VIRULENCE MECHANISMS OF PATHOGENIC YERSINIA
ASPECTS OF TYPE III SECRETION AND TWIN ARGinine TRANSLOCATION

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UMEÅ 2005
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ABSTRACT

The pathogenic bacteria *Yersinia pestis* and *Y. pseudotuberculosis* are related to the degree where the former is considered a subspecies of the latter, and still they cause disease of little resemblance in humans. *Y. pestis* is the causative agent of lethal bubonic and pneumonic plague, while *Y. pseudotuberculosis* manifests itself as mild gastroenteritis. An important virulence determinant for these species is their ability to secrete and inject toxins (Yop effectors) into immune cells of the infected host, in a bacterium-cell contact dependent manner. This ability depends on the extensively studied type III secretion system, a highly complex multicomponent structure resembling a needle. The induction of Yop secretion is a strictly controlled event. The two structural type III secretion components YscU and YscP are here shown to play a crucial role in this process, which is suggested to require an YscP mediated conformational change of the C-terminus of YscU. Proteolytic cleavage of YscU within this domain is further revealed to be a prerequisite for functional Yop secretion. The needle subcomponent itself, YscF, is recognised as a regulatory element that controls the induction of Yop effectors and their polarised delivery into target cells. Potentially, the needle might act as a sensor that transmits the inducing signal (i.e. target cell contact) to activate the type III secretion system. Secondly a, for *Yersinia*, previously unexplored system, the Twin arginine translocation (Tat) pathway, is shown to be functional and absolutely required for virulence of *Y. pseudotuberculosis*. A range of putative *Yersinia* Tat substrates were predicted *in silico*, which together with the Tat system itself may be interesting targets for future development of antimicrobial treatments.
This thesis is based on the following four articles, which are referred to in the text by their roman numerals.

I. Lavander, M., Ericsson, S. K., Bröms, J.E., and Forsberg, Å. The twin Arginine translocation system is essential for virulence of *Yersinia pseudotuberculosis*. *Submitted manuscript.*


1. BACKGROUND

1.1 Gram-negative bacteria in the pathogenic niche

Common to all bacteria are core functions, such as DNA replication, transcription and translation, synthesis of amino acids and nucleic acids. Moreover, bacteria utilise different strategies for survival depending on what ecological niche they occupy. An event that attracts particular interest from the scientific community is when the niche of choice is within the human body, especially when this host-bacterium relationship is harmful to the host. In this case the objectives for research are to understand, prevent and treat the infectious disease. To successfully invade and persist within a host, pathogenic bacteria depend on ‘virulence factors’, which includes elements important throughout the course of the infection. For instance, virulence factors enable bacteria to invade host tissues and protect the pathogens from the massive defence put up by the host immune system. Virulence factors also aid in the survival and proliferation of the bacteria under unfavourable conditions such as low pH or under oxidative stress and permit efficient transmission to new hosts. Intriguing and highly varying infection strategies are employed by different pathogens, resulting in a wide range of symptoms of infectious diseases.

A characteristic feature of a Gram-negative bacterial cell is the presence of a double membrane, separated by a periplasmic space, protecting the integrity of the
cytoplasm (fig. 1). Since pathogenic species largely rely on factors located on the bacterial surface, secreted into the extracellular milieu or translocated directly into the host cells during infection, specialised secretion systems are highly related to the ability of bacteria to cause disease. This has been the focus of the present study, where two secretion pathways – type III secretion (T3S) and twin arginine translocation (Tat) – have been analysed in pathogenic *Yersinia* species, causative agents of mild gastroenteritis or aggressive plague in humans. The different secretion systems of Gram-negative bacteria are reviewed below (chapter 1.3), with a more thorough description of T3S and Tat in chapters 1.4-1.6.

**1.2 A MOLECULAR VIEW ON *YERSINIA* INFECTIONS**

Characterised as Gram-negative, facultative anaerobic rod-shaped Proteobacteria of the family Enterobacteriacea, the notoriety of one species of *Yersinia* hugely overshadows all others, i.e. *Yersinia pestis* the agent of Plague.

**1.2.1 Plague: historic Reaper and potential tool of terror**

No infectious disease has left such an imprint on history as the dreaded plague. Having occurred as devastating pandemics, reaping millions of lives, it has had profound impact on human civilisation. During the 14th century, plague alone claimed one third of the European population. Referred to as the Black Death, this is to present day the most dramatic pandemic in history. The culprit behind the disease is the bacterium *Y. pestis* that manifests itself as bubonic or pneumonic plague, aggressive infections that are lethal and relentless. *Y. pestis* inhabits rodent populations, with transmission of the disease occurring via fleas feeding off an infected animal (Brubaker, 1991; Smego *et al.*, 1999). While plague today is endemic in Africa, Asia and the Americas, improved health and sanitation as well as the development of antibiotics have dramatically reduced the impact of plague infections. Outbreaks of *Y. pestis* infections do however still occur, with the
greatest risk of epidemics found in developing countries where plague is endemic and people live under insalubrious conditions (Duplantier et al., 2005; Gage and Kosoy, 2005; Perry and Fetherston, 1997). Further, with the rise of large scale terrorism, there is intensified awareness of the potential threat in use of Y. pestis as a biological warfare agent (Inglesby et al., 2000).

1.2.2 The enteropathogenic ancestry of Y. pestis

Of the 11 members of the genus Yersinia, Y. pestis, Y. pseudotuberculosis and Y. enterocolitica are known to cause disease in humans (Cornelis et al., 1998; Sulakvelidze, 2000). The latter two cause comparatively mild gastrointestinal infections presented as fever, vomiting, acute diarrhoea and abdominal pains due to mesenteric lymphadenitis. These infections are usually cleared from the host within 7-14 days and do not require antibiotic treatment, although septicaemia do occur in immunocompromised patients. The enteropathogenic yersiniae may further induce post infection complications such as reactive arthritis (Hannu et al., 2003; Smego et al., 1999). This thesis focuses on the remarkably closely related Y. pestis and Y. pseudotuberculosis. Having evolved from the much less malevolent ancestor Y. pseudotuberculosis as recently as 1,500-20,000 years ago, Y. pestis actually represents a clone within the Y. pseudotuberculosis species, making this an intriguing evolutionary event (Achtman et al., 1999; Achtman, 2004; Bercovier et al., 1980; Chain et al., 2004; Wren, 2003). The profoundly altered mode of transmission and pathogenesis of Y. pestis is reflected in dramatic changes within its genome (Achtman et al., 1999; Chain et al., 2004; Perry and Fetherston, 1997; Smego et al., 1999). Horisontal acquisition of two, for Y. pestis, unique plasmids pPla (≈100 kb) and pFra (≈9.6 kb) and a number of genomic islands presumably prompted the rapid evolution of the plague bacterium. Further, the Y. pestis chromosome contains a large number of pseudogenes, insertion sequences and extensive genomic rearrangements, corresponding to the abandonment of the
enteropathogenic lifestyle and adaptation into a highly efficient killer (Chain et al., 2004; Deng et al., 2002; Parkhill et al., 2001).

1.2.3 Plague for the mouse; a useful animal model

The holy grail of Yersinia research has for a long time been, and still is, the invention of new cures for plague. To achieve this goal, studies of Yersinia virulence mechanisms are frequently carried out with the mouse as the animal model, and Y. pseudotuberculosis as a model organism for Y. pestis. The genetic similarities between the yersiniae, and the fact that Y. pseudotuberculosis causes systemic plague-like disease in mice, makes this a convenient system to study more severe infections (Gemski et al., 1980b). Even if deductions such as mouse = human and Y. pseudotuberculosis = plague, should be regarded with a healthy amount of scepticism, this model system has proven useful to learn more about the molecular mechanisms of the disease.

1.2.4 Yersinia reservoirs

Y. pseudotuberculosis is a resilient bacterium, well adapted to persist for long periods in low-nutrient conditions and at temperatures ranging from 4 to 45°C, thus increasing the opportunities of being taken up by a host (Smego et al., 1999). Humans are infected via contaminated food and water, or by contact with animals that can be asymptomatic carriers, suffering from prolonged enteritis or dying from the infection (Fukushima et al., 1989; Hodges et al., 1984; Laukkanen et al., 2003). In contrast to the enteric Yersinia species, Yersinia pestis is an obligate pathogen that will perish fairly promptly outside an infected host (Smego et al., 1999). The mammalian reservoir of Y. pestis is mainly represented by many different species of rodents, with the most important arthropod vector being the rat flea Xenopsylla cheopis (Hinnebusch, 2005; Lowell et al., 2005; Mencher et al., 2004). When the flea feeds on a mammal that has developed septicemic plague, the bacteria
accompanying the engulfed blood will grow into a gelatinous biofilm that blocks the flea proventriculus (the valve that connects the oesophagus to the midgut). As blood is thus prevented from flowing into its stomach, the flea is starved, resulting in frantic but futile feeding attempts. Efficient regurgitation and inoculation of bacteria at the bite site will occur, thereby causing spread of the disease (Hinnebusch, 2005). Bacterial colonisation of the flea depends on the chromosomally encoded hemin storage system, Hms, which is suggested to synthesise extracellular polysaccharides required for biofilm formation (Hinnebusch et al., 1996; Jarrett et al., 2004). Further, the Yersinia murine toxin, Ymt, a phospholipase D encoded on the pFra plasmid is required for Y. pestis to persist within the flea (Du et al., 1995; Hinnebusch et al., 2002; Hinnebusch et al., 2000; Rudolph et al., 1999). Importantly, both Hms and Ymt are redundant for virulence in the mammalian host (Du et al., 1995; Kutyrev et al., 1992; Lillard et al., 1999). Interesting gene candidates with unresolved roles in vector colonisation are found within the Y. pestis genome, such as pathogenicity islands encoding products resembling insecticidal toxins (Chain et al., 2004; Deng et al., 2002; Parkhill et al., 2001).

1.2.5 First contact: adhere and invade

Shortly after ingestion, enteric Yersinia species leave the lumen of the gut to invade deep tissue sites as they traverse the M cells of the intestinal epithelium, a port of entry for many enteropathogenic microorganisms with the ability to adhere to and invade these cells (Autenrieth and Firsching, 1996; Boyle and Finlay, 2003; Clark et al., 1998; Neutra et al., 1996). Two adhesins identified for the gastrointestinal Yersinia species are invasin and YadA (Finlay and Caparon, 2005).

Chromosomally encoded by the inv gene, Yersinia invasin is expressed at ambient temperature or at 37°C at pH 5.5 and under high Na\(^{2+}\) levels, suggesting that the conditions of the small intestine can maintain invasin expression after ingestion of
the bacteria (Pepe et al., 1994). Anchored in the bacterial outer membrane with an elongated, rod-like protrusion on the surface, invasin binds with extraordinarily high affinity to β1 integrins exposed on the luminal side of the host M-cells to mediate entry into the cells (Clark et al., 1998; Hamburger et al., 1999; Isberg and Falkow, 1985; Isberg and Barnes, 2001; Marra and Isberg, 1997; Pepe and Miller, 1993; Van Nhieu and Isberg, 1991). Y. pseudotuberculosis inv mutants reveal defective colonisation of Peyer’s patches and the mesenteric lymph nodes, unable to transmigrate over the epithelium these strains show a tendency to colonise the luminal side of the intestine (Marra and Isberg, 1997). Encoded on the pYV plasmid, yadA expression is upregulated at 37°C. The autotransporter YadA then forms a multimeric lollipop-shaped protrusion from the outer bacterial membrane and suggestedly binds to extracellular matrix (ECM), thus contributing to adhesion and invasin independent uptake (Bolin et al., 1982; Eitel and Dersch, 2002; El Tahir and Skurnik, 2001; Hoiczyk et al., 2000; Roggenkamp et al., 2003; Yang and Isberg, 1993).

Consistent with abandoning the enteropathogenic lifestyle, yadA and inv are merely present as pseudogenes in Y. pestis (Deng et al., 2002; Parkhill et al., 2001; Rosqvist et al., 1988; Simonet et al., 1996). Instead, the multifunctional Pla protein (plasminogen activator), encoded on the pPla plasmid, enables tissue invasion of Y. pestis from the initial site of the flea bite. Subcutaneous infections in mice with a pla mutant strain results in only localised infections, indicating a role for Pla in systemic dissemination of the bacterium (Cowan et al., 2000; Sodeinde et al., 1992; Welkos et al., 1997). Pla also mediates adherence of Y. pestis to ECM, and induces activation of plasminogen into plasmin resulting in uncontrolled proteolysis and damage to host tissues (Lahteenmaki et al., 1998; Lahteenmaki et al., 2005). Pla has also proved able to sponsor invasion of epithelial cells.

The pH6 antigen is a fibrillar putative adhesin found in both Y. pestis and Y. pseudotuberculosis. It is expressed at 37°C and below pH 6.7 (conditions similar to
the macrophage phagolysosome) depends on the chaperone/usher pathway for assembly and has been shown to interact with eukaryotic cells and host lipoproteins (Ben-Efraim and Bichowsky-Slomnichi, 1964; Huang and Lindler, 2004; Lindler and Tall, 1993; Makoveichuk et al., 2003; Yang et al., 1996).

A crucial skill for pathogenic bacteria is the acquisition of iron, which is sequestered by the animal host. Yersiniabactin (Ybt), found in all three human pathogenic yersiniae, expresses a system for secretion of a siderophore that scavenges ferric iron from the host prior to being transported back into the bacterial cytoplasm. This system is required for the initial colonisation of the host, as a Y. pestis Ybt defective mutant strain is unable to spread from the initial subcutaneous locus of infection. In contrast, Ybt is dispensable for later septicemic stages of plague, as intravenous injection of the same mutant strain will result in full-blown disease (Lesic and Carniel, 2004; Perry and Fetherston, 2004).

1.2.6 Multiplication within macrophages

Although Yersinia infections primarily are extracellular, in vivo studies have revealed that all three human pathogenic species can be found within macrophages during early stages of colonisation (Pujol and Bliska, 2005). Y. pestis and Y. pseudotuberculosis share the ability to survive and multiply within macrophages, and in vitro assays place the pathogens within the phagolysosomes, where bacteria are challenged with a hostile environment of acidic pH, reactive oxygen and nitrogen species, antimicrobial peptides and proteases (Pujol and Bliska, 2003; Straley and Harmon, 1984a, b). Y. pseudotuberculosis has been shown able to inhibit acidification of the phagosome and, as Y. pestis, the production of nitric oxide (Pujol and Bliska, 2005; Tsukano et al., 1999). PhoP/PhoQ, a pleiotropic regulatory two-component system of Gram-negative bacteria is important for intracellular survival of Yersinia within macrophages. In vitro stress assays with phoP mutant strains of Y. pestis and Y. pseudotuberculosis revealed the PhoP/PhoQ
system to be important to resist acidic conditions, oxidative stress and Mg\textsuperscript{2+} starvation (Grabenstein et al., 2004; Oyston et al., 2000).

The ability of *Yersinia* to subvert the macrophage functions from within is suggested to be a strategy for acclimatisation to the mammalian host, prior to facing the host immune defence, with a range of genes coding for virulence related proteins being upregulated at 37°C (Pujol and Bliska, 2005). One such virulence factor, exclusive for *Y. pestis* and expressed from pFra, is the F1 antigen, which is exported and assembled via a chaperone/usher pathway to form a capsule-like structure that gives increased resistance against uptake by macrophages (Du et al., 2002).

### 1.2.7 Extracellular persistence

For colonisation of the extracellular compartments, the bacteria have to be able to thwart the different aspects of the immune defence, including recruitment of phagocytic cells, secretion of cytokines and complement mediated lysis. Even if route of infection and nature of the disease differ widely between the enteropathogenic *Yersinia* species and *Y. pestis*, they share tropism for lymphoid tissues where they resist the host innate immune response (Brubaker, 1991; Koornhof et al., 1999). This ability depends on the ~70kDa plasmid, pYV (plasmid of *Yersinia* virulence) shared exclusively between these three species (Ben-Gurion and Shafferman, 1981; Ferber and Brubaker, 1981; Gemski et al., 1980a; Gemski et al., 1980b). The plasmid codes for a secretion system, designated ‘type III’, and so called Yop effectors (*Yersinia* outer proteins), delivered by this system into eukaryotic cells (Rosqvist et al., 1994; Salmond and Reeves, 1993; Sory and Cornelis, 1994). Encoded on the plasmid are also the V-antigen (LcrV), and the adhesin YadA, which together with the Yops contribute to inhibition of phagocytosis and downregulation of the inflammatory response (Cornelis and Wolf-Watz, 1997; Cornelis et al., 1998; Nakajima et al., 1995; Sing et al., 2002).
Being a main focus of this thesis the type III secretion system will be discussed in more detail below (chapters 1.5-1.6).

If yersiniae reach the bloodstream (an unusual event for the enteric species unless infecting an immunocompromised host) they have to evade the complement mediated lysis, also known as **serum resistance**. Factors that protect against the complement are generally located on the bacterial surface and the adhesin YadA (chapter 1.2.5) has been shown to contribute to serum resistance (Biedzka-Sarek *et al.*, 2005). Lipopolysaccharides (LPS) are the major outer membrane components of Gram-negative bacteria, and an important virulence factor (Erridge *et al.*, 2002). The LPS consists of the lipid A, which is anchored in the outer membrane, an oligosaccharide core and the O-antigenic polysaccharide. The O-antigen has been shown to be significant for virulence of enteric yersiniae, proposedly by contributing to serum resistance as seen for many other pathogens (Skurnik and Bengoechea, 2003). Interestingly, *Y. pestis*, which has to efficiently colonise the blood stream to promote transmission via the flea, has ‘rough’ LPS, i.e. it is devoid of the O-antigen (Bruneteau and Minka, 2003; Skurnik *et al.*, 2000). However, this rough LPS is required for the activity of Pla (chapter 1.2.5) that has been shown to degrade components of the complement, thus contributing to *Y. pestis* serum resistance (Kukkonen *et al.*, 2004).

**1.2.8 Outcome of disease and strategies for transmission**

Usually, pathogenic bacteria do not benefit from killing their hosts, but will increase the opportunities for multiplication and transmission by causing a prolonged balanced infection. This is the strategy employed by the entero-pathogenic *Yersinia* species, which cause self-limiting infections in healthy humans and can reside asymptomatically in other animal hosts for long periods of time (Smego *et al.*, 1999). A very different pattern is seen for the dramatic progress of a *Y. pestis* infection. Here, the bacteria disseminate aggressively throughout the
host’s lymphatic system, entering the bloodstream in 2-6 days, causing heavy septicaemia and infecting vital organs. Proliferation of *Y. pestis* within lymph nodes provokes a massive inflammatory response, resulting in characteristic swelling buboes in armpits, groin and neck; hence ‘bubonic plague’. *Y. pestis* can also spread to and colonise the lungs (pneumonic plague), facilitating aerosol transmission of the bacterium. While mortality for untreated bubonic plague is around 60%, the pneumonic form has a mortality rate close to 100%, most likely due to that the bacteria are already adapted to the mammalian host (Brubaker, 1991; Smego *et al.*, 1999). The maintenance of the hypervirulent phenotype of *Y. pestis* is proposed to be a consequence of that even the most efficient flea vector, *X. cheopis*, is difficult for the bacterium to colonise and also transmits the disease with low prevalence. This promotes extremely high numbers of bacteria within the bloodstream of the host to improve the odds for transmission (Lorange *et al.*, 2005).

1.3 SECRETION SYSTEMS IN GRAM-NEGATIVE BACTERIA

Protein secretion is not only a prerequisite for life, but also highly related to pathogenesis. An overview of secretion system used by Gram-negative bacteria is given below, with a more detailed description of type III secretion and twin arginine translocation in chapters 1.4-1.6.

1.3.1 Secretion across the plasma membrane

Two systems work in parallel for protein secretion across the inner membrane: the general secretion pathway (GSP or Sec, below) and twin arginine translocation (Tat, chapter 1.4). The autotransporters, two-partner secretion, chaperone/usher and type II secretion systems only account for secretion across the outer membrane and thus rely on Sec or Tat for the initial step of export from cytoplasm to periplasm.
1.3.1.1 The general secretion pathway (GSP/Sec)

Sec is responsible for the major part of the export of proteins over the plasma membrane (de Keyzer et al., 2003; Muller et al., 2001). Found in bacteria, archaea and eukarya, the Sec pathway is the only secretion system shown to be a required for life, e.g. due to its essential role in membrane biogenesis (Dalbey and Chen, 2004; Pohlschroder et al., 1997). Substrates exported via Sec are defined by an N-terminal signal peptide, recognised by the chaperone SecB that targets the substrate to the SecYEG secretion channel. The secretion process is energised by ATP hydrolysis and the proton motive force, whereupon the signal peptide is cleaved off by a signal peptidase and the mature protein released into the periplasm (Dalbey and Von Heijne, 1992; de Keyzer et al., 2003; Driessen, 2001; Economou, 1999, 2000; Veenendaal et al., 2004).

1.3.2 Secretion across the outer membrane

Secretion across the outer membrane can occur via different systems as depicted in figure 2.

**Figure 2. Secretion systems of Gram-negative bacteria.** Schematic representation of secretion systems; T1S-T5S, denoting type I-V secretion. T2S and T5S depend on Sec or Tat for export over the inner membrane. AT = autotransporter, TPS = two partner secretion, CU = chaperon/shearer. IM = inner membrane, PP = periplasm, OM = outer membrane and CM = host cell membrane.
1.3.2.1 Autotransporters (AT)

These minimalistic secretion systems consist of substrates that mediate their own transport across the outer membrane. AT proteins have an N-terminal Sec signal peptide followed by a passenger domain and a C-terminal translocation domain (Jacob-Dubuisson et al., 2004). The latter supposedly forms a β-barrel structure in the outer membrane, enabling delivery of the passenger to perform its exoprotein functions (Henderson et al., 2004). The first AT secretion system identified was the IgA protease of Neisseria gonorrhoeae (Pohlner et al., 1987). Since this initial discovery, many AT proteins have been shown to play a role in pathogenesis of Gram-negative bacteria, contributing to e.g. serum resistance, attachment to eukaryotic cells and biofilm formation (Henderson et al., 1998; Henderson and Nataro, 2001; Newman and Stathopoulos, 2004).

1.3.2.2 Two-partner secretion (TPS)

While autotransporter proteins provide for their own secretion, the TPS secreted proteins, commonly referred to as TpsA, depend on a dedicated partner protein, TpsB, to form the β-barrel conduit over the outer membrane (Guedin et al., 2000; Jacob-Dubuisson et al., 2004; Konninger et al., 1999). The typical TpsA is a large protein, 100-500kDa or more, involved in bacterial virulence (Jacob-Dubuisson et al., 2001). Haemolysins of Serratia marcescens (nosocomial infection) and Proteus mirabilis (urinary tract infection) are part of a growing family of pore forming TpsA toxins (Hertle, 2005; Ondraczek et al., 1992). Further, many Gram-negative pathogens utilize this pathway to deliver adhesins to the bacterial surface (Julio and Cotter, 2005; Rojas et al., 2002; Smith et al., 2001).

1.3.2.3 Chaperon/usher pathways (CU)

Chaperon/usher systems provide a means to assemble adhesins on the bacterial surface, as filamentous or afimbrial structures. A periplasmic chaperone is
responsible for substrate recognition and interacts with the substrates to prevent premature aggregation and degradation while guiding them to the secretion machinery (Sauer et al., 2004). Examples of surface organelles assembled via the CU system include: the P pilus of uropathogenic E. coli and the F1 and pH6 antigens of Y. pestis (Chapman et al., 1999; Hull et al., 1981; Sauer et al., 2000; Zav'yalov et al., 1996).

1.3.2.4 Type I secretion (T1S)

The T1S system consists of three subcomponents: i) an inner membrane ATP-binding cassette (ABC) that energises translocation and is responsible for substrate recognition and recruitment; ii) a pore forming outer membrane protein (OMP) and iii) a membrane fusion protein (MFP) that bridges the other two components (Delepelaire, 2004). The substrates are recognised by a C-terminal secretion signal and delivered from the cytoplasm to the exterior milieu without periplasmic intermediates (Remaut and Waksman, 2004). T1S has been shown to be involved in toxin delivery and can also constitute multidrug efflux pumps that contribute to bacterial multiresistance (Paulsen, 2003). Examples of type I secreted virulence determinants include haemolysin A from uropathogenic E. coli, Vibrio cholerae RtxA toxin and adenylate cyclase toxin CyaA of Bordetella pertussis (whooping cough) (Delepelaire, 2004; Thanassi and Hultgren, 2000).

1.3.2.5 Type II secretion (T2S)

Type II secretion is responsible for the bulk export of Sec substrates destined for the extrabacterial compartment. Assembled from 12-15 components, the T2S structure spans the bacterial envelope, thus enabling use of the proton motive force of the inner membrane to energise substrate export (Pugsley et al., 1997; Sandkvist, 2001b). Type II exoprotein substrates carry a signal peptide for delivery over the plasmamembrane via either Sec or Tat, and are upon folding within the
periplasmic space probably recognised by the T2S system via their highly specific three-dimensional structure (Nunn, 1999; Pugsley et al., 1997; Rossier and Cianciotto, 2005; Voulhoux et al., 2001). Many T2S components share homology with the type IV pathway for pilus biogenesis, and T2S systems have also been shown to assemble pilus-like structures (Durand et al., 2003; Sauvonnet et al., 2000; Vignon et al., 2003). These do however primarily span the periplasmic space and only form rudimentary structures on the bacterial surface. The role of the T2 pilus has not been determined, but may include gating of the T2S apparatus or a piston-like function in protein expulsion (Nunn, 1999; Sandkvist, 2001b). Many virulence factors are secreted via the T2 system, such as cholera toxin from *Vibrio cholerae*, cell-wall degrading enzymes of *Erwinia* species (plant pathogens causing soft rot) and a range of virulence related exoproteins from the opportunistic pathogen *Pseudomonas aeruginosa*, including exotoxin A and phospholipase C (Sandkvist, 2001a). A T2S system has been identified for some strains of *Y. enterocolitica*, denoted Yts1 (*Yersinia* type II secretion), that has been shown to be important for virulence in mice (Iwobi et al., 2003).

### 1.3.2.6 Type IV secretion (T4S)

Type IV secretion machineries are assembled from at least 12 proteins forming a core complex anchoring the structure in the bacterial membranes, spanning the periplasmic space, with a pilus protruding from the bacterial surface. T4S systems support delivery of substrates in an uninterrupted route across both bacterial and host cell membranes (Remaut and Waksman, 2004). These organelles, related to bacterial conjugation systems, are utilised by Gram-negative bacteria for interbacterial DNA-transfer and adapted by pathogens for translocation of anti-host factors (proteins or DNA) into eukaryotic target cells (Christie, 1997, 2004; Remaut and Waksman, 2004). Virulence strategies that depend on T4S include delivery of oncogenic DNA into host cells by the plant pathogen *Agrobacterium*
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*erge et al.* and pertussis toxin secretion by *B. pertussis* (Burns, 2003; Christie, 1997; Nagai and Roy, 2003). In a number of *Y. pseudotuberculosis* strains a type IV pilus gene cluster, that contributes to virulence via the oral route, has recently been identified (Collyn *et al.*, 2002; Collyn *et al.*, 2004).

### 1.4 Twin Arginine Translocation (TAT)

The twin arginine translocation (Tat) system has attracted a vast amount of research interest during the past decade. A lot of the curiosity surrounding the Tat system stems from its unique ability to export proteins in an already folded state across the bacterial inner membrane. The Tat pathway was originally discovered in plant chloroplasts, where it supports protein transport from stroma into thylakoids. Energized by the transmembrane H⁺ gradient, this system was initially named the ΔpH pathway (Dalbey and Robinson, 1999; Settles *et al.*, 1997; Settles and Martienssen, 1998). In bacteria, the Tat system was originally referred to as ‘Membrane targeting and translocation’ Mtt (Weiner *et al.*, 1998) and shortly thereafter as ‘Twin arginine translocation’, a name that stuck (Sargent *et al.*, 1998).

#### 1.4.1 The Tat translocase

In Gram-negative bacteria the typical Tat translocase, as characterised in the model organism *E. coli*, is mainly built up from the components TatA, TatB and TatC encoded by the *tatABCD* operon. The remaining gene product, TatD, has been shown to be a cytoplasmic DNase redundant for Tat function, and without any known connection to the system apart from its co-localisation with the operon (Wexler *et al.*, 2000). While TatABC are required for functional Tat, a fourth component, TatE, encoded by a monocistronic gene is redundant (Ize *et al.*, 2002; Sargent *et al.*, 1998; Yahr *et al.*, 1995). TatA and TatB are both inserted into the bacterial inner membrane and share largely similar structures: an N-terminal...
membrane spanning hydrophobic $\alpha$-helix and a C-terminal amphipatic $\alpha$-helix protruding into the bacterial cytoplasm (Palmer and Berks, 2003; Porcelli et al., 2002; Sargent et al., 2001). TatC, the most conserved Tat component, has six membrane spanning domains, the N- and C-termini of the protein being located in the cytoplasm (Behrendt et al., 2004; Ki et al., 2004). The translocation of Tat substrates is hypothesized to occur in the following manner: TatC forms a 1:1 complex with TatB that may include low levels of TatA, although the bulk of TatA forms a homooligomeric channel in the inner membrane (Bolhuis et al., 2000; de Leeuw et al., 2002; Oates et al., 2003; Porcelli et al., 2002; Sargent et al., 2001). TatC is responsible for recognition of the Tat signal sequence (chapter 1.4.2) and when a substrate is present the TatA complex is transiently recruited to TatBC, providing a conduit for substrate export to the periplasmic space (Alami et al., 2003; Jongbloed et al., 2000). The translocation event occurs independently of ATP and is energised by the transmembrane proton gradient (Yahr and Wickner, 2001). Recent findings have shown that TatA forms different size complexes, probably assembled from modules of three or four TatA subunits. This would suggestedly allow the Tat translocation channel to adapt to the size of the substrate at hand (Gohlke et al., 2005; Oates et al., 2005).

1.4.2 The Tat signal peptide

Substrates exported via the Tat system are recognised by an N-terminal signal sequence, between 26 and 58 amino acids long, compared to 18-26 amino acids for the classical Sec signal peptides (Berks, 1996; von Heijne, 1985). Further, while the Sec signal peptide is devoid of consensus sequences, Tat substrates typically carry a (S/T)-R-R-x-FLK motif (Berks, 1996). This conserved sequence has a very strong amino acid bias, the twin arginines (RR) giving the system its name being the most invariable, but not completely irreplaceable for Tat function (Ize et al., 2002). Substrates with atypical signal sequences, including variations of the
signature twin arginine have been found: *Salmonella enterica* TrxB has a motif, SKRQFLQ, where one of the two arginines is missing (Hinsley *et al*., 2001). Also, RNR instead of the typical RR is seen for the Rieske Fe/S protein, an essential component of the photosynthetic electron transport chain of chloroplasts in *Spinacia oleracea* (Molik *et al*., 2001). Similar to Sec, the Tat signal peptide is commonly cleaved off after successful export to the periplasmic space (Berks, 1996).

### 1.4.3 Faith and function of the Tat substrates

In contrast to Sec, Tat exports substrates that acquire their folding within the bacterial cytoplasm (Bogsch *et al*., 1998; Pugsley, 1993). The Tat pathway is believed to have evolved for delivery of cofactor-containing proteins across the plasmamembrane, and the vast majority of Tat substrates in *E. coli* undergo cytosolic cofactor incorporation (Berks *et al*., 2003; Halbig *et al*., 1999; Palmer *et al*., 2005). Cofactorless substrates also exist, as exemplified by halophilic archaea. These organisms have extremely high salt levels in their cytosol, and rapid folding is required to prevent protein damage under these conditions. As a result, the majority of exported proteins in these archaea take the Tat route (Hutcheon and Bolhuis, 2003; Rose *et al*., 2002). A third variant of Tat substrates are proteins that lack the typical signal peptide, but still are routed to Tat by a “piggy-back” mechanism. In other words, they form heteromeric complexes in the cytoplasm with Tat-signal containing partners (Palmer *et al*., 2005).

Proteins translocated by Tat to the periplasm either remain there, are integrated into the outer or inner membrane or secreted to the extrabacterial milieu (Hatzixanthis *et al*., 2003; Ochsner *et al*., 2002; Sargent *et al*., 1998; Sargent *et al*., 2002). Twin arginine translocation has been shown to be connected to the ability to cause disease in a number of bacteria, for example phospholipase toxins exported via type II secretion in *Legionella pneumophila* (Legionnaire’s disease) and *P.*
**BACKGROUND**

*aeruginosa* have been shown to be Tat substrates (Rossier and Cianciotto, 2005; Voulhoux *et al.*, 2001). Disruption of Tat by mutagenesis commonly results in strains displaying pleiotropic defects. Apart from impaired phospholipase delivery, a *P. aeruginosa* Tat mutant was also deficient for motility, biofilm formation, ability to endure osmotic stress, respiration under anaerobic conditions, iron-metabolism and was also shown to be attenuated for virulence in a rat lung model (Ochsner *et al.*, 2002).

### 1.5 TYPE III SECRETION (T3S)

A general overview of type III secretion is given here, with a more in depth review of the *Yersinia* type III secretion system (T3SS) in chapter 1.6. Providing a means for delivery of numerous structurally and functionally diverse virulence proteins into eukaryotic cells, the T3SS is an important tool for interkingdom communication utilised by both pathogenic and symbiotic bacteria to establish a relationship with their eukaryotic hosts (Alfano and Collmer, 2004; Dale *et al.*, 2001; Dale *et al.*, 2002; Francis *et al.*, 2004; He *et al.*, 2004; Viprey *et al.*, 1998). Since the initial discovery of T3S, a range of infectious diseases have been correlated to bacteria that employ this secretion system as a virulence strategy (Aizawa, 2001; Hueck, 1998; Pallen *et al.*, 2003). With the number of sequenced genomes rapidly increasing, it is also evident that T3S systems are encoded by many pathogens for which its role in virulence remains to be determined (Pallen *et al.*, 2005). Many infections in animals, including humans, are caused by T3SS carrying pathogens, such as: the previously mentioned *Yersinia* spp, *Salmonella* spp (food poisoning and typhoid fever), *Shigella* spp (dysentery), *P. aeruginosa* (opportunistic infections), *Burkholderia pseudomallei* and *B. mallei* (melioidosis and glanders), *Bordetella* spp. (respiratory tract infections) and pathogenic species of *E. coli* that cause gastrointestinal disease of varying severity (Hueck, 1998;
Also plants suffer from the attacks of bacterial pathogens, such as *Pseudomonas syringae*, *Ralstonia solanacearum* or species of *Erwinia* and *Xanthomonas*, which utilise T3S to induce wilt, rot, necroses, galls and blight (Buttner and Bonas, 2002).

### 1.5.1 The flagellar T3SS and the substrate specificity switch

The components of the bacterial flagellum are secreted by a type III secretion system that largely is regarded as the mother of all T3S systems (Macnab, 1999; Saier, 2004). The flagellum is a tripartite organelle consisting of: the inner membrane basal body (containing a central rod), the hook, and the helical filament protruding from the bacterial cell like a rotary tail used for propulsion (fig. 3 and fig. 4, left panel). Reflecting their evolutionary relatedness, similarities between the flagellar T3SS and the injectisome-type T3SS is seen on the levels of sequence, structure as well as function (Aizawa, 2001; Blocker *et al.*, 2003; Hueck, 1998; Macnab, 1999).

Electron microscope studies of type III injectisomes from *E. coli*, *Salmonella enterica* and *Shigella flexneri* reveals basal bodies highly resembling those of the flagellum (fig. 4, inset and fig 5) (Blocker *et al.*, 2001; Kimbrough and Miller, 2000; Kubori *et al.*, 1998; Kubori *et al.*, 2000; Sekiya *et al.*, 2001).
Flagellar assembly is a strictly controlled process. The organelle is built from base to tip; new components are exported through the conduit formed by the flagellum itself and added on the growing end only after the earlier parts have been completed (Aizawa, 1996; Macnab, 1996, 2004). One critical event is the so called \textbf{substrate specificity switch} that occurs when assembly of the hook is completed and the flagellar T3S system ceases to export and assemble hook/rod type substrates in favour of the filament subcomponent FliC. Strains mutated in FliK are deficient for this switch and can not initiate FliC secretion but instead assemble hook after hook, a so called polyhook (fig. 4, middle) (Patterson-Delafield \textit{et al.}, 1973; Suzuki and Iino, 1981). Further investigation of this phenomenon revealed that FliK interacts with the inner membrane component FlhB to control the switch, since the polyhook phenotype of the \textit{fliK} mutant was partially suppressed by second site mutations in the FlhB. As a result a polyhook-filament phenotype is seen, i.e. the strain is still impaired for termination of hook assembly but able to form a filament (fig. 4, right) (Hirano \textit{et al.}, 1994; Kutsukake \textit{et al.}, 1994; Williams \textit{et al.}, 1996). The T3S injectisomes of \textit{Shigella}, \textit{Salmonella} and \textit{Yersinia} have all
revealed a corresponding switch deficiency when mutated in the FliK analogues Spa32, InvJ and YscP respectively. These mutants can not terminate assembly of the needle structure, resulting in superlong needles, and are also unable to initiate secretion of effector proteins (Paper II) (Agrain et al., 2005a; Journet et al., 2003; Kubori et al., 2000; Magdalena et al., 2002).

1.5.2 Type III systems, tailored for their hosts

While the basal bodies of secretion systems are homogenous in appearance, not only the delivered effectors but also the surface structures vary depending on the nature of the host-pathogen interaction. For organisms that come into close contact with the host cell membranes, the T3S systems form comparatively small needle-like protrusions, for Shigella (~45 nm), Salmonella (~80nm) and Yersinia (varying results of ~42nm or 60-80nm) (fig. 5) (Hoiczyk and Blobel, 2001; Journet et al., 2003; Kubori et al., 2000; Tamano et al., 2000; Tamano et al., 2002).

Enteropathogenic E. coli, on the other hand, utilises T3S to come into close contact with cells of the intestinal epithelium and accordingly supports a long filamentous type III protrusion that spans the mucus layer surrounding these cells (fig. 6)
(Knutton et al., 1998). Similarly, the T3S system of the plant pathogen *P. syringae* assembles a sturdy pilus for effector delivery, corresponding to the thick plant cell wall (fig. 7A) (He and Jin, 2003; Roine et al., 1997).

### 1.5.3 Delivery of effector proteins by the type III secretion system

The exact mechanism by which the effectors reach the host cell cytosol has not been resolved, but the common hypothesis is that the delivery occurs via a hollow channel formed by the secretion apparatus itself (Ghosh, 2004). In *P. syringae*, secretion of effectors has in deed been shown to occur through the Hrp-pilus type III apparatus, as shown in figure 7 (Jin and He, 2001).

![Figure 7. Evidence of secretion through the T3S Hrp pilus of *P. syringae*.](image)

1.6 THE PLASMID ENCODED *YERSINIA* TYPE III SECRETION SYSTEM

#### 1.6.1 The common virulence plasmid

The ~70 kb plasmid pYV (plasmid of *Yersinia* virulence) encodes a type III secretion system shared by *Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica*
The plasmid contains genes for: the **Ysc** (*Yersinia* secretion) proteins that build up the secretion organelle, **effector Yops** (*Yersinia* outer proteins) that interfere with host cell signalling, the **translocators** responsible for delivery of the effectors across the host cell membrane, **chaperones** for the effectors and the translocators, and also **regulatory components** required for the delicate regulation of the system (fig. 8).

**Figure 8. The plasmid encoded *Yersinia* T3SS.** The common virulence plasmid is here represented by the map of pIB1 of *Y. pseudotuberculosis*. Based on unpublished data (P. Cherepanov and T. Svensson). Reprinted with permission from (Schesser *et al.*, 2000). Copyright 2004 ASM Press.

### 1.6.2 The Ysc apparatus

The Ysc proteins (table 1) represent the most highly conserved group of T3S proteins, and are predicted to build up the *Yersinia* T3S organelle as depicted in figure 9.

While electron microscopy has been used to visualise the external parts of the organelle, including the YscF needle structures and the ring-shaped outer membrane pores formed by YscC, the membrane spanning basal body has never been isolated or visualised for *Yersinia* (Hoiczyk and Blobel, 2001; Koster *et al.*, 1997). Thus, the model in fig 9 is based on observations made for related T3SSs (chapter 1.5.1) including the flagellar.
### Table 1. Ysc proteins encoded by the virA, virB, virC and virG operons of pYV.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Size (kDa)</th>
<th>Comments</th>
<th>Localisation*</th>
<th>Required for Yop secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>virC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YscA</td>
<td>3.8</td>
<td>Hypothetical protein.</td>
<td>Unknown</td>
<td>N</td>
</tr>
<tr>
<td>YscB</td>
<td>15.4</td>
<td>Together with SycN forms a heterodimeric chaperone for YopN.</td>
<td>CP</td>
<td>yscB mutant derepressed</td>
</tr>
<tr>
<td>YscC</td>
<td>67.3</td>
<td>Forms oligomeric secretin OM ring of Ysc apparatus. Secreted via Sec.</td>
<td>OM</td>
<td>Y</td>
</tr>
<tr>
<td>YscD</td>
<td>46.9</td>
<td></td>
<td>IM/PP</td>
<td>Y</td>
</tr>
<tr>
<td>YscE</td>
<td>7.4</td>
<td>Interacts with YscG.</td>
<td>CP/IM</td>
<td>Y</td>
</tr>
<tr>
<td>YscF</td>
<td>9.4</td>
<td>Structural needle subcomponent.</td>
<td>Surface</td>
<td>Y</td>
</tr>
<tr>
<td>YscG</td>
<td>13.0</td>
<td>Chaperone-like properties, binds YscE.</td>
<td>CP/IM</td>
<td>Y</td>
</tr>
<tr>
<td>YscH</td>
<td>18.4</td>
<td>Secreted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YscI</td>
<td>12.6</td>
<td>Chaperone-like properties.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YscJ</td>
<td>27.0</td>
<td>Lipoprotein, possible membrane linker. Secreted via Sec.</td>
<td>IM/PP/CP</td>
<td>Y</td>
</tr>
<tr>
<td>YscK</td>
<td>23.9</td>
<td>Interacts with YscQ (Yeast 2/3 hybrid data).</td>
<td>CP</td>
<td>Y</td>
</tr>
<tr>
<td>YscL</td>
<td>24.9</td>
<td>Interacts with YscN and YscQ (Yeast 2/3 Hybrid data).</td>
<td>CP</td>
<td>Y</td>
</tr>
<tr>
<td><strong>virB</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YscN</td>
<td>47.8</td>
<td>Predicted ATPase.</td>
<td>IM/CP</td>
<td>Y</td>
</tr>
<tr>
<td>YscO</td>
<td>19.0</td>
<td>Secreted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YscP</td>
<td>50.4</td>
<td>Controls needle length and substrate specificity switch.</td>
<td>Secreted</td>
<td>Y</td>
</tr>
<tr>
<td>YscQ</td>
<td>34.4</td>
<td>Interacts with YscL and YscK (Yeast 2/3 hybrid data).</td>
<td>CP</td>
<td>Y</td>
</tr>
<tr>
<td>YscR</td>
<td>24.4</td>
<td>Structural component, protrudes into CP.</td>
<td>IM</td>
<td>Y</td>
</tr>
<tr>
<td>YscS</td>
<td>9.6</td>
<td>Structural component.</td>
<td>IM</td>
<td>Y</td>
</tr>
<tr>
<td>YscT</td>
<td>28.4</td>
<td>Structural component.</td>
<td>IM</td>
<td>?</td>
</tr>
<tr>
<td>YscU</td>
<td>40.4</td>
<td>Structural component, protrudes into CP. Regulates substrate specificity switch with YscP.</td>
<td>IM</td>
<td>Y</td>
</tr>
<tr>
<td><strong>virA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YscX</td>
<td>13.6</td>
<td>Interacts with YscY.</td>
<td>Secreted</td>
<td>Y</td>
</tr>
<tr>
<td>YscY</td>
<td>13.1</td>
<td>Possible chaperone for YscX, interacts with LcrH.</td>
<td>CP</td>
<td>Y</td>
</tr>
<tr>
<td>YscV/LcrD</td>
<td>77.8</td>
<td>Structural component, protrudes into CP.</td>
<td>IM</td>
<td>Y</td>
</tr>
<tr>
<td><strong>virG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YscW</td>
<td>14.6</td>
<td>Lipoprotein piloting YscC to OM and stabilising the secretin structure. Secreted via Sec.</td>
<td>OM</td>
<td>Y</td>
</tr>
</tbody>
</table>

* For some proteins, location is predicted but not proven. OM = outer membrane, PP = periplasm, IM = inner membrane, CP = cytoplasm. References: Allaoui et al., 1994; Allaoui et al., 1995; Bergman et al., 1994; Burghout et al., 2004; Day and Plano, 1998; Day et al., 2000; Day and Plano, 2000; Edqvist et al., 2003; Fields et al., 1994; Francis et al., 2001; Goguen et al., 1984; Haddix and Straley, 1992; Hoiczyk and Blobel, 2001; Iriarte and Cornelis, 1999; Jackson et al., 1998; Jackson and Plano, 2000; Journet et al., 2003; Koster et al., 1997; Michiels et al., 1991; Payne and Straley, 1998, 1999; Plano et al., 1991; Plano and Straley, 1993, 1995; Woestyn et al., 1994.
The localisation of different Yscs is predicted by a combination of *in silico* structure analysis, experimental data as well as by homology to known components from other TTS systems. A large number of the Ysc proteins are assembled in the inner membrane, and their exact roles not resolved but may be primarily structural. YscN is a peripheral inner membrane protein, and suggested to be the ATPase that energises the Ysc machinery (Woestyn et al., 1994). Some inner membrane Yscs possess large soluble domains that protrude into the cytoplasm, suggesting a possible function for them within this compartment (table 1, fig. 9). As for the flagellar T3SS (chapter 1.5.1), assembly of the Ysc apparatus has to occur in an ordered manner. This is apparent as some of the components of the machinery are exported via the Sec system, while others rely on the T3S system itself for their delivery. The outer membrane secretin protein, YscC, and its pilot, YscW, are examples of Sec dependent substrates while the needle component YscF, as well as the Ysc proteins released into the extrabacterial milieu, depends on a functional T3S machinery for export (table 1, fig. 9). Apart from being building blocks of the secretion apparatus, some Yscs have regulatory roles. One example is the soluble exported protein YscP that, like its homologue
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FliK of the flagellar T3SS, is involved in the T3S substrate specificity switch (chapter 1.5.1, Paper II).

1.6.3 The Yop effectors

The Yop effectors delivered by the Ysc machinery work in concert to inhibit the phagocytic ability of host cells and to repress the inflammatory response, thus enabling extracellular colonisation (Juris et al., 2002). An overview of the Yop effectors and their biological roles is given in table 2.

Table 2. The *Yersinia* Yop effectors.

<table>
<thead>
<tr>
<th>Effector</th>
<th>Chaperone</th>
<th>Activity</th>
<th>Cellular effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>YopH</td>
<td>SycH</td>
<td>PTPase</td>
<td>Disruption of focal complexes, inhibition of pro-inflammatory signalling Anti-phagocytosis, anti-inflammation</td>
</tr>
<tr>
<td>YopE</td>
<td>SycE</td>
<td>GAP</td>
<td>Disruption of actin microfilaments Anti-phagocytosis</td>
</tr>
<tr>
<td>YpkA</td>
<td>-</td>
<td>Serine-Threonine kinase</td>
<td>Disruption of actin microfilaments Anti-phagocytosis</td>
</tr>
<tr>
<td>YopT</td>
<td>SycT</td>
<td>Cysteine protease</td>
<td>Disruption of actin microfilaments Anti-phagocytosis</td>
</tr>
<tr>
<td>YopJ</td>
<td>-</td>
<td>Cysteine protease</td>
<td>Inhibition of MAPK and NF-κB signalling pathways Induction of apoptosis of macrophages, anti-inflammation</td>
</tr>
<tr>
<td>YopM</td>
<td>-</td>
<td>Leucin-rich repeat protein</td>
<td>Locates to nucleus Interferes with eukaryotic cell cycle</td>
</tr>
</tbody>
</table>

Review: Bliska, 2000; Cornelis et al., 1998; Juris et al., 2002; Navarro et al., 2005.

Virulence studies in mice suggest that YopH, YopE, YpkA and YopM are essential for virulence while YopT is not, and YopJ data are contradictory (Bolin and Wolf-Watz, 1988; Forsberg and Wolf-Watz, 1988; Galyov et al., 1993; Galyov et al., 1994; Leung et al., 1990; Monack et al., 1998; Trulzsch et al., 2004).

1.6.4 The *Yersinia* type III secretion chaperones

Syc proteins (specific Yop chaperones) bind to one or more specific partner with high affinity and are characteristically small, acidic proteins with a C-terminal
amphiphilic α-helix structure (Feldman and Cornelis, 2003; Marenne et al., 2004). There are multiple suggested roles for the type III chaperones, including stabilisation, anti-aggregation, prevention of premature folding, establishing a hierarchy for secretion and regulation of the T3SS (Feldman and Cornelis, 2003; Marenne et al., 2004). Consistent with their important roles, several substrates depend on chaperones, including the effector proteins YopE, YopH and YopT (table 2). The secreted negative regulators YopN and LcrQ are also chaperoned, by YscB+SycN and SycH respectively, while the translocators YopB and YopD require the LcrH (SycD) chaperone (Day and Plano, 1998; Jackson et al., 1998; Neyt and Cornelis, 1999; Wattiau et al., 1994; Wulff-Strobel et al., 2002). Further, there are Ysc proteins with chaperone-like properties, such as YscI and YscY, which are both encoded adjacent to genes for secreted proteins, YscH and YscX respectively, a common pattern seen for chaperones and their partners (table 1).

1.6.5 A signal for the *Yersinia* type III secretion substrates

Not only the effector Yops but also the translocators, some Ysc components and regulatory proteins depend on the T3S for export. While primarily studied for the effector Yops, the nature of the secretion signal is not clear. Residing within the N-terminus it has been proposed to lie either within the mRNA or the amino acid sequence and may be characterised by a specific pattern of amphipaticity. Recognition could occur directly by the Ysc apparatus itself, or via chaperones that will recognise and target the substrate to the machinery for delivery (Anderson and Schneewind, 1997; Lloyd et al., 2001a; Lloyd et al., 2001b; Lloyd et al., 2002; Michiels et al., 1990; Sorg et al., 2005; Sory et al., 1995). Consensus remains to be reached, and quite possibly alternative secretion signals might exist for different substrates.
1.6.6 The low calcium response, *in vitro* triggering of *Yersinia* T3S

While inhibited by mM levels of Ca\(^{2+}\), *in vitro* expression and secretion of Yop proteins can be triggered by cultivation of *Yersinia* at 37°C in calcium depleted medium (Forsberg *et al.*, 1987; Heesemann *et al.*, 1986; Michiels *et al.*, 1990). The induction of type III secretion in the absence of Ca\(^{2+}\) is accompanied by growth arrest, a phenomenon referred to as ‘the low calcium response’ (LCR) (Goguen *et al.*, 1984; Straley and Bowmer, 1986). Due to this phenotype, the growth of yersiniae at 37°C is denoted calcium dependent (CD). Mutants defective for LCR are classified as either calcium independent (CI) or temperature sensitive (TS). CI strains never enter growth restriction and are usually impaired for Yop expression and secretion. This is the phenotype for most *ysc* mutant strains and mutants defective for positive regulation. TS, or ‘calcium blind’, strains are restricted for growth at 37°C irrespective of calcium and commonly unable to repress both Yop expression and secretion in the presence of Ca\(^{2+}\). This phenotype reflects mutation in the negative regulation of Yop expression and, usually, secretion (Perry and Fetherston, 1997). It is difficult to appreciate the biological importance of the calcium response, which may even be an artifactual *in vitro* effect, but it has nonetheless been very valuable for *in vitro* investigation of the *Yersinia* T3SS.

1.6.7 *In vivo* induction of *Yersinia* type III secretion

A simplified model for the *in vivo* regulation of *Yersinia* type III secretion is presented below, without dwelling on controversies.

At 37°C, transcription of the genes encoded by the pYV plasmid is induced due to i) release of the histone-like repressor YmoA from the DNA, leading to conformational changes that favours transcription ii) expression of the positive regulator LcrF/VirF (Cornelis *et al.*, 1991; Hoe and Goguen, 1993; Jackson *et al.*, 2004; Lambert de Rouvroit *et al.*, 1992; Yother *et al.*, 1986). The Ysc machinery is assembled and basal levels of Yop proteins are expressed, but prior to contact with
the target cell a negative feed-back mechanism prevents secretion as well as elevated expression of the effectors. This mechanism relies on LcrG and a complex formed by YopN, TyeA and the heterodimeric YopN chaperone YscB+SycN, which obstructs the secretion channel prior to cell contact (Cheng and Schneewind, 2000; Cheng et al., 2001; Ferracci et al., 2005; Schubot et al., 2005). During these conditions negative regulators will be contained within the bacterium; a complex of LcrQ, YopD and LcrH, together repress Yop synthesis by an unknown mechanism. Upon contact with the host cell, the obstruction of the Ysc apparatus is relieved, allowing LcrQ and YopD to be secreted. Thus, bacterium-cell contact lead to derepression of the *Yersinia* type III secretion system, followed by subsequential translocation of Yop effectors into the host cells (Francis et al., 2001; Pettersson et al., 1996; Rimpilainen et al., 1992; Williams and Straley, 1998). The translocation depends on the three translocator proteins LcrV, YopB and YopD to form a pore in the host cell membrane, thus supporting directed delivery of the effectors into the cytosol of the eukaryotic cell to exert their functions (Forsberg et al., 1994; Holmstrom et al., 2001; Marenne et al., 2003; Persson et al., 1995; Pettersson et al., 1999; Rosqvist et al., 1994; Rosqvist et al., 1995; Sarker et al., 1998; Sory and Cornelis, 1994).
2. AIMS

The aim of this thesis was to determine the roles played by the components YscF, YscP and YscU in *Yersinia* type III secretion.

A second objective was investigation of the putative twin arginine translocation system of pathogenic *Yersinia* and its potential role in virulence.
3. RESULTS AND DISCUSSION

3.1 CONCLUSIONS IN BRIEF FROM PAPERS I-IV

I. Both Yersinia pestis and Y. pseudotuberculosis express a functional twin arginine translocation system that in the latter is shown to be required for motility, acid resistance and virulence. Several putative Tat substrates can be identified within the genome sequences of both species.

II. A Y. pseudotuberculosis yscP mutant strain secretes excess amount of the needle subcomponent YscF and is impaired for Yop secretion. This phenotype can be suppressed by 2nd site mutations in the cytoplasmic domain of YscU, suggesting that these two proteins together control the type III secretion substrate specificity switch.

III. YscU is cleaved within a conserved site (Asn-Pro-Thr-His) in the cytoplasmic domain. Introduction of mutations at this site results in uncleavable YscU variants. When expressed in trans, these proteins restore Yop secretion to an yscU mutant strain, suggesting that cleavage is redundant for YscU function.

IV. Expression of the uncleavable yscU variants in cis, however, reveal that cleavage is indeed required for Yersinia type III secretion. This illustrates the importance of the strictly conserved site as well as that the complex regulation of the components of the T3SS depends on expression of YscU in its native context.
RESULTS AND DISCUSSION

3.2 YERSINIA TWIN ARGinine TRANSLOCATION

3.2.1 Yersinia Tat is functional

Facilitated by the ever increasing number of sequenced genomes, putative twin arginine translocation systems have been identified in a wide range of bacteria (Dilks et al., 2003). As shown in Paper I, the presence of a fully functional Tat system could be confirmed for Y. pestis and Y. pseudotuberculosis. Not surprisingly, the closely related Y. enterocolitica also encodes a complete Tat system (Sanger institute; www.sanger.ac.uk/Projects/Y_enterocolitica), although its functionality remains to be confirmed.

3.2.2 A connection between Tat and Type III secretion?

My first incentive to study the Yersinia Tat system was due to an intriguing observation of a connection between Tat and the type III secretion system as reported by Michael Vasil and coworkers (unpublished results). Their studies on the opportunistic pathogen P. aeruginosa revealed that type III secretion requires a functional Tat system. Despite being pathogens of dissimilar life styles, with very different outcomes of infection, the virulence plasmid encoded T3SS of Yersinia is remarkably similar to the chromosomally encoded system of P. aeruginosa, with a number of components being functionally interchangeable between the two systems (Bröms et al., 2003a, b; Frithz-Lindsten et al., 1998). With Tat in the inner membrane and the T3S apparatus spanning the bacterial envelope, crosstalk between the two systems also in Yersinia was a distinct possibility, in particular since PscO, the YscO homologue (table 1), of P. aeruginosa was considered a putative Tat substrate (M. Vasil, unpublished results). As seen in table 3 both these proteins possess a twin arginine (bold) motif in the N-terminus.
RESULTS AND DISCUSSION

Table 3. N-terminal amino acid sequences of PscO and YscO

<table>
<thead>
<tr>
<th>Protein</th>
<th>Residue 1-36</th>
</tr>
</thead>
<tbody>
<tr>
<td>PscO</td>
<td>1-MSLALLLRRRRLDRAER AQGRQLLVRRAAAQEHT---</td>
</tr>
<tr>
<td>YscO</td>
<td>1-MI RR LHRVKVLVRAEKA IKTQACLQAAHRRHQE---</td>
</tr>
</tbody>
</table>

Genebank accession numbers: PscO: NP_250387; YscO: CAF25411.

However, with increased understanding of the Tat signal peptide, more accurate predictions of putative substrates can now be made. Analysis of these two putative signal sequences with the TatP software, or with Pedant pattern search (methods described in Paper I) suggests that they are not true Tat substrates. This implies that the Tat system of *P. aeruginosa* must affect the function of the T3SS in some other way than by impairing secretion of PscO. Moreover, the search for a Tat-T3SS connection in *Yersinia* turned out negative, since the *Yersinia tatC* mutant strain proved indistinguishable from wild type *Yersinia* in all aspects of T3S analysed (Paper I).

### 3.2.2 Tat is required for *Yersinia* virulence

Oral infection of mice revealed the *Y. pseudotuberculosis* tatC mutant to be highly attenuated for virulence and impaired for colonisation of lymphoid organs like the Peyer’s patches and spleen (Paper I). Since the mutant also exhibited increased *in vitro* sensitivity to acid, a possible explanation to the severe attenuation could be that the bacteria were eradicated by the low pH within the mouse gastrointestinal tract. To resolve this issue, the virulence of the tatC strain was studied by infection via the intraperitoneal route. The results showed that, also for this route, the Tat system is essential for virulence. Fascinatingly, the tatC mutant was equally attenuated as the virulence plasmid cured strain, i.e. lethal at a dose of $10^7$ bacteria/ml compared to $10^3$ for wild type infections (S.K. Ericsson and M. Lavander, unpublished). The acid sensitivity is an interesting phenotype also for this mode of infection, since it may deprive *Y. pseudotuberculosis* of its ability to survive and multiply within macrophages (chapter 1.2.6). Still, the tatC mutant showed survival within J774 macrophage-like cells equally well as the wild type
strain, suggesting that this is not the case, although the outcome might be different if activated macrophages were used.

### 3.2.3 Yersinia Tat substrates in relation to virulence

*In silico* methods were used to identify putative Tat substrates for *Y. pestis* and *Y. pseudotuberculosis* (table 3 in Paper I). Some of these are promising candidates that could explain the phenotypes of the *tatC* mutant. The resistance to acidic conditions could possibly be mediated by the carbonic anhydrase (CA) identified in the genomes of both *Y. pestis* and *Y. pseudotuberculosis* that may constitute an important virulence factor. CAs are zinc metalloenzymes, found in both pro- and eukaryotes, that catalyze the reversible hydration of CO₂ \[\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+\]. They are known to be involved in many physiological processes and have also been proposed to contribute to intracellular survival of bacteria. Interestingly, a recent publication on *Helicobacter pylori* demonstrated a periplasmic CA that was required for *in vitro* acid resistance (Marcus *et al.*, 2005; Smith and Ferry, 2000). Other interesting putative Tat substrates included YbtP and YbtQ (table 3 in Paper I), two proteins involved in iron acquisition, an ability absolutely crucial for colonisation of a host (chapter 1.2.5). Both YbtP and YbtQ have been shown to be essential for iron uptake by Yersiniabactin, and a *Y. pestis ybtP* mutant strain was avirulent via the subcutaneous route (Fetherston *et al.*, 1999). Further, proteins involved in transport and a number of proteins of unknown functions are interesting for future investigations of their potential roles in *Yersinia* virulence (table 3 in Paper I).

### 3.2.4 Future perspectives of Tat

The *in silico* screening for putative Tat substrates turned up a number of interesting proteins that may contribute to the ability of *Yersinia* to cause disease. Proteomic comparison of fractions of the extracytoplasmic bacterial compartments derived from the *tatC* mutant and the isogenic wild type strain will contribute to the search
for Tat substrates. Verification and complementation of these screens will determine which proteins are i) true substrates of \textit{Yersinia} Tat, and ii) important for virulence.

The severe attenuation of the \textit{tatC} mutant strain makes Tat an interesting target for development of novel antimicrobial therapeutics. One could envisage two different approaches: either targeting of a Tat substrate identified as crucial for virulence or, alternatively, to block the Tat system directly. The latter approach would be the method of choice, since the substrates may have human homologues (for example the carbonic anhydrase), while the system itself is found in prokaryotes, archaea and plants but, importantly, not in mammals. Utilisation of compound libraries has previously been successfully employed to locate substances that suppress the T3SS of \textit{Yersinia} and could constitute a useful method also in the present case (Kauppi \textit{et al.}, 2003; Nordfelth \textit{et al.}, 2005).

3.3 \textbf{ROLE OF YSCP AND YSCU IN \textit{YERSINIA} TYPE III SECRETION}

3.3.1 \textbf{YscP}

\textit{YscP} is a 50 kDa soluble protein found both intrabacterially and secreted in a type III dependent manner (Payne and Straley, 1999; Stainier \textit{et al.}, 2000). \textit{YscP} homologues exist in several T3SSs. While their overall sequence conservation is low, they possess a globular domain, essential for function, within the C-terminus. This structure is conserved and interchangeable between the closest related \textit{YscP} homologues (Agrain \textit{et al.}, 2005a).

3.3.1.1 \textbf{YscP, a protein of dual functions}

\textit{Paper II} shows that \textit{YscP} is required for substrate specificity switching (chapter 1.5.1), as an \textit{yscP} mutant exports excessive amounts of the needle component \textit{YscF} and is impaired for secretion of Yop effectors. The function for the switch is located within the C-terminus of \textit{YscP}, as revealed by introduction of deletions in
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this domain resulting in a non-functional protein (M. Lavander, unpublished). This is consistent with results from Agrain and coworkers, where function for Yop secretion was mapped between amino acids 385-500 of the 515 amino acid YscP protein of *Y. enterocolitica* coinciding with the conserved globular domain (Agrain *et al.*, 2005a). A second function of YscP is regulation of the length of the YscF needle. Apparently, the length of the needle is in direct relation to the length of the YscP peptide, suggesting that YscP acts as a molecular ruler (Journet *et al.*, 2003).

3.3.1.2 The role of YscP export

The very role of YscP export has not been determined. The signal for YscP export was hypothesised to be located within the extreme N-terminus, since amino acid 4-7 in InvJ (the YscP homologue from the *S. enterica* SPI-1 encoded T3SS), had been shown to be necessary and sufficient for T3S dependent export of this protein (Russmann *et al.*, 2002). To determine the minimal secretion signal of YscP, small deletions were introduced within the N-terminus of *Y. pseudotuberculosis* YscP (table 4). The YscPΔ2-34 variant, although stably expressed, was not secreted and could not restore Yop secretion to an yscP deletion mutant.

<table>
<thead>
<tr>
<th>Construct</th>
<th>N-terminal amino acid sequence</th>
<th>Yop secretion</th>
<th>YscP expression</th>
<th>YscP secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type YscP</td>
<td>MNKITTRSPIEYQLPGKPHLDACVDFEQALLHNNKGNCHPK</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>YscP (Δ2-34)</td>
<td>M-------------------------------HNNKGNCHPK</td>
<td>–</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>YscP (Δ6-34)</td>
<td>MNKIT----------------------------HNNKGNCHPK</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>YscP (Δ9-34)</td>
<td>MNKITTRS-----------------------HNNKGNCHPK</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>YscP (Δ11-34)</td>
<td>MNKITTRSPS----------------------HNNKGNCHPK</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 4. Effect of deletions within the YscP N-terminus

a) YscP variants with the described deletions were expressed in trans under control of the tac promoter of pMMB66HE in the yscP mutant strain pIB69 (Paper II). The number of plus (+) indicates levels of protein in supernatant (secretion) or total samples (expression) as detected with α-Yop and α-YscP antisera.

In contrast, by keeping the first 5, 8 or 10 residues, the same deletion variant could now be secreted as well as support Yop secretion, suggesting that the first five
amino acids of YscP constitute a minimal secretion signal. Curiously, Agrain and coworkers recently used sequential deletions to reveal that YscP of *Y. enterocolitica* may actually contain two secretion signals, within amino acid 1-35 and 97-137 respectively. To prevent YscP secretion both signals had to be disrupted, resulting in a protein able to support Yop secretion but not to control needle length (Agrain *et al.*, 2005b). This proposed second secretion signal seems to be missing in *Y. pseudotuberculosis* YscP, where deletion of residues 2-34 efficiently prohibits export of the protein (table 4).

Even if my results indicate that the ability to export YscP is required for Yop secretion, it is conceivable that the export may be a side effect of localising in the type III secretion apparatus during induction of the system, rather than a prerequisite for YscP function. YscP is secreted to the extracellular milieu, primarily under type III secretion inducing conditions (minus Ca\(^{2+}\)). However, overexpression of the protein results in high levels of YscP export also under non-inducing conditions (plus Ca\(^{2+}\)). This shows that there is a hierarchy of secretion, as the type III dependent delivery of YscP across the bacterial double membrane occurs under conditions when Yops are still restrained from secretion (M. Lavander, unpublished).

3.3.2 YscU

YscU is a 40 kDa inner membrane protein, with four transmembrane domains and a C-terminal tail protruding into the cytoplasm. YscU belongs to a highly conserved family of core components that are absolutely essential for type III secretion (Allaoui *et al.*, 1994; Hueck, 1998). Schematic representations of YscU can be found in Paper III, fig. 1 and Paper IV, fig. 6.

3.3.2.1 YscU and the role of cleavage

An early observation when expressing YscU *in trans* was that the protein is cleaved between Asn-263 and Pro-264 within a strictly conserved four amino acid
RESULTS AND DISCUSSION

site (Asparagine-Proline-Threonine-Histidine) in the C-terminus, and that mutation within this site abolished proteolysis (Papers III-IV). The conserved nature of the site, together with the observation of cleavage, indicated that the proteolysis may be of importance for YscU function. While initial studies in trans suggested that uncleavable variants of YscU could still promote Yop secretion, identical mutants expressed in cis could not (Papers III-IV). This is a clear illustration of the importance of working in the native context to avoid misleading high-copy effects, even more so when studying a highly complex apparatus where ratios between individual components may be crucial. If cleavage is indeed coupled to functional Yop-secretion, how does this occur? One possibility is that YscU generally adopts a conformation that is required for its function, but makes the protein prone to cleavage, i.e. cleavage as a secondary effect.

The proteolysis may on the other hand play a more direct role, as a primary event for functional T3S. For example, the role of proteolysis could be to set the YscU\textsubscript{CC} (amino acid 264-354, i.e. the C-terminal cleavage product) domain free to perform a function at a different locus of the T3S system. In flagellar T3SSs a protein, FlhX, homologous to the YscU\textsubscript{CC} domain, has been identified in many bacteria, suggesting a biological role for this peptide (Pallen et al., 2005). Alternatively, the parting of YscU\textsubscript{CC} from YscU\textsubscript{CN} (amino acid 211-263, extending from the transmembrane domain of YscU) could allow the latter to promote Yop secretion from within the apparatus. In this model, YscU\textsubscript{CC} would function as an inhibitor of YscU\textsubscript{CN}.

3.3.3 The YscP and YscU connection

Previous studies have indicated a direct interaction between YscU and YscP. For example, the observation of an interaction between the analogous proteins FlhB and FliK from the flagellar T3SS as seen in protein overlay assays supports the notion of a physical interaction also between YscU and YscP (Minamino and Macnab, 2000). Additionally, 2\textsuperscript{nd} site mutations within the C-terminal domain of
YscU allow for substrate specificity switching even without the presence of YscP (Paper II). However, attempts to confirm this interaction with various methods, including yeast two-hybrid, Flag pull-downs, and protein overlays, have been unsuccessful (M. Lavander, unpublished). This may suggest that the interaction for some reason is difficult to capture: either it is very transient, easily disassociates under less-than-optimal in vitro conditions or possibly that some third component is needed to stabilise the interaction between YscU and YscP. Then again, there is the scenario where the two proteins actually do not physically interact, and that another yet unidentified component may be responsible for the crosstalk between YscU and YscP in the in vivo situation. My belief is however that the two do indeed interact and that YscP thereby under permissive conditions induces a conformational change in the C-terminus of YscU that allows it to support Yop secretion. The hypothesis presented in Paper II suggests that the YscP dependent conformational change may be simulated by 2nd site mutations in YscU.

3.3.4 Role of the needle in type III secretion

A key element in T3S is the needle component YscF. The exact role of the needle in T3S is not clear, although the general belief is that it acts as a conduit through which the Yop effectors reach the target cell. To study the role of the needle, variants of YscF mutagenised with alanine substitutions were engineered and investigated for their ability to complement an yscF mutant strain. While a number of substitutions at conserved residues resulted in non-functional YscF variants (data not shown), the substitution mutant YscF_D46A gave a more intriguing phenotype. Whereas wild type YscF supported Yop secretion only under inducing in vitro conditions (minus Ca^{2+}), the YscF_D46A mutant showed constitutive expression and secretion of Yop effectors and translocators (fig. 10 A). Furthermore, the mutant did not display the polarised translocation observed for wild type Yersinia but instead delivered high amounts of Yops to the culture
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medium during cell infection assays (data not shown). Consequently, the mutant strain was severely delayed in YopE mediated cytotoxicity (fig. 10B).

Taken together, these observations suggest that the needle component is somehow involved in regulation of the T3SS, possibly by acting as a sensor of the extracellular milieu transmitting the inducing signal (cell contact in vivo, calcium depletion in vitro) to the T3SS. The role of YscF as a sensor was also recently suggested in a publication on Y. pestis (Torruellas et al., 2005).

3.3.5 A model of substrate specificity switching by YscP and YscU

A speculative model for how YscP and YscU could contribute to induction of Yop secretion is presented here.

During non secretion permissive conditions at 37°C (i.e. in presence of calcium or prior to target cell contact) Yop secretion is physically prohibited: A complex formed by YopN, its heterodimeric chaperon YscB+SycN and TyeA, which
anchors the complex at the inner membrane, is responsible for obstructing the secretion apparatus (Ferracci et al., 2005; Schubot et al., 2005). Upon cell contact, or depletion of calcium, the needle acts as a sensor and transmits the signal to the system (Torruellas et al., 2005). This signal is conferred to YscP, which alters the conformation of the C-terminal domain of YscU to a secretion-permissive state. YscU then interacts, directly or indirectly, with the ATPase YscN to alter the substrate specificity of the system. This interaction enables docking of chaperones to YscN, followed by dissociation of the chaperone and ATPase mediated unfolding and secretion of the substrate (Akeda and Galan, 2005; Gauthier and Finlay, 2003; Thomas et al., 2004). The first substrate to be secreted is YopN, thus unblocking the channel, which allows for secretion of negative regulatory elements (LcrQ and YopD) that normally suppress expression of the Yop effectors. This results in elevated Yop expression and Yop secretion completing the substrate specificity switch.

### 3.3.5 Future perspectives for YscU and YscP

One interesting and unidentified factor, that potentially could be a regulator of type III secretion, is the protease responsible for YscU cleavage. The ClpXP and Lon proteases have been shown to be involved in the regulation of type III secretion. More precisely, these proteases have been shown to be required for degradation of the histone-like protein YmoA, which represses transcription of T3S genes (chapter 1.6.7) (Jackson et al., 2004). The observation of YscU cleavage occurring in T3SS negative backgrounds (Paper III) implies that the responsible protease is ubiquitously expressed, or possibly that YscU might induce autolysis. However, no domains suggesting a proteolytic activity are found within YscU.

Further, interactions between YscP, YscU and other components of the *Yersinia* T3SS would be very valuable for an understanding of the substrate specificity switch. This would facilitate future work with placing the two proteins in a larger context.
context and designing experiments to establish the functions of the interactions involved in induction of Yop secretion.

3.4 Final Remarks

As the discovery of antibiotics offered a cure also for plague, the threat from *Y. pestis* may seem like ancient history. However, pathogens evolve ways to overcome the obstacles in their way and, consequently, multiresistant strains of *Y. pestis* have been isolated from patients in Madagascar, where plague is endemic (Galimand *et al.*, 1997; Guiyoule *et al.*, 2001). Currently, a sub-unit vaccine based on the *Y. pestis* V- and F1-antigens is raising the hopes for protection against both bubonic and pneumonic plague (Heath *et al.*, 1998; Titball and Williamson, 2001, 2004; Williamson *et al.*, 1995). While the V-antigen (LcrV) is present in all virulent *Yersinia* strains, there is evidence of fully virulent naturally occurring *Y. pestis* strains devoid of the F1-antigen (Davis *et al.*, 1996). Efficiency of the new sub-component vaccine against these isolates is an unresolved issue.

The type III secretion system and, as shown in this thesis, the twin arginine translocation system are required for *Yersinia* cause disease. Since both T3S and Tat are essential virulence determinants for a number of pathogens, these secretion systems are interesting targets for the development of treatments that may be active against a wide range of bacteria. While progress has already been made for chemical suppression of T3S (Kauppi *et al.*, 2003; Nordfelth *et al.*, 2005), the Tat system is unexplored as an antimicrobial target. Future investigations to identify key drugs that block the Tat system would be a valuable contribution to the development of novel therapies, which potentially could be efficient against a diversity of infectious diseases caused by bacterial pathogens.
ACKNOWLEDGEMENTS


Eva-Christine som fyllt i ALLA mina aktivitetsrapporter, du är en pärla!

Rumskompisen, Charlotta, som fått timmar att flyga till surr när vi borde ha gjort något vettigt. Mästerkocken och spådamen, Lena S. Emelie som introducerade irish coffee på menyn. Sandra som så gärna ville smaka… Min favvotjej Jeanette, som det på något konstigt sätt är helt omöjligt att ha träkigt tillsammans med. Ok, kanske lite småsegt på O’Hare, men i övrigt kommer jag inte på ett enda tillfälle.

Stort tack till alla andra tomtar, änglar, nördar, sångfåglar, ölbyggare och coola katter som fixat, trixt, flyyttat, knattrat, hämtat, gycklat, jazzat, bjussat och skrönat.

Rötterna: norra provinsernas allra bästa föräldrar Mommy och Lill-Paapei, superduperFarmor, lillbrosan Björn med Frida och Isse (han är då för fantastisk
TACK!

den pojken), allra minsta brorsan Mats och Lotta. Tack för allt. Samt övriga
Lundinare och Lavandrar (inkusive Westins), ni är en förträfflig skara! Kompisar
från förr, T&K, TBAFC, Anna-Karin, Ulf, Jocke, Anders, Henrik och Bröllarna,
samt gästpelarna Anna och Frida. Resesällskapet och konspiratörerna
Semmelmeister och Sellle. Och sist men störst, han som får mitt hjärta att slå ett
extra slag: Anders, ti-tick!

här kan du skriva ditt namn om jag
glömde bort dig, vilket naturligtvis absolut inte var min mening och jag ber genuint
om ursäkt för min tankspriddhet. (Ring 090-106639 så bjuder jag på en öl (leg. 18
år) som plåster på såren).
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