

**Role of YopE and LcrH in effector translocation, HeLa  
cell cytotoxicity and virulence**

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# **TABLE OF CONTENTS**

|  |           |
|--|-----------|
| <b>ABSTRACT .....</b>  | <b>5</b>  |
| <b>PAPERS IN THIS THESIS.....</b>  | <b>6</b>  |
| <b>INTRODUCTION .....</b>  | <b>7</b>  |
| <i>Yersinia spp. and their diseases.....</i>                                     | <i>7</i>  |
| Protective antigens and treatment of <i>Yersinia</i> infections .....            | 8         |
| <i>Virulence properties of Yersinia.....</i>                                     | <i>8</i>  |
| Motility.....  | 8         |
| Adhesion proteins.....   | 9         |
| Capsule and LPS .....  | 9         |
| <i>Target cells and cellular model systems.....</i>                              | <i>10</i> |
| <i>Type III Secretion System .....</i>   | <i>11</i> |
| The Secretion Apparatus .....  | 11        |
| T3SS chaperones .....  | 12        |
| The secretion signal.....  | 13        |
| Substrate specificity switch.....  | 13        |
| Translocation of effector proteins into eukaryotic cells.....                    | 14        |
| <i>Regulation of the T3SS.....</i>   | <i>15</i> |
| Positive regulation of T3S.....  | 15        |
| The Low calcium response.....  | 15        |
| De-repression of Yop synthesis by secretion of negative regulatory elements..... | 16        |
| LcrH is a negative regulator in <i>Yersinia</i> .....                            | 17        |
| <i>Effector proteins of Yersinia .....</i>                                       | <i>18</i> |
| <i>The family of RhoGTPases .....</i>  | <i>20</i> |
| Regulation of RhoGTPases .....   | 20        |
| RhoGTPases and the actin cytoskeleton .....                                      | 21        |
| Control of endocytosis by RhoGTPases.....  | 22        |
| RhoGTPases as targets for bacterial toxins.....                                  | 23        |
| <b>GENERAL AND SPECIFIC AIMS .....</b>   | <b>24</b> |

**RESULTS AND DISCUSSION ..... 25**

*In vitro* GAP activity towards RhoA, Rac1 and Cdc42 can be uncoupled from cytotoxicity ..... 25

*In vivo* substrate specificity of YopE in infected cells ..... 26

The role of YopE in prevention of pore formation..... 28

Regulation of effector translocation into target cells – a role for YopE..... 29

Functional differences between YopE and ExoS ..... 31

Another potential mechanism of translocation control in *Yersinia*..... 33

Delineation of YopD ..... 34

Low levels of the translocators is sufficient for effector translocation ..... 35

**ACKNOWLEDGEMENTS ..... 37**

**REFERENCES ..... 39**

**PAPERS I-V**

## **ABSTRACT**

In order to establish an extra-cellular infection the gram-negative bacteria *Yersinia pseudotuberculosis* uses a type III secretion system (T3SS) to translocate a set of anti-host effectors into eukaryotic cells. The toxins disrupt signalling pathways important for phagocytosis, cytokine production and cell survival. Secretion and translocation via this T3SS is strictly regulated on several levels. In this context, the function of YopE and LcrH during *Yersinia* infections has been analysed.

YopE is an essential translocated effector that disrupts the actin cytoskeleton of infected eukaryotic cells, by inactivating small GTPases through its GTPase activating protein (GAP) activity. However, cytotoxicity can be uncoupled from *in vitro* GAP activity towards the RhoA, Rac1 and Cdc42 GTPases. Furthermore, *in vivo* studies of the YopE GAP activity revealed that only RhoA and Rac1 are targeted, but this is not a pre-requisite for *Yersinia* virulence. Hence, YopE must target one or more additional GTPases to cause disease in mice.

YopE was the only *Yersinia* effector that blocked LDH release from infected cells. Moreover, translocated YopE could regulate the level of subsequent effector translocation by a mechanism that involved the YopE GAP function and another T3S component, YopK. Loss of translocation control elevated total T3S gene expression in the presence of eukaryotic cells. This indicated the existence of a regulatory loop for feedback control of T3S gene expression in the bacteria that originates from the interior of the eukaryotic cell after effector translocation is completed. This might represent the true virulence function of YopE.

Exoenzyme S (ExoS) of *Pseudomonas aeruginosa* has a YopE-like GAP domain with similar activity towards RhoA, Rac1 and Cdc42. However, ExoS is unable to complement hyper-translocation resulting from loss of YopE. This indicates a unique function for YopE in translocation control in *Yersinia* that might be dependent on correct intracellular localisation. It follows that the Membrane Localisation Domain in YopE was important for translocation control, but dispensable for cytotoxicity and blockage of LDH release.

YopD and its cognate chaperone LcrH are negative regulatory elements of the T3S regulon and together with YopB, are involved in the effector translocation process. Randomly generated point mutants in LcrH specifically effected stability and secretion of both the YopB and YopD substrates *in vitro* and prevented their apparent insertion as translocon pores in the membranes of infected cells. Yet, these mutants still produced stable substrates in the presence of eukaryotic cells and most could mediate at least partial effector translocation. Thus, only minimal amounts of the YopB and YopD translocator proteins are needed for translocation and the LcrH chaperone may regulate this process from inside the bacteria.

## PAPERS IN THIS THESIS

This thesis is based on the following publications referred to in the text by their roman numerals (I-V).

**I.** 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): 297-308. Reprinted with permission from Elsevier.

**II.** Aili, M., Isaksson, E.L., Hallberg, B., Wolf-Watz, H. and Rosqvist, R. 2005. Functional analysis of the YopE GAP activity of *Yersinia pseudotuberculosis*. *Cell. Microbiol.* *Submitted manuscript.*

**III.** Aili, M., Isaksson, E.L., Carlsson, S., Rosqvist, R., and Francis, M.S. 2005. Regulation of *Yersinia* Yop-effector delivery by translocated YopE. *Manuscript.*

**IV.** Francis, M.S., Aili, M., Wiklund, M-L., and Wolf-Watz, H. 2000. A study of the YopD-LcrH interaction from *Yersinia pseudotuberculosis* reveals a role for hydrophobic residues within the amphipathic domain of YopD. *Mol. Microbiol.* 38(1): 85-102. Reprinted with permission from Blackwell Publishing.

**V.** Edqvist, P.J., Aili, M., and Francis, M.S. 2005. Minimal secretion of the YopB and YopD translocators is sufficient for Yop-effector translocation by *Yersinia*. *Manuscript.*

Contribution by the author to the studies listed below will not be extensively discussed in this thesis.

Aili, M., Hallberg, B., Wolf-Watz, H., and Rosqvist, R. 2002. GAP activity of *Yersinia* YopE. *Methods Enzymol.* 358:359-370.

Henriksson, M.L., Francis, M.S., Peden, A., Aili, M., Stefansson, K., Palmer, R, Aitken, A. and Hallberg, B. 2002. A nonphosphorylated 14-3-3 binding motif on exoenzyme S that is functional *in vivo*. *Eur. J. Biochem.* 269(20):4921-4929.

## **INTRODUCTION**

As human beings living on the planet Earth, we are constantly in contact with organisms too small for us to see: microbes. They are everywhere and in many ways dictate the life we live. Some are beneficial to us, some do not care about us, and some are harmful to us, yet many generally see us as perfect food and housing sources. One group of micro-organisms we encounter causes diseases, ranging from mild to life-threatening. Disease-causing bacteria come in many variants and have developed a multitude of strategies to inflict harm on humans, and other host organisms. Two broad avenues of bacterial life-styles can be identified within a human host; extra-cellular and intra-cellular. Each bacterial species has developed specific mechanisms for survival, in addition to general ones shared by all bacteria, which arise from their choice of habitat e.g. the human body. The work described in this thesis has dealt with the use of an ingenious toxin distribution apparatus used by a wide array of bacterial pathogens, to infect an equally impressive number of hosts within the plant and animal kingdoms. This thesis primarily focuses on the human gastro-intestinal pathogen *Yersinia pseudotuberculosis*.

### ***Yersinia spp. and their diseases***

Gram-negative *Yersinia* spp. belong to the family *Enterobacteriaceae* and include 11 species, of which three are human pathogens [211, 274]. *Y. pestis*, the causative agent of plague, is transmitted by flea bites or aerosols, and can produce three clinical manifestations; bubonic plague with a mortality rate of between 40-60% (if left untreated), septicemic plague without bubo manifestation and pneumonic plague, both with a mortality rate close to 100% [211].

*Y. pestis* is closely related to the enteropathogenic *Y. pseudotuberculosis*, a food- and water-borne pathogen which causes self-limiting gastrointestinal infections in healthy humans [186]. The divergence of *Y. pestis* from *Y. pseudotuberculosis* may be as recent as 1500-20000 years ago [1]. Compared to *Y. pseudotuberculosis*, *Y. pestis* has down-sized its genome, thereby restricting itself to a life-style as a strict pathogen dependent upon the infected host for a number of essential nutrients, including amino acids and nucleotide precursors [56, 203].

*Y. enterocolitica* is a more distant relative to *Y. pestis* and *Y. pseudotuberculosis*, but causes a similar disease in humans as does *Y. pseudotuberculosis* [41, 42]. Enteric *Yersinia* causes terminal septicemia in mice, termed yersiniosis [55], which closely resembles the human disease. The mouse model of yersiniosis have been a

valuable tool in identifying virulence determinants in *Yersinia* [39, 102, 185, 289]. Non-pathogenic *Yersinia* have been isolated from food and water as well as sick and healthy humans [274].

### **Protective antigens and treatment of *Yersinia* infections**

Survivors of plague and humans exposed to enteric *Yersinia*, develop protective antibodies towards primarily the F1 capsule of *Y. pestis* and the V antigen (LcrV) of all three species [42, 211]. Historically, either live attenuated strains or killed whole cell vaccines have been used to protect against primarily plague although with limited success, especially against pneumonic plague [286]. Several of the *Yersinia* outer proteins (discussed below) are antigenic, but do not provide protection against plague in mice [159]. Today *Y. pestis* is considered a potential biological weapon, and this emphasizes the need for an effective vaccine.

Identified *Yersinia* infections can usually be successfully treated in the early stages by administration of classical antibiotics such as streptomycin and tetracycline [41, 211]. However, new anti-bacterial agents should be considered [146], as multi-resistant isolates of *Y. pestis* have been identified [101].

### **Virulence properties of *Yersinia***

The three human pathogenic species of *Yersinia* share several common virulence determinants despite the very different diseases they cause in humans. Motility enhances infection by enteric *Yersinia* [313, 314]. Attachment to the extra-cellular matrix and eukaryotic cells is facilitated by several different bacterial cell surface adhesins. A Type III Secretion System (T3SS) is used by the bacteria to disarm immune cells [66]. The T3SS is used to introduce toxins, anti-host effector proteins commonly named Yersinia outer proteins (Yops), into target cells, thereby preventing activation of the immune system [183, 198, 249, 298, 309]. All pathogenic *Yersinia* harbour a common, inter-changeable virulence plasmid of roughly 70 kb [23, 83, 220, 303], which encodes the components of the T3SS as well as the translocated Yops [24, 37, 40, 65, 116, 163, 220, 238, 269]. In addition, *Y. pestis* carries two specific plasmids encoding important virulence traits such as the F1 capsule and Plasminogen activator protease (Pla) [135] and the *ymt* gene, the product of which facilitates survival in, and spread via the flea [128].

### **Motility**

All *Yersinia* spp. possess the genes required for synthesis of flagella which confer motility to enteric *Yersinia* at low temperatures [62]. The expression of flagella

genes is repressed at high temperatures (37°C) [144, 145] and is possibly under the control of a quorum sensing system [18]. Motility of *Yersinia* plays a role in invasion of host cells and expression of the flagella genes is coordinated with that of the adhesin invasin [20, 313]. Motility is likely to enhance the ability of the bacteria to reach the intestinal epithelium during the early stages of infection. One of the flagella gene clusters in *Y. pestis* contains multiple mutations and would appear to be non-functional, whereas the other cluster seems functional [203]. However, the conditions during which it is expressed remain elusive, since motility has not been observed for *Y. pestis* [203].

### **Adhesion proteins**

*Yersinia* expresses several adhesins that exhibit diverse functions during infection. Adhesins allow enteric *Yersinia* to attach to host epithelial cells in the gastrointestinal tract and initiate uptake through M cells [63]. However, several adhesins are non-functional in *Y. pestis* [56], perhaps as a result of the radically different infection route of this species compared to the enteropathogenic *Yersinia*. No adhesin so far studied in enteric *Yersinia* is absolutely required for virulence in mice, although adhesin deficient mutants are slower in penetrating the intestinal epithelium [210, 291].

Four adhesins have been studied in *Yersinia*; Invasin [140], YadA [260], pH6 antigen [308] and Ail [179]. **Invasin** is a chromosomally encoded adhesin which interacts with host cell  $\beta$ 1-integrins to promote uptake of *Yersinia*, presumably during the initial phase of infection when enteric *Yersinia* penetrates the M cells of the intestinal epithelium [63, 251]. **YadA** promotes tight binding to eukaryotic cells and efficient uptake of the bacteria [34, 78] at 37°C when invasin is repressed [20]. In addition, YadA confers serum resistance to the bacteria [26], which is presumably important for their dissemination. **Ail** was identified by its ability to confer invasive properties to non-invasive *Escherichia coli* [179], however its role in *Yersinia* virulence is not clear [291]. **pH6 antigen** promotes adhesion to eukaryotic cells and is involved in hemagglutination [308], but a role of pH6 antigen in preventing phagocytosis is also proposed [136]. pH6 antigen is required for full virulence in *Y. pestis* [165], likely due to the absence of the other three adhesins in this species [56]. Recent genome projects have revealed a number of potential adhesin molecules in *Y. pestis*, but their functions are still unknown [203].

### **Capsule and LPS**

The capsule produced by *Y. pestis* at 37°C is a unique feature not shared with enteric *Yersinia* [135, 211]. The capsule is implicated in the ability of the bacterium

to resist phagocytosis by monocytes [74, 211] and expression obstructs bacterial binding to eukaryotic cells [74]. Enteric *Yersinia* produces a protective lipopolysaccharide (LPS) layer which confers resistance to complement-mediated killing as well as protection from antimicrobial peptides [259, 261]. In addition, LPS plays a role in effective colonisation of the intestinal tract [259]. A rough type of LPS is produced by *Y. pestis* [203, 259], where presumably the capsule is sufficient protection against the innate immune system.

### ***Target cells and cellular model systems***

Historically, *Yersinia* was thought of as an intra-cellular pathogen, residing in phago-lysosomes of macrophages [211, 224]. However, this view changed with the observation that virulent *Yersinia* prevents phagocytosis by professional phagocytes [51, 123, 238, 239, 278]. All pathogenic *Yersinia* have tropism for lymphoid tissues in infected hosts [66]. Lymphoid organs are not easily accessible for the bacteria, as they need to cross specialised barriers in order to reach this niche. Enteric *Yersinia* have the ability to promote uptake by specialised M cells in the intestinal epithelium [63, 112, 172]. By utilizing the unique features of antigen sampling exhibited by M cells, *Yersinia* is delivered to the Peyer's patches underneath the epithelial layer in the intestine [63, 172]. In this immune cell dense milieu, the T3SS is presumed to be activated and the translocated Yop effectors disarm the innate immune response to *Yersinia*, thus enabling extra-cellular growth of the pathogen [122, 187]. In a recent murine infection study, macrophages and dendritic cells were identified as the *in vivo* target cells for translocated Yop effectors [171], presumably as an indirect effect of the phagocytic nature of these cells. However, all pathogenic species of *Yersinia* are considered to be facultative intra-cellular bacteria and can replicate within macrophages [224, 271, 272, 320]. This ability might be of importance during initial stages of infection.

Several *in vitro* cell infection models for *Yersinia* have been developed over the years. HeLa cells have been extensively used to study both the invasive properties and the ability of the bacteria to prevent uptake [73, 213, 290]. Upon infection with wild type *Yersinia*, HeLa cells undergo a morphology change resulting in disassembly of the actin cytoskeleton [240, 242]. Macrophages have also been a favoured cell type for functional studies of *Yersinia* to stimulate infection-like conditions *in vitro* [108, 290]. Both HeLa cells and immune cell lines have been used to identify eukaryotic targets of essential virulence effectors of *Yersinia* [30, 72, 106, 213]. Yeast has also been successfully used to reveal functions of translocated effector proteins [188, 304].

### ***Type III Secretion System***

Type III Secretion Systems (T3SS) can be viewed upon as syringes used by numerous gram-negative bacteria to directly transport toxins from the bacterial cytoplasm across two bacterial membranes and a eukaryotic cell membrane to the interior of a eukaryotic cell [100]. Among the proteins necessary for building up the secretion machinery spanning the bacterial envelope is a core set with high identity in all known T3SS [6, 137, 191, 200]. A second set of proteins with lesser identity between systems most likely reflects a level of built-in specificity needed to sense the environment so that the bacteria knows where and when the injection apparatus should be used to modulate host responses [100, 200]. This would be different for each species, because they normally occupy unique niches defined by a set of distinctive environmental cues [137].

T3SSs can be separated from other bacterial secretion systems [285] by four distinguishable features; **1)** the N-terminal secretion signal is not cleaved upon secretion [60, 177] **2)** customised intra-bacterial chaperones are needed for many of the secreted proteins [141, 190, 293, 302] **3)** high induction of the system requires tight interaction with target cells during infection [214, 241]. **4)** one-step introduction of toxins from the bacterial cytoplasm to the interior of eukaryotic cells [161].

### **The Secretion Apparatus**

Analysis of secretion-deficient *Yersinia* revealed a role for about 20 *ysc* (*Yersinia secretion*) genes, located on the common *Yersinia* virulence plasmid, in secretion of virulence determinants [9, 24, 86, 116, 178, 217, 301]. Ysc products is believed to assemble in the bacterial envelope into a syringe-like structure to enable transport of T3SS secreted substrates over the bacterial double-membrane, as has been visualised for T3SS from other bacteria [36, 152, 252, 281]. The Ysc-T3SS is responsible for secretion and translocation into eukaryotic cells of at least six different Yop effectors (discussed below). The *ysc* genes were indirectly identified as regulators of the Low calcium response (discussed below) [24, 86, 178, 217], since a non-functional T3SS prevents induction of secreted substrates [24]. In this way, correct regulation of the system requires a secretion competent apparatus, a process referred to as feedback inhibition.

Recently, a second T3SS was identified in *Yersinia*, the Ysa (*Yersinia secretion apparatus*) system. It is encoded on the chromosome and contains at least 13 Ysa proteins which build up the Ysa secretion apparatus, independently of the Ysc T3SS [90, 121]. Initial studies showed that at least 8 proteins, called Ysps (*Yersinia secreted proteins*), were secreted by the Ysa T3SS [121]. Interestingly, three of

these Ysps have been identified as known secretion substrates of the Ysc T3SS; YopN, YopJ/P and YopE [311]. Four novel Ysps are known only to be substrates of the Ysa T3SS, specificity provided in part by a Ysp specific chaperone, all of which are encoded on the chromosome in close proximity to the *ysa* operon [91]. Nevertheless, to be able to secrete common substrates, the Ysa-T3SS and the Ysc-T3SS must engage in regulatory cross-talk in a way that is not yet known.

### **T3SS chaperones**

Functional Type III Secretion (T3S) depends on two classes of cytosolic chaperones; effector class chaperones and translocator class chaperones [293, 302]. Common to both classes is their ability to confer increased stability and efficient secretion of a cognate substrate(s) (PAPER IV, [68, 69, 89, 96, 247, 293, 294]). T3SS chaperones are small, acidic globular proteins [167, 293] that bind to their substrate generally as homodimers, or as in the case of YscB and SycN, heterodimers [27, 68, 216].

**Effector class chaperones** bind to translocated effectors of the T3SS and consist of SycH, YerA (SycE) and SycT in *Yersinia*. YerA and SycT bind YopE and YopT [96, 138, 294], respectively, while SycH binds not only YopH, but also LcrQ (YscM1 and YcsM2 in *Y. enterocolitica*) [216, 293]. A regulatory role has been proposed for the SycH/LcrQ complex in establishing a secretion hierarchy through the Ysc T3SS, allowing for rapid secretion of YopH before YopE [305]. Similarly, the YerA and SycT chaperones would secure secretion of YopE and YopT before secretion of the chaperone-less effectors YopM, YopJ and YpkA [167]. Only moderate sequence similarity exist between the known effector chaperones from different T3SSs, which is surprising in light of their structural similarities – a common mix of  $\beta$ -sheet and  $\alpha$ -helical folds [27, 169, 216, 268]. In general, the effector class chaperones keep the N-terminus of its substrate in an unfolded state, whereas in contrast the C-terminal enzymatic domain of the substrate often is folded and active [7, 28, 268].

**Translocator class chaperones** usually bind at least two substrates, the translocator proteins essential for effector translocation into eukaryotic cells (PAPER IV, [197, 293]). Structurally, this class of chaperones harbour a distinct tetratricopeptide repeat motif involved in protein-protein interactions [201], which are of functional importance to the *Yersinia* chaperone LcrH [76]. LcrH (SycD) of *Y. pseudotuberculosis* binds and stabilises YopB and YopD in the bacterial cytoplasm prior to secretion (PAPER IV, [190, 197]). A YopD-LcrH complex is also required for proper regulation of secretion and translocation (discussed below) [92].

## **The secretion signal**

Proteins secreted by the T3SS are not processed [177]. The secretion signal of T3S substrates is located in the very N-terminus of the protein [160, 177, 247], but its nature (peptide or mRNA) is still under debate [12, 168, 226-228]. Recently, evidence for promiscuous secretion of substrates by both T3SS and the flagellar system of *Yersinia* have been reported [292, 311, 312], suggesting a general secretion targeting signal for secreted substrates of both systems. This is not surprising in light of evidence that flagella assembly requires its own T3SS, from which it is believed the pathogenic T3SSs have evolved [110, 200, 244]. *Yersinia* encodes three structurally independent T3SSs that all secrete a common subset of substrates. Thus, bacteria might well regulate the secretion specificity by only expressing one system at a certain time [86, 91, 155, 295]. This suggests a cross-talk between the regulatory circuits of the three systems in *Yersinia*. A further illustration of this is the activation of the *yop* regulon in the absence of the flagellar regulatory genes [33]. However, secretion from the bacteria via a different T3SS is not always followed by translocation into eukaryotic target cells [160]. Yet, studies have shown that in *Yersinia* the effector protein YopJ is targeted by both the Ysc and the Ysa T3SS into the cytosol of eukaryotic cells [311].

A second secretion signal has been described for T3SS substrates that is dependent on specific chaperones [44, 197, 247, 293]. Chaperone-dependent secretion of pre-made proteins is rapid [168] and has been suggested to confer a hierarchy to secretion, such that proteins essential for the translocation step are secreted prior to the translocated effectors [167]. Recently a study in *Salmonella enterica* proposed a model for chaperone-dependent secretion of effectors [7]. The effector-chaperone complex docks to the T3SS ATPase in the cytoplasm of the bacteria via a binding domain in the chaperone. ATP hydrolysis by the ATPase unfolds the enzymatic domain of the effector and releases the effector from the chaperone for secretion through the T3SS [7]. The T3SS ATPase is a highly conserved component [137], so that the observed functional relationship between chaperone-ATPase is likely to be a universal feature among all T3SSs. It is also very likely that it is this relationship that enables chaperones to confer secretion specificity, so that substrates are secreted by the appropriate T3SS [160].

## **Substrate specificity switch**

A common theme for T3SS is a change in substrate secretion between components constituting the secretion apparatus, regulatory substrates, the translocators and finally the translocated effector proteins. How this specificity switch is achieved is not completely clear, but a key role is played by YscP.

YscP is a secreted component of the T3SS affecting *yop* expression and secretion of Yops [205, 265]. Deletion of *yscP* results in an increase in the amount of YscF (the needle protein of the T3S apparatus) on the surface of the bacteria and longer YscF-filaments [4, 77]. This indicates that an *yscP* mutant fails to switch from secretion of needle components to translocator/effector substrates. YscP must control secretion specificity together with YscU, an inner-membrane protein with a large cytosolic domain [9], since suppressor mutants in YscU restore controlled secretion of Yops in a *yscP* mutant [77]. YscU can be cleaved in the C-terminus [157], which is essential for Yop secretion [156]. Consistent with this regulatory effect, is the decrease in *yop* expression and secretion when YscU is over-expressed [157].

### **Translocation of effector proteins into eukaryotic cells**

The final goal for pathogenic bacteria utilizing a T3SS is the introduction of anti-host effector proteins into the cytosol of eukaryotic target cells [67, 100]. In *Yersinia*, the translocon operon *lcrGVHyopBD* encodes the translocator proteins YopB, YopD and LcrV [25, 212, 222]. Secretion of these proteins by the T3SS is essential for subsequent translocation of Yop effectors into eukaryotic cells [118, 119, 125, 192, 195, 214, 243, 264, 283]. The translocators are believed to form a pore in the eukaryotic cell membrane through which the effector proteins gain access to the eukaryotic cell [133, 170, 197, 243, 297].

LcrV was first recognized as a protective antigen against *Y. pestis* produced at 37°C [19, 50], and subsequently identified as part of the Low calcium response (Lcr) [212], meaning that it promotes effector gene expression upon deprivation of Ca<sup>2+</sup> (*in vitro*) [221]. More recently however, LcrV was shown to be involved in the translocation process *per se* by being the only purified component of the translocation apparatus capable of pore formation [48, 133, 170]. Consistent with this is localisation of LcrV at the bacterial surface, presumably at the zone of contact between bacteria and target cell, including the tip of the YscF needle [85, 184, 215]. LcrV also interacts with YopB and YopD [190, 245], the two other essential secreted components of the translocation machinery that are also considered to be pore forming proteins [117, 119, 197]. LcrG has no direct role in formation of the translocation pore [189], rather LcrG is a negative regulator of T3SS (discussed below) [173, 193, 255].

Finally, LcrH is also encoded by the translocon operon. It was first identified as a negative regulator of translocation and essential for virulence [25, 189, 212, 223]. Later it was demoted to a “mere” chaperone function when it was identified as essential for the stability of the translocator proteins YopB and YopD inside

bacterial cells and for their subsequent efficient secretion [190, 293]. As essential components of the translocation apparatus, YopB and YopD also form pores in plasma membranes of eukaryotic cells [119, 189, 283]. In addition, YopD has a regulatory role inside bacteria, where it acts as a negative regulator of *yop* expression in partnership with LcrH and another regulatory element, LcrQ [11, 92, 197, 299]. Furthermore, YopD is itself translocated into target cells [93], but the role of translocated YopD is unknown.

### ***Regulation of the T3SS***

Regulation of the T3SS is achieved on several levels. In response to physical and chemical cues, such as temperature and other extra-cellular signals, the bacteria can determine a spatial reference during infection and decide if this is where it needs to induce the *yop* regulon. In addition, functional completion of the secretion apparatus is an important check-point, as is the monitoring of translocation of the effectors in order to recognize when sufficient intoxication is achieved.

### **Positive regulation of T3S**

The T3SS in *Yersinia* is repressed at 26°C, the optimal growth temperature for *Yersinia*, and activated at 37°C, host temperature [88, 129, 130]. Positive transcriptional regulation of the *yop* regulon at 37°C is achieved by activation of LcrF (VirF in *Y. enterocolitica*), an AraC-like transcriptional activator [24, 64, 129, 130, 155, 310]. In simulated *in vivo* like conditions, production and translocation of Yops by *Yersinia* requires close contact with the eukaryotic target cell [119, 134, 214, 241].

### **The Low calcium response**

During *in vitro* growth, pathogenic *Yersinia* exhibits growth restriction at 37°C in the absence of calcium, termed the Low calcium response (Lcr) [49, 99, 109, 120, 127, 154]. This directly coincides with high level production and T3SS-mediated export of Yops and LcrV to the culture supernatant [38, 39, 86, 175, 176, 215, 220, 270, 273]. For the most part, all components necessary for this temperature and calcium regulation are encoded on the common 70 kb virulence plasmid of *Yersinia* [23, 83, 104, 105, 218, 219].

Growth restriction is a fascinating phenomenon in *Yersinia*, presenting an intriguing paradox – no growth when the key virulence determinant, T3S, is maximally induced – which is not yet solved. Nevertheless, growth restriction and the Lcr have allowed the characterisation of several T3S genes. Wild type *Yersinia*,

which require calcium for growth at 37°C *in vitro* (termed Calcium Dependent, CD) maintain normal Yop regulatory control under these conditions [39, 127, 154]. On the other hand, strains able to grow at 37°C even without the addition of calcium (termed Calcium Independent, CI), are usually avirulent in virulence models of yersiniosis, since they do not express the T3SS [99, 109, 196]. These strains are likely defective in a positive activator of T3S or fail to assemble a functional secretion system. In contrast, strains unable to grow at 37°C regardless of calcium condition, are termed Temperature Sensitive (TS). These strains are constitutively producing Yops, likely due to loss of negative regulation [87, 92, 223, 255, 299]. Of late, these broad groups appear inadequate, since loss of some regulatory proteins result in a similar growth phenotype, although the proteins act on different levels of T3S regulation. In addition, studies reported here clearly highlight that *in vitro* regulation phenotypes are not always mirrored by regulatory phenotypes observed *in vivo* (PAPER III and PAPER V).

### **De-repression of Yop synthesis by secretion of negative regulatory elements**

Negative regulation of the T3SS in the absence of inductive signals at 37°C is believed to be achieved by blocking of the secretion system. The cytosolic protein LcrG controls the *yop* regulon during non-inducing conditions [255], presumably by forming a complex together with LcrV hindering secretion through the T3SS [71, 158, 173, 193]. Upon T3SS activation, LcrG is secreted and the negative regulation is lifted, allowing positive regulatory elements to induce expression and secretion of the translocation machinery and subsequently translocation of the anti-host effectors [193, 255, 257]. In addition, YopN forms a complex in the cytoplasm with TyeA, SycN and YscB which has the potential to function as a block for secretion through the T3SS from the inside [61, 84, 87, 139, 250, 275]. The regulatory relationship between the LcrV-LcrG complex and the YopN-TyeA-SycN-YscB complex is unclear.

Yet another degree of repression of the *yop* regulon is asserted by the negative element LcrQ [8, 236, 266]. Repression by LcrQ can be lifted by an interaction with the cytosolic chaperone SycH, since this promotes secretion of LcrQ [52, 54, 277, 305]. An emerging idea is that LcrQ acts together with a complex of YopD and LcrH by binding to certain *yop* mRNA in order to prevent translation during non-inducing conditions [11, 53]. Presumably, during inducing conditions SycH would bind LcrQ and direct it for secretion, as LcrH would do for YopD. This suggests a model in which a cytoplasmic YopD-LcrH complex hinders transcription of *yop* genes and de-repression would require YopD secretion.

The sequential building up of the T3SS is in itself a regulatory event. The needle protein YscF is the last protein to assemble into the T3S apparatus. This generates a signal for assembly completion and a subsequent switch to secrete Yop substrates [4, 132]. YscF may also function as a regulator of T3S by potentially sensing extra-cellular calcium levels [8]. It also has a crucial role in translocation of secreted proteins into target cells in a cell contact dependent manner [287], perhaps by allowing for correct localisation of LcrV in close contact to the target cell [184].

### **LcrH is a negative regulator in *Yersinia***

Further analysis of the LcrH-YopD complex has revealed a regulatory role for LcrH, separate from its chaperone function in stabilising YopD and YopB (PAPER IV, [190, 293]). Regulation in an *lcrH* null mutant cannot be restored by overproduction of stable YopD [92]. Hence, the LcrH-YopD interaction is required for correct regulation in *Yersinia*. Significantly, however, some LcrH mutants can still bind and stabilise YopD, but remain deregulated for Yop expression. These mutants fail to bind YscY (T3SS chaperone to the secreted component YscX), indicating that this interaction must also be important for the regulatory effect of LcrH [45, 92].

Collectively, these data illustrate a fundamental role of LcrH and YopD in regulation of T3S genes. In fact, the secretion of YopD may even be a molecular switch that signals gene expression to be turned on [305]. This secretion event, and also that of YopB, is dependent in part on the chaperone LcrH (PAPER IV, [76, 92, 190]). In addition, Yop effector translocation depends on YopB and YopD secretion and even low levels of substrate secretion appears sufficient for successful translocation (PAPER V, [46, 47, 124, 197]). It follows that LcrH may have the capacity to regulate translocation from within the bacterial cytoplasm.

All in all, regulatory control of T3S occurs on several levels, with a strict hierarchy in order to ensure proper activation of the components of the system only when they are needed. Why *Yersinia* T3S displays such a multi-layered regulation is still an enigma. Perhaps the different regulatory proteins blocking the secretion apparatus respond to different extra-cellular signals providing spatial and temporal information to the bacteria. If all regulatory proteins would interpret the same signals, it is surprising that redundancy is not displayed in the system. Our limited understanding of the *in vivo* signals for T3S is likely to lead to an over-simplified notion of when and where T3S is employed by *Yersinia*.

## Effector proteins of *Yersinia*

The T3SS of *Yersinia* is used by the bacteria to translocate a minimum of six anti-host effector proteins into target cells (figure 1). Taken together the joint action of these toxins allows the bacteria to escape the host's immune system and remain in an extra-cellular milieu to replicate. At least four effector proteins are essential for *Yersinia* to cause systemic infections in mice; YopM, YopH, YopE and YpkA [40, 88, 102, 162]. The role of YopJ and YopT in virulence is still unclear [103, 181, 289].

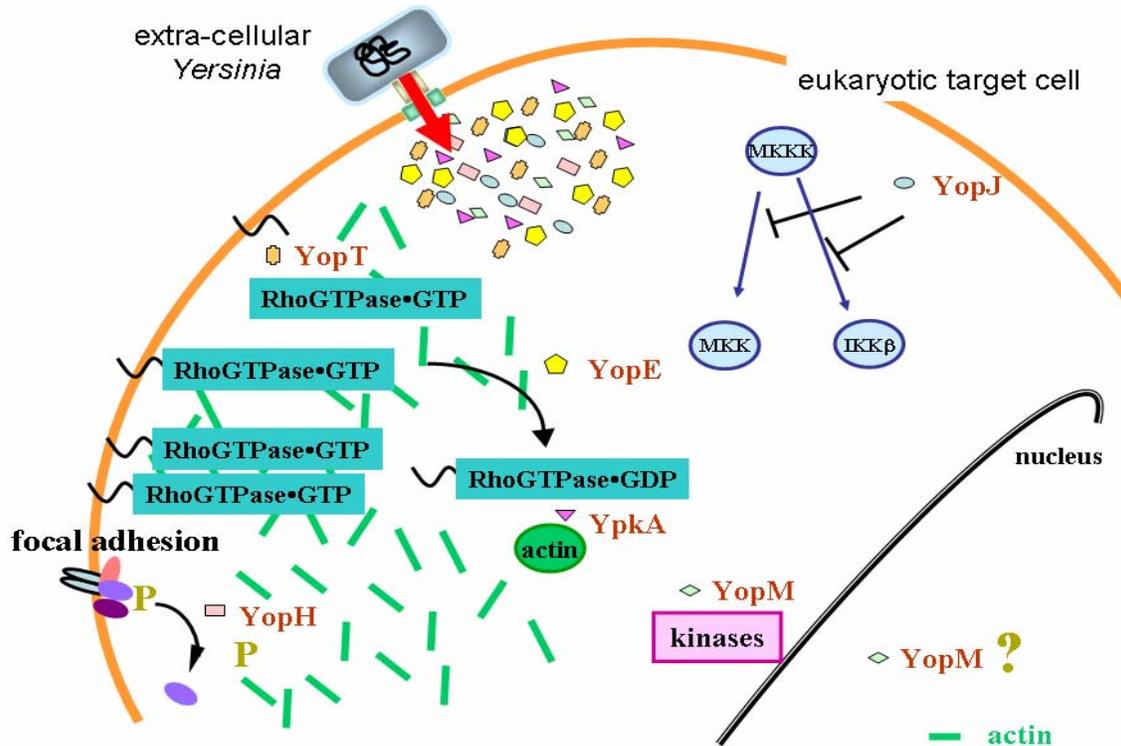


Figure 1. Translocated effectors of *Yersinia* disrupt cellular signalling. The co-ordinated attack on the actin cytoskeleton and focal adhesions by YopH, YopT, YopE and YpkA is believed to prevent phagocytosis. YopJ inhibits MAP kinase induction of cytokines and induces apoptosis. The function of YopM is unknown, but the protein can be found associated with cellular kinases and in the nucleus.

**YopJ** (YopP in *Y. enterocolitica*) induces apoptosis in macrophages [180, 182, 317] by suppression of pro-inflammatory cytokines via inhibition of the MAP kinase pathways (figure 1) [199, 202, 248, 317] and is involved in inhibition of the adaptive immunity [288]. **YopM** is a Leucine Rich Repeat (LRR) protein [80, 163] which upon secretion can inhibit platelet aggregation [162] and also binds  $\alpha$ -thrombin *in vitro* [230, 258]. It is thereby thought to promote dispersion of the bacteria in the blood. The function of YopM in eukaryotic cells is not known, but it

interacts with two cellular protein kinases [174] and can also be detected in the nucleus (figure 1) [256]. YopM may, however, interfere with cytokine-dependent activation of vital cellular components of the innate immunity [148].

The remaining four effector proteins (YopH, YpkA, YopT and YopE) all target different aspects of actin dynamics in the eukaryotic cell (figure 1). **YopH** is one of the most potent tyrosine phosphatases described [318] and an essential virulence determinant of *Yersinia* [40] involved in anti-phagocytosis [238], inhibition of calcium signalling in neutrophils [14] and inhibition of T and B cell activation [10, 309]. YopH de-phosphorylates eukaryotic signalling proteins important for focal adhesion function and T cell activation such as p130Cas, FAK, Fyb, LAT and Slp-76 [13, 32, 35, 72, 106, 213]. **YpkA** (YopO in *Y. enterocolitica*) is a serine/threonine protein kinase [102] found at the inner surface of the eukaryotic cell membrane after translocation [118]. YpkA requires actin as an activating co-factor in infected cells (figure 1) [143] and although YpkA interacts with the small GTPases RhoA and Rac1 (important regulators of the actin cytoskeleton – discussed below) [22, 75], there exists no evidence of YpkA-dependent phosphorylation of these proteins. It may be that the actin disruption by YpkA is due to its ability to block activation of RhoA without interfering with GEF-induced GDP/GTP exchange [75, 143, 188].

In contrast to YopH and YpkA, direct effects on the distribution and activity of small GTPases have been identified for YopT and YopE [2]. **YopT** is a cysteine protease responsible for removing the C-terminus of RhoA [3, 254]. This induces a re-distribution of membrane-bound RhoA to the cytosol (figure 1) and uncoupling of the RhoA-effector interaction necessary for RhoA regulation of the actin cytoskeleton [262]. The end result of YopT activity is cellular cytotoxicity [138]. **YopE** is a GTPase Activating Protein (GAP) with *in vitro* activity towards RhoA, Rac1 and Cdc42 (figure 1) [31, 304]. Upon bacterial infection of eukaryotic cells or micro-injection of purified protein, YopE induces a rapid collapse of the actin cytoskeleton [240], presumably via its ability to down-regulate small RhoGTPases [5, 15, 31, 304]. Together with YopH, YopE was initially recognized as one of the Yop effectors responsible for blocking phagocytosis of *Yersinia* [238, 239]. Other effects of translocated YopE have also been attributed to the GAP activity, such as prevention of *Yersinia*-induced pore formation in target cell membranes [296], inhibition of pro-inflammatory signalling [183, 249] and disruption of tight junctions [279]. Taken together, this emphasises YopE as a key effector in *Yersinia* virulence. By targeting members of the small RhoGTPase family, one effector can have multiple effects on signalling pathways in target cells. This is evident from results reported in this thesis (see Results and Discussion section).

## ***The family of RhoGTPases***

The family of small RhoGTPases were identified as important regulators of the actin cytoskeleton in eukaryotic cells [17, 280]. To date, 22 members have been identified [17], which can be divided into eight subgroups based on sequence

| <b>Sub-groups</b> | <b>Members</b>                |
|-------------------|-------------------------------|
| <b>Cdc42</b>      | Cdc42, TC10, TCL, Chp, Wrch-1 |
| <b>Rac</b>        | Rac1, Rac2, Rac3, RhoG        |
| <b>Rho</b>        | RhoA, RhoB, RhoC              |
| <b>RhoD</b>       | RhoD, Rif                     |
| <b>Rnd</b>        | Rnd1, Rnd2, Rnd3/RhoE         |
| <b>RhoH/TFF</b>   | RhoH/TFF                      |
| <b>RhoBTB</b>     | RhoBTB1, RhoBTB2              |
| <b>Miro</b>       | Miro-1, Miro-2                |

Table 1. Subgroups of RhoGTPases.

similarities (table 1). RhoGTPases have many other functions in eukaryotic cells besides regulation of actin dynamics, these include induction of nitric oxide production, cell cycle progression, microtubule organisation, regulation of cell adhesion and transcriptional control [29, 280]. This thesis will focus on the regulation of RhoGTPases and their role in actin dynamics and will not discuss other features of these regulatory proteins.

## **Regulation of RhoGTPases**

RhoGTPases function as molecular switches, alternating between a GTP-bound active conformation and a GDP-bound inactive conformation (figure 2) [280]. A post-translational modification of the C-terminal CAAX-domain confers membrane-localisation to the GTPase [280]. Important regulators of RhoGTPase activity are Guanine Exchange Factors (GEF), GTPase Activating Proteins (GAP) and Guanine nucleotide Dissociation Inhibitors (GDI) (figure 2) [280]. The proteins regulating RhoGTPases out-number the GTPases by at least 3 to 1 for the GEFs and the GAPs [206], showing the importance of tight regulation of RhoGTPases for correct function in eukaryotic cells. The GEF and GAP functions are often found as discrete domains in multi-functional proteins [206]. GEFs activate RhoGTPases by exchanging the GDP for a GTP upon an activation signal (figure 2) [280]. RhoGDIs bind to GDP-bound GTPases and block spontaneous release of the nucleotide, thereby preventing activation (figure 2) [246]. Extra-cellular signalling results in release of GDI from the GTPase thereby allowing for subsequent activation by GEFs [246, 280].

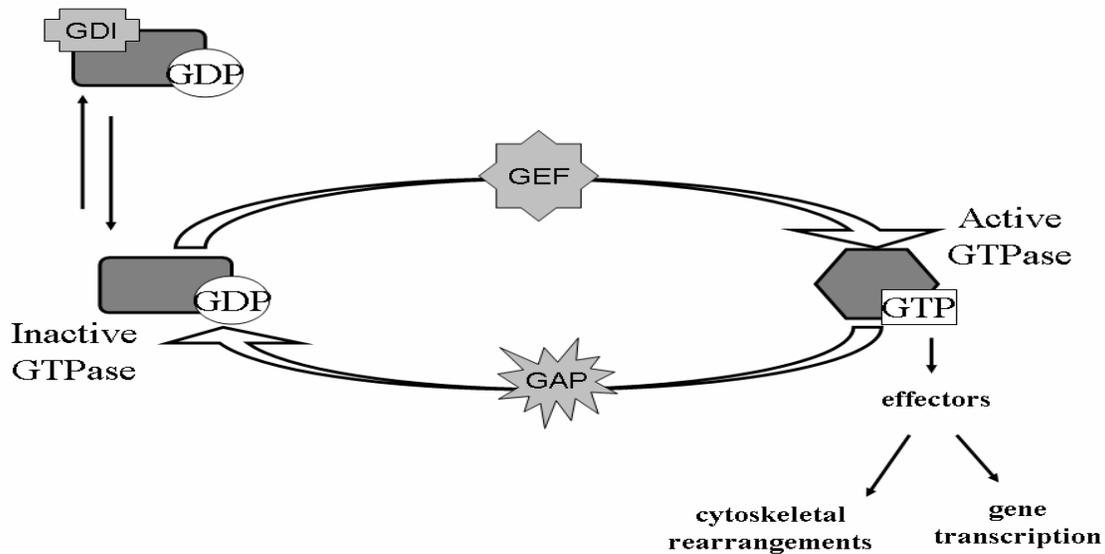


Figure 2. Regulation of small RhoGTPases. Guanine Exchange Factors (GEFs) activate the GTPase upon stimulation. GTPase Activating Proteins (GAPs) increases the intrinsic GTP-hydrolysis, thereby inactivating the GTPase. Guanine nucleotide Dissociation Inhibitors (GDIs) sequester inactive GTPases and prevent spontaneous exchange of GDP to GTP. Only active GTPases interact with down-stream effector proteins.

RhoGTPases have an intrinsic GTP hydrolysis activity, which can be increased by GAPs (figure 2) [206]. Hydrolysis of GTP to GDP inactivates the RhoGTPase by inducing a conformational change resulting in loss of interaction with downstream effector proteins [29, 206]. Common to all eukaryotic RhoGAPs is an essential arginine residue, positioned to stabilize the transition state during hydrolysis of the GTP nucleotide [315].

### **RhoGTPases and the actin cytoskeleton**

RhoGTPases were first discovered as regulators of the actin cytoskeleton, with distinct morphological changes dependent upon activation of Rac1 (lamellipodia), RhoA (stress fibre formation) or Cdc42 (filopodia) [17, 194, 233, 234]. Most studies have focused on these three members, but successive identification of numerous other RhoGTPase family members have painted a picture of sophisticated cross-talk within the eukaryotic cell for control of the actin cytoskeleton and cellular processes dependent upon functional actin dynamics. Such processes include motility, endocytosis, cell cycle progression, adhesion and phagocytosis [225, 231, 232, 280].

RhoGTPases do not directly interact with the actin cytoskeleton. Rather regulation occurs via down-stream effector proteins (figure 2) [29]. Most members of the RhoGTPase family have been implicated in direct regulation of the actin

cytoskeleton [17]. However, RhoH and the Rnd proteins are GTPase deficient [58, 164]. RhoH rather seems to function as an inhibitor of the NF $\kappa$ B and p38 pathways, which are activated by other members of the Rho family [164]. Rnd1-3 are thought to antagonize the action of RhoA on stress fibre formation [17, 58]. In addition, phosphorylation of Rnd3 by a RhoA-effector increases its stability and activity, thereby suggesting a way of down-regulating RhoA induced pathways in the cells [235].

Most members of the Cdc42-group affect the formation of filopodia in cells, albeit to different extent [17]. In addition, Chp and Wrch-1 both act as activators of the JNK MAP kinase pathway [16, 282]. Rif induces the formation of long filopodia in a Cdc42-independent manner [209], and both RhoD and Rif reorganise the actin cytoskeleton extensively [17]. The RhoA-family induce stress fibres when over-expressed in cells [17]. All members of the Rac1-family induce lamellipodia in cells and Rac1 and Rac2 can also induce thick bundling of actin filaments [17].

RhoBTB1 and 2 have a modular organisation with a RhoGTPase domain followed by other domains important for protein-protein interactions [229]. No role for the RhoBTB proteins in actin regulation has been identified [17]. Miro proteins are atypical RhoGTPases and function within mitochondrial homeostasis, rather than actin regulation [17, 94].

### **Control of endocytosis by RhoGTPases**

Several GTPases regulate endocytic pathways, including phagocytosis and receptor recycling [79, 225, 232]. RhoB closely associates with late endosomes and retards receptor trafficking through the endosomal pathway [232]. RhoD localises to endosomes and is proposed to modulate endosome distribution and motility [232]. TCL is implicated in regulation of receptor endocytosis and recycling [70]. TC10 also plays a role in recycling of receptors via the endocytic pathway, perhaps by remodelling of cortical actin [57, 59, 113]. RhoGTPases may also function in exocytosis in a variety of cell types and are implicated in the release of neurotransmitters [232].

Phagocytosis is a specialised form of endocytosis and the first line of defence against invading micro-organisms by the innate immune system. Two major types of phagocytosis by professional phagocytes have been identified and both processes are regulated by small GTPases of the Rho family. Rac1 and Cdc42 are involved in Fc-receptor phagocytosis (type I) whereas RhoA has been identified as an important regulator of complement receptor phagocytosis (type II) [225]. *Yersinia* translocated effectors can block both kinds of phagocytosis by disrupting

signalling pathways down-stream of the Fc- and complement receptors [13, 82, 300].

### **RhoGTPases as targets for bacterial toxins**

Many pathogens exploit the actin cytoskeleton of eukaryotic cells either to promote attachment to cells and uptake into intra-cellular compartments for replication [111, 319], or as is the case with *Yersinia*, the actin cytoskeleton is targeted in order to prevent phagocytosis and ensure an extra-cellular milieu for bacterial replication [67, 111]. A common way used by many bacterial species to interfere with the actin cytoskeleton is by targeting members of the RhoGTPase family [111]. Significantly, these are not necessarily substrates of T3SS. Cytotoxic Necrotising Factor (CNT) of *Escherichia coli* and Dermonecrotic toxin (DNT) of *Bordetella* species activate members of the Rho family by constitutive modifications preventing nucleotide hydrolysis [131]. Exoenzyme S (ExoS) of *Pseudomonas aeruginosa* has a C-terminal ADP-ribosyltransferase activity [153, 166], which modifies members of several different GTPase families, either resulting in activation (in the case of Rac1 [237]) or deactivation (in the case of Ras and RhoA [126]). *Clostridium* spp. localise toxins into target cells to constitutively inactivate RhoGTPases [131].

Another group of bacterial toxins mimic the eukaryotic regulatory proteins of RhoGTPases (figure 2). By interfering with the proper control of the GTPase activity, the bacteria can modulate the host response to suit its purposes [111]. YopE of *Yersinia* has been shown to act as a GAP towards RhoA, Rac1 and Cdc42 *in vitro* [5, 31, 304] and disrupt Rac1 dependent signalling pathways in eukaryotic cells [15], resulting presumably in prevention of phagocytosis [15, 31, 238]. *Salmonella* spp. promotes its own uptake into epithelial cells by actin remodelling and then subsequent host cell recovery [319]. Uptake is mediated by translocation of the T3SS effectors SopE and SopE2, GEFs for Cdc42 and Rac1 [95]. Cell recovery requires translocation of SptP that possesses a YopE-like GAP domain that targets Rac1 and Cdc42 *in vitro* [97, 98, 267]. Another GAP domain is found in ExoS, which targets Rac1, RhoA and Cdc42 both *in vitro* and during infection of eukaryotic cells [126, 150, 208, 306]. *P. aeruginosa* also encodes a second GAP-containing toxin, Exoenzyme T (ExoT) with 76% identity to ExoS [21]. The GAP activity of ExoT targets the same substrates as ExoS [147], making it hard to understand why the bacteria carries two proteins with seemingly the same function. Several other identified T3SS effector proteins can modulate the activity of small RhoGTPases in infected eukaryotic cells, but their exact mechanisms of action are unknown [111].

## GENERAL AND SPECIFIC AIMS

One general aim of this study was to investigate the function of the translocated protein YopE, an essential virulence determinant of *Yersinia pseudotuberculosis*. YopE belongs to a small group of bacterial GTPase Activating Proteins (GAPs). My work focused on trying to understand the biological functions of YopE in relation to its GAP activity.

Specific Aims:

- I. Define regions in YopE essential for its known properties; *in vitro* GAP activity towards small GTPases and disruption of the actin cytoskeleton?
- II. Identify the *in vivo* targets of YopE in infected eukaryotic cells.
- III. Identify amino acids essential for the *in vivo* activity of YopE, focusing on virulence in mice and inactivation of small GTPases in infected cells.

A second general aim was to study the control of translocation during *Yersinia* infection of eukaryotic cells. Not only did this examine potential regulatory roles of pore-forming components, since this feature is a pre-requisite for translocation, but also the idea that translocated effectors could exhibit regulatory effects.

Specific Aims:

- IV. Investigate the molecular mechanisms underlining the LcrH-YopD interaction.
- V. Examine whether LcrH-dependent secretion of YopB and YopD can influence efficiency of effector translocation.
- VI. Determine if any translocated effector can control further toxin delivery.

## RESULTS AND DISCUSSION

YopE is an essential virulence determinant of *Yersinia pseudotuberculosis* with an actin disrupting activity and the ability to prevent bacterial phagocytosis by macrophages [88, 239, 240].

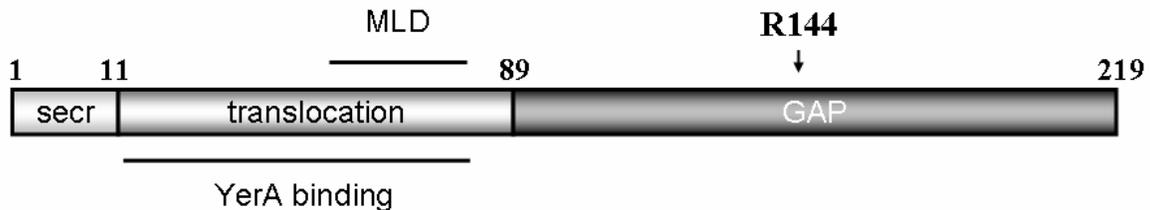


Figure 3. Organisation of the YopE protein. Amino acids 1 to 11 are essential for secretion. The YerA chaperone binds to amino acids 11 to 74 as a homodimer. The C-terminal (amino acids 89 to 219) is a GTPase Activating Protein (GAP) towards small RhoGTPases. Amino acids 54 to 75 constitute a Membrane Localisation Domain (MLD) in YopE for membrane targeting inside eukaryotic cells.

YopE is a 219 amino acids long protein (figure 3) [88], with a T3SS secretion signal in the first 11 amino acids [168, 247]. Stability, secretion and translocation of YopE is facilitated by binding to the cytosolic chaperone YerA to a domain in the N-terminus of YopE (figure 3) [247, 263, 264, 293, 294, 302]. In recent years YopE has been shown to function as a GTPase Activating Protein (GAP) towards three members of the RhoGTPase family *in vitro* and to prevent Rho dependent signalling in yeast [304]. Moreover, mutation of the essential arginine at position 144, renders the YopE protein non-functional towards RhoA, Rac1 and Cdc42 *in vitro* and the corresponding mutant bacteria is avirulent in mice hence the GAP activity of YopE is essential for virulence [5, 31, 304]. A Membrane Localisation Domain (MLD) for targeting to eukaryotic membranes have been identified in the N-terminus of the protein (figure 3), overlapping in part with the domain necessary for translocation and YerA binding [44, 151].

### ***In vitro GAP activity towards RhoA, Rac1 and Cdc42 can be uncoupled from cytotoxicity***

We wanted to characterise regions in YopE important for cytotoxicity towards HeLa cells and determine if this correlated to *in vitro* GAP activity towards RhoA, Rac1 and Cdc42. By deletion analysis of the N- and C- terminal ends of YopE we defined an essential minimal region of amino acids 90-215 for YopE dependent cytotoxicity (figure 2, PAPER I). Further dissection of the C-terminal domain (aa

90-215) of YopE was carried out by smaller in frame deletions. Using this approach, all except four mutants lost the cytotoxicity phenotype. The four regions dispensable for cytotoxicity encompassed the residues 131 to 135, 162 to 165, 189 to 192 and 211 to 215 (figure 2 and 3, PAPER I). Despite this wild-type phenotype, none of these four deletion mutants could inactivate RhoA, Rac1 or Cdc42 *in vitro* (figure 5, PAPER I). These results show for the first time that it is possible to genetically separate the cytotoxic function of YopE from its *in vitro* GAP activity towards RhoA, Rac1 and Cdc42. This brings into question the essential nature of RhoA, Rac1 and Cdc42 as targets for YopE during cell infection.

Even small deletions can cause changes to a protein's overall tertiary structure. With the help of structural modelling [114, 115, 149] and by using the combined knowledge from the solved structures of YopE, ExoS and SptP [81, 267, 306], we showed that changes in the poly-peptide structure could occur in at least four of our deletion mutants. Therefore, we generated more subtle mutations using alanine substitution to further analyse the GAP domain of YopE. Remarkably, alanine mutagenesis revealed only one amino acid triplet essential for cytotoxicity (aa 181-183), supporting the idea that our original deletions caused disturbances in the overall structure (table 1, PAPER I). In contrast, only one triplet substitution mutant (aa 210-212) approached the level of the GAP activity of the wild type protein (figure 5 and table 1, PAPER I). This further supports the notion that cytotoxicity is not dependent on functional *in vitro* GAP activity towards RhoA, Rac1 and Cdc42.

The finding that *in vitro* YopE GAP activity towards RhoA, Rac1 and Cdc42 could be separated from its cytotoxic effect on target cells is important. This offers a way to dissect the molecular mechanisms of one function in isolation from the other. However, as a note of interest, the data could also imply that the main *in vivo* target of YopE does not include RhoA, Rac1 and/or Cdc42. Thus, the question really is what the critical target of YopE is during infection of the host?

### ***In vivo substrate specificity of YopE in infected cells***

To better understand the function of YopE during infection, we employed pulldown assays to look at the activation state of RhoA, Rac1 and Cdc42 in infected HeLa cells. This approach has previously identified RhoA and Cdc42 as targets for the GAP activity of wild type ExoS [126]. We showed that YopE does not target Cdc42 in cells, although it is an efficient GAP *in vitro* towards this GTPase (PAPER II, [5, 304]). However, inactivation of Rac1 and RhoA by wild type

*Yersinia* occurs as early as 5 and 30 minutes, respectively, after initiation of infection (figure 7, PAPER II).

Extensive mutagenesis of the GAP domain of YopE revealed a function of amino acids 181-183 in separating *in vitro* GAP activity towards RhoA, Rac1 and Cdc42 from cytotoxicity (PAPER I). This amino acid triplet lies in a domain (aa 178 to aa 187) with a high degree of similarity in the three bacterial GAPs and this region will be referred to as the “homology domain” (figure 1, PAPER II). The homology domain could be involved in target binding by SptP [267]. We created six single alanine substitutions of amino acids 178 to 183 to further dissect the functions of YopE during *Yersinia* infections. A range of unique phenotypes was revealed when these mutants were analysed.

First, we showed that the cytotoxicity of YopE is dose-dependent. In other words, a cytotoxic phenotype was observed when the mutant protein (W181A) was expressed *in trans* from a multi-copy plasmid, but not when this same mutant protein was produced under native conditions *in cis* on the *Yersinia* virulence plasmid (table 1, PAPER II). This was not seen with the R144A GAP mutant, devoid of the catalytically essential arginine residue (table 1, PAPER II). Perhaps the W181A mutant can sequester an essential regulator of the actin cytoskeleton when hyper-translocated. Yet, if this was true, the GAP mutant would be expected to behave similarly, since mutation of the arginine 144 is not believed to alter the tertiary structure of YopE. We also considered the possibility that W181A still possessed some residual GAP activity, but at least *in vitro*, it displayed no activity towards RhoA, Rac1 and Cdc42 at concentrations at least 100 times higher than that required by wild type YopE to completely hydrolyse bound GTP to GDP (figure 6, PAPER II). The other five mutants in the homology domain were all cytotoxic towards HeLa cells irrespective of their level of expression (table 1, PAPER II). All further studies were conducted with mutants where the mutation was introduced on the virulence plasmid.

Only two mutants created in the “homology domain” (F178A and S179A) were virulent in mice (table 1, PAPER II), while all the mutant proteins tested were defective for *in vitro* GAP activity towards RhoA, Rac1 and Cdc42 (figure 6, PAPER II). This result further substantiates our conclusions from Paper I that there is little correlation between cytotoxicity, *in vitro* GAP activity and virulence.

It is prudent to consider that *in vitro* GAP activity profiles of YopE are only a guide to what might be happening *in vivo*. After all, studies on eukaryotic GAP proteins show differences between *in vitro* and *in vivo* GAP activity [253, 316]. Thus, to

clarify the effect of the single alanine mutants on endogenous RhoA, Rac1 and Cdc42 during cell infections, we performed pulldown assays of active GTPases at 30 minutes post-infection (figure 8, PAPER II). Surprisingly, one virulent mutant (F178A) was severely reduced in its ability to inactivate RhoA and Rac1 in infected cells at 30 minutes post-infection. In addition, the attenuated mutant G182A was indistinguishable from wild type at this time point (figure 8, PAPER II). Furthermore, the avirulent mutant T183A, which showed wild type activity towards Rac1, was unable to inactivate RhoA (figure 8, PAPER II). Taken together these mutants suggest that additional target(s) for YopE inside eukaryotic cells are necessary for virulence, and although YopE targets RhoA and Rac1 during cell infection, these GTPases are not the essential targets.

This is the first evidence that the “homology domain” of YopE is involved in target recognition and that individual amino acids in a bacterial GAP domain can influence binding to distinct GTPases. If the proposed anti-phagocytic function of YopE [31, 239] is critical for *Yersinia* virulence, a defect in GAP activity as seen with the F178A mutant, should have had severe consequences on bacterial virulence. However, this is not the case, suggesting that alternative targets of YopE are essential to cause disease or that YopE possesses an alternative, as yet undescribed, function during infection.

### ***The role of YopE in prevention of pore formation***

We also analysed the single alanine mutants for their ability to prevent pore formation in the eukaryotic cell membrane, analysed as release of lactate dehydrogenase (LDH) from infected cells (figure 5, PAPER II), an activity dependent on the GAP function of YopE (PAPER III, [296]). Whether this phenotype has relevance in the context of *in vivo* YopE function is clouded. However, it correlates well with the ability of YopE to cause cytotoxicity and LDH release is dependent on functional actin cytoskeleton regulation [296], which suggests an additional role of YopE that could be investigated with our new assortment of phenotypically distinctive mutants. Indeed, only non-cytotoxic mutants of YopE (R144A and W181A) have lost the ability to block LDH release (figure 5, PAPER II), presumably by an inability to prevent actin dependent pore formation in the eukaryotic plasma membrane [296]. All cytotoxic mutants of YopE blocked LDH release (figure 5, PAPER II). This establishes a clear link between the YopE-dependent functions of cytotoxicity and prevention of pore formation. This is consistent with the role for host cell actin in both phenotypes. Interestingly, the relationship between pore prevention (blockage of LDH release)

and virulence is less clear, because the avirulent T183A mutant is still cytotoxic and efficiently blocks LDH release (table 1 and figure 5, PAPER II).

We also analysed the contribution of all known translocated effector Yops with respect to LDH release from infected cells. No other Yop effector was found to be involved (figure 2, PAPER III). Control of LDH release is a phenomenon specific to YopE. Thus, the YopH- and YpkA-induced cytotoxicity must be distinct from that induced by YopE and generates different cell fates [2, 43, 143]. In addition, although a *yopK* null mutant induces bigger pores in sheep erythrocytes and hyper-translocates effector proteins (figure 3, PAPER III, [134]), it still prevents LDH release to the same extent as wild type *Yersinia* (figure 2, PAPER III). This data elevates YopE towards a specific role in preventing *Yersinia*-induced pore formation, an activity dependent on the polymerization/de-polymerization cycle of the actin cytoskeleton [296]. On the basis of our avirulent T183A mutant, which is still cytotoxic, prevents pore formation and functions as a GAP *in vivo* towards Rac1 (PAPER II), we propose a role for Rac1 in YopE-mediated control of LDH release and cytotoxicity. However, the fully cytotoxic F178A mutant is defective for Rac1 inactivation in infected cells (PAPER II). This suggests that an additional GTPase is targeted for the actin disruption phenotype associated with YopE or that very low GAP activity is sufficient for cytotoxicity to occur, since F178A has a low *in vitro* GAP activity towards Rac1, RhoA and Cdc42 (PAPER II).

So are these phenotypes a true reflection of the role of YopE inside eukaryotic cells? The answer might be both yes and no – yes, because YopE targets Rac1 and RhoA *in vivo*, and no, because one virulent mutant (F178A) could not inactivate Rac1 and RhoA in infected cells. Perhaps YopE targets a number of GTPases in eukaryotic cells during infection. Although YopE-targeting of Rac1 leads to actin cytoskeleton disruption, we do not believe that Rac1 is the target ultimately responsible for the contribution of YopE in *Yersinia* virulence. The family members of RhoGTPases have a high degree of identity and eukaryotic GAPs often target more than one member [206, 316]. Both Rac1 and RhoA possess close homologous counterparts that function within vesicle transport and cellular immune responses (e.g. RhoB, RhoD and Rac2) [142, 225, 307]. Thus, the effect of YopE on Rac1 and RhoA could be a secondary effect of its ability to target another closely related GTPase.

### ***Regulation of effector translocation into target cells – a role for YopE***

The lack of correlation between control of pore formation and virulence is intriguing. As a result we initiated studies to try and uncover the consequences of

the inability of YopE to prevent pore formation. Interestingly, the YopE GAP mutant, which does not prevent LDH release (figure 5, PAPER II), had also lost control of translocation into eukaryotic cells – translocating elevated levels of itself and YopH (figure 3, PAPER III). Moreover, the GAP mutant maintained constitutive Yop synthesis in the presence of eukaryotic cells (figure 4, PAPER III). In contrast, wild type *Yersinia* demonstrates a tight control of effector translocation levels and also produced less Yops in the presence of cells than the GAP mutant (figure 3 and 4, PAPER III). This suggested a role for the GAP activity of translocated YopE in sending a signal back to the bacterium to turn off further synthesis, secretion and translocation of Yops. This highlights one mechanism by which *Yersinia* can strike a balance between paralyzing target cells and avoiding activation of the innate immune response through excessive toxin-induced cellular damage. This notion of YopE-dependent translocation control was further strengthened by the observation that hyper-translocation also occurred in the avirulent mutants W181A and T183A (figure 3, PAPER II). This is the first time a translocated T3SS effector has been shown to be involved in regulation of translocation of itself and other effectors. Importantly, this regulatory effect is specific to the presence of eukaryotic cells because during *in vitro* growth conditions, all *yopE* mutants regulate Yop synthesis normally (figure 4, PAPER II).

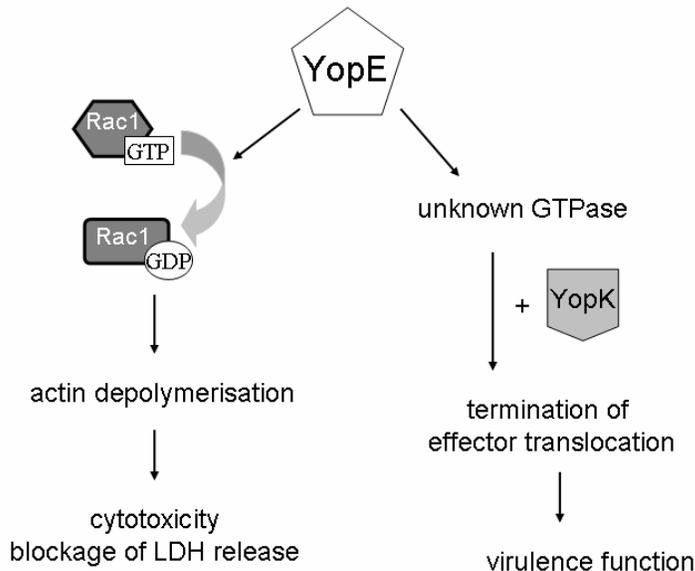


Figure 4. Model of YopE activities inside eukaryotic cells. Inactivation of Rac1 (and to some extent RhoA) by YopE accounts for the actin disruption activity of YopE. YopE regulates the level of effector translocation together with YopK by inactivation of an unknown GTPase. The latter function of YopE is essential for virulence in mice.

If regulation of translocation initiation is understudied in the T3SS, almost nothing is known about the signals that control termination of translocation. One study has observed hyper-translocation of effector proteins by a *yopK* mutant that renders the bacteria avirulent in the mouse model of yersiniosis [134]. This finding has significant similarities to our observation with YopE. It appears that *Yersinia* utilises YopE and YopK to determine when enough translocated effectors have been introduced into target cells (figure 4). The absence

of this mechanism spells doom for the infecting bacteria. Thus, bacteria must weigh the energetic cost of infinite Yop production against the programmed functions of the translocated effectors through a function of translocated YopE. The net result is translocation control during infection and effective colonisation of the host. This unique function for YopE in translocation control requires GAP activity. However, what might be the target of this activity is still unclear, for avirulent mutants in other parts of YopE, having lost regulatory control, still maintain some functional GAP activity towards established targets *in vivo* (PAPER II).

We therefore propose a model whereby YopE exerts a role on two functional pathways inside eukaryotic cells, targeting at least two different GTPases (figure 4). One pathway influences cytotoxicity and control of pore formation, and at least in part is mediated by Rac1 targeting. The other pathway involves effector translocation control, which is an essential virulence trait of YopE (PAPER II), and is mediated by targeting of an as yet unknown target (figure 4). The second pathway controlling translocation also requires functional YopK [134].

### ***Functional differences between YopE and ExoS***

Exoenzyme S (ExoS) of *Pseudomonas aeruginosa* is a bi-functional T3SS translocated effector with an N-terminal YopE-like GAP domain. ExoS has been shown to act as a GAP towards Rac1, RhoA and Cdc42 [107, 126]. We wanted to investigate if ExoS has the same function as YopE in effector translocation control (PAPER III). Wild type ExoS has two enzymatic activities, so we analysed mutants devoid of either GAP activity or the ADP-ribosyltransferase (ADPRT) activity that also targets small GTPases [126]. The GAP domains of YopE and ExoS share 40% identity. Surprisingly, the ExoS GAP activity was not sufficient to complement a *yopE* null mutant phenotype with respect to elevated LDH release or hypertranslocation of YopH during infection of HeLa cells (figure 6 & 7, PAPER III). ExoS was translocated efficiently because all ExoS variants induced cytotoxicity on HeLa cells after 1 hour (table 1, PAPER III), fully complementing the *yopE* null mutant for this activity.

The reason behind an inability of ExoS to complement a *yopE* mutant defective in pore formation control or translocation control is very fascinating, especially since ExoS targets Rac1 both *in vitro* and *in vivo* [107, 126, 150] and Rac1 targeting appears to be important for at least YopE-mediated pore formation control (PAPER II and PAPER III). The ADP-ribosyltransferase activity of ExoS has been suggested to mask the GAP activity [126, 237]. However, it would then be expected that ADPRT negative ExoS should be able to complement the *yopE* null

mutant – this was not the case (figure 6 and 7, PAPER III). Interestingly, a recent study claimed that the effect of ExoS on Rac1 is cell type specific, perhaps due to the subcellular localisation of Rac1 in different cell lines [237]. In this context, it is worth noting that both ExoS and YopE contain a MLD, rich in hydrophobic amino acids (figure 5A), but with low amino acid homology, in the N-terminal part of the protein [151, 207]. Moreover, they appear functionally inter-changeable, in that domain swapping experiments between ExoS and YopE still enable hybrid proteins to be translocated and cause cytotoxicity [151]. We have also identified a similar sequence in the N-terminus of ExoT and SptP (figure 5A). This indicates a conserved localisation targeting mechanism for the bacterial GAP proteins.

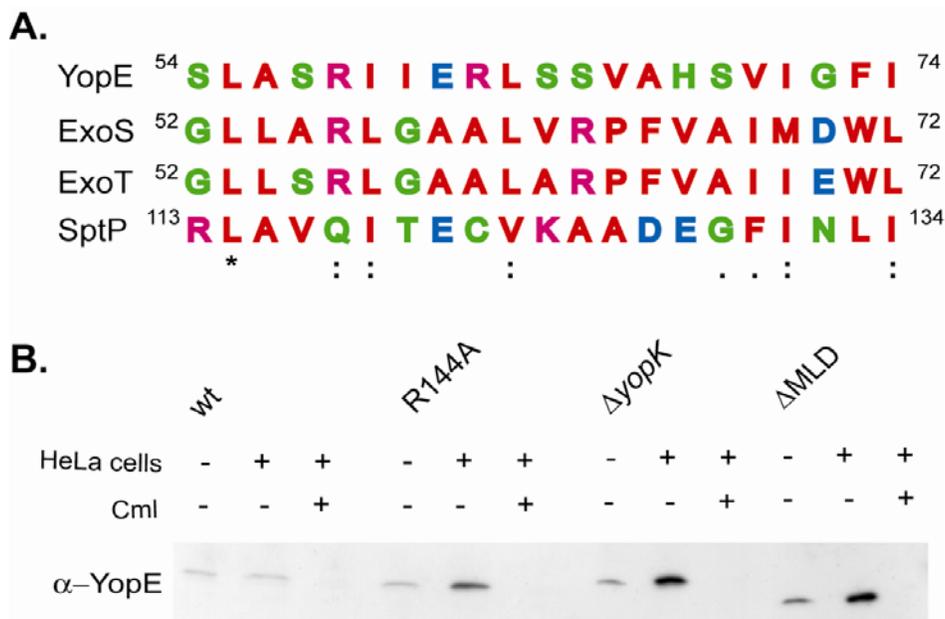


Figure 5. The Membrane Localisation Domain (MLD) of YopE is involved in regulatory control of *yop* expression. **(A)** Alignment of MLDs from bacterial GAPs. Red (small, hydrophobic and aromatic side groups), blue (acidic side groups), green (hydroxyl, amine and basic (Q) side groups) and magenta (basic side groups). **(B)** Control of Yops production during bacteria-cell contact by YopE. Wild type (YPIII/pIB102), *yopER144A* (YPIII/pIB562) GAP defective mutant, *yopK* (YPIII/pIB155) or *yopEΔMLD* (YPIII/pIB529) were added to wells with (+) or without (-) HeLa cell monolayers for 90 min. Analysis performed as described in PAPER II and PAPER III.

Intriguingly, the MLD of YopE renders YopE dependent on YerA binding for protein secretion and translocation [44], although sufficient information for secretion and translocation of YopE is encoded in the first 50 amino acids of the protein [44, 247, 302]. Perhaps conservation of the MLD reflects the essential nature of this domain in targeting of YopE inside eukaryotic cells. The significance of the MLD in translocation control has so far not been investigated. We are in the

process of analysing a YopE mutant devoid of its MLD. The MLD mutant displayed wild type cytotoxicity and pore prevention, as measured by LDH release from infected cells, in addition to normal *in vitro* regulatory control of Yop synthesis. Interestingly, the control of Yop synthesis in the presence of eukaryotic cells was lost (figure 5B). Virulence has yet to be determined for this mutant. However, these preliminary results indicate an important functional role for the MLD of YopE. Future research should focus on the possibility that the MLD of YopE and ExoS confer different intracellular localisations that are biologically relevant, reflecting functionally distinct molecular targets. In this context, it is also important to consider ExoT, which also functions as a GAP towards small RhoGTPases [147, 276].

Another consideration is that ExoS may not complement these phenotypes in *Yersinia* because the feedback signal(s) requires native components. Such observations have been shown for the translocon components – complete functionality is only observed when they are co-expressed with native components [48]. Thus, it is prudent to remember that YopK – which functions together with YopE in the translocation control pathway (figure 4) – is actually unique to *Yersinia*, having no known homologues in protein data bases. ExoS may not complement a *yopE* null mutant simply because the signal induced by ExoS can not be interpreted by YopK. Future experiments should therefore address the concept of translocation control in *P. aeruginosa*, with particular focus on the possible role of translocated GAP proteins (ExoS and ExoT) in preventing excessive pore formation and effector translocation. Ultimately, further studies into the *in vivo* targets of each toxin will give key insights to the function of these intriguing T3SS effectors.

One can only speculate which additional RhoGTPase(s) YopE targets and how this results in feedback signalling to the bacteria to terminate effector translocation. Perhaps it occurs via receptor changes on the surface of the target cell or a more direct effect on the translocon components. At this stage, the mechanism remains illusive.

### ***Another potential mechanism of translocation control in Yersinia***

Apart from the studies of YopE (PAPER III) and YopK (PAPER III, [134]), there is little known about the control mechanisms for translocation initiation and termination during *Yersinia* infections. We were interested in identifying other possible mechanisms for translocation control. Translocation of effector proteins by *Yersinia* is dependent on the translocon operon *lcrGVHyopBD* [25, 134, 212, 222].

The dual role of YopD in regulation and translocation together with the coupling of this regulatory function with LcrH [92, 125, 189, 197, 223, 299], lead us to believe that the YopD-LcrH complex may represent a critical check-point for regulatory control during *Yersinia* infections. Indeed, this might even be a form of translocation control. The last part of this thesis explores this idea further.

### ***Delineation of YopD***

Our long-term goal was to define regions in YopD that are essential for its many important functions during *Yersinia* infection. Perhaps this would also allow isolation of distinct functional domains in the protein. We analysed sequential deletions within YopD, which revealed a need for the first 90 amino acids for efficient YopD secretion (figure 4, PAPER IV). Binding to the LcrH chaperone, at least in the Yeast Two Hybrid System (Y2HS), required a central domain (aa 53-149) (figure 5, PAPER IV). The C-terminal amphipathic domain [284], was also necessary for interaction with LcrH (figure 5, PAPER IV).

We were intrigued by this observation, since this amphipathic domain is critical for all known functions of YopD [93, 197]. Thus, we continued to analyse the role of the YopD amphipathic domain in LcrH binding. Amino acid substitution mutagenesis identified hydrophobic residues to be most important for establishing the LcrH-YopD interaction in the Y2HS (figure 7 and table 2, PAPER IV). In addition, we identified mutants in LcrH that suppressed these non-binding YopD mutants, thereby restoring complex formation (table 4, PAPER IV). For the first time, we also isolated LcrH mutants defective in binding wild type YopD (figure 8, PAPER IV). These mutations spanned the entire *lcrH* coding region, meaning that no single domain of LcrH could be identified as essential for binding to YopD. Taken together, this study revealed distinct domains in YopD responsible for chaperone binding. A subsequent follow up study using the YopD mutants generated herein, was able to identify distinct functional domains of YopD with respect to pore formation and regulatory control [197]. Furthermore, this study and others (PAPER V, [92, 201]) has capitalised on the many LcrH mutants isolated. With the use of these mutants, we now know that the LcrH-YopD interaction is essential for YopD and YopB stability and dramatically affects the level of YopB and YopD secretion and subsequent effector translocation (PAPER IV & PAPER V, [76, 92, 197]).

### ***Low levels of the translocators is sufficient for effector translocation***

The importance of the LcrH-like chaperones, including PcrH of *Pseudomonas aeruginosa*, in assembly of the translocon is evident [47, 204]. During *in vitro* conditions both chaperones are essential for the pre-secretory stability and efficient secretion of their binding partners (PAPER V, [47, 76, 190, 293]). However, this is not the case in the presence of eukaryotic cells, where stable YopB and YopD are easily detected in an *lcrH* null mutant (figure 5, PAPER V). In addition, a portion of the chaperone substrates can also be secreted in these conditions (P. J. Edqvist, personal communication). Despite this, an *lcrH* null mutant is non-cytotoxic towards HeLa cells (figure 3, PAPER V). From our earlier study, several substitution mutants in LcrH have been identified, which display no or reduced binding to YopB and YopD in the Y2HS (PAPER IV and V). Further characterization of these mutants revealed poorly stabilized and secreted substrates during *in vitro* conditions (figure 2, PAPER V). In fact, many mutants were indistinguishable from the *lcrH* null mutant (PAPER V). Remarkably however, all except one of these mutants were still able to induce sufficient levels of the YopB and YopD translocators to promote effector translocation into target cells (figure 3 and 4, PAPER V), but in many cases this translocation was severely reduced (figure 4, PAPER V). A changed level of pore formation by secreted YopB and YopD in these LcrH mutants could explain the different degree of translocation. Therefore pore formation in infected eukaryotic cell plasma membranes was investigated. In contrast to wild type *Yersinia*, several LcrH mutants failed to induce measurable pore formation in target cell membranes (figure 6 & 7, PAPER V). This supports our view that minimal amount of translocators is needed for effector translocation by *Yersinia*.

In conclusion, we have engineered LcrH to secrete less substrate and thereby translocate less Yop effectors into target cells. Our interpretation of this data is two fold: 1) only very low levels of secreted translocators are required for efficient effector translocation, and 2) levels of secretion could be regulated by the LcrH chaperone to control the extent of translocation.

This study raises interesting questions for the future. Does substrate secretion by an *lcrH* null mutant associated with eukaryotic cells indicate that LcrH confers substrate secretion specificity? Perhaps only in the presence of LcrH, are the translocator substrates specifically targeted to and secreted through the correct T3SS to allow for subsequent effector translocation into eukaryotic cells. This is relevant since *Yersinia* encodes at least three different T3SS [56, 203]. Does low level translocation by bacteria poorly secreting YopB and YopD imply that this is an LcrH-dependent mechanism to control translocation? After all, it is conceivable

that the regulatory role of LcrH could be to ensure that the correct levels of pore-forming proteins are secreted. Lastly, why is YopB and YopD stable during infection with an *lcrH* null mutant? This suggests that the primary role of LcrH is not as a “stabiliser” of the YopB and YopD protein in the bacterial cytoplasm, since the substrates are stable and can be secreted in an *lcrH* mutant in the presence of eukaryotic cells. Could lack of effector translocation in an *lcrH* mutant indicate that LcrH is actually required to interpret signals resulting from target cell contact? Recognition of these signals by LcrH could then be the trigger for appropriate secretion of the translocator proteins YopB and YopD. Answers to these questions, and others, will require much more investigation.

My work is now finished. Here at last, on the shores of the sea... comes the end of our Fellowship. I will not say, do not weep... not all tears are an evil.

(Gandalf – The Lord of the Rings)

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“I don't know half of you half as well as I should like, and I like less than half of you half as well as you deserve.” (Bilbo – Lord of the Rings)

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