The multifunctional GAP protein YopE of *Yersinia* is involved in effector translocation control and virulence

Elin Isaksson

Department of Molecular Biology
Laboratory for Molecular Infection Medicine Sweden (MIMS)
Umeå Centre for Microbial Research (UCMR)
Umeå University, Sweden
Umeå 2010

ISBN: 978-91-7459-100-2
Cover graphics by Ulf Lundkvist
Printed by Arkitektkopia Umeå
Umeå, Sweden 2010

To my family

Table of Contents

Abstract

The Gram-negative bacterium *Yersinia pseudotuberculosis* employs a type 3 secretion system (T3SS) to establish infections. The T3SS translocates a diverse set of effector proteins directly into the host cells. The coordinate action of the translocated effectors blocks the innate immune system of the host and ensures extracellular proliferation of the bacterium. YopE is an essential effector that disrupts the actin cytoskeleton of infected host cells. This cytotoxicity is caused by the inactivation of RhoGTPases by the GTPase Activating Protein (GAP) activity of YopE. YopE was demonstrated to inactivate the RhoGTPases Rac1 and RhoA *in vivo*. However, Rac1 and RhoA inactivation was not a prerequisite for cytotoxicity or virulence. Thus, YopE must have additional targets during infection. Surprisingly, avirulent *yopE* mutants had lost the control of Yop expression in the presence of target cells and they all overtranslocated effectors. It appeared as if translocated YopE was able to control Yop expression and effector translocation via a feedback inhibition mechanism. This feedback inhibition was dependent on functional GAP activity. Translocation control could also be mediated by exogenous GAP activity, suggesting that effector translocation control might be a general property of all bacterial GAP proteins. Besides YopE, the regulatory protein YopK was also found to be involved in the effector translocation control process. Clearly, as demonstrated in virulence, the roles for YopE and YopK are intimately related.

Further, YopE possesses a membrane localization domain (MLD) required for proper localization. A *yopE*ΔMLD mutant had lost the feedback inhibition of YopE expression and was avirulent. Hence, the effector translocation control of YopE requires both proper localization as well as functional GAP activity.

In addition, fish keratocytes were established as a novel model system for *Y. pseudotuberculosis* infections. YopE was found to be the sole effector responsible for cytotoxicity towards the keratocytes. Further, induction of cytotoxicity required fully native YopE protein which indicated that the keratocytes would be useful as a sensitive model system for further studies of YopE mediated phenotypes.

In summary, this thesis work has sought to unravel the multiple functions of translocated YopE. A novel role was elucidated where *Yersinia* utilizes translocated YopE to control the process of effector translocation into host cells. This regulatory control was connected to virulence in the mouse model of disease. Thus, perhaps YopE should be considered also as a regulatory protein besides being a classical effector.

Papers included in this thesis

This thesis is based upon the following publications and a manuscript, which are referred to in the text by their roman numerals (I-IV).

I. Aili M., Isaksson E.L., Hallberg B., Wolf-Watz H., Rosqvist R., Functional analysis of the YopE GTPase-activating protein (GAP) activity of *Yersinia pseudotuberculosis*. Cell Micro. 2006 Jun;8(6):1020-33

II. Aili M, Isaksson E.L., Carlsson S. E. Wolf-Watz H., Rosqvist R., Francis M.S., Regulation of *Yersinia* Yop-effector delivery of translocated YopE. Int J Med Microbiol. 2008 Apr;298(3-4):183-92

III. Isaksson E.L., Aili M, Fahlgren A. Carlsson S. E., Rosqvist R., Wolf-Watz H., The Membrane Localization Domain is required for the intracellular localization and auto-regulation of YopE in *Yersinia pseudotuberculosis*. Infect Immun. 2009 Nov; 77(11):4740-9

IV. Isaksson E.L., Lindell K., Aili M., Milton D.L., Wolf-Watz H. Fish scale keratocytes constitute a sensitive model system for YopE mediated phenotypes in *Yersinia pseudotuberculosis* infections. Manuscript in preparation.

Introduction

The *Yersinia* genus

Species within the *Yersinia* genus are Gram-negative rod-shaped bacteria that belong to the family of *Enterobacteriaceae*. The genus has 11 known members. Out of these 11, *Yersinia pestis*, Y*ersinia pseudotuberculosis* and *Yersinia enterocolitica* are pathogenic to humans. Despite their differences in infection routes and disease severity, the pathogenic species share a common tropism for lymphoid tissue, where they resist phagocytosis by disrupting the action of the innate immune system [40, 139]. The ability to remain extracellular is mainly due to the presence of the virulence plasmid, pYV (plasmid of *Yersinia* virulence) [62, 98].

The remaining eight species, *Y. fredriksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. bercovieri*, *Y. mollaretii*, *Y. rohdei*, *Y. ruckeri* and *Y. aldovae*, are classified as secondary invaders or saprophytes. They have not been extensively studied, but are generally considered to be non-pathogenic. [234]

Yersinia pestis- the causative agent of plague

Historical background

Y. pestis is the causative agent of plague. Plague has been estimated to have killed up to 200 million people, making *Y. pestis* one of the deadliest and feared bacteria known to man. Plague has occurred during at least three pandemics. The first, Justinian plague, struck during the 6th to 8th century during the reign of the Roman emperor Justinian. This pandemic rapidly spread from Africa, to eventually affect the whole world known at that time. Records state that up to 50-60% of the population was lost, and the pandemic is considered as one of the events leading to the weakening of the Byzantine era.[185, 205]

Plague reappeared during the Middle Ages in Europe, this time as a pandemic known as the Black Death. The Black Death killed approximately one third of the population, causing enormous social and economical devastation. The impact was to that extent, where plague is believed to have shaped the genetic makeup of the surviving population. [100, 193]

The last plague pandemic started in mainland China in the 1850-ies and is still ongoing. During this outbreak, Alexandre Yersin and Shibasaburo Kitasato both reported the isolation of the plague bacillus within weeks of each other in 1894. Kitasato was initially credited for the discovery. However, later it was clear that the bacteria he described most likely was a contaminant of the analyzed sample. Instead, in 1896, Yersin could successfully cure a plague patient using serum raised against his organism.

In honour of his discovery, the bacterial genus was later given the name *Yersinia*. [146]

The plague disease

Plague is a zoonosis primarily affecting wild rodent populations. The disease is vector-borne, almost always transmitted by the flea *Xenopsylla cheopsis* (Figure 1). [121] Within hours of the flea bite, bacteria disseminate from the bite site to local lymph nodes, where they cause an inflammatory response. This causes swelling of the lymph nodes, also known as buboes (hence the name bubonic plague). Bacteria can spread from the lymph nodes into the bloodstream, resulting in severe bacteraemia and sepsis. The mortality rate for bubonic plague is above 50% when left untreated and patients generally die from septicaemic chock and multi-organ failure. In rare cases, bacteria can spread to the lungs, causing pneumonic plague. For pneumonic plague, the mortality rate is close to 100%, and this form of disease is highly contagious, spreading rapidly from human to human.

Y. pestis can for diagnosis be recovered from bubo aspirates, blood and sputum. However, the bacterium is rather slow growing and therefore laboratory diagnostics take time. Once diagnosed, plague can be treated by most antibiotics. [193, 224]

The plague disease is often considered as a problem of the past, but the third pandemic is still ongoing. WHO (World Health Organization) records from the years 2004-2009 show a total of 12503 plague cases, including 843 deaths. The plague cases are reported from 16 countries in Africa, Asia and the Americas and plague is endemic in many countries in Africa. [177] Naturally antibiotic resistant *Y. pestis* strains have been reported from Madagascar, which would upon spread pose a significant threat to man health worldwide [94].

Y. pestis is also listed as a potential biological warfare agent. Biological warfare is not a modern invention. Starting in ancient times, plague corpses were catapulted over town walls of besieged cities. During World War II, Japanese troupes experimented with different ways to spread plague, by for instance dropping infected fleas from airplanes. Fortunately, their attempts gave futile results. Today, biotechnology advances might provide the potential of aerosol formation. In the hands of terrorists, aerosolized *Y. pestis* would cause wide spread panic and perhaps high mortalities in pneumonic plague. [65]

Y. pestis has recently emerged from *Y. pseudotuberculosis*

Y. pestis is estimated to have emerged as a clone from *Y. pseudotuberculosis* only 1500-20000 years ago [1]. In fact, the two species are so similar that *Y. pestis* rather should be categorized as a subspecies of *Y. pseudotuberculosis*.

However, due to the historical impact of plague and for health security reasons, the current separate classification remains [185].

1500-20000 years is a dramatically short time period, especially when considering that *Y. pestis* transformed from being a soil present enterobacterium into a vector-borne pathogen, not able to survive outside the host [48]. *Y. pestis* has undergone significant changes in order to establish this new infection route. Essential for flea transmission is the ability to block the proventriculus, a blockage valve of the flea midgut. *Y. pestis* forms a biofilm on the valve which stops the blood meal from reaching into the stomach of the flea. Thus, the starving flea repeatedly tries to feed on new hosts, regurgitating bacteria present within the foregut content into the bite site. [121]

During evolution, *Y. pestis* has acquired two additional plasmids called pMT1(pFra) and pPCP1(pPla) in addition to the shared virulence plasmid pYV. pMT1 encodes for the F1 protein, a highly immunogenic protein that forms a capsule on the bacterial surface [90]. Also present on pMT1 is the *ymt* gene that encodes for a phospholipase D (PLD). *ymt* is essential for bacterial colonisation and replication within the flea midgut [120, 122].

Dissemination of bacteria from the flea bite site requires the plasminogen activator, encoded on the pPCP1 plasmid. The plasminogen activator is a surface protease that activates mammalian plasminogen, degrades complement and adheres to laminin. The activator is required in subcutaneous infections, in the formation of buboes, and for primary pneumonic plague. [106]

Besides the additional plasmids, the chromosomal genome of *Y. pestis* has undergone some rearrangements. A number of genes have been inactivated and a few genes have been obtained [180, 259]. The presence of these extra plasmids, the pseudogenes or the new genetic material does not entirely explain the extreme difference in virulence when comparing *Y. pestis* with its ancestor *Y. pseudotuberculosis*. Instead, the striking difference in virulence is more likely due to differential regulation of pre-existing genetic elements. [48]

Yersinia pseudotuberculosis and *Yersinia enterocolitica*- enteropathogens present in the environment

In contrast to the strict vector-borne *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* have the ability to survive in nature. They are normally present in soil and water and cause disease by accidental ingestion of contaminated food or water [221]. In humans, enteropathogenic *Yersinia* cause a disease often referred to as yersiniosis. Yersiniosis is an infection characterized by gastrointestinal symptoms ranging from abdominal pain, diarrhea, and enterocolitis to the more severe mesenteric lymphadenitis. The mesenteric lymphadenitis can easily be misdiagnosed as appendicitis [21, 33,

64]. In rare cases, infections with enteropathogenic *Yersinia,* and especially *Y. enterocolitica*, can cause reactive arthritis in humans [102].

To cause disease, enteropathogenic *Yersinia* first adheres and attaches to the specialized M-cells (Microfold cells) present in the small intestine. (Figure 1) The M-cells are specialized epithelial cells only present in the follicle associated epithelia that overlay lymphoid tissue [29, 179]. Larger aggregates of lymphoid tissue are organized into Peyer´s patches. M-cells sample foreign material and microorganisms from the inside of the small intestine and deliver them to the immune cells present in the underlying lymphoid tissue [8, 150, 222]. This sampling mechanism is exploited by a number of pathogens. Enteropathogenic *Yersinia* binds β1-integrins on the apical surface of the M-cell, via the adhesins Invasin and YadA, to mediate transport to the underlying lymphoid tissue (Leong, Fournier et al. 1990)[184, 213]. Once delivered within the Peyer´s patch, most other bacteria would rapidly be ingested by professional phagocytes. *Yersinia* is however equipped with effector proteins delivered via the type 3 secretion system (T3SS) that prevents bacterial uptake. Thus, *Yersinia* replicate as extracellular aggregates within necrotic lesions. All pathogenic *Yersinia* also have the ability to survive and replicate within macrophages, but the importance of intracellular proliferation is not fully understood. [194] Bacteria can spread from the Peyer´s patches and reach the mesenteric lymph node (MLN). In rare cases, *Y. pseudotuberculosis* can enter the bloodstream and cause septicaemia in humans [154].

In rodents, enteropathogenic *Yersinia* causes a lethal systemic infection that resembles plague. In mice, bacteria spread from the MLN to the liver and spleen and hence establishe a systemic infection.

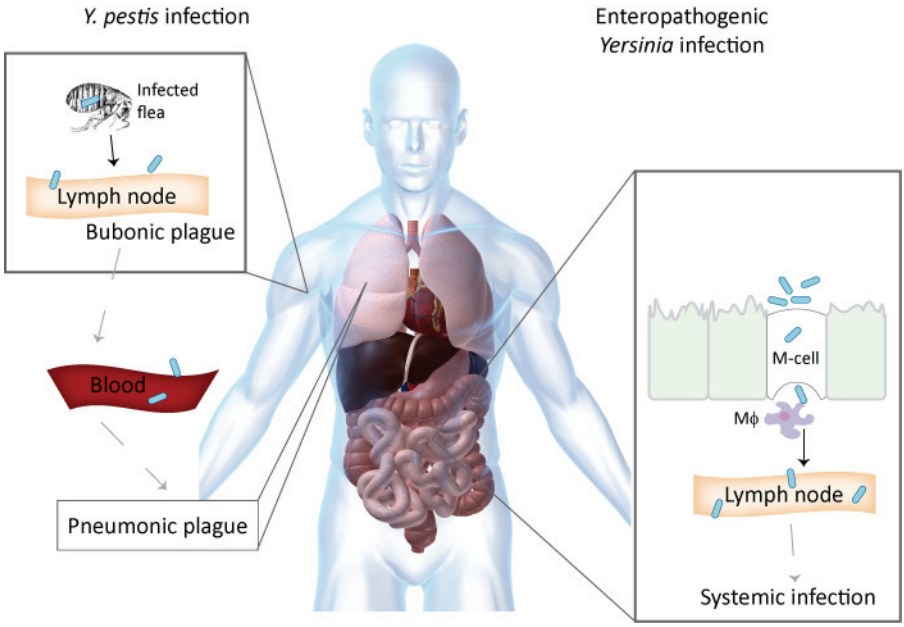

Figure 1: The different infection routes for pathogenic *Yersinia*. Adopted from [259]

The virulence plasmid pYV encodes a type 3 secretion system (T3SS)

Crucial for the disease causing ability of bacteria is the ability to transport virulence associated proteins from the inside of the bacterium where they are made to their intended site of action. To execute this task, there are a large number of dedicated bacterial secretion systems.

One of these secretion systems, first identified in *Yersinia*, is denoted the type three secretion system (T3SS) [63, 232]. Over 25 different Gram-negative bacterial species are equipped with one or several T3SS and these species can be divided into seven subfamilies.

As shown in table 1, species encoding for T3SS includes strict animal pathogens as well as plant pathogens or symbionts. The T3SS consists of a protein secretion apparatus, often called the injectisome due to the structural resemblance of a syringe. The secretion apparatus is responsible for the delivery of a diverse set of effector proteins. The effectors are believed to be delivered directly into the target cell by the injectisome. Over 100 different effectors have been identified and one species often delivers from 6 to 20 proteins. Depending on the life style of the bacterium, the effectors have different functions, as preventing uptake by phagocytes or promoting uptake by non-phagocytic cells or modulating pro-inflammatory signalling and more. [59, 87, 115, 126, 240]

T3SS family	Species	Description
Ysc	Pathogenic *Yersinia*	Human pathogen
	Pseudomonas aeruginosa	Potential human pathogen
	Aeromonas salmonicida	Fish pathogen
	Photorabdus luminescens	Mutualistic bacteria
	Vibrio parahaemolyticus	Human pathogen
	Bordetella pertussis	Human pathogen
	Desulfovibrio vulgaris	Environmental bacteria
SPI-I	*Salmonella enterica*	Human pathogen
	Shigella flexneri	Human pathogen
	Burkholderia pseudomallei	Human pathogen
	Chromobacterium violaceum	Human pathogen
	Sodalis glossinidius	Symbiont
	Yersinia enterocolitica	Human pathogen
SPI-II	*Escherichia coli* (EPEC,EHEC)	Human pathogen
	Salmonella enterica	Human pathogen
	Citrobacter rodentium	Mouse pathogen
	Chromobacterium violaceum	Human pathogen
	Edwardsiella tarda	Human pathogen
	Yersinia pestis	Human pathogen
	Yersinia pseudotuberculosis	Human pathogen
Clamydiales	*Chlamydia trachomatis*	Human pathogen
	Chlamydia pneumoniae	Human pathogen
Hrp-I	*Pseudomonas syringae*	Plant pathogen
	Erwinia amylovora	Plant pathogen
	Pantoea agglomerans	Commensal
	Vibrio parahaemolyticus	Human pathogen
Hrp-II	*Burkholderia pseudomallei*	Human pathogen
	Ralstonia solanacearum	Plant pathogen
	Xanthomonas campestris	Plant pathogen
Rhizobium	*Mesorhizobium loti*	Plant symbiont
	Rhizobium	Plant symbiont

Table 1: The seven T3SS families. Adopted from [59]

In *Yersinia*, the T3SS is encoded on the 70 kB pYV virulence plasmid, shared by the pathogenic species. The plasmid encodes for the structural components of the system, as well as the virulence-associated effector proteins delivered, or translocated, into the host. Also present on the plasmid are genes encoding for the translocator proteins and chaperones that are required for the effector translocation process. Further, the plasmid encodes for regulatory elements controlling T3SS assembly and activation.[62]

When discussing the concept of T3SS, it is important to distinguish between the two processes of secretion and translocation. Secretion refers to the active transport of proteins from the bacterial cytosol, over the inner and outer membrane, to the bacterial surface or the supernatant. The term

translocation describes the transport from the inside of the bacterium directly across the eukaryotic membrane. Even though translocation requires the process of secretion, these processes can be separated. Effector proteins often have separate secretion and translocation domains, suggesting that information required for secretion differs from information required for translocation. Further, translocation occurs only upon target cell contact, while secretion can be induced *in vitro*.

Environmental regulation of the T3SS

A successful infection would require the bacterium to sense and interpret its surrounding environment, so virulence genes can be turned on or off when appropriate. The *Yersinia* T3SS genes are under strict regulation, controlled by several different environmental signals.

Temperature regulation- positive regulation of the T3SS

Temperature within the host is often different compared to the temperature outside of the host. Therefore, many bacteria use temperature or changes in temperature, as a key regulator of virulence gene expression.

Yersinia is not an exception, and the T3SS is controlled by the temperature induced master regulator LcrF (Low calcium response F). LcrF (VirF in Y. *enterocolitica*) belongs to the family of AraC transcriptional regulators [57]. When *Yersinia* is outside the mammalian host, the T3SS is inactive. Upon infection, and a temperature shift to 37°C, the T3SS is activated by LcrF binding to T3SS gene promoter regions. [144, 165]

The low calcium response- regulation by calcium

Another way for bacteria to sense the environment is to monitor the level of divalent cations. The concentration of cations often differs from extracellular to intracellular compartments and can hence be exploited as a localization cue. In this way, *Salmonella typhimurium* utilizes the levels of magnesium ions as a mean for localization and regulation of different virulence genes[103].

In *Yersinia*, and in *Pseudomonas aeruginosa*, the T3SS genes are at least *in vitro* controlled by the presence of calcium in the growth medium [128, 243]. Early observations demonstrated that *Yersinia* stopped growing when calcium was depleted from the growth medium. Initially, the growth defect was interpreted as a metabolic requirement for calcium. [119, 143] Later it was clear that the growth arrest coincided with the induction of the T3SS, and that the growth arrest most likely was due to the massive cost of producing and secreting all T3SS related proteins. [32, 85, 117, 231, 265] The exact mechanism behind the low calcium response (LCR) remains clouded. However, it is a useful tool for mimicking *in vivo* cell contact. Further, the

LCR has been important for the characterization of T3SS mutants. In respect to their LCR, mutants can be divided into three groups.
Wt *Yersinia* requires calcium for growth at 37°C. Depletion of calcium results in massive secretion of T3SS substrates into the growth medium. This phenotype has therefore been coined Calcium Dependent (CD).
Mutants that are able to grow at 37°C irrespective of calcium levels are termed Calcium Independent (CI). These mutant strains often have a defect in positive regulation, or secretion system build up. Hence, they do not secrete substrates, rendering them avirulent in animal models. The third calcium phenotype is the Temperature Sensitive (TS) phenotype. Here, mutant strains unable to grow at 37°C irrespective of calcium levels are classified. These strains are constitutively producing the secreted proteins, due to defects in negative regulatory loops. [62, 265]

Regulation by target cell contact
Target cell contact is sensed by many pathogenic bacteria and utilized as an important regulatory cue. For *Yersinia,* target cell contact is a strict requirement for effector translocation *in vivo*. Cell contact has been demonstrated to induce transcription, secretion, and the subsequent translocation of effectors into the host cell. [187, 189, 201] How *Yersinia* senses target cells is unknown. The needle protein YscF is suggested to sense the presence of the target cell and to relay a signal back to the bacteria. For the *in vitro* mimic of cell contact, YscF is suggested to interpret calcium levels. [68, 123, 239] The concept of target cell contact and regulation will be revisited in the Results and Discussion section.

Structural components of the T3SS- the injectisome

The T3SS secretion apparatus is one of the most complex multi-protein assemblies known in bacteria. The secretion apparatus of *Yersinia* is built up by approximately 25 Ysc (*Yersinia* secretion) proteins.[58, 59]

Genetic analysis has shown that the structural components of the T3SS shares ancestral origin with the flagella system [206]. The flagellum, besides providing motility, is also an export system that first secretes hook proteins followed by filaments [157, 158]. During evolution, the T3SS appears to have been acquired via horizontal gene transfer [99].

The structure of the secretion apparatus, or the injectisome, has been visualized by transmission electron microscopy (TEM) for *S. typhimurium*, *S. flexneri* and EPEC (Enteropathogenic *E. coli*) [142, 214, 237]. In common for these structures is two cylindrical structures anchored in the inner and outer membrane, joined together with a rod. Depending on bacteria, the basal body is completed with a hollow needle, a pilus or a filament. (Figure 2)

In *Yersinia,* the structure is completed by a straight hollow needle, consisting of multimerized YscF subunits. The average needle length is 60-80 nm with an external diameter of 6-7 nm and an inner diameter of 2 nm [123]. On every bacterium, approximately 50-100 needles are found. Substrates destined for secretion trough the needle are believed to travel unfolded, or at least partially unfolded, trough the hollow inner tube since the inner diameter do not encompass folded proteins [58]. For proper function, the needles must have a certain regulated length, which is controlled by the YscP protein. Deletions or insertions in YscP results in shorter or longer needles respectively. [134]

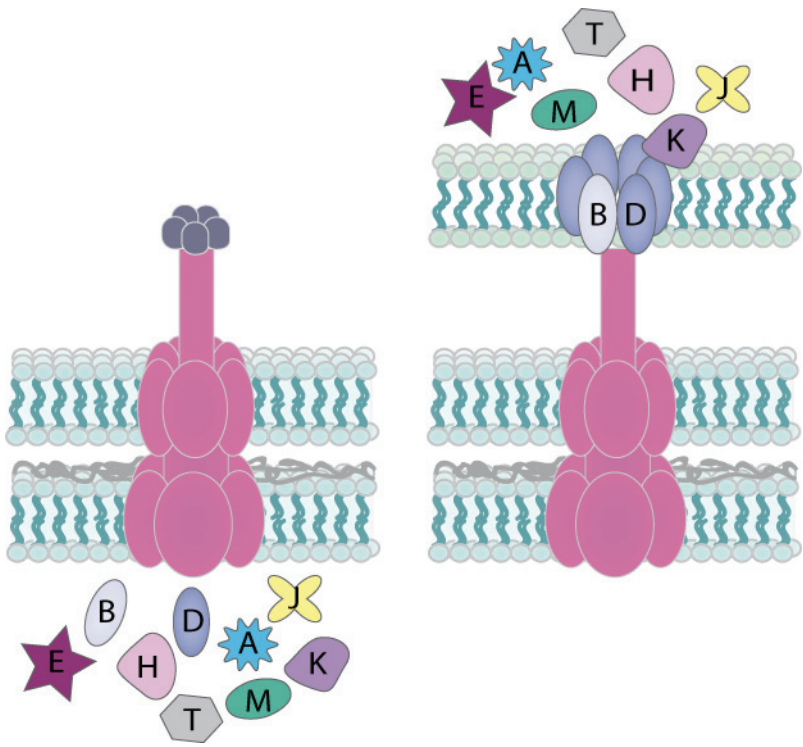

Figure 2: Schematic illustration of the T3SS of *Yersinia*. After a shift to host cell temperature but prior to cell contact the secretion apparatus is built up in the inner and outer membrane of the bacterium. The structure is completed with a needle with an LcrV complex on the tip. The secreted substrates, the translocators and effectors are kept within the bacterial cytosol. Upon target cell contact, the translocators YopB and YopD are presumed to be secreted first to form a pore in the eukaryotic cell membrane. Effector proteins are delivered directly into the cytoplasm via the needle and trough the pore. The effectors have different anti-host functions to ensure extracellular proliferation of the bacterium.

Control of secretion

The T3SS is an impressive virulence determinant with very powerful anti-host effects. This potent system is under tight regulatory control to prevent miss-directed induction and premature secretion. As described previously,

the system is tightly regulated on the induction and assembly level. But also the process of secretion and translocation of the effectors is tightly regulated.

Gate keeping of the secretion channel

Prior to cell contact, or in the presence of calcium, the Ysc secretion machinery is closed. Several proteins are suggested to be involved in blocking this channel.

YopN (*Yersinia* outer protein N) is crucial to prevent premature secretion. Two models exist for how YopN controls secretion. YopN was found to be localized to the bacterial surface prior to host cell contact [86]. A *yopN* mutant secretes Yop substrates into the supernatant regardless of cell contact or calcium levels. This is in contrast to wild-type *Yersinia* that displays what is known as polarized translocation, where no effectors are found in the surrounding media. Since the polarized translocation is lost in the *yopN* mutant, YopN might act as an outer gate keeper of the T3SS channel. According to this hypothesis, YopN would be secreted upon target cell contact and thus removing the plug of the T3S machinery, allowing for effector secretion.

The other hypothesis states that YopN prior to induction of secretion is kept in a complex consisting of the two chaperones of YopN, SycN and YscB, as well as TyeA in the bacterial cytoplasm. YopN can not be secreted when bound to TyeA, and is in this model believed to plug the secretion channel from the inside. Upon cell contact, TyeA would disassociate from the complex, releasing YopN for secretion and thereby the secretion channel is opened. [51, 67, 130, 131]

Another protein important for preventing premature secretion is LcrG. LcrG is postulated to act as an inner gate keeper, blocking the Ysc secretion apparatus either directly or indirectly. According to the titration model, YopN would be secreted upon cell contact, allowing for secretion of LcrQ (see next section). Upon LcrQ secretion, the levels of LcrV increase and LcrV can then form a complex with LcrG [69]. Formation of this complex will titre away LcrG from blocking the secretion channel, allowing for full induction and secretion of Yops.[159, 176]

A negative regulatory loop controlling secretion

Secretion is not only controlled by proteins blocking the secretion channel. During non-permissive secretion conditions, a negative feedback mechanism blocks transcription of *yop* genes (The secreted substrates are designated Yops). This negative regulatory loop involves the LcrQ protein [196]. LcrQ accumulates in the cytoplasm during non-permissive conditions and is part of a tripartite complex together with YopD and LcrH. This complex can bind to 5´-untranslated regions of *yop* mRNA. Presumably, host cell contact

causes disassociation of this complex and LcrQ will be secreted, and the *yop* mRNA can be translated into Yop proteins. [10, 44-46, 89, 252, 260]

The secretion signal

The T3SS apparatus secretes several different substrates. According to the substrate specificity switch theory, external Ysc proteins, such as the needle protein YscF, are secreted first [77]. When the secretion apparatus is completed, the specificity of the apparatus switches to secretion of the translocator proteins followed by the effector proteins [3, 77]. To be recognized by the apparatus, substrates destined for secretion via the T3SS must encompass a specific secretion signal. Since a secretion apparatus from one bacterial species can secrete substrates originating from another species, the secretion signal seems to be conserved. Unlike substrates designated for Sec-dependent secretion, T3S substrates do not have an N-terminal signal sequence that later is cleaved off. [52, 60]

The secretion signal of T3S substrates is located in the very N-terminus of the protein. Despite much effort, the exact nature of the signal is unknown. Comparisons of the N-termini of different substrates do not reveal any conserved amino acids or structural motifs. The secretion signal for the effector protein YopE has been extensively studied using various fusion constructs. The 11 first amino acids are sufficient for secretion, but the nature of the signal is still under debate [210]. One hypothesis states that information for secretion is contained within the mRNA sequence [9]. The mRNA model is challenged by a more recent study. Here, the mRNA sequence was disrupted while the wild-type amino acid sequence was kept intact. This mRNA disruption did not block secretion [155]. Thus, the secretion signal appears to be contained within the amino acid sequence. Further, a second secretion signal is proposed to be located within chaperone binding region of the effectors [53, 147, 244]. The N-terminal secretion signal is sufficient for secretion, but translocation requires the presence of the second secretion signal.

Dedicated chaperones of the T3SS

Many of the secreted proteins of the T3SS rely on chaperones for proper function. The chaperones have been implicated to serve as secretion signals, to prevent premature association and aggregation, to infer stability, to prevent degradation, or to maintain effectors in a secretion competent, unfolded state. Chaperones are usually small, acidic cytoplasmic proteins that associate with one specific substrate. Despite low primary sequence identity, the structure of T3SS chaperones often adopts a similar globular fold. In *Yersinia,* the chaperones are called Syc proteins for specific *Yersinia* chaperone. [27, 50, 53, 61, 227]

The chaperones of the T3SS can be divided into three distinct classes depending on their cognate substrate. Class I bind to effectors, and can bind one (Class IA) or multiple effectors (Class IB). SycE, SycH, SycO and SycT of *Yersinia* are examples of class IA and they are the cognate chaperones of YopE, YopH, YpkA (YopO) and YopT respectively [84, 129, 151, 245]. SycE, first identified as YerA (*yopE*-regulating gene A), binds as a dimer to amino acids 15-75 of YopE in the bacterial cytosol. The crystal structure demonstrates that the chaperone binding region of YopE is wrapped around the SycE dimer. Upon YopE secretion, SycE is dissociated and remains in the cytosol. [27, 84, 210]

The effectors YopM and YopJ do not appear to require a cognate chaperone. The absence of a chaperone for YopM and YopJ implicates that the chaperones might infer a secretion hierarchy, however evidence are scarce. [27, 35] According to the secretion hierarchy theory, the translocators should be secreted first, followed by effectors equipped with chaperones. Lastly secreted would be effectors lacking a chaperone.

Class II chaperones associate to translocator proteins, exemplified by LcrH (SycD) that binds to both YopB and YopD [245]. Interestingly, translocator chaperones often have an additional role in regulation. [89, 260].

In the last class, class III, chaperones of the flagella system are grouped, since the flagella system is evolutionary related to the T3SS [25]. [181]

The secreted proteins

After complete assembly of the T3SS apparatus, and upon target cell contact, the T3S system starts to secrete two sets of substrates, the translocator proteins and the effectors. According to the above mentioned secretion hierarchy theory, translocator proteins are assumed to be secreted before the effector proteins. The secreted substrates are collectively designated Yops for *Yersinia* outer proteins [31]. Initially, the Yops were detected in the outer membrane of *Y. pseudotuberculosis*, but later they were found to also be secreted [63].

The translocator proteins YopB, YopD and LcrV

YopB, YopD and LcrV are the translocators of *Yersinia* and they are all encoded on the same operon. [26, 108, 190, 225]

YopB and YopD

In related T3SS from other bacteria, YopB and YopD protein analogs are present, suggesting that the role for the translocators is preserved. The analogs are not related in sequence, but rather share structural similarities.

YopB and YopD both contain hydrophobic domains and are hence predicted to be membrane spanning proteins [108]. They have been

demonstrated to form pores in artificial membranes as well as in membranes of red blood cells and macrophages [39, 175, 238]. However, this pore has not yet been visualized *in vivo*, and the distinction between real pore forming activity and general lysis is difficult.

What however is clear is that both YopB and YopD are required for the translocation process. No translocation occurs in *yopB* or *yopD* mutants, rendering them avirulent in the mice model. YopB and YopD are also localized in the interface between the bacterium and the target cell during infection which is consistent with a role in translocation process. [109, 201, 225] Both YopB and YopD activity depend on the same chaperone, LcrH. In the absence of LcrH, no effector translocation occurs [174].

Besides being a translocator protein, YopD has multiple functions. YopD also has a role in regulation, in collaboration with the cognate chaperone LcrH and the negative regulatory element LcrQ [252]. Further, YopD has been demonstrated to be translocated into target cells. The role for translocated YopD remains enigmatic, since no effector function has been attributed to YopD. YopD can however interact with the effector YopE, and one hypothesis suggests that YopD might act as an intracellular chaperone to protect translocated YopE from degradation. [88, 112]

YopB also has additional functions besides the essential role in translocation. YopB induces a pro-inflammatory response resulting in the production of the cytokine IL-8 and activation of the MAP kinase Jnk, Erk and the small GTPase Ras. How, and why, YopB elicits this response, and the role for YopB in signalling versus the role in translocation, is not known. This pro-inflammatory signalling is later counteracted by the action of the translocated effectors YopE, YopH and YopJ.

LcrV

LcrV was discovered in the mid 1950-ies and was first recognized as a protective antigen against plague, so undoubtedly, LcrV is a major virulence marker in *Yersinia*. Later, LcrV was found to be located on the virulence plasmid, and a part of the low calcium response (LCR). [41]

The role for LcrV in the translocation process is a more recent discovery, where LcrV is required for the pore forming activity by YopB and YopD [101]. LcrV has been shown to be located on the outside of the bacterium before cell contact, and recently, LcrV was found to be localized to the tip of the YscF needle [170, 190].

Further, LcrV has been demonstrated to be immunomodulatory by down-regulating the pro-inflammatory cytokines TNFα and IFNγ and up-regulating the anti-inflammatory cytokine IL-10 [218]. LcrV is suggested to perform the immunomodulatory effects via a TLR-2 (Toll-like receptor) interaction on the host cell surface. However, the significance, or existence of a TLR-2 interaction, is under debate due to conflicting results [20, 192, 195].

The effector proteins

The T3SS of *Yersinia* is known to translocate six effector proteins into the target cell cytosol where they all have different anti-host functions.

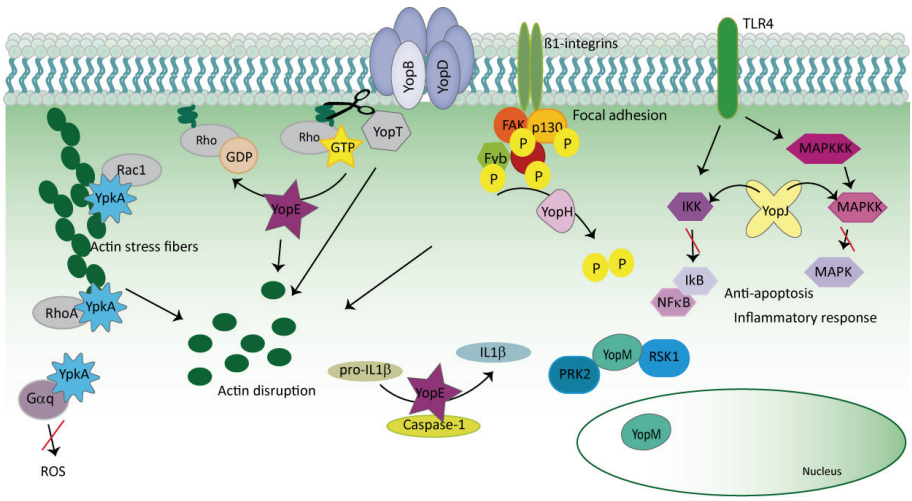

Figure 3: Scematic illustration of translocated Yop effector functions within the host cell

YopM

The role for YopM during infection remains elusive. All other effector Yops have been assigned an enzymatic function, which appears to be lacking in YopM. Despite the lack of enzymatic activity, YopM is clearly important for virulence for all pathogenic *Yersinia* strains [152, 160, 241].

YopM is a leucine-rich repeat (LRR) protein [79]. LRR is a structural motif involved in protein-protein interactions and LRR proteins adopt a horseshoe like fold where the inside bend form a binding surface for interacting proteins [23]. YopM interacts with two eukaryotic kinases, RSK1 (ribosomal protein S6 kinase) and PRK2 (protein kinase C-related kinase) [161]. RSK1 is a substrate of Erk in the MAPK (Mitogen-activated protein kinase) signal transduction pathway and hence involved in events regulating cell survival and growth [133]. PRK2 is involved in cytoskeletal changes, receptor tyrosine kinase signalling and activation of translation [254]. One hypothesis states that YopM recruits RSK1 and PRK2 to a novel signalling complex and alters signalling to modulate innate immune responses to promote bacterial virulence. This hypothesis is based on the finding that mice infected with a *yopM* mutant show higher levels of IFNγ and decreased levels of IL-10. IL-10 is an anti-inflammatory cytokine that dampens the production of both TNFα and IFNγ and is normally increased during wild-type *Yersinia* infection to dampen the response of the innate immune system. [163]

YopM is also, as the sole effector, localized to the nucleus of infected cells, and seem to exploit the endosomal vesicular trafficking system for the transport to the nucleus. However, the significance for nuclear localization is unknown.[24, 219]

YpkA

YpkA (YopO in *Y. enterocolitica*) is a multi-domain protein. The very N-terminus targets the protein to the inner surface of the target cell plasma membrane. In the C-terminal, a serine/threonine kinase domain is located. This region was identified due to the very high similarity with eukaryotic Ser/Thr kinases. (Hence the name YpkA for *Yersinia* protein kinase A). In the most C-terminal part, a GDI-like (Guanosine nucleotide dissociation inhibitor) domain is localized that interacts with the GDP-bound form of the small RhoGTPases RhoA and Rac1 and prevents their activation. The GDI domain is followed by an actin-binding domain that binds actin and auto-activates the kinase activity. [74, 135]

YpkA is essential for virulence and a kinase deficient mutant is attenuated [95]. Recently is has been demonstrated that YpkA targets Gαq proteins and thereby inhibits multiple signalling pathways. Gαq proteins couple with G protein-coupled receptors to convey multiple extracellular signals to the inside of the cell, hence playing a central regulatory role. Especially, the Gαq family stimulates PLC-β activity which will in the end generate the production of reactive oxygen species (ROS). Since YpkA inhibits the Gαq proteins, YpkA might inhibit ROS production. [173]

Previously, YpkA has been demonstrated to cause cell rounding and to disrupt actin stress fiber formation and to be involved in anti-phagocytosis. These functions are requiring both N- and C-terminal activities in concert.[104, 135, 251]

YopJ

Upon bacterial infection, the MAPK and NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) signalling pathways are rapidly activated. Activation of these pathways would result in activation of the innate immune system and cytokine production [248]. In *Yersinia* infections, these pathways are quickly inhibited by the YopJ protein (YopP in *Y. enterocolitica*). [178, 211] Besides inhibiting signalling pathways, YopJ has also been demonstrated to induce apoptosis in infected macrophages and dendritic cells. [30, 166]

The exact mechanism of YopJ has been enigmatic until recently when it was discovered that YopJ is an acetyltransferase. YopJ employs CoA (Acetyl-coenzyme A) as a cofactor to acetylate critical serine and threonine residues of MAPKK (Mitogen-activated protein kinase kinase). This acetylation prevents phosphorylation of these residues, hence blocking downstream

signalling. [171] YopJ also acetylates IKK (IκB kinase), a key player in the NFκB pathway [167].

Despite the role of YopJ as a key player in down-regulation of the pro-inflammatory response, the role in virulence is less clear. YopJ appears to be important only for virulence in oral infections with enteropathogenic *Yersinia*, where YopJ exerts its role in the later stages of systemic infections. [37, 148, 156, 169, 241, 266]

YopH

YopH is a potent tyrosine phosphatase. [105] Tyrosine phosphorylation is a reversible post-translational modification important for many cellular processes and it is controlled by tyrosine kinases and tyrosine phosphatases. YopH is localized to focal adhesions within infected cells [188]. Focal adhesions provide a link between the actin cytoskeleton and the extracellular matrix (ECM). They are large dynamic macromolecular assemblies that not only anchor the cell to the substratum, but also concentrate and transmit signals at the sites of integrin binding and clustering [73]. Since *Yersinia* is assumed to bind β1-integrins on target cells, it is not surprising that YopH targets phosphotyrosine proteins found in the focal adhesions [132]. Some of the reported targets include $p130^{Cas}$, focal adhesion kinase (Fak), paxillin, Fyn-binding protein (Fyb) and SKAP-HOMO. Inactivation of these targets leads to the disruption of integrin signalling, the focal adhesions, and the actin cytoskeleton and eventually disruption of phagocytosis. [70, 110, 186, 188] The effect of YopH is remarkably rapid, within 30 s after initial bacterial- cell contact, dephosphorylation of host proteins can be observed [12].

Besides dephosphorylation of focal adhesion proteins, YopH has additional functions. YopH has been demonstrated to block calcium release from infected neutrophils induced by bacterial attachment to β1-integrins [11]. Further, YopH suppresses the oxidative burst of infected macrophages, and is capable of inducing apoptosis in T-cells and to block T- and B-cell activation.[7, 208] However, it is unknown whether these latter effects are due to the phosphatase activity towards known targets or are due to activity towards unknown targets. It is also difficult to validate the involvement of YopH in adaptive immune responses *in vivo*, since a *yopH* mutant fails to cause systemic infections in the mouse model of disease. [248]

YopE

The YopE protein is essential for virulence in *Yersinia* [83]. YopE is a GTPase activating protein (GAP) towards members of the family of small RhoGTPases. Other bacterial GAP effector proteins are found in *Pseudomonas aeruginosa* and in *Salmonella enterica*.

YopE mimics functionally, but not structurally, eukaryotic GAP proteins, and interacts with the target RhoGTPase by inserting an arginine finger motif into the catalytic site of the GTPase. This speeds up the hydrolysis of GTP into GDP and inactivates the GTPase and hence blocks downstream effects and signalling. [209, 262]

In vitro, YopE has been demonstrated to target the RhoGTPases Rac1, RhoA and Cdc42 [14, 28, 258]. Via the GAP activity, YopE disrupts the actin cytoskeleton of the infected cell. This phenotype is referred to as cytotoxicity, and YopE mediates cytotoxicity towards a wide range of cell types originating from multiple organisms [36, 200]. When the arginine finger motif, the arginine at position 144, is mutated into an alanine, cytotoxicity is abolished and the mutant is avirulent [4, 28, 258].

YopE, together with YopH, YpkA and YopT, contributes to the anti-phagocytic effect of *Y. pseudotuberculosis* to counteract actions of the innate immunity in epithelial cells, macrophages and neutrophils [81, 104, 202].

YopE is also involved in prevention of pore formation. According to the microinjection hypothesis for T3SS mediated translocation, the translocator proteins form a pore in the target cell membrane. A multi-effector mutant displays higher pore-forming activities than wild-type, suggesting that effectors would block the translocation pore. As confirmation of this hypothesis, the pore-forming activity was reduced when YopE was over-expressed in the multi-effector background.[109] When pore-forming was examined by measuring LDH release from infected cells, YopE was found to be responsible for pore formation prevention. Further, the blockage was mediated by the GAP activity, since a GAP deficient mutant could not prevent pore formation. [247]

Further, the GAP activity of YopE also influences cytokine production, where caspase-1 mediated maturation of prointerleukinβ in macrophages is inhibited. This inhibition is mediated via the inactivation of Rac1 [212]. In epithelial cells, YopE has been demonstrated to inhibit pro-inflammatory IL-8 release.[250]

Translocated YopE localizes to the perinuclear membrane region in mammalian cells [201]. Important for this localization is the membrane localization domain (MLD). The MLD covers amino acids 54-75, which is a region overlapping the YerA chaperone binding domain. [140] Besides providing proper localization, potential additional roles for the MLD of YopE are not studied.

Clearly, YopE contributes to virulence by multiple mechanisms, mediated by itself or in synergy with other effectors. Likely, there exist additional, still unknown functions of translocated YopE. The YopE protein is therefore the subject for further investigations, as presented in the Results and Discussion section.

YopT

YopT, as YopE, targets the small RhoGTPases. YopT is a cysteine protease that cleaves off the lipid modification of the target RhoGTPase [216]. The lipid modification mediates membrane attachment of the RhoGTPase and is required for the interaction with effectors and regulators. After cleavage by YopT, the RhoGTPase is no longer associated with the membrane and is thereby inactivated.

YopT has been shown to inactivate Rac1, RhoA and Cdc42 *in vitro* [215, 216], however RhoA appears to be the preferred *in vivo* target [2]. Recently, RhoG has also been demonstrated to be a target for proteolytic cleavage by YopT. RhoG targeting is interesting, since RhoG is located in the close proximity within β1-integrin receptors to which enteropathogenic *Yersinia* bind. This inactivation of RhoG leads to disruption of actin structures and impaired phagocytosis. [168]

A *yopT* mutant has no effect on virulence in *Y. enterocolitica*. It appears as if the YopT function is redundant to the more potent effects of YopE. Virulence can only be partially restored by introducing YopT in a *yopET* mutant in *Y. pseudotuberculosis*. [249]

Some *Y. pseudotuberculosis* strains have inactivated the *yopT* gene. A *yopT* mutant was found to be more effective in colonization of certain tissues and therefore it might be advantageous to delete the gene. [241]

YopK- a translocated regulator

YopK (YopQ in *Y. enterocolitica*) is an enigmatic protein. YopK is only present in the *Yersinia* species, with no known homolog in any other bacteria expressing a T3SS. YopK has recently been identified to be translocated, but has no effector function assigned [97]. Rather, YopK appears to regulate Yop effector translocation. A *yopK* mutant strain overtranslocates effectors into the target cell, while a strain over-expressing YopK shows the opposite, with reduced translocation of effectors. A *yopK* mutant is avirulent in the mice model and the infection is cleared already at stage within the Peyer´s patches. YopK is expressed at considerable lower levels than the other effector Yops which perhaps is a reflection of a role in regulation, rather than acting as an effector.

YopK has been implicated to regulate the pore forming process of YopB and YopD. In infected erythrocytes, YopK modulates the size of the pore, and a *yopK* mutant display higher pore forming activities, consistent with the overtranslocating phenotype.[124, 125]

Other bacterial GAP effector proteins besides YopE

ExoS and ExoT from Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative opportunistic human pathogen and is the most common hospital acquired infection. Immuno-compromised patients, such as people suffering from cystic fibrosis, cancer, HIV, or severe burn wounds, are at high risk for *P. aeruginosa* infections.

P. aeruginosa encodes for a T3SS similar to the Ysc system of *Yersinia*, where substrates of *Pseudomonas* can be secreted by *Yersinia*, and vice versa [92]. The T3SS is dedicated to translocate four effectors into the target cell; ExoS, ExoT, ExoU and ExoY.[114] ExoU is a very potent phospholipase that causes severe cytotoxicity of infected cells [82, 207]. ExoY is an adenylate cyclase that elevates the levels of cAMP within the target cell, causing actin rearrangements [264].

ExoS and ExoT are two highly similar proteins. In fact, they are so similar that ExoT was first assumed to be a pre-form of ExoS. [263] Both are bi-functional toxins, with an N-terminal GAP activity and a C-terminal ADP-ribosyltransferase activity. ADP-ribosylation is the covalent transfer of ADP-ribose from NAD+ to host proteins, catalyzed by bacterial toxins which block the interaction between the small Rho GTP-binding protein and their GEF (Guanine Exchange Factors) proteins. This prevents interaction with downstream effectors [128]. ExoS and ExoT display the same RhoGAP specificity, targeting Rac1, RhoA and Cdc42 both *in vivo* and *in vitro*. [137, 141] The GAP activity disrupts the actin cytoskeleton and the phagocytic process [183]. In ADP-ribosylation, they display different specificities, where ExoS targets multiple proteins including Ras and Rab proteins while ExoT seem to target only Crk-I and Crk-II [55, 56, 235].

ExoS has a membrane localization domain (MLD) that covers amino acids 51-77 [182, 267]. The MLD do not influence T3SS secretion or translocation of ExoS, however, it affects the intracellular localization and influences both RhoGAP activity and ADP-r activity. Clearly, correct intracellular localization is important for proper activity [182, 269]. ExoS, via the MLD, travels from the plasma membrane to the perinuclear region of the cell. ExoS co-immunoprecipitates with Rab5, Rab6 and Rab9 which suggests that ExoS exploits endosomal trafficking pathways to reach the perinuclear region. The Rab proteins with 60 members also belong to the family of small GTPases, and are involved in endocytosis and exocytosis [230]. ExoS displays ADPr activity towards Rab5, Rab7, Rab8 and Rab11 to inhibit host cell vesicle trafficking and endocytosis.[71, 72, 268] By sequence alignment, and the similar host localization pattern, ExoT is also assumed to posses a similar MLD [22].

SptP of Salmonella enterica

Salmonella enterica has a different life style compared to *Yersinia*. Instead of preventing uptake, *Salmonella* is an intracellular pathogen that invades non-phagocytic cells. The invasion is a unique feature, where *Salmonella* induces ruffles in non-phagocytic cells that lead to bacterial internalization by macropinocytosis. Once internalized, bacteria multiply within the SCV (*Salmonella* containing vacuole) that traffics to a perinuclear region that provides a niche for intracellular bacterial replication.

Salmonella encodes for two separate T3SS, SPI-1 (*Salmonella* pathogenicity island) and SPI-2. SPI-1 is important for the early events of pathogen entry, while SPI-2 is important for intracellular survival within the SCV. [127, 162]

SptP is part of the SPI-1 and inactivates RhoGTPases to reverse the membrane ruffling induced by the effectors SopE, SopE2, and SopB [96, 111, 229, 270]. SptP is demonstrated to be a GAP protein towards Rac1 and Cdc42. By inactivating Rac1 and Cdc42, SptP down-regulates the activation of MAP kinases and thereby blocks the production of pro-inflammatory cytokines.[93, 226]

SptP, as ExoS and ExoT, is a bifunctional toxin. The C-terminal possesses a tyrosine phosphatase activity, resembling the activity of YopH. [136]

The family of small RhoGTPases- attractive targets for pathogens

Classical RhoGTPases	Members
Cdc42	Cdc42, TC10, TCL
Rac	Rac1, Rac2, Rac3, RhoG
Rho	RhoA, RhoB, RhoC
RhoD/F	RhoD, RhoF (Rif)
Atypical RhoGTPases	
RhoBTB	RhoBTB1, RhoBTB2
RhoH	RhoH
RhoU/V	RhoU (Wrch1), RhoV (Chp)
Rnd	Rnd1, Rnd2, Rnd3

Table 2: The 20 human RhoGTPases[19, 34]

The family of small RhoGTPases has twenty members in humans and belongs to the large Ras superfamily. (Table 3) RhoGTPases are essential regulators in many important cellular processes, such as cell division, actin cytoskeleton regulation, gene transcription control, apoptosis and endocytosis. Their role as regulators of so many crucial cellular events makes them very attractive as targets for pathogens. Interestingly, bacterial factors that affect Rho proteins are only found in Gram-negative bacteria [149].

The RhoGTPases can be subdivided into two groups depending on how they themselves are regulated. The classical Rho GTPases are regulated by the GTPase cycle described below (figure 3). The atypical RhoGTPases are not regulated by this simple mechanism, instead they appear to be regulated by the level of expression, by protein-protein interactions or by degradation. [19]

The classical RhoGTPases act as molecular switches, being active when bound to GTP and inactive when bound to GDP. The activity of RhoGTPases is highly regulated and the switch is mainly controlled by three sets of regulatory proteins. GDI (Guanine nucleotide dissociation inhibitors) proteins sequester GDP-bound RhoGTPases from the cell membrane and keep the protein inactive in the cytosol. The bound GDI masks the isoprenyl group. An isoprenyl group is found in the C-terminal of all RhoGTPases which allows for membrane association that is essential for activity. Several stimulatory molecules cause the release of the GDI from the Rho protein, exposing the isoprenyl group which redirects the protein to the membrane. Once the GDI is released, GEFs (Guanine Exchange Factor) can associate with the RhoGTPase. GEF proteins accelerate the exchange of GDP into GTP. When the GTPase is activated, the conformation of two regions, switch I and switch II, is changed to provide an interaction surface for downstream effector proteins. [76] There are a vast number of downstream effectors described, most being protein or lipid kinases. The RhoGTPases are switched off by GAPs (GTPase Activating Protein). GAP proteins increase the low intrinsic hydrolysis of the bound GTP into GDP by introducing a catalytic arginine into the active site.

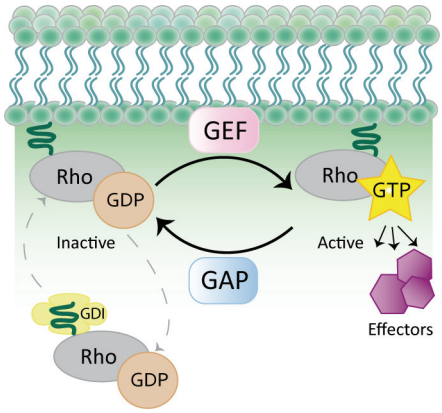

Figure 3: The RhoGTPases. The classical RhoGTPases act as molecular switches, controlling many crucial events in the eukaryotic cell. The switch function is dependent on a GDP-GTP cycle controlled by GEF, GAP and GDI proteins.

Since the discovery of the *Rho* gene in 1985, most focus so far has been on RhoA, Cdc42 and Rac1. RhoA is important for stress fiber and focal adhesion formation, Cdc42 is crucial for filopodia formation. Filopodia, or microspikes, are thin cytoplasmic projections that extend from the leading edge of the cell. Rac1 is regulating the lamellipodium which also is a cytoskeletal projection found in the leading edge of migrating cells. [34, 220]

Cell culture and animal models of *Yersinia* infection

Cell culture models used for Yersinia infections

To study bacterial virulence, *in vitro* cell infection models are extensively used. Much of our basal knowledge of bacterial virulence and the interaction between pathogen and host are deduced from cell culture experiments.

For *in vitro* studies, numerous cell lines have been used for *Yersinia* infections. Enteropathogenic *Yersinia* binds β1-integrins present on the

target cell for bacterial attachment and subsequent effector translocation. β1-integrins are present on a wide variety of cells which perhaps explains the successful translocation of Yop effectors into so many different cell types. [36] Epithelial cells, predominantly HeLa cells, have been extensively used for studying the anti-host effects of Yop effectors, bacterial internalization, and cytokine response [15, 107, 187, 200, 201]. To study inhibition of phagocytosis, respiratory burst, and TNFα and IFNγ, as well as apoptosis induction, macrophages have been exploited [113, 199, 203, 204]. Polymorphonuclear leukocytes (PMNs) have been a tool for studying resistance towards phagocytosis, killing and antimicrobial peptides [153, 255, 256].

During *in vivo* infection of mice, *Y. pseudotuberculosis* displays specificity for phagocytic cells and especially for neutrophils present in the Peyer´s patches, MLN and spleens of infected mice. If neutrophils are depleted, the overall translocation is reduced. [75]

Fish scale keratocytes as a putative model for Y. pseudotuberculosis infections

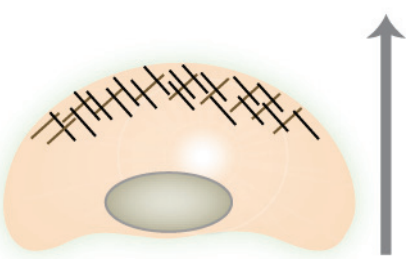

Figure 4: An illustration of a single moving keratocyte. The single moving keratocyte often adopts the "inverted canoe shape". The rapid movement of the cell occurs in the direction indicated by the arrow.

The fish scale keratocytes constitute the main structural component of the fish epidermis. They are known under many names, such as Malphigian cells, pavement cells, squamous cells or filamentous containing cells. [43, 118, 197, 217, 246] Keratocytes are involved in the quick-wound repair mechanism, where they migrate and rapidly cover wounds on the fish surface [42, 191]. Besides providing a mechanical barrier against opportunistic infections, the cells have been demonstrated to be phagocytic, engulfing Gram-negative bacteria and latex beads [16, 17]. Keratocytes display an unusual high motility and therefore they have been used as a model system for actin based motility and locomotion [138, 145, 236, 253]. Basically, the cell is constituted of a large extended two-dimensional actin network, the lamellipodium, and a nucleus located in the rear of the cell body. The lamellipodium is controlled by the small RhoGTPases, and especially members of the Rac subgroup, Rac1, Rac2, Rac3 and RhoG [18].

The presence of this large extended actin network, in combination with the intrinsic high motility, makes the keratocytes interesting as a putative model for *Yersinia* infections.

Non-mammalian Yersinia models

The use of non-mammalian models for bacterial infection is becoming more and more attractive. By using non-mammalian models it is possible to study more complex interactions between host and pathogen then what is possible in a cell culture system. The use of non-mammalian models is often cost-effective and is not associated with ethical constraints. Usually, it is possible to include a larger number of animals, the model is often genetically tractable and mutant collections are available.[228]

The nematode *C. elegans* has been widely used to study bacterial virulence factors, and two models exist for *Yersinia* infections. One major disadvantage in using *C. elegans* is the fact that the worms do not survive at 37°C. Therefore, the effect of virulence factors only expressed at 37°C might be undetected.[66, 233]

The use of insects and larvae as model systems has the advantage that they survive at 37°C. Further, the innate immune system of insects is rather similar to the innate immune system of humans and insects possess a phagocytic-like cell type called the haemocyte. The haemocyte, like phagocytes, is capable of ingesting bacterial pathogens and generates bactericidal compounds. [172]

Larvae of the wax moth, *Galleria mellonella*, have been used as a model system for *Y. pseudotuberculosis*. Larvae are susceptible to infection and bacteria are found within the haemocyte, suggesting that this model can be useful for studying factors important for intracellular survival of *Yersinia*.[49]

The amoeba *Dictyostelium discoideum* has phagocytic ability and the mechanism of phagocytosis is well conserved compared to humans. The amoeba is interesting for *Yersinia* pathogenesis since a large part of the effectors is directed towards actin cytoskeleton and hence phagocytosis prevention. [257]

Mammalian Yersinia models

During the decades, numerous animal models have been established for *Yersinia* infections. To study the potential of vaccine candidates for *Y. pestis* non-human primates have been used [78]. Animals more commonly used in *Yersinia* research are small rodents, including mice, rats, guinea pigs and rabbits. The small rodents have been an invaluable tool for our understanding of basic molecular mechanisms in bacterial pathogenicity. All pathogenic *Yersinia* cause a systemic lethal infection in mice that resembles the human plague disease. Therefore, *Y. pseudotuberculosis* or *Y. enterocolitica* are relevant models for studying plague, without needing to use the human lethal *Y. pestis* [242]. Mouse models of *Yersinia* infections have elucidated many important processes such as the infectious route, the subsequent bacterial dissemination, host responses and more.[116]

In contrast to many animal models for human enteropathogens, experimental enteropathogenic *Yersinia* infections in rodents resemble well the human yersiniosis disease. Rabbits are a suitable model for studying *Yersinia* induced enteritis. The secondary reactive arthritis can be studied using susceptible rats.

For animal research, the IVIS (*In vivo* imaging system) is a valuable invention. The IVIS technology allows for real-time imaging of events, such as a progressing bacterial infection. IVIS captures photons of light emitted from live bacteria that have been engineered to produce bioluminescence. This allows for non-invasive monitoring of the infected animals. Thus, this technology reduces the number of animals used and thereby also reduces the costs significantly. The data collected is also highly reliable, since the same animal is followed throughout the infection period.

Aim of thesis

The major aim of this study was to investigate the functions of translocated YopE in *Yersinia pseudotuberculosis*. YopE is a GTPase Activating Protein (GAP) and is an essential effector required for virulence of the bacterium.
A second aim was to examine the potential of using fish scale derived keratocytes as a novel model system for *Y. pseudotuberculosis* infections.

Results & Discussion

The YopE protein of *Yersinia pseudotuberculosis*

The YopE protein is one of the translocated effectors of *Yersinia*. YopE mediates a cytotoxic effect towards host cells and contributes to the overall anti-phagocytic effect. YopE has been extensively studied and the protein of 219 amino acids can be functionally divided into several distinct domains. (Figure 5).

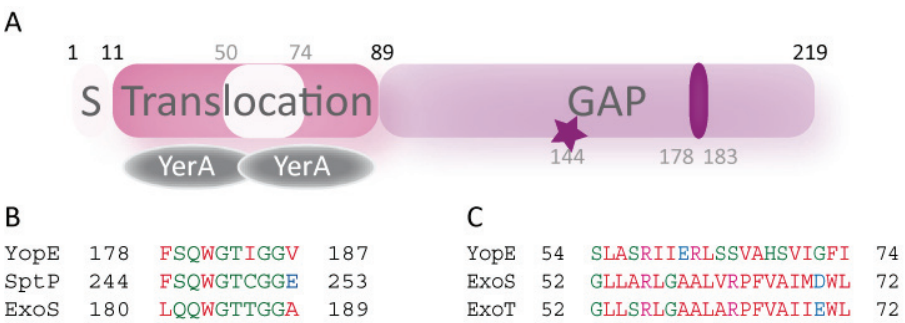

Figure 5A) The YopE protein. The first 11 amino acids are required for secretion. The translocation domain is required for translocation and encompasses the binding site of the cognate chaperone YerA. Within the translocation domain, the membrane localization domain is found (aa 50-74). Last is the effector domain. The R at position 144 is essential for GAP activity. Amino acids 178-183 are implicated in the substrate recognition process and is hence called the homology domain. B) Multiple sequence alignment of the homology domain from the related GAP proteins YopE, SptP and ExoS. C) Multiple sequence alignment of the membrane localization domain of YopE, ExoS and ExoT. *(Color code: red- hydrophobic residues, blue- acidic, magenta- basic, green- hydroxyl, amine or basic)*

The 11 first amino acids are required for secretion of YopE over the bacterial double membranes. The secretion sequence is followed by the translocation domain which encompasses the binding site for the cognate chaperone YerA. YerA is required for stability, secretion and translocation of YopE and binds YopE as a dimer covering amino acids 15-75 [27, 210]. Within the chaperone binding region, a membrane localization domain (MLD) has been identified. The MLD is important for correct intracellular membrane localization of YopE within the eukaryotic cell. [140] Last is the GAP (GTPase Activating

Protein) domain that encodes for the effector activity of YopE. Before this study was initiated, YopE was known to target the small RhoGTPases Rac1, RhoA and Cdc42 *in vitro* [14, 28, 258]. The RhoGTPases are attractive targets for bacterial pathogens since they are important regulators of many crucial cellular processes. The activity of RhoGTPases is controlled by a GTP-GDP cycle. When bound to GTP, the Rho protein is active and can interact with multiple downstream effectors. [6] The GAP activity of YopE is dependent on the arginine residue at position 144, called the arginine finger motif. The arginine finger inserts into the catalytic site of the RhoGTPase and increases the hydrolysis of the bound GTP into GDP [76]. When R144 is mutated into an alanine, GAP activity is abolished and the mutant is avirulent in the mice model [4, 28].

Besides YopE, the effectors ExoS and ExoT from *P. aeruginosa* and SptP from *Salmonella enterica* are also GAP proteins targeting small RhoGTPases. The crystal structures of the GAP domains of YopE, ExoS and SptP have all been solved [80, 226, 261]. Despite low sequence homology, the structures superimpose remarkably well. The structure of SptP has been solved in complex with the *in vitro* substrate Rac1, which identified a stretch of amino acids in SptP putatively involved in the substrate recognition process [226]. This stretch of amino acids (consisting of amino acids 178-187 in YopE) is highly conserved for YopE, ExoS and SptP, implicating that they might be important for substrate recognition also in YopE. Due to the high identity, this stretch was termed the homology domain in Paper I. Further, this region (aa 166-180) was previously shown to be important for YopE *in vitro* GAP activity and HeLa cell cytotoxicity, showing the importance of these amino acids [5].

To examine the putative role for the homology domain in respect to substrate recognition and GAP activity, single alanine point mutations were created for amino acids 178-183 in YopE.

Point mutants of the homology domain show reduced GAP activity *in vitro*

In vitro GAP assays were performed to investigate if the point mutations within the homology domain affected the YopE GAP activity *per se*. *In vitro* GAP activity was measured as the hydrolysis of ^{32}P-GTP into GDP using purified proteins. Here, we used YopE constructs only consisting of the GAP domain with the single alanine point mutations, engineered with a His-tag for simpler purification.

The *in vitro* GAP activity towards all three reported targets of YopE, Rac1, RhoA and Cdc42 was examined, and the same result emerged for all three. Point mutants *yopE*F178A, *yopE*S179A and *yopE*Q180A were still able to increase GTPase activity towards the examined RhoGTPases, albeit at 100-fold reduced levels compared to wild-type YopE. Thus, these amino acids did

not appear to be directly involved in substrate specificity *in vitro*, however they were reduced in activity. In contrast, *yopE*W181A did not at all increase GTPase activity, and hence displayed the same phenotype as the GAP deficient *yopE*R144A mutant. (Paper I, fig. 6)
Unfortunate for a complete analysis, we were unable to purify the YopE variants *yopE*G182A and *yopE*T183A. Nonetheless, the results obtained demonstrate that the region of amino acids 178-181 of YopE is central for *in vitro* GAP activity towards Rac1, RhoA and Cdc42.

YopE targets Rac1 and RhoA *in vivo*, but not Cdc42

YopE has previously been shown to target the small Rho proteins Rac1, RhoA and Cdc42 *in vitro* [14, 28, 258]. However, to gain deeper insight into the functional role of translocated YopE, the targets of YopE during infection were examined. Pull-downs of downstream targets of active forms of Rac1, RhoA and Cdc42 were performed in HeLa cells to investigate the *in vivo* targets of YopE.

Already 5 min post infection with wild-type *Y. pseudotuberculosis*, 90% of active Rac1 was abolished. As expected, HeLa cells infected with the GAP deficient *yopE*R144A mutant could not inactivate Rac1. For RhoA, the inactivation by wild-type bacteria was delayed compared to Rac1 and occurred 30 min post-infection. Thus, YopE appears to target Rac1 in the early immediate stage of infection and RhoA during the later stages. (Paper I, fig. 7)

No inactivation of Cdc42 was detected, which indicated that Cdc42 is not an *in vivo* target of YopE, at least not in the HeLa cell model. The discrepancy between *in vitro* and *in vivo* data shows that data obtained by *in vitro* methods are not always compatible with *in vivo* data. This further strengthens the importance of performing experiments in the most relevant biological model available.

The homology domain point mutants were employed in the pull-down assays to explore the involvement of the domain in substrate recognition. Interestingly, the *yopE*F178A and *yopE*W181A mutants had lost their ability to inactivate Rac1. *yopE*Q180A retained some ability to inactivate Rac1, while *yopE*S179A, *yopE*G182A and *yopE*T183A inactivated Rac1 at wild-type levels.

For RhoA, *yopE*S179A and *yopE*G182A were as efficient as wild-type in targeting RhoA and *yopE*F178A, *yopE*W181A and *yopE*T183A were as inefficient as *yopE*R144A. Again, also for RhoA, *yopE*Q180A displayed an intermediate phenotype. These pull-down assay results clearly demonstrates that the homology domain is involved in target recognition during *in vivo* conditions. One especially interesting result was that *yopE*T183A inactivated Rac1, but failed to target RhoA, showing that amino acid T183 is essential for RhoA recognition. (Paper I, fig. 8)

HeLa cell cytotoxicity can be disconnected from Rac1/RhoA activity *in vivo*

HeLa cell cytotoxicity is a hallmark effect caused by translocated YopE and is dependent on the arginine at position 144 [28, 200, 258]. Surprisingly, mutants devoid of GAP activity towards Rac1, RhoA and Cdc42 *in vitro* were still cytotoxic, suggesting that these three proteins can not be the sole targets responsible for cytotoxicity [5].

The homology domain point mutants were tested for cytotoxicity to investigate the connection between cytotoxicity and GAP activity further.

All point mutants, except *yopE*W181A, induced cytotoxicity to the same level as wild-type YopE. When *yopE*W181A was over-expressed, cytotoxicity was restored. Thus, cytotoxicity induced by the *yopE*W181A mutant is dependent on the level of YopE protein translocated. (Paper I, table 1)

In conclusion, several of the point mutants are still able to induce cytotoxicity but fail to inactivate Rac1 and/or RhoA *in vivo* further strengthen the notion that HeLa cell cytotoxicity can not solely by caused by RhoA or Rac1 inactivation

Rac1/RhoA activity is redundant for virulence in the mice model

Yersinia readily infects small rodents and therefore it is possible to use laboratory mice as a model system for infections.

The homology domain of YopE appeared to be important for substrate recognition. The relevance of the domain during *in vivo* infections was next examined. Mice were infected intraperitoneally with increasing doses (10^4-10^7 cfu/ml) (colony forming units) of the point mutants. An ID_{50} (Infectious dose affecting 50% of animals) was calculated, rather than the classical LD_{50} (Lethal dose killing 50% of animals). ID_{50} corresponds to the bacterial dose where 50% of the animals showed signs of severe disease and therefore were euthanized. Wild-type infected mice had an ID_{50} of $2{,}8*10^3$ bacteria, while the avirulent *yopE*R144A showed an ID_{50} of $1*10^7$ bacteria. The mutants *yopE*F178A and *yopE*S179A were as virulent as wild-type, with ID_{50}-values of $1{,}3*10^4$ and $1{,}1*10^4$ respectively. Virulence was defined as a value within five times of the wild-type ID_{50}. By that classification, *yopE*Q180A and *yopE*G182A were attenuated with values of $4{,}3*10^5$ and $9{,}2*10^4$ bacteria. *yopE*W181A ($1*10^7$) and *yopE*T183A ($8*10^6$) were as avirulent as the *yopE*R144A mutant.(Paper I, table 1)

The animal experiment demonstrates that additional targets must exist for YopE during *in vivo* infections since neither Rac1 nor RhoA inactivation is directly connected to mice virulence. This can be exemplified with the *yopE*F178A mutant that is as virulent as the wild-type, despite the failure of RhoA and Rac1 inactivation. In contrast, the attenuated *yopE*G182A shows wild-type inactivation of both RhoA and Rac1.

YopE displays feedback inhibition of Yop expression and controls the extent of translocation

We were surprised that activity towards RhoA and/or Rac1 was not required for virulence in the mice model of infection. When levels of Yops expressed were investigated, a fascinating pattern started to unravel. *In vitro*, all homology domain mutants produced Yops at the same level as wild-type. However, during *in vivo* conditions in the presence of eukaryotic cells, the avirulent mutants *yopE*R144A, *yopE*W181A and *yopE*T183A produced twice the amounts of Yops than wild-type. The attenuated mutants (*yopE*Q180A and *yopE*G182A) also showed a slight increase in total Yop protein production (intrabacterial and intracellular), while the virulent mutants *yopE*F178A and *yopE*S179A displayed wild-type levels of Yop expression in the presence of cells. (Paper I, fig. 4)

Interestingly, it appeared as if YopE originating from the virulent strains could control Yop expression during *in vivo* conditions by some kind of feedback inhibition mechanism. The exact mechanism underlying the observed inhibition remains to be elucidated, nonetheless it is clearly not active during *in vitro* cultivation of *Y. pseudotuberculosis*.

The finding that more Yops were produced by the avirulent mutants in the presence of cells was intriguing. Would the avirulent mutants also translocate elevated levels of Yops? In a HeLa cell translocation assay, it was clear that mutants lacking feedback control also overtranslocated YopE. The levels of overtranslocation were similar to the hypertranslocation phenotype previously described for the *yopK* mutant [125]. (Paper I, fig. 3)

Thus, there is a clear connection between virulence in the animal model and the ability to control the extent of effector translocation. To our knowledge, this is the first time a translocated effector has been implicated to be involved in controlling the extent of its own translocation. This raises the question that perhaps YopE should be considered as a regulator of effector translocation as well as a traditional effector protein.

Summary of important phenotypes for the homology domain point mutants

The mutants of the homology domain of YopE have been investigated in several assays described above, that have identified several interesting phenotypes for translocated YopE. To clarify the results for the reader, the obtained results are summarized in table 3 below.

Alanine substitutions of single amino acids	144 R	178 F	179 S	180 Q	181 W	182 G	183 T	wt
Rac1 inactivation	No	No	Yes	Yes	No	Yes	Yes	Yes
RhoA inactivation	No	No	Yes	Yes	No	Yes	No	Yes
HeLa cytotoxicity *In cis*	-	+++	+++	+++	-	+++	+++	+++
HeLa cytotoxicity *In trans*	-	+++	+++	+++	+++	+++	+++	+++
Virulence	No	Yes	Yes	Att.	No	Att.	No	Yes
Regulation of translocation	No	Yes	Yes	Yes	No	Yes	No	Yes
Pore formation prevention	No	Yes	Yes	Yes	No	Yes	Yes	Yes

Table 3: Overview of the homology domain mutants and their corresponding phenotypes. For clarity, *yopE* R144A and wild-type YopE phenotypes are included.

YopE is the sole effector responsible for pore formation prevention

Yersinia is known to translocate six effectors into the target cell. Out of these six, five are essential for virulence. [248] To ensure a successful infection, the effector translocation process must be tightly regulated. Our knowledge of how *Yersinia* exerts translocation control is however limited.

In Paper I, we started to unravel the potential of translocated YopE acting as a regulator of effector translocation. According to the microinjection hypothesis for T3SS mediated translocation, the effectors are delivered through a pore formed in the eukaryotic membrane by the translocators. One mechanism of translocation control would be by controlling the translocation pore formation process, as has been reported for the YopK protein [125].

Previous data demonstrated that YopE prevents pore formation, measured as blockage of LDH release from infected cells [247]. This finding was corroborated in Paper II, where YopE prevented LDH release mediated by the inherent GAP activity. Further, no other effector was involved in pore formation prevention. (Paper II, fig. 1) Surprisingly, a *yopK* mutant behaved as wild-type, not releasing LDH. This is in contrast to earlier observations where YopK was found to be involved in the pore formation process [125].
One explanation could be due to the fact that a *yopK* mutant overtranslocates YopE and the excessive amounts of YopE would prevent pore formation. Another explanation for the discrepancy regarding the regulatory role for YopK in pore formation control could be that different assays and different cell types were used. For YopK pore formation, erythrocytes were used, while HeLa cells were assayed for LDH release. The erythrocytes are inert cells, without any cell signalling events. HeLa cells, on the other hand, are proliferating cells equipped with multiple signalling mechanisms. Also important, LDH is a large molecule with a diameter of ~4,3 nm [13]. This exceeds the estimated radius of the translocation pore, which is estimated to be ~2,5 nm [39, 109, 175]. Clearly, LDH could not be secreted directly trough the translocation pore when released from infected cells, so LDH release is not a direct measurement of translocation pore formation. LDH release is however connected to actin rearrangements and hence cytotoxicity. Only the non-cytotoxic mutants *yopE*R144A and *yopE*W181A failed to block LDH release. (Paper I, fig. 5)

YopE and YopK regulates translocation into target cells

YopE appeared to control the level of protein produced in the presence of target cells as well as controlling the level of its own translocation. Further, YopE was not only controlling the extent of its own translocation, but also controlled the level of YopH, suggesting that YopE mediated a general translocation control mechanism, controlling the extent of translocation of multiple effectors. No other effector shared this capacity, since all other tested effector mutants translocated YopE and YopH at the same level as wild-type. (Paper II, fig. 2) This translocation control mechanism involved the regulatory protein YopK. A *yopK* mutant is known to overtranslocate effectors into HeLa cells [125], and according to expectations, overdelivered YopE and YopH also in our assay. The underlying mechanism behind the translocation control, involving both YopE and YopK, is unknown. Presumably, translocated YopE might act as a sensor of translocation. When sufficient amounts of effectors are translocated, YopE sends a signal back to the extracellular located bacteria mediated via the GAP activity. The signal might be relayed via YopK that is demonstrated to interact with the translocon constituents YopB and YopD in the eukaryotic cell membrane. [38] Further, the needle protein YscF has itself been implicated in signalling

extracellular events and perhaps the feedback signalling can be transmitted via YscF [239]. Once the signal reaches into the bacteria, the message is interpreted and further Yop effector synthesis, secretion and translocation is switched off. By using a translocated effector as a sensor, *Yersinia* would be able to modulate the levels of effectors introduced. The lymph tissue tropism of *Yersinia* must require a certain degree of paralysation of the host immune system. This is achieved by the introduction of a controlled amount of anti-host effectors. But an over-paralyzing effect would be detrimental for the bacterium. Overt translocation would quickly alert the host immune system of the bacterial presence, and the infection will rapidly be cleared. This might be the case for the avirulent mutants *yopK*, *yopE*R144A or *yopE*T183A, that all have lost feedback control of Yop effector expression and translocation.

General bacterial GAP activity can control effector translocation

So, it was clear that the GAP activity of YopE was required for successful feedback inhibition of both Yop effector expression and translocation. Is GAP activity in general all what was required? The related GAP proteins of *P. aeruginosa*, ExoS and ExoT, are translocated by the *Yersinia* T3S apparatus [91]. ExoS and ExoT are both bifunctional toxins with N-terminal GAP activity and C-terminal ADP-ribosyltransferase activity [22]. Wild-type ExoS or ExoT as well as GAP- or ADPr- deficient constructs were expressed in parallel with wild-type YopE or YopER144A in a *yopE* mutant background. Constructs expressing GAP active versions of YopE, ExoS or ExoT could control expression of YopD and YopE in the presence of target cells, while all GAP deficient constructs failed to control production. (Paper II, fig. 4) The same were true for translocation control, where GAP active constructs of YopE, ExoS and ExoT could limit the extent of YopH and YopE translocation. (Paper II, fig. 5) It appears if the exogenous effectors ExoS and ExoT can control the extent of translocation when ectopically expressed in *Yersinia*, suggesting a general ability of the GAP domain to sense and control the level of translocation. In support of this hypothesis, ExoS has recently been demonstrated to be involved in a similar feedback mechanism, preventing further secretion and translocation of effectors in *P. aeruginosa*. In contrast to YopE, the inhibition could be achieved by either one of the two enzymatic activities of ExoS. [54]

YopE and YopK act in concert in virulence

YopE and YopK clearly play interrelated roles in effector translocation regulation. Animal experiments were performed with *yopE* and *yopK* single mutants as well as a double *yopEyopK* mutant in an attempt to increase the understanding of the underlying mechanism. [47]

Here, the IVIS technology was employed. IVIS allows for *in vivo* bioluminescent imaging of a progressing bacterial infection in real-time.

The desired mutant strains were obtained by introducing the appropriate deletion constructs into the bioluminescent wild-type *Y. pseudotuberculosis* strain YPIII/XEN4 that has the *luxCDABE* operon integrated on the virulence plasmid. BALB/c female mice, 6-8 weeks old, were divided into four groups with 9 mice per bacterial strain. Animals were orally infected with a high dose of bacteria (1,1-1,3*10^8 cfu/ml) in the drinking water. The animals were anaesthetised on day 1, 3, 5 and 7 post-infection and imaged for bioluminescence in the IVIS chamber. On day 1, it was clear that all animals were infected at similar levels, with bioluminescent signals of the same intensity originating from the abdominal region. In figure 6, one representative animal per strain is shown. Wild-type infected mice started to show signs of disease on day 3 which correlated with high bioluminescence signals. Surprisingly, *yopK* infected mice emitted the same bioluminescence as wild-type although the mice appeared completely healthy. Also apparent on day 3 was the synergistic contributions of both YopE and YopK in virulence. The double *yopEyopK* mutant appeared to be cleared from the infected mice, since no emitted photons could be visualized. When organs from infected mice were examined on day 3, it was clear that wild-type bacteria had spread systemically, with bioluminescence signals originating from the small intestine, the MLN as well as from the liver and spleen. For both *yopK* and *yopE* single infections, signals could be detected only from the small intestine and the MLN, while the double mutant infected organs did not emit any bioluminescent signals.

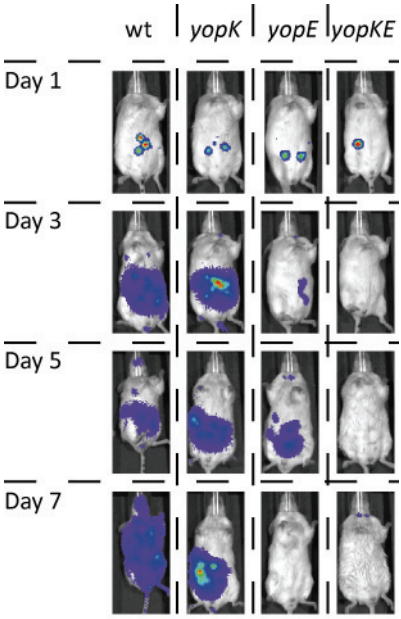

Figure 6: YopE and YopK are interconnected in mouse virulence. On the indicated days post-infection, infected mice were imaged for bioluminescence. One representative animal per strain is shown.

On day 5, the condition of the wild-type infected mice started to deteriorate with severe signs of disease. Again, *yopK* infected mice displayed wild-type levels of bioluminescence signals without any visual signs of disease. Neither the *yopK* nor *yopE* mutant infection had spread beyond the MLN, demonstrating their inability to cause systemic disease.

The experiment was terminated on day 7, when both *yopE* and *yopEyopK* infected mice had cleared the infection. Still, the *yopK* infected mice emitted strong signals, however bacteria were cleared from the MLN and were only recovered from the small intestine region.

The mice infection strengthens the conclusion that YopE and YopK are interconnected key players in virulence and demonstrates the importance of maintaining effector translocation control for the establishment of a successful *Yersinia* infection.

Further dissection of the YopE protein- the membrane localization domain (MLD)

The role for YopE in regulation of translocation was novel and exciting. To further elucidate this regulatory role and to perhaps gain better understanding of the mechanism behind, we wanted to further examine and dissect the YopE protein. In ExoS, Barbieri and co-workers have identified the presence of a membrane localization domain (MLD) that targets ExoS to intracellular membranes [182]. Further, a MLD for YopE was also identified, and this domain covering amino acids 54-75, was found to be both required and sufficient for correct intracellular localization of YopE [140].

We wanted to investigate the MLD further, to examine whether correct intracellular localization was required for the newly discovered regulatory role of YopE.

The MLD is essential for intracellular membrane localization of YopE

Translocated YopE locates to the perinuclear, membrane-rich region of infected cells [201]. However, HeLa cells intoxicated with wild-type YopE is severely affected in morphology, which makes determination of localization difficult. The GAP deficient mutant *yopE*R144A has been shown to localize to the same perinuclear region as wild-type YopE (Paper I, fig. 2), but without any morphological effect towards the cells. Therefore, the localization experiments were conducted in the GAP deficient background. By performing indirect immunofluorescence stainings, it was clear that the MLD was required for correct intracellular localization of translocated YopE. YopEΔMLD protein was detected dispersed within the cytoplasm of infected HeLa cells. This was in sharp contrast to YopER144A that displayed the expected perinuclear staining pattern. (Paper III, fig 4.)

To confirm the findings from the immunofluorescence experiment, a biochemical fractionation on infected cells was also performed, where intracellular membranes were separated from the cytosol. For wild-type *Y. pseudotuberculosis,* almost all detected translocated YopE ended up in the predicted membrane fraction, while YopEΔMLD ended up in the cytosolic

fraction. The levels of translocated protein were however rather low, so the overtranslocating GAP deficient background was also employed. The over-translocated YopEΔMLD-R144A was only detected within the cytosolic fraction, with no ability to associate with intracellular membranes. YopER144A ended up both in the membrane fraction as well as in the cytosolic fraction. The level of translocation was highly elevated, and perhaps the appearance of YopER144A also in the cytosolic fraction was due to a saturation effect of the membranes. (Paper III, fig. 3B)
By both immunofluorescence stainings and a biochemical approach, we have demonstrated that the MLD is important for correct intracellular localization of YopE, corroborating previous data from the Barbieri group[140].

YopE mediated HeLa cell cytotoxicity requires the MLD

HeLa cell cytotoxicity has long been the characteristic effect of translocated YopE [200]. Despite the fact that cytotoxicity is not directly connected to virulence, cytotoxicity is still connected to functional GAP activity [5]. The *yopE*ΔMLD was examined for cytotoxicity to see whether correct intracellular localization was required for induction of a cytotoxic response towards HeLa cells. The *yopE*ΔMLD was very much delayed in the onset of cytotoxicity and failed to elicit a full cytotoxic response, regardless the duration of infection. (Paper III, fig 5.) The lack of cytotoxicity could be due to two reasons, either YopEΔMLD protein has lost the inherent GAP activity due to the deletion, or YopE needs to be membrane associated to interact with the eukaryotic target protein responsible for cytotoxicity. To examine the first hypothesis, *in vivo* GAP activity assays were performed.

*yopE*ΔMLD retains full GAP activity towards Rac1 and RhoA

As a consequence of construction of the *yopE*ΔMLD mutant, half the YerA chaperone binding domain, amino acids 50-74 was lost. It was plausible that the deletion itself could interfere with the GAP activity residing in the C-terminal part of YopE and this needed to be investigated.

In paper I, we identified Rac1 and RhoA as *in vivo* targets of YopE. By employing the same pull-down technique here, we could verify that YopEΔMLD inactivates Rac1 and RhoA with the same kinetics as wild-type YopE protein. Thus, deletion of the amino acids corresponding of the MLD does not affect the GAP activity *per se*. It rather shows the importance of proper intracellular localization for interaction with the biological significant eukaryotic targets for YopE. Clearly, Rac1 and RhoA can not be the main RhoGTPases responsible for the cytotoxicity or the virulence associated functions mediated by YopE.

Auto-regulation of YopE expression is lost in the *yopE*ΔMLD, yet translocation control is retained

Studies on the MLD of YopE have so far demonstrated that the domain was required for proper intracellular localization of YopE, but it did not interfere with the GAP activity *per se*. In paper I and II, we discovered, and verified, a role for translocated YopE in effector translocation control. Most importantly, this feedback control mechanism was directly connected to virulence in the mice model. Next, we wanted to see if proper intracellular targeting was required for translocation control. The *yopE*ΔMLD mutant showed a clear defect in the ability to down-regulate the expression of YopE in the presence of target cells, thus it displayed the same phenotype as a *yopE*R144A or a *yopK* mutant. (Paper III, fig. 2)

All mutants defective for auto-regulation of YopE expression *in vivo* examined so far, also overdelivered effectors into HeLa cells. However, the *yopE*ΔMLD mutant did not overtranslocate YopE. In fact, *yopE*ΔMLD translocated equal amounts of YopE as wild-type *Y. pseudotuberculosis* into HeLa cells. The double mutant *yopE*ΔMLD-R144A displayed the same level overtranslocation as the *yopE*R144A single mutant, so deletion of the MLD did not interfere with the overtranslocation phenotype. (Paper III, fig. 3A)

The *yopE*ΔMLD mutant appeared to lack the auto-regulatory control of YopE expression, yet translocational control was retained. In conclusion, for the first time it is possible to separate auto-regulation of expression from the extent of effector translocation. Why *yopE*ΔMLD translocated equal amounts of YopE protein as wild-type, bearing in mind the fact that more YopE protein is made, could have a simple explanation. Since *yopE*ΔMLD is deleted in half the YerA chaperone binding domain, this could result in impaired YopE translocation. However, this is not necessary the case, as the MLD has been demonstrated to be the region that creates the actual need for the chaperone. The role for YerA might be to protect an aggregation prone region from premature membrane association within the bacterium [35].

The group of Bliska has nicely demonstrated the involvement of both YopE GAP activity and active RhoA in the translocation control process in *Yersinia* [164]. However, the *yopE*ΔMLD mutant inactivates RhoA and retains the translocation control, which is a finding that suggests that additional proteins besides RhoA should be involved in the process of translocation control. Further, from the translocation assay results, it is clear that the reduced cytotoxicity observed with the *yopE*ΔMLD mutant is not due to lesser amounts of YopE translocated. Instead, the reduced cytotoxicity rather reflects the importance of correct localization for induction of cytotoxicity towards HeLa cells.

Proper localization and auto-regulation of YopE is required for virulence

A mice infection was performed to investigate the biological significance of proper intracellular localization and auto-regulation of YopE expression. As for the previously described experiment, investigating the differential contributions of YopE and YopK during infection, the IVIS technology was used. Here, the *yopE*ΔMLD mutant was examined in parallel with wild-type *Y. pseudotuberculosis*, a *yopE* mutant and a *yopE*R144A GAP deficient mutant (Paper III, fig. 7). The *yopE*ΔMLD was as avirulent as the full *yopE* deletion or as the *yopE*R144A mutation. None of the mutant infected mice showed any signs of disease and they had all cleared the infection on day 13 post-infection. Thus, both the MLD and a functional GAP domain is required for virulence in the mice model of infection, showing that translocated YopE need to be at the right place and display proper GAP activity during infection in order to cause full disease.

The establishment of a novel model system for *Yersinia pseudotuberculosis* infections

Our study of the multiple roles for translocated YopE has generated many interesting results. Here, we wanted to develop a novel model system in an attempt to further elucidate the multiple YopE phenotypes further.

Y. pseudotuberculosis causes cytotoxicity towards fish scale keratocytes

Fish scale keratocytes are found on the outermost surface on the fish skin [43]. They are responsible for the quick wound repair mechanism of fish where they rapidly migrate to cover wounds to provide a mechanical barrier [42, 191]. The cell consists of a large extended lamellipodium, a two-dimensional actin network. The lamellipodium provides the forward motion to the cell and is regulated by small RhoGTPases. Keratocytes are extremely motile and are among the fastest moving eukaryotic cells known. [17, 18, 220]

The presence of this large actin network made these cells attractive as a putative model system for *Yersinia* infections. Four of the translocated effectors of *Yersinia*, YpkA, YopE, YopH and YopT, are known to affect the actin cytoskeleton. [248]

Single migrating keratocytes were infected with wild-type *Y. pseudotuberculosis*. The infection process was monitored by live cell microscopy. Within the first minutes of infection, the actin network of the keratocytes started to rapidly collapse. This actin destruction was similar to the cytotoxic effect inflicted by *Yersinia* in many other cell types. Therefore, we have chosen to use the term cytotoxicity also here. Interestingly, the onset of cytotoxicity was much more rapid then what is seen in the conventional

HeLa cell model. In HeLa cells, cytotoxicity is often scored 45 minutes post-infection.

Importantly, the cytotoxic effect was caused by an active T3SS. A T3SS deficient strain did not affect cell morphology, instead these bacteria were rapidly internalized. (Paper IV, fig. 2) Further, the effect was mediated by the action of a translocated effector, since a translocation deficient strain did not cause any visual effect towards the cells.

YopE is the sole effector responsible for cytotoxicity

To examine which effector that was responsible for cytotoxicity, mutants of all effectors were investigated. A *yopK* mutant was also included, since YopK is known to regulate the effector translocation process and displays overt cytotoxicity in HeLa cells [125](Paper II). YopE was found to be the sole effector responsible for the cytotoxic effect towards the keratocytes. No other effector mutant, or the *yopK* mutant, caused any effect towards the cells. This is in good agreement with other cell infection models, where YopE is known to responsible for cytotoxicity [36, 200].

Cytotoxicity towards keratocytes requires fully native YopE protein

To investigate the requirements for the cytotoxic effect, several mutants in different regions of YopE were employed in the keratocyte model.
Essential for the effector activity of YopE is a functional GAP domain. When the arginine at position 144 is mutated into alanine, GAP activity is abolished and the mutant is non-cytotoxic toward HeLa cells [4, 28] Consistently, GAP activity was required for cytotoxicity towards the keratocytes as well.

The membrane localization domain is required for correct intracellular membrane localization of translocated YopE and cytotoxicity in HeLa cells (Paper III). A *yopE*ΔMLD mutant failed to induce cytotoxicity in the keratocytes, thus demonstrating the importance of correct localization of YopE also in the fish cells. Further, mutants of the homology domain were investigated. The homology domain was important for recognition of the target RhoGTPase [226](Paper I). None of the homology domain mutants could evoke a cytotoxic response in the keratocytes. Neither could over-expression of homology domain mutants restore cytotoxicity. This is in sharp contrast to what is observed in the HeLa cell model, where all but one mutation induces cytotoxicity. Further, in HeLa cells, over-expression of the non-cytotoxic *yopE*W181A restores cytotoxicity to wild-type levels. (Paper I) Thus, the keratocytes appears to be more responsive towards YopE mediated cytotoxicity, since induction of cytotoxicity requires fully native YopE. We conclude that the keratocytes could constitute a model system for further studies of YopE mediated phenotypes.

YopE is capable of causing cytotoxicity towards multiple cell types derived from diverse organisms. Further, YopE has been demonstrated to target multiple RhoGTPases during *in vivo* conditions. Besides inactivating Rac1 and RhoA, YopE has recently been demonstrated to inactivate Rac2 and RhoG (Paper I)[198, 223]. Based on these multiple targets identified, as well as the ability to cause cytotoxicity towards multiple cell types, we speculate that perhaps YopE has no preferred RhoGTPase target protein. Instead, YopE activity might be promiscuous and target multiple RhoGTPases present in all the different cell types encountered during an infection. Surely, from the bacterial point of view, having an effector capable of inactivating multiple host proteins at multiple locations would be beneficial for promoting bacterial survival, proliferation and virulence.

Conclusions

- YopE inactivates the small RhoGTPases Rac1 and RhoA *in vivo*. However, Rac1 and RhoA inactivation is not required for virulence

- Avirulent *yopE* mutants have lost auto-regulatory control of Yop expression and overtranslocate effectors into HeLa cells. Thus, YopE appear to control the effector translocation process, via the GAP activity.

- Exogenous GAP activity can control effector translocation in *Yersinia*, suggesting a conserved novel function for all bacteria GAP proteins.

- YopE-mediated effector translocation control also requires YopK. Animal experiments demonstrate the individual and mutual contributions of both YopE and YopK in *Yersinia* virulence.

- The membrane localization domain of YopE is vital for correct intracellular membrane localization, auto-regulation of YopE expression and virulence, demonstrating the importance of correct localization for proper regulatory function of YopE.

- Fish keratocytes could be a sensitive model system for YopE mediated phenotypes for further studies

- We speculate that YopE is a promiscuous GAP protein with no preferred RhoGTPase target protein. This conclusion is based on the multiple, and different, YopE phenotypes observed in numerous cell types derived from different organisms, as well as the identification of several different RhoGTPase targets for YopE.

Acknowledgements

Finally, I have reached the page that will be read by most people. It was also by far the most difficult one to write! My five years at the department of Molecular Biology have come to an end, and there are a lot of people I wish to thank and express my gratitude to.

Först ut, **Hasse**. Tack för att jag har fått utveckla en hög grad av självständighet, fast du samtidigt funnits där när det har behövts. Och för att jag fick prata i USA, min hittills roligaste dag i forskningsvärlden. Undra bara om den 13 december kommer att bli ännu bättre?!

Present members in the **HWW** lab: **Ann-Catrin**, en kall norrbottning som gömmer det största och varmaste hjärtat! Vad skulle jag gjort utan dig i gruppen?! (Nästan alldeles säkert något annat…) **Frederic**, the only French-Caribbean Same to my knowledge. Thanks for bringing in a lot of laughter to the lab. **Stefan**, for demonstrating how a lab bench should be organized and for your kind questions asking how we are doing? **Tomas**, jag avvundas din passion för att snacka science. **Helen**, jag avvundas den coola snabbhet som du skrev din avhandling med. Grattis till fina baby **Harry**! **Roland N.** Som om det inte var nog med att behöva stå ut med min far på labb, så dök även hans avkomma upp ett antal år senare. Tack för hjälp med alla stammar och tusen frågor! **Tidigare medlemmar**: **Margareta**, vilken tur att du behövde hjälp där på slutet. Annars hade denna bok haft en helt annan titel. Jag lärde mig dock snabbt att inte låna tippar ur din låda. **Janne**, för att du stod ut med mig som frågvis projektstudent, för cykelreparationer och oväntade lärdomar. Jag hade aldrig annars vetat vad som händer när man eldar western-filter…**Sara G**. För att du besvarade tusentals frågor som började med Du, Sara…**Sara C.S.B,** Hasses hemliga tredje doktorand. Tack för att du gick igenom denna process precis innan mig och för att du är en så fin vän! **Andra *Yersinia*-människor att tacka**:**Roland R.** För medförfattarskap, mikroskopi i alla former och otaliga antikroppar, **Anna** för en fantastiskt bra grundkurs i musanatomi. **Karen**, for help with live microscopy software. **Kristina**, för lysande mutanter. **Sara**, rara **C**. Du kommer vara en av få som faktiskt läst hela denna bok, särskilt tack för det! Att det ska vara så svårt med såna här singular och plural, eller vad det nu heter…Fantastiskt roligt att du och **Andreas** också blir föräldrar! **The lunching ladies,** för ett välkommet avbrott prick 11.30. **Katrin**, jag hoppas bli en lika bra mamma som du **& Barbara,** vänligast av alla! **All members at journal clubs**, **group seminars** and **meetings**, and especially **Matt**, thanks for your contributions to the papers, and for thoughtful comments and suggestions during group meetings. **Tiago**, for sharing teaching duties. **Debbie**, for invaluable help with the fish manuscript and for advice regarding both science as well as life in more general. **Kristoffer**, för dina

grundläggande upptäckter om fiskcellerna. **Vicky**, for questions and comments on seminars, and for lately signing endless numbers of papers.
Other people to thank: Bengt för medförfattarskap, **Jonas**, **Bernt-Eric & Sun** for being part of my yearly evaluation committee, **Tord** för tusen LADOK-papper, **Sven** för koncernöverblick, **administrativ personal,** mest **Helena,** som fått svara på otaliga frågor, **personal i media och disk** som underlättar vår vardag, **all PhD colleagues**, **past & present!**

Stort tack till **alla i stora gänget** med ursprung från molekylärbiologen, för sista aprilfirande, midsommarfirande, nyårsfirande, födelsedagsfirande, fest helt enkelt! **Jeanette & Maria** för oväntat 30-årsfirande, **Sofia**, för att du är så omtänksam. Tack för dina tappra försök att motionera mig. **Petra**, finaste vännen som gav mig tak över huvudet när det krisade. Det är jag evigt tacksam för. Ses i Sthlm i vår, då vi måste få bjuda dig och **Anders** på middag.

Alla vänner från den riktiga världen, Elin, saknar dig massvis! Tur att det inte är så långt till Norrköping från Sthlm! **Katarina**, bästa kurslabbpartner någonsin. **Pia,** Uppsala är ju också nära Sthlm.
Alla Lars fantastiska vänner som nu också är mina.

Familjen: Mamma, bäst av alla. Du skämmer bort mig! **Pappa**, för att du på något sätt fick Ulf Lundkvist att rita ett omslag till din avhandling, som 23 år senare även löste mitt omslagsdilemma. **Anneli**, inte alla som får ha tre föräldrar. Älskade lillasyster **Sofia** och **Mattias,** våra bästa middagsgäster.
Lillebror **Jonas,** bra på allt det där storasyster inte alls kan. **Farmor Rut** för pysselgenen. **Familjen Isaksson-Nilsson**, bästa hundvakterna till världens bästa **Smilla**. Raraste **svärföräldrar** för omtanke (om än något orolig sådan), **Catharina**, **Jarl** & **Sofie**, för att ni välkomnar Egyptens gräshoppor.
Stora hjärtat, **Lars**, för att det är så lite som du inte kan, och för att du är bäst i världen nästan jämt. Jag älskar dig!
Och till sist, **Lilla hjärtat**, för att du har satt din egna, alldeles obönhörliga deadline. Det har fått din mamma att hålla sin. Längtar efter dig.

Äntligen!/ G. Fylking

This work was performed within the Laboratory of Molecular Infection Medicine Sweden (MIMS) and the Umeå Centre for Microbial Research (UCMR) and was supported by fundings from Swedish Research Council and the J C Kempe Memorial Fund.

References

1. **Achtman, M., Zurth, K., Morelli, G., Torrea, G., Guiyoule, A., and Carniel, E.**,1999. *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. Proc Natl Acad Sci U S A. **96**(24): p. 14043-8.
2. **Aepfelbacher, M., Trasak, C., Wilharm, G., Wiedemann, A., Trulzsch, K., Krauss, K., Gierschik, P., and Heesemann, J.**,2003. Characterization of YopT effects on Rho GTPases in *Yersinia enterocolitica*-infected cells. J Biol Chem. **278**(35): p. 33217-23.
3. **Agrain, C., Callebaut, I., Journet, L., Sorg, I., Paroz, C., Mota, L. J., and Cornelis, G. R.**,2005. Characterization of a Type III secretion substrate specificity switch (T3S4) domain in YscP from *Yersinia enterocolitica*. Mol Microbiol. **56**(1): p. 54-67.
4. **Aili, M., Hallberg, B., Wolf-Watz, H., and Rosqvist, R.**,2002. GAP activity of *Yersinia* YopE. Methods Enzymol. **358**: p. 359-70.
5. **Aili, M., Telepnev, M., Hallberg, B., Wolf-Watz, H., and Rosqvist, R.**,2003. *In vitro* GAP activity towards RhoA, Rac1 and Cdc42 is not a prerequisite for YopE induced HeLa cell cytotoxicity. Microb Pathog. **34**(6): p. 297-308.
6. **Aktories, K. and Barbieri, J. T.**,2005. Bacterial cytotoxins: targeting eukaryotic switches. Nat Rev Microbiol. **3**(5): p. 397-410.
7. **Alonso, A., Bottini, N., Bruckner, S., Rahmouni, S., Williams, S., Schoenberger, S. P., and Mustelin, T.**,2004. Lck dephosphorylation at Tyr-394 and inhibition of T cell antigen receptor signaling by *Yersinia* phosphatase YopH. J Biol Chem. **279**(6): p. 4922-8.
8. **Amerongen, H. M., Weltzin, R., Mack, J. A., Winner, L. S., 3rd, Michetti, P., Apter, F. M., Kraehenbuhl, J. P., and Neutra, M. R.**,1992. M cell-mediated antigen transport and monoclonal IgA antibodies for mucosal immune protection. Ann N Y Acad Sci. **664**: p. 18-26.
9. **Anderson, D. M. and Schneewind, O.**,1997. A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. Science. **278**(5340): p. 1140-3.
10. **Anderson, D. M., Ramamurthi, K. S., Tam, C., and Schneewind, O.**,2002. YopD and LcrH regulate expression of *Yersinia enterocolitica* YopQ by a posttranscriptional mechanism and bind to yopQ RNA. J Bacteriol. **184**(5): p. 1287-95.
11. **Andersson, K., Magnusson, K. E., Majeed, M., Stendahl, O., and Fallman, M.**,1999. *Yersinia pseudotuberculosis*-induced calcium signaling in neutrophils is blocked by the virulence effector YopH. Infect Immun. **67**(5): p. 2567-74.
12. **Andersson, K., Carballeira, N., Magnusson, K. E., Persson, C., Stendahl, O., Wolf-Watz, H., and Fallman, M.**,1996. YopH of *Yersinia pseudotuberculosis* interrupts early phosphotyrosine signalling associated with phagocytosis. Mol Microbiol. **20**(5): p. 1057-69.
13. **Andersson, M. M., Hatti-Kaul, R., Brown, B.**,2000. Dynamic and Static Light Scattering and Fluorescence Studies of the Interactions between Lactate Dehydrogenase and Poly(ethyleneimine). The Journal of Physical Chemistry B **104**(15): p. 3660-3667.
14. **Andor, A., Trulzsch, K., Essler, M., Roggenkamp, A., Wiedemann, A., Heesemann, J., and Aepfelbacher, M.**,2001. YopE of *Yersinia*, a GAP for Rho GTPases, selectively modulates Rac-dependent actin structures in endothelial cells. Cell Microbiol. **3**(5): p. 301-10.
15. **Arnold, R., Scheffer, J., Konig, B., and Konig, W.**,1993. Effects of *Listeria monocytogenes* and *Yersinia enterocolitica* on cytokine gene expression and release from human polymorphonuclear granulocytes and epithelial (HEp-2) cells. Infect Immun. **61**(6): p. 2545-52.
16. **Asbakk, K.**,2001. Elimination of foreign material by epidermal malpighian cells during wound healing in fish skin. J. Fish Biol. **58**: p. 953-966.
17. **Asbakk, K., Dalmo, R.A:**,1998. Atlantic salmon (*Salmo salar* L.) epidermal Malpighian cells- motile cells clearing away latex beads *in vitro*. J. Mar. Biotechnol. . **6**: p. 30-34.

18. **Aspenstrom, P., Fransson, A., and Saras, J.**,2004. Rho GTPases have diverse effects on the organization of the actin filament system. Biochem J. **377**(Pt 2): p. 327-37.
19. **Aspenstrom, P., Ruusala, A., and Pacholsky, D.**,2007. Taking Rho GTPases to the next level: the cellular functions of atypical Rho GTPases. Exp Cell Res. **313**(17): p. 3673-9.
20. **Auerbuch, V. and Isberg, R. R.**,2007. Growth of *Yersinia pseudotuberculosis* in mice occurs independently of Toll-like receptor 2 expression and induction of interleukin-10. Infect Immun. **75**(7): p. 3561-70.
21. **Baert, F., Peetermans, W., and Knockaert, D.**,1994. Yersiniosis: the clinical spectrum. Acta Clin Belg. **49**(2): p. 76-85.
22. **Barbieri, J. T. and Sun, J.**,2004. *Pseudomonas aeruginosa* ExoS and ExoT. Rev Physiol Biochem Pharmacol. **152**: p. 79-92.
23. **Bella, J., Hindle, K. L., McEwan, P. A., and Lovell, S. C.**,2008. The leucine-rich repeat structure. Cell Mol Life Sci. **65**(15): p. 2307-33.
24. **Benabdillah, R., Mota, L. J., Lutzelschwab, S., Demoinet, E., and Cornelis, G. R.**,2004. Identification of a nuclear targeting signal in YopM from *Yersinia* spp. Microb Pathog. **36**(5): p. 247-61.
25. **Bennett, J. C. and Hughes, C.**,2000. From flagellum assembly to virulence: the extended family of type III export chaperones. Trends Microbiol. **8**(5): p. 202-4.
26. **Bergman, T., Hakansson, S., Forsberg, A., Norlander, L., Macellaro, A., Backman, A., Bolin, I., and Wolf-Watz, H.**,1991. Analysis of the V antigen l*crGVH-yopBD* operon of *Yersinia pseudotuberculosis:* evidence for a regulatory role of LcrH and LcrV. J Bacteriol. **173**(5): p. 1607-16.
27. **Birtalan, S. C., Phillips, R. M., and Ghosh, P.**,2002. Three-dimensional secretion signals in chaperone-effector complexes of bacterial pathogens. Mol Cell. **9**(5): p. 971-80.
28. **Black, D. S. and Bliska, J. B.**,2000. The RhoGAP activity of the *Yersinia pseudotuberculosis* cytotoxin YopE is required for antiphagocytic function and virulence. Mol Microbiol. **37**(3): p. 515-27.
29. **Bockman, D. E. and Cooper, M. D.**,1973. Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix, and Peyer's patches. An electron microscopic study. Am J Anat. **136**(4): p. 455-77.
30. **Boland, A. and Cornelis, G. R.**,1998. Role of YopP in suppression of tumor necrosis factor alpha release by macrophages during *Yersinia* infection. Infect Immun. **66**(5): p. 1878-84.
31. **Bolin, I., Portnoy, D. A., and Wolf-Watz, H.**,1985. Expression of the temperature-inducible outer membrane proteins of yersiniae. Infect Immun. **48**(1): p. 234-40.
32. **Bolin, I., Forsberg, A., Norlander, L., Skurnik, M., and Wolf-Watz, H.**,1988. Identification and mapping of the temperature-inducible, plasmid-encoded proteins of *Yersinia* spp. Infect Immun. **56**(2): p. 343-8.
33. **Bottone, E. J.**,1997 *Yersinia enterocolitica*: the charisma continues. Clin Microbiol Rev. **10**(2): p. 257-76.
34. **Boureux, A., Vignal, E., Faure, S., and Fort, P.**,2007. Evolution of the Rho family of ras-like GTPases in eukaryotes. Mol Biol Evol. **24**(1): p. 203-16.
35. **Boyd, A. P., Lambermont, I., and Cornelis, G. R.**,2000. Competition between the Yops of *Yersinia enterocolitica* for delivery into eukaryotic cells: role of the SycE chaperone binding domain of YopE. J Bacteriol. **182**(17): p. 4811-21.
36. **Boyd, A. P., Grosdent, N., Totemeyer, S., Geuijen, C., Bleves, S., Iriarte, M., Lambermont, I., Octave, J. N., and Cornelis, G. R.**,2000. *Yersinia enterocolitica* can deliver Yop proteins into a wide range of cell types: development of a delivery system for heterologous proteins. Eur J Cell Biol. **79**(10): p. 659-71.
37. **Brodsky, I. E. and Medzhitov, R.**,2008. Reduced secretion of YopJ by *Yersinia* limits in vivo cell death but enhances bacterial virulence. PLoS Pathog. **4**(5): p. e1000067.
38. **Brodsky, I. E., Palm, N. W., Sadanand, S., Ryndak, M. B., Sutterwala, F. S., Flavell, R. A., Bliska, J. B., and Medzhitov, R.**,2010. A *Yersinia* effector protein promotes virulence by preventing inflammasome recognition of the type III secretion system. Cell Host Microbe. **7**(5): p. 376-87.

39. **Broms, J. E., Sundin, C., Francis, M. S., and Forsberg, A.**,2003. Comparative analysis of type III effector translocation by *Yersinia pseudotuberculosis* expressing native LcrV or PcrV from *Pseudomonas aeruginosa*. J Infect Dis. **188**(2): p. 239-49.
40. **Brubaker, R. R.**,1991. Factors promoting acute and chronic diseases caused by yersiniae. Clin Microbiol Rev. **4**(3): p. 309-24.
41. **Brubaker, R. R.**,2003 Interleukin-10 and inhibition of innate immunity to Yersiniae: roles of Yops and LcrV (V antigen). Infect Immun. **71**(7): p. 3673-81.
42. **Bullock, A. M., Marks, R., Roberts, R.J.**,1978. The cell kinetics of teleost skin epidermis: epidermal mitotic activity in relation to wound healing at varying temperatures in plaice (*Pleuronectes platessa*). J. Zool. London. **185**: p. 197-204
43. **Bullock, A. M., Roberts, R.J.**,1974. The dermatology of marine teleost fish. I. The normal integument. Oceanogr. Mar. Biol. Annu. Rev. **13**: p. 383-411.
44. **Cambronne, E. D. and Schneewind, O.**,2002. *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. **184**(21): p. 5880-93.
45. **Cambronne, E. D., Cheng, L. W., and Schneewind, O.**,2000. LcrQ/YscM1, regulators of the *Yersinia yop* virulon, are injected into host cells by a chaperone-dependent mechanism. Mol Microbiol. **37**(2): p. 263-73.
46. **Cambronne, E. D., Sorg, J. A., and Schneewind, O.**,2004. Binding of SycH chaperone to YscM1 and YscM2 activates effector yop expression in *Yersinia enterocolitica*. J Bacteriol. **186**(3): p. 829-41.
47. **Carlsson, S. E., Isaksson, E.L.**,Unpublished data.
48. **Chain, P. S., Carniel, E., Larimer, F. W., Lamerdin, J., Stoutland, P. O., Regala, W. M., Georgescu, A. M., Vergez, L. M., Land, M. L., Motin, V. L., Brubaker, R. R., Fowler, J., Hinnebusch, J., Marceau, M., Medigue, C., Simonet, M., Chenal-Francisque, V., Souza, B., Dacheux, D., Elliott, J. M., Derbise, A., Hauser, L. J., and Garcia, E.**,2004. Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. Proc Natl Acad Sci U S A. **101**(38): p. 13826-31.
49. **Champion, O. L., Cooper, I. A., James, S. L., Ford, D., Karlyshev, A., Wren, B. W., Duffield, M., Oyston, P. C., and Titball, R. W.**,2009. *Galleria mellonella* as an alternative infection model for *Yersinia pseudotuberculosis*. Microbiology. **155**(Pt 5): p. 1516-22.
50. **Cheng, L. W. and Schneewind, O.**,1999. *Yersinia enterocolitica* type III secretion. On the role of SycE in targeting YopE into HeLa cells. J Biol Chem. **274**(31): p. 22102-8.
51. **Cheng, L. W. and Schneewind, O.**,2000. *Yersinia enterocolitica* TyeA, an intracellular regulator of the type III machinery, is required for specific targeting of YopE, YopH, YopM, and YopN into the cytosol of eukaryotic cells. J Bacteriol. **182**(11): p. 3183-90.
52. **Cheng, L. W. and Schneewind, O.**,2000. Type III machines of Gram-negative bacteria: delivering the goods. Trends Microbiol. **8**(5): p. 214-20.
53. **Cheng, L. W., Anderson, D. M., and Schneewind, O.**,1997. Two independent type III secretion mechanisms for YopE in *Yersinia enterocolitica*. Mol Microbiol. **24**(4): p. 757-65.
54. **Cisz, M., Lee, P. C., and Rietsch, A.**,2008. ExoS controls the cell contact-mediated switch to effector secretion in *Pseudomonas aeruginosa*. J Bacteriol. **190**(8): p. 2726-38.
55. **Coburn, J. and Gill, D. M.**,1991. ADP-ribosylation of p21ras and related proteins by *Pseudomonas aeruginosa* exoenzyme S. Infect Immun. **59**(11): p. 4259-62.
56. **Coburn, J., Wyatt, R. T., Iglewski, B. H., and Gill, D. M.**,1989. Several GTP-binding proteins, including p21c-H-ras, are preferred substrates of *Pseudomonas aeruginosa* exoenzyme S. J Biol Chem. **264**(15): p. 9004-8.
57. **Cornelis, G., Sluiters, C., de Rouvroit, C. L., and Michiels, T.**,1989. Homology between *virF*, the transcriptional activator of the *Yersinia* virulence regulon, and AraC, the *Escherichia coli* arabinose operon regulator. J Bacteriol. **171**(1): p. 254-62.
58. **Cornelis, G. R.**,2002. *Yersinia* type III secretion: send in the effectors. J Cell Biol. **158**(3): p. 401-8.

59. **Cornelis, G. R.**,2006. The type III secretion injectisome. Nat Rev Microbiol. **4**(11): p. 811-25.
60. **Cornelis, G. R. and Wolf-Watz, H.**,1997. The *Yersinia* Yop virulon: a bacterial system for subverting eukaryotic cells. Mol Microbiol. **23**(5): p. 861-7.
61. **Cornelis, G. R. and Van Gijsegem, F.**,2000. Assembly and function of type III secretory systems. Annu Rev Microbiol. **54**: p. 735-74.
62. **Cornelis, G. R., Boland, A., Boyd, A. P., Geuijen, C., Iriarte, M., Neyt, C., Sory, M. P., and Stainier, I.**,1998. The virulence plasmid of *Yersinia*, an antihost genome. Microbiol Mol Biol Rev. **62**(4): p. 1315-52.
63. **Cornelis, G. R., Biot, T., Lambert de Rouvroit, C., Michiels, T., Mulder, B., Sluiters, C., Sory, M. P., Van Bouchaute, M., and Vanooteghem, J. C.**,1989. The *Yersinia yop* regulon. Mol Microbiol. **3**(10): p. 1455-9.
64. **Cover, T. L. and Aber, R. C.**,1989. *Yersinia enterocolitica*. N Engl J Med. **321**(1): p. 16-24.
65. **Cristopher, G. W., Cieslak, T.J., Pavlin, J.A., Eitzen, E.M.**,1997. Biological warfare. A historical perspective. JAMA. **278**: p. 351-56.
66. **Darby, C., Hsu, J. W., Ghori, N., and Falkow, S.**,2002. *Caenorhabditis elegans*: plague bacteria biofilm blocks food intake. Nature. **417**(6886): p. 243-4.
67. **Day, J. B. and Plano, G. V.**,1998. A complex composed of SycN and YscB functions as a specific chaperone for YopN in *Yersinia pestis*. Mol Microbiol. **30**(4): p. 777-88.
68. **Deane, J. E., Roversi, P., Cordes, F. S., Johnson, S., Kenjale, R., Daniell, S., Booy, F., Picking, W. D., Picking, W. L., Blocker, A. J., and Lea, S. M.**,2006. Molecular model of a type III secretion system needle: Implications for host-cell sensing. Proc Natl Acad Sci U S A. **103**(33): p. 12529-33.
69. **DeBord, K. L., Lee, V. T., and Schneewind, O.**,2001. Roles of LcrG and LcrV during type III targeting of effector Yops by *Yersinia enterocolitica*. J Bacteriol. **183**(15): p. 4588-98.
70. **Deleuil, F., Mogemark, L., Francis, M. S., Wolf-Watz, H., and Fallman, M.**,2003. Interaction between the *Yersinia* protein tyrosine phosphatase YopH and eukaryotic Cas/Fyb is an important virulence mechanism. Cell Microbiol. **5**(1): p. 53-64.
71. **Deng, Q. and Barbieri, J. T.**,2008. Modulation of host cell endocytosis by the type III cytotoxin, *Pseudomonas* ExoS. Traffic. **9**(11): p. 1948-57.
72. **Deng, Q., Zhang, Y., and Barbieri, J. T.**,2007. Intracellular trafficking of *Pseudomonas* ExoS, a type III cytotoxin. Traffic. **8**(10): p. 1331-45.
73. **Dubash, A. D., Menold, M. M., Samson, T., Boulter, E., Garcia-Mata, R., Doughman, R., and Burridge, K.**,2009. Chapter 1. Focal adhesions: new angles on an old structure. Int Rev Cell Mol Biol. **277**: p. 1-65.
74. **Dukuzumuremyi, J. M., Rosqvist, R., Hallberg, B., Akerstrom, B., Wolf-Watz, H., and Schesser, K.**,2000. The *Yersinia* protein kinase A is a host factor inducible RhoA/Rac-binding virulence factor. J Biol Chem. **275**(45): p. 35281-90.
75. **Durand, E. A., Maldonado-Arocho, F. J., Castillo, C., Walsh, R. L., and Mecsas, J.**,2010. The presence of professional phagocytes dictates the number of host cells targeted for Yop translocation during infection. Cell Microbiol. **12**(8): p. 1064-82.
76. **Dvorsky, R. and Ahmadian, M. R.**,2004. Always look on the bright site of Rho: structural implications for a conserved intermolecular interface. EMBO Rep. **5**(12): p. 1130-6.
77. **Edqvist, P. J., Olsson, J., Lavander, M., Sundberg, L., Forsberg, A., Wolf-Watz, H., and Lloyd, S. A.**,2003. YscP and YscU regulate substrate specificity of the *Yersinia* type III secretion system. J Bacteriol. **185**(7): p. 2259-66.
78. **Ehrenkranz, N. J. and Meyer, K. F.**,1955. Studies on immunization against plague. VIII. Study of three immunizing preparations in protecting primates against pneumonic plague. J Infect Dis. **96**(2): p. 138-44.
79. **Evdokimov, A. G., Anderson, D. E., Routzahn, K. M., and Waugh, D. S.**,2001. Unusual molecular architecture of the *Yersinia pestis* cytotoxin YopM: a leucine-rich repeat protein with the shortest repeating unit. J Mol Biol. **312**(4): p. 807-21.
80. **Evdokimov, A. G., Tropea, J. E., Routzahn, K. M., and Waugh, D. S.**,2002. Crystal structure of the *Yersinia pestis* GTPase activator YopE. Protein Sci. **11**(2): p. 401-8.

81. **Fallman, M., Andersson, K., Hakansson, S., Magnusson, K. E., Stendahl, O., and Wolf-Watz, H.**,1995. *Yersinia pseudotuberculosis* inhibits Fc receptor-mediated phagocytosis in J774 cells. Infect Immun. **63**(8): p. 3117-24.
82. **Finck-Barbancon, V., Goranson, J., Zhu, L., Sawa, T., Wiener-Kronish, J. P., Fleiszig, S. M., Wu, C., Mende-Mueller, L., and Frank, D. W.**,1997. ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. Mol Microbiol. **25**(3): p. 547-57.
83. **Forsberg, A. and Wolf-Watz, H.**,1988. The virulence protein Yop5 of *Yersinia pseudotuberculosis* is regulated at transcriptional level by plasmid-plB1-encoded trans-acting elements controlled by temperature and calcium. Mol Microbiol. **2**(1): p. 121-33.
84. **Forsberg, A. and Wolf-Watz, H.**,1990. Genetic analysis of the *yopE* region of *Yersinia* spp.: identification of a novel conserved locus, *yerA,* regulating *yopE* expression. J Bacteriol. **172**(3): p. 1547-55.
85. **Forsberg, A., Bolin, I., Norlander, L., and Wolf-Watz, H.**,1987. Molecular cloning and expression of calcium-regulated, plasmid-coded proteins of *Y. pseudotuberculosis*. Microb Pathog. **2**(2): p. 123-37.
86. **Forsberg, A., Viitanen, A. M., Skurnik, M., and Wolf-Watz, H.**,1991. The surface-located YopN protein is involved in calcium signal transduction in *Yersinia pseudotuberculosis*. Mol Microbiol. **5**(4): p. 977-86.
87. **Foultier, B., Troisfontaines, P., Muller, S., Opperdoes, F. R., and Cornelis, G. R.**,2002. Characterization of the *ysa* pathogenicity locus in the chromosome of *Yersinia enterocolitica* and phylogeny analysis of type III secretion systems. J Mol Evol. **55**(1): p. 37-51.
88. **Francis, M. S. and Wolf-Watz, H.**,1998. YopD of *Yersinia pseudotuberculosis* is translocated into the cytosol of HeLa epithelial cells: evidence of a structural domain necessary for translocation. Mol Microbiol. **29**(3): p. 799-813.
89. **Francis, M. S., Lloyd, S. A., and Wolf-Watz, H.**,2001. The type III secretion chaperone LcrH co-operates with YopD to establish a negative, regulatory loop for control of Yop synthesis in *Yersinia pseudotuberculosis*. Mol Microbiol. **42**(4): p. 1075-93.
90. **Friedlander, A. M., Welkos, S. L., Worsham, P. L., Andrews, G. P., Heath, D. G., Anderson, G. W., Jr., Pitt, M. L., Estep, J., and Davis, K.**,1995. Relationship between virulence and immunity as revealed in recent studies of the F1 capsule of *Yersinia pestis*. Clin Infect Dis. **21 Suppl 2**: p. S178-81.
91. **Frithz-Lindsten, E., Du, Y., Rosqvist, R., and Forsberg, A.**,1997. Intracellular targeting of exoenzyme S of *Pseudomonas aeruginosa* via type III-dependent translocation induces phagocytosis resistance, cytotoxicity and disruption of actin microfilaments. Mol Microbiol. **25**(6): p. 1125-39.
92. **Frithz-Lindsten, E., Holmstrom, A., Jacobsson, L., Soltani, M., Olsson, J., Rosqvist, R., and Forsberg, A.**,1998. Functional conservation of the effector protein translocators PopB/YopB and PopD/YopD of *Pseudomonas aeruginosa* and *Yersinia pseudotuberculosis*. Mol Microbiol. **29**(5): p. 1155-65.
93. **Fu, Y. and Galan, J. E.**,1999. A *salmonella* protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. Nature. **401**(6750): p. 293-7.
94. **Galimand, M., Carniel, E., and Courvalin, P.**,2006. Resistance of *Yersinia pestis* to antimicrobial agents. Antimicrob Agents Chemother. **50**(10): p. 3233-6.
95. **Galyov, E. E., Hakansson, S., Forsberg, A., and Wolf-Watz, H.**,1993. A secreted protein kinase of *Yersinia pseudotuberculosis* is an indispensable virulence determinant. Nature. **361**(6414): p. 730-2.
96. **Galyov, E. E., Wood, M. W., Rosqvist, R., Mullan, P. B., Watson, P. R., Hedges, S., and Wallis, T. S.**,1997. A secreted effector protein of *Salmonella dublin* is translocated into eukaryotic cells and mediates inflammation and fluid secretion in infected ileal mucosa. Mol Microbiol. **25**(5): p. 903-12.
97. **Garcia, J. T., Ferracci, F., Jackson, M. W., Joseph, S. S., Pattis, I., Plano, L. R., Fischer, W., and Plano, G. V.**,2006. 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. **74**(10): p. 5645-57.
98. **Gemski, P., Lazere, J. R., Casey, T., and Wohlhieter, J. A.**,1980. Presence of a virulence-associated plasmid in *Yersinia pseudotuberculosis*. Infect Immun. **28**(3): p. 1044-7.

99. **Gophna, U., Ron, E. Z., and Graur, D.**,2003. Bacterial type III secretion systems are ancient and evolved by multiple horizontal-transfer events. Gene. **312**: p. 151-63.
100. Gottfried, R. S., *The black death. Natural and human disaster in medieval Europe*. 1983: The Free Press, New York, N.Y.
101. **Goure, J., Broz, P., Attree, O., Cornelis, G. R., and Attree, I.**,2005. Protective anti-V antibodies inhibit *Pseudomonas* and *Yersinia* translocon assembly within host membranes. J Infect Dis. **192**(2): p. 218-25.
102. **Granfors, K. and Toivanen, A.**,1986. IgA-anti-*yersinia* antibodies in *yersinia* triggered reactive arthritis. Ann Rheum Dis. **45**(7): p. 561-5.
103. **Groisman, E. A.**,1998. The ins and outs of virulence gene expression: Mg2+ as a regulatory signal. Bioessays. **20**(1): p. 96-101.
104. **Grosdent, N., Maridonneau-Parini, I., Sory, M. P., and Cornelis, G. R.**,2002. Role of Yops and adhesins in resistance of *Yersinia enterocolitica* to phagocytosis. Infect Immun. **70**(8): p. 4165-76.
105. **Guan, K. L. and Dixon, J. E.**,1990. Protein tyrosine phosphatase activity of an essential virulence determinant in *Yersinia*. Science. **249**(4968): p. 553-6.
106. **Haiko, J., Suomalainen, M., Ojala, T., Lahteenmaki, K., and Korhonen, T. K.**,2009. Invited review: Breaking barriers--attack on innate immune defences by omptin surface proteases of enterobacterial pathogens. Innate Immun. **15**(2): p. 67-80.
107. **Hakansson, S., Galyov, E. E., Rosqvist, R., and Wolf-Watz, H.**,1996. The *Yersinia* YpkA Ser/Thr kinase is translocated and subsequently targeted to the inner surface of the HeLa cell plasma membrane. Mol Microbiol. **20**(3): p. 593-603.
108. **Hakansson, S., Bergman, T., Vanooteghem, J. C., Cornelis, G., and Wolf-Watz, H.**,1993. YopB and YopD constitute a novel class of *Yersinia* Yop proteins. Infect Immun. **61**(1): p. 71-80.
109. **Hakansson, S., Schesser, K., Persson, C., Galyov, E. E., Rosqvist, R., Homble, F., and Wolf-Watz, H.**,1996. The YopB protein of *Yersinia pseudotuberculosis* is essential for the translocation of Yop effector proteins across the target cell plasma membrane and displays a contact-dependent membrane disrupting activity. Embo J. **15**(21): p. 5812-23.
110. **Hamid, N., Gustavsson, A., Andersson, K., McGee, K., Persson, C., Rudd, C. E., and Fallman, M.**,1999 YopH dephosphorylates Cas and Fyn-binding protein in macrophages. Microb Pathog. **27**(4): p. 231-42.
111. **Hardt, W. D., Chen, L. M., Schuebel, K. E., Bustelo, X. R., and Galan, J. E.**,1998. *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. Cell. **93**(5): p. 815-26.
112. **Hartland, E. L. and Robins-Browne, R. M.**,1998. *In vitro* association between the virulence proteins, YopD and YopE, of *Yersinia enterocolitica*. FEMS Microbiol Lett. **162**(2): p. 207-13.
113. **Hartland, E. L., Green, S. P., Phillips, W. A., and Robins-Browne, R. M.**,1994. Essential role of YopD in inhibition of the respiratory burst of macrophages by *Yersinia enterocolitica*. Infect Immun. **62**(10): p. 4445-53.
114. **Hauser, A. R.**,2009 The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. Nat Rev Microbiol. **7**(9): p. 654-65.
115. **He, S. Y., Nomura, K., and Whittam, T. S.**,2004. Type III protein secretion mechanism in mammalian and plant pathogens. Biochim Biophys Acta. **1694**(1-3): p. 181-206.
116. **Heesemann, J., Gaede, K., and Autenrieth, I. B.**,1993. Experimental *Yersinia enterocolitica* infection in rodents: a model for human yersiniosis. Apmis. **101**(6): p. 417-29.
117. **Heesemann, J., Gross, U., Schmidt, N., and Laufs, R.**,1986. Immunochemical analysis of plasmid-encoded proteins released by enteropathogenic *Yersinia* sp. grown in calcium-deficient media. Infect Immun. **54**(2): p. 561-7.
118. **Henrikson, R. C., Matoltsy, A.G.** ,1968. The fine structure of teleost epidermis. I. Introduction and filament-containing cells. J. Ultrastruct. Res. . **21**: p. 194-212.
119. **Higuchi, K., Kupferberg, L. L., and Smith, J. L.**,1959. Studies on the nutrition and physiology of Pasteurella pestis. III. Effects of calcium ions on the growth of virulent and avirulent strains of *Pasteurella pestis*. J Bacteriol. **77**(3): p. 317-21.
120. **Hinnebusch, B. J.**,2003. Transmission factors: *Yersinia pestis* genes required to infect the flea vector of plague. Adv Exp Med Biol. **529**: p. 55-62.

121. **Hinnebusch, B. J.**,2005. The evolution of flea-borne transmission in *Yersinia pestis*. Curr Issues Mol Biol. **7**(2): p. 197-212.
122. **Hinnebusch, B. J., Rudolph, A. E., Cherepanov, P., Dixon, J. E., Schwan, T. G., and Forsberg, A.**,2002. Role of *Yersinia* murine toxin in survival of Yersinia pestis in the midgut of the flea vector. Science. **296**(5568): p. 733-5.
123. **Hoiczyk, E. and Blobel, G.**,2001. Polymerization of a single protein of the pathogen *Yersinia enterocolitica* into needles punctures eukaryotic cells. Proc Natl Acad Sci U S A. **98**(8): p. 4669-74.
124. **Holmstrom, A., Rosqvist, R., Wolf-Watz, H., and Forsberg, A.**,1995. Virulence plasmid-encoded YopK is essential for *Yersinia pseudotuberculosis* to cause systemic infection in mice. Infect Immun. **63**(6): p. 2269-76.
125. **Holmstrom, A., Petterson, J., Rosqvist, R., Hakansson, S., Tafazoli, F., Fallman, M., Magnusson, K. E., Wolf-Watz, H., and Forsberg, A.**,1997. YopK of Yersinia *pseudotuberculosis* controls translocation of Yop effectors across the eukaryotic cell membrane. Mol Microbiol. **24**(1): p. 73-91.
126. **Hueck, C. J.**,1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol Mol Biol Rev. **62**(2): p. 379-433.
127. **Ibarra, J. A. and Steele-Mortimer, O.**,2009. *Salmonella*--the ultimate insider. *Salmonella* virulence factors that modulate intracellular survival. Cell Microbiol. **11**(11): p. 1579-86.
128. **Iglewski, B. H., Sadoff, J., Bjorn, M. J., and Maxwell, E. S.**,1978. *Pseudomonas aeruginosa* exoenzyme S: an adenosine diphosphate ribosyltransferase distinct from toxin A. Proc Natl Acad Sci U S A. **75**(7): p. 3211-5.
129. **Iriarte, M. and Cornelis, G. R.**,1998. YopT, a new *Yersinia* Yop effector protein, affects the cytoskeleton of host cells. Mol Microbiol. **29**(3): p. 915-29.
130. **Iriarte, M. and Cornelis, G. R.**,1999. Identification of SycN, YscX, and YscY, three new elements of the *Yersinia yop* virulon. J Bacteriol. **181**(2): p. 675-80.
131. **Iriarte, M., Sory, M. P., Boland, A., Boyd, A. P., Mills, S. D., Lambermont, I., and Cornelis, G. R.**,1998. TyeA, a protein involved in control of Yop release and in translocation of *Yersinia* Yop effectors. Embo J. **17**(7): p. 1907-18.
132. **Isberg, R. R. and Leong, J. M.**,1990. Multiple beta 1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell. **60**(5): p. 861-71.
133. **Jensen, C. J., Buch, M. B., Krag, T. O., Hemmings, B. A., Gammeltoft, S., and Frodin, M.**,1999. 90-kDa ribosomal S6 kinase is phosphorylated and activated by 3-phosphoinositide-dependent protein kinase-1. J Biol Chem. **274**(38): p. 27168-76.
134. **Journet, L., Agrain, C., Broz, P., and Cornelis, G. R.**,2003. The needle length of bacterial injectisomes is determined by a molecular ruler. Science. **302**(5651): p. 1757-60.
135. **Juris, S. J., Rudolph, A. E., Huddler, D., Orth, K., and Dixon, J. E.**,2000. A distinctive role for the Yersinia protein kinase: actin binding, kinase activation, and cytoskeleton disruption. Proc Natl Acad Sci U S A. **97**(17): p. 9431-6.
136. **Kaniga, K., Uralil, J., Bliska, J. B., and Galan, J. E.**,1996. A secreted protein tyrosine phosphatase with modular effector domains in the bacterial pathogen *Salmonella typhimurium*. Mol Microbiol. **21**(3): p. 633-41.
137. **Kazmierczak, B. I. and Engel, J. N.**,2002. *Pseudomonas aeruginosa* ExoT acts in vivo as a GTPase-activating protein for RhoA, Rac1, and Cdc42. Infect Immun. **70**(4): p. 2198-205.
138. **Keren, K., Pincus, Z., Allen, G. M., Barnhart, E. L., Marriott, G., Mogilner, A., and Theriot, J. A.**,2008. Mechanism of shape determination in motile cells. Nature. **453**(7194): p. 475-80.
139. **Koornhof, H. J., Smego, R. A., Jr., and Nicol, M.**,1999. Yersiniosis. II: The pathogenesis of *Yersinia* infections. Eur J Clin Microbiol Infect Dis. **18**(2): p. 87-112.
140. **Krall, R., Zhang, Y., and Barbieri, J. T.**,2004. Intracellular membrane localization of *pseudomonas* ExoS and *Yersinia* YopE in mammalian cells. J Biol Chem. **279**(4): p. 2747-53.
141. **Krall, R., Sun, J., Pederson, K. J., and Barbieri, J. T.**,2002. *In vivo* rho GTPase-activating protein activity of *Pseudomonas aeruginosa* cytotoxin ExoS. Infect Immun. **70**(1): p. 360-7.

142. **Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galan, J. E., and Aizawa, S. I.**,1998. Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. Science. **280**(5363): p. 602-5.
143. **Kupferberg, L. L. and Higuchi, K.**,1958. Role of calcium ions in the stimulation of growth of virulent strains of *Pasteurella pestis*. J Bacteriol. **76**(1): p. 120-1.
144. **Lambert de Rouvroit, C., Sluiters, C., and Cornelis, G. R.**,1992. Role of the transcriptional activator, VirF, and temperature in the expression of the pYV plasmid genes of *Yersinia enterocolitica*. Mol Microbiol. **6**(3): p. 395-409.
145. **Lee, J. and Jacobson, K.**,1997. The composition and dynamics of cell-substratum adhesions in locomoting fish keratocytes. J Cell Sci. **110 (Pt 22)**: p. 2833-44.
146. **Lee Ligon, B.**,2006. Plague: A review of its history and potential as a biological weapon. Semin Pediatr. Infect. Dis. . **17**: p. 161-170.
147. **Lee, V. T., Anderson, D. M., and Schneewind, O.**,1998. Targeting of *Yersinia* Yop proteins into the cytosol of HeLa cells: one-step translocation of YopE across bacterial and eukaryotic membranes is dependent on SycE chaperone. Mol Microbiol. **28**(3): p. 593-601.
148. **Lemaitre, N., Sebbane, F., Long, D., and Hinnebusch, B. J.**,2006. *Yersinia pestis* YopJ suppresses tumor necrosis factor alpha induction and contributes to apoptosis of immune cells in the lymph node but is not required for virulence in a rat model of bubonic plague. Infect Immun. **74**(9): p. 5126-31.
149. **Lemonnier, M., Landraud, L., and Lemichez, E.**,2007. Rho GTPase-activating bacterial toxins: from bacterial virulence regulation to eukaryotic cell biology. FEMS Microbiol Rev. **31**(5): p. 515-34.
150. **Leong, J. M., Fournier, R. S., and Isberg, R. R.**,1990. Identification of the integrin binding domain of the Yersinia pseudotuberculosis invasin protein. Embo J. **9**(6): p. 1979-89.
151. **Letzelter, M., Sorg, I., Mota, L. J., Meyer, S., Stalder, J., Feldman, M., Kuhn, M., Callebaut, I., and Cornelis, G. R.**,2006. The discovery of SycO highlights a new function for type III secretion effector chaperones. Embo J. **25**(13): p. 3223-33.
152. **Leung, K. Y., Reisner, B. S., and Straley, S. C.**,1990. YopM inhibits platelet aggregation and is necessary for virulence of *Yersinia pestis* in mice. Infect Immun. **58**(10): p. 3262-71.
153. **Lian, C. J., Hwang, W. S., and Pai, C. H.**,1987. Plasmid-mediated resistance to phagocytosis in *Yersinia enterocolitica*. Infect Immun. **55**(5): p. 1176-83.
154. **Ljungberg, P., Valtonen, M., Harjola, V. P., Kaukoranta-Tolvanen, S. S., and Vaara, M.**,1995. Report of four cases of *Yersinia pseudotuberculosis* septicemia and a literature review. Eur J Clin Microbiol Infect Dis. **14**(9): p. 804-10.
155. **Lloyd, S. A., Norman, M., Rosqvist, R., and Wolf-Watz, H.**,2001. *Yersinia* YopE is targeted for type III secretion by N-terminal, not mRNA, signals. Mol Microbiol. **39**(2): p. 520-31.
156. **Logsdon, L. K. and Mecsas, J.**,2006. The proinflammatory response induced by wild-type *Yersinia pseudotuberculosis* infection inhibits survival of yop mutants in the gastrointestinal tract and Peyer's patches. Infect Immun. **74**(3): p. 1516-27.
157. **Macnab, R. M.**,1999. The bacterial flagellum: reversible rotary propellor and type III export apparatus. J Bacteriol. **181**(23): p. 7149-53.
158. **Macnab, R. M.**,2003. How bacteria assemble flagella. Annu Rev Microbiol. **57**: p. 77-100.
159. **Matson, J. S. and Nilles, M. L.**,2001. LcrG-LcrV interaction is required for control of Yops secretion in *Yersinia pestis*. J Bacteriol. **183**(17): p. 5082-91.
160. **McCoy, M. W., Marre, M. L., Lesser, C. F., and Mecsas, J.**,2010. The C-terminal tail of *Yersinia pseudotuberculosis* YopM is critical for interacting with RSK1 and for virulence. Infect Immun. **78**(6): p. 2584-98.
161. **McDonald, C., Vacratsis, P. O., Bliska, J. B., and Dixon, J. E.**,2003. The *yersinia* virulence factor YopM forms a novel protein complex with two cellular kinases. J Biol Chem. **278**(20): p. 18514-23.
162. **McGhie, E. J., Brawn, L. C., Hume, P. J., Humphreys, D., and Koronakis, V.**,2009. *Salmonella* takes control: effector-driven manipulation of the host. Curr Opin Microbiol. **12**(1): p. 117-24.

163. **McPhee, J. B., Mena, P., and Bliska, J. B.**,2010. 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. **78**(8): p. 3529-39.
164. **Mejia, E., Bliska, J. B., and Viboud, G. I.**,2008. *Yersinia* controls type III effector delivery into host cells by modulating Rho activity. PLoS Pathog. **4**(1): p. e3.
165. **Michiels, T., Vanooteghem, J. C., Lambert de Rouvroit, C., China, B., Gustin, A., Boudry, P., and Cornelis, G. R.**,1991. Analysis of *virC*, an operon involved in the secretion of Yop proteins by *Yersinia enterocolitica*. J Bacteriol. **173**(16): p. 4994-5009.
166. **Mills, S. D., Boland, A., Sory, M. P., van der Smissen, P., Kerbourch, C., Finlay, B. B., and Cornelis, G. R.**,1997. *Yersinia enterocolitica* induces apoptosis in macrophages by a process requiring functional type III secretion and translocation mechanisms and involving YopP, presumably acting as an effector protein. Proc Natl Acad Sci U S A. **94**(23): p. 12638-43.
167. **Mittal, R., Peak-Chew, S. Y., and McMahon, H. T.**,2006. Acetylation of MEK2 and I kappa B kinase (IKK) activation loop residues by YopJ inhibits signaling. Proc Natl Acad Sci U S A. **103**(49): p. 18574-9.
168. **Mohammadi, S. and Isberg, R. R.**,2009. *Yersinia pseudotuberculosis* virulence determinants invasin, YopE, and YopT modulate RhoG activity and localization. Infect Immun. **77**(11): p. 4771-82.
169. **Monack, D. M., Mecsas, J., Bouley, D., and Falkow, S.**,1998. *Yersinia*-induced apoptosis in vivo aids in the establishment of a systemic infection of mice. J Exp Med. **188**(11): p. 2127-37.
170. **Mueller, C. A., Broz, P., Muller, S. A., Ringler, P., Erne-Brand, F., Sorg, I., Kuhn, M., Engel, A., and Cornelis, G. R.**,2005. The V-antigen of *Yersinia* forms a distinct structure at the tip of injectisome needles. Science. **310**(5748): p. 674-6.
171. **Mukherjee, S., Keitany, G., Li, Y., Wang, Y., Ball, H. L., Goldsmith, E. J., and Orth, K.**,2006. *Yersinia* YopJ acetylates and inhibits kinase activation by blocking phosphorylation. Science. **312**(5777): p. 1211-4.
172. **Mylonakis, E., Casadevall, A., and Ausubel, F. M.**,2007. Exploiting amoeboid and non-vertebrate animal model systems to study the virulence of human pathogenic fungi. PLoS Pathog. **3**(7): p. e101.
173. **Navarro, L., Koller, A., Nordfelth, R., Wolf-Watz, H., Taylor, S., and Dixon, J. E.**,2007. Identification of a molecular target for the *Yersinia* protein kinase A. Mol Cell. **26**(4): p. 465-77.
174. **Neyt, C. and Cornelis, G. R.**,1999. Role of SycD, the chaperone of the *Yersinia* Yop translocators YopB and YopD. Mol Microbiol. **31**(1): p. 143-56.
175. **Neyt, C. and Cornelis, G. R.**,1999. Insertion of a Yop translocation pore into the macrophage plasma membrane by *Yersinia enterocolitica*: requirement for translocators YopB and YopD, but not LcrG. Mol Microbiol. **33**(5): p. 971-81.
176. **Nilles, M. L., Williams, A. W., Skrzypek, E., and Straley, S. C.**,1997. *Yersinia pestis* LcrV forms a stable complex with LcrG and may have a secretion-related regulatory role in the low-Ca2+ response. J Bacteriol. **179**(4): p. 1307-16.
177. World Health Organization (WHO)., *Weekly epidemiological record*, in *http://www.who.int/wer*. 2010. p. 37-48.
178. **Orth, K., Palmer, L. E., Bao, Z. Q., Stewart, S., Rudolph, A. E., Bliska, J. B., and Dixon, J. E.**,1999. Inhibition of the mitogen-activated protein kinase kinase superfamily by a *Yersinia* effector. Science. **285**(5435): p. 1920-3.
179. **Owen, R. L. and Jones, A. L.**,1974. Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. Gastroenterology. **66**(2): p. 189-203.
180. **Parkhill, J., Wren, B. W., Thomson, N. R., Titball, R. W., Holden, M. T., Prentice, M. B., Sebaihia, M., James, K. D., Churcher, C., Mungall, K. L., Baker, S., Basham, D., Bentley, S. D., Brooks, K., Cerdeno-Tarraga, A. M., Chillingworth, T., Cronin, A., Davies, R. M., Davis, P., Dougan, G., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Karlyshev, A. V., Leather, S., Moule, S., Oyston, P. C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S., and Barrell, B. G.**,2001. Genome sequence of *Yersinia pestis*, the causative agent of plague. Nature. **413**(6855): p. 523-7.

181. **Parsot, C., Hamiaux, C., and Page, A. L.**,2003. The various and varying roles of specific chaperones in type III secretion systems. Curr Opin Microbiol. **6**(1): p. 7-14.
182. **Pederson, K. J., Krall, R., Riese, M. J., and Barbieri, J. T.**,2002. Intracellular localization modulates targeting of ExoS, a type III cytotoxin, to eukaryotic signalling proteins. Mol Microbiol. **46**(5): p. 1381-90.
183. **Pederson, K. J., Vallis, A. J., Aktories, K., Frank, D. W., and Barbieri, J. T.**,1999. The amino-terminal domain of *Pseudomonas aeruginosa* ExoS disrupts actin filaments via small-molecular-weight GTP-binding proteins. Mol Microbiol. **32**(2): p. 393-401.
184. **Pepe, J. C. and Miller, V. L.**,1990. The *Yersinia enterocolitica inv* gene product is an outer membrane protein that shares epitopes with *Yersinia pseudotuberculosis* invasin. J Bacteriol. **172**(7): p. 3780-9.
185. **Perry, R. D. and Fetherston, J. D.**,1997. *Yersinia pestis*--etiologic agent of plague. Clin Microbiol Rev. **10**(1): p. 35-66.
186. **Persson, C., Carballeira, N., Wolf-Watz, H., and Fallman, M.**,1997. The PTPase YopH inhibits uptake of *Yersinia*, tyrosine phosphorylation of p130Cas and FAK, and the associated accumulation of these proteins in peripheral focal adhesions. Embo J. **16**(9): p. 2307-18.
187. **Persson, C., Nordfelth, R., Holmstrom, A., Hakansson, S., Rosqvist, R., and Wolf-Watz, H.**,1995. Cell-surface-bound *Yersinia* translocate the protein tyrosine phosphatase YopH by a polarized mechanism into the target cell. Mol Microbiol. **18**(1): p. 135-50.
188. **Persson, C., Nordfelth, R., Andersson, K., Forsberg, A., Wolf-Watz, H., and Fallman, M.**,1999. Localization of the *Yersinia* PTPase to focal complexes is an important virulence mechanism. Mol Microbiol. **33**(4): p. 828-38.
189. **Pettersson, J., Nordfelth, R., Dubinina, E., Bergman, T., Gustafsson, M., Magnusson, K. E., and Wolf-Watz, H.**,1996. Modulation of virulence factor expression by pathogen target cell contact. Science. **273**(5279): p. 1231-3.
190. **Pettersson, J., Holmstrom, A., Hill, J., Leary, S., Frithz-Lindsten, E., von Euler-Matell, A., Carlsson, E., Titball, R., Forsberg, A., and Wolf-Watz, H.**,1999. The V-antigen of *Yersinia* is surface exposed before target cell contact and involved in virulence protein translocation. Mol Microbiol. **32**(5): p. 961-76.
191. **Phromsuthirak, P.**,1977. Electron microscopy of wound healing in the skin of *Gasterosteus aculeatus*. J. Fish Biol. **11**: p. 193-206.
192. **Pouliot, K., Pan, N., Wang, S., Lu, S., Lien, E., and Goguen, J. D.**,2007. Evaluation of the role of LcrV-Toll-like receptor 2-mediated immunomodulation in the virulence of *Yersinia pestis*. Infect Immun. **75**(7): p. 3571-80.
193. **Prentice, M. B., Rahalison, L.**,2007. Plague. The Lancet. **369**: p. 1196-1207.
194. **Pujol, C. and Bliska, J. B.**,2005. Turning *Yersinia* pathogenesis outside in: subversion of macrophage function by intracellular yersiniae. Clin Immunol. **114**(3): p. 216-26.
195. **Reithmeier-Rost, D., Hill, J., Elvin, S. J., Williamson, D., Dittmann, S., Schmid, A., Wilharm, G., and Sing, A.**,2007. The weak interaction of LcrV and TLR2 does not contribute to the virulence of *Yersinia pestis*. Microbes Infect. **9**(8): p. 997-1002.
196. **Rimpilainen, M., Forsberg, A., and Wolf-Watz, H.**,1992. A novel protein, LcrQ, involved in the low-calcium response of *Yersinia pseudotuberculosis* shows extensive homology to YopH. J Bacteriol. **174**(10): p. 3355-63.
197. **Roberts, R. J., Young, H., Milne, J.A.**,1971. Studies on the skin of plaice (*Pleuronectes platessa* L.). 1. The structure and ultrastructure of normal plaice skin. J. Fish Biol. . **4**: p. 87-98.
198. **Roppenser, B., Roder, A., Hentschke, M., Ruckdeschel, K., and Aepfelbacher, M.**,2009. *Yersinia enterocolitica* differentially modulates RhoG activity in host cells. J Cell Sci. **122**(Pt 5): p. 696-705.
199. **Rosqvist, R., Bolin, I., and Wolf-Watz, H.**,1988. Inhibition of phagocytosis in *Yersinia pseudotuberculosis*: a virulence plasmid-encoded ability involving the Yop2b protein. Infect Immun. **56**(8): p. 2139-43.
200. **Rosqvist, R., Forsberg, A., and Wolf-Watz, H.**,1991. Intracellular targeting of the *Yersinia* YopE cytotoxin in mammalian cells induces actin microfilament disruption. Infect Immun. **59**(12): p. 4562-9.

201. **Rosqvist, R., Magnusson, K. E., and Wolf-Watz, H.**,1994. Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. Embo J. **13**(4): p. 964-72.
202. **Ruckdeschel, K., Roggenkamp, A., Schubert, S., and Heesemann, J.**,1996. Differential contribution of *Yersinia enterocolitica* virulence factors to evasion of microbicidal action of neutrophils. Infect Immun. **64**(3): p. 724-33.
203. **Ruckdeschel, K., Roggenkamp, A., Lafont, V., Mangeat, P., Heesemann, J., and Rouot, B.**,1997. Interaction of *Yersinia enterocolitica* with macrophages leads to macrophage cell death through apoptosis. Infect Immun. **65**(11): p. 4813-21.
204. **Ruckdeschel, K., Machold, J., Roggenkamp, A., Schubert, S., Pierre, J., Zumbihl, R., Liautard, J. P., Heesemann, J., and Rouot, B.**,1997. *Yersinia enterocolitica* promotes deactivation of macrophage mitogen-activated protein kinases extracellular signal-regulated kinase-1/2, p38, and c-Jun NH2-terminal kinase. Correlation with its inhibitory effect on tumor necrosis factor-alpha production. J Biol Chem. **272**(25): p. 15920-7.
205. **Russel, J. C.**,1968. That earlier plague. Demography. **5**: p. 174-184.
206. **Saier, M. H., Jr.**,2004. Evolution of bacterial type III protein secretion systems. Trends Microbiol. **12**(3): p. 113-5.
207. **Sato, H., Frank, D. W., Hillard, C. J., Feix, J. B., Pankhaniya, R. R., Moriyama, K., Finck-Barbancon, V., Buchaklian, A., Lei, M., Long, R. M., Wiener-Kronish, J., and Sawa, T.**,2003. The mechanism of action of the *Pseudomonas aeruginosa*-encoded type III cytotoxin, ExoU. Embo J. **22**(12): p. 2959-69.
208. **Sauvonnet, N., Lambermont, I., van der Bruggen, P., and Cornelis, G. R.**,2002. YopH prevents monocyte chemoattractant protein 1 expression in macrophages and T-cell proliferation through inactivation of the phosphatidylinositol 3-kinase pathway. Mol Microbiol. **45**(3): p. 805-15.
209. **Scheffzek, K., Ahmadian, M. R., and Wittinghofer, A.**,1998. GTPase-activating proteins: helping hands to complement an active site. Trends Biochem Sci. **23**(7): p. 257-62.
210. **Schesser, K., Frithz-Lindsten, E., and Wolf-Watz, H.**,1996. Delineation and mutational analysis of the *Yersinia pseudotuberculosis* YopE domains which mediate translocation across bacterial and eukaryotic cellular membranes. J Bacteriol. **178**(24): p. 7227-33.
211. **Schesser, K., Spiik, A. K., Dukuzumuremyi, J. M., Neurath, M. F., Pettersson, S., and Wolf-Watz, H.**,1998. 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. **28**(6): p. 1067-79.
212. **Schotte, P., Denecker, G., Van Den Broeke, A., Vandenabeele, P., Cornelis, G. R., and Beyaert, R.**,2004. Targeting Rac1 by the *Yersinia* effector protein YopE inhibits caspase-1-mediated maturation and release of interleukin-1beta. J Biol Chem. **279**(24): p. 25134-42.
213. **Schulte, R., Kerneis, S., Klinke, S., Bartels, H., Preger, S., Kraehenbuhl, J. P., Pringault, E., and Autenrieth, I. B.**,2000. Translocation of *Yersinia entrocolitica* across reconstituted intestinal epithelial monolayers is triggered by *Yersinia* invasin binding to beta1 integrins apically expressed on M-like cells. Cell Microbiol. **2**(2): p. 173-85.
214. **Sekiya, K., Ohishi, M., Ogino, T., Tamano, K., Sasakawa, C., and Abe, A.**,2001. Supermolecular structure of the enteropathogenic *Escherichia coli* type III secretion system and its direct interaction with the EspA-sheath-like structure. Proc Natl Acad Sci U S A. **98**(20): p. 11638-43.
215. **Shao, F., Merritt, P. M., Bao, Z., Innes, R. W., and Dixon, J. E.**,2002. A *Yersinia* effector and a *Pseudomonas* avirulence protein define a family of cysteine proteases functioning in bacterial pathogenesis. Cell. **109**(5): p. 575-88.
216. **Shao, F., Vacratsis, P. O., Bao, Z., Bowers, K. E., Fierke, C. A., and Dixon, J. E.**,2003. Biochemical characterization of the *Yersinia* YopT protease: cleavage site and recognition elements in Rho GTPases. Proc Natl Acad Sci U S A. **100**(3): p. 904-9.
217. **Shepard, K. L.**,1994. Functions of fish mucus. Rev. Fish Biol. Fish. **4**: p. 401-429

218. **Sing, A., Rost, D., Tvardovskaia, N., Roggenkamp, A., Wiedemann, A., Kirschning, C. J., Aepfelbacher, M., and Heesemann, J.**,2002. *Yersinia* V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. J Exp Med. **196**(8): p. 1017-24.
219. **Skrzypek, E., Cowan, C., and Straley, S. C.**,1998. Targeting of the *Yersinia pestis* YopM protein into HeLa cells and intracellular trafficking to the nucleus. Mol Microbiol. **30**(5): p. 1051-65.
220. **Small, J. V., Stradal, T., Vignal, E., and Rottner, K.**,2002. The lamellipodium: where motility begins. Trends Cell Biol. **12**(3): p. 112-20.
221. **Smego, R. A., Frean, J., and Koornhof, H. J.**,1999. Yersiniosis I: microbiological and clinicoepidemiological aspects of plague and non-plague *Yersinia* infections. Eur J Clin Microbiol Infect Dis. **18**(1): p. 1-15.
222. **Sneller, M. C. and Strober, W.**,1986. M cells and host defense. J Infect Dis. **154**(5): p. 737-41.
223. **Songsungthong, W., Higgins, M. C., Rolan, H. G., Murphy, J. L., and Mecsas, J.**,2010. ROS-inhibitory activity of YopE is required for full virulence of *Yersinia* in mice. Cell Microbiol. **12**(7): p. 988-1001.
224. **Sorabjee, J. S.**,2005. Plague. Medicine. **33**(7): p. 30-31.
225. **Sory, M. P. and Cornelis, G. R.**,1994. Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells. Mol Microbiol. **14**(3): p. 583-94.
226. **Stebbins, C. E. and Galan, J. E.**,2000. Modulation of host signaling by a bacterial mimic: structure of the *Salmonella* effector SptP bound to Rac1. Mol Cell. **6**(6): p. 1449-60.
227. **Stebbins, C. E. and Galan, J. E.**,2001. Maintenance of an unfolded polypeptide by a cognate chaperone in bacterial type III secretion. Nature. **414**(6859): p. 77-81.
228. **Steinert, M., Leippe, M., and Roeder, T.**,2003. Surrogate hosts: protozoa and invertebrates as models for studying pathogen-host interactions. Int J Med Microbiol. **293**(5): p. 321-32.
229. **Stender, S., Friebel, A., Linder, S., Rohde, M., Mirold, S., and Hardt, W. D.**,2000. Identification of SopE2 from *Salmonella typhimurium*, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell. Mol Microbiol. **36**(6): p. 1206-21.
230. **Stenmark, H.**,2009. Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol. **10**(8): p. 513-25.
231. **Straley, S. C.**,1991. The low-Ca2+ response virulence regulon of human-pathogenic Yersiniae. Microb Pathog. **10**(2): p. 87-91.
232. **Straley, S. C., Plano, G. V., Skrzypek, E., Haddix, P. L., and Fields, K. A.**,1993. Regulation by Ca2+ in the *Yersinia* low-Ca2+ response. Mol Microbiol. **8**(6): p. 1005-10.
233. **Styer, K. L., Hopkins, G. W., Bartra, S. S., Plano, G. V., Frothingham, R., and Aballay, A.**,2005. *Yersinia pestis* kills *Caenorhabditis elegans* by a biofilm-independent process that involves novel virulence factors. EMBO Rep. **6**(10): p. 992-7.
234. **Sulakvelidze, A.**,2000. Yersiniae other than *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*: the ignored species. Microbes Infect. **2**(5): p. 497-513.
235. **Sun, J. and Barbieri, J. T.**,2003. *Pseudomonas aeruginosa* ExoT ADP-ribosylates CT10 regulator of kinase (Crk) proteins. J Biol Chem. **278**(35): p. 32794-800.
236. **Svitkina, T. M., Verkhovsky, A. B., McQuade, K. M., and Borisy, G. G.**,1997. Analysis of the actin-myosin II system in fish epidermal keratocytes: mechanism of cell body translocation. J Cell Biol. **139**(2): p. 397-415.
237. **Tamano, K., Aizawa, S., Katayama, E., Nonaka, T., Imajoh-Ohmi, S., Kuwae, A., Nagai, S., and Sasakawa, C.**,2000. Supramolecular structure of the *Shigella* type III secretion machinery: the needle part is changeable in length and essential for delivery of effectors. Embo J. **19**(15): p. 3876-87.
238. **Tardy, F., Homble, F., Neyt, C., Wattiez, R., Cornelis, G. R., Ruysschaert, J. M., and Cabiaux, V.**,1999. *Yersinia enterocolitica* type III secretion-translocation system: channel formation by secreted Yops. Embo J. **18**(23): p. 6793-9.
239. **Torruellas, J., Jackson, M. W., Pennock, J. W., and Plano, G. V.**,2005. The *Yersinia pestis* type III secretion needle plays a role in the regulation of Yop secretion. Mol Microbiol. **57**(6): p. 1719-33.

240. **Troisfontaines, P. and Cornelis, G. R.**,2005. Type III secretion: more systems than you think. Physiology (Bethesda). **20**: p. 326-39.
241. **Trulzsch, K., Sporleder, T., Igwe, E. I., Russmann, H., and Heesemann, J.**,2004. Contribution of the major secreted yops of *Yersinia enterocolitica* O:8 to pathogenicity in the mouse infection model. Infect Immun. **72**(9): p. 5227-34.
242. **Une, T. and Brubaker, R. R.**,1984. *In vivo* comparison of avirulent Vwa- and Pgm- or Pstr phenotypes of yersiniae. Infect Immun. **43**(3): p. 895-900.
243. **Vallis, A. J., Yahr, T. L., Barbieri, J. T., and Frank, D. W.**,1999. Regulation of ExoS production and secretion by *Pseudomonas aeruginosa* in response to tissue culture conditions. Infect Immun. **67**(2): p. 914-20.
244. **Wattiau, P. and Cornelis, G. R.**,1993. SycE, a chaperone-like protein of *Yersinia enterocolitica* involved in Ohe secretion of YopE. Mol Microbiol. **8**(1): p. 123-31.
245. **Wattiau, P., Bernier, B., Deslee, P., Michiels, T., and Cornelis, G. R.**,1994. Individual chaperones required for Yop secretion by *Yersinia*. Proc Natl Acad Sci U S A. **91**(22): p. 10493-7.246. **Whitear, M.**,1970. The skin surface of of bony fishes. J Zool London. **160**: p. 437-454.
247. **Viboud, G. I. and Bliska, J. B.**,2001. A bacterial type III secretion system inhibits actin polymerization to prevent pore formation in host cell membranes. Embo J. **20**(19): p. 5373-82.
248. **Viboud, G. I. and Bliska, J. B.**,2005. *Yersinia* outer proteins: role in modulation of host cell signaling responses and pathogenesis. Annu Rev Microbiol. **59**: p. 69-89.
249. **Viboud, G. I., Mejia, E., and Bliska, J. B.**,2006. Comparison of YopE and YopT activities in counteracting host signalling responses to *Yersinia pseudotuberculosis* infection. Cell Microbiol. **8**(9): p. 1504-15.
250. **Viboud, G. I., So, S. S., Ryndak, M. B., and Bliska, J. B.**,2003. Proinflammatory signalling stimulated by the type III translocation factor YopB is counteracted by multiple effectors in epithelial cells infected with *Yersinia pseudotuberculosis*. Mol Microbiol. **47**(5): p. 1305-15.
251. **Wiley, D. J., Nordfeldth, R., Rosenzweig, J., DaFonseca, C. J., Gustin, R., Wolf-Watz, H., and Schesser, K.**,2006. The Ser/Thr kinase activity of the *Yersinia* protein kinase A (YpkA) is necessary for full virulence in the mouse, mollifying phagocytes, and disrupting the eukaryotic cytoskeleton. Microb Pathog. **40**(5): p. 234-43.
252. **Williams, A. W. and Straley, S. C.**,1998. YopD of *Yersinia pestis* plays a role in negative regulation of the low-calcium response in addition to its role in translocation of Yops. J Bacteriol. **180**(2): p. 350-8.
253. **Wilson, C. A., Tsuchida, M. A., Allen, G. M., Barnhart, E. L., Applegate, K. T., Yam, P. T., Ji, L., Keren, K., Danuser, G., and Theriot, J. A.**,2010. Myosin II contributes to cell-scale actin network treadmilling through network disassembly. Nature. **465**(7296): p. 373-7.
254. **Vincent, S. and Settleman, J.**,1997. The PRK2 kinase is a potential effector target of both Rho and Rac GTPases and regulates actin cytoskeletal organization. Mol Cell Biol. **17**(4): p. 2247-56.
255. **Visser, L. G., Annema, A., and van Furth, R.**,1995. Role of Yops in inhibition of phagocytosis and killing of opsonized *Yersinia enterocolitica* by human granulocytes. Infect Immun. **63**(7): p. 2570-5.
256. **Visser, L. G., Hiemstra, P. S., van den Barselaar, M. T., Ballieux, P. A., and van Furth, R.**,1996. Role of YadA in resistance to killing of *Yersinia enterocolitica* by antimicrobial polypeptides of human granulocytes. Infect Immun. **64**(5): p. 1653-8.
257. **Vlahou, G., Schmidt, O., Wagner, B., Uenlue, H., Dersch, P., Rivero, F., and Weissenmayer, B. A.**,2009. *Yersinia* outer protein YopE affects the actin cytoskeleton in *Dictyostelium discoideum* through targeting of multiple Rho family GTPases. BMC Microbiol. **9**: p. 138.
258. **Von Pawel-Rammingen, U., Telepnev, M. V., Schmidt, G., Aktories, K., Wolf-Watz, H., and Rosqvist, R.**,2000. GAP activity of the *Yersinia* YopE cytotoxin specifically targets the Rho pathway: a mechanism for disruption of actin microfilament structure. Mol Microbiol. **36**(3): p. 737-48.
259. **Wren, B. W.**,2003. The yersiniae--a model genus to study the rapid evolution of bacterial pathogens. Nat Rev Microbiol. **1**(1): p. 55-64.

260. **Wulff-Strobel, C. R., Williams, A. W., and Straley, S. C.**,2002. LcrQ and SycH function together at the Ysc type III secretion system in *Yersinia pestis* to impose a hierarchy of secretion. Mol Microbiol. **43**(2): p. 411-23.
261. **Wurtele, M., Renault, L., Barbieri, J. T., Wittinghofer, A., and Wolf, E.**,2001. Structure of the ExoS GTPase activating domain. FEBS Lett. **491**(1-2): p. 26-9.
262. **Wurtele, M., Wolf, E., Pederson, K. J., Buchwald, G., Ahmadian, M. R., Barbieri, J. T., and Wittinghofer, A.**,2001. How the *Pseudomonas aeruginosa* ExoS toxin downregulates Rac. Nat Struct Biol. **8**(1): p. 23-6.
263. **Yahr, T. L., Barbieri, J. T., and Frank, D. W.**,1996. Genetic relationship between the 53- and 49-kilodalton forms of exoenzyme S from *Pseudomonas aeruginosa*. J Bacteriol. **178**(5): p. 1412-9.
264. **Yahr, T. L., Vallis, A. J., Hancock, M. K., Barbieri, J. T., and Frank, D. W.**,1998. ExoY, an adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III system. Proc Natl Acad Sci U S A. **95**(23): p. 13899-904.
265. **Yother, J. and Goguen, J. D.**,1985. Isolation and characterization of Ca2+-blind mutants of Y*ersinia pestis*. J Bacteriol. **164**(2): p. 704-11.
266. **Zhang, Y. and Bliska, J. B.**,2010. YopJ-promoted cytotoxicity and systemic colonization are associated with high levels of murine interleukin-18, gamma interferon, and neutrophils in a live vaccine model of *Yersinia pseudotuberculosis* infection. Infect Immun. **78**(5): p. 2329-41.
267. **Zhang, Y. and Barbieri, J. T.**,2005. A leucine-rich motif targets *Pseudomonas aeruginosa* ExoS within mammalian cells. Infect Immun. **73**(12): p. 7938-45.
268. **Zhang, Y., Deng, Q., and Barbieri, J. T.**,2007. Intracellular localization of type III-delivered *Pseudomonas* ExoS with endosome vesicles. J Biol Chem. **282**(17): p. 13022-32.
269. **Zhang, Y., Deng, Q., Porath, J. A., Williams, C. L., Pederson-Gulrud, K. J., and Barbieri, J. T.**,2007. Plasma membrane localization affects the RhoGAP specificity of *Pseudomonas* ExoS. Cell Microbiol. **9**(9): p. 2192-201.
270. **Zhou, D., Chen, L. M., Hernandez, L., Shears, S. B., and Galan, J. E.**,2001. A *Salmonella* inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. Mol Microbiol. **39**(2): p. 248-59.