Type III Secretion System of Phytopathogenic Bacterium *Pseudomonas syringae*: From Gene to Function

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Academic Dissertation

To be presented, with the permission of the Faculty of Biosciences of the University of Helsinki, for public criticism in auditorium 2 at Viikki Infocenter Korona (Viikinkaari 11, Helsinki) on March 28th, 2007, at 12 o'clock noon.

Helsinki 2007

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Cover illustration: Bacterial type III secretion apparatus.

ISSN 1795-7079
ISBN 978-952-10-3783-2
ISBN 978-952-10-3784-9 (PDF)

Contents

ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by the respective Roman numerals.

I Li, C.M., Brown, I., Mansfield, J., Stevens, C., Boureau, T., Romantschuk, M. and Taira, S. (2002) The Hrp pilus of *Pseudomonas syringae* elongates from its tip and acts as a conduit for translocation of the effector protein HrpZ. *EMBO J*., 21, 1909-1915.

II Li, C.M., Hienonen, E., Haapalainen, M., Kontinen, V.P., Romantschuk, M. and Taira, S. (2007) Type III secretion system-associated pilus of *Pseudomonas syringae* as an epitope display tool. *FEMS Microbiol Lett*., 269, 104-9.

III Li, C.M., Haapalainen, M., Lee, J., Nurnberger, T., Romantschuk, M. and Taira, S. (2005) Harpin of *Pseudomonas syringae* pv. *phaseolicola* harbors a protein binding site. *Mol. Plant Microbe Interact*., 18, 60-66.

ABBREVIATIONS

ABC	ATP-binding cassette
Amp	ampcilin
cv.	cultivar
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
GSP	general secretion pathway
HR	hypersensitive reaction
Hrc	hypersensitive reaction and pathogenesis conserved
Hrp	hypersensitive reaction and pathogenesis
IM	inner (cytoplasmic) membrane
JA	jasmonic acid
kb	kilo base pairs
KB	King's medium B
Km	kanamycin
LB	Luria-Bertani broth (LB broth) medium
MAMPs	microbe-associated molecular patterns
NB-LRR	nucleotide binding, leucine-rich repeat containing protein
OM	outer membrane
PAI	pathogenicity island
PCD	programmed cell death
PP	periplasm
Pph.	*Pseudomonas syringae* pv. *phaseolicola*
Pst	*Pseudomonas syringae* pv. *tomato*
Pss	*Pseudomonas syringae* pv. *syringae*
pv.	pathovar
R	resistance (gene or protein)
Rif	rifampicin
RT	room temperature
SA	salicylic acid
SAR	system acquired resistance
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
spp.	subspecies

T1SS	type I secretion system
T2SS	type II secretion system
T3SS	type III secretion system
T4SS	type IV secretion system
T5SS	type V secretion system
Tat	twin-arginine translocation
Tc	tetracycline
wt	wild type
Ysc	*Yersinia* secretion
Yops	*Yersinia* outer proteins

SUMMARY

The type III secretion system (T3SS) is an essential requirement for the virulence of many Gram-negative bacteria which infect plants, animals and mankind. Pathogens use the T3SS to deliver effector proteins from the bacterial cytoplasm to the eukaryotic host cells, where the effectors subvert host defenses. The best candidates for directing effector protein traffic are the bacterial type III-associated appendages, called needles or pili.

In plant pathogenic bacteria, the best characterized example of a T3SS-associated appendage is the HrpA pilus of the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000. The components of the T3SS in plant pathogens are encoded by a cluster of *hrp* (hypersensitive reaction and pathogenicity) genes. Two major classes of T3SS-secreted proteins are: harpin proteins such as HrpZ which are exported into extracellular space, and avirulence (Avr) proteins such as AvrPto which are translocated directly to the plant cytoplasm.

This study deals with the structural and functional characterization of the T3SS-associated HrpA pilus and the T3SS-secreted harpins. By insertional mutagenesis analysis of HrpA, we located the optimal epitope insertion site in the amino-terminus of HrpA, and revealed the potential application of the HrpA pilus as a carrier of antigenic determinants for vaccination. By pulse-expression of proteins combined with immuno-electron microscopy, we discovered the Hrp pilus assembly strategy as addition of HrpA subunits to the distal end of the growing pilus, and we showed for the first time that secretion of HrpZ occurs at the tip of the pilus. The pilus thus functions as a conduit delivering proteins to the extracellular milieu. By using phage-display and scanning-insertion mutagenesis methods we identified a conserved HrpZ-binding peptide and localized the peptide-binding site to the central domain of HrpZ. We also found that the HrpZ specifically interacts with a host bean protein. Taken together, the current results provide deeper insight into the molecular mechanism of T3SS-associated pilus assembly and effector protein translocation, which will be helpful for further studies on the pathogenic mechanisms of Gram-negative bacteria and for developing new strategies to prevent bacterial infection.

A. INTRODUCTION

In Gram-negative bacteria, the cell envelope consists of an inner membrane (IM), periplasm, cell wall, and an outer membrane (OM). Often the transport of water and small nutrient molecules between the cytoplasm and extracellular milieu is relatively easy due to the semi-permeable membrane, but the transport of large proteins such as toxins and enzymes are much more complex. However, the translocation of protein across biological membranes is a fundamental part of cellular life. The secretion of proteins from the cytoplasm through the IM, and in some cases through the OM to the extracellular space, requires dedicated machineries, which are classically divided into five categories: type I, II, III, IV, and V secretion pathway (Thanassi & Hultgren, 2000; Table 1). The pathways differ from each other mainly by the presence or absence of a signal peptide on the secreted protein and by the characteristics of different translocation steps (reviewed by Fath & Kolter, 1993; Pugsley, 1993; Salmond & Reeves, 1993). Protein secretion across the two membranes takes place either in one continuous step (for type I and type III protein secretion pathways, and for T-DNA transfer of type IV secretion pathway), or in two separate steps (for type II pathway and pertussis toxin secretion of type IV pathway). In the two-step secretion pathways, transportation of protein across the IM and OM requires separate protein machineries, and periplasmic intermediates occur between the two protein translocation steps (reviewed by Henderson *et al*., 2004; Wandersman, 1996).

Transportation of the precursor protein from cytoplasm to or across the IM is often referred to as protein export. In the literature, the term “export” also often refers to protein secretion. In this thesis, “protein secretion” denotes the process where protein is translocated across the OM to the extracellular space in Gram-negative bacteria.

1. Protein export across the bacterial inner membrane

Prokaryotes contain two parallel pathways for the export of proteins across the cytoplasmic membrane, the Sec-dependent pathway and the Tat (twin-arginine translocation) pathway.

1.1. Sec-dependent protein export pathway

The Sec pathway is found in prokaryotes, archaea and eukaryotes, and it is needed for the translocation of proteins across the cytoplasmic membrane or endoplasmic reticulum (ER) membrane. The amino terminal signal peptide of the preprotein is about 20-30 amino acids long and composed of mainly hydrophobic amino acid residues. The core elements of the Sec system, composed of SecYEG (Sec61αβγ in the case of archaea and eukaryotic organelles), are well conserved and are likely to have functionally equivalent roles in the translocation process. In *Escherichia coli*, during or shortly after synthesis, the chaperone SecB binds the pre-protein, maintains it in a translocation-competent state, and targets it to the SecYEG-bound SecA located in cytoplasmic membrane. Subsequently, the pre-protein is translocated through the SecYEG pore using the energy from ATP hydrolysis and the proton-motive force which drives the pre-protein into and across the membrane. Signal peptide of the preprotein is cleaved on the periplasmic side by leader peptidase, encoded by LepB in *E. coli*, releasing the mature protein from the Sec machinery to periplasmic space or integrated in cytoplasmic membrane (reviewed by Economou, 1999; de Keyzer *et al*., 2003; Mori & Ito, 2001; Pugsley, 1993; Rusch & Kendall, 2006; Stephenson, 2005).

1.2. Tat pathway for transporting folded proteins

The Tat pathway is found both in eukaryotes for proteins transported across the chloroplast thylakoid membrane (Settles *et al*., 1997) and in many bacteria such as *E. coli* and *Pseudomonas aeruginosa* for proteins translocated into or across the IM (Berks *et al*., 2000; Thomas *et al*., 2001; Voulhoux *et al*., 2001; Wu *et al*., 2000). This pathway differs from the Sec pathway in terms of its remarkable ability to transport fully folded proteins, and the amino-terminal signal peptides of the Tat-dependent proteins possess a SRRxFLK consensus motif. In *E. coli*, the simplest Tat system consists of three protein components: TatA, TatB, and TatC (Sargent *et al*., 1998). During the export process, Tat secretion substrates are initially bound by the TatBC complex while the TatC binds to the consensus motif of the signal peptide. The TatBC-substrate complex then moves to the TatA channel and associates with it. By utilizing the proton-motive force, the substrate is transported to the periplasm and the

signal peptide is cleaved off by the signal peptidase in the periplasm.

The coexistence of Tat and Sec export pathways as well as different secretion pathways in one organism has been found in many bacterial systems. Andre Filloux' group reported for the first time that in *P. aeruginosa* the secretion of phospholipase C is Tat-dependent while the secretion of exotoxin A is Sec-dependent (Filloux *et al*., 1998, Voulhoux *et al*., 2001). The virulence contribution of the Tat pathway in the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000, has also been reported (Bronstein *et al*., 2005).

2. Protein secretion pathways in Gram-negative bacteria

As mentioned previously, there are at least five different secretion systems present in Gram-negative bacteria (Figure 1 & Figure 2), each of them will be introduced briefly below, except the type III secretion system (T3SS). Since T3SS is the main focus of current study, it will be described in more detail in Section 2.6. Pathogenic bacteria use various virulence factors to invade and survive in their host, and may eventually destroy the host cell. Hence, extracellular secretion of proteins is often a major virulence mechanism in bacterial infection (Table 1).

2.1. Type I protein secretion pathway

One of the strategies used by Gram-negative bacteria to secrete proteins across the entire envelope is the type I secretion system (T1SS), also known as the ATP-binding cassette (ABC) transporter. *E. coli* α-hemolysin (HlyA) was the first protein shown to be secreted through the T1SS (Goebel *et al*., 1982). The HlyA is a toxin protein interacting with the target cell and causing pore-formation in the host cell plasma membrane. Another example is the antibacterial toxin colicin V encoded by the *cvaC* gene, which is a small substrate with 103 residues and secreted via the CvaAB/TolC machinery in *E. coli* (Gilson *et al*., 1990). The largest known protein secreted through the T1SS is the *Pseudomonas fluorescence* LapA, containing more than 8000 amino acid residues. LapA is known to be involved in adhesion and biofilm formation (Hinsa *et al*., 2003). The secretion of other proteins, such as metalloproteases (*Erwinia chrysanthemi, P. aeruginosa* and *Serratia marcescens* proteases), haemophores (*Serratia marcescens* HasA and *P. aeruginosa* HasAp haemoproteins), lipases (*S. marcescens* LipA) and S-layer proteins, are all T1SS-dependent. Although

these proteins vary greatly in size, from 78 to 8682 residues, they are generally very acidic and often enzymatic or toxic to host cells (Delepelaire, 2004). Most proteins are rich in the glycine-repeated motif GGXGXD close to the carboxyl terminus. The secretion signal is typically located within the last 50-60 residues of carboxyl terminus of the secreted protein and is not cleaved as they cross the membrane. The three-dimensional structure of the metalloprotease of *P. aeruginosa* shows that the repeats form a 'parallel beta roll' structure that is believed to facilitate the passage of the secreted protein (Baumann *et al*., 1993).

The T1SS machinery is composed of three membrane proteins (Figure 1), which are all required for secretion (reviewed by Wandersman, 1996). The first is an inner membrane ATP-binding cassette (ABC) protein which typically comprises a nucleotide-binding domain and a transmembrane domain. The ABC protein restricts the substrate binding and ensures that only T1SS specific substrates are recognized via their secretion signal. The second protein is a membrane fusion protein which contains a short cytoplasmic domain, an inner membrane anchor, and a large periplasmic domain. It is believed that the membrane fusion protein links the inner membrane and outer membrane components of the T1SS and is responsible for the binding of the substrate on the cytoplasmic side. The third protein is a pore forming outer membrane protein. The T1SS machinery spans the entire cell envelope and the protein is secreted from cytoplasm directly to extracellular medium in a single step. Proteins secreted by the ABC transporter lack the N-terminal cleavable signal sequences typical for proteins exported by Sec system (Delepelaire & Wandersman 1990; Ghigo & Wandersman 1994; Mackman *et al*., 1986; Pugsley, 1993).

Table 1. Comparison of the protein secretion pathways in gram-negative bacteria (Modified from Salmond & Reeves, 1993; Thanassi *et al*., 1998; and Henderson *et al*., 2004)

Pathway / Character	Type I	Type II	Type III	Type IV	Type V		Chaperone/usher
					AT	TPS	
Sec-dependent	no	yes (or Tat-dependent)	no	yes (*pertussis* toxin) /no (all others)	yes	yes	yes
Amino-terminal signal sequence	no	yes	Yes (non-cleavable)	Yes / no	yes	yes	yes
Number of genes encoding secretion system	3	12-16	>20	⩾11	1	2	2
Contact-dependent secretion	no	no	yes	no	no	no	no
Surface appendage (Prototype)	no	Type IV pili	Hrp pili Needle complex	T pilus F pilus	no	no	P pili Type 1 pili
Secretion Representative	HlyA	PulA Exotoxin (ETA) Phospholipase (Plc)	HrpZ Avr proteins	Pertussis toxin (Ptx) T-DNA	IgAp	ShlA FHA	PapD/PapC FimC/FimD
Location of Secretion-system proteins	IM, OM	CP, IM, OM	CP, IM, OM	CP, IM, OM	PP, OM	PP OM	PP OM
ATP-dependent in translocation across the OM	yes	yes	yes	yes	no	no	no

AT: autotransporter; **TPS**: two-partner secretion; **CP**: cytoplasm; **IM**: inner membrane; **OM**: outer membrane; **PP**: periplasm; **IgAp:** the neisserial IgA1 protease**; ShlA**: cytolysin of *S. marcescens*; **FHA:** filamentous hemagglutinin of *B. pertussis*.

2.2. Type II protein secretion pathway

Type II protein secretion system (T2SS) is widely distributed among most Gram-negative bacteria for secretion of extracellular degradative enzymes and toxins (reviewed by Johnson *et al*., 2006). Before the discovery of existence of Tat export system, it was regarded as the main branch of the general secretion pathway (GSP) as an extension of the Sec export pathway. Secretion of proteins through T2SS occurs in two separate steps. In the first step, the protein precursors are exported through cytoplasmic membrane to the periplasm using either the Sec-dependent pathway or Tat pathway, depending on the nature of the signal peptide. As discussed in Section 1.1 and 1.2, the signal sequence is cleaved off by a periplasmic signal peptidase when it reaches the periplasm and the mature protein is released. In the second step, the proteins are secreted from the periplasm through the outer membrane to the extracellular space using T2SS apparatus, which are encoded by approximately 12 to 16 genes (Filloux, 2004; Sandkvist, 2001). Based on experimental data with *P. aeruginosa* T2SS component proteins (Bally *et al*., 1992; Voulhoux *et al*., 2001), a type II secretion apparatus model formed by the multi-protein complex that spans the entire cell envelope has been proposed and shown in Figure 1.

T2SS was first discovered in *Klebsiella pneumoniae*, where it was found to be required for secretion of lipoprotein pollulanse (d' Enfert, *et al*., 1987). The conservation of T2SS was then identified in many Gram-negative bacteria such as *E. coli*, *Erwinia carotovora*, *Yesiniae entrocolitica*, and so on (reviewed by Filloux, 2004). The genes encode type II secreton components are usually clustered (reviewed by Pugsley, 1993). So far the precise functions of individual component proteins are not very well characterized. It is clear that mutations in most of the T2SS component genes result in abortion of the secretion process and accumulation of the protein in the periplasm (Andro *et al*., 1984). The T2SS apparatus spans entire cell envelop without extracellular filamentous appendage, the only exception is the type IV pilus as indicated in Table 1.

Various experimental approaches have been used for studying the secretion signal of the protein secreted through T2SS, and it turns out that the multiple sites in the T2SS-secreted proteins are, instead of being treated as secretion signal, needed for recognition of and targeting to the T2SS apparatus (reviewed by Palomäki, 2003).

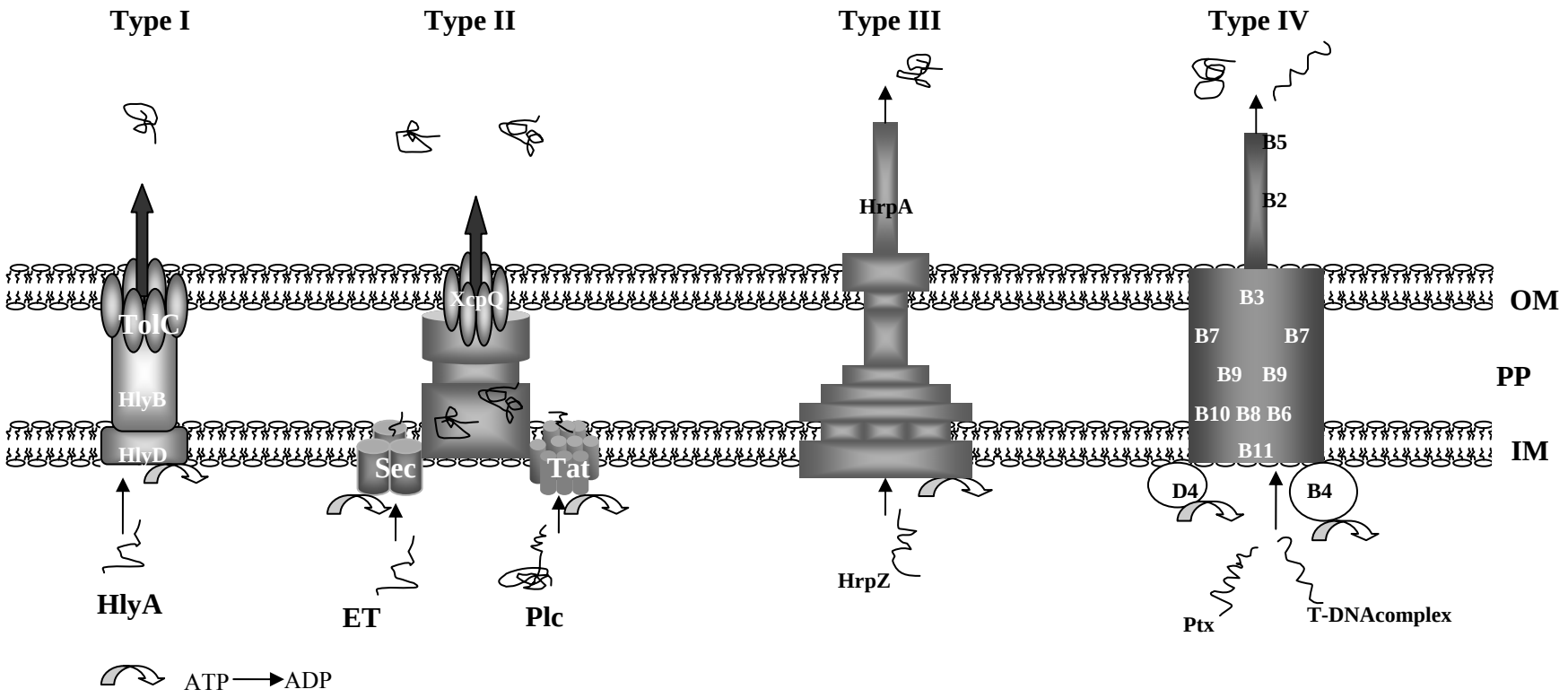

Figure. 1. Schematic representation of the type I, II, III, and IV protein secretion systems. The type I pathway is exemplified by *Escherichia coli* HlyA secretion; the type II pathway is exemplified by *Pseudomonas aeruginosa* exotixin A (ETA, Sec exported) and phospholipase (PlcTat exported) secretions; the type III pathway is exemplified by *Pseudomonas syringae* HrpZ harpin secretion; the type IV pathway is exemplified by *Agrobacterium tumefaciens* VirB secretion system [Adopted from Backert & Meyer, 2006 (T4SS); Hueck, 1998 (T1SS); Voulhoux *et al*., 2001 (T2SS)].

Several T2SS-dependent proteins have been shown to induce plant defense responses, including hypersensitive response-like reactions. Bacterial pathogens can suppress these defense responses, and the recent results indicate that suppression is mediated by proteins secreted through the type III secretion system (reviewed by Jha *et al*., 2005).

2.3. Type IV protein secretion pathway

The Type IV protein secretion system (T4SS) is widely distributed in the following biological aspects: the conjugation of plasmid and other mobile DNA (Christie & Vogel, 2000; Lai, *et al*., 2000; Lessl & Lanka, 1994); the translocation of effector molecules into target cells; the uptake of DNA; and the release of DNA-protein complex and effector protein into the extracellular milieu (reviewed by Backert & Meyer, 2006).

T4SS has been ancestrally related to bacterial conjugation systems, through which plasmids or other mobile DNA elements are transferred between different cells. *Agrobacterium tumefaciens* T-DNA transfer is one of the best characterized T4S systems. It is also a unique system for trans-kingdom DNA transfer. In the Ti system, pilin are associated with T4SS, and it is believed to form a conduction channel. Although the morphology and the function of the *Agrobacterium* T-pilus appear very similar to the type III secretion system-associated pili (see Section 3.3.3), there is no sequence similarity at all between the two systems. The T4SS-dependent VirB/D4 machinery consists of 11 proteins encoded by *virB*1-11 and the so-called coupling protein VirD4. The T-pilus is built up with the VirB2 as the major subunit and VirB5 as the minor subunit which was found only in the tip of the T-pilus. In association with the OM protein VirB9 and by consuming ATP energy, the conformational adaptation and stabilization of the two inner membrane associated proteins, VirD4 and VirB11, takes place. VirB7 is an OM-associated lipoprotein which forms a disulfide-bonded heterodimeric complex with VirB9, and this complex stabilizes other VirB proteins (reviewed by Christie, 2004; Figure 1).

Like the type III secretion system, T4SS is used by many pathogens to deliver effector proteins to eukaryote cells during infection. In *Helicobacter pylori*, CagA is translocated through the T4SS. In host cells CagA interferes with actin cytoskeletal

rearrangement and induces proinflammatory response (Brandt *et al*., 2005; Selbach *et al*., 2003). *Bordetella pertussis* toxin (Ptx), the causative agent of whooping cough, is composed of five protein subunits named as S1-S5. The toxin is also secreted through T4SS, but instead of directly entering a target cell like most of other effector proteins, it is translocated into the extracellular medium. Once secreted, Ptx itself mediates host cell binding and delivery of the catalytic S1 subunit into host cytosol. Thus, protein secretion and host cell translocation of the effector are not linked in this particular T4SS (Weiss *et al*., 1993). Unlike single step DNA transfer through T4SS, The translocation of Ptx across the OM was found to be Sec-dependent two-step process as mentioned in Table 1 (reviewed by Christie, 2004).

2.4. Type V protein secretion: Autotransporter and Two-partner secretion pathways

Both autotransporter and two-partner-secretion systems are present in a wide range of Gram-negative bacteria for transportation of large virulence proteins across the OM. Many proteins secreted by these two systems have an amino-terminal extension of about 25 amino acid residues located in the otherwise typical Sec-dependent signal peptide. The precise function of the amino-terminal extension is not known but it may aid in the co-translational translocation of the precursors across the inner membrane (Chevalier *et al*., 2004; Henderson *et al*., 2004). After the Sec-dependent precursors are exported across the inner membrane, the autotransporter-dependent and two-partner-secretion-dependent passengers reach the periplasm before being further translocated across the outer membrane. The periplasmic intermediates have to be kept unfolded or partly unfolded at low abundance until they reach the outer membrane (Klauser *et al*., 1990; Klauser *et al*., 1992). Resident periplasmic chaperones may help the proper folding and insertion of the passenger domain in the OM.

2.4.1. Autotransporter pathway

Autotransporter (AT) pathway is probably the simplest protein secretion system since all the necessary secretion components are included in one polypeptide which typically consists of three domains: cleavable amino-terminal signal sequence which is needed for the preprotein translocation across the IM, a C-terminal translocator domain (β-domain) which inserts in the OM and serves as a channel for the third part

of AT, and an internal passenger domain. The passenger domain includes an amino-terminal translocation activity part and a C-terminal auto-chaperone part which are necessary for translocation to the OM (Figure 2). However, the nature of the passenger domain does not seem to be important, as it can be substituted by foreign proteins and be subsequently secreted (Klauser *et al*., 1990; Suzuki *et al*., 1995). As there is no ATP as an energy source in the periplasm, the driving force for the protein translocation across the OM comes not only from the release of autochaperone from the secreted protein but possibly also from either the folding of the passenger domains as they reach the cell surface or the folding of the β-domain in the OM (reviewed by Jacob-Dubuisson *et al*., 2004). After translocation, according to the biological function of the mature proteins, the final localization can be either the cell surface as adhesins, or in extracellular space as the case of most proteases.

The neisserial protease IgA1 (IgAp) was the first identified AT protein (Pohlner *et al*., 1987). Many other IgA-like proteins have since been discovered. Numerous AT-dependent proteins are adhesins such as Hsf of *H. influenzae*, pertactin (Prn) of *B. pertussis*, YadA of *Y. enterocolitica* and *Y. pseudotuberculosis*. Two AT-dependent proteins are major constituents of bacterial surface structures: S-layer forming rOmpB of *Rickettsia typhi* (Sleytr & Messner, 1983) and Hsr of *Helicobacter mustelae* (Forester *et al*., 2001; Schauer & Fox, 1994).

Recently, a subfamily of AT-dependent proteins, with YadA and the *H. influenzae* Hia as prototypic examples, were found to require trimerization in order to promote their secretion. They are termed AT-2 secretion system (Roggenkamp *et al*., 2003; Surana *et al*., 2004).

The AT system has been widely used as a tool for surface display of peptides and proteins, for vaccine development and other biological purposes. It can display small peptides of 10-15 amino acids to full length protein of 613 amino acids long (reviewed by Rutherford & Mourez, 2006).

2.4.2. Two-partner secretion pathway

Two-partner secretion (TPS) pathway is functionally similar to the AT secretion system, except that two independent polypeptides encode the passenger TpsA and

translocator TpsB (Figure 2). The genes encoding these two proteins tend to be located within the same operon. With the similar function as AT β-domain, TpsB protein is predicated to form a channel with β-barrel in the OM (reviewed by Thanassi *et al*., 2005). The secretion of the filamentous haemagglutinin (FHA) of *B. pertussis*, adhesins HMW1 and HMW2 of *H. influenzae*, and the haemolysin ShlA of *S. marcescens* have been extensively studied and are often referred to as the TPS model systems (reviewed by Henderson *et al*., 2004).

2.5. The chaperone/usherpathway

The chaperone/usher pathway (CU) is dedicated to the assembly and secretion of a superfamily of adhesive virulence-associated filamentous structures on the surface of Gram-negative bacteria (Thanassi *et al*., 1998). The proteins secreted through this pathway typically assemble into a rod-shaped fiber, termed fimbriae. The prototypes of the CU pathway are the adhesive type 1 and P pili expressed by uropathogenic *E. coli*, but some capsular proteins such as F1 subunits of *Y. pestis* F1-capsule (a major protective antigen with an antiphagocytic role) are also secreted through this pathway.

The components of this pathway consist of a periplasmic chaperone and an integral outer membrane protein termed usher (Figure 2). During transportation, the substrates are exported across the inner membrane via the Sec system, followed by the immediate binding of the chaperone; then the amino-terminal signal sequences of the substrates are cleaved off and the substrates are released into the periplasm. The protein folds in the periplasm before the translocation across the outer membrane. The chaperone-substrate complexes then target the outer membrane usher assembly sites. The targeting of the complexes to the usher triggers chaperone dissociation from the substrate, and induces the opening of the usher channel, which is about 2-3 nm in diameter. The narrow usher channels would allow the partly folded fimbrillin but not the helical rod form to pass. The final conformation is established when the substrate reaches the cell surface (reviewed by Thanassi & Hultgren, 2000).

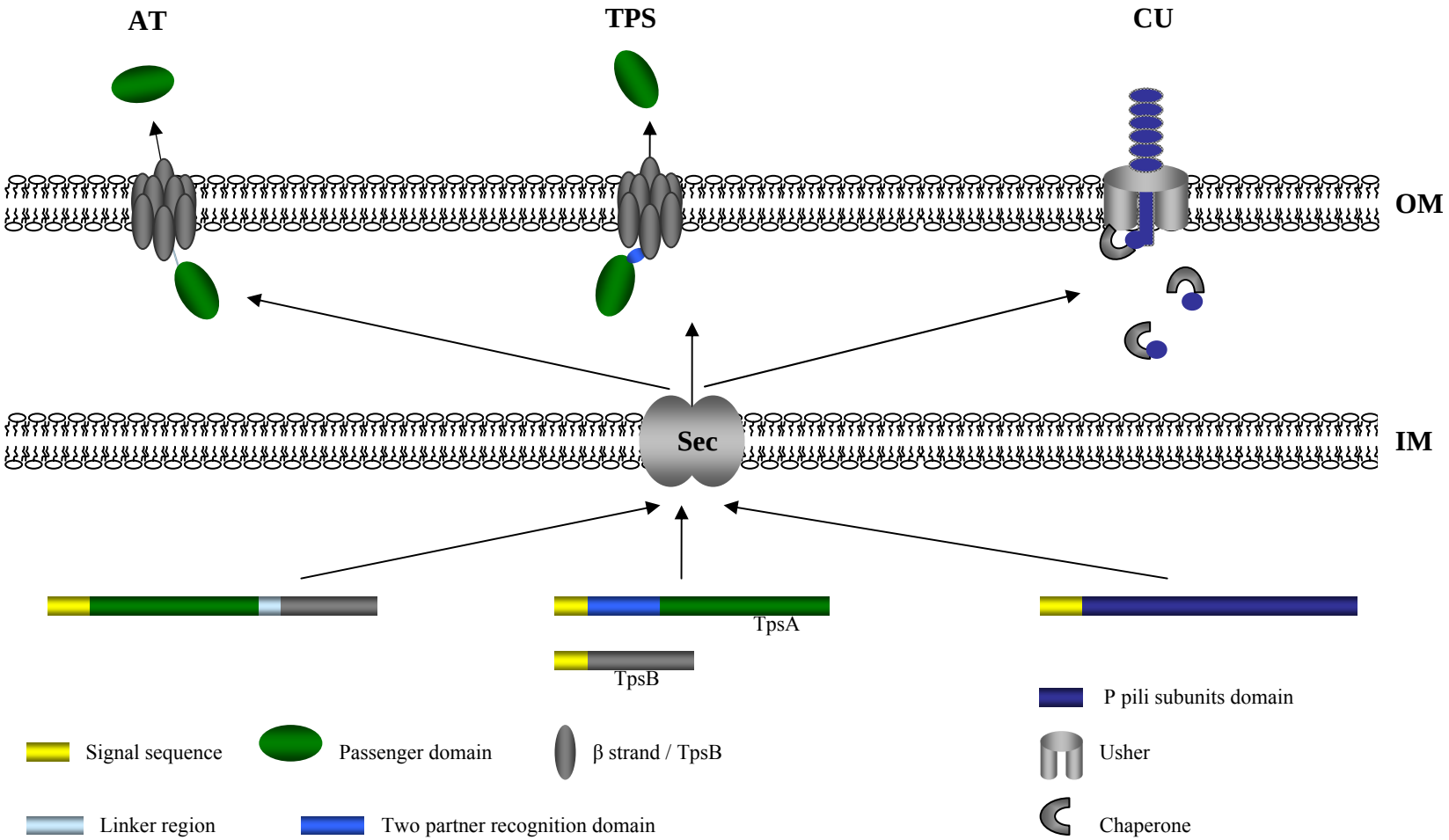

Figure. 2. Schematic representations of the autotransporter (AT), two partner secretion (TPS) and chaperone/usher (CU) protein secretion systems. The polyproteins are exported through the IM via the Sec machinery. Once in the periplasm, the signal sequences are cleaved, the β domains of both AT and TPS insert into the OM and form a pore, the passenger domains insert into the pore and are translocated to the cell surfaces. For protein secreted through the CU pathway, exemplified by P pili, the periplasmic chaperone binds to each subunit to keep their proper folding and to prevent premature subunit-subunit interactions. Chaperone-subunit complexes then migrate to the OM usher, the folded subunits (liner fiber) are translocated through the usher and the pilus is assembled on the cell surface [adopted from Henderson *et al*., 2004; Jacob-Dubuisson *et al*., 2004; Thanassi & Hultgren, 2000]

2.6. Type III protein secretion pathway

The type III secretion system (T3SS) was first discovered by Guy Cornelis' group from the study of the animal bacterial pathogen *Yersinia* outer proteins in early 1990s (Michiels *et al*., 1990). Soon after, researchers found similar secretion mechanisms existing in many Gram-negative bacterial pathogens from both animals and plants (reviewed by Alfano & Collmer, 1997; He, 1997; Hueck, 1998; Keen, 1990; Romantschuk *et al*., 2001). *Agrobacterium* is about the only genus of Gram-negative phytopathogens in which this system has not been found. This system functions as a molecular syringe delivering bacterial virulence proteins, termed effectors, from the cytoplasm directly into the extracellular milieu or the cytosol of host cells. However, in *Rhizobium* spp. this system was found to serve no pathogenic but as symbiotic purposes (Viprey *et al*., 1998). T3SS is the most complex protein secretion system in bacteria judged by the number of proteins constituting the secretion apparatus, and it is distinguished from the other secretion systems by several features. First, unlike the cleavable signal peptide for Sec-dependent protein secretion, the non-cleavable amino terminal secretion signal is localized either in the mRNA of or directly on the secreted protein (Anderson & Schneewind, 1997; Lloyd *et al*., 2001). Secondly, specific chaperones are needed for the secretion of many effectors. Thirdly, the secretion apparatus consists of two parts: the cylindrical base, which spans the entire cell envelope, and the extracellular filamentous appendage, which is termed needle in animal pathogens and pilus in plant pathogens. The cylindrical base is genetically and morphologically similar to the bacterial flagellar secretion machinery but, unlike the flagellum, filamentous appendage facilitates host cell contact (reviewed by He, 1997; Knutton, *et al*. 1998; Kubori *et al*., 1998).

Although T3SS is called the 'contact-dependent' protein secretion system (Aldon *et al*., 2000; Ginocchio *et al*., 1994; Pettersson *et al*., 1996; Zierler & Galan, 1995), the secretion of many T3SS-dependent proteins can be artificially induced *in vitro* by mimicking host environmental conditions (Demers *et al*., 1998; He *et al*., 1993; Michiels *et al*., 1990; Vallis *et al*., 1999). However, the secretion and translocation of some bacterial effector proteins are strongly induced only upon contact with target cells. So far this 'contact-dependent' phenomenon has been shown for animal pathogens *Shigella* spp., *Yesinia* spp. and *P. aeruginosa* (Pettersson *et al*., 1996;

Watarai *et al*., 1995) and only one plant pathogen *Ralstonia solanacearum* (Aldon *et al*., 2000).

2.6.1. Ysc-Yops

The *Yersinia* secretion-*Yersinia* outer proteins (Ysc-Yops) system is the best studied T3SS example. In *Yersinia* spp., the T3SS is encoded on a virulence plasmid. Mutations in any of the *ysc* genes can abolish the secretion of Yops. The secretion of Yops into the extracellular space can be induced artificially by incubation of the bacteria in the culture medium in the absence of Ca^{2+} at 37°C. However, purified secreted Yops have no cytotoxic effect on cultured cells, indicating that physical contact of *Yersinia* with the target cells and translocation of Yops into the host cytosol is required for *Yersinia* pathogenesis *in vivo* (reviewed by Cornelis, 1998; Hueck, 1998).

Translocation of effector Yops into host cytosol requires coordination between T3SS-dependent needle complex and the translocon comprised of YopB, YopD, and LcrV. The function and assembly of the T3SS needle complex will be described in more detail in a separate paragraph below. Upon contact with the target cell, YopB and YopD were found to form a translocation pore in the host cell plasma membrane (Tardy *et al*., 1999). LcrV was found to be required for the pore assembly and pore size control (Holmstrom *et al*., 2001). LcrV is localized to the distal tip of the needle and it forms a bridge that connects the needle with the translocon (Mueller *et al*., 2005). In the absence of any of the translocon components, Yops are secreted out of the bacteria but fail to enter the host cell (Lee & Schneewind, 1999; Pettersson *et al*., 1999).

2.6.2. Secretion signal and T3SS-substrate-specific chaperones

Yops and other proteins destined for secretion through T3SS are generally composed of two domains: the secretion signal domain and the translocation domain, which specifically target the secretion apparatus. The secretion signals of the T3SS-secreted proteins are either located on the 5' end of mRNA or within the first 15-20 amino acids of the secreted proteins. In contrast to the Sec-dependent signal, the putative T3SS signal domain has no clear consensus sequence on either the 5' end of the mRNA or amino acid level, and no cleavage of amino terminus or carboxyl terminus

occurs. The T3SS-specific chaperones facilitate the secretion of protein by binding to their amino termini within the first 140 amino acids, guiding them to the T3SS-dependent apparatus and holding them in unfolded state prior to secretion. The chaperones are generally small (around 15 kDa) acidic proteins without sequence similarity among them, and act as dimers, typically binding only to its partner protein (reviewed by Ghosh, 2004).

2.6.3. Structure and length of T3SS appendage

The macromolecular structure of T3SS apparatus in animal pathogenic bacteria is composed of two distinct parts: the needle complex and the translocon. The needle is anchored to a base that spans the inner and outer bacterial membranes. The base itself consists of two sets of ring complexes embedded in each of the two membranes (Kubori *et al*., 1998; Tamano *et al*., 2000). The translocon proteins, in contrast, are secreted through the needle complex and associate with the host cell membrane, where they function to transfer proteins into the cytosol of the host cell (Blocker *et al*., 2001; Cordes *et al*., 2003; Davis & Mecsas, 2007).

The needle structures of *Yersinia*, *Salmonella*, *Shigella*, and *E. coli* are each primarily composed of a single protein (YscF, PrgI, MxiH, and EscF, respectively) (Blocker *et al*., 1999; Hoiczyk & Blobel, 2001; Kubori *et al*., 1998; Sekiya *et al*., 2001), which polymerizes to form a tube. The needle proteins from different species are all small (7 to 10 kDa) and are mostly alpha helical in structure, but share only between 20 and 30% sequence identity (see also Section 3.3 for T3SS of plant pathogens).

Needle length varies from 45 to 80 nm in different bacterial species. However, in plant pathogenic bacteria, the length of the T3SS-associated pilus can extend to several micrometers. The length of the needle is controlled by a specific protein in each system, for example, by YscP in *Yersinia*, Spa32 in *Shigella*, and InvJ in *Samonella*. In *Yersinia*, export of the *Yersinia* needle subunit continues until the needle reaches the length of the extended YscP protein. YscP then switches the secretion process from the needle subunit to the effector. The precise length of the needle is adjusted according to the dimensions of the other structures on the host cell surface and on the bacterial cell surface (reviewed by Cornelis 2006; Galan & Wolf-Watz, 2006). However, the T3SS in EPEC (enteropathogenic *E. coli*) and EHEC

(enterohemorrhagic *E. coli*) is unique. The needle complex encoded by EscF is extended by an additional larger structure, the EspA filament (Crepin *et al*., 2005; Daniell *et al*., 2001).

3. Phytopathogens

3.1. The cause of plant disease and plant innate immunity

Plant disease can be caused by either biotic or abiotic factors. The abiotic factors include environmental extremes, mineral deficiencies or toxicities, imbalance of essential nutrients, or chemical pollutants. Physiological disorders in plant are often followed by invasion of the real pathogens: fungi, bacteria, viruses, nematodes, protozoa, and viroids. Development and establishment of infectious disease depends on the combination of the pathogen, the host, and the environmental condition, which are referred to as the "disease triangle" (reviewed by Agrios, 2005). Various pathogens invade plant hosts in different ways and to different extents. Fungi invade and grow directly through or between the host cells by producing mycelium. Viruses and viroids invade plant cells intracellularly through wounds or by the help of vectors and also move from cell to cell in tissues. Bacteria and most nematodes generally invade plant tissues intercellularly. Bacterial infection of a plant is the main focus of this thesis and will be discussed in more detail below.

In the long history of co-evolution of plants and pathogens, plants have developed various defense mechanisms against potential pathogens. The innate immunity or non-host resistance mechanisms in plants can result from successful passive defenses, such as structural and metabolic (biochemical) defenses. These include constitutive barrier structures such as wax and cuticle that cover the epidermal cells and thick-walled cells, also, inhibitory substances such as phenolic compounds, tannins, cell wall-degrading enzymes like glucanases and chitinases, toxic phytoalexins, defensins and reactive oxygen species (reviewed by Agrios, 2005; Nurnberger *et al*., 2004). Non-host resistance can also result from active defenses induced upon pathogen recognition. For example, upon inoculation with necrosis-inducing pathogens or various nonpathogenic root-colonizing *Pseudomonads*, or treatment with salicylic acid (SA), plants acquire enhanced resistance to a broad spectrum of pathogens, and the induced resistance occurs not only at the site of the initial treatment but also in distal, untreated plant parts, which is called the

systemic-acquired resistance. Induced defense responses are regulated by a network of complex signal transduction pathways in which the hormonal signals SA, jasmonic acid (JA) and ethylene play major roles. The various induced resistance phenomena are all associated with an enhanced capacity for the rapid and effective activation of plant cellular defense responses to pathogens (He *et al*., 2006; Melotto *et al*., 2006). These responses include the hypersensitive reaction (HR), cell-wall strengthening, the oxidative burst and the expression of various defense-related genes. The HR resembles the programmed cell death of animal cells and aims at preventing the spread of disease into healthy tissues (Dangl & Jones, 2001). The combination of different plant innate defenses eliminates many of the potential pathogens and prevents plant from disease.

3.2. Gram-negative bacterial pathogen

Pseudomonas, as well as four other Gram-negative bacterial genera*, Xanthomonas*, *Ralstonia*, *Erwinia*, and *Agrobacterium*, are the main Gram-negative bacterial pathogens in plant. The bacteria infect plants by invading plant tissues through natural openings such as stomata or wounds, multiplying in the intercellular space outside of the plant cell wall (Beattie & Lindow, 1994; Boureau *et al*., 2002; Romantschuk & Bamford, 1986; Wilson *et al*., 1999), and producing virulence factors which contribute to the formation of symptoms. Different plant pathogenic bacteria can cause a wide range of different kinds of symptoms, for example, soft rots caused by *Erwinia carotovora* and *E. chrysanthemi*, vascular wilts of solanaceous plants by *Ralstonia solanacearum*, foliar spots and blight of pepper and tomato by *Xanthomonas campestris*, bacterial speck by *Pseudomonas syringae* and tumors caused by *Agrobacterium tumefaciens*.

3.2.1. Host specificity of *P. syringe*

P. syringae is a host-specific pathogen that is capable of infecting the aerial parts of its host plant. There are more than 40 different *P. syringae* pathovars classified on the basis of their host range. Many pathovars including *P. syringae* pv. *syringae* (*Pss*), *P. syringae* pv. *tomato* (*Pst*), and *P. syringae* pv. *phaseolicola* (*Pph*) have been widely used as model organisms to study bacterial pathogenesis in plant (reviewed by Hirano & Upper, 2000). Particularly, *Pst* DC3000 is a well-studied plant pathogen of tomato and *Arabidopsis thaliana*, the model organism of the plant kingdom. *Pst* DC3000

causes bacterial speck on tomato, and is taxonomically quite divergent from pathovar *syringae* (Manceau & Horvais, 1997). The total genome sequence of *Pst* DC3000 has been determined (Buell *et al*., 2003). Hence, it has gained a special status as the main model strain for genetic and molecular studies of the plant pathogenicity of *P. syringae*.

3.2.2. Virulence factors of *P. syringae* that contribute to plant pathogenesis

Nearly all bacterial virulence factors are located on the bacterial surface or are secreted to the extracellular milieu or inside the plant cell. Therefore, bacterial protein secretion systems are important virulence determinants. *P. syringae* pathovars produce a large number of protein and non-protein virulence factors that are directly or indirectly toxic to plant cells or protect from plant defenses, such as phytotoxins, extracellular polysaccharides, and other effectors. They have been considered to be virulence factors, since their production results in increased disease severity (reviewed by Bender *et al*., 1999). For example, extracellular polysaccharides like alginate may protect the bacterium from oxidative stress and promote tissue colonization (Keith *et al*., 2003). Phytotoxins (coronatine, syringomycin, syringopeptin, tabtoxin, and phaseolotoxin) inhibit specific enzymes surrounding the host cells. These substances suppress some host defense and facilitate movement and multiplication of the pathogen in the host. However, functions of these substances in pathogenesis all depend on their secretion from bacteria and contact with plant cells. The bacterial secretion system and cell surface structures, like pili, fimbriae and flagella, are important virulence factors both in human and plant pathogens (reviewed by Finlay & Falkow, 1997). The plant pathogenic bacterial T3SS-associated pili are believed to contact the host cell and mediate the movement of virulence and avirulence proteins into the host cells. The function of T3SS-dependent proteins is to suppress the host defense and thus promote bacterial survival and multiplication inside the host tissue (Mudgett, 2005). Analysis of the *Pst* DC3000 genome has revealed that there may be more than fifty T3SS-secreted effectors in one single strain (reviewed by Greenberg & Vinatzer, 2003). T3SS effectors define the host range of a certain *P. syringae* pathovar (see Section 3.3.4).

3.3. Hrp secretion system of *P. syringae*

In plant pathogenic bacteria, the T3SS is called the Hrp secretion system and is encoded by the *hrp* gene cluster. The *hrp* genes are so named because they are involved in plant pathogenic bacteria to elicit **h**ypersensitive **r**eaction in resistant plants and to cause disease (**p**athogenesis) in susceptible host plants. Genetic analysis of several plant pathogenic bacterial genomes has shown that pathogens are distinguished from their non-pathogenic relatives by presence of a so-called pathogenicity island (PAI) encoding T3SS and pathogenesis related genes (Alfano *et al*., 2000; Jackson *et al*., 1999). Related pathogens harbor similar clusters of T3SS effector genes, earlier termed *avr*, which are often located in or adjacent to the *hrp* gene cluster. The proteins encoded by *avr* genes and some *hrp* genes are secreted through T3SS (reviewed by Lindeberg *et al*., 2006). Recently, a unified nomenclature for *P. syringae* T3SS-secreted proteins was established (reviewed by Lindeberg *et al*., 2005). The detailed naming system can be found in the website: www.pseudomonas-syringae.org.

So far the *hrp* genes have been described in *P. syringae*, *X. campestris*, *R. solanacearum*, *E. amylovora* (reviewed by Collmer, 1998), *E. carotovora* (Rantakari *et al*., 2001), and *R. solanacearum* (Arlat *et al*., 1992; Van Gijsegem *et al*., 1995). The *hrp* gene clusters are either located in the chromosome or in a plasmid. Based on the degree of conservation of their encoded protein components, the *hrp* clusters can be divided into two groups: group I contains *hrp* gene clusters of *E. amylovora* and *P. syringae*, and group II for *hrp* gene clusters of *X. campestris* and *R. solanacearum* (Alfano & Collmer, 1997).

3.3.1. The organization of *hrp* genes and function of Hrp proteins in *P. syringae*

The first report on *hrp* genes in plant pathogenic bacteria was made over 20 years ago (Lindgren *et al*., 1986). Typically the *hrp* genes in *P. syringae* pathovars are clustered in a single 25 kb chromosomal region containing up to 27 *hrp* genes organized in 7 operons encoding either regulatory, secretory, or effector proteins (Figure 3). A cosmid carrying this region is sufficient to enable nonpathogenic *E. coli* and *P. fluorescence* to elicit HR *in planta* (Huang *et al*., 1988).

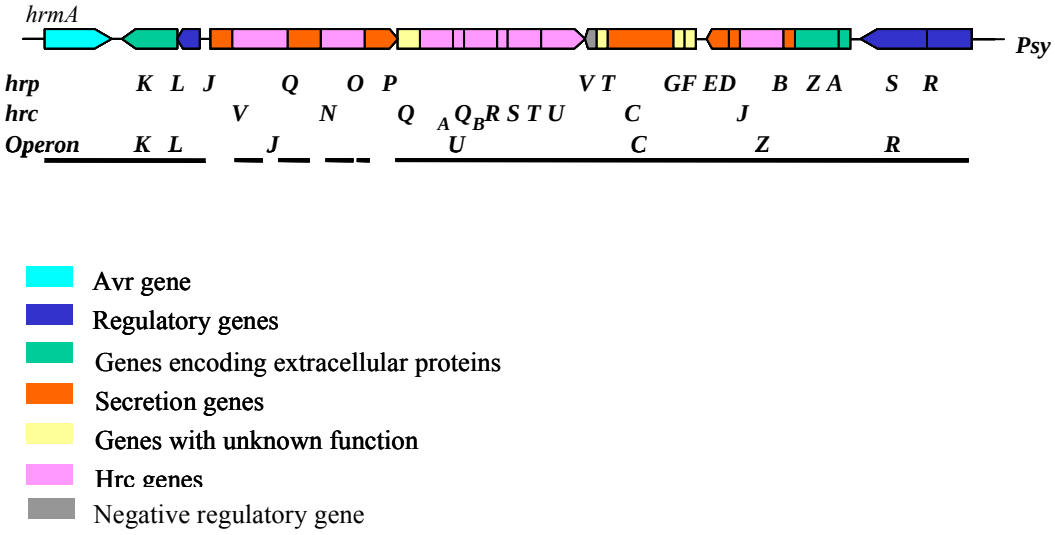

Figure 3. The *hrp* gene cluster of *Pst* DC3000 and their functions. The cluster contains 27 *hrp* genes and the *hrmA* gene. Arrows indicate the direction of transcription. Regions sequenced in DC3000 are indicated by lines beneath the *hrp* cluster of *P. syringae* [adopted from Galan & Collmer, 1999; He *et al*., 1997].

There are about 20 Hrp proteins that are involved in the protein secretion apparatus indicated by the genes colored with red and purple in Figure 3. Nine of the *hrp* genes are conserved among diverse bacterial pathogens of plants and animals and have been renamed *hrc* (HR and conserved) according to homology with *Yersinia ysc* genes (Gough, *et al*., 1992; Winans, *et al*., 1996). These *hrc* genes are *hrcC*, *hrcJ*, *hrcN*, *hrcQ*, *hrcR*, *hrcS*, *hrcT*, *hrcU*, and *hrcV* (Bogdanove *et al*., 1996; Figure 3). Eight of these *hrc* encoded proteins show high similarity to flagellar basal body components. These Hrc proteins are located in the inner membrane surface and form a conserved core resembling the flagellar basal body. It has been suggested that the core could be involved in the recognition of a universal secretion signal (Anderson *et al*., 1999). However, *hrcC* is the only exception not homologous with flagellar genes as it belongs to the so called secretin family and functions as a pore forming protein in the outer membrane (reviewed by Alfano & Collmer, 1997).

3.3.2. Regulation of *hrp* gene expression and secretion

Expression of *hrp/hrc* genes is tightly controlled. They are expressed at a very low level *in vitro* in nutrient-rich media but can be induced in infected plant tissues (Boureau *et al*., 2002) or in artificial Hrp-inducing minimal media that mimic the *in planta* conditions, that being a minimal medium with low osmotic strength and a pH of about 5.8, supplemented with simple sugar such as fructose or sucrose (Huynh, *et al*., 1989; Salmeron & Staskawicz, 1993; Xiao *et al*., 1992). The maximal induction conditions, however, vary from species to species, between different pathovars and even from one *hrp* gene or operon to another. Activation of *hrp* gene expression *in planta* occurs within 2-3 hours after inoculation (Boureau *et al*., 2002; Wei, *et al*., 1992; Xiao *et al*., 1992).

Three intracellular positive regulatory proteins HrpR, HrpS and HrpL are required for expression of *hrc/hrp* genes. These proteins appear to function in a regulatory cascade in which HrpS and HrpR consist of two-component regulator system and activate the expression of HrpL *in vivo* in response to a signal present in host tissue or *in vitro* in Hrp-inducing minimal medium (Xiao *et al*., 1994). HrpL is a sigma factor that activates all *hrp* and *avr* genes by recognizing a 26 bp conserved sequence GGAACC-N16-CCAC, the so called *hrp*-box, present in the upstream regions of many *hrp* and *avr* genes (Deng *et al*., 1998; Innes *et al*., 1993; Shen & Keen, 1993).

HrpV was found to be a negative regulator of the *hrp* regulon (Preston *et al*., 1998). In Hrp-inducing minimal medium, overexpression of the *hrpV* gene down-regulates *hrp/hrc* gene expression whereas *hrp/hrc* gene expression is elevated in a *hrpV* mutant. Recent studies by Alfano and co-workers (Fu *et al*., 2006; Petnicki-Ocwieja *et al*., 2005) have revealed that HrpK1 and HrpJ encoded by the genes on the neighboring operons of the *hrp* gene cluster of *P. syringae* act as translocators, comparable to the role of YopN in *Yersinia*, for the proper translocation of the T3SS-dependent accessory protein and effectors.

3.3.3. Hrp pilus structure & function

The HrpA pilus of *Pst* DC3000 is 6-8 nm in diameter and its assembly on the bacterial surface depends on the T3SS (Roine *et al*., 1997a). It is required for *Pst* DC3000 to

cause disease in *Arabidopsis* and tomato, and HR in tobacco. The HrpA is a 113-amino-acid protein, and it has been shown that HrpA alone is sufficient for the formation of the filament structures, indicating that HrpA is the sole or main structural protein of the Hrp pilus (Roine *et al*., 1997a). The Hrp pilus has been suggested to act as the physical pipeline directing proteins across the plant cell wall into the plant cytoplasm (He, *et al*., 1997; Roine, *et al*., 1997a; Roine *et al*., 1997b). Wei and colleagues (2000) showed that the Hrp pilus is an integral component of a protein secretion structure. Brown and colleagues (2001) showed the Hrp pilus enables *Pst* DC3000 to translocate virulence proteins at the right place and time during bacterial infection of plant. Wei and colleagues showed that the functional HrpA protein is required for secretion of HrpW harpin and AvrPto in culture (Wei *et al*., 2000). They also showed that a *hrpA* mutation affected the transcript level of the two positive regulatory genes *hrpR* and *hrpS*, and the full expression of all core *hrc/hrp* gene operons as well as *hrpW* and *avrPto* that reside outside the core *hrc/hrp* gene cluster.

By transposon mutagenesis analysis of the *hrpA* gene of *Pst*. DC3000, our group found previously that most of the insertions, as well as deletions of a large portion in the N-terminal half of the pilin, were tolerated without affecting protein secretion, pilus assembly and pathogenicity to plants (Taira *et al*., 1999). On the other hand, almost all the insertions in the C-terminal half abolished pilus formation while protein production and secretion was not affected. These observations indicate that it is the C-terminal half that is involved in and essential for pilus assembly. All insertions between the promoter and start codon as well as one insertion in codon 10 resulted in mutants that did not produce pilin at all. However, further analysis revealed that lack of pilin production was due to a failure in mRNA transcription or instability of the messenger RNA (Taira *et al*., 1999). Further research in our group has revealed that the secretion signal of HrpA, as with many other T3SS-secreted proteins, is in the first 15 codons of mRNA or in the 15 amino-terminal amino acids of the protein (Hienonen *et al*., 2002).

Morphologically Hrp pili appear to be flexible whereas needles of T3SS in animal pathogens (see section 2.6) appear to be rigid. The length of the Hrp pilus of *Pst* DC3000 is much longer than the needle (generally less than 80 nm), which is probably a necessary feature for phytopathogens in traversing the thick (>100 nm)

plant cell wall. Unlike needles, which are dispersed over the entire bacterial surface, the Hrp pilus of *Ralstonia solanacearum* was found to emanate only from one pole (Van Gijsegem *et al*., 2000).

Despite the similar biological functions, the Hrp pilin genes of different plant pathogenic bacteria are much less conserved than the other genes involved in the Hrp secretion systems. For example, HrcC proteins of *P syringae* pvs. *tomato* and *syringae* share 80% sequence similarity (Deng *et al*., 1998). In contrast, HrpA from *Pst* DC3000 has only 30% identity to HrpA from *Pss* or *Pph*, and HrpA from these pathovars has about 20% sequence similarity to YscF (reviewed by Ghosh, 2004). Furthermore, the major subunit of *E. amylovora* Hrp pilus shares only 30% identity to HrpA of *Pst* DC3000 (Jin *et al*., 2001), whilst the structure protein of *R. solanacearum* Hrp pilus have no detectable similarity with other Hrp pilus proteins (Van Gijsegem *et al*., 2000).

3.3.4. Proteins secreted through the Hrp secretion system

The Hrp secretion system of *P. syringae* has been shown to secrete two major families of proteins. The first family includes harpins such as HrpZ and HrpW (encoded within PAI) that are secreted in the apoplast (intercellular space). Harpins can elicit HR in non-host plants when administered extracellularly in high concentrations. The second family consists of effector/Avr proteins (such as AvrPto, AvrRpt2 and AvrB of *P. syringae*) that function inside the plant cells and are believed to contribute to pathogenicity in susceptible host plants (reviewed by Alfano & Collmer, 2004). Some Avr proteins are thought to suppress host defenses by interaction with intracellular targets (Leach & White, 1996; Tsiamis *et al*., 2000).

Harpin proteins

Harpins are heat-stable, acidic, glycine-rich proteins. They are secreted into culture medium when the Hrp system is expressed, and elicit HR when infiltrated into the leaves of tobacco and several other non-host plants (Krause & Durner, 2004; Wei *et al*., 1992). Although harpins are expressed by different plant pathogens, the genes encoding the harpins do not appear to be highly conserved among different genera, which is indicated by the dissimilarity of the harpin-encoding-genes like *hrpN* and *HrpW* of *E. carotovora* and *E. amylovora*, *hrpZ* and *hrpW* of *Ps. syringae*, *hrpF* of *X.*

campestris, *hpaG* of *X. axonopodis* pv. *glycines*, and *popA* of *R. solanacearum*. The amino acid sequences of harpins do not share significant homology with other known proteins either. Harpin was initially defined as elicitors of HR in non-host plant as mutations in some harpin genes abolished the induction of HR (Alfano *et al*., 1996; Bauer *et al*., 1995; Ham *et al*., 1998). Nevertheless, mutations of the *hrpZ* harpin gene in various *P. syringae* strains has little or no effect on HR elicitation on reisitance host plants, whereas a double mutant of *hrpZ* and *hrpW* retains part of the virulence (Alfano *et al*., 1996; Charkowski *et al*., 1998). Therefore, the natural function of harpins in pathogenesis as well as their ability to elicit the HR when introduced artificially into the apoplast of plant is unclear. HrpZ of *P. syringae*, HrpF of *X. campestris* and PopA of *R. solanacearum* have been shown to bind to the plant plasma membrane and form ion-conducting pore in artificial lipid bilayers (Buttner *et al*., 2002; Lee *et al*., 2001a; Lee *et al*., 2001b; Racape *et al*., 2005), suggesting that it would function on the host plasma membrane. HrpZ has been also shown to form multimers in solution (Chen *et al*. 1998). Therefore, it is possible that HrpZ could function as a membrane-associated complex. The HrpN harpin produced by *Erwinia* spp. (Wei *et al*. 1992) was shown to affect plasma membrane ion channels in *A. thaliana* suspension cells (El-Maarouf *et al*., 2001). Surprisingly, the association of either HrpZ or HrpN harpin with plant cell membrane seems to be a reversible event (Lee *et al*. 2001b; Pike *et al*. 1998). These findings suggest that the harpins could be involved in the release of nutrients from the host cell, or they could be a putative secretion system accessory protein and function in the modification of the plant cell wall during transport of Avr proteins (Collmer *et al*., 2002).

Interestingly, some evidence indicated that harpins functioned as signaling molecules with multiple functions, and most importantly, the group of proteins could induce plant systematic resistance (Bauer *et al*., 1997; Dong *et al*., 1999; Jang *et al*., 2006; Qiu *et al*., 1997; Wei & Beer, 1996; Zitter & Beer, 1998). Therefore, it seems that harpins have dual or multiple roles in the interaction process with plant cells.

Effector/Avr proteins

Avr proteins represent another family of secreted Hrp-dependent proteins. A typical *P. syringae* strain contains multiple *avr* genes located not only in the *hrp/hrc* gene cluster but also in the area adjacent to it (Alfano *et al*., 2000; Lorang & Keen, 1995).

Avr genes have no phenotype when expressed in *hrp* mutant pathogens or in non-pathogenic bacteria like *E. coli* which lacks the Hrp system (Gopalan, 1996; Pirhonen *et al*., 1996). The purified avirulence proteins have no effect on plant when it was infiltrated into plant tissue, whereas HR was induced when it was delivered through Hrp system and expressed inside the host cells (Scofield *et al*., 1996; Tang *et al*., 1996), indicating that a functional Hrp system is needed for full activation of the Avr proteins.

Many Hrp-dependent effectors appear to suppress host defence responses by inhibiting PCD elicited by other effectors (Abramovitch *et al*., 2003; Jamir *et al*., 2004). Some effectors target host cell GTP-binding proteins (GTPases) by mimicking eukaryotic enzymes and therefore are able to alter cellular signalling pathways (Aepfelbacher & Heesemann, 2001). Some effectors interfere with host signalling pathways and possess cysteine protease activity (Buttner & Bonas, 2003; Shao *et al*., 2002). Some effectors are proposed to target the host transcription machinery in the plant cell nucleus based on the presence of active nuclear localization signals (NLS) (Szurek *et al*., 2002; Van den Ackerveken *et al*., 1996; Yang & Gabriel, 1995).

Gene-for-gene interaction

Plants have evolved genetically controlled resistance against their true pathogens by matching their *R* (resistance) genes with the *avr* (avirulence) genes possessed by the pathogen in the so-called "gene-for-gene interaction" manner (Flor, 1971; Fig. 4). Avr proteins contribute to bacterial virulence when lacking a cognate *R* gene in the host (reviewed by Cook, 1998; Van den Ackervaken & Bonas, 1997). The most studied gene-for-gene interaction is the defense through HR at the pathogen entry sites, where plant cells die rapidly (programmed cell death) and local necrotic lesions are formed in response to bacterial attack. More and more studies indicated that plant disease resistance proteins do not interact directly with their cognate T3SS-secreted effectors in a simple receptor-ligand manner. In resistant plants, when pathogen-derived elicitors are recognized by plant cells, a complex signaling cascade is triggered in the host, which results in gene activation, *de novo* protein synthesis, the production of antimicrobial compounds, and cell death at the infection sites (Leister & Katagiri, 2000; Scofield *et al*., 1996; Tang *et al*., 1996).

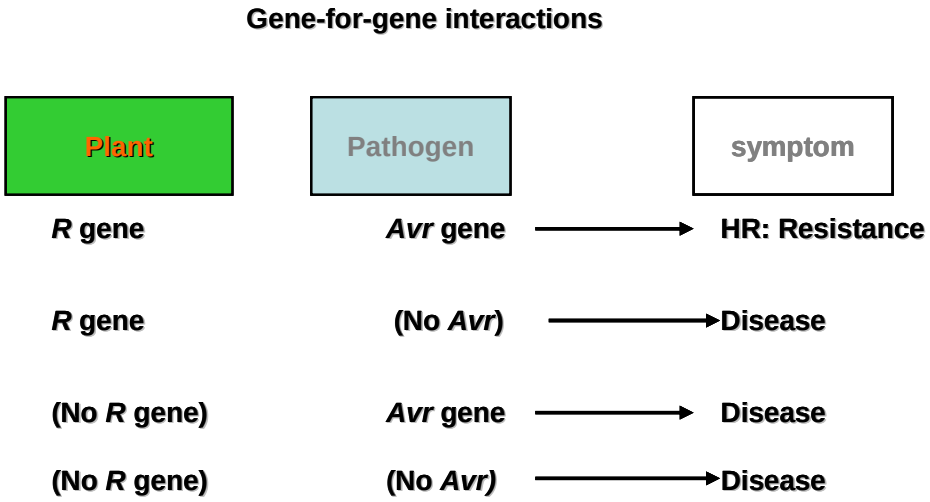

Figure 4. Schematic representation of gene-for-gene interaction between plant and pathogen. R: Resistance, Avr: Avirulence

Many plant resistance proteins contain a nucleotide binding (NB) motif and a leucine-rich repeat (LRR) at the carboxyl terminus and a Toll-like receptor at the amino terminus. NB-LRR proteins are structurally similar to human proteins containing NOD (nucleotide binding and oligomerization domain)-Toll-like receptor (NLRs) which are involved in innate immunity in response to reorganization of PAMPs or MAMPs (pathogen or microbe-associated molecular patterns) including LPS, flagellin, harpin, and so on (da Cunha *et al*., 2006; Dangl &Jones 2001; Inohara *et al*., 2001). Functional NB-LRR proteins recognize the presence of specific bacterial T3SS-secreted effectors during bacterial infection, and trigger the defense responses that are nearly always associated with HR at the infection site (reviewed by Belkhadir, *et al*., 2004).

B. AIMS OF THE STUDY

The Gram-negative bacterial T3SS system is the pathogenic determinant in both animal and plant pathogens, and it has been extensively studied in animal pathogenic bacteria for the last 20 years. Many effector proteins have been shown to be translocated to the host cytosol through the T3SS needle complex. However, at the time when this project started, there were still open questions related to the mechanisms of needle complex assembly. The studies involving plant pathogenic bacterial T3SS-dependent secretion were few due to the difficulty in monitoring protein secretion in plant tissue. Very little was known about the assembly of the Hrp pilus, the function of harpin in pathogenesis, and the dedicated function of many effectors. The main goals of this study were the following:

1. Characterization of the structure and the mechanism of assembly of the Hrp pilus
2. Genetic dissection of the HrpA topology of the Hrp pilus
3. Biotechnological applications of HrpA pilus
4. Characterization of the translocation of effector proteins through the pilus
5. Searching the plant target of harpin by studying *in vitro* protein–protein interaction using phage display method
6. Functional domain analysis of harpin by studying transposon mutagenized *hrpZ* insertional mutants

C. MATERIALS AND METHODS

The bacterial strains and plant materials used in this study are listed in Table 2, and plasmids and constructs used are listed in Table 3. The experimental methods are described in detail in the original publications and manuscripts, and are summarized in Table 4.

Table 2. Bacterial strains and plant materials used in this study

Bacterial Strains/	**Origin or relevant characteristics**	**Reference**
Escherichia coli		
Escherichia coli DH5α	*hsdR,recA,lacZYA,Δ80dlacZΔM15*	Gibco BRL
Escherichia coli BL21(DE3)	expression vector	Studier *et al*., 1990
Escherichia coli K91	Km^R	Smith & Scott, 1993
Pseudomonas syringae		
Pst DC3000	Wild type (wt) Rif^R	Cuppels, 1986
DC3000 *hrpA*$^-$	Rif^RKm^R	Roine *et al*.,1997a
DC3000 *hrpZ*$^-$	Rif^RKm^R	Alfano *et al*., 1996
DC3000 *hrpA*$^-$/phrpA259	phenotype as wt DC3000 $Rif^RTc^RKm^R$	Taira *et al*.,1999
DC3000 *hrpA*$^-$/phrpA221	phenotype as wt DC3000 $Rif^RTc^RKm^R$	Taira *et al*.,1999
DC3000 *hrpA*$^-$/phrpA256	phenotype as wt DC3000 $Rif^RTc^RKm^R$	Taira *et al*.,1999
DC3000 *hrpA*$^-$/phrpA222	phenotype as wt DC3000 $Rif^RTc^RKm^R$	Taira *et al*.,1999
Plant material		
Tobacco (*Nicotiana tabacum* cv. Samsun)		
Tomato (*Lycopersicon esculentum* cv. Agriset)		
Bean (*Phaseolus vulgaris* cv. Red Mexican)		
Parsley (*Petroselinum crispum* cv. Hamburger Schnitt)		
Arabidobsis thaliana (var.Colombia)		

Table 3. Plasmids and constructs used in this study

Plasmid (construct)	Property or description	Reference
pDN18	Broad-host-range RK2-derived cloning vector with *lacZ*α and MCS from pUC18 (pDN18), Tc^R	Nunn *et al*. 1990
pDN18-N	The original *NotI* site on pDN18 was removed, Tc^R	Taira *et al*.,1999
pRK2013	Conjugation helper plasmid, Km^R	Figurski & Helinski, 1979
pJC40	Expression vector, Amp^R	Clos & Brandau, 1994
pSYH10	Expression vector, Amp^R	He *et al.*, 1993
pBBR1MCS	Broad-host-range cloning vector, Tc^R	Kanter-Smoler *et al*., 1994
pTPT11	P*mer* and merR of R100 in pPP driving of *lucGR*, Rif^R, Tc^R	Petänen *et al*., 2001
pFLAG-A^{15}	Φ (*flag-hrpA*^{15}) in pDN18-N, Tc^R	This study
p FLAG-A^{23}	Φ (*flag-hrpA*^{23}) in pDN18-N, Tc^R	This study
p FLAG-A^{24}	Φ (*flag-hrpA*^{24}) in pDN18-N, Tc^R	This study
p FLAG-A^{48}	Φ (*flag-hrpA*^{48}) in pDN18-N, Tc^R	This study
pMerFLAGHrpA	Plasmid harboring mercury inducible promoter driving FLAG-tagged *hrpA*, Rif^R, Tc^R	This study
pMerFLAGHrpZ	Plasmid harboring mercury inducable promoter driving FLAG-tagged *hrpZ*, Rif^R, Tc^R	This study
p Men-A^{23}	Φ (*Men-hrpA*^{23}) in pDN18-N, Tc^R	This study
p Men-FLAG-A^{23}	Φ (*Men-flag-hrpA*^{23}) in pDN18-N, Tc^R	This study
p ST-A^{28}	Φ (*serine/threonine-hrpA*^{28}) in pDN18-N, Tc^R	This study
p GFP-A^{28}	Φ (*gfp-hrpA*^{28}) in pDN18-N, Tc^R	This study
p ΔA(15-53)-A^{15}	Φ (Δ*A*$^{15\text{-}53}$-*hrpA*^{15}) in pDN18-N, Tc^R	This study
p ΔA(15-57)-A^{15}	Φ (Δ*A*$^{15\text{-}57}$-*hrpA*^{15}) in pDN18-N, Tc^R	This study
p ΔA(15-79)-A^{15}	Φ (Δ*A*$^{15\text{-}79}$-*hrpA*^{15}) in pDN18-N, Tc^R	This study
p ΔA(15-84)-A^{15}	Φ (Δ*A*$^{15\text{-}84}$-*hrpA*^{15}) in pDN18-N, Tc^R	This study
p ΔA(15-88)-A^{15}	Φ (Δ*A*$^{15\text{-}88}$-*hrpA*^{15}) in pDN18-N, Tc^R	This study

Amp^R, ampicillin-resistant; Km^R, kanamycin-resistant; Rif^R, rifampicin-resistant; Tc^R, tetracycline-resistant; Φ, fusion; Δ, deletion; aa, amino acid.

Men: a fragment of *Neisseria meningitides* B gene, encoding the surface loop IV of the porin PorA of *N. meningitidis* B:12:P1.7,16.

ST: serine/threonine-rich epitope.

GFP: The first β-hairpin fragment of the green fluorescent protein.

Table 4. Methods used in this study

Method	**Used & described in**
Conjugations	I, II
DNA sequencing and sequence analysis	I, II, III
ELISA	II
Gene fusion	I, II
Genetic complementation analysis	I, II
HR assay	II, III
Immunoblotting	I, II, III
Immuno fluorescence microscopy	I, II
Immuno electron microscopy	I, II
Molecular cloning techniques	I, II, III
Phage display	III
Plant proteins extraction	III
Protein expression & purification	I, II, III
Transmission electron microscopy	I, II
Transposon mutagenesis	II
Virulence tests	I, II, III

D. RESULTS AND DISCUSSIONS

D.1. The optimal epitope insertion site in HrpA is the middle part of the N-terminal region (I & II).

The previous studies in our group have shown that the carboxyl-terminal part of the HrpA pilin is responsible for pilus assembly, whereas the amino terminus tolerates short, 15 amino acid insertions and large deletions (Taira *et al*., 1999). Therefore, the amino terminus, between codons 15 to 57 of HrpA, is a putative region for inserting heterologous amino acid sequences. A FLAG epitope was cloned at four different amino terminus-encoding sites, at amino acids 15, 23, 24 and 48 in the *hrpA* gene. Complementation of *P. syringae* DC3000 *hrpA*$^-$ with pDN18 derivatives encoding the tagged pilins showed that a FLAG insertion at all four sites permitted pilin secretion, pilus assembly and function *in planta*. The immunofluoresence microscopy study illustrated that the Hrp pili with a FLAG tag immediately downstream of positions 23 and 24 were well labeled with anti-FLAG monoclonal antibodies, while the pili with the tag in positions 15 and 48 were much less efficiently labeled (**II, Figure 1**), suggesting that the FLAG epitopes tagged at codon 23 and 24 are surface exposed. Hence the middle part of the amino terminal region of HrpA is optimal for displaying epitopes on the pilus surface.

D.2. HrpA pilus is assembled *in vivo* by adding HrpA subunits to the distal end of the growing pilus (I).

The molecular mechanism of T3SS needle/pilus assembly has been studied for a long time. By pulse-expression of FLAG-tagged pili, we managed to follow the development of Hrp pili using transmission electron microscope. *Pst* DC3000 harboring a FLAG-tagged *hrpA* construct driven by a mercury inducible promoter (pMerFLAGHrpA, both FLAG-HrpA and wild type HrpA were designed to be expressed and secreted in this construct) was grown for 8 h in *hrp* inducing minimal medium before induction of FLAG-HrpA by addition of $HgCl_2$. At time points 15, 30 and 60 min after mercury induction, the samples were collected, fixed, and then examined. Few pilus–associated gold particles were observed at the distal end of the

appendage at the first time point, 15 min after mercury induction. Immuno-gold labeling of the pilus increased only at the distal end of the pilus at later time points (**I, Figure 3)**. The time-course also allowed determination of the rate of pilus extension to be around 50 nm/min (**I, Figure 4)**. The results clearly demonstrated rapid growth of the pilus by the incorporation of HrpA subunits at the tip of the filament and suggest that HrpA monomers are translocated acropetally through the growing pilus.

Distal extension of the Hrp pilus indicates that the mechanism of pilus assembly is simililar to that of the bacterial flagellum, since newly made flagellin subunits are also added to the tip of the flagellum (Emerson *et al*., 1970). The flagellar apparatus has been suggested to be a member of the T3SS family (Macnab, 1999) and the flagellin subunits travel through the flagellar hollow structure (Namba *et al*., 1989). Our observation supports the idea of a common evolutionary origin of the flagellum and the Hrp pilus.

D.3. The effector protein HrpZ is secreted through the Hrp pilus (I).

The translocation of T3SS-dependent effector proteins through Hrp pilus has been proposed long time ago. We next addressed the possibility that effector proteins may also travel through the pilus. As Avr and Vir proteins are secreted *in vitro* with low efficiency (Jackson *et al*., 1999; Jin *et al*., 2001), we used the harpin protein HrpZ as a model. We cloned the $hrpZ_{Pph}$ gene under the control of the mercury promoter and transformed the resulting plasmid, pMerHrpZ, into DC3000Δ*hrpZ*. The bacteria were grown on electron microscopy grids as described above. When the mercury induction was performed 8 h after Hrp induction, newly made HrpZ was first detected at the tip of the pilus, and along time shifting HrpZ coated the distal portion of the growing appendage (**I**, **Figure 5A & 5B**). All parts of the emerging pilus were decorated with the HrpZ antiserum when bacteria were exposed to $HgCl_2$ throughout the incubation (**I**, **Figure 5C**). As observed with HrpA, a secretion route through the pilus was demonstrated. Our finding is in accordance with that the extrusion model of effector protein AvrPto from the Hrp pilus tip of *P. syringae* (Jin & He, 2001).

D.4. Hrp pilus, as a carrier of antigen for vaccination (II).

As described in **D.1.,** the amino terminal part of HrpA can well bear the insertion. We then studied the possibility to use the Hrp pilus as an epitope display tool. The advantages of the Hrp pilus, compared with conventional fimbrial epitope display tools, are that the pili can auto-assemble *in vitro* and can be strongly expressed in a noncomplex minimal medium, and the plant pathogen *P. syringae* is not pathogenic to humans. In this study, various peptides were inserted into the pilin subunit, and the secretion, assembly and surface properties of the modified pili were monitored (**II, Table 2 & Figure 2**). We concluded that the outwards-projecting N-terminal region of the pilin can tolerate insertions of up to 43 amino acids without losing the assembly and protein translocation competence, adding the Hrp pilus display system to a list of potential vaccine display tools.

The Hrp pilus of *P. syringae* has an external diameter of 6-8 nm (Brown *et al*., 2001; Roine *et al*., 1997b) and it is likely that the internal diameter is as narrow as a *Yersinia* needle, that being 2 nm (Hoiczyk & Blobel, 2001). Considering pilus dimensions, proteins destined for secretion would be expected to be at least partially unfolded to enter and travel through Hrp pilus. As discussed previously, chaperones play an important role in keeping the protein unfolded prior to secretion. For HrpA, such a chaperone has not been described. Given that HrpA pilin can auto-assemble to form filaments *in vitro* (Roine *et al*., 1997b), it seems that HrpA itself has an inbuilt ability to stay in an unfolded form prior to secretion. However, the three-dimensional structure of the epitope may restrict the use of the pilus as an epitope display tool. Since the epitope forming a β-hairpin structure impaired the secretion of HrpA (**II**, **Figure 2**).

D.5. Interaction of HrpZ$_{Pph}$ harpin with a host protein (III)

D.5.1. HrpZ$_{Pph}$ binds to a defined peptide sequence.

As discussed previously, HrpZ probably exerts its function as an integral membrane protein. Therefore, the possible interactions between HrpZ and host proteins are likely to take place within a lipid environment. Thus, the putative interactions might be hidden by the lipids and hard to detect directly. We chose an indirect method to study the protein-binding characteristics of HrpZ involving the affinity of random peptides displayed on phage particles. Phage clones carrying a peptide with affinity to HrpZ$_{Pph}$ were sequenced. The sequences were found to encode only eight peptides (**III**, **Table 1**), which are strikingly similar, all containing a hydrophobic amino acid motif with tryptophan and leucine, the consensus being W(L)ARWLL(G/L). A similar strategy with HrpZ$_{Pto}$ was performed, and the sequences of phage-binding peptides were found to be similar to HrpZ$_{Pph}$ phage binding peptides. The similarity of the HrpZ$_{Pph}$-binding peptide sequences suggests that HrpZ$_{Pph}$ has a binding site for a protein and not just for any hydrophobic molecule, and the protein-protein interaction was confirmed by a similar approach with HrpZ$_{Pto}$. However, no sequences with a perfect match to the consensus peptide were found in the available sequence databases.

D.5.2. Peptide-binding site maps in the middle of the HrpZ$_{Pph}$ sequence.

To locate the peptide-binding site within HrpZ$_{Pph}$, a library of randomly distributed 5-amino-acid insertion mutations on HrpZ$_{Pph}$ was constructed. We found that 12 out of 276 mutants were distinct non-binding mutants (**III**, **Figure 1A**). Sequence analysis revealed that all of the inhibiting mutations resided in the central part of HrpZ$_{Pph}$, between amino acids 86 and 194. However, several non-inhibiting insertion mutations were localized in the non-binding domain between the inhibiting mutations (**III**, **Figure 1B**). The peptide-binding site was also mapped within the central domain of HrpZ$_{Pto}$ by using same mapping strategy.

A 69-85 amino acid long homology domain, which is common to all of the HrpZ and HrpN harpins, was found within the non-binding region (**III**, **Figure 2A**). In addition

to this conserved region, both HrpZ$_{Pph}$ and HrpN have a domain with a low-complexity amino acid sequence rich in glycine, which is characteristic for harpins. In the HrpZ$_{Pph}$ sequence, the glycine-rich domain is downstream of the conserved region, whereas the glycine-rich domain is at the amino terminal side of HrpN. Thus, HrpZ$_{Pph}$ and HrpN seem to have two similar domains but in a reverse order (**III**, **Figure 2B**).

D.5.3. HrpZ$_{Pph}$ binds to an acidic, heat-sensitive, host-specific protein of bean.

To test the specificity of HrpZ$_{Pph}$-binding-peptides, the binding affinity of phage-displayed peptide-1 and peptide-3 was assayed for HrpZ$_{Pst}$, HrpZ$_{Pss}$, and HrpN$_{Ecc}$ harpins. The result (**III**, **Figure 3**) suggests that HrpZ$_{Pph}$ harpin binds specifically to a peptide, which would probably be an HrpZ$_{Pph}$-interaction region on host protein.

When plant protein samples were probed with antiserum raised against the HrpZ$_{Pph}$ -binding peptide-3, the antiserum recognized small and acidic proteins in bean, tomato, parsley, and *Arabidopsis* leaf protein samples on both SDS-PAGE blots (**III, Figure 4A)** and native IEF gel blots (**III, Figure 4B**). HrpZ$_{Pph}$ did not bind to denatured proteins (data not shown) but did bind to an acidic bean protein under non-denaturing conditions (**III, Figure 4B)**. Furthermore this interaction was strengthened after extraction of the lipids from the bean sample, but HrpZ$_{Pph}$ did not bind the non-host proteins purified from tomato, parsley, and *Arabidopsis* under non-denaturing conditions (**III, Figure 4B)**. These data further support our conclusion that HrpZ$_{Pph}$ binds to a host protein.

From our results we conclude that HrpZ$_{Pph}$ binds in a host-specific manner to a small and acidic plant protein containing a peptide-3-like epitope.

E. CONCLUDING REMARKS

This thesis work reveals the route for construction of the Hrp pilus and delivery of the protein HrpZ$_{Pph}$ to host cells. The interaction of HrpZ$_{Pph}$ with the host cell target and the molecular mechanisms of its function have also been partly addressed. By answering the fundamental and longstanding question concerning the molecular mechanism of the inter-kingdom protein traffic and the bacterial pathogenicity, it will help us to eventually reveal new approaches for development of disease control.

The finding that the FLAG-tag is displayed on the pilus surface led us to investigate the possibility to use the Hrp pilus as a general epitope display tool. Our results clearly show that the Hrp pilus has potential to be used as an epitope display carrier, although there are some size and conformation limitations. The auto-assembling nature of the pilus also allows combination of different epitopes in the same polymeric structure. This could be an additional advantage of the Hrp pilus as an epitope carrier, compared with the traditional fimbrial and flagellar epitope display tools. In addition, the changing of the pilus surface properties after adding epitopes should help future structural studies of the pilus.

ACKNOWLEDGMENTS

This work was carried out at the Department of Biological and Environmental Sciences, Division of General Microbiology, University of Helsinki under supervision of Professor Martin Romantschuk and Docent Suvi Taira. I wish to express my gratitude to Professor Kielo Haahtela, head of the department, and Professor Timo Korhonen, director of the division, for providing excellent work facilities and pleasant research atmosphere. I would also like to acknowledge the Graduate School of Biotechnology and Molecular Biology (GSBM) and University of Helsinki for financial supports.

I owe my deepest gratitude to my supervisors Professor Martin Romantschuk and Docent Suvi Taira for their enlightening guidance, generous support and constant encouragement during all stages of my PhD study. My sincere thanks go to Mara for granting me the opportunity to join his excellent research group and for leading me to the high-standard scientific research. My special thanks belong to Suvi, my direct supervisor, for her scientific supervision and personal friendship. It has been a great pleasure to have her as my supervisor. She is not only scientifically bright and broad-minded, but also a person of exceptional kindness and integrity.

I am grateful to Docent Elina Roine and Minna Pirhonen, members of my thesis follow-up committee, for their fruitful discussions and suggestions during the follow-up meeting. I want to express my sincere thanks to Docent Elina Roine and Dr. Stéfane Genin, my thesis reviewers, for their critical reading, valuable suggestions and insightful comments which have been very important for the improvement of this thesis.

I give my warmest thanks to all the former and present members of Mara's lab: Dr. Inga Sarand for the opportunity to work with her to start my microbiological research and for her friendship throughout all these years; Dr. Kim Yjälä for leading me to step in the *Sphingomonas* research; Anssi, Tristan, Elina, Minna, and Jukka for all their stimulating discussions and friendly working atmosphere.

I would like to thank other collaborators for their fruitful collaboration: Prof. John Mansfield, Dr. Ian Brown & Dr. Conrad Stevens of Imperial College, UK; Prof. Thorsten Nürnberger of University of Tübingen & Dr. Justin Lee of Institute of Plant Biochemistry of Halle, Germany; and Dr. Vesa P. Kontinen of KTL, Helsink.

My special appreciation goes to Lissu for instructing me on electron microscopy, Raili, Riitta and Merja for the excellent technical support, and all the other colleagues in YMBO who have helped me in a way or another over the years, Thank you!

I gratefully acknowledge Dr. Sara Butcher and Pasi Laurinmaki for their guidance on the cryo-EM, and the members in the EM unit: Eija, Arja, Mervi, Pirkko, for their friendship and great help whenever I needed.

I am thankful to Dr. Erkki Raulo and Dr. Anita Tienhaara, the coordinator and the secretary of GSBM, respectively, and Dr. Eeva Sievi, the coordinator of Viikki Graduate School of Biosciences, for organizing excellent courses and international symposiums.

I am also thankful to China Scholarship Council (CSC) and the Education Section of China Embassy in Finland for awarding me the prize for "Outstanding Overseas Chinese PhD Students" in 2004.

I address my great thanks to my close friends: Tian Li, Wei Hong, Xuejiao, Xiaolu, Xuqian for their great help whenever I needed and nice company during free time.

I am utmost indebted to my parents for not only bringing the life but also giving me unlimited support and love! 亲爱的爸爸妈妈，正是从您们身上，我理解了什么是：爱的伟大，爱的无私! I am wholeheartedly grateful to my younger brothers and sisters-in-law: Xiaodong & Yingying, Xiaoguang & Bingbing for their love!

Last but not least, my deepest gratitude goes to my husband Deyin for his love and everlasting support and to our lovely children Willi & Nancy for making my life wonderful, and I never doubt that they are my greatest achievement!

Chunmei Li

Helsinki, March 2007

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