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Substrate Selectivity and Molecular Adaptation in the Outer Membrane Proteases of *Yersinia pestis* and *Salmonella enterica*

Johanna Haiko

Division of General Microbiology

Department of Biosciences

Faculty of Biological and Environmental Sciences

and

Viikki Graduate School in Molecular Biosciences

University of Helsinki

Academic dissertation

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Supervisor	Professor Timo Korhonen, Ph.D. Department of Biosciences Faculty of Biological and Environmental Sciences University of Helsinki, Finland
Reviewers	Docent Pentti Kuusela, M.D., Ph.D. Department of Bacteriology and Immunology Haartman Institute University of Helsinki, Finland
	Professor Mikael Skurnik, Ph.D. Department of Bacteriology and Immunology Haartman Institute University of Helsinki, Finland
Opponent	Associate Professor Dieter Schifferli, Dr. Med. Vet., Ph.D. Department of Pathobiology School of Veterinary Medicine University of Pennsylvania, Philadelphia, USA
Custos	Professor Kielo Haahtela, Ph.D. Department of Biosciences Faculty of Biological and Environmental Sciences University of Helsinki, Finland

Cover figure: Pla structure

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List of original publications

The thesis is based on the following articles, which are referred with their Roman numerals.

I **Johanna Haiko**, Liisa Laakkonen, Katri Juuti, Nisse Kalkkinen, and Timo K. Korhonen. The omptins of *Yersinia pestis* and *Salmonella enterica* cleave the reactive center loop of plasminogen activator inhibitor 1. ***Journal of Bacteriology*, 2010, 192:4553-61**.

II Mercedes Valls Serón, **Johanna Haiko**, Timo K. Korhonen, Philip G. de Groot, and Joost C.M. Meijers. Thrombin-activatable fibrinolysis inhibitor is degraded by *Salmonella enterica* and *Yersinia pestis*. ***Journal of Thrombosis and Haemostasis,* 2010, 8:2232-40.**

III **Johanna Haiko**, Maini Kukkonen, Janne J. Ravantti, Benita Westerlund-Wikström, and Timo K. Korhonen. The single substitution I259T, conserved in the plasminogen activator Pla of pandemic *Yersinia pestis* branches, enhances fibrinolytic activity. ***Journal of Bacteriology*, 2009, 191:4758-66**.

IV **Johanna Haiko**, Liisa Laakkonen, Benita Westerlund-Wikström, and Timo K. Korhonen. Molecular adaptation of a plant-bacterium outer membrane protease towards plague virulence factor Pla. **Submitted manuscript**.

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Johanna Haiko's contribution to the articles:

I Cleavage and inactivation assays; participation in planning and writing

II Initial observations with *E. coli*; experiments with *Y. pestis* and *S. enterica*; participation in planning and writing

III Initial observation; laboratory experiments; participation in planning and writing

IV Laboratory experiments; participation in planning and writing

Summary

Proteolysis is important in bacterial pathogenesis and colonization of animal and plant hosts. In this work I have investigated the functions of the bacterial outer membrane proteases, omptins, of *Yersinia pestis* and *Salmonella enterica*. *Y. pestis* is a zoonotic pathogen that causes plague and has evolved from gastroenteritis-causing *Yersinia pseudotuberculosis* about 13 000 years ago. *S. enterica* causes gastroenteritis and typhoid fever in humans. Omptins are transmembrane β-barrels with ten antiparallel β-strands and five surface-exposed loops. The loops are important in substrate recognition, and variation in the loop sequences leads to different substrate selectivities between omptins, which makes omptins an ideal platform to investigate functional adaptation and to alter their polypeptide substrate preferences. The omptins Pla of *Y. pestis* and PgtE of *S. enterica* are 75% identical in their amino acid sequences. Pla is a multifunctional protein with proteolytic and non-proteolytic functions, and it increases bacterial penetration and proliferation in the host. Functions of PgtE increase migration of *S. enterica in vivo* and bacterial survival in mouse macrophages, thus enhancing bacterial spread within the host.

Mammalian plasminogen/fibrinolytic system maintains the balance between coagulation and fibrinolysis and participates in several cellular processes, e.g., cell migration and degradation of extracellular matrix proteins. This system consists of activation cascades, which are strictly controlled by several regulators, such as plasminogen activator inhibitor 1 (PAI-1), α_2-antiplasmin (α_2AP), and thrombin-activatable fibrinolysis inhibitor (TAFI).

This work reveals novel interactions of the omptins of *Y. pestis* and *S. enterica* with the regulators of the plasminogen/fibrinolytic system: Pla and PgtE inactivate PAI-1 by cleavage at the reactive site peptide bond, and degrade TAFI, preventing its activation to TAFIa. Structure-function relationship studies with Pla showed that threonine 259 of Pla is crucial in plasminogen activation, as it prevents degradation of the plasmin catalytic domain by the omptin and thus maintains plasmin stability. In this work I constructed chimeric proteins between Pla and Epo of *Erwinia pyrifoliae* that share 78% sequence identity to find out which amino acids and regions in Pla are important for its functions. Epo is neither a plasminogen activator nor an invasin, but it degrades α_2AP and PAI-1. Cumulative substitutions towards Pla sequence turned Epo into a Pla-like protein. In addition to threonine 259, loops 3 and 5 are critical in plasminogen activation by Pla. Turning Epo into an invasin required substitution of 31 residues located at the extracellular side of the Epo protein above the lipid bilayer, and also of the β1-strand in the N-terminal transmembrane region of the protein. These studies give an example of how omptins adapt to novel functions that advantage their host bacteria in different ecological niches.

Abbreviations

aa	Amino acid
α_2AP	α_2-antiplasmin
BHI	Brain-heart infusion
ε-ACA	ε-aminocaproic acid
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
HGT	Horizontal gene transfer
IgG	Immunoglobulin G
IPTG	Isopropyl-β-D-thiogalactopyranoside
IS	Insertion sequence
kb	Kilobase pairs
kDa	Kilodalton
LD_{50}	Lethal dose 50
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-assisted laser desorption ionization – time of flight
MMP	Matrix metalloproteinase
MS	Mass spectrometry
NADH	Nicotinamide adenine dinucleotide
PAI	Plasminogen activator inhibitor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDB	Protein Data Bank
RCL	Reactive center loop
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SCV	*Salmonella* containing vacuole
SNP	Single-nucleotide polymorphism
SPI	*Salmonella* pathogenicity island
T3SS	Type III secretion system
TAFI(a)	(Activated) thrombin-activatable fibrinolysis inhibitor
TBS	Tris-buffered saline
TFPI	Tissue factor pathway inhibitor
TIMP	Tissue inhibitor of metalloproteinase
TLR4	Toll-like receptor 4
tPA	Tissue-type plasminogen activator
uPA	Urokinase-type plasminogen activator
Vn	Vitronectin
WHO	World Health Organization
wt/vol	weight/volume
Å	Ångström

1 Introduction

This thesis work addresses how two bacterial pathogens, *Yersinia pestis* and *Salmonella enterica,* interfere with the human fibrinolytic cascade. The significance of proteolysis in bacterial pathogenesis is generally approved, and proteolytic activity is widespread among pathogens (Travis *et al.*, 1995; Lantz, 1997; Potempa *et al.*, 2000). Bacterial proteases target several host factors during infection to gain nutrients, to lyse host tissues directly or indirectly, to activate or inactivate host protease cascades, to attenuate host defense, or to process other bacterial virulence factors, such as toxins (reviewed in Travis *et al.*, 1995; Lantz, 1997). Examples of proteolytic bacteria include *Legionella pneumophila*, streptococci, staphylococci, and species in the genera *Neisseria*, *Clostridium*, and *Vibrio.* Proteolysis has also been shown to be important in *Y. pestis* pathogenesis (reviewed in Lantz, 1997; Degen *et al.*, 2007). Proteolytic functions related to pathogenesis of *S. enterica* have been studied less, and *S. enterica* is usually not regarded as a proteolytic organism (Lähteenmäki *et al.*, 2005a; Ramu *et al.*, 2007; Ramu *et al.*, 2008; Yun *et al.*, 2009). Both *Y. pestis* and *S. enterica* express outer membrane proteases, omptins, on their surface (see chapter 1.4 below), and in this study the targets and functions of these and other omptins were investigated.

Bacterial infection begins when the bacteria come in contact with the host, bypass the host's immune defenses and start to proliferate. Invasive bacterial pathogens pass the first line of defense, such as skin or mucosal layers, and migrate from the primary infection site deeper into tissues where they multiply. In addition to physical barriers, the mammalian protection includes leukocytes, the normal microbial flora, and the complement system. Also the haemostatic system, which regulates the balance between coagulation and fibrinolysis, participates in the battle: fibrin clots are formed after tissue damage or inflammation, and they restrain the migration of the bacteria in the host. Some pathogenic bacteria have evolved to utilize the haemostatic system in order to penetrate through host tissue barriers and to circumvent the host defense mechanisms (Lähteenmäki *et al.*, 2005b). Also commensal and probiotic bacteria have been shown to interact with the haemostatic system, suggesting that these interactions provide benefit for these species, possibly by enabling colonization or by providing nutrients (Antikainen *et al.*, 2007; Candela *et al.*, 2007; Hurmalainen *et al.*, 2007). The interactions of the bacteria with the plasminogen/fibrinolytic system and the role of these interactions in bacterial infections are discussed in more detail in chapter 1.3.

1.1 *Yersinia pestis*

Y. pestis is the causative agent of plague, a disease that has killed millions of humans in three pandemics (Justinian, 6th-8th centuries; the Black Death, 14th-19th centuries; modern, 19th century onwards) and is still enzootic in the endemic plague areas. According to World Health Organization (WHO), there are about 2 000 cases and 200 deaths per year, mostly in Africa and Asia, although the actual number may be higher due to the lack of diagnostics and statistics in remote areas. Because the occurrence of human cases and local epidemics has increased during the last decades, plague has been classified as a re-emerging disease (WHO). The genomics of *Y. pestis* is of broad interest because it is considered a model species of rapid evolution of a severe bacterial pathogen (Wren, 2003), discussed more detailed below. Also the threat of bioterrorism and identification of strains with antibiotic resistance have raised concern and motivated plague research recently (Galimand *et al.*, 1997; Inglesby *et al.*, 2000; Galimand *et al.*, 2006; Welch *et al.*, 2007).

Plague is a zoonosis transmitted mainly by rodents and their fleas. Its endemic reservoirs occur in Asian, African, and American rodent populations. Also other mammalian species, including humans, are susceptible to plague infection. Mammalians get *Y. pestis* infection via a bite by an infected blood-sucking insect vector, usually flea. The bacteria block the flea's foregut, and in an attempt to suck blood from mammals, the starving flea injects the bacteria under the mammalian skin (Bacot & Martin, 1914). *Y. pestis* penetrates through tissues from the primary infection site to lymph nodes where it proliferates, causing bubonic plague. Bacterial proliferation causes swollen lymph nodes, called buboes. Another route of infection is via respiratory droplets from an infected mammal to another. The bacteria spread to lungs within the droplets and multiply causing primary pneumonic plague. The third form of plague is primary septicaemic plague, where a flea injects the bacteria directly into a blood vessel (Sebbane *et al.*, 2006a). Secondary pneumonic or septicaemic plague occurs if the bacteria spread from buboes to lungs or to the blood stream, respectively.

The main symptoms of bubonic plague are fever, headache, and the formation of buboes. The first symptoms of pneumonic plague resemble that of flu. In septicaemic plague the bacteria spread in the blood, liver, spleen, and other organs. The symptoms are similar to other Gram-negative sepsis and finally lead to excessive bleeding. The mortality in untreated septicaemic and pneumonic plague is almost 100% and about 50% in untreated bubonic plague. The antibiotic treatment with streptomycin, tetracycline, or gentamicin is usually effective if started at the early stage of infection.

The genus *Yersinia* belongs to the family *Enterobacteriaceae* and consists of 15 species, of which three are human pathogens: *Y. pestis, Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*. *Y. pseudotuberculosis* and *Y. enterocolitica* reside in the environment, and

consumption of contaminated food or water causes gastroenteritis, a localized infection that rarely requires antibiotic treatment. The evolution of *Y. pestis* is quite a recent event: *Y. pestis* has diverged from its ancestor, *Y. pseudotuberculosis* serotype O:1b, only about >13 000 years ago (Achtman *et al.*, 1999; Skurnik *et al.*, 2000; Achtman *et al.*, 2004; Achtman, 2008). *Y. enterocolitica* is more distantly related to these two species (Achtman *et al.*, 1999). The disease outcome, the host range, and the capability to use a flea vector differentiate *Y. pestis* from *Y. pseudotuberculosis*. Despite the phenotypic differences, the hybridization studies showed already thirty years ago that the genomes of these two species are over 90% identical (Bercovier *et al.*, 1980). Several *Y. pestis* and *Y. pseudotuberculosis* genomes have been sequenced to date (Parkhill *et al.*, 2001a; Deng *et al.*, 2002; Chain *et al.*, 2004; Song *et al.*, 2004; Chain *et al.*, 2006; Eppinger *et al.*, 2007; Garcia *et al.*, 2007; Eppinger *et al.*, 2009; Eppinger *et al.*, 2010), and they have revealed that *Y. pestis* has gained new genes via horizontal (or lateral) gene transfer (HGT), and that its genome has undergone rearrangements and accumulated several pseudogenes compared to *Y. pseudotuberculosis* (reviewed in Wren, 2003).

The genome of *Y. pestis* consists of a chromosome and three virulence plasmids, a 70-kb pCD (or pYV), a 96-kb pMT1, and a 9.5-kb pPCP1 (Ferber & Brubaker, 1981). pCD is common to all human-pathogenic yersiniae, and it encodes several virulence determinants, e.g., type III secretion system (T3SS; reviewed in Cornelis *et al.*, 1998). The other two plasmids are *Y. pestis*-specific and have probably been acquired via HGT; overall, *Y. pestis* has gathered only a few virulence factors and they are mostly encoded in plasmids (Prentice *et al.*, 2001; Wren, 2003). Atypical GC content is an indication of the acquisition of DNA via HGT (Parkhill *et al.*, 2001a; Song *et al.*, 2004; Liang *et al.*, 2010). pMT1 shares more than 50% of its sequence with pHCM2, a plasmid of *S. enterica* serovar Typhi CT18, suggesting common ancestry (Parkhill *et al.*, 2001b; Prentice *et al.*, 2001). pMT1 encodes two virulence factors: an antiphagocytic F1 capsule and a murine toxin that facilitates colonization of the flea (Ferber & Brubaker, 1981; Du *et al.*, 2002). pPCP1 contains genes encoding the plasminogen activator Pla, a crucial virulence factor (see chapter 1.4.1), pesticin, and the pesticin immunity protein (Ferber & Brubaker, 1981; Sodeinde & Goguen, 1988).

DNA microarray analysis of 22 *Y. pestis* and 10 *Y. pseudotuberculosis* genomes revealed the abundance of insertion sequence (IS) elements in *Y. pestis* genome compared to *Y. pseudotuberculosis* (Hinchliffe *et al.*, 2003). The amount of IS elements in *Y. pestis* genome enables homologous recombination and genomic rearrangements, i.e., insertions and inversions, that can make genes nonfunctional (Parkhill *et al.*, 2001a; Deng *et al.*, 2002; Radnedge *et al.*, 2002; Chain *et al.*, 2004; Song *et al.*, 2004; Garcia *et al.*, 2007; Gu *et al.*, 2007; Darling *et al.*, 2008). About 5% of *Y. pestis* genes have been inactivated by point mutations, insertions, or deletions (Chain *et al.*, 2004). For example, *inv* and *yadA*, whose expression is crucial for invasion and adhesion of *Y. pseudotuberculosis*, are inactive in

Y. pestis: *inv* contains an IS element and *yadA* a frameshift mutation (Rosqvist *et al.*, 1988; Simonet *et al.*, 1996). *Ure* operon in *Y. pestis* contains a premature stop codon in *ureD* and is inactivated; urease is crucial for *Y. pseudotuberculosis* as it contributes nitrogen production from urea (Sebbane *et al.*, 2001). *Y. pestis* produces rough lipopolysaccharide (LPS) with short O side chain, as the O side chain gene cluster in *Y. pestis* genome is inactivated by insertions and deletions (Skurnik *et al.*, 2000; Prior *et al.*, 2001). LPS consists of lipid A, core region, and O side chain. Smooth LPS protects several pathogens, including *Salmonella*, by providing serum resistance (Joiner *et al.*, 1982; Rautemaa & Meri, 1999). On the other hand, smooth LPS hinders the function of surface proteins, such as Pla (Kukkonen *et al.*, 2004; see chapter 1.4.1), and could thus be disadvantageous to *Y. pestis*. Lipid A provokes the host immune response because it is recognized by Toll-like receptor 4 (TLR4; Miller *et al.*, 2005). *Y. pestis* expresses tetra-acylated lipid A at 37 °C, and this type of LPS does not activate TLR4; hence inflammatory response is not provoked (Kawahara *et al.*, 2002; Montminy *et al.*, 2006; Dentovskaya *et al.*, 2008; Matsuura *et al.*, 2010). *Y. pestis* has lost several gene products as they have become pseudogenes, a typical feature of certain type of pathogens, such as *Rickettsia prowazekii* and *Mycobacterium leprae* (Andersson *et al.*, 1998; Wren, 2000; Parkhill *et al.*, 2001a; Moran, 2002; Chain *et al.*, 2004; Song *et al.*, 2004; Tong *et al.*, 2005; Chain *et al.*, 2006; Pallen & Wren, 2007; Ahmed *et al.*, 2008). Pseudogenes accumulate in the genome when the bacterium adapts to new environment and genes that become dispensable are spontaneously inactivated; this is abundant in obligate intracellular pathogens (reviewed in Wren, 2000). The pseudogene amount in *Y. pestis* is lower than in obligate intracellular bacteria but nevertheless reflects the recent change in the ecological niche of this bacterium (Wren, 2000; Wren, 2003).

Altogether, there are only minor differences in *Y. pestis* and *Y. pseudotuberculosis* genomes (Wren, 2003). *Y. pestis* could actually be classified as a subspecies of *Y. pseudotuberculosis*, but due to historical and practical reasons and because their ecological niches and diseases they cause are so different, it is still considered a distinct species (Bercovier *et al.*, 1980; Achtman *et al.*, 1999).

Y. pestis has been subdivided into biovars that differ in their abilities to ferment glycerol and reduce nitrate to nitrite. The three classical biovars, Antiqua, Medievalis, and Orientalis, are thought to be associated with the three plague pandemics (Devignat, 1951; Guiyoule *et al.*, 1994). However, this remains controversial as novel molecular analyses have shown that biovars are not monophyletic and do not directly correlate to molecular groupings that are based on synonymous single-nucleotide polymorphisms (SNPs; Achtman *et al.*, 2004; Drancourt *et al.*, 2004; Drancourt *et al.*, 2007). Pestoides isolates belong to biovar Antiqua and are atypical and ancestral *Y. pestis* because they can ferment rhamnose and melibose and sometimes lack the pPCP1 plasmid (Achtman *et al.*, 2004; Anisimov *et al.*, 2004; Garcia *et al.*, 2007; Bearden *et al.*, 2009). There is also a novel fourth biovar, Microtus, which belongs

to biovar Medievalis on the basis of glycerol fermentation and nitrate reduction (Song *et al.*, 2004). However, Microtus differs phenotypically from Medievalis as it cannot utilize arabinose and because it is thought to be avirulent for humans (Song *et al.*, 2004). Microtus has several large deletions in its genome and a unique pseudogene distribution, and it is considered ancient form of *Y. pestis* (Achtman *et al.*, 2004; Song *et al.*, 2004; Zhou *et al.*, 2004a; Zhou *et al.*, 2004b; Zhou *et al.*, 2004c). Frequency of SNPs in *Y. pestis* is low; however, the ancient isolates, such as Microtus and Pestoides, contain larger amounts of SNPs, indicating that they have longer evolutionary history (Achtman *et al.*, 2004; Eppinger *et al.*, 2010).

Different virulence factors are expressed during the plague life cycle in the flea and in the mammal. The temperature shift from the ambient temperature of the flea (20–25 °C) to mammalian 37 °C triggers the expression of several virulence factors, in addition to LPS alterations. Hemin storage (*hms*) locus is essential in the formation of a blockage in a flea (Hinnebusch *et al.*, 1996). Pigmentation (*pgm*) locus and a siderophore yersiniabactin (*ybt*) are important in utilization of host iron. T3SS encodes translocation machinery and secreted virulence factors LcrV and Yop effector proteins that are injected inside the host cells via the machinery. Fibrillar pH 6 antigen (PsaA) that is expressed at 37 °C at acidic pH inhibits phagocytosis and adheres to respiratory tract epithelial cells (Huang & Lindler, 2004; Liu *et al.*, 2006). *Y. pestis* is considered facultatively intracellular as it resides mainly extracellular during infection, but it can multiply within macrophages at the early stages of infection (Cavanaugh & Randall, 1959; Finegold, 1969; Janssen & Surgalla, 1969; Straley & Harmon, 1984; Charnetzky & Shuford, 1985). Plasminogen activator Pla has an important role in the pathogenesis of *Y. pestis*: it harnesses the mammalian plasminogen/fibrinolytic system in multiple ways, and this is dealt in chapter 1.4.1.

1.2 *Salmonella enterica*

Salmonella belongs to the family *Enterobacteriaceae* along with *Yersinia*. Both *S. enterica* and *Y. pestis* are invasive pathogens, and *Y. pestis* and gastroenteritis-causing *Salmonella* can infect rodents and cats. Both species have been used as bioweapons (Bhalla & Warheit, 2004). HGT has been an important event in the evolution of *Y. pestis* and *S. enterica* (Whittam & Bumbaugh, 2002; Porwollik & McClelland, 2003), and as described above for pMT1 and pHCM2, *S. enterica* serovar Typhi and the ancestor of *Y. pestis* have been partners in at least one HGT event, conceivably in a double-infected mammal or in soil. Both Typhi and *Y. pestis* have recently evolved from gastroenteritis-causing bacteria to more severe pathogens and can thus be used as paradigms of pathogen evolution. *S. enterica* and *Y. pestis* possess some virulence strategies in common, e.g., they both inject the effector proteins to their host cells through a T3SS (reviewed in Finlay & Falkow, 1997). In contrast to *Y. pestis,*

S. enterica is mainly intracellular during infection (Janssen & Surgalla, 1969; Ohl & Miller, 2001).

Genus *Salmonella* has diverged from the genus *Escherichia* about 120-160 million years ago (Ochman & Wilson, 1987). *Salmonella* has been divided into two species: *S. enterica* and *Salmonella bongori*. Only *S. enterica* is related to human disease; *S. bongori* is a reptile commensal. *S. enterica* contains six subspecies that are classified into serogroups and over 2 400 serovars that are divided on the basis of the O (LPS) and H (flagellar) antigens. *S. enterica* ssp. *enterica* causes 99% of human infections: serovars Typhimurium, Enteritidis, Typhi, and Paratyphi are the most common and most studied serovars. *S. enterica* serovars Typhimurium and Enteritidis cause localized gastroenteritis, and serovars Typhi and Paratyphi cause typhoid and paratyphoid fever, which are severe systemic infections. Serovars are genetically closely related but vary in their host specificities (Edwards *et al.*, 2002). Gastroenteritis-causing *Salmonella* is zoonotic, affecting also other species than humans, such as pigs, cattle, and chickens (Stevens *et al.*, 2009). On the contrary, serovar Typhi is a human-specific pathogen. Serovar Typhi has evolved quite recently, about 10 000-50 000 years ago (Kidgell *et al.*, 2002; Roumagnac *et al.*, 2006), and its genome has accumulated several pseudogenes (5%), inversions, transpositions, and insertions compared to non-typhoidal *S. enterica* (Liu & Sanderson, 1995; Parkhill *et al.*, 2001b; Baker & Dougan, 2007; Sabbagh *et al.*, 2010). One important virulence factor present in Typhi but absent in the non-typhoidal serovars is the Vi capsule that is responsible for serum resistance and inhibition of phagocytosis (Looney & Steigbigel, 1986; Hashimoto *et al.*, 1993).

S. enterica infection occurs via contaminated water or food, usually eggs or raw meat. Especially in a case of typhoid fever the disease can spread in a contact with an infected person or an asymptomatic carrier. According to WHO, *Salmonella*-gastroenteritis affects millions of people annually, especially in developing countries, and causes thousands of deaths, and Typhi affects 16-33 million people with 216 000 deaths per year. Gastroenteritis is usually mild and self-limiting, but the bacteria can spread to distant organs and cause systemic infection, particularly in immunocompromised patients (Coburn *et al.*, 2007). Typhoid fever requires antibiotic treatment, and vaccines made of attenuated bacteria or of Vi capsule are also available (Crump & Mintz, 2010). In mice, serovar Typhimurium causes systemic infection and similar symptoms as serovar Typhi in humans, and mice have been used to study systemic salmonellosis; however, a mouse model for *Salmonella*-gastroenteritis also exists (Boyle *et al.*, 2007).

S. enterica infection begins when the bacteria reach the ileum after oral ingestion. In the ileum, the bacteria enter the epithelium via M cells, epithelial cells, or dendritic cells. Bacteria adhere the cells via adhesins, which include several fimbrial types, and inject effector proteins inside the host cells via a T3SS, which is encoded in the *Salmonella*

pathogenicity island 1 (SPI-1; Ochman *et al.*, 1996; van der Velden *et al.*, 1998; Guo *et al.*, 2007). SPIs are chromosomal regions that encode several virulence factors, and are obtained via HGT and flanked by mobile elements, such as prophages (Groisman & Ochman, 1996; Vernikos & Parkhill, 2006). Altogether 21 SPIs have been recognized in *Salmonella* so far; SPI-1 is present in both *S. enterica* and *S. bongori*, but SPI-2 is lacking in *S. bongori* genome (Ochman & Groisman, 1996; Hensel *et al.*, 1997). There are also differences in SPI compositions in different *S. enterica* serovars (reviewed in Sabbagh *et al.*, 2010). SPI-1 genes are expressed in gut lumen, where the osmolarity is high and O_2 level is low (Bajaj *et al.*, 1996). SPI-1 T3SS effectors, such as SipA, SipC, SopB, and SopE, modify the host cell's actin cytoskeleton and disrupt tight junctions, which causes membrane ruffles and affects host signaling pathways, leading to bacterial endocytosis (Haraga *et al.*, 2008). SptP is responsible for the down-regulation of host signaling pathways and restoration of the actin cytoskeleton (Fu & Galán, 1998; Murli *et al.*, 2001), and AvrA inhibits activation of nuclear factor κB, hence decreasing the immune response (Collier-Hyams *et al.*, 2002). *S. enterica* is usually not regarded as a proteolytic organism, but increased *S. enterica*-induced proteolysis, caused by host and bacterial proteases, has been observed in infected macrophages and in the colon of infected rats (Lähteenmäki *et al.*, 2005a; Rodenburg *et al.*, 2007; Ramu *et al.*, 2008).

After traversing the epithelial cell barrier, *S. enterica* is subsequently phagocytosed by macrophages. Inside the cells the bacteria form a modified phagosome called *Salmonella*-containing vacuole (SCV), where they replicate (reviewed in Gorvel & Méresse, 2001). SPI-1-encoded PhoP/Q two-component regulatory system is activated inside murine macrophages (Alpuche Aranda *et al.*, 1992). PhoQ sensor on the outer membrane senses the acidification in phagosomes, which leads to increased expression of PhoP-activated genes and reduced expression of PhoP-repressed genes (Alpuche Aranda *et al.*, 1992). Genes encoded in SPI-2, e.g., another T3SS, are expressed at this phase of infection, when Mg^{2+} and phosphate concentrations are low (Deiwick *et al.*, 1999). SPI-2-encoded gene products prevent the fusion of SCV with lysosomes (Buchmeier & Heffron, 1991), protect the bacteria from reactive oxygen and nitrogen species, and hence enable the bacteria to survive and replicate inside macrophages and to cause systemic infection (Steele-Mortimer *et al.*, 1999; Gorvel & Méresse, 2001; Chakravortty *et al.*, 2002). Finally *S. enterica* induces the death of the host epithelial cells and macrophages by apoptosis and pyroptosis, respectively, releasing the bacteria that can then spread further to migrating macrophages. During the extracellular phase, the serum resistance caused by several factors, e.g., smooth LPS, and Rck that inhibits the membrane attack complex and binds the complement regulatory factor H, protects *S. enterica* from the attack of the complement system (Vandenbosch *et al.*, 1989; Heffernan *et al.*, 1992a; Heffernan *et al.*, 1992b; Sukupolvi *et al.*, 1992; Murray *et al.*, 2003; Ho *et al.*, 2010). *S. enterica* spreads inside circulating macrophages via blood stream into mesenteric

lymph nodes, spleen, liver, and other organs, to cause systemic disease (Brown *et al.*, 2006; Mastroeni *et al.*, 2009).

1.3 Plasminogen/fibrinolytic system and its function in bacterial infections

Human plasminogen/fibrinolytic system regulates blood coagulation and fibrin clot degradation after injury, but it also participates in several other processes, such as cellular migration, tissue remodeling and repair, ovulation, and embryo implantation (reviewed in Rijken & Lijnen, 2009). There is a subtle homeostatic balance between coagulation and fibrinolysis: uncontrolled coagulation causes thrombosis, and unrestrained fibrinolysis leads to excessive bleeding. The plasminogen/fibrinolytic system consists of activation cascade with several proteins that are tightly controlled by specific activators and inhibitors (Fig. 1). Also the synthesis of the participating factors is controlled by, e.g., growth factors, hormones, cytokines, and cyclic nucleotides (Lijnen & Collen, 1995; Lantz, 1997). The homeostasis of this system is disrupted in several cancer types and also during invasive bacterial infections (Degen, 1999).

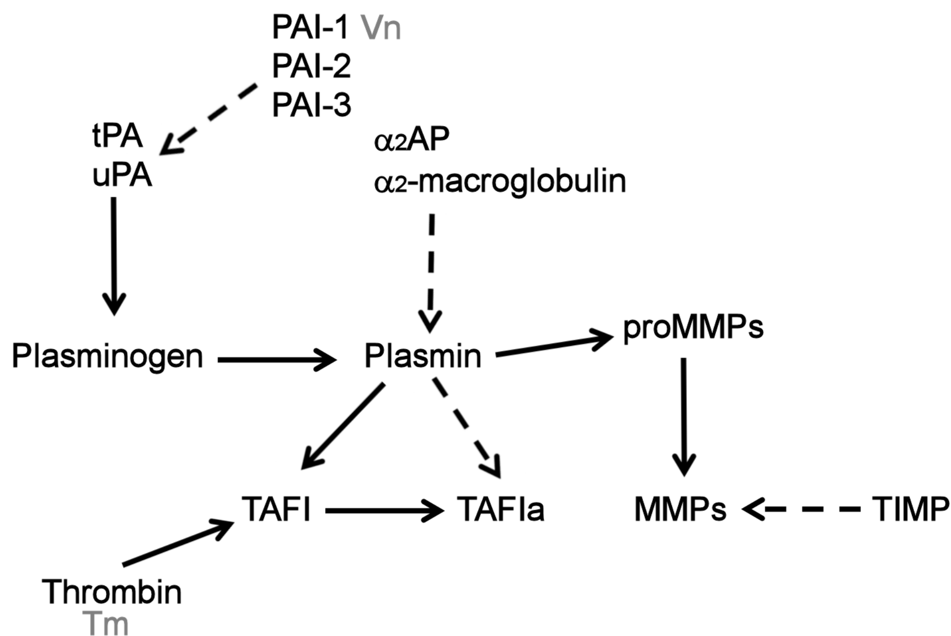

Figure 1. Overview of the plasminogen/fibrinolytic system. Arrow with solid line indicates activation; arrow with dashed line indicates inactivation. Cofactor proteins that do not directly participate in fibrinolysis are shown in gray. α_2AP, α_2-antiplasmin; MMPs, matrix metalloproteinases; PAI, plasminogen activator inhibitor; TAFI(a), (activated) thrombin-activatable fibrinolysis inhibitor; TIMP, tissue inhibitor of metalloproteinase; Tm, thrombomodulin; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; Vn, vitronectin.

1.3.1 Plasminogen and plasmin

Plasminogen is an abundant precursor of plasmin with a concentration of 2 μM in plasma (Rijken & Lijnen, 2009). Plasmin is a broad-spectrum serine protease that participates in

degradation of extracellular matrix (ECM) proteins fibrin, fibronectin, vitronectin and laminin, and activation of pro-matrix metalloproteinases (proMMPs) to MMPs (Lijnen & Collen, 1995; Myöhänen & Vaheri, 2004). MMPs degrade collagens and gelatin in ECM to enable cellular migration (Parks *et al.*, 2004). Plasminogen is activated to plasmin in humans by two serine proteases, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), by cleavage between R561-V562 peptide bond (Summaria *et al.*, 1967a). Fibrin enhances tPA activity, and tPA is the major plasminogen activator in fibrinolysis, whereas uPA functions mostly at cell surfaces via uPA receptor that is present in several cell types, including monocytes (Hoylaerts *et al.*, 1982; Blasi *et al.*, 1986; Ellis *et al.*, 1989; Ellis *et al.*, 1991).

Plasmin consists of two chains that are linked with disulfide bonds: a heavy chain with five kringle domains and a light chain containing the catalytic Ser-His-Asp triad (Robbins *et al.*, 1967; Summaria *et al.*, 1967b; Sottrup-Jensen *et al.*, 1975; Peisach *et al.*, 1999; Wang *et al.*, 2000). Native plasminogen (Glu-plasminogen) exists in tight conformation, has glutamic acid at the N-terminus, and can be converted to Lys-plasminogen that has more relaxed conformation and is a preferred substrate for the plasminogen activators (reviewed in Castellino & Ploplis, 2005). The kringle domains contain the lysine and aminohexyl binding sites that mediate the attachment of plasminogen to fibrin and to cell surfaces (Miles *et al.*, 1988; Wu *et al.*, 1990). The kringle domains also contain the binding sites for plasmin inhibitors, main inhibitor α_2AP and α_2-macroglobulin (Collen, 1976; Moroi & Aoki, 1976; Müllertz & Clemmensen, 1976; Frank *et al.*, 2003).

Plasminogen/fibrinolytic system is a target for several bacterial pathogens that cause invasive infections (reviewed in Lähteenmäki *et al.*, 2005b; Sun, 2006; Bergmann & Hammerschmidt, 2007; Degen *et al.*, 2007). The bacteria utilize human plasminogen/fibrinolytic system in order to penetrate through tissues or to escape from the attack of the immune system (Tillett & Garner, 1933; Mullarky *et al.*, 2005; Sun, 2006; Bergmann & Hammerschmidt, 2007; Beaufort *et al.*, 2008; Crane *et al.*, 2009; Rijken & Lijnen, 2009; Clinton *et al.*, 2010). Bacteria can activate plasminogen either proteolytically or via complex formation, or they can bind plasminogen via plasminogen receptors leading to subsequent activation by host activators tPA and uPA (Bergmann & Hammerschmidt, 2007; Table 1). Streptococcal streptokinase and staphylokinase of *Staphylococcus aureus* are the most studied bacterial plasminogen activators, but in contrast to tPA, uPA, and *Y. pestis* Pla, they do not cleave plasminogen. Instead, streptokinase forms a complex with plasminogen, which induces conformational changes that result in activation of other plasminogen molecules (Buck *et al.*, 1968). Staphylokinase uses similar strategy, but it requires fibrin as a cofactor, and the complex is not protected from inhibition by α_2AP in contrast to streptokinase (Lijnen *et al.*, 1992; Lottenberg *et al.*, 1992; Bergmann & Hammerschmidt, 2007). The importance of staphylokinase during infection is unclear (Kwiecinski *et al.*, 2010). Most of the bacteria

listed in Table 1 are mainly extracellular during infection, but also *Francisella tularensis,* a mainly intracellular pathogen, has been shown to interact with plasminogen and plasmin, which leads to degradation of opsonizing antibodies and to bacterial penetration through ECM (Crane *et al.*, 2009; Clinton *et al.*, 2010). In addition to pathogens, also commensal species in the genus *Lactobacillus* express plasminogen receptors on their surface, resulting in enhanced tPA-mediated plasminogen activation, which may help colonization and gain of nutrients in the intestinal tract (Antikainen *et al.*, 2007; Hurmalainen *et al.*, 2007).

Table 1. Examples of pathogenic bacterial interactions with the plasminogen/fibrinolytic system.

Bacterial species	Protein	Function	Reference
Yersinia pestis	Pla	Plasminogen activation, inactivation of α_2AP	Sodeinde *et al.*, 1992; Kukkonen *et al.*, 2001
Porphyromonas gingivalis	Trypsin-like protease	Plasminogen activation, inactivation of α_2AP and α_2-macroglobulin	Grenier, 1996
Streptococci, *Staphylococcus aureus*	Streptokinase, staphylokinase	Plasminogen activation	Lack, 1948; Davidson, 1960; Lottenberg *et al.*, 1992
Streptococci, *Borrelia burgdorferi, Escherichia coli, Francisella tularensis, Haemophilus influenzae, Helicobacter pylori, Neisseria gonorrheae, Neisseria meningitidis, Salmonella enterica, Staphylococcus aureus*	Plasminogen receptors	Plasminogen binding to activate plasminogen	Kuusela & Saksela, 1990; Parkkinen *et al.*, 1991; Kuusela *et al.*, 1992; Ullberg *et al.*, 1992; Fuchs *et al.*, 1994; Sjöström *et al.*, 1997; Kukkonen *et al.*, 1998; Pantzar *et al.*, 1998; Clinton *et al.*, 2010
Staphylococcus aureus	Aureolysin	Activation of pro-uPA, inactivation of PAI-1 and α_2AP	Beaufort *et al.*, 2008
Bacillus subtilis	Subtilisin NAT	Inactivation of PAI-1	Urano *et al.*, 2001
Pseudomonas aeruginosa	LasB (PsE)	Activation of pro-uPA and proMMP-2, inactivation of PAI-1	Boudier *et al.*, 2005; Beaufort *et al.*, 2010

α_2AP, α_2-antiplasmin; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor 1; uPA, urokinase-type plasminogen activator

1.3.2 Plasminogen activator inhibitor 1

The balance of coagulation and fibrinolysis is critical for normal homeostasis, and therefore the plasminogen/fibrinolytic system is strictly controlled by specific activators and inhibitors whose function leads to either promotion or inhibition of fibrinolysis (see Fig. 1). As the majority of the enzymes involved in the plasminogen/fibrinolytic cascade are serine proteases, their inhibitors are serine protease inhibitors, serpins. tPA and uPA are inhibited by plasminogen activator inhibitors (PAI) 1-3, of which PAI-1 is the primary inhibitor (Kruithof *et al.*, 1984; Rijken & Lijnen, 2009). PAI-1 is synthesized by several mammalian cell types,

and its concentration in plasma is 400 pM (Rijken & Lijnen, 2009). In addition to fibrinolysis, PAI-1 regulates cell migration by modulating ECM, regulating cell adhesion, and modifying the formation of chemoattractant gradients (Deng *et al.*, 1996; Czekay *et al.*, 2003; Marshall *et al.*, 2003). PAI-1 levels are increased in patients with gastric cancer, severe pneumonia, or sepsis (Pralong *et al.*, 1989; Günther *et al.*, 2000; Kaneko *et al.*, 2003; Zeerleder *et al.*, 2006). LPS has been shown to upregulate PAI-1 expression in murine lungs, leading to neutrophil recruitment (Arndt *et al.*, 2005). PAI-1 expression is also stimulated by inflammatory cytokines (reviewed in Kruithof, 2008).

PAI-1 is a glycoprotein of 50 kDa (43 kDa as a recombinant protein without glycosylation) and 379 amino acid residues. Similarly to other serpins, PAI-1 consists of three β-sheets and nine α-helices (Gils & Declerck, 1998). PAI-1 exists in three different conformations: active, latent, and cleaved form (Hekman & Loskutoff, 1985; Declerck *et al.*, 1992; Mottonen *et al.*, 1992; Aertgeerts *et al.*, 1995; Sharp *et al.*, 1999; Nar *et al.*, 2000; Stout *et al.*, 2000; Jensen & Gettins, 2008; Dewilde *et al.*, 2009). Active PAI-1 inactivates tPA and uPA by inserting its reactive center loop (RCL) into the catalytic center of the plasminogen activator and forming a covalent 1:1 complex with it. RCL is about 20-amino-acid-long flexible loop that contains the reactive site peptide bond R346-M347, which is accessible in the active conformation (Sharp *et al.*, 1999; Nar *et al.*, 2000; Stout *et al.*, 2000). Conversion of active PAI-1 into a latent, inactive form includes large conformational changes: the N-terminus of RCL inserts into a central β-sheet A where the RCL is inaccessible (Mottonen *et al.*, 1992). Latent PAI-1 can become activated by treatment with denaturing agents (Hekman & Loskutoff, 1985). PAI-1 can also become cleaved at RCL between R346-M347 by its substrates, leading to conformation that resembles the latent form (Aertgeerts *et al.*, 1995; Dewilde *et al.*, 2009). The actual significance of the cleavage is unclear, but it might be related to the regulation of PAI-1 (Declerck *et al.*, 1992).

Active PAI-1 is spontaneously converted to a latent form in only 2-3 hours at 37 °C, but in a complex with vitronectin (Vn), PAI-1 is stabilized in its active conformation, and its half-life is increased by about 2.5 fold (Declerck *et al.*, 1988; Lindahl *et al.*, 1989). Vn is an abundant (4 µM) plasma glycoprotein that exists in a single-chain (78 kDa) and a two-chain (65 kDa and 10 kDa) form (Preissner *et al.*, 1985; Preissner, 1991). Vn binds to PAI-1 with the N-terminal high-affinity binding site somatomedin B domain and the C-terminal hemopexin-like domain, and the binding site of Vn in PAI-1 is located in the β-strand 1A and the helices C and E, at another end of the molecule than the RCL (Lawrence *et al.*, 1994; Nar *et al.*, 2000; Arroyo De Prada *et al.*, 2002; Jensen *et al.*, 2002; Schroeck *et al.*, 2002; Zhou *et al.*, 2003; Schar *et al.*, 2008a; Schar *et al.*, 2008b). Vn decreases the latency transition of PAI-1 by affecting the conformation of RCL so that it is fully exposed and more optimally located to cleave tPA and uPA (Li *et al.*, 2008).

PAI-1 can be inhibited with antibodies or small-molecule inhibitors, but their affinity is often low and many of them cannot inactivate PAI-1 when it is bound to Vn (Gils *et al.*, 2002; Crandall *et al.*, 2004; Elokdah *et al.*, 2004; Gopalsamy *et al.*, 2004; Liang *et al.*, 2005; Izuhara *et al.*, 2008). PAI-1 inhibitors are of interest because of their potential use in the treatment of thrombosis and different cancer types.

PAI-1 levels are increased in lungs during severe *Klebsiella pneumoniae* infection in mice, in gastric epithelial cells during *H. pylori* infection, and in *Salmonella*-infected Caco-2 cells (Park *et al.*, 1997; Renckens *et al.*, 2007; Keates *et al.*, 2008; Kenny *et al.*, 2008). PAI-1 deficiency in knockout mice is related to impaired host defense against *K. pneumoniae*, and overexpression of PAI-1 protects mice from *K. pneumoniae* (Renckens *et al.*, 2007). Secreted bacterial proteases, serine protease subtilisin NAT of *Bacillus subtilis,* metalloprotease aureolysin of *S. aureus,* and metalloprotease/elastase LasB (PsE) of *Pseudomonas aeruginosa,* have been observed to degrade PAI-I (Urano *et al.*, 2001; Boudier *et al.*, 2005; Beaufort *et al.*, 2008; Beaufort *et al.*, 2010). *S. aureus* thus harnesses the plasminogen/fibrinolytic system in several ways: staphylokinase activates plasminogen, and aureolysin activates pro-uPA and inactivates PAI-1 and α_2AP (Beaufort *et al.*, 2008). Also, LasB, a virulence factor of *P. aeruginosa*, has been shown to interact with the plasminogen system in many ways (Boudier *et al.*, 2005; Beaufort *et al.*, 2010).

1.3.3 Thrombin-activatable fibrinolysis inhibitor

TAFI, a.k.a. plasma procarboxypeptidase B/R/U, is a 56 kDa glycoprotein with 401 amino acids, and belongs to the family of zinc-containing metallocarboxypeptidases (Campbell & Okada, 1989; Hendriks *et al.*, 1989; Hendriks *et al.*, 1990; Eaton *et al.*, 1991; Wang *et al.*, 1994; Bajzar *et al.*, 1995). TAFI is a precursor that in its active form, TAFIa, regulates fibrinolysis and participates in inflammatory processes by hydrolyzing bradykinin, osteopontin, and complement components C3a and C5a (Shinohara *et al.*, 1994; Myles *et al.*, 2003; reviewed in Leung *et al.*, 2008). TAFI is synthesized in the liver and circulates in plasma at a concentration of 75 nM (Eaton *et al.*, 1991; Rijken & Lijnen, 2009).

TAFI is converted to active TAFIa by cleavage of the activation peptide between R92-A93, and the remaining 309 amino acids form the catalytic domain (Eaton *et al.*, 1991). TAFI can be activated by plasmin, thrombin, trypsin and neutrophil elastase (Eaton *et al.*, 1991; Marx *et al.*, 2009). The activation of TAFI by thrombin can be accelerated by about 1250 fold with thrombomodulin, and this complex is probably the physiological activator of TAFI (Bajzar *et al.*, 1996). Thrombin is a procoagulant protein, and thus TAFI represents a link between fibrinolysis and coagulation. TAFIa inhibits fibrinolysis by removing C-terminal lysines from fibrin (Wang *et al.*, 1998). C-terminal lysines are the binding sites for plasminogen and tPA, and their removal prevents plasmin generation by tPA, leading to reduced lysis of fibrin clots (Wang *et al.*, 1998). The half-life of TAFIa is about 10 min at 37 °C because of an

“instability region” in its structure, and TAFIa is rapidly inactivated to TAFIai by temperature-induced conformational change, or proteolytically by plasmin at arginines 302, 327, and 330 (Wang *et al.*, 1994; Marx *et al.*, 2000; Marx *et al.*, 2002; Anand *et al.*, 2008). TAFIa can also be inhibited by lysine and arginine analogs, carboxypeptidase inhibitor, chelating and reducing compounds, synthetic inhibitors, and anti- or nanobodies; however, no physiological inhibitors are known (Wang *et al.*, 1994; Lazoura *et al.*, 2002; Barrow *et al.*, 2003; Mao *et al.*, 2003; Polla *et al.*, 2004; Suzuki *et al.*, 2004; Wang *et al.*, 2007; Develter *et al.*, 2009; Buelens *et al.*, 2010).

Not much is known about the role of TAFI in bacterial infections, but TAFI levels are decreased in sepsis patients (Zeerleder *et al.*, 2006). In contrast, increased gastric TAFI levels have been observed in patients infected with *H. pylori,* and it has been speculated that TAFI may be a protective factor against *H. pylori* (Ikeda *et al.*, 2009). *Streptococcus pyogenes* binds TAFI on its surface by the collagen-like surface proteins SclA and SclB, which might lead to increased fibrin formation and protection of the bacteria from the host immune system (Påhlman *et al.*, 2007).

1.4 Omptin family

Omptins are widely spread outer membrane proteases of the Gram-negative bacteria. Omptins are highly similar in their structures and share at least 40% sequence identity. The evolutionary tree of omptins is shown in Fig. 2. Omptin genes are thought to have transferred via HGT, as many of the genes identified to date are encoded in plasmids, some in conjugative or mosaic plasmids. Of the chromosomal omptin genes, *ompT* of *E. coli* is located on a prophage and *pgtE* of *S. enterica* is flanked by IS elements. *ompT* is encoded also in a plasmid, denoted $ompT_P$, and the mature amino acid sequences of these OmpT variants differ by about 25%.

OmpT of *Escherichia coli* has been crystallized about ten years ago (Vandeputte-Rutten *et al.*, 2001), and the structures of the other omptins have been modeled on the basis of OmpT coordinates. The crystal structure of *Y. pestis* Pla became available during preparation of this thesis, revealing that the overall structure is highly similar to OmpT but considerable differences are found in the loops (Eren *et al.*, 2010). Omptins are monomeric β-barrels with ten antiparallel β-strands, four periplasmic turns and five surface-exposed loops (Vandeputte-Rutten *et al.*, 2001; Fig. 3). Omptin barrel size is about 70 Å in length, of which 40 Å is located above the lipid bilayer, determined by the two girdles of hydrophobic aromatic amino acids (Vandeputte-Rutten *et al.*, 2001). Mature omptins have 290-300 amino acids, and they have conserved catalytic residues D84, D86, D206, and H208 (Pla numbering) that form the active site groove (Kramer *et al.*, 2001; Vandeputte-Rutten *et al.*, 2001).

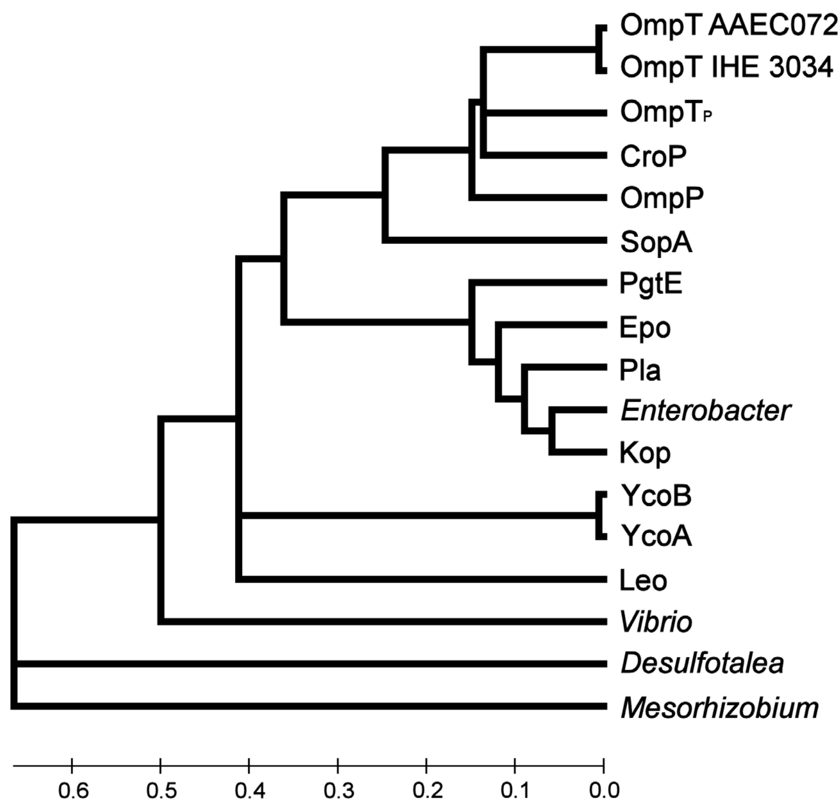

Figure 2. Cladogram of the omptin family (Haiko *et al.*, 2010; published with the permission of the American Society for Microbiology). Phylogenetic tree was created with the MEGA4 program (Tamura *et al.*, 2007). The evolutionary distances are in the units of the number of amino acid substitutions per site. The most characterized omptins are presented with their protein names, the uncharacterized are presented with the names of their host bacteria. Different OmpT variants are the chromosomal OmpT of *E. coli* strains AAEC072 and IHE 3034, and $OmpT_P$ denotes the plasmid OmpT of IHE 3034.

The proteolytic mechanism of omptins combines features of serine and aspartic proteases: D206-H208 dyad activates nucleophilic water that hydrolyses the scissile peptide bond of the substrate, and D84-D86 couple might participate in coordinating the water molecule (Kramer *et al.*, 2001; Vandeputte-Rutten *et al.*, 2001). The orientation of D84-D86 couple is similar to that in the aspartic proteases, but it participates in activating the water molecule only indirectly (Kramer *et al.*, 2001). Omptins also lack the consensus sequence characteristic of aspartic proteases, and their protein fold is different from typical aspartic proteases (Kramer *et al.*, 2001). The orientation of D206-H208 catalytic dyad resembles that of Ser-His-Asp triad of serine proteases, but the proposed catalytic S99 is too far to form the catalytic triad with D206 and H208 (Kramer *et al.*, 2001; Vandeputte-Rutten *et al.*, 2001). Although omptins were first classified as serine proteases and are nowadays usually regarded as aspartic proteases, they do not easily fit into either class on the basis of the OmpT and Pla crystal structures, and they probably form a protease class of their own (Kramer *et al.*, 2001; Vandeputte-Rutten *et al.*, 2001; Eren *et al.*, 2010).

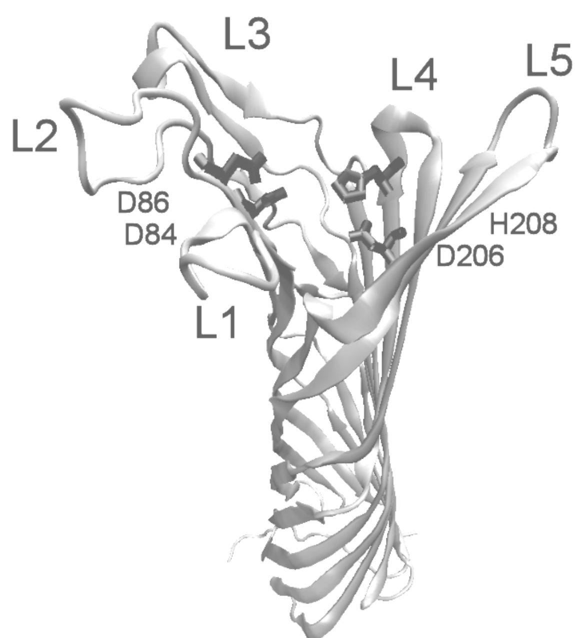

Figure 3. Structure of Pla of *Y. pestis,* seen as a tilted side view. Catalytic amino acids D84, D86, D206, and H208 are shown. L1-L5 denote the surface loops. The structure was visualized with the VMD program (Humphrey *et al.*, 1996) and is based on the crystal structure of Pla D86A (Protein Data Bank [PDB] code 2X4M; Eren *et al.*, 2010), where A86 was manually substituted to aspartate.

To be active and functional, omptins require rough LPS (Kramer *et al.*, 2000; Kramer *et al.*, 2002; Kukkonen *et al.*, 2004; Brandenburg *et al.*, 2005; Pouillot *et al.*, 2005; Suomalainen *et al.*, 2010). Smooth LPS, which has long O side chain, sterically hinders the substrate recognition of the omptin loops as the loops protrude just above the core region of LPS (Kukkonen *et al.*, 2004). Pla and OmpT bind LPS with Y134, E136, R138, and R171 (Pla numbering), but exactly how LPS induces the activity of omptins remains unknown, and LPS binding does not induce considerable conformational changes in OmpT or Pla (Kramer *et al.*, 2002; Brandenburg *et al.*, 2005; Eren *et al.*, 2010). It has been suggested that LPS binding induces pushing of the β7-strand inward, thus affecting the active site geometry in L4 (Eren *et al.*, 2010).

Omptins studied so far tend to cleave their substrates between basic amino acids (Dekker *et al.*, 2001; McCarter *et al.*, 2004; Hwang *et al.*, 2007; Agarkov *et al.*, 2008). OmpT prefers arginine or lysine at P1 and P1' positions, and also residues further from the cleavage site contribute to the substrate specificity of OmpT (Dekker *et al.*, 2001; McCarter *et al.*, 2004). OmpP is similar in its preferences towards arginine and lysine, but it can also cleave between the S-R peptide bond (Hwang *et al.*, 2007). However, the preferred polypeptide substrates of omptins differ and their functions are diverse, mainly due to the differences in their surface loops (reviewed in Kukkonen & Korhonen, 2004; Hritonenko & Stathopoulos, 2007; Haiko *et al.*, 2009). The purported virulence-associated functions identified for the most characterized omptins, OmpT of *E. coli*, Pla of *Y. pestis*, and PgtE of *S. enterica*, are listed in Table 2. In addition, Pla has been shown to degrade *Yersinia* outer membrane proteins (Yops), but it is unknown whether this is related to virulence (Sodeinde *et al.*, 1988). Degradation of antimicrobial peptides is common to all these three omptins: OmpT degrades protamine of salmon milt, Pla degrades human LL-37 and also rCRAMP and RBD-1 from rat bronchoalveolar lavage fluid, and PgtE cleaves synthetic C18G (Stumpe *et al.*, 1998; Guina *et al.*, 2000; Galván *et al.*, 2008). OmpT, Pla, and PgtE also degrade tissue-factor pathway

inhibitor (TFPI), an anticoagulant protein (Yun *et al.*, 2009). Some studies have indicated that also OmpT is a plasminogen activator (Mangel *et al.*, 1994; Varadarajan *et al.*, 2008), but according to studies by our group, it activates plasminogen only poorly (Kukkonen *et al.*, 2001). Comparison of the crystal structures of Pla and OmpT provides explanation for the observed difference: loops 3, 4, and 5 that participate in recognition of plasminogen differ markedly between Pla and OmpT (Eren *et al.*, 2010). *ompT* gene has been shown to be present at high frequency in *E. coli* strains that cause urinary tract infections (Foxman *et al.*, 1995), but the pathogenetic role of OmpT remains unknown and it has rather been thought to be a housekeeping protease (Kukkonen & Korhonen, 2004). The functions of Pla and PgtE and their contribution to the virulence of their host bacteria are described in chapters 1.4.1 and 1.4.2.

Table 2. Some functions of Pla of *Y. pestis*, PgtE of *S. enterica*, and OmpT of *E. coli*.

Function	Pla	PgtE	OmpT	Reference(s)
Plasminogen activation	+	+/−	−	McDonough & Falkow, 1989; Sodeinde & Goguen, 1989; Sodeinde *et al.*, 1992; Kukkonen *et al.*, 2001; Kukkonen *et al.*, 2004
Inactivation of α_2AP	+	+	−	Kukkonen *et al.*, 2001; Lähteenmäki *et al.*, 2005a
Degradation of antimicrobial peptides	+	+	+	Stumpe *et al.*, 1998; Guina *et al.*, 2000; Galván *et al.*, 2008
Degradation of complement components	+	+	−	Sodeinde *et al.*, 1992; Ramu *et al.*, 2007; unpublished
Degradation of gelatin	−	+	−	Ramu *et al.*, 2008
Activation of proMMP-9	−	+	−	Ramu *et al.*, 2008
Degradation of TFPI	+	+	+	Yun *et al.*, 2009
Adhesion to laminin, ECM, and eukaryotic cells	+	+	−	Kienle *et al.*, 1992; Lähteenmäki *et al.*, 1998; Lähteenmäki *et al.*, 2001; Kukkonen *et al.*, 2004
Invasion into human endothelial and epithelial cells	+	−	−	Cowan *et al.*, 2000; Lähteenmäki *et al.*, 2001

+/−, poor activation; α_2AP, α_2-antiplasmin; ECM, extracellular matrix; proMMP-9, pro-matrix metalloproteinase-9; TFPI, tissue-factor pathway inhibitor

The functions of the other omptins have been studied less. OmpP of *E. coli* has been shown to degrade SecY, a component of the protein translocation machinery (Matsuo *et al.*, 1999). SopA (IcsP) of *Shigella flexneri* is required for polar localization of IcsA (VirG) on the bacterial surface, and is important in intracellular actin-based motility of *Shigella* (Egile *et al.*, 1997). CroP of *Citrobacter rodentium* increases the resistance of its host to α-helical antimicrobial peptides and is regulated by the PhoP/Q system (Le Sage *et al.*, 2009). Leo (Lpa) of *L. pneumophila* slowly activates plasminogen (Vranckx *et al.*, 2007). Chromosomally encoded YcoA of *Y. pestis* has been suggested to be a virulence factor in infection of *Caenorhabditis elegans* (Styer *et al.*, 2005). Epo (PlaA) of a pear blight pathogen

E. pyrifoliae is closely related to Pla, and it is encoded on a mosaic plasmid pEP36 that has homology to a virulence plasmid pEA29 of *Erwinia amylovora*, but the functions of Epo are unknown (McGhee *et al.*, 2002).

1.4.1 *Yersinia pestis* Pla

Plasminogen activator Pla is a multifunctional virulence factor of *Y. pestis*. The *pla* gene is carried by the virulence plasmid pPCP1, and *pla* deletion increases the LD_{50} value in subcutaneously infected mice millionfold (Sodeinde *et al.*, 1992). On the contrary, the deletion does not cause detectable change in the LD_{50} when the mice are infected intravenously or intraperitoneally (Sodeinde *et al.*, 1992). This indicates that Pla is important at the first stage of infection when the bacteria reach lymph nodes by penetrating through tissue barriers (Sodeinde *et al.*, 1992). In primary septicaemic plague Pla is dispensable (Sebbane *et al.*, 2006a). During pneumonic plague Pla promotes bacterial replication in lungs: proteolytically active Pla is essential in *Y. pestis* proliferation but unnecessary in bacterial dissemination, and thus Pla does not increase bacterial motility in pneumonic plague (Lathem *et al.*, 2007). *pla* deletion mutants cause reduced inflammatory response in mice lungs, and the infection does not progress to the pro-inflammatory phase (Lathem *et al.*, 2007).

pla is conserved and its predicted amino acid sequence is 100% identical in all pandemic *Y. pestis* branches (Rawlings *et al.*, 2008). *pla*-encoding pPCP1 is usually absent from Pestoides strains; these strains can still disseminate into lymph nodes, but the infection does not proceed (Welkos *et al.*, 1997; Worsham & Roy, 2003; Garcia *et al.*, 2007; Revazishvili *et al.*, 2008; Eppinger *et al.*, 2010). The sequences of biovar Microtus and an atypical Pestoides isolate Angola contain *pla* whose predicted amino acid sequence has substitution T259I at L5 (Song *et al.*, 2004; Eppinger *et al.*, 2010). Microtus and Angola are thought to represent the ancient *Y. pestis* branches because their genomic features are highly similar to *Y. pseudotuberculosis* in contrast to modern *Y. pestis* isolates (Achtman *et al.*, 2004; Song *et al.*, 2004; Eppinger *et al.*, 2010).

Pla exists in a premature form and in three different mature isoforms: α, β, and γ (Sodeinde & Goguen, 1988; Sodeinde *et al.*, 1988; Kutyrev *et al.*, 1999). The premature form (ca. 35 kDa in SDS-PAGE) is unprocessed and contains the signal peptide that is cleaved when the protein is inserted into the outer membrane. The biological relevance of the different mature forms is unknown. α-Pla (ca. 33 kDa) and γ-Pla (ca. 31 kDa) are thought to be full-length proteins with different conformations, and these isoforms have been observed also with OmpT and PgtE (Kramer *et al.*, 2000; Kukkonen *et al.*, 2001; Kukkonen *et al.*, 2004). Conformational differences are thought be the reason for the observed different migration of unboiled protein samples in the gel, because the β-barrel fold is stable and tolerates

detergents (Nakamura & Mizushima, 1976; Kramer *et al.*, 2000). β-Pla (ca. 33 kDa) is the cleaved form that has been autoprocessed at K262 at L5 (Kukkonen *et al.*, 2001).

The functions of Pla include both proteolytic and non-proteolytic functions, as listed in Table 2. Proteolytic and non-proteolytic activities are distinct, since proteolytically inactive Pla mutant, where the catalytic amino acid D206 has been substituted with alanine, retains its adhesive and invasive abilities (Lähteenmäki *et al.*, 2001). Pla-mediated adhesion enables the bacteria to target tissue barriers, and fibrinolytic actions help the bacteria to penetrate through them. *Y. pestis* harnesses the human plasminogen/fibrinolytic system by using the proteolytic functions of Pla. Pla activates plasminogen to plasmin in a similar way as human plasminogen activators tPA and uPA, i.e., by cleavage between R561-V562 (Sodeinde *et al.*, 1992). Pla also inhibits the plasmin inhibitor α_2AP. Plasminogen activation and inactivation of α_2AP lead to uncontrolled plasmin activity. The importance of the plasminogen/fibrinolytic system in plague infection is highlighted by the fact that plasminogen deficient mice are more resistant to *Y. pestis* infection, although their general health is poor (Goguen *et al.*, 2000; Degen *et al.*, 2007). Fibrinogen deficiency, in contrast, does not provide any survival benefit (Degen *et al.*, 2007). Increased fibrin deposition and accumulation of inflammatory cells are observed in the lungs and the liver of mice infected with *pla*-negative *Y. pestis* intranasally or intravenously (Degen *et al.*, 2007; Lathem *et al.*, 2007). This indicates that the expression of Pla causes degradation of fibrin and decreased neutrophil recruitment, which leads to diminished inflammatory response (Degen *et al.*, 2007; Lathem *et al.*, 2007).

The degradation of complement component C3 by Pla may inhibit the chemoattractation of leukocytes and increase the serum resistance of *Y. pestis;* however, also *pla*-negative *Y. pestis* remain serum resistant (Sodeinde *et al.*, 1992). The inactivation of TFPI and the subsequent increase in coagulation were proposed to occur at a different stage of infection than the fibrinolysis-promoting functions, and it may protect the bacteria from the human immune system at some stage of infection (Yun *et al.*, 2009). Invasiveness might be needed when *Y. pestis* spreads from the infection site to lymph nodes, spleen, and liver, and further to other organs (Lähteenmäki *et al.*, 2001). It has not been determined whether binding to laminin enhances invasiveness of Pla, and it is not known whether Pla-mediated invasion into endothelial cells is polar, i.e., occurs at apical or basolateral surface of the cells. Pla has been shown to use DEC-205 receptor to be phagocytosed by macrophages (Zhang *et al.*, 2008). Altogether Pla is a multifunctional protease with several targets, and it also possesses various non-proteolytic functions. Together these functions increase the proteolysis of host tissues that enables bacterial penetration and proliferation.

Pla expression is transcriptionally regulated by the cyclic adenosine monophosphate receptor protein (Kim *et al.*, 2007). Pla is slightly up-regulated at 37 °C compared to 25 °C (Motin *et*

al., 2004; Chromy *et al.*, 2005). Pla is highly expressed inside buboes of infected rats (Sebbane *et al.*, 2006b), and although Pla is expressed also at high extent in fleas, the proteolytic activity of Pla is higher in cells cultivated at 37 °C compared to cells grown at 25 °C, and the reason lies at least partly in the LPS structure (Han *et al.*, 2004; Lawson *et al.*, 2006; Suomalainen *et al.*, 2010; Vadyvaloo *et al.*, 2010). Lower acylation and lower substitution of aminoarabinose in lipid A at 37 °C correlate with higher proteolytic activity and the isoform pattern of Pla (Suomalainen *et al.*, 2010). *Y. pestis* is naturally rough, which enables the activity of Pla (Skurnik *et al.*, 2000; Prior *et al.*, 2001; Kukkonen *et al.*, 2004). Thus, efficient proteolysis by *Y. pestis* depends on both Pla and LPS. In a mouse model of pneumonic plague, expression of Pla is down-regulated after 48 h post infection (Lathem *et al.*, 2005).

1.4.2 *Salmonella enterica* PgtE

PgtE (also known as the E protein) of *S. enterica* shares about 75% sequence identity to Pla, and these two proteases belong to the Pla subfamily of omptins together with Epo of *E. pyrifoliae*, Kop (*K*lebsiella outer membrane protease) of *K. pneumoniae*, and the omptin of *Enterobacter* sp. (see Fig. 2). PgtE expression has been observed so far only in serovar Typhimurium, but conserved *pgtE* genes with >98% identity at amino acid level have been found in the genomes of other *S. enterica* serovars, including Typhi and Paratyphi (Rawlings *et al.*, 2008).

Similarly to Pla, PgtE displays three mature molecular forms, α (34 kDa), β (17 kDa), and γ (14 kDa; Kukkonen *et al.*, 2004). β and γ are not detected with the proteolytically inactive PgtE derivatives, and these forms are significantly smaller than the corresponding Pla forms, indicating that they are differently processed (Kukkonen *et al.*, 2004). The possible site for autoprocessing is R154 in L3: cleavage at this site leads to two peptides with approximate sizes of β- and γ-PgtE (Kukkonen *et al.*, 2004).

PgtE has been shown to increase the survival of *S. enterica* serovar Typhimurium in mice and in human serum and to promote bacterial growth inside vacuoles of murine macrophages, but it does not enhance the survival of the bacteria inside human macrophages or dendritic cells (Lähteenmäki *et al.*, 2005a; Pietilä *et al.*, 2005; Ramu *et al.*, 2007; Ramu *et al.*, 2008). Degradation of the key complement components by PgtE, function shared with Pla, may increase serum resistance of the bacteria that are released from macrophages and are not protected by smooth LPS (Ramu *et al.*, 2008). PgtE, unlike Pla, is able to promote gelatinase activity by directly degrading gelatin and indirectly by activating macrophage-secreted proMMP-9 to active gelatinase (Ramu *et al.*, 2008). Activation of proMMP-1, proMMP-8, and proMMP-9 has been previously observed also by the proteinases of *P. aeruginosa* and *Vibrio cholerae* (Okamoto *et al.*, 1997). Degradation of gelatin and increased activation of plasminogen might enable *S. enterica* to spread through tissues during the extracellular phase

when the bacteria are released from macrophages (Lähteenmäki *et al.*, 2005a; Ramu *et al.*, 2008). Enhanced cell migration also increases the likelihood of the contacts between the bacteria and macrophages (Lähteenmäki *et al.*, 2005a; Ramu *et al.*, 2008). Activation of MMPs may also be related to macrophage apoptosis and modulation of the inflammatory responses (Opdenakker *et al.*, 2001; Parks *et al.*, 2004; Tamura *et al.*, 2004). PgtE provides resistance to antimicrobial peptides, which is critical during colonization and inside macrophages (Guina *et al.*, 2000; Prost *et al.*, 2007; Santos *et al.*, 2009). Hence PgtE possesses several functions that are beneficial to *S. enterica* during its multiplication in the SCV, during macrophage migration, and when the bacteria are released from the macrophages.

pgtE belongs to a phosphoglycerate transfer operon, but PgtE is not essential in phosphoglycerate transport (Yu & Hong, 1986). The expression of *pgtE* is under SlyA control that is in turn regulated by the PhoP/Q system, and PgtE is highly expressed in SCVs inside macrophages (Guina *et al.*, 2000; Eriksson *et al.*, 2003; Lähteenmäki *et al.*, 2005a; Navarre *et al.*, 2005).

For *S. enterica*, smooth LPS is a virulence factor that protects the bacterium against the complement, as deletion of *wzz* genes that control the length of the O side chain attenuates the bacterium (Murray *et al.*, 2003). However, expression of PhoP/Q in the SCV induces modifications to the LPS structure: the length of the O antigen is reduced and aminoarabinose and 2-hydroxymyristate are added to a phosphate group of lipid A (Groisman *et al.*, 1997; Guo *et al.*, 1997; Gunn *et al.*, 1998). These changes in lipid A decrease the immunostimulatory effect of LPS and increase resistance to antimicrobial peptides (Guo *et al.*, 1997). Inside murine macrophages *S. enterica* serovar Typhimurium is rough, which enables the proteolytic function of PgtE (Guo *et al.*, 1997; Lähteenmäki *et al.*, 2005a).

1.5 Omptins as evolvable proteases

Protein evolvability is associated with broad substrate range, catalytic promiscuity, i.e., ability to catalyze different types of reactions, and mutational robustness (O'Loughlin *et al.*, 2006). Robust proteins tolerate mutations and can easily change their function under selective pressure (Wagner, 2005; Romero & Arnold, 2009). Because several proteolytic enzymes, e.g., *Bacillus* subtilisin, are important in biotechnology, their catalytic properties have been improved in several studies (reviewed in Yuan *et al.*, 2005; Kaur & Sharma, 2006). Nevertheless, few studies have been devoted to molecular adaptation of bacterial virulence factors.

Omptins are ideal proteins to study molecular adaptation: they form a large family and have diverse functions, suggesting that they are highly evolvable (Tracewell & Arnold, 2009). The β-barrel structure is robust and stable: it tolerates large deletions in the loops and periplasmic

turns without losing its membrane topology and orientation, as shown with the eight-stranded β-barrel OmpA (Ried *et al.*, 1994; Koebnik, 1999a; Koebnik, 1999b). The cleavage preference of OmpT of *E. coli* has been successfully changed from R-R bond towards non-basic amino acids (Varadarajan *et al.*, 2005; Varadarajan *et al.*, 2008). The polypeptide substrate selectivities of the omptins have also been altered by substitutions in the surface loops: OmpT has been turned into an efficient plasminogen activator and Pla into a gelatinase by creating OmpT-Pla and Pla-PgtE chimeras, respectively (Kukkonen *et al.*, 2001; Ramu *et al.*, 2008).

2 Aims of the study

At the beginning of this project it was already known that Pla of *Y. pestis* and PgtE of *S. enterica* interact with the human plasminogen/fibrinolytic system by activating plasminogen and by inactivating α_2AP, and PgtE in addition by activating proMMP-9. However, very little was known about possible interactions of omptins with the regulatory proteins of the plasminogen/fibrinolytic system; such interactions would have considerable impact on the infectious processes of *Y. pestis* and *S. enterica*. Kukkonen *et al.* (2001) and Ramu *et al.* (2008) had previously investigated the substrate selectivities and the structure-function relationships of Pla and PgtE by creating omptin chimeras. MEROPS database (Rawlings *et al.*, 2008) has continuously introduced new members for the omptin family, and the amount of omptins increases hand in hand with the growing number of finished bacterial sequencing projects. Omptins have spread by HGT, but molecular details of their evolution remain largely obscure. My studies can provide insight into omptin evolution in different host bacteria.

The goals of this thesis work were

1) to study the novel functions of omptins within the plasminogen/fibrinolytic system;

2) to investigate the substrate selectivity of Pla and to identify amino acids or regions of the protein that are important for its functions; and

3) to reveal novel aspects about the evolution of omptins.

3 Materials and methods

3.1 Bacterial strains (I-IV)

Bacterial strains used in this study are listed in Table 3.

Recombinant *E. coli* XL1 with pSE380 vector and its pMRK derivatives were grown at 37 °C in Luria Bertani supplemented with ampicillin (100 µg/ml), tetracycline (12.5 µg/ml) and glucose (0.2% wt/vol). For the expression of omptins, the strains were cultivated in Luria broth supplemented as described above, collected in PBS, and grown overnight on plates containing 5 µM isopropyl-β-D-thiogalactopyranoside (IPTG) and antibiotics as above.

Y. pestis KIM D27 and KIM D34 were cultivated over two nights at 37 °C on brain-heart infusion (BHI) plates, inoculated in 10 ml BHI broth supplemented with hemin (40 µg/ml) and cultivated twice over two nights at 37 °C. Recombinant *Y. pestis* strains were cultivated over two nights at 37 °C in BHI broth supplemented with hemin as above and with glucose and ampicillin as with *E. coli*. For the expression of Pla, the bacteria were collected and plated on BHI plates containing IPTG, hemin, and ampicillin as above, and grown over two nights.

S. enterica 14028R and 14028R-1 were cultivated overnight at 37 °C in PhoP/Q-inducing N-minimal medium (pH 7.4), supplemented with 38 mM glycerol, 0.1% casamino acids, 2 mg/ml thiamine, and 8 mM $MgCl_2$. *S. enterica* 14028R-1 complemented with pSE380 or pMRK3 were cultivated overnight at 37 °C in Luria broth supplemented with IPTG and ampicillin as above.

Y. pseudotuberculosis strains were cultivated overnight at 37 °C in 10 ml Luria; PB1 strains were supplemented with kanamycin (50 µg/ml). *E. coli* IHE 3034, *K. pneumoniae*, and *S. flexneri* were cultivated in Luria Bertani overnight at 37 °C. *E. pyrifoliae* was cultivated in Luria Bertani at 28 °C overnight in broth and over two nights on a plate.

For the assays, the bacteria were collected in phosphate-buffered saline (PBS; pH 7.1) unless otherwise indicated, pelleted, and adjusted to optical density (600 nm) of 1.2 (corresponding to ca. 10^9 cells/ml) or 2.0 (2×10^9 cells/ml).

Table 3. Bacterial strains used in this study.

Bacterial strain	Description	Article	Reference(s) or source
Erwinia pyrifoliae Ep1/96	Wild type	IV	Rhim *et al.*, 1999, DSMZ12162
Escherichia coli IHE 3034	Wild type, meningitis isolate	I	Pouttu *et al.*, 1999
Escherichia coli XL1 Blue MRF'	Δ(*mcrA*) 183 Δ(*mcrCB-hsdSMR-mrr*) 173 *endA1 supe44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB lacI*^{q}*ZΔ M15* Tn 10 (tet)], rough LPS	I- IV	Stratagene
Klebsiella pneumoniae 342	Wild type	I	Fouts *et al.*, 2008
Salmonella enterica serovar Typhimurium 14028R	Rough LPS derivative of 14028	I, II	Wick *et al.*, 1994
Salmonella enterica serovar Typhimurium 14028R-1	Δ*pgtE* derivative of 14028R	I, II	Lähteenmäki *et al.*, 2005a
Shigella flexneri M90T	Wild type	I	Sansonetti *et al.*, 1982
Yersinia pestis KIM D27	pPCP1$^+$ Δ*pgm* pYV$^+$ derivative of *Y. pestis* KIM-10	I, II	Finegold *et al.*, 1968; Une & Brubaker, 1984
Yersinia pestis KIM D34	pPCP1$^-$ Δ*pgm* pYV$^+$ derivative of *Y. pestis* KIM-10	I, II, III	Finegold *et al.*, 1968; Une & Brubaker, 1984
Yersinia pseudotuberculosis IP 32953	Wild type	I	Chain *et al.*, 2004
Yersinia pseudotuberculosis PB1 Δ*wb* pYV$^+$	Rough derivative of PB1, with the 20 kb *ddhD-wzz* chromosomal fragment deleted	I	J. A. Bengoechea
Yersinia pseudotuberculosis PB1 Δ*wb* pYV$^-$	Rough derivative of PB1, with the 20 kb *ddhD-wzz* chromosomal fragment deleted, cured of pYV	I	Kukkonen *et al.*, 2004

3.2 Recombinant DNA techniques (I, III, IV)

Plasmid constructs used in this study are listed in Table 4. The omptin genes were cloned with polymerase chain reaction (PCR) using DNA from *E. coli* IHE 3034 (*ompT*), *E. pyrifoliae* Ep1/96 (*epo*), *K. pneumoniae* 342 (*kop*), *S. flexneri* M90T (*sopA*), *Y. pestis* KIM D34 (*ycoA*), and *Y. pseudotuberculosis* IP 32953 (*ycoB*) as templates. The omptin mutants and hybrids were cloned with recombinant PCR using the internal primer pair including the substitutions. The genes were cloned in the pSE380 vector under the IPTG-inducible *trc* promoter and transformed into an appropriate bacterial strain. The nucleotide sequences of the plasmid constructs were verified by sequencing.

Table 4. Plasmid constructs used in this study.

Plasmid construct	Description	Article	Reference
pSE380	Expression vector, *trc* promoter, *lacO* operator, *lacI, bla*	I- IV	Invitrogen
pMRK1	*pla* in pSE380	I- IV	Kukkonen *et al.*, 2001
pMRK2	*ompT* of *E. coli* AAEC072 in pSE380	I	Kukkonen *et al.*, 2001
pMRK2b	Chromosomal *ompT* of *E. coli* IHE 3034 in pSE380	I	This study
pMRK2c	Plasmid *ompT* of *E. coli* IHE 3034 in pSE380	I	This study
pMRK3	*pgtE* in pSE380	I-III	Kukkonen *et al.*, 2004
pMRK4	*epo* in pSE380	IV	This study
pMRK7	*sopA* in pSE380	I	This study
pMRK8	*ycoA* in pSE380	I	This study
pMRK9	*ycoB* in pSE380	I	This study
pMRK10	*kop* in pSE380	I	This study
pPlaT259I	Microtus *pla* in pSE380	III	This study
pMRK111	*pla,* D206A	I, II	Kukkonen *et al.*, 2001
pMRK1.51	*pla*, ^{259}TIDKN→IIDKT	III	Ramu *et al.*, 2008
pMRK31	*pgtE*, D206A	I, II	Kukkonen *et al.*, 2004
pMRK3.51	*pgtE*, ^{259}IIDKT →TIDKN	III	This study
pMRK105	Pla with 5 aa from Epo: **^{161}KGVRV→NQRPG**	IV	This study
pMRK405	Epo with 5 aa from Pla: **^{159}NQRPG→KGVRV**	IV	This study
pMRK110	Pla with 10 aa from Epo: ^{161}KGVRV→NQRPG, **^{262}KN→TI, ^{268}VSIG→ASLD**	IV	This study
pMRK410	Epo with 10 aa from Pla: ^{159}NQRPG→KGVRV, **^{260}TI→KN, ^{266}ASLD→VSIG**	IV	This study
pMRK117	Pla with 17 aa from Epo: **^{35}ETG→SNA, ^{88}MNE→QNS, ^{155}YT→SV**, ^{161}KGVRV→NQRPG, ^{262}KN→TI, ^{268}VSIG→ASLD	IV	This study
pMRK417	Epo with 17 aa from Pla: **^{33}SNA→ETG, ^{86}QNS→MNE, ^{153}SV→YT**, ^{159}NQRPG→KGVRV, ^{260}TI→KN, ^{266}ASLD→VSIG	IV	This study
pMRK431	Epo with 31 aa from Pla: ^{33}SNA→ETG, ^{86}QNS→MNE, ^{153}SV→YT, ^{159}NQRPG→KGVRV, ^{260}TI→KN, ^{266}ASLD→VSIG, **^{26}KELV→HEML, ^{101}GTDVNY→ATNVNH, I257T, H79N, D92E, S210D, S167N, D219G, E249D, G22S**	IV	This study
pMRK442	Epo with 42 aa (as in pMRK431 and N-terminal amino acids 1-45) and the signal sequence from Pla	IV	This study

Bold text indicates the novel substitutions compared to a previous hybrid; aa, amino acid

3.3 Protein and peptide detection

3.3.1 Detection of proteins with anti-omptin sera (III, IV)

Expression of Pla, Epo and their derivatives were analyzed from the cell envelope preparations or from the whole-cell samples. The cell envelope samples were prepared by sonicating the bacteria (10^9 cells/ml) in PBS on ice with 2.5 mM ethylene diamine tetra-acetic acid. The remaining cells were pelleted, and the supernatants were centrifuged further. The cell envelopes in the supernatants were pelleted and suspended in PBS and SDS-PAGE loading buffer. Whole-cell samples were prepared by mixing the bacteria (10^9 cells/ml) with SDS-PAGE loading buffer, and boiled. The samples were run in a 12% (wt/vol) SDS-PAGE gel, transferred onto a nitrocellulose membrane, and detected by Western blotting with anti-Pla-His_6-antisera (1:500), anti-Pla loop sera (1:500; Kukkonen *et al.*, 2001), or anti-Epo-His_6-antisera (1:1000), and with alkaline phosphatase-conjugated anti-rabbit IgG (1:1000; Dako) and phosphatase substrate.

3.3.2 Degradation of PAI-1/Vn complex, TAFI, plasminogen, and α_2AP (I-IV)

In general, the protein degradations were studied by mixing the protein with the bacteria in PBS (unless otherwise indicated), incubated at 37 °C for 2-8 hours, centrifuged, and analyzed the supernatants in 12% SDS-PAGE gels and by Western blotting with appropriate antibodies. The detection was done with alkaline phosphatase-conjugated anti-rabbit IgG (1:1000) and alkaline phosphatase substrate, except with plasminogen degradations.

Degradation of PAI-1/Vn complex was assessed as follows: 2.5 µg recombinant active human PAI-1 (American Diagnostica) was incubated with 5 µg human plasma Vn (Promega) for 1 h at 37 °C. The bacteria (4×10^7) were added and incubated with the proteins for 2-8 h. The detection was done with polyclonal anti-PAI-1 antibody (1:5000; Calbiochem) or with polyclonal anti-Vn antibody (1:1000; Calbiochem).

In TAFI degradation analysed with Western blotting, 820 nM TAFI (purified basically as in Marx *et al.*, 2000) was incubated for 2 h with the bacteria (4×10^7). Detection was done with polyclonal anti-TAFI antibody (1:2000; Mosnier *et al.*, 1998). To analyze the degradation in SDS-PAGE, 1.9 µM TAFI was incubated with the bacteria, adjusted in Tris-buffered saline (TBS, pH 7.4) as above and incubated in 100 mM Hepes/0.01% Tween-20 (pH 8.0). The samples were run in 10% SDS-PAGE gel that was stained with Coomassie Brilliant Blue. In experiments with ε-aminocaproic acid (ε-ACA; Sigma-Aldrich), its concentration was 5 mM.

Plasminogen degradation detected with anti-human plasminogen antibody (American Diagnostica) was assessed by incubating 2.5 µg human Glu-plasminogen and 4×10^7 bacteria for 2-7 h. The detection was done with polyclonal anti-human plasminogen antibody (1 mg/ml, diluted 1:1000), peroxidase-conjugated anti-rabbit IgG (1:2500; GE Healthcare),

and enhanced chemiluminescence (ECL) detection reagents (GE Healthcare) according to the manufacturer's instructions. Plasminogen cleavage detected with monoclonal anti-human plasminogen catalytic domain antibody (R&D Systems) was performed as above, with the following modifications: Glu-plasminogen (3 µg) and 4.8×10^7 bacteria were incubated for 3 h, and detected with the monoclonal antibody (500 µg/ml, diluted 1:500), peroxidase-conjugated anti-mouse IgG (GE Healthcare; 1:1000), and ECL detection reagents.

Degradation of human α_2AP (0.8 µg; Calbiochem) was assayed as detailed earlier (Kukkonen *et al.*, 2001; Lähteenmäki *et al.*, 2005a), with 3×10^7 bacteria and incubation time of 2 h.

3.3.3 Peptide analysis of degraded PAI-1 and TAFI (I, II)

The Pla- or PgtE-expressing *E. coli* XL1 were incubated with PAI-1 or TAFI at 37 °C for 2 h, except Pla with TAFI for 5 h. The samples were run in a 12% SDS-PAGE gel. For N-terminal sequencing the gel was blotted and sequenced by Edman degradation. For mass spectrometric analysis the samples were cut from the gel, digested with trypsin, and exposed to matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF-MS). The resulting peptides were analyzed with the Mascot search engine (www.matrixscience.com). To determine the exact cleavage site of PAI-1 by Pla and PgtE, the degradation products were isolated by reversed-phase chromatography and analyzed with MALDI-TOF-MS.

3.4 Protease activity assays

3.4.1 PAI-1 activity (I)

PAI-1 (0.5 µg) and Vn (1.0 µg) were incubated on 96-well microtitre plates (Nunc) in PBS for 1 h at 37 °C to form a complex. Bacteria (3.2×10^7 cells in PBS) were incubated with the complex for 4 h. High-molecular weight uPA (160 ng, 12.8 IU; American Diagnostica) was added and incubated for 30 min. Chromogenic uPA substrate pyro-Glu-Gly-Arg-p-nitroaniline hydrochloride (S-2444; 2.1 mg/ml; Chromogenix) was added and A_{405} nm was measured after 3 h. Significances were calculated with Student's *t* test.

3.4.2 TAFIa activation (II)

TAFI (3 µg/ml with *Y. pestis* and *S. enterica*; 10 µg/ml with *E. coli;* 40 µg/ml in time- and dose-dependent assays) was incubated with the bacteria (2×10^7 or 4×10^7 in TBS) in 100 mM Hepes/0.01% Tween-20 at 37 °C for 2 h. In experiments with ε-ACA, its concentration was 2.5 mM and TAFI concentration was 40 µg/ml. In plasma assays, 165 nM TAFI was added to 20 µl of three times diluted TAFI-depleted plasma and incubated with the bacteria (4×10^9) as above.

TAFIa activity was measured from the supernatants containing 40 nM TAFI by mixing 8 nM thrombin (from Dr. W. Kisiel, University of New Mexico, Albuquerque, NM) and 16 nM thrombomodulin (American Diagnostica) in 5 mM $CaCl_2$ and Hepes buffer as above, and incubating 15 min at room temperature. Reaction mixture (90 µl) containing 2.7 mM $MgSO_4$, 10.9 mM KCl, 30 µM H-D-Phe-Pro-Arg-chloromethylketone (Bachem), 2.4 mM phosphoenolpyruvate (Biopool AB), 0.5 mM nicotinamide adenine dinucleotide (NADH; Biopool AB), 2.7 mM adenosine triphosphate (Biopool AB), 6 mM hippuryl-Arg (Bachem), pyruvate kinase (45 µg/ml; Biopool AB), lactate dehydrogenase (15 µg/ml; Biopool AB), and excess of arginine kinase, was mixed with 10 µl of activated TAFI. TAFIa activity was measured as a loss of NADH absorbance at A_{340} nm.

3.4.3 Clot-lysis assay (II)

TAFI (10 µg/ml; 40 µg/ml in time- and dose-dependent assays) was incubated with the bacteria (2×10^7 or 4×10^7 in TBS) in 100 mM Hepes/0.01% Tween-20 at 37 °C for 2 h. The clot-lysis times were determined from the supernatants using purified system, where 10 nM thrombin, recombinant tPA (0.3 µg/ml; Biopool AB), 20 mM $CaCl_2$, and 5 nM thrombomodulin were mixed with TAFI (40 nM) in buffer (pH 7.4) containing 25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, and 0.1% (wt/vol) bovine serum albumin. Fibrinogen (4.5 µM; Kordia Life Sciences) and plasminogen (90 nM) were added, and the turbidity was measured at A_{405} nm. The clot-lysis time was determined as the time difference between the half-maximal lysis and the half-maximal clotting.

3.4.4 Plasminogen activation (III, IV)

Human Glu-plasminogen (4 µg or 0.4 µg) was mixed with 8×10^7 bacteria (in PBS). For cumulative analysis of initial plasmin formation, chromogenic plasmin substrate H-D-Val-Leu-Lys-p-nitroaniline dihydrochloride (S-2251; 2.5 mg/ml; Chromogenix) was added, and plasmin formation was measured at 37 °C at A_{405} nm. The time course of plasmin formation was analyzed similarly, except that the plasmin substrate was added at time points of 0 h, 1 h, 2 h, 5 h, 10 h, and 22 h, and the plasmin activity was measured after 15 min incubation.

3.4.5 Fibrinolysis plate method (III)

Human fibrinogen (1.73 mg/ml; depleted of plasminogen, von Willebrand factor, and fibronectin; Kordia Life Sciences) was suspended in H_2O with human Glu-plasminogen (5 µg/ml). Bovine thrombin (5 NIH units; MP Biomedicals) in 100 mM sodium borate buffer (pH 7.74) was added by gently mixing. The solution was poured onto a six-well plate (Nunc). Bacteria (10^7) in PBS were pipetted on the plate, and the plate was incubated at 37 °C overnight (*E. coli*) or over two nights (*Y. pestis*).

3.4.6 Inactivation of α_2AP and plasmin (III)

In α_2AP inactivation, human α_2AP (5.75 µg/ml) was incubated with the bacteria (8×10^7) in PBS at 37 °C for 2 h. Human plasmin (0.5 µg; Sigma-Aldrich) was then added and incubated for 30 min. In plasmin inactivation, the bacteria were incubated with plasmin for 30 min (amounts as above). Chromogenic plasmin substrate was then added, and plasmin activity was measured at A_{405} nm after 90 min.

3.5 Invasion into eukaryotic cells (IV)

Endothelial-like cell line ECV304 (T-24 urinary bladder carcinoma cell line; MacLeod *et al.*, 1999) was cultivated in Medium 199 (Gibco) supplemented with 10% fetal calf serum (Gibco) and 2 mM L-glutamine (Gibco) for 3-4 days at 37 °C with 5% CO_2. Bacterial invasion was studied using the gentamicin protection assay (Tang *et al.*, 1993). ECV304 cells were grown at a 24-well plate (Nunc) and washed with PBS. The bacteria (10^5) in Medium 199 were added onto the wells. The plate was centrifuged and incubated for 2 h at 37 °C with 5% CO_2. The wells were washed, and the extracellular bacteria were killed with gentamicin (100 µg/ml). Eukaryotic cells were lysed with 0.2% Triton X-100, and the number of intracellular bacteria was determined by viable counting. The amount of intracellular bacteria was compared to the amount of the bacteria incubated with the eukaryotic cells to calculate the invasion percentages.

3.6 Sequence alignment and protein structure models (I, III, IV)

Pairwise alignment of Pla and PgtE sequences was done with the needle program from the EMBOSS package (Rice *et al.*, 2000). The multiple sequence alignment of several omptins was done with ClustalW (Chenna *et al.*, 2003), and the secondary structure elements were determined on the basis of OmpT structure (Vandeputte-Rutten *et al.*, 2001), or in article IV, on the basis of Pla structure (Eren *et al.*, 2010). A cladogram presentation of the omptin family sequences was done with the MEGA4 program (Tamura *et al.*, 2007). The amino acid sequences were obtained from the NCBI GenBank protein database, and their accession numbers are: Pla AAA27667 (Sodeinde & Goguen, 1989); PgtE AAF85951 (Guina *et al.*, 2000); Epo YP_003208082 (Kube *et al.*, unpublished); Kop YP_002235876 (Fouts *et al.*, 2008); Enterobacter omptin YP_001176628 (Copeland *et al.*, unpublished); YcoA NP_670257 (Deng *et al.*, 2002); YcoB YP_069801 (Chain *et al.*, 2004); *E. coli* K-12 (AAEC072) OmpT CAA30008 (Grodberg *et al.*, 1988); *E. coli* IHE 3034 OmpT ADE89723 (Moriel *et al.*, 2010); IHE 3034 $OmpT_P$ HM210637; SopA AAC45084 (Egile *et al.*, 1997); OmpP BAA97899 (Kaufmann *et al.*, 1994); Leo YP_124757 (Cazalet *et al.*, 2004); *Vibrio fischeri* omptin AAW85741 (Ruby *et al.*, 2005); *Desulfotalea psychrophila* omptin YP_066438 (Rabus *et al.*, 2004); *Mesorhizobium loti* omptin NP_106686 (Kaneko *et al.*, 2000); CroP YP_003365570 (Petty *et al.*, 2010).

The Pla, Epo and Pla-Epo hybrid models were built with the Modeller program (Sali & Blundell, 1993) by using the OmpT structure (article I; PDB code 1i78) or Pla structure (article IV; PDB code 2x4m) as a template. PAI-1 RCL (residues 340-351) was modeled on the basis of the existing structure (PDB code 1dvm). The structures were visualized with the VMD program (Humphrey *et al.*, 1996).

4 Results

4.1 Pla and PgtE inactivate the regulators of the fibrinolytic system (I, II)

Previous studies had shown that Pla and PgtE interact with several proteins involved in the plasminogen/fibrinolytic system (Kukkonen *et al.*, 2001; Lähteenmäki *et al.*, 2005a; Ramu *et al.*, 2008), which led me to investigate whether these omptins also target the important control proteins of this system, i.e., PAI-1, a key regulator of plasminogen activation, and TAFI, a regulator of fibrinolysis. Such interactions would disrupt the balance of the system and enhance proteolysis at the infection site.

4.1.1 Inactivation of PAI-1 (I)

I studied the interaction of PAI-1 with *Y. pestis* KIM D27, *Y. pestis* KIM D34, *S. enterica* 14028R, and *S. enterica* 14028R-1 by incubating the bacteria with the PAI-1/Vn complex, and detecting the resulting peptides by Western blotting with polyclonal anti-PAI-1 and anti-Vn antibodies. *Y. pestis* KIM D27 with *pla*-encoding plasmid pPCP1 and rough *S. enterica* strain 14028R degraded PAI-1 into smaller fragment that was named PAI-1*. These strains also degraded Vn into several smaller fragments. In contrast, *S. enterica* 14028R-1, where *pgtE* has been deleted, and *Y. pestis* KIM D34, cured of pPCP1, did not cleave PAI-1 or Vn. We also investigated whether an ancestor of *Y. pestis,* a rough *Y. pseudotuberculosis* strain PB1 Δ*wb* (Achtman *et al.*, 1999; Skurnik *et al.*, 2000) with or without the virulence plasmid pYV, degraded PAI-1 or Vn. Both *Y. pseudotuberculosis* strains were unable to cleave these proteins.

The role of Pla and PgtE in PAI-1 degradation was confirmed with the recombinant *E. coli* XL1 (pMRK1) expressing Pla and *E. coli* XL1 (pMRK3) expressing PgtE. The bacteria were incubated with the PAI-1/Vn complex, and degradation of PAI-1 was detected as above. Pla and PgtE seemed to be responsible for the cleavage of PAI-1, as no degradation of PAI-1 was observed by the empty vector strain *E. coli* XL1 (pSE380). In *E. coli* XL1 background the cleavage of PAI-1 by Pla was similar to *Y. pestis* background, but PgtE in *E. coli* degraded PAI-1 into two smaller fragments of about 25 kDa in addition to PAI-1*, while in *S. enterica* only one degradation product, PAI-1*, was observed.

The omptin-generated cleavage site in PAI-1 was determined by N-terminal sequencing and mass spectrometric analyses. The N-terminus of PAI-1* was intact, and MALDI-TOF-MS analysis of the reversed-phase purified PAI-1* gave the sizes of 38 982 Da (Pla-generated) and 38 972 Da (PgtE-generated), corresponding to the mass of the PAI-1-fragment V1-R346. Also the peptide mass fingerprint analysis of the trypsin-cleaved PAI-1* resulted in

sequences from only V1-R346. The cleavage site in PAI-1 by Pla and PgtE was thus located at the reactive site peptide bond R346-M347 in the RCL.

It was next investigated how the degradation of PAI-1 by Pla and PgtE affected its serpin activity towards its target protease uPA. When *Y. pestis* KIM D27 or *S. enterica* 14028R were incubated with the PAI-1/Vn complex, PAI-1 was no longer able to inhibit uPA. On the contrary, the serpin activity of PAI-1 remained intact when the PAI-1/Vn complex was incubated either with *Y. pestis* KIM D34 or *S. enterica* 14028R-1, or without the bacteria. *E. coli* XL1 (pMRK1) and *E. coli* XL1 (pMRK3) impaired the activity of PAI-1, in contrast to *E. coli* XL1 (pSE380). The protease activity of Pla or PgtE was required for the inactivation, because the activity of PAI-1 was not abolished with the protease negative mutants, Pla D206A or PgtE D206A, expressed in *E. coli* XL1.

I compared Pla and PgtE to other omptins, Epo of *E. pyrifoliae*, Kop of *K. pneumoniae*, YcoA of *Y. pestis*, YcoB of *Y. pseudotuberculosis*, chromosomal OmpT variants of *E. coli* AAEC072 and *E. coli* IHE 3034, plasmid-encoded $OmpT_P$ of *E. coli* IHE 3034, and SopA of *S. flexneri.* The omptins were expressed in *E. coli* and the cleavage of PAI-1 was detected as above. Only the omptins that belong to the Pla subfamily, including Pla, PgtE, Epo, and Kop (see Fig. 2 in the introduction), cleaved PAI-1. No degradation of PAI-1 was observed with *E. coli* expressing the Pla or PgtE mutants with the catalytic site substitution D206A. *Yersinia* chromosomal omptins, YcoA and YcoB that form the Yco subfamily, were negative in PAI-1 degradation. The members of the OmpT subfamily, including the chromosomally and plasmid-encoded OmpT variants and SopA, were unable to cleave PAI-1. Analysis of the 16 known complete omptin family member sequences and structure modeling revealed plausible reasons for this functional difference between the omptin subfamilies: although the catalytic groove of the omptins seems to be conserved and suitable for binding a large and basic amino acid, such as R346 in PAI-1, distinction at the loop regions could provide explanation for the observed differences. The potentially important amino acid differences between the Pla subfamily and the OmpT/Yco subfamilies are found in the loops 1, 4, and 5 in residues T36, L213, I260, D266 and D273 (Pla numbering).

4.1.2 Degradation of TAFI (II)

To determine the effects of *S. enterica* and *Y. pestis* to the activatability of TAFI, the bacteria were incubated with TAFI, and TAFI was then activated to TAFIa by thrombin-thrombomodulin complex. Changes in the activatability of TAFI were measured indirectly with TAFIa activity. *S. enterica* 14028R and *Y. pestis* KIM D27 impaired the activity of TAFIa by 85% and 97%, respectively, compared to the maximal activity of TAFIa in the buffer. Also *S. enterica* 14028R (pMRK3), complemented with the *pgtE*-encoding plasmid, clearly reduced the TAFIa activity. *S. enterica* 14028R-1 or *S. enterica* 14028R (pSE380) did

not cause change in the activity of TAFIa. *Y. pestis* KIM D34 decreased the activity of TAFIa by 59%, clearly less than KIM D27.

Recombinant *E. coli* XL1 expressing PgtE or Pla were assayed to confirm that the omptins were responsible for the inhibition of TAFIa activity. *E. coli* XL1 (pMRK3) and (pMRK1) reduced TAFIa activity by 80% and 61%, respectively. *E. coli* XL1 expressing the proteolytically negative PgtE D206A (pMRK31) and Pla D206A (pMRK111) decreased the activity of TAFIa by about 30%. TAFIa activity was also measured from TAFI-depleted plasma supplemented with TAFI. *E. coli* XL1 (pMRK3) and (pMRK1) reduced the activity of TAFIa in plasma by 49% and 60%, respectively, and the proteolytically negative D206A mutants decreased the activity by 31%. Clot-lysis assay with recombinant *E. coli* XL1 supported results obtained with the TAFIa activity assays. When *E. coli* XL1 expressing PgtE or Pla were incubated with TAFI, the clot-lysis times were reduced, indicating that the anti-fibrinolytic potential of TAFIa was impaired. D206A mutants had only minor effect on the clot-lysis times. Inhibition of TAFIa activity and decrease in clot-lysis times by PgtE- or Pla-expressing bacteria were dependent on bacterial dose and incubation time.

I investigated the reason for the inhibition of TAFIa function by PgtE and Pla by Western blotting with polyclonal anti-TAFI antibody and by SDS-PAGE. Strains expressing PgtE or Pla, *S. enterica* 14028R, *S. enterica* 14028R-1 (pMRK3), *Y. pestis* KIM D27, *E. coli* XL1 (pMRK3), and *E. coli* XL1 (pMRK1), degraded TAFI into smaller molecular weight fragments. In contrast, bacteria without *pgtE* or *pla* and *E. coli* XL1 expressing the D206A mutants caused only minor degradation of TAFI or no degradation at all. The omptin-cleaved TAFI migrated fuzzily in the SDS-PAGE gel as do all glycosylated proteins, and because all the N-glycosylation sites of TAFI are located in the N-terminal activation peptide, it was hypothesized that the cleavage of TAFI by the omptins occurs at the C-terminus. Addition of the lysine analog ε-ACA that restrains plasmin-mediated C-terminal cleavage of TAFI prevented the degradation of TAFI by PgtE- or Pla-expressing *E. coli* XL1 and increased the activatability of TAFI to TAFIa in the presence of PgtE or Pla. N-terminal sequencing and mass spectrometric analysis also suggested that degradation of TAFI by PgtE and Pla is C-terminal, although the exact cleavage site remains unknown.

4.2 Residues critical for plasminogen activation and invasiveness of Pla (III, IV)

It was already known that the loop structures are important for the functions of the omptins (Kukkonen *et al.*, 2001; Ramu *et al.*, 2008), so I further analyzed the importance of the specific loop regions and amino acids in the plasminogen activation and invasiveness mediated by Pla. Synonymous and nonsynonymous single-nucleotide polymorphisms have been involved in the evolution of *Y. pestis*, and I analyzed the effect of a nonsynonymous substitution T259I in Pla that differentiates pandemic and ancestral *Y. pestis* lineages. The

functions of common Pla were compared to those of Pla T259I and to those of Epo, whose mature protein sequence (290 aa) differs from Pla by 65 amino acids.

4.2.1 T259 and loops 3 and 5 are important for plasminogen activation (III, IV)

Ancestral *Y. pestis* isolates, biovar Microtus and the Pestoides strain Angola, contain substitution T259I in their predicted Pla sequences (Song *et al.*, 2004; Eppinger *et al.*, 2010). I substituted threonine 259 in Pla of *Y. pestis* biovar Medievalis to isoleucine, and expressed mutated Pla in a pSE380 vector in *Y. pestis* KIM D34 and *E. coli* XL1 hosts, and compared Pla T259I to wild type Pla in functional assays. I included PgtE in the assays for comparison, because PgtE also possesses isoleucine at position 259, and I also included the Pla-PgtE hybrids PgtE ^{259}IIDKT/TIDKN (Ramu *et al.*, 2008) and Pla ^{259}TIDKN/IIDKT.

The amount of Pla expression was similar with Pla and Pla T259I in *Y. pestis* and *E. coli* backgrounds on the basis of whole cell and cell envelope preparations, but the Pla isoform patterns of Pla T259I and Pla ^{259}TIDKN/IIDKT were different from that of wild type Pla: autoprocessed β-Pla was detected only with the wild type Pla but not with the mutants. Furthermore, wild type Pla was almost completely in the γ-Pla form in the unboiled cell envelope sample, whereas the mutants also showed pre- and α-Pla forms. Also, the γ-Pla of the mutants migrated faster in the SDS-PAGE gel.

Y. pestis KIM D34 (pPlaT259I) was less efficient than *Y. pestis* KIM D34 (pMRK1) in a fibrinolysis plate assay with purified components. *E. coli* XL1 (pMRK1.51) expressing Pla ^{259}TIDKN/IIDKT, and *E. coli* XL1 (pMRK3) expressing PgtE exhibited only limited fibrinolysis, while *E. coli* XL1 (pMRK1) efficiently lysed fibrin. In comparison to PgtE, *E. coli* XL1 (pMRK3.51) expressing PgtE ^{259}IIDKT/TIDKN showed minor improvement in fibrinolysis.

The initial, cumulative plasminogen activation was similar with *Y. pestis* KIM D34 (pMRK1) and *Y. pestis* KIM D34 (pPlaT259I) with the plasminogen concentration of 20 µg/ml, but difference in initial plasminogen activation was observed with low (2 µg/ml) plasminogen concentration. The same difference between Pla and Pla T259I was observed in *E. coli* XL1 background. No plasminogen activation was observed with the negative controls, *Y. pestis* KIM D34 (pSE380) and *E. coli* XL1 (pSE380). *E. coli* XL1 (pMRK3) was quite inefficient in initial plasminogen activation in both plasminogen concentrations, and the substitution ^{259}IIDKT/TIDKN in pMRK3.51 clearly improved the plasminogen activation of PgtE. Plasmin formation was also measured by incubating the bacteria with plasminogen and adding the plasmin substrate at different time intervals in order to find out whether plasmin activity is maintained during the incubation. The plasmin activity was lower with Pla T259I than with Pla, expressed in *Y. pestis* or in *E. coli*. Similar results were obtained with Pla ^{259}TIDKN/IIDKT. Plasmin activity was very low when plasminogen was incubated with

PgtE-expressing *E. coli*, but substitution ^{259}IIDKT/TIDKN in PgtE caused increase in plasminogen activation.

The reason for the poor plasminogen activation by Pla T259I was reduced plasmin stability: plasminogen was activated by Pla T259I and Pla ^{259}TIDKN/IIDKT, but the formed plasmin became subsequently inactivated, as observed when the bacteria were incubated with plasmin. *E. coli* XL1 (pMRK3) was the most efficient in plasmin inactivation. In contrast, *E. coli* XL1 (pMRK1) was similar to *E. coli* XL1 (pSE380); these strains did not inactivate plasmin. *E. coli* XL1 (pMRK3.51) with PgtE ^{259}IIDKT/TIDKN caused only minor inactivation, and *E. coli* XL1 (pMRK1.51) and *E. coli* XL1 (pPlaT259I) were slightly more efficient in inactivation of plasmin.

Similar plasminogen cleavage patterns were observed with Pla, Pla T259I, Pla ^{259}TIDKN/IIDKT, and PgtE when expressed in *E. coli* and detected with polyclonal anti-human plasminogen antibody that recognizes the kringle structures of plasminogen and plasmin. Similar pattern was observed when plasminogen was incubated with uPA. *E. coli* XL1 (pMRK3.51) was weaker than *E. coli* XL1 (pMRK3) in plasminogen cleavage. In contrast, when plasminogen degradation was observed with anti-human plasminogen catalytic domain antibody that recognizes the catalytic domain in the plasmin light chain, differences between the cleavage patterns by the omptin variants were observed. Plasmin light chain was observed when plasminogen was incubated with uPA, *E. coli* XL1 (pMRK1), or *E. coli* XL1 (pMRK3.51). On the contrary, no plasmin light chain was detected with Pla T259I, Pla ^{259}TIDKN/IIDKT, or PgtE. These results correlated well with the plasmin stability observed in the stepwise plasminogen activation assay.

α_2AP degradation patterns by *E. coli* XL1 (pPlaT259I) and *E. coli* XL1 (pMRK1.51) were similar to degradation patterns by Pla and PgtE expressed in *E. coli* XL1; all these proteins efficiently degraded the molecule. PgtE ^{259}IIDKT/TIDKN was weaker in the cleavage of α_2AP. Inactivation of α_2AP was dramatically increased due to T259I substitution: Microtus Pla and PgtE were more efficient than Pla in inactivation of α_2AP. Surprisingly, Pla and PgtE ^{259}IIDKT/TIDKN were both at the same level as *E. coli* XL1 (pSE380) in α_2AP inactivation.

In order to learn more about the interactions of Pla and its substrates, I compared Pla to Epo of *E. pyrifoliae* by expressing the proteins in *E. coli*. When this study was initiated, Epo was the closest homolog of Pla. Despite the similarities in sequences, functional differences between Pla and Epo were observed. Epo was found to be a poor plasminogen activator – hence the name Epo, *Erwinia pyrifoliae* omptin, instead of PlaA – and it did not mediate invasion either, both of which are functions characteristic of Pla. Epo degraded plasminogen, but inefficiently compared to Pla, and Epo did not form detectable amounts of the plasmin light chain by Western blotting. However, Epo was found to degrade the serpins α_2AP and PAI-1. In order to elucidate the regions and amino acids responsible for the different

properties of Pla and to study molecular adaptation in the omptin family, I constructed Pla-Epo chimeras and expressed them in *E. coli* XL1. The substitutions were selected on the basis of the sequence differences and knowing that loop regions are critical for the substrate selectivity (Fig. 4 and Table 1). Pla and Epo differ by 65 amino acids, of which 31 are located on the extracellular side above the lipid bilayer, and of these, 18 are in the surface loops.

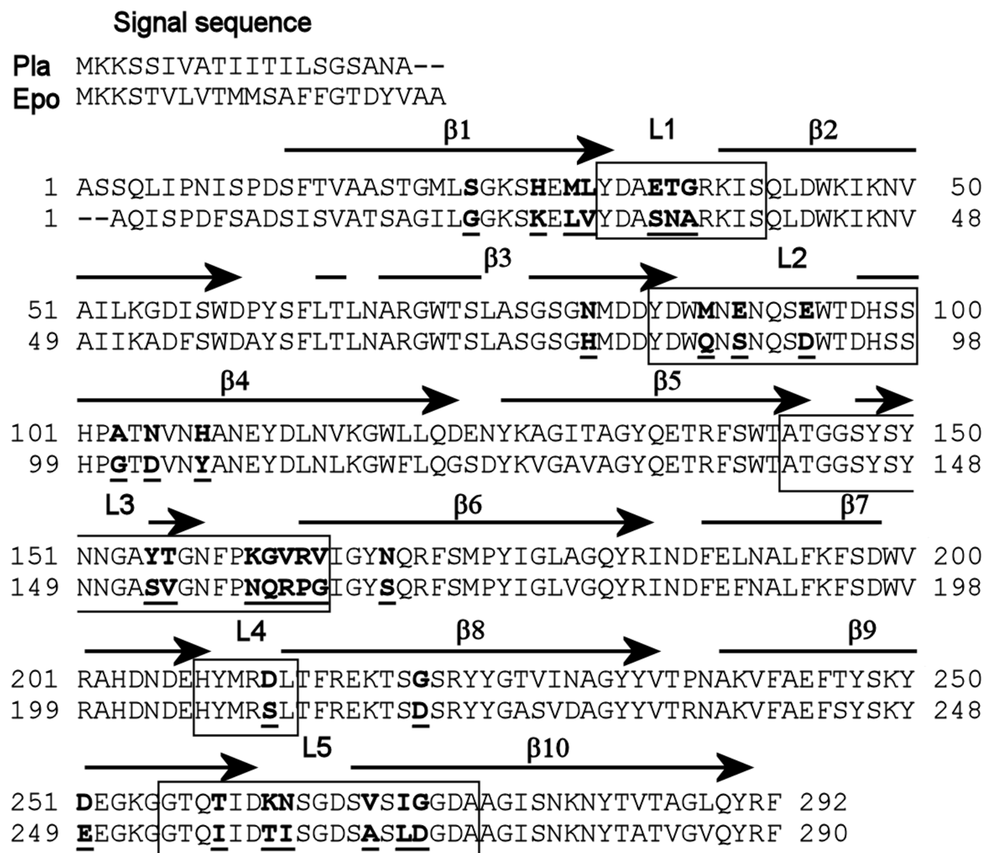

Figure 4. Sequence alignment of the amino acid sequences of Pla (above) and Epo (below). Signal sequence consists of 20 (Pla) or 22 (Epo) amino acids. The sequences were aligned with ClustalW (Chenna *et al.*, 2003). Secondary structure elements are based on the Pla crystal structure (Eren *et al.*, 2010) and are defined by Stride (Frishman & Argos, 1995). L1-L5 denote the extracellular loops. β-strands (β1-β10) are indicated with arrows. The gaps in β3 and β7 indicate irregularities in the structure. The 31 differing amino acids that are located at the extracellular side above the lipid bilayer are shown with bold face and underlined.

Results obtained with the Pla-Epo hybrid proteins confirmed the importance of surface loop residues in plasminogen activation. Initial plasminogen activation in Epo was obtained by substituting five residues, ^{159}NQRPG at L3, in Epo to corresponding residues ^{161}KGVRV of Pla (pMRK405). Five reverse substitutions at L3 in Pla in pMRK105 decreased the plasminogen activation by Pla dramatically. Further substitutions in Epo at L5, ^{260}TI/KN and ^{266}ASLD/VSIG, encoded in pMRK410, resulted in plasminogen activation similar to Pla. Further cumulative substitutions in Epo in pMRK417 at L1, L2, and L3 (^{33}SNA/ETG, ^{86}QNS/MNE, ^{153}SV/YT; L4 has only one amino acid difference) slightly enhanced plasminogen activation. Reverse 10 or 17 substitutions in Pla (pMRK110 and pMRK117)

abolished plasminogen activation completely. Further cumulative substitutions in Epo until all the 31 differing residues located at the extracellular surface were substituted (pMRK431; see Fig. 4), did not enhance the ability to activate plasminogen. No substantial differences were found in the expression levels of the hybrid proteins, as analyzed by Western blotting with anti-His_6-Pla, anti-His_6-Epo, and anti-Pla loop antisera.

Explanations for the differences of Pla and Epo in plasminogen activation were analyzed by comparing the Pla structure (Eren *et al.*, 2010) and the model structure of Epo. It was found out that the residues at L3 and L5 interact with L4, which contains the active site residues D206 and H208. In Pla, V163 at L3 interacts with residues at L4 and L5, while ^{159}NQR in Epo point towards the water phase. These interactions may cause differences in the substrate binding pocket and electrostatic properties between Pla and Epo. The only difference at L4 between Pla and Epo is D212 (S210 in Epo), and it forms a hydrogen bond with N263 at L5 in Pla. K262 (L5) and R164 (L3) in Pla strengthen the β-barrel by interacting with L4, unlike the corresponding residues T260 and P162 in Epo.

I also tested the chimeric proteins in PAI-1 degradation, and interestingly, proteins encoded in the plasmids pMRK405, pMRK410, and pMRK417 degraded PAI-1 more efficiently than Pla or Epo. Further substitutions in Epo, encoded in plasmids pMRK431 and pMRK442 (with 42 substitutions), resulted in similar degradation as observed with Pla and Epo. Substitutions in Pla, encoded in pMRK105, pMRK110, and pMRK117, abolished the ability of Pla to degrade PAI-1.

4.2.2 Invasiveness requires several regions in Pla (IV)

In addition to plasminogen activation, Pla functions as an invasin, and is the only omptin studied so far that mediates invasion into human endothelial-like cell line ECV304. Also Epo-expressing *E. coli* XL1 was negative in the gentamicin protection assay that was used to study bacterial invasion. The plasminogen-activating Pla-Epo hybrids, expressed in *E. coli*, were tested in the invasion assay, but even when all the differing 31 residues that are located at the extracellular part of the protein were substituted in Epo, only a low level of invasiveness was detected. However, when the N-terminus of Pla, including the β1-strand, was added to this hybrid protein (42 substitutions in total, encoded in pMRK442), the invasiveness increased to the level of Pla.

5 Discussion

5.1 Substrate specificities of the omptins

Differences in the omptin functions with polypeptide substrates have been shown to be dictated by sequence variation in their surface loops that restrain or enable the formation of the protease-substrate complexes. Overall, evolutionary plasticity of proteins is higher in the loop regions than in the core structure (Panchenko *et al.*, 2005), and this holds for omptins as well and explains the variation in the polypeptide target selectivity between omptins. In this and previous work our group has shown that Pla and PgtE have evolved towards different functions: Pla to an efficient plasminogen activator and an invasin and PgtE to a gelatinase and a proMMP-9 activator (Lähteenmäki *et al.*, 2001; Ramu *et al.*, 2008). In addition, both Pla and PgtE inactivate α_2AP (Kukkonen *et al.*, 2001; Lähteenmäki *et al.*, 2001) and PAI-1, serpins that regulate plasmin activity. PgtE was more effective than Pla in serpin cleavage, and more efficient serpinolytic activity was seen with Microtus Pla. All the tested omptins in the Pla subfamily (Pla, PgtE, Epo, and Kop of *K. pneumoniae*) were able to degrade PAI-1. Further, the Epo hybrids with a few amino acids from Pla were more effective in PAI-1 degradation than either Pla or Epo. These findings are compatible with the hypothesis that serpinolytic activity is a property of the progenitor molecule in this subfamily. My results further indicate that the effect of PgtE on plasminogen activation is indirect rather than direct, while Pla activates plasminogen directly. Although I have shown here that PgtE-mediated plasmin activity is transient, it is still possible that *in vivo* PgtE-generated plasmin is able to perform proteolytic functions before its inactivation by PgtE. Interestingly, the consequences of the actions of Pla and PgtE are similar: enhanced, uncontrolled proteolysis at host tissues. However, the target tissues and pathogenetic consequences differ.

As Microtus and Angola are thought to represent the ancient *Y. pestis* branches (Achtman *et al.*, 2004; Eppinger *et al.*, 2010), their Pla mutant T259I might represent the ancestral form of Pla that has later on evolved into a more efficient plasminogen activator and an important factor in plague pathogenesis. On the other hand, it can be speculated that attenuated *Y. pestis* strain with Pla T259I has evolved to not kill its hosts and thus spread more effectively. However, this seems unlikely, since mutant Pla is present in the ancient *Y. pestis* branches, not in the pandemic strains. In this work I compared these two Pla mutants and showed that this nonsynonymous SNP dramatically changes the protein function. This may also affect the virulence of the host bacterium. The reasons why substitution I259T affects the functions of Pla – and also PgtE – are unknown. This residue is located near the Pla autoprocessing site K262 at L5, and both these residues are situated near the catalytic groove. Substitution of T259 with isoleucine causes lack of the autoprocessed β-Pla isoform. However, the functions

of β- and γ-Pla are not known. The explanation of the observed functional differences between Pla and Pla T259I might lie in the differential recognition of the plasminogen/plasmin by these two protein variants, leading to either degradation of the catalytic domain of plasmin or formation of undetectable amounts of it by Pla T259I. The effect of this substitution in α_2AP inactivation was the opposite, indicating that the substitution in common Pla diverged its function towards direct plasmin formation.

I observed that altering the substrate selectivity of Epo towards plasminogen required substitution of only a few surface loop residues. Turning Epo into an invasin, on the contrary, required substitution of all the 31 differing residues located at the extracellular side on the protein, and the N-terminus including the β1-strand. The mechanism how these membrane-embedded, highly distant residues affect the target recognition remains perplexing. Invasion probably consists of various steps, including adhesion and membrane fusion, and may thus require several regions in Pla. My results confirm the earlier data that plasminogen activation and invasion abilities of Pla are distinct from each other (Lähteenmäki *et al.*, 2001). The invasion target of Pla in human endothelial cells is not known, which makes it difficult to compare the mechanisms of invasiveness and plasminogen activation. The model system for endothelial cell invasion, i.e., the ECV304 cell line, can be questioned: this widely used cell line has been shown to be cross-contaminated with the T-24 human urinary bladder carcinoma cell line and is thus not a real endothelial cell line (MacLeod *et al.*, 1999). Nevertheless, this does not invalidate the differences observed between Pla and Epo.

E. pyrifoliae is associated with a pear blight disease, and *epo* is encoded in a mosaic plasmid that closely resembles a virulence plasmid pEA29 of *E. amylovora* (McGhee *et al.*, 2002), so it is possible that Epo participates in proteolytic functions and tissue damage that benefit its host during infection. The omptins that belong to the Pla subfamily degraded PAI-1, and it was concluded that serpin inactivation is a function shared by this subfamily. However, the functions of Epo *in vivo* remain unknown. Plants have serpin homologs with highly similar overall structures with the mammalian serpins (Marshall, 1993; Irving *et al.*, 2000; Rawlings *et al.*, 2008; Roberts & Hejgaard, 2008; Lampl *et al.*, 2010), and these might be the targets of Epo. Plant serpins may inhibit serine and cysteine proteases encoded in plant genomes (Roberts & Hejgaard, 2008), but most experiments for the activity of the plant serpins have been performed with mammalian serine or cysteine proteases as substrates (Christeller & Laing, 2005). Suggested functions of plant serpins are regulation of host immune response and programmed cell death, and the protection of storage proteins in seeds from parasites (Roberts & Hejgaard, 2008).

K. pneumoniae is part of human normal microbial flora but can also cause diseases, such as pneumonia and urinary tract infections. *kop* was cloned from a large plasmid pKP187 of *K. pneumoniae* 342. This strain is a plant symbiont, so Kop might have functions that

promote symbiosis with the host plant. However, *K. pneumoniae* 342 can cause urinary tract infections and pneumonia in mice, although the infection in lungs is milder than with the clinical isolate *K. pneumoniae* C3091 (Fouts *et al.*, 2008). It is not known whether Kop is expressed in other *K. pneumoniae* strains. PAI-1 levels have been detected to increase during *K. pneumoniae* infection in mice, and PAI-1 seems to protect mice from the disease (Renckens *et al.*, 2007). PAI-1 degradation by Kop might thus promote *K. pneumoniae* infection.

Functions of the *Yersinia* YcoA and YcoB remain to be discovered. They are 99% identical to each other. It is not known whether they are proteolytically active, because the catalytic residue D84 (Pla numbering) has been substituted with asparagine. However, YcoA was found to promote *Y. pestis* infection in *C. elegans* (Styer *et al.*, 2005).

Sequence alignment and structural modeling of the omptin sequences suggested that some amino acid substitutions can prevent or promote the complex formation with the targets, leading to completely different outcome in a reaction of different omptins with the same substrate. My studies give insight into how the evolution of the omptin family may have occurred, and how different functions within a protein family can evolve. To determine in detail how omptins target different polypeptide substrates, however, requires structure determination of the omptin-substrate complexes.

5.2 Interactions of Pla and PgtE with the plasminogen/fibrinolytic system

Pla and PgtE disrupt the control of the plasminogen/fibrinolytic system by inactivating PAI-1 and by inhibiting the activatability of TAFI to TAFIa, which probably leads to increased levels of active plasmin and uncontrolled fibrinolysis and proteolysis of host tissues. This may be beneficial when the bacteria penetrate through tissues and spread to distant organs. The importance of the plasminogen/fibrinolytic system in plague infection has been widely accepted, and it is a clear difference between *Y. pestis* and its ancestor *Y. pseudotuberculosis*. The significance of the plasminogen/fibrinolytic system in *S. enterica* infections is less clear.

Pla and PgtE degrade PAI-1 at RCL between R346-M347. The cleavage site in α_2AP by the omptins remains to be determined, but it is possible that it occurs at the RCL similarly to cleavage of PAI-1. Subtilisin NAT of *B. subtilis* belongs to a family of subtilisin-like serine proteases and degrades PAI-1 at the same site as Pla and PgtE (Urano *et al.*, 2001). Also metalloprotease aureolysin of *S. aureus* and metalloprotease/elastase LasB of *P. aeruginosa* degrade PAI-I (Boudier *et al.*, 2005; Beaufort *et al.*, 2008; Beaufort *et al.*, 2010). Aureolysin degrades PAI-1 completely, but LasB cleaves PAI-1 at C-terminus, probably at or near the RCL (Beaufort *et al.*, 2008; Beaufort *et al.*, 2010). It thus seems that these bacterial pathogens that are associated with different diseases have similar functions but they use different catalytic mechanisms to cleave PAI-1.

TAFI has been earlier linked to two bacterial pathogens: *S. pyogenes* that causes skin and throat infections binds TAFI, and increased TAFI levels have been observed during the infection with *H. pylori*, a cause of gastric ulcer (Påhlman *et al.*, 2007; Ikeda *et al.*, 2009). Both these phenomena lead to activation of TAFI to TAFIa and inhibition of fibrinolysis, which has been hypothesized to protect the bacteria from the human immune system (Påhlman *et al.*, 2007; Ikeda *et al.*, 2009). On the contrary, my results show that the levels of functional TAFIa are decreased due to the degradation of TAFI by Pla and PgtE. This is the first time when bacterial proteases have been observed to degrade TAFI and to restrain its activation to TAFIa. The mechanism how lysine analog ε-ACA prevents the degradation of TAFI by the omptins is not clear, but it refers to the importance of the lysine residues in the interaction. The exact cleavage site in TAFI remains to be determined, but it is probable that the cleavage occurs at C-terminal arginine or lysine residues.

Further assays in plasma with PAI-1 and TAFI could reveal more about what happens during plague or salmonellosis *in vivo*. However, as plasma contains several omptin substrates, such as plasminogen, α_2AP, and complement components, the results of these assays will be complicated to interpret. Plasminogen is an abundant protein in plasma, and plasmin activates TAFI and subsequently inactivates TAFIa, and the direct effect on TAFI by the omptins is difficult to differentiate from the actions of plasmin. The situation is complicated also because the D206A mutants, deficient in plasminogen activation, were not completely negative in the plasma assays with TAFI; it is possible that they still have activity towards some substrates. Ongoing work in our laboratory (H. Tossavainen, unpublished) favors this hypothesis.

My thesis work shows that in addition to plasminogen activation, Pla and PgtE attack the plasminogen/fibrinolytic system also in other ways in order to generate uncontrolled plasmin activity and proteolysis. Increased Pla-generated plasmin activity and fibrinolysis is associated with the observed increase in proteolysis and bacterial proliferation in plague *in vivo* (Degen *et al.*, 2007; Lathem *et al.*, 2007). Monocytes and macrophages have been shown to utilize plasmin during their migration, and plasmin can act as a chemoattractant to monocytes (Syrovets *et al.*, 1997; Ploplis *et al.*, 1998). Activated macrophages show increased plasminogen activation at their surface (Vassalli *et al.*, 1992). Enhanced plasmin activity and proteolysis during systemic salmonellosis can thus recruit monocytes to the site of infection and improve the migration of macrophages and *S. enterica*.

6 Conclusions

In this study I have revealed two novel targets for the omptins Pla of *Y. pestis* and PgtE of *S. enterica*: they degrade and inactivate PAI-1 and degrade TAFI by preventing its activation to TAFIa. I have also shown that T259 at L5 is critical for the stability of plasmin formed by Pla, and, on the other hand, I259 makes PgtE more active towards α_2AP. This is one of the few described biological consequences of an SNP in a bacterial pathogen. I have further detected that efficient plasminogen activation by Pla is dictated in particular by the surface loops 3 and 5, and that plasminogen activation and invasiveness are distinct properties. Invasiveness requires both the residues located at the extracellular side on the protein and the N-terminal β1-strand of Pla, and it would be fascinating to identify the target of Pla in endothelial cells. I have revealed that Epo and Kop function as serpin inhibitors; investigating the functions of these omptins and their possible relevance in diseases that their hosts cause might be an interesting task. It would be appealing to find out novel functions for the OmpT variants and see if they differ from each other in their substrate selectivities.

Omptins have evolved to different directions to provide proteolytic and non-proteolytic functions that benefit their host bacteria during the infectious process. My studies showed that acquisition of novel proteolytic functions in the omptin fold occurs easily, as substitution of only a few amino acids can change their substrate selectivity, either to improve the existing function or to gain novel targets. The omptin fold might offer a promising platform for directed enzyme evolution and biotechnological applications. My studies proposed that serpinolytic activity could have been the selection advantage in the evolution of the progenitor omptin of the Pla subfamily.

It is likely that the interactions of the omptins with the plasminogen/fibrinolytic system are not restricted to molecules detected in this and previous studies, which may well prove a fruitful avenue for further studies.

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Johanna, autumn 2010

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