Delivery of TypeIII Secreted Toxins by *Yersinia pseudotuberculosis*
-the Role of LcrV, YopD, and Free Lipids in the Translocation Process

av

Jan Olsson

Akademisk avhandling

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Abstract
Bacteria that infect humans and animals face a hard combat with the host’s immune system and in order to establish infection, pathogenic bacteria has evolved mechanisms to avoid being cleared from the host tissue. Many Gram-negatives carry a Type 3 secretion (T3S) system that is used to deliver effector proteins (toxins) into host cells. The toxins exhibit a broad range of intra cellular effects that has in common that they increase the chances of the bacteria to establish infection, multiply in infected tissue or spread to other tissues or hosts. The object of this study was to analyse the mechanisms behind the T3S effectors delivery into target cells. Two bacterial proteins, LcrV and YopD, which are involved in the translocation of effectors were analyzed by mutagenesis. LcrV was found to affect the efficiency of the translocation, probably by determining the size of the pore in the target cell membrane through which the effectors pass. Truncated variants of the multi-functional YopD revealed that defined regions of the protein were important for pore-formation and translocation. Effectors and translocators were demonstrated to form complexes with free acyl chains (lipids) at the bacterial surface. These complexes –termed Yop-lipid complexes, (YLC)– are released from the surface and can act as discrete units that are able to promote translocation of effectors even when separated from the bacterium from which they originate. These findings shed new light on the process of effector translocation by Yersinia and possibly by other gram-negative bacterial pathogens with a similar T3S setup.

Key words: Yersinia, T3SS, translocation, YopD, LcrV, YLC, fatty acids.
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ABSTRACT

Bacteria that infect humans and animals face a hard combat with the host’s immune system and in order to establish infection, pathogenic bacteria has evolved mechanisms to avoid being cleared from the host tissue. Many Gram-negatives carry a Type 3 secretion (T3S) system that is used to deliver effector proteins (toxins) into host cells. The toxins exhibit a broad range of intracellular effects that has in common that they increase the chances of the bacteria to establish infection, multiply in infected tissue or spread to other tissues or hosts. The object of this study was to analyze the mechanisms behind the T3S effectors delivery into target cells. Two bacterial proteins, LcrV and YopD, which are involved in the translocation of effectors, were analyzed by mutagenesis. LcrV was found to affect the efficiency of the translocation, probably by determining the size of the pore in the target cell membrane through which the effectors pass. Truncated variants of the multi-functional YopD revealed that defined regions of the protein were important for pore-formation and translocation. Effectors and translocators were demonstrated to form complexes with free acyl chains (lipids) at the bacterial surface. These complexes – termed Yop-lipid complexes, (YLC) – are released from the surface and can act as discrete units that are able to promote translocation of effectors even when separated from the bacterium from which they originate. These findings shed new light on the process of effector translocation by Yersinia and possibly by other gram-negative bacterial pathogens with a similar T3S setup.
PAPERS IN THIS THESIS

This thesis is based on the following articles referred to in the text by their roman numerals.


Front cover: A Yersinia pseudotuberculosis bacterium surrounded by Yop-lipid complexes. The picture is captured with atomic force microscopy by the author. See paper III for details.

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CD-</td>
<td>cluster designation or cluster of differentiation</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxy-terminal</td>
</tr>
<tr>
<td>EPEC</td>
<td>enteropathogenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>HPI</td>
<td>high-pathogenicity island</td>
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<tr>
<td>GAP</td>
<td>GTP activating protein</td>
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<tr>
<td>GDP</td>
<td>guanosine 5’-diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL1</td>
<td>interleukin</td>
</tr>
<tr>
<td>LD50</td>
<td>lethal dose 50</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>heat-labile toxin</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>M-cells</td>
<td>Microfold cells</td>
</tr>
<tr>
<td>NF-kB</td>
<td>nuclear factor of chain κ of B cells</td>
</tr>
<tr>
<td>Mg</td>
<td>Magnesium</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>Myf</td>
<td>mucoid <em>Yersinia</em> factor</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino-terminal</td>
</tr>
<tr>
<td>OM</td>
<td>outer membrane</td>
</tr>
<tr>
<td>OMV</td>
<td>outer membrane vesicles</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer’s Patches</td>
</tr>
<tr>
<td>Psa</td>
<td>pH 6 antigen</td>
</tr>
<tr>
<td>PTPase</td>
<td>protein tyrosine phosphatase</td>
</tr>
<tr>
<td>T1S</td>
<td>Type I Secretion</td>
</tr>
<tr>
<td>T2S</td>
<td>Type II Secretion</td>
</tr>
<tr>
<td>T3S</td>
<td>Type III Secretion</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>YLC</td>
<td>Yop-lipid complex</td>
</tr>
<tr>
<td>Yop</td>
<td><em>Yersinia</em> outer protein</td>
</tr>
<tr>
<td>Ypk</td>
<td><em>Yersinia</em> protein kinase</td>
</tr>
<tr>
<td>YPM</td>
<td><em>Yersinia pseudotuberculosis</em>-derived mitogen</td>
</tr>
<tr>
<td>wt</td>
<td>wild-type</td>
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</tbody>
</table>
BACKGROUND

Yersinia

The genus *Yersinia* belongs to the family Enterobacteriaceae, which includes several of the most thoroughly elucidated gram-negative bacteria, such as members of the genera *Enterobacter, Escherichia, Salmonella,* and *Shigella* (62). There are 11 *Yersinia* species (95), three of which are pathogenic to humans and animals: *Y. pestis*, the causative agent of plague, and the two enteropathogens *Y. pseudotuberculosis* and *Y. enterocolitica*. *Y. pseudotuberculosis* diverged from the other *Yersinia* species about 42–187 million years ago. *Y. pestis* evolved from *Y. pseudotuberculosis* much more recently—less than 20,000 years ago (2, 28).

Pathogenic isolates of *Y. enterocolitica* stem primarily from infected animals or vegetables contaminated with feces (213). In human clinical cases, pigs are often the source, which is illustrated by the results of a study conducted in Norway showing that 83% of examined swine were carriers (152). *Y. pseudotuberculosis* is isolated mainly from farm animals and pets (69), and it is widely found in soil and water polluted with feces of infected animals (70, 212). A recent investigation performed in Finland tracked the source of a major outbreak in humans to lettuce that was contaminated with *Y. pseudotuberculosis* in water spread by a sprinkler system (156).

Routes of infection

As mentioned above, *Y. pestis* causes plague, and this bacterial species has evolved intriguing mechanisms to infect humans via a flea bite (168, 246). The enteropathogens *Y. enterocolitica* and *Y. pseudotuberculosis* gain access to the host by the oral route, that is, through ingestion of contaminated food or water. The bacteria pass through the stomach and further into the small intestine to reach their niche in the terminal part of the ileum and in the cecum (Fig. 1) (37, 162, 201). This region of the digestive tract is rich in Peyer's patches (PPs), which constitute the gateway for pathogenic *Yersinia* to enter the gut tissue. In mice that undergo oral infection, the PPs can contain up to 1,000 times more bacteria per milligram of tissue than the surrounding mucosa (82). Peyer's patches consist of lymphoid follicles overlaid by follicle-associated epithelium. Intersected in the epithelium are microfold cells (M cells) that collect antigens from the intestinal lumen and transport them to lymphocytes and macrophages.
adjacent to their basolateral parts. This is the port of entry for many enteroinvasive pathogens, and both *Y. pseudotuberculosis* (39, 134) and *Y. enterocolitica* (15, 82) have been shown to utilize M cells to breach the epithelial barrier. Following passage through M cells, the pathogenic yersiniae multiply in the PPs. Microscopic examination reveals that PPs can contain extracellular bacterial colonies that are apparently ignored by the phagocytic cells (15, 82, 122, 179, 198). In otherwise healthy human patients, infection of PPs with enteropathogenic yersiniae rarely causes symptoms in areas beyond the intestinal tissues and regional lymph nodes (mesenteric lymphadenitis) (150). Systemic infections have been observed chiefly in immunosuppressed patients and people being treated for iron imbalance (33). In mice and rabbits, yersiniae infections are lethal, and the bacteria spread from PPs via lymphatic vessels to lymph nodes and then further into the major lymphatic organs, where the liver and spleen exhibit a heavy bacterial burden, which finally kills the affected animal (15, 122). Yersiniae are considered to be extracellular throughout the entire infection process, even at late stages in the liver and spleen (198). However, under certain conditions, intracellular spreading cannot be excluded, since it has been shown that *Y. pseudotuberculosis* can multiply in macrophages as the result of active blocking of phagosome acidification (174, 222).
Attachment

Bacterial adhesins play an important role in several steps of the infection process. In support of that conclusion, studies have shown that a *Y. enterocolitica* mutant devoid of the three defined *Yersinia* adhesins Invasin, Ail, and YadA is rapidly eliminated from the host, and it is unable to invade the gut epithelium (179, 224). Invasin is a member of the bacterial intimin/invasin family, which consists of adhesion proteins found on the surface of a variety of enteropathogens (139). Invasin binds to β1-integrins located on the apical surface of M cells and thereby promotes internalization of the bacteria (39, 100, 196). Indeed, an invasin-negative strain has been found to be essentially incapable of reaching PPs (134). By intraperitoneal injection, the initial interaction with M cells that is necessary for uptake of the microbes from the intestine is not required. Therefore, an invasin *Y.*
*pseudotuberculosis* mutant had the same LD50 as the wild-type (wt) *Y. pseudotuberculosis* in an intraperitoneal challenge study (140). After invading PPs, yersiniae seem to require functional YadA to be able to multiply extracellularly in the form of clusters (59). Pepe and colleagues found that a *Y. enterocolitica yadA* mutant was cleared from PPs, thus they considered this strain to be avirulent (167). In contrast, the virulence of *Y. pseudotuberculosis* is not impaired by deletion of *yadA* (24, 81), and *Y. pestis* naturally lacks that gene. It has been demonstrated that YadA from both *Y. pseudotuberculosis* and *Y. enterocolitica* binds collagen (60) and fibronectin (215). Besides functioning in cell adhesion, YadA protects the pathogens from humoral elements of the innate immune system, such as defensins and serum complement lysis (180).

Considering the results discussed above, it is tempting to suggest that Invasin is involved in the initial step of uptake of yersiniae from the gut, but that YadA exerts its (redundant) effect later in this process. However, it has been observed that an *inv/yadA* double mutant was just as efficient as wt *Y. pseudotuberculosis* at colonizing PPs (134). Hence it is apparent that the Inv/β1-integrin pathway is not the only route along which yersiniae can reach PPs.

**Yersinia and the chronic inflammation Reactive Arthritis**

Yersiniae have been found to be associated with reactive arthritis, a clinical manifestation that involves severe joint inflammation in humans (73). In an experimental model of this disease (145), yersiniae were found intracellularly in tissues of the arthritic joint. These bacteria were non-cultivable and in the process of being degraded. Bacterial debris persisted for several weeks and its presence coincided in time with the inflammation. No particular bacterial compound has been identified as the factor that triggers the immune response in this context, although it is known that the higher the virulence potential of a given *Yersinia* strain, the greater it’s potential to cause reactive arthritis (71).
Virulence factors

The ability of yersiniae to utilize PPs as a niche for proliferation requires the inactivation of phagocytes (181). Such inactivation constitutes a crucial virulence trait that is associated with the Type III secreted (T3S) *Yersinia* outer proteins (Yops) (23, 136, 172, 207), which are encoded on a plasmid referred to as the virulence plasmid. Strains that lack the virulence plasmid are rapidly cleared from PPs and are therefore avirulent (121, 179, 201). Specifically, the two Yops designated YopH and YopE are involved in the ability of *Yersinia* to inhibit phagocytosis (181, 182), and YopT and YopO (YpKA in *Y.pseudotuberculosis*) also participate in resistance to such engulfment. It has been demonstrated that these four proteins act synergistically to increase the resistance of *Y. enterocolitica* to phagocytosis by macrophages (78).

**YopH**

YopH is a tyrosine phosphatase that targets signaling pathways that are important for both innate and adaptive immunity (42, 104). The N-terminal end contains a T3S secretion signal, a chaperone-binding site, and a substrate-targeting domain that binds substrate proteins that are phosphorylated on tyrosine moieties (110). The catalytic domain is located in the C terminus, and it shows substantial similarity to eukaryotic protein tyrosine phosphatases (PTPases) (244). The PTP domain also contains an amino acid (aa) stretch that transports YopH to focal complexes (169) and a second substrate-targeting site that binds tyrosine-phosphorylated proteins (101). The virulence function of YopH that has been characterized most extensively is the ability to shut down the signaling pathways of host cells and thereby block their capacity to phagocytose the bacteria (64). One of the early signaling responses to bacterial encounter is triggered by interaction between the bacterial adhesion protein Invasin and a host β1 integrin molecule. This Ca$^{2+}$-mediated signal is central for the factors associated with phagocytosis, such as the oxidative burst and degranulation, and it is efficiently and very rapidly switched off by YopH (13, 14). Several eukaryotic proteins have been identified as targets for YopH (4, 42), many of which are associated with focal complexes where YopH has been shown to accumulate during infection (99, 232). YopH also counteracts other immune response pathways, such as those involving expression of chemoattractant protein 1 (190) and activation of T and B cells (42).
Kerschen and colleagues studied mice challenged intravenously with a *Y. pestis* *yopH* null mutant and found that the bacteria were markedly attenuated in an LD50 assay, and they were eliminated from the spleen within a few days of infection (108). It has also been observed in mice that *Y. pseudotuberculosis* and *Y. enterocolitica* *yopH* mutants are unable to establish systemic infections following oral challenge, and they are rarely found in the liver or spleen (128, 221). In addition, the LD50 of a *yopH* mutant intraperitoneally administrated was 1000-fold decreased compared with wild-type *Y. pseudotuberculosis* (25, 169).

**YopE**

YopE exhibits GAP (GTPase-activating protein) activity towards RhoA, Rac-1, and Cdc42 in vitro (4, 227). A recent study revealed Rac-1 and RhoA as in vivo targets, but the relevance of these enzymatic activities in virulence is unclear, as *yopE* mutants that lack GAP activity toward these targets were just as virulent as the wild-type *Y. pseudotuberculosis* (7). YopE binds to one of the mentioned GTPases and facilitates GTP hydrolysis (4). The GTP turnover leads to conformational changes that weaken binding of the GTPase to downstream components of signaling cascades. The classic phenotype of YopE action is the cytotoxic effect of this virulence factor on HeLa cells, which occurs through disruption of the actin cytoskeleton (183).

Research has provided considerable information about the effects of YopE. For example, it has been found that macrophages and neutrophils were more efficient at engulfing a *Y. enterocolitica* *yopE* mutant than a wt strain (78). Also, YopE is known to contain a membrane localization domain that consists of hydrophobic residues and targets the protein to the perinuclear membrane (114). YopE can counteract both the production of proinflammatory cytokines and phagocytosis (195, 226). In an early study, Straley and colleagues infected mice intravenously with a *Y. pestis* *yopE* null mutant and noted that the bacteria were able to reach the liver and proliferate, but they were cleared after six days (208). The LD50 was $10^4$ times higher with that mutant than with the wt bacteria. Other investigators have found that a YopE mutant of *Y. enterocolitica* spread systemically in mice following oral infection, but the bacteria were cleared from the liver and spleen after 12 days (221). By comparison, it has been reported that a *Y. pseudotuberculosis* *yopE* mutant exhibited defective systemic spreading after oral infection (128, 182). The differences between the *Yersinia* species with regard to the importance of YopE might be explained by the presence and complementary function of YopT in *Y. enterocolitica* and *Y. pestis*. 
YopT

YopT acts as a cysteine protease that inactivates GTPases by removing the lipid modification that is responsible for releasing the GTPase from the membrane (197). When delivered into cultured cells, YopT exerts effects that are similar to those of YopE, that is, it disrupts stress fibers, causes cell rounding, and inhibits phagocytosis. The role of YopT in *Yersinia* pathogenesis is unclear. Nonetheless, it has been observed that cultured macrophages and neutrophils internalized a *Y. enterocolitica yopT* mutant more efficiently than wt *Y. enterocolitica* (78). Moreover, in a mouse oral infection assay, it was found that a *Y. enterocolitica yopT* mutant was just as virulent as its parental strain (221), and YopT is obviously not required for virulence in *Y. pseudotuberculosis*, since it is naturally lacking in that species.

YpkA (YopO)

YpkA in *Y. pseudotuberculosis*, which is called YopO in *Y. enterocolitica* and *Y. pestis*, carries several functional domains. The N-terminal half of the protein contains a serine/threonine kinase catalytic domain (79) that is activated by host factors such as actin when YpkA is translocated into host cells (103). YpkA binds to GDP- or GTP-bound RhoA or Rac-1 with similar affinity. However, this interaction does not affect GDP/GTP exchange mediated by the GTPase, and it does not require YpkA kinase activity (57). Deletion of the last 20 residues prevents actin binding and abolishes actin-induced activation of YpkA (104). YpkA can disrupt the actin cytoskeleton of infected cells, which results in rounding up of the cells and complete disruption of the stress fibers (79, 104). Published findings regarding the importance of YpkA in virulence are conflicting. Galyov and colleagues noted that deletion of either the catalytic domain or the C-terminal half of *ypkA* in *Y. pseudotuberculosis* resulted in avirulence in a mouse oral infection model (72). However, in other studies, *Y. enterocolitica* and *Y. pseudotuberculosis ypkA* mutants were found to be just as efficient as their respective parental strains with respect to their ability to spread systemically and colonize murine liver or spleen (128, 221).
**YopJ**

YopJ (called YopP in *Y. enterocolitica*) is a cysteine protease that inhibits MAPK and NF-κB signaling pathways in host cells by binding MAPK kinases and IKK (5, 159, 192), which in turn suppresses the production of cytokines and induces apoptosis in macrophages (160, 243). It is assumed that YopJ removes ubiquitin modification from -yet unidentified- target proteins in host cells (159). Overexpression of YopJ in cultured cells prevents IKKβ ubiquitination (38). YopJ-induced activation of apoptosis in macrophages requires inhibition of the NF-κB pathway and activation of an apoptotic pathway (187, 208, 243). YopJ can also disrupt activation of the transcription factor CREB via p38 MAPK-mediated signaling and thereby inhibit the activation of a variety of immediate early response genes that are critical for the production of cytokines and growth factors (142). YopJ is not necessary for the virulence of *Y. pestis* in intravenously infected mice (208). It has also been observed that *yopJ* mutants of *Y. pseudotuberculosis* and *Y. enterocolitica* were somewhat attenuated when orally distributed (148, 221). Furthermore, YopJ might be involved in fighting the adaptive immune response, since it has been found to induce apoptosis in antigen-presenting dendritic cells (61).

**YopM**

YopM contains 20-aa leucine-rich repeat motifs, and it is transported to the nucleus via a vesicle-associated pathway (200). In addition, it has been suggested that this protein acts as a transcription regulator (88, 191), although further work is needed to confirm that assumption. A study has shown that YopM forms a complex with two cytoplasmic kinases, RSK1 and PRK2, which are thereby activated (138). In mice, a *Y. enterocolitica* yopM mutant was found to be avirulent after oral challenge (221), and the same was noted for a *Y. pestis* yopM mutant after intravenous challenge (108).

**Yersiniabactin**

Yersiniabactin is a siderophore that allows yersiniae to capture iron ions that are bound to eukaryotic carrier proteins (85). The genes that encode synthesis, transport, and regulation of yersiniabactin are located on the high-pathogenicity island (HPI) (34). *Y. enterocolitica* serotype 1B, *Y. pseudotuberculosis* serotype I and *Y. pestis* are highly virulent, in other
words they are capable of causing systemic infections in humans and are lethal to mice, and they all carry the HPI (35). \textit{Y. enterocolitica} strains mutated in the HPI locus are much less virulent than the wt bacteria when introduced subcutaneously and intragastrically, whereas they are still lethal after intravenous injection (85). Even strains that lack yersiniabactin can behave in a highly virulent manner, if soluble iron or drugs acting as exogenous siderophores are introduced into experimental animals along with the bacteria (119, 178).

\textbf{LipidA}

Lipid A is the hydrophobic anchor of lipopolysaccharide (LPS), thus it represents the toxic part of bacterial endotoxin (175). The process by which LPS is released from bacteria is poorly understood (48). Lipid A molecules are detected by the innate immune system via toll-like receptor 4 (TLR4), which is found on macrophages and endothelial cells (3). Activation of TLR4 by LPS (or lipid A) is mediated by formation of a complex with LPS-binding-protein present in serum. The LPS+LPS-binding-protein complex binds CD14, which in turn activates TLR4 (223) that triggers the release of inflammation mediators such as TNF-\(\alpha\), IL1-\(\beta\), and tissue factor (19, 56) and thereby activates the adaptive immune response (141). These processes are initiated to help the host to clear bacterial infections. However, if they are overly induced—as in the case of massive sepsis—there is a risk of damage to small blood vessels, intravascular coagulation, and organ failure. This severe complication of gram-negative bacterial infections is known as septic shock (27, 214). Certain antibiotics have been found to increase the release of endotoxin, and hence they may cause unexpected side effects when given to cure bacterial infections (90).

\textbf{Superantigen}

Superantigens are bound unprocessed to the major histocompatibility complex (MHC) on the surface of antigen-presenting cells and to the variable region of the \(\beta\) chain of the T cell receptor (135). Upon binding, superantigens stimulate proliferation of lymphocytes and trigger the release of large amounts of cytokines, and they are therefore a major cause of inflammation. \textit{Y. pseudotuberculosis}-derived mitogen (YPM) is a superantigen that is secreted by some strains of this \textit{Yersinia} species (1). In infected mice, a \textit{Y. pseudotuberculosis} strain mutated in \textit{ypm} has been found to exhibit reduced
virulence compared to the wt strain (36), but the mechanism underlying this reduction has not been studied in detail.

**pH6 antigen (Psa) or Mucoid *yersinia* factor (Myf)**

Psa form fine, 4 nm in diameter, fibrillar structures that extend up to 2µm from the surface of the bacterial cell (98, 124). It was suggested that Psa plays a role in virulence, because a *Y. pestis* psaA mutant was found to be attenuated when introduced by the intravenous route (123). Purified PsaA has been demonstrated to bind both the lipoprotein-containing apolipoprotein B found in human plasma (132) and β1-linked galactosyl residues in the glycosphingolipids present in many cell types (166). It has also been suggested that the protein is involved in binding of the bacteria to target cells to ensure effective delivery of Yops (205). Other studies have provided results indicating a direct antiphagocytic role for Psa (93).
Structure of the gram-negative bacterial cell-envelope

Inasmuch as prokaryotic cells lack organelles, all molecular events that dictate their lives take place in extremely local clusters of molecular substrates, enzymes, and cofactors. The few structures that can be considered as anchor points for such clusters include the DNA molecule for transcription, ribosomes for protein synthesis, and—perhaps the most intricate and diverse of all—the cell envelope. All communication with the surroundings must occur through the membranes, and most of the processes that are of interest from the perspective of pathogenesis concern the cell envelope. The envelope of gram-negative bacteria consists of three layers called the inner membrane, the periplasmic space, and the outer membrane (Fig. 2).

The inner membrane is in contact with the cytoplasm, and it is made up of phospholipids organized in a bilayer. Associated and intercalated with the lipids are membrane proteins that are involved in metabolic pathways and the transport of molecules across the membrane. These proteins constitute 60% of the mass of the inner membrane. Outside the inner membrane is the periplasmic space, a compartment that is separated from the cytoplasm and contains the periplasm, which harbors components of several biosynthetic and metabolic pathways and the cell wall. The cell wall of gram-negatives is built up of murein, a peptidoglycan that consists of a single, gigantic approximately 10-nm-thick polymeric molecule that forms a sack around the cytoplasmic membrane. Outside the periplasmic space is the outer
membrane, which, like the cytoplasmic membrane, is a lipid bilayer, although the two layers are not equal in composition. The inner layer consists of phospholipids and thereby resembles the cytoplasmic membrane, whereas the outer layer is primarily composed of LPS. The main protein constituents of the outer membrane are proteins involved in transport across the membrane or are part of surface structures (47, 155)

The gram-negative cell envelope polar lipids; Phospholipids and LPS

![Phospholipids](image)

**Phospholipids** are glycerol-based molecules that have two nonpolar fatty acid chains attached via ester bonds to two of the glycerol OH groups and a phosphate-linked polar group attached to the third. The most abundant polar groups are phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol. Most of the lipid chains are saturated or mono-unsaturated and are 12–18 carbons long (47).

![LPS](image)

**LPS** is made up of a core of polysaccharides to which nonpolar fatty acids (lipid A) and a variable polysaccharide chain (O-antigen) are attached. The region where the lipid binds to the core polysaccharide is negatively charged and attracts divalent cations, especially Mg$^{2+}$, which is important for stabilizing the LPS layer. Lipid A is composed of a backbone disaccharide to which 5–7 saturated fatty acids are attached by ester or amide linkage (176).
Delivery of virulence factors

Bacterial virulence is intimately associated with the ability to secrete virulence factors. Gram-negatives have evolved sophisticated secretion systems to solve the task of transporting proteins across the cell envelope. These systems are classified based on the mechanism of transport and its components, distinguishing between whether the secreted substrates pass the inner membrane via the Sec pathway and carry an amino-terminal signal sequence that is cleaved in the periplasm, or if they are moved directly from the cytosol to the outside without the involvement of a periplasmic intermediate (217). Several categories of Sec-dependent systems have been defined, which represent various strategies for passing proteins through the outer membrane (217). The Sec-independent variants, such as Type I and Type III secretion, include protein complexes that span the entire width of the cell envelope (94, 216).

Type III secretion

Several Yops in the bacterial cytosol are moved across the inner membrane, the periplasmic space, and the outer membrane in the process referred to as Type III secretion (T3S) (188). This designation stems from the underlying mechanism, which differs from the mechanisms of both Sec-dependant secretion and ABC (ATP-binding cassette; Type I) secretion (177) in that it allows secretion of substrates without signal-tag processing by a multi-component protein structure comprising about 25 protein species (45). Many gram-negative bacteria have homologous T3S systems, which are involved both in the secretion of virulence factors and as a component of the flagellar system (94, 102). Because T3S is involved in delivery of virulence factors from pathogenic bacteria into eukaryotic host cells, the concept of type III secretion has, in some cases, been extended to include transport across the plasma membrane of the target cell. The whole process has been conceptualized as an injection in which the T3S structure represents the syringe and needle, thus the term “injectisome” was coined to describe it (43). For the sake of clarity, in the following discussion a distinction is made between T3S, meaning secretion from the bacterial cytosol to outside the bacterial cell, and the next step in the journey of the effector protein, which comprises translocation across the host cell membrane (186, 203).

Electron micrographs of purified T3S organelles from *Salmonella* (111, 115), *Shigella* (21, 22, 210), and *E. coli* (52) have revealed a structure that includes the following elements (102): (i) a central part (the basal body) that
is anchored in the two membranes and spans the periplasmic space; (ii) a thin filament (the needle), which protrudes out from the outer membrane; (iii) components facing the cytoplasm (the bulb). In agreement with the supposed function of the T3S system (T3SS) as a continuous conduit from the bacterial to the host cell cytosol, it has been suggested that an extra subpart is included that functions as an extension of the needle and is involved in cell-cell contact and translocation (51, 149).

Figure 5. The T3S protein-organelle spans the entire bacterial envelope and contains a needle-like structure protruding out from the outer membrane.

The mechanism of T3S

T3S substrates lack a defined consensus secretion signal, although it has been reported that the N terminus is necessary (74, 94). No clear sequence or folding pattern can be seen among the N termini of substrates, and it has been suggested that the signal is an unfolded peptide, possibly with a special pattern of charges (125, 126). The signal has also been suggested to reside in the mRNA structure coding for the N-terminus of the polypeptide (11, 12). In addition, many of the substrates have chaperones whose binding sites are located immediately downstream of the putative secretion signal and they may be responsible for targeting the substrates for secretion (116, 161, 173).
The inner diameter of the needle is only 2–3 nm (41, 53, 89), thus it is too narrow to accommodate the effectors in a folded state; by comparison, the YopE catalytic domain (63) and the YopH catalytic domain (209) have diameters of at least 2.5 and 4 nm, respectively. Accordingly, it has been suggested that an “unfoldase” function is required for this type of secretion to occur (8, 234).

The energy required for secretion is provided both via hydrolysis of ATP by one of the cytoplasmic components (236) and by the proton motive force (233). Some of the proteins that make up the T3S structure (e.g., the OM secretin) contain a cleavable secretion signal and are transported over the inner membrane in a Sec-dependent manner (94), whereas other proteins (e.g., the needle subunit) presumably utilize the “almost complete” T3SS to achieve their own secretion. This process requires that the structural components are secreted in a certain order, and also that a decision is made when structural components should be succeeded by host-interacting substrates. It was proposed that a “substrate switch” is responsible for this function, originally for the flagellar system (87), although this was later found to apply to the virulence factor T3SS as well (58, 147). The essential components of this switch are the inner membrane protein (FlhB in flagella, YscU in *Yersinia ysc-yop* T3SS) and its secreted partner protein (FliK / YscP) (6, 131, 147).

### Translocation into target cells

Together with the virulence factors (effectors), the secretion of another class of proteins called the translocators is also achieved by the T3SS. In short, they are usually two or three proteins that are essential for the passage of the effectors across the target cell membrane and into the lumen of the target cell (74).

The translocators of different species are not generally homologous, although they do share structural elements such as hydrophobic stretches (31). They are also functionally similar in that they have membrane interacting capacity (21, 80, 225) and form pores (49, 50, 153, 194, 211). In many cases, the translocators have been shown not only to assist the effectors in entering the target cell, but also to exhibit intracellular localization and activities themselves, which is exemplified by Ipa B in *Shigella* (86), Esp B in *E. coli* (238), and Sip B and C in *Salmonella* (40). The translocators in *Yersinia* are YopB, YopD, and LcrV. It has been found that YopD itself is translocated (67), although this protein has no known
intra cellular role. It has been shown that LcrV is located at the tip of the T3SS needle prior to actual contact with a target cell (149), and proteins involved in translocation that form extensions of the needle have previously been identified, one example is EspA in *E. coli* (112).

The translocators are conveyed by the T3S system in the same manner as the effectors, but, because they are required for effector entry, they must be in place first. A hot potato in the concept of the “continuous conduit” is how this hierarchy among secreted proteins is achieved (9, 218). Various environmental cues induce secretion without contact with a host cell, which is illustrated by the effects of the hydrophobic compound Congo red on *Shigella* (16, 165) and a low calcium level on *Yersinia* (206). The low calcium response (76), causes in *Yersinia* both a dramatic upregulation of T3S virulence genes and massive secretion of Yops. For unknown reasons, this also causes the bacteria to cease growing. This calcium-dependency (for growth) when a functional T3SS is present has been of tremendous importance as a means to classify mutants of the T3S. Based on this, the Calcium Independent phenotype corresponds to T3S negative strains and the Calcium Blind (or Temperature Sensitive) phenotype are strains that are constitutively upregulated for T3S (168, 206). Whether or not this low calcium response is solely an *in vitro* phenomenon or if low levels of Ca$^{2+}$ represents an *in vivo* signaling cue is uncertain. The secretion of Yersinia Yops can also be made to occur in a sequential manner *in vitro*, because the translocators YopB and YopD respond to some extent to signals other than the effectors (117).

Calcium chelation in growth medium has been found to reduce T3S of the translocators and increase the secretion of the effector Tir in enteropathogenic *E. coli* (EPEC) and diffusely adhering EPEC strains (97, 107). Two proteins involved in sorting translocators from effectors in a particular order have been identified, SepL and Rorf6 (SepD), which differentially regulate the secretion of translocators and effectors in *Citrobacter rodentium* (55) and *E. coli* (54). SepD is needed only for secretion of translocators in *C. rodentium* (55), whereas it is apparently essential for secretion of both translocators and effectors in *E. coli* (157).

In *S. flexneri*, certain translocators, for example IpaB, have been claimed to outcompete effectors for the secretion channel, as demonstrated by the finding that strains with mutant genes for these translocators exhibit deregulated and increased secretion of a number of proteins, many of which are effectors (30, 165). It can not be ruled out, however, that this effect represent a general deregulation of the secretion system. Research has also
revealed the involvement of effector and translocator chaperones in a secretion hierarchy (26). It has been suggested that the *Yersinia* YopH chaperone SycH forms a complex with LcrQ to create a mediator of secretion hierarchy (239).

**Oddities in T3S research**

Even though most of the publications in the field of T3S research do apply to the ideas discussed above, a few studies have presented data that do not fit very well into the existing model(s). For instance, the translocators/effectors IpaB and IpaC produced by *Shigella* trigger uptake of the bacterium by a host cell when they are provided purified from the bacterium (143), that is, they do not have to be temporally and spatially connected to a T3S needle in order to function correctly. Instead, they are located at the surface of the bacterium, and, when the microbe comes in contact with a target cell, they are released from the surface (230). *E. coli* expresses the translocated intimin receptor (Tir), which is transported via the T3SS and is required for close binding of the bacterium to the target cell. It has recently been shown that the translocators EspB and C are not needed for Tir to get access to the cell lumen (146). Similar to Ipas, EspC can enter the target cell membrane in a bacteria-free system (96). Also the *Salmonella* translocators SipB and C are moved to the bacterial surface before contact with the host cell (40, 84).

The *Yersinia* effector YopH is functional inside the target cell already 30 s after onset of the bacterial encounter. According to the injection model, first the translocators (YopB, YopD, and LcrV) travel—in an unfolded form—through the secretion apparatus and fold to form a functional translocation pore in the eukaryotic plasma membrane. Thereafter, YopH must make the same journey, and, within the target cell cytosol, it has to fold and find its target protein(s).

As discussed in greater detail in the results and discussion section of this thesis, it is possible that these discrepancies can be explained by a translocation-competent protein complex that is located at the bacterial surface prior to contact with the eukaryotic cell.
Other translocation mechanisms

**A-B toxins**

A-B toxins are made up of two parts: the catalytically active subunit A, which is associated with the subunit B comprising one or more receptor- and membrane-interacting components. The A-B toxins exploit the normal mechanisms that mammalian cells use to take up materials from the extracellular environment and subsequent intracellular protein trafficking pathways. Some of these proteins, such as diphtheria toxin (118) and botulinum toxin, breach the membrane barrier around acidified endosomes, whereas others, including cholera toxin, *E. coli* heat-labile enterotoxin, Shiga and Shiga-like toxins, and *Pseudomonas* exotoxin A reach the cytosol of the host cell by translocating from the lumen of the endoplasmic reticulum (129). Some toxins enter eukaryotic cells by clathrin-independent endocytosis, as is the case for cholera toxin, which binds to the ganglioside GM₁ and crosses the membrane through cholesterol-rich plasma membrane domains (caveolae) (158).

The receptor-binding, translocation-competent B subunit of the well-studied diphtheria toxin consists of a C-terminal domain that forms two β-sheets that are responsible for receptor binding, and a second domain that contains hydrophobic helices that facilitate the membrane translocation step. Upon contact with the low pH in an endosome, diphtheria toxin undergoes a conformational change (219, 220). That alteration exposes the hydrophobic helices and thereby allows them to insert into the endosomal membrane, where they form a protein channel that functions as a translocation pore for the partially unfolded A fragment (242).

**Formation and functions of outer membrane vesicles**

Several species of gram-negative bacteria release material from their outer membrane in the form of vesicles (reviewed in (137, 245)). The mechanism responsible for generation of these outer membrane vesicles (OMVs) is not well known. Nonetheless, it has been suggested that lytic transglycosylases excise bits of the cell wall and liberate them from the peptidoglycan, and the accumulation of released muramyl peptides in the periplasmic space creates turgor pressure on the outer membrane, which triggers the blebbing that creates the OMVs (245).
It has been demonstrated that OMVs are used by the bacterium to reduce levels of toxic compounds and attacking phages (113, 127), and also as vehicles for delivery of bacterial molecules into foreign cells (20, 105). The mechanism for the entry of OMVs or its components into the target cell remain an intriguing challenge to reveal, but in a study by Kadurugamuwa and colleagues, LPS from OMVs were found integrated into the target bacterial cell envelope in a manner that likely requires some type of membrane fusion event (106). OMVs have been shown to mediate the delivery of several \textit{E. coli} toxins. This is illustrated by the pore-forming toxin ClyA, which is secreted over the inner membrane via an unknown mechanism and is packed from the periplasm into OMVs, where it shows higher specific toxicity than it does when prepared directly from the periplasm, possibly because it is in an oligomerized state when associated with the vesicles (228). For some \textit{E. coli} strains, a correlation has also been reported between secretion of heat-labile toxin (LT) and release of small vesicles (229). In the cited study, FITC-labeled LT was found to be internalized in cells by an endocytic mechanism that involved \textit{G}_{\text{ml}}\text{rafts and lipid rafts in the target cell} (109). In this case, the toxin was secreted across the outer membrane and was observed to be associated with vesicles in the extracellular milieu via binding to Kdo in LPS (91, 92). The T1S \alpha-hemolysin has also been detected in association with OMVs (17).
AIMS OF THIS THESIS

The aim of this thesis was to determine the molecular mechanisms involved in translocation of T3S effector proteins by *Yersinia pseudotuberculosis*.

RESULTS AND DISCUSSION

The responsibility for mediating the transport of *Yersinia* T3S effector proteins across the plasma membrane of the target cell has been attributed to the three translocator proteins LcrV, YopB, and YopD (67, 80, 133, 170, 186, 203), which are encoded by the same operon and are indispensable for functional translocation. Homologous translocator proteins based on identical genetic organization can be found in the T3SS of *Photorabdus* (231), *Aeromonas* (241), *Pseudomonas aeruginosa* (240), and *Vibrio parahemolyticus* (163). Generally speaking, translocator proteins in the T3SS of many other bacteria do not show a high degree of homology, although they are similar with regard to some physical properties, such as hydrophobic stretches and membrane-interacting capacity (for a review, see (31)). Interestingly, the translocators are not very specific for the particular effectors they naturally dispatch, as effectors from one species can be efficiently handled by translocators from another (185).

In addition to the presumed structural functions of YopD and LcrV as building blocks in a gateway into the target cell, both these proteins act in regulatory circuits that determine the expression of virulence factors: LcrV is needed for upregulation (18, 173) and YopD for downregulation (235). These regulatory aspects complicated the present investigation of the structural properties of these proteins, and hence the subsequent studies (papers I and II) were focused on finding ways to circumvent these problems.

LcrV and the translocation pore

It has been reported that *Pseudomonas aeruginosa* PcrV can complement LcrV in *Y. pseudotuberculosis* (170). However, the experiments described in paper I showed that a *Yersinia lcrV* mutant trans-complemented with *pcrV* was less efficient than the wt strain at translocating effector proteins. In that study, LcrV-PcrV hybrid proteins with wt regulatory properties were expressed in a *Yersinia lcrV* background, and the translocation efficiency was found to depend on a central region in the proteins. When that region was of *lcrV* origin, the hybrid protein complemented an *lcrV* mutant to wt
levels, but when it was of \textit{pcrV} origin, the effect was similar to complementation with \textit{pcrV}. The pore-forming properties of strains expressing hybrid proteins were scored as their ability to lyse erythrocytes. The sizes of pores can be estimated by adding extracellular carbohydrates of various dimensions that can act as osmoprotectants if they are too big to pass through membrane pores. Erythrocytes contain larger pores if they are infected with \textit{Yersinia} strains expressing LcrV or LcrV-like hybrids than if they contain strains generating PcrV or PcrV-like hybrids. Surprisingly, when purified LcrV, PcrV, and hybrid proteins were applied to a synthetic lipid bilayer, they were able to form well-defined membrane channels. In this purified system, the channels created by LcrV/LcrV-like molecules showed greater conductivity than those formed by PcrV/PcrV-like proteins. Thus LcrV has intrinsic membrane activity that can lead to channel formation even in the absence of its presumed partners YopB and YopD. LcrV purified by two separate techniques gave rise to pores with the same properties. By comparison, Bröms and colleagues have reported that the pore-size determining functions of LcrV and PcrV depend on the nature of their partners (29). More precisely, PcrV forms small pores only when it is associated with YopB and YopD; when it is coupled with its natural partners PopB and PopD, the pores are of the same size as those created by LcrV cooperating with YopB and YopD. In another study (77), it was found that PcrV is required for insertion of PopB and PopD into membranes but not for pore-formation \textit{per se}. The observation that LcrV can form channels in an artificial lipid bilayer is remarkable, because this protein does not contain any obvious membrane-interacting regions, and, unlike YopB and YopD, it has not been found to be associated with liposomes or purified membranes (153, 211). The channels made by LcrV do presumably not represent the functional translocation pore, but might have a role in a signaling event. LcrV is surface located prior to cell contact (170) as an extension of the T3S needle (149) and one way of the bacteria to sense cell contact could involve recognition of the low Ca$^{2+}$ condition in the target cell lumen via a channel made by LcrV. The LcrV channel may in addition serve as a foundation for the translocation pore to which YopB and YopD attach and make up a functional translocation unit.

The translocation competence of YopD requires more than pore formation

YopD has been shown to do the following: associate with and create channels in lipid bilayers (211); form a complex with LcrV and YopB (154, 189); interact with the translocated effector protein YopE (83); participate in regulatory processes that govern the expression of virulence determinants
Clearly, it is no overstatement to say that YopD is the *Yersinia* T3S protein that has the most diverse spectrum of functions. In the investigation reported in paper II, a series of *yopD* variants were generated in which 20-aa stretches were deleted throughout the length of YopD, and these variants were assessed regarding their ability to complement a full-length *yopD* mutant. Based on the results, it was possible to link certain regions in YopD to some of the above-mentioned functions. The areas of the molecule that enable pore formation and effector protein translocation were found to overlap each other. Despite that, almost the entire protein was required for translocation, whereas larger parts of the molecule were dispensable for pore formation. This finding of the separateness of these two functions is interesting, because it implies that, besides being a structural component of the pore, YopD plays a more active role in the translocation process. The reported interaction with YopE is intriguing in this respect, if, for example, such interplay is rendered impossible in translocation-negative but pore-formation-positive YopD variants. However, the concept that an active relationship between YopD and all the translocated effectors is necessary for translocation is not supported by experimental data, and it does not easily comply with the interchangeability of T3S substrates between species. YopD located at the surface of bacterial cells can be labeled with biotin via amino-reactive reagents (Olsson, J., unpublished). Together with the data presented in paper III concerning the translocation competent Yop-lipid complexes (YLC) and the accessibility of YopD for immunolabeling, it is tantalizing to think of YopD as a surface exposed component of the YLC ready to dock with its partner LcrV at the site of translocation.

There is no hierarchy in the secretion of translocators and effectors *in vitro* by an *lcrQ* mutant

The theory of translocation occurring through a pore created by a set of proteins specifically designated for this task includes the assumption of a hierarchy of secretion: the translocators must be allowed to form the pore before any effector proteins can pass through it. Models comprising the idea of sequential secretion have been suggested by several investigators (9, 44, 117, 218, 239). Moreover, a number of studies have addressed the question of secretion cues and a secretion hierarchy in *Yersinia*. For example, it is known that a low-calcium environment promotes secretion of Yops in a non-selective way. As an explanation for that effect it has been suggested that the lack of Ca\(^{2+}\) destabilizes the secretion structure so that even components that normally belong to the T3S core structure are instead released into the surrounding medium and are thus considered to be secreted.
Free amino acids, albumin, and a low calcium level have also been proposed to stimulate sequential steps in secretion (117). Mutational analyses have revealed that chaperones of the secreted substrates cooperate with LcrQ to regulate the secretion (32, 239).

LcrQ is indeed one of the key regulatory elements, because it represses the ysc/yop T3S system when it is overexpressed or not allowed to be released through a functional T3SS (171). Deletion of lcrQ leads to high expression of Yop proteins that is not induced by an external signal. Yet, no secretion can occur without a functional T3SS. We utilized this knowledge to produce a Yersinia strain with mutations in both lcrQ and yscF, the latter encoding an essential constituent of the T3SS. More specifically, YscF is the core component of the filamentous appendage that protrudes from the outer membrane (the needle) (89) and it is required for secretion, even in vitro. By trans-complementing the double lcrQ/yscF mutant strain with a plasmid containing yscF under the controllable arabinose-induced araBAD promoter, it is possible to regulate secretion of pre-made virulence proteins, because that process is initiated when the final component of T3SS (YscF) is expressed.

In the present experiments, bacteria were grown in LB medium or LB supplemented with 5 mM EGTA for 2 h at 37 °C, after which 0.02% arabinose was added (time point 0). Secreted proteins were precipitated from culture supernatants at various time points after induction by adding TCA, and they were subsequently analyzed by SDS-PAGE and Coomassie brilliant blue staining (Fig. 6A) or by immunoblotting (Fig. 6B and C). Precipitated proteins from bacteria grown in medium with EGTA (−Ca2+) exhibited the typical Yop profile 70 min after yscF expression, although detectable bands of several Yops could be seen 30 min after induction (Fig. 6A). Western blotting detected Yop D, YopE, and YopN as early as 10 min after addition of arabinose (Fig. 6B and C). Subsequently, the amounts accumulated in Ca2+-containing medium increased up to 40–60 minutes after induction, after which a plateau was reached (Fig. 6C). When the bacteria were grown in Ca2+-depleted medium, the secretion did not level off, but instead the rate increased dramatically to levels clearly detectable in a stained gel after 70 minutes (Fig. 6A). Inasmuch as YopD is a translocator, it did not differ from the translocated effector protein YopE with respect to secretion dynamics (Fig. 6B and C). Interestingly, proteins can be detected just as early, or even earlier, in +Ca2+ medium than in −Ca2+ medium. In this de-repressed experimental setup, the effect of −Ca2+ medium to heavily induce secretion seems to kick in as late as 70 minutes after secretion is allowed to occur. Low levels of LcrV were detected even without the addition of arabinose. This phenomenon involving secretion of LcrV despite a nonfunctional ysc/yop T3SS has also been described by Fields and Straley,
who proposed that it might be due to an alternative route of secretion for LcrV (65). Based on our findings, we concluded that, in an lcrQ background, there was no hierarchy in the secretion of translocators and effectors. This may agree with the proposed role for LcrQ in regulating the order of secretion. However, preliminary experiments introducing plcRF in an yscF strain have revealed a similar pattern of secretion, that is, the translocators were not secreted before the effectors (data not shown). In such a setup, secretion of the proteins is to a larger extent defined by their expression that is also triggered as the T3S apparatus is completed, which make studies of the early secretion events more difficult.

Figure 6. Proteins secreted by a Y. pseudotuberculosis yscF/lcrQ pyscF+ strain 0-80 minutes after induction of yscF. A heavy increase of secretion in a –Ca²⁺ medium can be seen 70 minutes after induction (A). Secretion of both effectors and translocators start as the expression of pyscF+ is induced, with no obvious hierarchy, neither in –Ca²⁺ (B), nor in +Ca²⁺ (C) medium.
Surface-located Yops associate with free lipids and are then released from the bacteria

The results of the controlled onset of secretion described above indicated that both translocator proteins (e.g. YopD) and translocated proteins (e.g. YopE) are secreted simultaneously, and that the early secretion is efficient in Ca$^{2+}$-containing medium. An electron microscopic study of the surface of wt bacteria revealed significant amounts of YopE and YopD on the surface of the bacterial envelope (paper III). In addition, spherical structures were found associated with the bacterial surface and released into the growth medium. When material was precipitated from the medium and purified in a density gradient, secreted Yops copurified with the spherical structures. The Yop-containing fraction was found to include free acyl chains with lengths of 14–18 carbons, along with the effectors YopH and YopE, the translocators LcrV and YopD, the outer-membrane-localized porin OmpA, and the adhesion protein Ail. Despite the differences in their hydrophobic properties, these protein species all accumulated together with the free lipids at a density of 1.08 g/ml, which is distinct from the densities of both the inner membrane (1.14 g/ml) and the outer membrane (1.22 g/ml), most likely due to the presence of the free acyl chains. This accumulated material was denoted Yop-lipid complex (YLC).

Purified YLC can translocate effector proteins in a bacteria-free system

It has been difficult to detect activity of Yersinia effectors that have been purified from the bacteria, even in the presence of the translocators. However, considering YopE as an example, the activity is retained when the protein is purified and in a soluble form, as indicated by its ability to induce the cytotoxic response when microinjected into a target cell (184). In fact, the need for a bacterium to be in close contact with the target cell in order to ensure functional translocation is one of the cornerstones in the hypothesis of a continuous conduit between the bacterial cytoplasm to the cytoplasm of the target cell. The findings that both translocator and effector Yops are located near the surface of the bacteria and that they are copurified in a lipid complex prompted us to assess the purified YLC in a bacteria-free translocation assay (paper III). Since the effect of translocation of YopE on its eukaryotic target cells is transient, we adapted an assay system based on translocation of the Pseudomonas aeruginosa effector exoenzyme S (ExoS),
a protein that irreversibly modifies intracellular targets such as Ras when translocated into host cells. We transformed plasmid pTS103 (68) expressing \textit{exoS} from its native promoter into the \textit{Yersinia yopE} mutant YPIII(pIB525) and the isogenic translocation-deficient mutant YPIII(pIB525/621) (\textit{yopE, yopD}). YopE-defective backgrounds were chosen to avoid complications that might arise due to the similar cytotoxicity of YopE and ExoS (68). YLC was added to K562 cells growing in suspension (130). Since translocation of ExoS result in cell death (68), the relative number of dead cells was first determined by FACS analysis of propidium iodide uptake. The results showed that cell death increased significantly in proportion to the concentration of YLC prepared from the translocation-competent strain that was added to the culture. In contrast, similar concentrations of YLC derived from the isogenic \textit{yopD} mutant did not induce cell death beyond the background levels. Importantly, the extent of Ras modification paralleled the cell death induced by YLC obtained from the translocation-competent strain, whereas YLC derived from the translocation-defective \textit{yopD} mutant did not modify Ras. This observation demonstrates that ExoS was indeed translocated after addition of YCF and that YopD was needed for the process.

The translocation of T3S substrates by \textit{Yersinia} was put in a new light by the findings that effectors and translocators are transported to the bacterial surface prior to contact with a target cell, and that translocation can be achieved by a protein-lipid complex. T3S translocators/effectors expressed in other species have been successfully purified in an active form that can, for instance, induce uptake of bacteria (143) or cause lysis (96), and the same has been demonstrated for bacterial toxins secreted by mechanisms other than T3S (75, 129, 144). A mechanism in which the T3SS is used to transport substrates to the bacterial surface for subsequent release and translocation into a target cell would allow a bacterium to exhibit a quick and versatile response upon coming in contact with a potential host cell. Furthermore, compared to the continuous conduit model, it would require a lower degree of hierarchy among the secreted substrates, since the translocators and effectors travel as complexes. The presence of free fatty acids in the YLC was completely unexpected, and the role of these compounds remains to be investigated. Free fatty acids are released when phospholipids are processed by phospholipase A (202). A virulence-associated protein (designated PldA) that displays phospholipase A activity has been identified in \textit{Yersinia} (193). Further work is needed to determine whether there is a connection between the formation or release of YLC and the activity of PldA.
Concluding remarks

The model indicating that T3S of effector proteins into a target cell occurs via a needle structure in the bacterium and a pore in the plasma membrane of the host cell is based on information that has been accumulated over a number of years. Much inspiration has come from the flagellar T3SS, in which the axial proteins are moved through a narrow channel (3 nm in diameter) in the growing filament to finally reach and consolidated into the distal end of that structure (151). The same applies to secreted proteins, which reach the distal end of the virulence-associated needle, as has been demonstrated in elegant studies of *Pseudomonas syringae* (120) and *E. coli* (46). In *E. coli*, the protein EspA is added at the tip of the needle to extend the hollow conduit (46). Although these results provide strong support for the conduit model, other interpretations are also possible. Albeit highly speculative, it is plausible that the needles and their extensions primarily play a role as bacterial sensors of contact with host cells. Such a function would also be compatible with the finding that it is necessary for the needle to extend beyond LPS structures on the bacterial surface (6). If that is the case, some of the proteins regarded as translocators may function primarily as sensory molecules for structures on the surface of a target cell. Indeed, this type of operation is supported by the detection of LcrV at the tip of the needle (149), as well as the reported role of this protein as a ligand for Toll-like receptor 2 (TLR2) (199). This function might induce a signal that is relayed through the needle structure to a site on the bacterial surface where translocator/effecter complexes are released and actually make contact with the target cell, and are subsequently either inserted into the plasma membrane or endocytosed.

Pores formed by the translocator proteins would allow the effectors to gain access to the target cell lumen through either the plasma membrane, or the membrane of endosomes. Beside their pore-forming properties, the translocators are, because of their hydrophobic regions, candidates for being involved in lipid interactions also in the YLCs. In fact, a *yopB* mutant strain exhibited an altered morphology of the surface material when studied in AFM. Instead of the spherically shaped material seen surrounding the wt bacteria, the material released by a *yopB* mutant had the appearance of a 5-10 nm thick sheet (Olsson, J., unpublished). The truncated YopD, studied in paper II that formed pores, but nevertheless did not promote translocation of YopE, may also be defective in interactions with proteins or lipids in the YLC. Further studies are, however, required to prove these theories.
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