuss, P. Dersch, M. Fallman, T. Edgren, and H. Wolf-Watz, "Increased plasmid copy number is essential for Yersinia T3SS function and virulence," *Science (80-.)*, vol. 353, no. 6298, pp. 492–495, 2016.
- [154] R. Rosqvist, Å. Forsberg, M. Rimpiläinen, T. Bergman, and H. Wolf-Watz, "The cytotoxic protein YopE of Yersinia obstructs the primary host defence.," *Mol. Microbiol.*, vol. 4, no. 4, pp. 657–67, Apr. 1990.
- [155] E. Skrzypek, P. L. Haddix, G. V. Plano, and S. C. Straley, "New Suicide Vector for Gene Replacement in Yersinia and Other Gram-Negative Bacteria," 1993.
- [156] F. Ferracci, F. D. Schubot, D. S. Waugh, and G. V. Plano, "Selection and characterization of Yersinia pestis YopN mutants that constitutively block Yop secretion.," *Mol. Microbiol.*, vol. 57, no. 4, pp. 970–87, Aug. 2005.
- [157] S. S. Joseph and G. V. Plano, "Identification of TyeA residues required to interact with YopN and to regulate Yop secretion.," *Adv. Exp. Med. Biol.*, vol. 603, no. i, pp. 235–45, Jan. 2007.
- [158] M. W. Jackson, J. B. Day, and G. V. Plano, "YscB of Yersinia pestis Functions as a Specific Chaperone for YopN," *J. Bacteriol.*, vol. 180, no. 18, pp. 4912–4921, Sep. 1998.
- [159] J. B. Day and G. V. Plano, "A complex composed of SycN and YscB functions as a specific chaperone for YopN in Yersinia pestis," *Mol. Microbiol.*, vol. 30, no. 4, pp. 777–788, Nov. 1998.
- [160] S. S. Joseph and G. V. Plano, "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.*, vol. 3, p. 1, Jan. 2013.

- [161] Y. Cherradi, L. Schiavolin, S. Moussa, A. Meghraoui, A. Meksem, L. Biskri, M. Azarkan, A. Allaoui, and A. Botteaux, "Interplay between predicted inner-rod and gatekeeper in controlling substrate specificity of the type III secretion system.," *Mol. Microbiol.*, vol. 87, no. 6, pp. 1183–99, Mar. 2013.
- [162] P.-C. Lee, S. E. Zmina, C. M. Stopford, J. Toska, and A. Rietsch, "Control of type III secretion activity and substrate specificity by the cytoplasmic regulator PcrG.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 19, pp. E2027–36, May 2014.
- [163] M. L. Nilles, a W. Williams, E. Skrzypek, and S. C. Straley, "Yersinia pestis LcrV forms a stable complex with LcrG and may have a secretion-related regulatory role in the low-Ca²⁺ response.," *J. Bacteriol.*, vol. 179, no. 4, pp. 1307–16, Feb. 1997.
- [164] M. L. Nilles, K. A. Fields, and S. C. Straley, "The V antigen of Yersinia pestis regulates Yop vectorial targeting as well as Yop secretion through effects on YopB and LcrG.," *J. Bacteriol.*, vol. 180, no. 13, pp. 3410–20, Jul. 1998.
- [165] J. S. Matson and M. L. Nilles, "LcrG-LcrV interaction is required for control of Yops secretion in Yersinia pestis.," *J. Bacteriol.*, vol. 183, no. 17, pp. 5082–91, Sep. 2001.
- [166] D. M. Anderson, K. S. Ramamurthi, C. Tam, and O. Schneewind, "YopD and LcrH regulate expression of Yersinia enterocolitica YopQ by a posttranscriptional mechanism and bind to yopQ RNA.," *J. Bacteriol.*, vol. 184, no. 5, pp. 1287–95, Mar. 2002.
- [167] C. R. Wulff-Strobel, A. W. Williams, and S. C. Straley, "LcrQ and SycH function together at the Ysc type III secretion system in Yersinia pestis to impose a hierarchy of secretion," *Mol. Microbiol.*, vol. 43, no. 2, pp. 411–423, Mar. 2002.
- [168] E. D. Cambronne, J. A. Sorg, and O. Schneewind, "Binding of SycH chaperone to YscM1 and YscM2 activates effector yop expression in Yersinia enterocolitica.," *J. Bacteriol.*, vol. 186, no. 3, pp. 829–41, Feb. 2004.
- [169] L. Li, H. Yan, L. Feng, Y. Li, P. Lu, Y. Hu, and S. Chen, "LcrQ blocks the role of LcrF in regulating the Ysc-Yop type III secretion genes in Yersinia pseudotuberculosis.," *PLoS One*, vol. 9, no. 3, p. e92243, 2014.
- [170] a Holmström, R. Rosqvist, H. Wolf-Watz, and a Forsberg, "Virulence plasmid-encoded YopK is essential for Yersinia pseudotuberculosis to cause systemic infection in mice.," *Infect. Immun.*, vol. 63, no. 6, pp. 2269–76, Jun. 1995.
- [171] S. E. Thorslund, T. Edgren, J. Pettersson, R. Nordfelth, M. E. Sellin, E. Ivanova, M. S. Francis, E. L. Isaksson, H. Wolf-Watz, and M. Fällman, "The RACK1 signaling scaffold protein selectively interacts with Yersinia pseudotuberculosis virulence function.," *PLoS One*, vol. 6, no. 2, p. e16784, Jan. 2011.
- [172] M. Cisz, P.-C. Lee, and A. Rietsch, "ExoS controls the cell contact-mediated switch to effector secretion in Pseudomonas aeruginosa.," *J. Bacteriol.*, vol. 190, no. 8, pp. 2726–38, Apr. 2008.
- [173] A. Holmstrom, J. Pettersson, R. Rosqvist, S. Hakansson, F. Tafazoli, M. Fallman, K.-E. Magnusson, H. Wolf-Watz, and A. Forsberg, "YopK of Yersinia pseudotuberculosis controls translocation of Yop effectors across the eukaryotic cell membrane," *Mol. Microbiol.*, vol. 24, no. 1, pp. 73–91, Apr. 1997.
- [174] S. Thorslund, "Role of the Yersinia protein YopK in microbe-host interactions," 2012.
- [175] K. Schesser, A.-K. Spiik, J.-M. Dukuzumuremyi, M. F. Neurath, S. Pettersson, and H. Wolf-Watz, "The yopJ locus is required for Yersinia-mediated inhibition of NF-kappaB activation and cytokine expression: YopJ contains a

- eukaryotic SH2-like domain that is essential for its repressive activity,” *Mol. Microbiol.*, vol. 28, no. 6, pp. 1067–1079, Jun. 1998.
- [176] R. Mittal, S.-Y. Peak-Chew, and H. T. McMahon, “Acetylation of MEK2 and I kappa B kinase (IKK) activation loop residues by YopJ inhibits signaling.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 103, no. 49, pp. 18574–9, Dec. 2006.
- [177] Y.-H. Hao, Y. Wang, D. Burdette, S. Mukherjee, G. Keitany, E. Goldsmith, and K. Orth, “Structural requirements for Yersinia YopJ inhibition of MAP kinase pathways.,” *PLoS One*, vol. 3, no. 1, p. e1375, Jan. 2008.
- [178] D. M. Monack, J. Meccas, N. Ghori, and S. Falkow, “Yersinia signals macrophages to undergo apoptosis and YopJ is necessary for this cell death.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 94, no. 19, pp. 10385–90, Sep. 1997.
- [179] Y. Zhang, A. T. Ting, K. B. Marcu, and J. B. Bliska, “Inhibition of MAPK and NF-kappa B pathways is necessary for rapid apoptosis in macrophages infected with Yersinia.,” *J. Immunol.*, vol. 174, no. 12, pp. 7939–49, Jun. 2005.
- [180] S. Gröbner, S. Schulz, I. Soldanova, D. S. J. Gunst, M. Waibel, S. Wesselborg, S. Borgmann, and I. B. Autenrieth, “Absence of Toll-like receptor 4 signaling results in delayed Yersinia enterocolitica YopP-induced cell death of dendritic cells.,” *Infect. Immun.*, vol. 75, no. 1, pp. 512–7, Jan. 2007.
- [181] S. Mukherjee, G. Keitany, Y. Li, Y. Wang, H. L. Ball, E. J. Goldsmith, and K. Orth, “Yersinia YopJ Acetylates and Inhibits Kinase Activation by Blocking Phosphorylation,” *Science (80-.)*, vol. 312, no. 5777, pp. 1211–1214, 2006.
- [182] R. Benabdillah, L. Jaime Mota, S. Lützelshwab, E. Demoinet, and G. R. Cornelis, “Identification of a nuclear targeting signal in YopM from Yersinia spp.,” *Microb. Pathog.*, vol. 36, no. 5, pp. 247–261, 2004.
- [183] E. Skrzypek, C. Cowan, and S. C. Straley, “Targeting of the Yersinia pestis YopM protein into HeLa cells and intracellular trafficking to the nucleus,” *Mol. Microbiol.*, vol. 30, no. 5, pp. 1051–1065, Dec. 1998.
- [184] C. McDonald, P. O. Vacratsis, J. B. Bliska, and J. E. Dixon, “The yersinia virulence factor YopM forms a novel protein complex with two cellular kinases.,” *J. Biol. Chem.*, vol. 278, no. 20, pp. 18514–23, May 2003.
- [185] J. B. McPhee, P. Mena, and J. B. Bliska, “Delineation of regions of the Yersinia YopM protein required for interaction with the RSK1 and PRK2 host kinases and their requirement for interleukin-10 production and virulence.,” *Infect. Immun.*, vol. 78, no. 8, pp. 3529–39, Aug. 2010.
- [186] Z. Ye, A. A. Gorman, A. M. Uittenbogaard, T. Myers-Morales, A. M. Kaplan, D. A. Cohen, and S. C. Straley, “Caspase-3 mediates the pathogenic effect of Yersinia pestis YopM in liver of C57BL/6 mice and contributes to YopM’s function in spleen.,” *PLoS One*, vol. 9, no. 11, p. e110956, 2014.
- [187] E. E. Galyov, S. Håkansson, Å. Forsberg, and H. Wolf-Watz, “A secreted protein kinase of Yersinia pseudotuberculosis is an indispensable virulence determinant,” *Nature*, vol. 361, no. 6414, pp. 730–732, Feb. 1993.
- [188] L. Navarro, A. Koller, R. Nordfelth, H. Wolf-Watz, S. Taylor, and J. E. Dixon, “Identification of a Molecular Target for the Yersinia Protein Kinase A,” *Mol. Cell*, vol. 26, no. 4, pp. 465–477, 2007.
- [189] W. L. Lee, J. M. Grimes, and R. C. Robinson, “Yersinia effector YopO uses actin as bait to phosphorylate proteins that regulate actin polymerization.,” *Nat. Struct. Mol. Biol.*, vol. 22, no. 3, pp. 248–55, Mar. 2015.
- [190] G. Prehna, M. I. Ivanov, J. B. Bliska, and C. E. Stebbins, “Yersinia Virulence Depends on Mimicry of Host Rho-Family Nucleotide Dissociation Inhibitors,” *Cell*, vol. 126, no. 5, pp. 869–880, 2006.
- [191] M. Iriarte and G. R. Cornelis, “YopT, a new Yersinia Yop effector protein, affects the cytoskeleton of host cells,” *Mol. Microbiol.*, vol. 29, no. 3, pp. 915–929, Aug. 1998.

- [192] R. Zumbihl, M. Aepfelbacher, A. Andor, C. A. Jacobi, K. Ruckdeschel, B. Rouot, and J. Heesemann, "The cytotoxin YopT of *Yersinia enterocolitica* induces modification and cellular redistribution of the small GTP-binding protein RhoA.," *J. Biol. Chem.*, vol. 274, no. 41, pp. 29289–93, Oct. 1999.
- [193] F. Shao, P. M. Merritt, Z. Bao, R. W. Innes, and J. E. Dixon, "A *Yersinia* Effector and a *Pseudomonas* Avirulence Protein Define a Family of Cysteine Proteases Functioning in Bacterial Pathogenesis," *Cell*, vol. 109, no. 5, pp. 575–588, 2002.
- [194] F. Shao, P. O. Vacratsis, Z. Bao, K. E. Bowers, C. A. Fierke, and J. E. Dixon, "Biochemical characterization of the *Yersinia* YopT protease: cleavage site and recognition elements in Rho GTPases.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 100, no. 3, pp. 904–9, Feb. 2003.
- [195] G. I. Viboud, E. Mejia, and J. B. Bliska, "Comparison of YopE and YopT activities in counteracting host signalling responses to *Yersinia* pseudotuberculosis infection," *Cell. Microbiol.*, vol. 8, no. 9, pp. 1504–1515, Sep. 2006.
- [196] I. Bölin and H. Wolf-Watz, "The plasmid-encoded Yop2b protein of *Yersinia* pseudotuberculosis is a virulence determinant regulated by calcium and temperature at the level of transcription.," *Mol. Microbiol.*, vol. 2, no. 2, pp. 237–45, Mar. 1988.
- [197] R. Rosqvist, I. Bölin, and H. Wolf-Watz, "Inhibition of phagocytosis in *Yersinia* pseudotuberculosis: a virulence plasmid-encoded ability involving the Yop2b protein.," *Infect. Immun.*, vol. 56, no. 8, pp. 2139–43, Aug. 1988.
- [198] K. L. Guan and J. E. Dixon, "Protein tyrosine phosphatase activity of an essential virulence determinant in *Yersinia*.," *Science*, vol. 249, no. 4968, pp. 553–6, Aug. 1990.
- [199] Z. Y. Zhang, J. C. Clemens, H. L. Schubert, J. A. Stuckey, M. W. Fischer, D. M. Hume, M. A. Saper, and J. E. Dixon, "Expression, purification, and physicochemical characterization of a recombinant *Yersinia* protein tyrosine phosphatase.," *J. Biol. Chem.*, vol. 267, no. 33, pp. 23759–66, Nov. 1992.
- [200] C. Persson, N. Carballeira, H. Wolf-Watz, M. Fällman, "The PTPase YopH inhibits uptake of *Yersinia*, tyrosine phosphorylation of p130^{Cas} and FAK, and the associated accumulation of these proteins in peripheral focal adhesions," *EMBO J.*, vol. 16, no. 9, pp. 2307–2318, May 1997.
- [201] D. S. Black and J. B. Bliska, "Identification of p130Cas as a substrate of *Yersinia* YopH (Yop51), a bacterial protein tyrosine phosphatase that translocates into mammalian cells and targets focal adhesions.," *EMBO J.*, vol. 16, no. 10, pp. 2730–44, May 1997.
- [202] N. Hamid, A. Gustavsson, K. Andersson, K. McGee, C. Persson, C. . Rudd, and M. Fällman, "YopH dephosphorylates Cas and Fyn-binding protein in macrophages," *Microb. Pathog.*, vol. 27, no. 4, pp. 231–242, 1999.
- [203] K. Andersson, K. E. Magnusson, M. Majeed, O. Stendahl, and M. Fällman, "*Yersinia* pseudotuberculosis-induced calcium signaling in neutrophils is blocked by the virulence effector YopH.," *Infect. Immun.*, vol. 67, no. 5, pp. 2567–74, May 1999.
- [204] L. K. Logsdon and J. Meccas, "Requirement of the *Yersinia* pseudotuberculosis effectors YopH and YopE in colonization and persistence in intestinal and lymph tissues.," *Infect. Immun.*, vol. 71, no. 8, pp. 4595–607, Aug. 2003.
- [205] K. Trülzsch, T. Sporleder, E. I. Igwe, H. Rüssmann, and J. Heesemann, "Contribution of the major secreted yops of *Yersinia enterocolitica* O:8 to pathogenicity in the mouse infection model.," *Infect. Immun.*, vol. 72, no. 9, pp. 5227–34, Sep. 2004.
- [206] D. S. Black and J. B. Bliska, "The RhoGAP activity of the *Yersinia*

- pseudotuberculosis cytotoxin YopE is required for antiphagocytic function and virulence.," *Mol. Microbiol.*, vol. 37, no. 3, pp. 515–27, Aug. 2000.
- [207] U. Von Pawel-Rammingen, M. V Telepnev, G. Schmidt, K. Aktories, H. Wolf-Watz, and R. Rosqvist, "GAP activity of the Yersinia YopE cytotoxin specifically targets the Rho pathway: a mechanism for disruption of actin microfilament structure.," *Mol. Microbiol.*, vol. 36, no. 3, pp. 737–48, May 2000.
- [208] M. Aili, B. Hallberg, H. Wolf-Watz, and R. Rosqvist, "GAP activity of Yersinia YopE," *Methods Enzymol.*, vol. 358, pp. 359–370, 2002.
- [209] M. Aili, E. L. Isaksson, B. Hallberg, H. Wolf-watz, and R. Rosqvist, "Functional analysis of the YopE GTPase-activating protein (GAP) activity of Yersinia pseudotuberculosis," vol. 8, no. January, pp. 1020–1033, 2006.
- [210] A. Fahlgren, L. Westermark, K. Akopyan, and M. Fällman, "Cell type-specific effects of Yersinia pseudotuberculosis virulence effectors.," *Cell. Microbiol.*, vol. 11, no. 12, pp. 1750–67, Dec. 2009.
- [211] E. L. Isaksson, M. Aili, A. Fahlgren, S. E. Carlsson, R. Rosqvist, and H. Wolf-Watz, "The membrane localization domain is required for intracellular localization and autoregulation of YopE in Yersinia pseudotuberculosis.," *Infect. Immun.*, vol. 77, no. 11, pp. 4740–9, Nov. 2009.
- [212] R. Dewoody, P. M. Merritt, A. S. Houppert, and M. M. Marketon, "YopK regulates the Yersinia pestis type III secretion system from within host cells.," *Mol. Microbiol.*, vol. 79, no. 6, pp. 1445–61, Mar. 2011.
- [213] R. Dewoody, P. M. Merritt, and M. M. Marketon, "YopK controls both rate and fidelity of Yop translocation.," *Mol. Microbiol.*, vol. 87, no. 2, pp. 301–17, Jan. 2013.
- [214] E. Mejia, J. B. Bliska, and G. I. Viboud, "Yersinia Controls Type III Effector Delivery into Host Cells by Modulating Rho Activity," *PLoS Pathog.*, vol. 4, no. 1, p. e3, 2008.
- [215] C. N. Berger, V. F. Crepin, K. Baruch, A. Mousnier, I. Rosenshine, and G. Frankel, "EspZ of enteropathogenic and enterohemorrhagic Escherichia coli regulates type III secretion system protein translocation.," *MBio*, vol. 3, no. 5, 2012.
- [216] J. T. Garcia, F. Ferracci, M. W. Jackson, S. S. Joseph, I. Pattis, L. R. W. Plano, W. Fischer, and G. V Plano, "Measurement of effector protein injection by type III and type IV secretion systems by using a 13-residue phosphorylatable glycogen synthase kinase tag.," *Infect. Immun.*, vol. 74, no. 10, pp. 5645–57, Oct. 2006.
- [217] F. D. Schubot, M. W. Jackson, K. J. Penrose, S. Cherry, J. E. Tropea, G. V Plano, and D. S. Waugh, "Three-dimensional structure of a macromolecular assembly that regulates type III secretion in Yersinia pestis.," *J. Mol. Biol.*, vol. 346, no. 4, pp. 1147–61, Mar. 2005.
- [218] J. E. Deane, P. Roversi, C. King, S. Johnson, and S. M. Lea, "Structures of the Shigella flexneri Type 3 Secretion System Protein MxiC Reveal Conformational Variability Amongst Homologues," 2008.
- [219] F. Ferracci, J. B. Day, H. J. Ezelle, and G. V Plano, "Expression of a functional secreted YopN-TyeA hybrid protein in Yersinia pestis is the result of a +1 translational frameshift event.," *J. Bacteriol.*, vol. 186, no. 15, pp. 5160–6, Aug. 2004.
- [220] A. A. A. Amer, T. R. D. Costa, S. I. Farag, U. Avican, Å. Forsberg, and M. S. Francis, "Genetically engineered frameshifted YopN-TyeA chimeras influence type III secretion system function in Yersinia pseudotuberculosis.," *PLoS One*, vol. 8, no. 10, p. e77767, Jan. 2013.
- [221] J. B. Day, F. Ferracci, and G. V Plano, "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.*, vol. 47, no. 3, pp. 807–23, Feb. 2003.
- [222] J. Huang, C. F. Lesser, and S. Lory, "The essential role of the CopN protein in Chlamydia pneumoniae intracellular growth.," *Nature*, vol. 456, no. 7218, pp. 112–5, 2008.
- [223] T. L. Archuleta, Y. Du, C. A. English, S. Lory, C. Lesser, M. D. Ohi, R. Ohi, and B. W. Spiller, "The Chlamydia effector chlamydial outer protein N (CopN) sequesters tubulin and prevents microtubule assembly," *J. Biol. Chem.*, vol. 286, no. 39, pp. 33992–33998, 2011.
- [224] G. A. BACON and T. W. BURROWS, "The basis of virulence in Pasteurella pestis: an antigen determining virulence.," *Br. J. Exp. Pathol.*, vol. 37, no. 5, pp. 481–93, Oct. 1956.
- [225] T. W. BURROWS and G. A. BACON, "V and W antigens in strains of Pasteurella pseudotuberculosis.," *Br. J. Exp. Pathol.*, vol. 41, no. 1, pp. 38–44, Feb. 1960.
- [226] P. B. Carter, R. J. Zahorchak, and R. R. Brubaker, "Plague virulence antigens from Yersinia enterocolitica.," *Infect. Immun.*, vol. 28, no. 2, pp. 638–40, May 1980.
- [227] W. D. LAWTON, R. L. ERDMAN, and M. J. SURGALLA, "BIOSYNTHESIS AND PURIFICATION OF V AND W ANTIGEN IN PASTEURELLA PESTIS.," *J. Immunol.*, vol. 91, pp. 179–84, Aug. 1963.
- [228] S. E. Leary, E. D. Williamson, K. F. Griffin, P. Russell, S. M. Eley, and R. W. Titball, "Active immunization with recombinant V antigen from Yersinia pestis protects mice against plague.," *Infect. Immun.*, vol. 63, no. 8, pp. 2854–8, Aug. 1995.
- [229] G. W. Anderson, S. E. Leary, E. D. Williamson, R. W. Titball, S. L. Welkos, P. L. Worsham, A. M. Friedlander, and A. M. Friedlander, "Recombinant V antigen protects mice against pneumonic and bubonic plague caused by F1-capsule-positive and -negative strains of Yersinia pestis.," *Infect. Immun.*, vol. 64, no. 11, pp. 4580–5, Nov. 1996.
- [230] T. Une and R. R. Brubaker, "Roles of V antigen in promoting virulence and immunity in yersiniae.," *J. Immunol.*, vol. 133, no. 4, pp. 2226–30, Oct. 1984.
- [231] V. L. Motin, R. Nakajima, G. B. Smirnov, and R. R. Brubaker, "Passive immunity to yersiniae mediated by anti-recombinant V antigen and protein A-V antigen fusion peptide.," *Infect. Immun.*, vol. 62, no. 10, pp. 4192–201, Oct. 1994.
- [232] J. Hill, S. E. Leary, K. F. Griffin, E. D. Williamson, R. W. Titball, J. I. M. Hill, S. E. C. Leary, K. F. Griffin, E. D. Williamson, R. W. Titball, C. B. D. P. Down, W. S. P. Ojq, and U. Kingdom, "Regions of Yersinia pestis V antigen that contribute to protection against plague identified by passive and active immunization . Regions of Yersinia pestis V Antigen That Contribute to Protection against Plague Identified by Passive and Active Immunizati," vol. 65, no. 11, 1997.
- [233] R. W. Titball and E. D. Williamson, "Vaccination against bubonic and pneumonic plague," *Vaccine*, vol. 19, no. 30, pp. 4175–4184, 2001.
- [234] E. D. Williamson and P. C. F. Oyston, "Protecting against plague: towards a next-generation vaccine.," *Clin. Exp. Immunol.*, vol. 172, no. 1, pp. 1–8, Apr. 2013.
- [235] S. B. Price, C. Cowan, R. D. Perry, and S. C. Straley, "The Yersinia pestis V antigen is a regulatory protein necessary for Ca2(+)-dependent growth and maximal expression of low-Ca2+ response virulence genes.," *J. Bacteriol.*, vol. 173, no. 8, pp. 2649–57, Apr. 1991.
- [236] M. R. Sarker, C. Neyt, I. Stainier, and G. R. Cornelis, "The Yersinia Yop virulon: LcrV is required for extrusion of the translocators YopB and YopD.,"

- J. Bacteriol.*, vol. 180, no. 5, pp. 1207–14, Mar. 1998.
- [237] U. Derewenda, A. Mateja, Y. Devedjiev, K. M. Routzahn, A. G. Evdokimov, Z. S. Derewenda, and D. S. Waugh, “The structure of Yersinia pestis V-antigen, an essential virulence factor and mediator of immunity against plague,” *Structure*, vol. 12, no. 2, pp. 301–6, Feb. 2004.
- [238] S. Chaudhury, K. P. Battaile, S. Lovell, G. V Plano, and R. N. De Guzman, “Structure of the Yersinia pestis tip protein LcrV refined to 1.65 Å resolution,” *Acta Crystallogr. Sect. F. Struct. Biol. Cryst. Commun.*, vol. 69, no. Pt 5, pp. 477–81, May 2013.
- [239] A. J. Blocker, J. E. Deane, A. K. J. Veenendaal, P. Roversi, J. L. Hodgkinson, S. Johnson, and S. M. Lea, “What’s the point of the type III secretion system needle?,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 105, no. 18, pp. 6507–13, May 2008.
- [240] M.-N. Marenne, L. Journet, L. J. Mota, and G. R. Cornelis, “Genetic analysis of the formation of the Ysc–Yop translocation pore in macrophages by Yersinia enterocolitica: role of LcrV, YscF and YopN,” *Microb. Pathog.*, vol. 35, no. 6, pp. 243–258, Dec. 2003.
- [241] T. R. D. Costa, P. J. Edqvist, J. E. Bröms, M. K. Ahlund, A. Forsberg, and M. S. Francis, “YopD self-assembly and binding to LcrV facilitate type III secretion activity by Yersinia pseudotuberculosis,” *J. Biol. Chem.*, vol. 285, no. 33, pp. 25269–84, Aug. 2010.
- [242] L. R. Allmond, T. J. Karaca, V. N. Nguyen, T. Nguyen, J. P. Wiener-Kronish, and T. Sawa, “Protein binding between PcrG-PcrV and PcrH-PopB/PopD encoded by the pcrGVH-popBD operon of the Pseudomonas aeruginosa type III secretion system,” *Infect. Immun.*, vol. 71, no. 4, pp. 2230–3, Apr. 2003.
- [243] A. Botteaux, M. P. Sory, L. Biskri, C. Parsot, and A. Allaoui, “MxiC is secreted by and controls the substrate specificity of the Shigella flexneri type III secretion apparatus,” *Mol. Microbiol.*, vol. 71, no. 2, pp. 449–60, Jan. 2009.
- [244] T. Kubori and J. E. Galan, “Salmonella Type III Secretion-Associated Protein InvE Controls Translocation of Effector Proteins into Host Cells,” *J. Bacteriol.*, vol. 184, no. 17, pp. 4699–4708, Sep. 2002.
- [245] C. B. O’Connell, E. A. Creasey, S. Knutton, S. Elliott, L. J. Crowther, W. Luo, M. J. Albert, J. B. Kaper, G. Frankel, and M. S. Donnenberg, “SepL, a protein required for enteropathogenic Escherichia coli type III translocation, interacts with secretion component SepD,” *Mol. Microbiol.*, vol. 52, no. 6, pp. 1613–25, Jun. 2004.
- [246] T. L. Yahr, L. M. Mende-Mueller, M. B. Friese, and D. W. Frank, “Identification of type III secreted products of the Pseudomonas aeruginosa exoenzyme S regulon,” *J. Bacteriol.*, vol. 179, no. 22, pp. 7165–8, Nov. 1997.
- [247] D.-K. Shen and A. J. Blocker, “MxiA, MxiC and IpaD Regulate Substrate Selection and Secretion Mode in the T3SS of Shigella flexneri,” *PLoS One*, vol. 11, no. 5, p. e0155141, Jan. 2016.
- [248] M. C. Schlumberger, A. J. Müller, K. Ehrbar, B. Winnen, I. Duss, B. Stecher, and W.-D. Hardt, “Real-time imaging of type III secretion: Salmonella SipA injection into host cells,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 35, pp. 12548–53, Aug. 2005.
- [249] S. E. Thorslund, D. Ermert, A. Fahlgren, S. F. Erttmann, K. Nilsson, A. Hosseinzadeh, C. F. Urban, and M. Fällman, “Role of YopK in Yersinia pseudotuberculosis resistance against polymorphonuclear leukocyte defense,” *Infect. Immun.*, vol. 81, no. 1, pp. 11–22, Jan. 2013.
- [250] J. Torruellas, M. W. Jackson, J. W. Pennock, and G. V Plano, “The Yersinia pestis type III secretion needle plays a role in the regulation of Yop secretion,” *Mol. Microbiol.*, vol. 57, no. 6, pp. 1719–33, Sep. 2005.
- [251] R. Kenjale, J. Wilson, S. F. Zenk, S. Saurya, W. L. Picking, W. D. Picking, and

A. Blocker, "The Needle Component of the Type III Secretion of *Shigella* Regulates the Activity of the Secretion Apparatus," *J. Biol. Chem.*, vol. 280, no. 52, pp. 42929–42937, Dec. 2005.