STRUCTURE-FUNCTION RELATIONSHIPS IN THE OMPTIN FAMILY OF ENTEROBACTERIAL PROTEASES/ADHESINS

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Cover illustration: A cladogram presention of omptin protein sequence alignment.

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LIST OF ORIGINAL PUBLICATIONS

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I **Kukkonen, M., Lähteenmäki, K., Suomalainen, M., Kalkkinen, N., Emödy, L., Lång, H., and Korhonen, T. K.** (2001) Protein regions important for plasminogen activation and inactivation of α_2-antiplasmin in the surface protease Pla of *Yersinia pestis*. Mol. Microbiol. **40**:1097-1111.

II **Lähteenmäki, K., Kukkonen, M., and Korhonen, T.K.** (2001) Pla surface protease/adhesin of *Yersinia pestis* mediates bacterial invasion into human endothelial cells. FEBS Lett. **504**:69-72

III **Kukkonen, M., Suomalainen, M., Kyllönen, P., Lähteenmäki, K., Lång, H., Virkola, R., Helander, I. M., Holst, O., and Korhonen, T. K.** (2003) Lack of O-antigen is essential for plasminogen activation by *Yersinia pestis* and *Salmonella enterica*. Mol. Microbiol. In press.

SUMMARY

The omptins are a family of structurally related surface proteases found in pathogenic species of the *Enterobacteriaceae*. This thesis work addressed the structure-function relationships in three members of the family: Pla of the plague bacterium *Yersinia pestis*, PgtE of *Salmonella enterica*, and OmpT from *Escherichia coli*. Their sequences are ca. 50 % identical, and structure modelling showed that the three proteases share a common β-barrel fold. The omptin barrel has 10 transmembrane β-strands and five surface-exposed, short loop structures. For analysis, the omptin genes *pla, pgtE* and *ompT* were expressed in recombinant *E. coli*. Pla was found to be autoprocessed at a specific residue in a surface loop of the molecule, but prevention of autoprocessing by substitution of the cleavage site had no effect on the proteolytic activity of Pla. Similarly, PgtE was autoprocessed once secreted to the cell wall. The three omptin molecules exhibited limited serological crossreactivity, as analyzed with antisera raised against His_6-fusion peptides of each omptin as well as against synthetic peptides mimicking the five surface loops of Pla.

The common property associated with omptins is the proteolytic activation of a host proenzyme, plasminogen. Plasmin formation by *Y. pestis* has been associated with the highly invasive character of plague, and we observed that Pla and PgtE, but not OmpT, efficiently cleaved and activated plasminogen. Further, Pla, but not OmpT, also proteolytically inactivated the main inhibitor of plasmin activity in humans, α_2-antiplasmin, which most likely is important for the ability of *Y. pestis* to create uncontrolled proteolysis and invasiveness during infection. Residues important for the proteolytic activity of Pla were identified by substitution analysis; some of the identified residues are homologs to the catalytic residues recently reported for OmpT, and some residues are involved in substrate binding and had more limited effects. The role of surface loops in substrate specificity was demonstrated by converting OmpT into a Pla-like enzyme by cumulative substitutions at surface loops. This conversion exemplifies how a housekeeping protease can evolve into a powerful virulence factor by cumulative substitutions at critical surface loops, without changing the overall β-barrel fold. Pla, but not OmpT or PgtE, was found to enhance bacterial invasiveness into human endothelial cells; Pla also was a more potent adhesin to basement membrane than the two other omptins. The adhesive and invasive functions of Pla did not involve its proteolytic activity. Pla and PgtE have a dual interaction with lipopolysaccharide (LPS). Reconstitution of His_6-Pla with LPS indicated that for activity Pla requires rough LPS. On the other hand, the virulence-associated functions of Pla, i.e. activation of plasminogen, adhesion to basement membrane and invasiveness, were sterically inhibited by the presence of an O-antigen on the bacterial surface. Plasminogen activation by PgtE was detectable in rough *Salmonella* only.

This study shows that the individual omptins exhibit significant variability in their functions which can be attributed to sequence differences at the surface-exposed loops of the omptins.
The results reveal a mechanism of targeted, uncontrolled proteolysis important in the pathogenesis of plague. The selective advantage of the unique lack of an O-antigen in *Y. pestis* has remained an open question, this thesis work shows that the lack of an O-antigen is important for a full utilization of the proteolytic potential of Pla. Conversely, the presence of an O-antigen in *Salmonella* renders its plasminogen activator PgtE cryptic. The results are important also in that they demonstrate that omptins are multifunctional surface proteins and give an example of the evolution of a virulence function in plague.

1. INTRODUCTION

For pathogenic bacteria, proteases and proteolytic functions are potential virulence factors. Bacterial proteases may directly degrade structural proteins of the host and cause massive tissue damage, or indirectly, they may influence - activate or inhibit - proteinase cascades of the human body. Proteolysis may aid the infecting pathogen by several mechanisms to survive or multiply in the host (reviewed in Goguen *et al*., 1995; Travis *et al*., 1995; Lamont and Jenkinson, 1998; Potempa *et al*., 2000; Edwards *et al*., 2003). Amino acids or peptides released from tissue or circulating proteins of the host may be utilized for nutritional demands of the pathogen. Proteolysis is known to be critical in the resistance of pathogenic bacteria against immune defences of the human body, at the levels of both native and acquired immunity. Degradation of antimicrobial cationic peptides produced by epithelial cells and phagocytes enhances survival of bacteria at epithelial surfaces and increases their resistance against phagocytic killing (reviewed Hancock and Scott, 2000; Peschel, 2002). Bacterial proteases are also known to inactivate a component or components of the complement system, which can result in poor chemotaxis of phagocytes to the infection site, failure of the complement cascade to form the membrane attack complex (MAC) on the bacterial surface, or poor opsonization by the C3b complement protein (reviewed in Rautemaa and Meri, 1999). Proteases attacking immunoglobulins are important virulence factor of some bacterial species. Finally, proteases acting directly or indirectly onto tissue barriers of the human body, i.e. the extracellular matrices (ECMs), have been found important for bacterial spread to secondary infection sites (Harrington, 1996; Lottenberg *et al*., 1994; Lähteenmäki *et al*., 2001; Edwards *et al*., 2003). Proteolytic activities within the human body are tightly regulated, and an important aspect of proteolysis in bacterial infections is the ability of several pathogens to inactivate control mechanisms of mammalian proteolysis.

This study addresses three homologous bacterial proteases, omptins, Pla of *Yersinia pestis*, PgtE of *Salmonella enterica* serovar Typhimurium, and OmpT of *Escherichia coli*. These proteases offer an interesting model for pathogen evolution as they are adapted to the life style of their host bacterium and several of the proteolytic aspects mentioned above are exhibited by these proteases. The omptins share a common structural backbone and have minor sequence variations in their surface-exposed regions; this results in differing specificities and functions in infectious diseases. Further, it appears that omptins are multifunctional. They may enhance bacterial virulence by nonproteolytic functions as well, i.e. by promoting bacterial adherence to tissue components and invasion into human cells.

The omptin-positive species addressed in this study cause different diseases varying in the route of infection, the tissues and organs affected in the infection, and the level of bacterial invasiveness. *Y. pestis* is the causative agent of plague, which is a highly fatal and invasive zoonosis. *Y. pestis* remains a potential health risk, it persists endemically on most continents (Perry and Fetherston, 1997), was recently found to contain self-transmissible multidrug resistance plasmids (Galimand *et al*., 1997; Guiyoule *et al*., 2001) and is a potential biological weapon (Inglesby *et al*., 2000). *Y. pestis* is transmitted from infected rodents by a flea vector or, during the pulmonary phase of the disease, by aerosols produced by a coughing patient. An essential feature in the zoonotic infection is the bacteria's ability to resist host defences at the subcutaneous infection site and to disseminate to local lymph nodes. After massive proliferation at lymphatic tissue, bacteria are released to circulation and disseminated to liver, spleen and other organs. Their eventual proliferation in circulation may cause sepsis. Life cycle of *Y. pestis* is completed as a flea has a blood meal on a septicaemic victim (reviewed

by Perry and Fetherston, 1997). Genetically, *Y. pestis* is uniform and estimated to have evolved from the intestinal pathogen *Y. pseudotuberculosis* O1:b only 1500-20 000 years ago (Achtman *et al*., 1999; Skurnik *et al*., 2000). Comparison of *Y. pestis* and *Y. pseudotuberculosis* offers a model to study the evolution of pathogenic species. During its evolution *Y. pestis* has gained genes and potential virulence functions through lateral gene transfer. Conversely, it has lost gene functions through several mechanisms. The genome sequence of *Y. pestis* contains 149 pseudogenes; many of their counterparts are important for host adaptation or virulence in other enteric bacteria (Parkhill *et al*., 2001; Deng *et al*., 2002; Radnedge *et al*., 2002; Hinchliffe *et al*, 2003; reviewed in Wren, 2003). The determinants inactive in *Y. pestis*, include those for the O side chain of lipopolysaccharide (LPS) (Skurnik *et al*., 2000; Prior *et al*., 2001) which is an important virulence factor of enteropathogenic *Yersinia* (Al-Hendy *et al*., 1992; Zhang *et al*., 1997; Darwin and Miller, 1999; Mecsas *et al*., 2001; Karlyshev *et al*., 2001; Najdenski *et al*., 2003). The selective advantage of gene inactivation for *Y. pestis* has not been characterized. On the other hand, acquisition of two plasmids, pFra and pPCP1, has been essential for the life cycle of *Y. pestis*. pFra encodes functions enabling bacterial colonization in the flea vector (Hinnebusch *et al*., 2002), whereas pPCP1 is needed for the dissemination of the plague bacterium from the initial infection site at dermis into deeper tissues (Brubaker *et al*., 1965; Sodeinde *et al*., 1992).

Salmonella enterica is an oral-faecal pathogen that invades the intestinal epithelium and may penetrate into deeper tissues. In systemic infections, *S. enterica* invades at ileum through the M cells in Peyer's patches and replicates inside submucosal phagocytes, and migration of infected macrophages is thought to facilitate bacterial dissemination within the host (reviewed in Ohl and Miller, 2001). In macrophages, *S. enterica* multiplies within *Salmonella*-containing vacuoles (SCVs), and the infection and survival of *Salmonella* within phagocytes are influenced by genes encoded in so-called *Salmonella* pathogenicity islands (SPIs) (Galán, 2001).

E. coli occurs as a harmless inhabitant of the human intestine, but certain strains are associated with infectious diseases and classified in distinct pathogroups: enteropathogenic (EPEC), enterohemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC) (reviewed in Natero and Kaper, 1998), uropathogenic (UPEC) (Väisänen-Rhen *et al*., 1984; Pere *et al*., 1988), and meningitis-associated (MENEC) *E. coli* (Selander *et al*., 1986). The pathogroups consist of conserved clonal groups of *E. coli* and are characterized by their pathogroup-specific virulence genes and functions. Available genomic sequences have revealed considerable variations in *E. coli* pathogroups, i. e. in the sizes of the genomes which range between 4100-5594 kb as well as in the number of ORFs (4289-5448 ORFs) (Blattner *et al*., 1998; Perna *et al*., 2001; Hayashi *et al*., 2001; Welch *et al*., 2002). In addition to a conserved core genome, each pathogroup contains characteristic virulence genes often located in the genomic elements called pathogenicity islands (PAIs) which probably have spread horizontally across bacterial species (Hacker *et al*., 1997; Dobrindt *et al*., 2003). On more general grounds, lateral gene transfer of PAIs probably has been very important in the evolution of the various enterobacterial pathogens, including *Y. pestis* and *S. enterica* (Groisman and Ochman, 1996; Hacker *et al*., 1997; Buchrieser *et al*., 1998; Bach *et al*., 1999; 2000; al-Hasani *et al*., 2001; Parkhill *et al*., 2001; Deng *et al*., 2002).

1.1. The omptin family of enterobacterial proteases

Omptins are a family of structurally related, integral outer membrane proteases detected in *Enterobacteriaceae*. Six members of the family, OmpT and OmpP of *E. coli*, Pla of *Yersinia pestis*, PgtE of *Salmonella*, Pla endopeptidase A of *Erwinia pyrifoliae*, and SopA of *Shigella flexneri* have been characterized and share high sequence identity (Table 1), which forms the basis for family assignment. The omptin sequences lack cysteine and have typical features of a β-barrel fold, which in Gram-negative bacteria is restricted to outer membrane proteins (Koebnik *et al*., 2000; Schulz, 2000). Functionally, the omptins are atypical proteases that lack the signature sequences of classical protease families and are resistant against inhibitors typical for a protease class (Bond and Butler, 1987; Sugimura and Higashi, 1988; Sugimura and Nishihara, 1988; White *et al*., 1995). Until recently, omptins formed the S18 family of serine peptidases, which as a group are characterized by the Ser-Asp-His catalytic triad (Rawlings and Barrett, 1994a). During preparation of this thesis work, the crystal structure of OmpT was resolved (Vandeputte-Rutten *et al*., 2001) and on the basis of a pair of aspartates at the catalytic site of OmpT, the omptin family was reclassified as aspartic proteases (Kramer *et al*., 2001 ; Vandeputte-Rutten *et al*., 2001; Rawlings *et al*., 2002).

Omptins cleave protein or peptide substrates preferentially between two successive basic amino acid residues. Substrate specificity of OmpT has been most exceedingly studied (Sugimura and Nishihara, 1988; Dekker *et al*., 2001; Okuno *et al*., 2002). The consensus sequence of OmpT cleavage site is (Arg/Lys)↓(Arg/Lys)-Ala. The amino acids flanking the cleavage site are numbered P1 to P*n* towards the N terminus and P1’ to P*n*’ towards the C terminus of the substrate (Rawlings and Barrett, 1994b). OmpT is highly selective towards a basic residue at position P1 but less exclusive at P1´ where several amino acids are tolerated. Presence of spacious aromatic or negatively charged side chain at P2, P1, P1’and P2’ prevents proteolysis by OmpT (Dekker *et al*., 2001). Other omptins show similar activity towards polypeptide substrates: OmpP cleaved a fusion protein at an Arg-Arg bond (Kaufmann *et al*., 1994) and IcsA of *Shigella* is cut by SopA between two arginines within the sequence Ser-Ser-Arg-Arg-Ala-Ser-Ser (Fukuda *et al*., 1995). Pla of *Y. pestis* cleaves plasminogen at an Arg-Val bond (Sodeinde *et al*., 1992). However, as will be shown in this thesis work, the omptins exhibit variable specificity against physiologically or medically important polypeptides, which probably explains why they affect enterobacterial virulence by varying efficiency and mechanisms.

Table 1. The omptin family of enterobacterial surface proteases

Bacterium	Omptin	Size of the mature peptide (amino acids)	Sequence identity with Pla (%)	Location of the gene	References
Yersinia pestis	Pla	292	100	9,5 kb plasmid	Sodeinde and Goguen, 1989 McDonough and Falkow, 1989
Erwinia pyrifoliae	PlaA	290*	78	36-kb plasmid	McGhee *et al*., 2002 Maxson-Stein *et al*., 2003
Salmonella enterica	PgtE	292*	75	chromosome	Yu and Hong, 1986 Guina *et al*., 2000
Escherichia coli	OmpT	297	50	chromosome, cryptic prophage	Grodberg *et al*., 1988 Nakata *et al*., 1993 Hayashi *et al*., 2001
Escherichia coli	OmpP	292	48	99 kb F-plasmid	Kaufmann *et al*., 1994 Matsuo *et al*., 1999 NC_002483
Shigella flexneri	SopA	301	40	210 kb virulence plasmid	Egile *et al*., 1997 Venkatesan *et al*., 2001

* the signal peptide cleavage site was predicted by using program SignalP V2.0.b2 (Nielsen *et al*., 1997; http://www.cbs.dtu.dk/services/SignalP-2.0/)

1.2. Structure of omptins

OmpT is the only omptin (Vandeputte-Rutten *et al*., 2001) for which the crystal structure has been solved. Characteristically for an integral outer membrane protein, OmpT is a β-barrel and has ten antiparallel β-strands connected by four short periplasmic turns and five extracellular loops (Figure 1A and B). In common with outer membrane proteins, two girdles of aromatic residues border the membrane-embedded hydrophobic zone on the barrel surface and determine the position of OmpT in the membrane (Figure 1A). OmpT barrel is long, ~70 Å in its longest dimension, and protrudes on the extracellular side ~40Å from the lipid bilayer. The outermost loops are located just above the rim of the LPS core region (Vandeputte-Rutten *et al*., 2001; Figure 1A).

The active site of OmpT was identified by the crystal structure determination and substitution analysis (Kramer *et al*., 2000a; 2001; Vandeputte-Rutten *et al*., 2001; see Figure 1A). The catalytic residues reside in a negatively charged cleft surrounded by the five loops. The deep negatively charged pocket that is formed by 18 residues is conserved within the omptin family (Vandeputte-Rutten *et al*., 2001; Figure 1). On the other hand, the loop regions on the top of the barrel vary in sequence, the possible role of this variability in substrate specificity was one topic of this work.

The catalytic mechanism for peptide bond cleavage by OmpT seems novel (Kramer *et al*., 2000a; 2001 Vandeputte-Rutten *et al*., 2001). Substitution of four residues, Asp83 and Asp85 at loop 2 and Asp210 and His212 at loop 4 (Figure 1A) decreased enzymatic activity by at least 10 000-fold which is expected for residues directly participating in catalysis (Kramer *et al*., 2000a; 2001). The configuration of the proposed catalytic pairs Asp210-His212 and Asp83-Asp85 resembles the structure of the catalytic sites in serine proteases as well in aspartic proteases, but OmpT does not easily fit into either class. The His 212-Asp210 pair lacks the correctly positioned nucleophilic serine of serine proteases (Vandeputte-Rutten *et al*., 2001) as well as the D(T/S)G consensus sequence of aspartic proteases. OmpT is not active at acidic pH (Kramer *et al*., 2000b) and not inhibited by known protease inhibitors specific for a peptide bond cleavage pathway (Sugimura and Higashi, 1988; Sugimura and Nishihara, 1988; White *et al*., 1995). It was suggested that the Asp-His catalytic dyad activates a water molecule and subsequently performs the nucleophilic attack on the scissile bond. The Asp83-Asp85 pair may position the water molecule correctly or stabilize the oxyanion intermediate during the catalysis (Kramer *et al*., 2001; Vandeputte-Rutten *et al*., 2001).

LPS forms the outer leaflet of outer membrane in most Gram-negative bacteria and contains three parts: the lipid A, the core oligosaccharide and the highly variable O side chain (O antigen) consisting of oligosaccharide repeats. Lipid A and the inner core are highly conserved within *Enterobacteriaceae* (Heinrichs *et al*., 1998; Bruneteau and Minka, 2003). O-chain is important for pathogenesis of *E. coli*, *Salmonella* and enteropathogenic *Yersinia* (Liang-Takasaki *et al*., 1983; Al-Hendy *et al*., 1992; Zhang *et al*., 1997; Darwin and Miller, 1999; Karlyshev *et al*., 2001; Mecsas *et al*., 2001; Najdenski *et al*., 2003) as it is required for colonization, as well as for resistance against cationic peptides, complement lysis and phagocytosis (Joiner, 1985; Burns and Hull, 1998; 1999; Wachter and Brade, 1989; Rautemaa and Meri, 1999; Skurnik *et al*., 1999; Skurnik, 2003). Rather surprisingly, *Y. pestis* is phenotypically rough; it has no O-side chain due to several mutations in the O-chain biosynthesis genes (Skurnik *et al*., 2000; Prior *et al*., 2001).

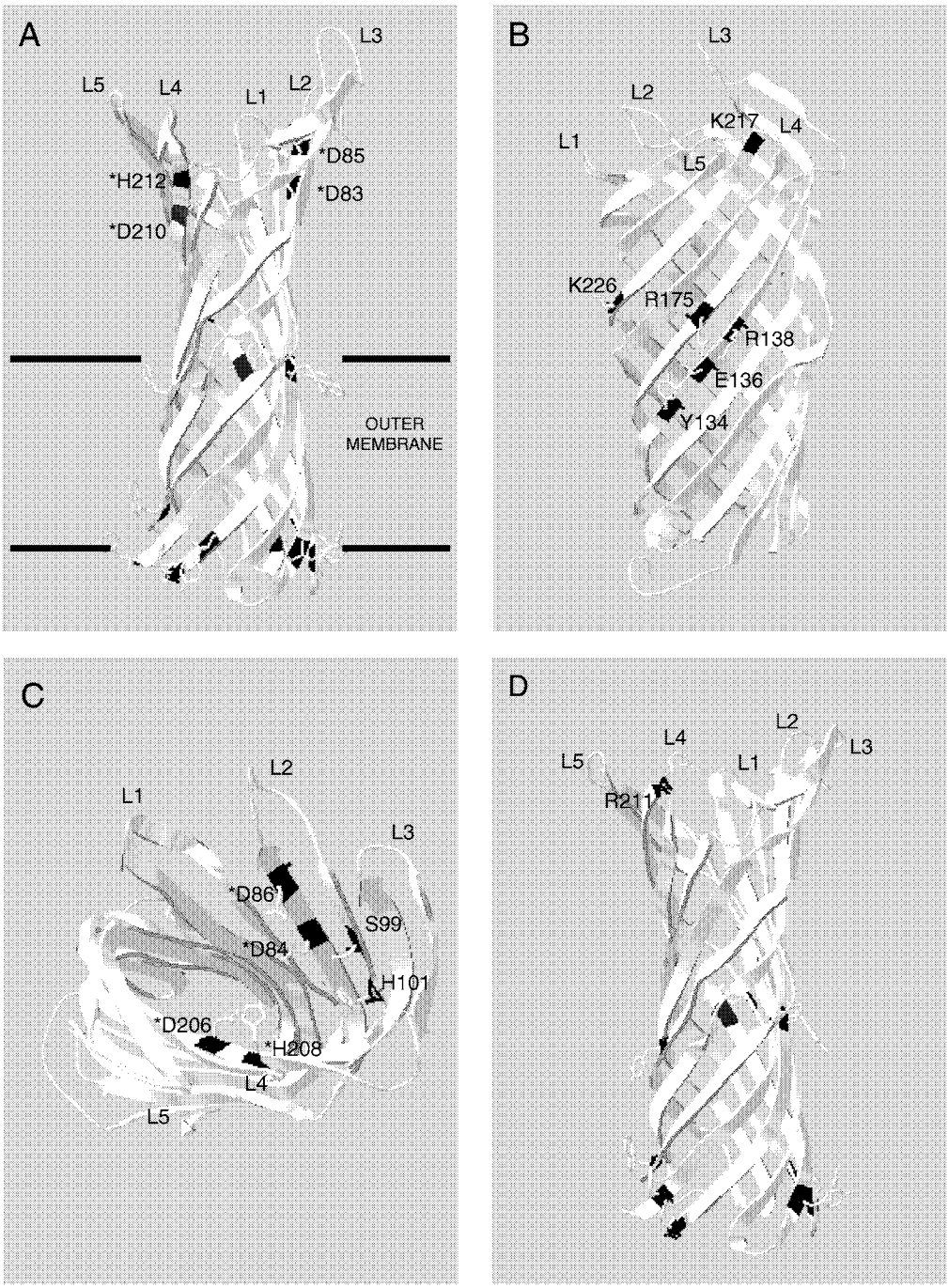
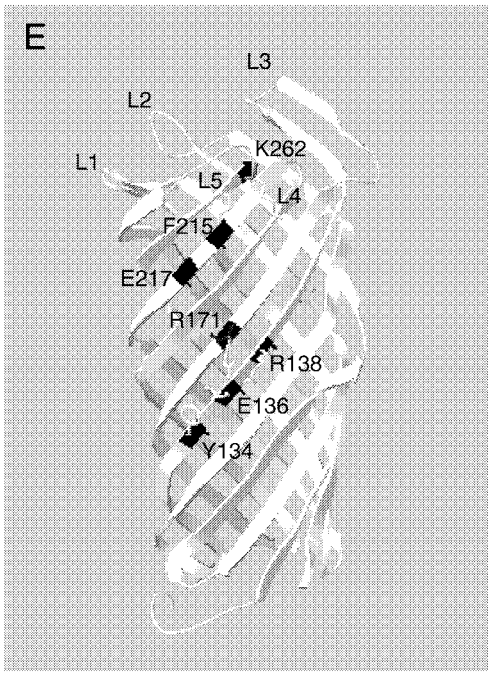
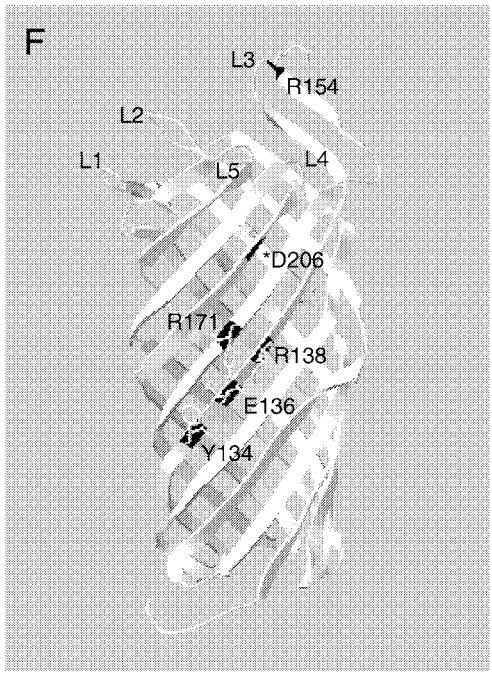


Figure 1. Ribbon models of OmpT (A and B), Pla (C, D and E) and PgtE (F), and alignment of the mature amino acid sequences (G). The loops (L1-L5) in the models are indicated and the functionally important residues are specified with one letter code and their residue number. A and B show sideviews of OmpT rotated 90° along the barrel axis. A) The active site residues: the D83-D85 pair at loop 2 and the D210-H212 dyad at loop 4. The approximate position of the outer membrane is indicated by horizontal lines, and the two girdles of aromatic residues residing at the borders of outer membrane are shown. B) Residues important in LPS binding and autoprocessing by OmpT. R138, R175 and K226 form part of the consensus LPS-binding motif, the conserved Y134 and E136 have lipid A -binding counterparts in FhuA (Ferguson *et al.*, 2000; Vandeputte-Rutten *et al.*, 2001). C) Top view of the predicted active site groove of Pla. D84 and D86 at loop 2 and D206 and H208 at loop 4 are putative active-site residues; S99 and H101 in loop 2 probably participate in substrate binding (Vandeputte-Rutten *et al.*, 2001; paper I). D and E show sideviews of Pla rotated 90 ° along the barrel axis. D) R211 at the tip of loop 4 is important for the substrate specificity of Pla (paper I). The aromatic girdle residues are shown. E) LPS-binding and autoprocessing sites of Pla. The residues of the consensus LPS-binding motif, R138 and R171, are indicated. The autoprocessing site K262 in loop 5 and the residues E215 and F217 involved in self–recognition are also shown (paper I).





G

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                                                    L1
OmpT ST ETLS--FTPD NINADISLGT LSGKTKERVY LAEEGGRKVS QLDWKFNNAA IIKGAINWDL   60
Pla  AS SQLIPNISPD SFTVAASTGM LSGKSHEMLY DAET-GRKIS QLDWKIKNVA ILKGDISWDP   61
PgtE ES ALFIPDVSPD SVTTSLSVGV LNGKSRELVY DTDT-GRKLS QLDWKIKNVA TLQGDLSWEP   61

                                  L2
OmpT    MPQISIGAAG WTTLGSRGGN MVDDWMDSS NPGTWTDESR HPDTQLNYAN EFDLNIKGWL   120
Pla     YSFLTLNARG WTSLASGSGN MDDYDWMNEN QSE-WTDHSS HPATNVNHAN EYDLNVKGWL   120
PgtE    YSFMTLDARG WTSLASGSGH MVDHDWMSSE QPG-WTDRSI HPDTSVNYAN EYDLNVKGWL   120
                                  * *
                                                    L3
OmpT    LNEPNYRLGL MAGYQESRYS FTARGGSYIY SSEEGFRDDI GSFPNGERAI GYKQRFKMPY   180
Pla     LQDENYKAGI TAGYQETRFS WTATGGSYSY -NN-G-A-YT GNFPKGVRVI GYNQRFSMPY   176
PgtE    LQGDNYKAGV TAGYQETRFS WTARGGSYIY -DN-G-R-YI GNFPHGVRGI GYSQRFEMPY   176

                                                    L4
OmpT    IGLTGSYRYE DFELGGTFKY SGWVESSDNG EHYDPGKRIT YRSKVKDQNY YSVAVNAGYY   240
Pla     IGLAGQYRIN DFELNALFKF SDWVRAHDNG EHY--MRDLT FREKTSGSRY YGTVINAGYY   234
PgtE    IGLAGDYRIN DFECNVLFKY SDWVNAHDNG EHY--MRKLT FREKTENSRY YGASIDAGYY   234
                                                * *
                                  L5
OmpT    VTPNAKVYVE GAWNRVTNKK GNTSLYDHNN -NTSDYSKNG AGIENYNFIT TAGLKYTF    297
Pla     VTPNAKVFAE FTYSKYDEGK GGTQTIDKNS GDSVSIGGDA AGISNKNYTV TAGLQYRF    292
PgtE    ITSNAKIFAE FAYSKYEEGK GGTQIIDKTS GDTAYFGGDA AGIANNNYTV TAGLQYRF    292
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F) LPS-binding and autoprocessing sites of PgtE. The putative autoprocessing site at R154 in loop 3 (paper III). The models of the omptins were made with the help of the Swiss Model homology modeling server using the coordinates of 3D model of OmpT (ExPDB 1I78) and the figures were drawn using Swiss-Pdb Viewer (Guex and Peitsch, 1997; http://www.expasy.org/spdbv/)

G) Sequence alignment of the mature sequences of OmpT, Pla and PgtE. The β-strand regions (black arrows) and the loops are indicated. Residues critical for proteolytic activity are shown with white letters on black background; the catalytic residues are pointed out with asterisks (Kramer *et al.*, 2000b; 2001; paper I and III). The putative LPS binding residues are indicated by gray background. Residue R211 important for proteolytic specificity at Pla (paper I) is boxed. The autoprocessing sites of the omptins and the residues involved in self-recognition in Pla are underlined (papers I and III). The numbering of the amino acids is shown on the right. The aligning of the sequences was made by using Clustal W program (http://www.ebi.ac.uk/clustalw/index.html).

LPS also is an important structural component of the Gram-negative cell wall and known to interact with outer membrane proteins. FhuA is a ferric siderophore receptor and an β-barrel outer membrane protein of *E. coli*, and the only protein whose structure in complex with bound LPS has been solved (Ferguson *et al*., 1998; Ferguson *et al*., 2000). A single LPS molecule of *E. coli* K-12 is non-covalently bound to a FhuA monomer so that lipid A is located just above the outer aromatic girdle at the barrel. At one side of the barrel, hydrophobic side chains of membrane-spanning amino acids of FhuA settle five of the six lipid A acyl chains parallel to the barrel axis. Eleven charged or polar residues tightly bind the β-barrel of FhuA with the negatively charged phosphates of the inner core and the diglucosamine of the lipid A. Structure-based search in the Protein Data Bank revealed that a subset of four residues involved in the FhuA-LPS interactions is conserved in several LPS-binding prokaryotic and eukaryotic proteins (Ferguson *et al*., 2000). Comparison of the crystal structures of OmpT and the LPS-FhuA complex suggested that also OmpT interacts with LPS (Ferguson *et al*., 1998; 2000; Vandeputte-Rutten *et al*., 2001). OmpT has three of the four residues of the consensus LPS-binding motif similarly positioned as in the FhuA-LPS complex (Vandeputte-Rutten *et al*., 2001; Figure 1B). Pla and PgtE sequences, on the other hand, contain two arginines of the motif (see Figure 1E and F), these are predicted to interact electrostatically and through a hydrogen bond with the lipid A 4' phosphate.

Specific interactions with LPS are known to contribute to correct protein folding and insertion into the outer membrane. Certain outer membrane proteins require inner core phosphates for their assembly in the outer membrane. In deep-rough mutants of *Salmonella* and *E. coli*, the amount of the OmpF porin in the outer membrane is strongly reduced, whereas OmpA is much less affected (reviewed in Nikaido and Vaara, 1985; Ried *et al*., 1990). Trimerization of OmpF (Sen and Nikaido, 1991) and folding of *E.coli* porin PhoE (de Cock *et al*., 1999) are efficiently supported by LPS with non-substituted phosphates in the inner core. Lipid A acyl chains are needed for PhoE folding *in vitro* (de Cock *et al*., 1999).

Recent evidence strongly suggests that LPS indeed is essential for the enzymatic activity of OmpT (Kramer *et al*., 2000b; 2002). Denatured OmpT can be refolded in the presence of several detergents to adopt the β-barrel conformation. However, the refolded OmpT is not enzymatically active unless LPS is added (Kramer *et al*., 2000b). As judged by circular dicroism analysis, addition of LPS does not cause large structural changes in OmpT, and the activation probably is gained through subtle conformational changes in the preformed β-barrel (Kramer *et al*., 2002). Presence of at least one of the heptose-bound phosphates in the inner core of LPS is important for enzymatic activity of the reconstituted OmpT. However, the core region is not indispensable for the enzymatic activity of OmpT, as OmpT is activated to some extent also in the presence of the lipid A only. Instead, the full acylation of the lipid A was found necessary for the *in-vitro* activation of OmpT (Kramer *et al*., 2002).

1.3. Expression and assembly of omptins

The genes encoding omptins are located either on plasmids or on the chromosome (Table 1). The regulation of omptin expression as well as the mechanisms of their assembly at the outer membrane have not been extensively studied. The omptin peptide sequences contain a typical approximately 20-residue-long signal sequence (Grodberg *et al*., 1988; McDonough and Falkow, 1989; Sodeinde and Goguen, 1989; Kaufmann *et al*., 1994; Egile *et al*., 1997) of proteins secreted by the general Sec-machinery. No specific chaperones have so far been

detected in omptin expression, the omptin sequences however contain a C-terminal phenylalanine (see Figure 1G), which has been found critical for the correct secretion of the porin PhoE from the periplasm of *E. coli* (de Cock *et al*., 1997; Jansen *et al*., 2000).

Some differences in the regulation of omptin gene expression have been found. Transcription of *ompP*, but not of *ompT*, is under catabolite repression, and a potential binding site for the catabolite activator protein is located upstream of the gene (Kaufmann *et al*., 1994). Production of OmpT and OmpP is temperature dependent and takes place at 37 °C but not at 27 °C (Manning and Reeves, 1977; Kaufmann *et al*., 1994), whereas Pla is produced at both temperatures (Straley and Brubaker, 1982; McDonough and Falkow, 1989). *sopA* has been suggested to be regulated by VirB (Fukuda *et al*., 1995) which at 37 °C activates transcription of various invasion genes in *Shigella* (Tobe *et al*., 1991). In *Salmonella* expression of several virulence functions is regulated at the transcriptional level by the PhoPQ two component system as a response to Mg^{2+} and Ca^{2+} concentration (reviewed in Ernst *et al*., 2001; Groisman, 2001). The PhoPQ system is needed for efficient expression of PgtE in the bacterial outer membrane (Adams *et al*., 1999; Guina *et al*., 2000), the regulation was proposed to be posttranscriptional (Guina *et al*., 2000). Analysis of transcriptional fusions between *pgtE* and firefly luciferase gene *(f-luc*) in wild type and PhoPQ mutant backgrounds showed that the transcription of *pgtE* was independent from *phoPQ*, and, moreover, the PhoPQ regulation did not affect translation initiation or translocation of PgtE into periplasm. The PhoPQ and the PmrAB regulatory systems respond to intracellular conditions and affect the expression of several genes, in particular, they regulate also modifications in the LPS structure of *S. enterica* (Guo *et al*., 1997; 1998; Gunn *et al*., 1998; Gibbons *et al*., 2000; Trent *et al*., 2001; Ernst *et al*., 2001; Groisman, 2001). Ability to substitute phosphates in the lipid A and the core with basic components probably is important for enteric bacteria, as it has been found to increase bacterial resistance against antimicrobial cationic peptides from phagocytes or epithelial cells (Gunn and Miller, 1996; Gunn *et al*., 1998; Gunn *et al*., 2000; reviewed in Ernst *et al*., 2001; Kawahara *et al*., 2002). As LPS influences assembly and/or activity of several outer membrane proteins, including omptins, it is possible that the effect of the PhoPQ system on surface expression of PgtE is an indirect result from cell surface changes regulated by the two-component system(s). It is interesting to note that transcription of *pgtE* is upregulated in *Salmonella enterica* serovar Typhimurium isolated from SCVs of murine macrophage-like J774-A.1 cells (Eriksson *et al*., 2003). This suggests that PgtE has a function in the intracellular survival of *S. enterica*.

1.4. Functions of omptins

1.4.1. Association with virulence

Pla of *Y. pestis* is the only omptin for which a role in virulence has been clearly documented. Virulent isolates of *Y. pestis* harbour the plasmid pPCP1 which is needed for the invasive character of plague. Loss of pPCP1 increases the median lethal dose of *Y. pestis* by a million-fold in subcutaneously infected mice, whereas no effect on virulence is seen in intravenously infected mice (Brubaker *et al*., 1965; Ferber and Brubaker, 1981). pPCP1 is specific to *Y. pestis*, and encodes three proteins: the bacteriocin pesticin, the protein conferring immunity to pesticin, and Pla (Sodeinde and Goguen, 1988; Hu *et al*., 1998). Sodeinde *et al*. (1992) showed by mutagenesis and complementation analysis of pPCP1 that the virulence function is encoded by *pla*. When bacteria were injected subcutaneously into mice, the LD_{50} value of a

Pla-negative mutant strain was close to 10^7 bacteria, which is very high when compared to the LD_{50} value of less than 50 bacteria for the isogenic Pla-positive strain. In intravenous infections, however, no difference between the virulence of the strains was observed. These data strongly indicate that Pla specifically enables the dissemination of *Y. pestis* from the subcutaneous infection site into the circulation. However, a less dramatic role for Pla has been suggested in pneumonic plague (Welkos *et al*., 2002).

Although the central role of Pla in the invasiveness of plague is well documented, it is important to note that the introduction of pPCP1 into *Y. pseudotuberculosis* does not increase the bacterium's virulence in a subcutaneous infection of mice (Kutyrev *et al*., 1999). This indicates that also other factors are needed to turn the intestinal pathogen into a systemic pathogen. These factors have not been identified (Parkhill *et al*., 2002). Intrestingly, *Y. pestis* strains such as Pestoides F (Motin *et al*., 2002; Worsham and Roy, 2003) have been described to be virulent in mice although they lack *pla* (Welkos *et al*., 1997; Worsham and Roy, 2003). These strains are interesting in the sense that they might represent transitional forms in the evolution of *Y. pestis* from *Y. pseudotuberculosis* (Motin *et al*., 2002).

The role of other omptins in pathogenesis is less obvious. SopA is encoded by the virulence plasmid in *Shigella* (Egile *et al*., 1997; Venkatesan *et al*., 2001) and EIEC (Santapaola *et al*., 2002) and indirectly potentiates intracellular movement needed for the bacterial virulence by cleaving the IcsA surface protein of *Shigella* (Egile *et al*., 1997). OmpT and OmpP are most often referred to as obstacles in expression and purification of recombinant proteins (Grodberg and Dunn, 1988; Baneyx and Georgiou, 1990; Hanke *et al*., 1992; Klauser *et al*., 1992; White *et al*., 1995; Matsuo *et al*., 1999; Miller, 1996 and references therein). OmpT cleaves fusion proteins which have unfolded regions and come into contact with the outer membrane in periplasm or are secreted to cellular surface, and it has been suggested to function in turnover and/or degradation of cell envelope proteins (Baneyx and Georgiou, 1990; Hanke *et al*., 1992; White *et al*., 1995; Miller, 1996 and references therein; Oliver *et al*., 2003). However, presence of *ompT* in *E. coli* isolates correlates with complicated urinary tract infections (UTIs) (Webb and Lundrigan, 1996), and a higher prevalence of *ompT* in isolates from UTI and newborn meningitis than in faecal strains has been reported (Lundrigan and Webb, 1992; Foxman *et al*., 1995; Marrs *et al*., 2002; Johnson *et al*., 2002). These studies suggest that OmpT may a have a role in pathogenesis of extraintestinal *E. coli* infections. Association of F-plasmid or OmpP with pathogenic situations has not been reported, neither has a possible role of PlaA in plant diseases caused by erwinias been reported.

1.4.2. Proteolytic targets for omptins

Several potential virulence-related functions have been proposed for omptins. Of these, the **plasminogen activation** by Pla of *Y. pestis* has been documented to be directly involved in pathogenesis of an infectious disease. The role of Plg in the pathogenesis of *Y. pestis* infection is underlined by the finding that Plg-deficient mice are a hundred-fold more resistant to *Y. pestis* infection than normal mice (Goguen *et al*., 2000). Plg is 90kDa proenzyme found in body fluids and extracellular matrices (ECM) and present in high concentrations (2μM) in human plasma. Plg is proteolytically activated to plasmin (Pln), which is a broad specificity serine protease that degrades several circulating and tissue proteins and activates latent proenzymes, prohormones and growth factors (reviewed in Saksela, 1985; Stephens and Vaheri, 1993). Pln is a key enzyme in fibrinolysis (Lijnen and Collen, 1995) and functions in

tissue remodelling and cellular movement through tissue barriers formed by basement membranes (BMs) and ECM. Pln directly degrades non-collagenous components of ECM and BM and on the other hand activates procollagenases, which degrade the collagen fibers or networks. Migrating cells, e.g. metastatic tumor cells, use surface-bound Pln to disintegrate tissue barriers and to disseminate into new locations in the body (reviewed in Liotta *et al*., 1986; Mignatti and Rifkin, 1993; Plow *et al*., 1999).

Uncontrolled plasmin activity would be disastrous, and the Plg system is tightly regulated through several mechanisms. This results in correct timing and targeting of the proteolytic activity in several physiological processes. The physiological activators, tissue-type plasminogen activator (tPA) and urokinase (uPA) activate Plg by a single proteolytic cleavage. Immobilization of Plg through its kringle structures to lysine-containing cellular receptors or target molecules causes a major conformational change in the proenzyme, this leads to exposure of the cleavage site and dramatically enhances activation by tPA (Mangel *et al*., 1990). The Plg system is also controlled by inhibitors of the Plg activators as well as of the Pln activity (reviewed in Rijken1995; Lijnen and Collen, 1995). The major inhibitor of Pln activity in circulation is α2-antiplasmin (α2AP). α2AP inhibits Pln activity by a two-step process which involves rapid formation of a reversible 1:1 complex between Plg and α2AP, and slower formation of a covalent bond between the active site serine in Pln and the reactive site in α2AP. α2AP binds to the Plg kringle structures, that also are involved in the immobilization of Plg/Pln to receptors, and hence α2AP inhibits Pln only in solution (reviewed in Lijnen and Collen, 1995). Immobilization of Plg/Pln onto a Plg receptor is a mechanism to create localized and transient proteolysis, best exemplified in the degradation of fibrin clots (reviewed in Lijnen and Collen, 1995).

Pla activates Plg similarly to human activators by cleaving the peptide bond between Arg560-Val561 in the proenzyme (Sodeinde *et al*., 1992). It has been estimated that the efficiency of Plg activation per cell surface area are essentially similar for Pla on the surface of *Y. pestis* and for urokinase on mammalian cells (Sodeinde *et al*., 1992). Pla also forms enzymatically active Pln in human and rat plasma (Sodeinde *et al*., 1992), which contain the inhibitors of Plg system, in particular α2AP. Plg or Pln receptors have not been reported for *Y. pestis*, and the mechanism by which Pla forms Pln activity in the presence of physiological inhibitors was not known when this thesis work was initiated.

The capacity of PgtE and OmpT to activate Plg has remained controversial. Original observations associating OmpT with Plg activation involved long incubations (9 h) of Plg with cell wall preparations from *E. coli* (Leytus *et al*., 1981). Lundrigan and Webb (1992) observed that of 282 clinical isolates of *E. coli*, majority were positive for *ompT* but only one strain exhibited detectable Plg activation in a fibrin lysis assay. Taken together, these data suggest that the Plg activator activity of OmpT is rather poor. Similarly, Plg activator activity has not been observed in wild-type isolates of *S. enterica* (Sodeinde and Goguen, 1989; Lähteenmäki *et al*., 1995). In contrast, recombinant *E. coli* strain expressing *pgtE* from a high-copy-number plasmid activated Plg in a 18-h long fibrin lysis assay. In the same assay, a thousand-fold lower number of *Y. pestis* cells was sufficient to cause detectable lysis of fibrin film (Sodeinde and Goguen, 1989). These observations indicate that PgtE is capable of Plg activation, perhaps with a lower efficiency than Pla, but for unknown reasons the activity is not detectable in *S. enterica* isolates.

Pla of *Y. pestis* has been reported to cleave the **C3 component of serum complement** (Sodeinde and Goguen, 1992). The cleavage of C3 may inactivate the downstream

antibacterial activities of the complement cascade, such as formation of the C5a fragment needed for chemotaxis of phagocytic cells as well as opsonization of bacteria with C3b (Rautemaa and Meri, 1999). Indeed, peripheral lesions of Pla-positive *Y. pestis* KIM-10 cells contained relatively few inflammatory cells, whereas the lesions containing isogenic Pla-negative cells become surrounded by phagocytic cells, mainly polymorphonuclear neutrophils (Sodeinde *et al*., 1992). However, in another study no difference in inflammatory response at subcutaneous infection sites was found between isogenic Pla-positive and Pla-negative *Y. pestis*; the Pla-negative derivative however spread much less efficiently from the initial infection site (Welkos *et al*., 1997). *Y. pestis* lacks the O-antigen which contributes to serum resistance of enteropathogenic *Yersinia* (Wachter and Brade, 1989; Skurnik, 2003) and cleavage of a complement component might therefore be important for serum resistance of *Y. pestis*. However, Sodeinde *et al*. (1992) found that Pla-negative *Y. pestis* was resistant to high concentrations of human serum and concluded that Pla does not provide resistance to complement-mediated lysis. Possible inactivation of complement proteins by the other omptins has not been studied.

Cationic antimicrobial peptides (CAMPs) play an important role in innate immunity against Gram-negative bacteria by enhancing the immune response in the host and by directly killing the bacteria (reviewed in Hancock and Scott, 2000; Hancock, 2001). CAMPs are short amphipathic peptides found on skin and mucosal surfaces as well as in neutrophils and macrophages (Hiemstra *et al*., 1993;1999; Boman, 1995).CAMPs penetrate the outer membrane, permeabilize the cytoplasmic membrane and may act on cytoplasmic anionic targets (Hancock and Scott, 2000; Hancock, 2001). Pathogenic bacteria use various resistance mechanisms against the direct bactericidal activity of CAMPs, these include outer membrane modification and efflux pumps (Bengoechea *et al*., 2000; Ernst *et al*, 2001; reviewed by Peschel, 2002). CAMPs are rich in Lys and Arg and hence potential targets for proteolysis by omptins. HPLC analysis of bacterial supernatants showed that CAMPs protamine and G18C (Darveau *et al*., 1992) were degraded in the presence of OmpT-expressing *E. coli* and PgtE-expressing *Salmonella*, and strains expressing omptins were more resistant to the killing by CAMPs than were their isogenic omptin-negative derivatives (Stumpe *et al*., 1998; Guina *et al*., 2000). Deletion of *pgtE* decreased survival of *Salmonella* in the presence of two α-helical CAMPs but did not alter sensitivity of bacteria to CAMPs with a β-sheet structure (Guina *et al*., 2000). OmpT also degrades another α-helical CAMP, mastoparan (Sugimura and Nishihara, 1988). These findings indicate selectivity of omptins for α-helical CAMPs.

Omptins have also been implicated in degradation of **outer membrane proteins**. Pathogenic *Yersinia* species share 70 kb plasmid which encodes virulence factors called Yops (*Yersinia* outer membrane proteins) and their dedicated type III secretion system (TTSS). Yops include effector molecules injected to host cytosol as well as proteins needed for their translocation through eukaryotic plasma membrane and they are found in outer membranes of enteropathogenic *Yersinia* (reviewed in Cornelis *et al*., 1998). In *Y. pestis* Yops are detected only in outer membranes of strains lacking pPCP1 or functional Pla, and it was shown that Pla degrades Yops (Sodeinde *et al*., 1988). Pla-mediated degradation of Yops does not impair function of TTSS, and Pla-positive and Pla- negative *Y. pestis* target Yops equally well into human HeLa cells (Skrzypek *et al*., 1998). The role of Pla-mediated degradation of Yops for pathogenesis of *Y. pestis* has remained unclear. SopA processes the outer membrane protein IcsA needed for intra- and intercellular actin-based movement and virulence in *Shigella* (Makino *et al*., 1986; Bernardini *et al*., 1989; Sansonetti *et al*., 1991; Goldberg and Theriot, 1995). IcsA is an autotransporter (Henderson *et al*., 1998) and contains a β-barrel membrane anchor and an extracellular functionally active domain which is released from bacterial

surface by SopA (Suzuki *et al*., 1995; Fukuda *et al*., 1995; Egile *et al.*, 1997). IcsA is delivered at the old pole of the bacterium (Steinhauer *et al*., 1999), at which an actin tail is assembled (Goldberg *et al*., 1993). Smooth LPS and SopA maintain the polar distribution of IcsA. *Shigella* is very closely related to *E. coli* and resembles EIEC in pathogenesis in that both are oral pathogens and employ actin-based movement for bacterial spread into intestinal epithelium (Sansonetti, 2001). *Shigella* and isolates of EIEC lack *ompT* and introduction of *ompT* into *Shigella* causes total cleavage, rather than processing, of IcsA and thus abolishes actin-based motility (Nakata *et al*., 1993). This offers an example of different substrate specifities of omptins.

1.4.3. Non-proteolytic functions of omptins

Bacterial adhesion to mucins covering epithelia, to epithelial receptors or to ECM is important in nearly all bacterial infections. ECM consists mainly of collagens, laminins, fibronectins, proteoglycans and elastin assembled into complex networks (Hay, 1991). BMs are specialized sheet-like structures of ECM which underline epithelia and endothelia and form tissue barriers which pathogenic bacteria and tumor cells must penetrate in order to enter into circulation or adjacent tissue compartments. BMs are also considered as reservoirs of the Plg system and contain Plg activators as well as Plg which can be activated to functional plasmin (Farina *et al*., 1996). BMs also contain procollagenases that are proteolytically activated by Pln and further disrupt the barrier functions of ECMs. Metastatic tumor cells adhere to ECMs and gain Pln activity which is used directly and through activated procollagenases for cellular migration across tissue barriers (Liotta *et al*., 1986; Mignatti and Rifkin, 1993).

Pla-expressing bacteria adhere to the mouse BM preparation Matrigel and the ECM prepared from cultures of the human lung cell line NCI-H292 (Lähteenmäki *et al*., 1998). Pla efficiently mediates adhesion to laminin and to a lesser extent also to heparan sulphate proteoglycan (HSPG). Also, a low-affinity binding of Pla to mouse type IV collagen has been reported (Kienle *et al*., 1992) but this was not seen with human collagens (Lähteenmäki *et al*., 1998). Pla was also found to enhance bacterial adhesion to human epithelial cell lines and extracted glycolipids (Kienle *et al*., 1992). It remains to be determined whether the Pla-mediated adhesion to BM and to epithelial cell cultures involve the same target molecules, e.g. laminin or heparan-containing compounds. Pla is not able to degrade laminin or lung epithelial cells matrix directly (Lähteenmäki *et al*., 1998), which, on the other hand, are targets for plasmin proteolysis (Mignatti and Rifkin, 1993). Indeed, degradation of laminin and ECM from human lung cells via Pla-mediated plasmin formation has been demonstrated (Lähteenmäki *et al*., 1998). Thus, the adhesion of *Y. pestis* to laminin and BM may potentiate penetration of the bacteria through tissue barriers by localizing proteolysis onto plasmin-susceptible tissue targets.

While this study was being initiated, Cowan *et al*. (2000) reported an efficient *in-vitro* **invasion** of *Y. pestis* into human epithelial HeLa cell line and that the presence of the plasmid pPCP1 was responsible for over 90% of the observed invasiveness. The authors did not determine the role of Pla in the invasion, but an interesting observation was that serum at low concentration inhibited the invasion into HeLa cells. This suggests that Pla does not mediate invasion once *Y. pestis* has reached the circulation.

2. AIMS OF THE STUDY

Y. pestis is one of the most fatal bacterial pathogens to humans known to date, and Pla is a central virulence molecule in the pathogenesis of plague. This study was initiated to analyze the structure-function features in the Pla molecule that enable the bacterium's spread through tissue barriers. Bacteria were known to intervene with the Plg/Pln system at various levels (reviewed by Lähteenmäki *et al*., 2001), and the central roles Pla and Plg in the pathogenesis of plague had become clear (Sodeinde *et al*., 1992; Goguen *et al*., 2000). On the other hand, function of the other omptins of *Enterobacteriaceae* in Plg activation and in bacterial virulence remained less clear, and speculations about the evolution of Pla from PgtE had been suggested (Sodeinde and Goguen, 1989). Pla is a medically important plasminogen activator, and comparison of its functional and structural features to those of other omptins offered an evolutionarily and medically interesting question. Pla had been found to be multifunctional in that it mediates bacterial adherence to laminin and BMs (Lähteenmäki *et al*., 1998), and this work was inspired by a report suggesting that Pla could be involved in invasion of *Y. pestis* into human cells (Cowan *et al*., 2000). The crystal structure of OmpT (Vandeputte-Rutten *et al*., 2001) became available during preparation of this thesis and shaped the work by giving better grounds for structure-function analysis. More specifically, this study addressed the functional comparison of the omptins and structure-function relationships in Pla.

3. MATERIALS AND METHODS

Bacterial strains, eukaryotic cell lines and plasmids used in this study are listed in Tables 3 and 4. The methods are described in detail in the original articles and are summarized in Table 5.

Table 3. Bacterial strains and cultured eukaryotic cell lines used in this study

Bacterial strain	Characteristics	Article	Reference
E. coli			
XL1 Blue MRF´	Δ(*mcrA*) 183 Δ(McrCB-hsdSMR-mrr) 173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB lacI*^{q} *Z*Δ *M15* Tn *10* (tet)]	I, II, III	Stratagene
BL21 λDE	F$^-$ *hsd ompT lon*	I	Novagen
M15		III	Qiagen
AAEC072	Δ*fim* derivative of *E. coli* MG1655	I	Blomfield *et al.*, 1991
IHE307	Rough LPS	III	Selander *et al*., 1986
IHE3074	Rough LPS	III	Selander *et al*., 1986
IHE3034	O18 LPS	III	Selander *et al*., 1986
IHE1049	O1 LPS	III	Pere *et al*., 1988
S. enterica			
Typhimurium SL1102	Re LPS	III	Wilkinson *et al*., 1972
Typhimurium SL1102-1	Δ*pgtE* derivative of SL1102	III	This study
Typhimurium 14028	Smooth LPS	III	ATCC
Typhimurium 14028-1	Δ*pgtE* derivative of 14028	III	This study
Typhimurium SH401	Rough LPS	III	Kukkonen *et al*., 1993
Typhimurium SL696	Smooth LPS	III	Wilkinson *et al*., 1972
Typhimurium SL901	Semirough LPS	III	Wilkinson *et al*., 1972
Minnesota SF1112	Ra LPS	III	Schmidt and Lüderitz, 1969
Minnesota SF1127	Rb_1 LPS	III	Schmidt and Lüderitz, 1969
Minnesota SF1119	Rc LPS	III	Schmidt and Lüderitz, 1969
Minnesota SF1121	Rd_1 LPS	III	Schmidt and Lüderitz, 1969
Minnesota SF1118	Rd_2 LPS	III	Schmidt and Lüderitz, 1969
Minnesota SF1167	Re LPS	III	Schmidt and Lüderitz, 1969
Y. pestis			
KIM D27	pPCP1$^+$ *pgm* pYV$^+$ derivative of *Y. pestis* KIM-10	I	Finegold *et al*., 1968; Une and Brubaker, 1984
KIM D34	pPCP1$^-$ *pgm* pYV$^+$ derivative of *Y. pestis* KIM-10	I	Finegold *et al*.,1968; Une and Brubaker, 1984 Sample *et al*., 1987
Y. pseudotuberculosis			
Pa3606	O1b LPS, pYV$^-$	III	Tsukobura and Aleksic, 1995
PB1	O1b LPS, pYV$^-$	III	Perry and Brubaker, 1983
PB1Δ*wb*	lacks Oag due to Δ*wb*, pYV$^-$	III	J.A. Bengoechea M.Skurnik
ECV-304	endothelial-like cell line	II,III	Takahashi *et al*., 1990 ATCC (CRL-1998)
HUV-EC-C	human endothelial cell line	III	ATCC (CRL-1730)
HUVEC	primary human endothelial cells	III	Technoclone

Table 4. Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pC4006	*pla* of *Y. pestis* cloned in a 1.2 kb *Pst* I-*Hin*d III fragment	Kienle *et al*., 1992
pSE380	*trc* promoter, *lacO* operator, *lacI*	Invitrogen
pMRK1*	*pla* in pSE380	I, II, III
Hy1	Pla L1-L4 - OmpT L5 hybrid	I
Hy4	Pla L1- OmpT L2-L5 hybrid	I
Hy5	OmpT L1 – Pla L2-L5 hybrid	I
Hy6	OmpT L1-L2 – Pla L3-L5 hybrid	I
Hy7	OmpT L1-L3 – Pla L4-L5 hybrid	I
Hy8	OmpT L1-L4 – Pla L5 hybrid	I
Hy9	Pla L2 - OmpT L1, L3-L5 hybrid	I
Hy10	OmpT L2 – Pla L1, L3-L5 hybrid	I
Hy11	Pla L3 – OmpT L1-L2, L4-L5 hybrid	I
Hy12	OmpT L3 – Pla L1-L2, L4-L5 hybrid	I
Hy13	Pla L4 – OmpT L1-L3, L5 hybrid	I
Hy14	OmpT L4 – Pla L1-L3, L5 hybrid	I
Hy17	Residues 210-229 in Pla L4 substituted	I
Hy18	Residues 191-209 in Pla L4 substituted	I
pMRK2	*ompT* in pSE380	I, II, III
OmpT/ΔDP	ΔD214 P215 in OmpT	I
OmpT/ΔDP/K217R	ΔD214 P215 and substitution K217R in OmpT	I
OmpT/ΔDP/K217R/L3	ΔD214 P215, substitution K217R and substitution of L3 in OmpT	I
pMRK3	*pgtE* in pSE380	III
pMRK31	PgtE D206A	III
pMRK32	PgtE R138E	III
pMRK33	PgtE R171E	III
pMRK34	PgtE R138E R171E	III
pQE30	T5 promoter, *lac* operator	Qiagen
pREP4	*lacI*	Qiagen

*In paper I, following single amino acid substitutions were made in Pla: H28V, H98V, H101V, H108V, H203V, H280V, S77A, S99A, S267A, D84A, D86A, D97A, D206A, D212R, M210G, R211K, L213I, F215Y, E217S, K218A, K240A, K249A, K254A, K262A, K280A. Also, insertion D211 P212 was made. The constructs were otherwise identical to pMRK1. Pla variants S99A and D206A were also used in paper II.

Table 5. Methods used in this study

Method	described and /or used in
Cultivation of bacteria	
Induction of omptin production in recombinant strains	I, II, III
Induction of *pgtE* expression in wt *Salmonella*	III
Genetic methods	
Allelic replacement	III
Construction of expression vectors	I, III
Construction of hybrid molecules	I
DNA sequencing	I, III
Generation of point mutations	I, III
Protein work	
Amino acid sequencing	I
Production of antibodies	I, III
Purification of His_6-tag-omptins	I, III
Reconstitution of Pla	III
Characterization of omptin expression in bacterial strains	
Heat modifiability of omptins	III
Immunofluorescence	I
Isolation of cell envelopes	I, III
Western blotting	I, III
Characterization of omptin functions	
α2AP cleavage	I
α2AP inactivation	I
Adhesion to ECM	II
Adhesion to Matrigel	III
Autoprocessing	I, III
Invasion	II, III
Plasminogen activation	I, II, III
Plasminogen cleavage	I
Purification of LPS	III

4. RESULTS AND DISCUSSION

4.1. Structure of Pla and PgtE (I, III)

4.1.1. Topology and structure modelling (I, III)

This study was initiated by a structural modelling of Pla and OmpT, which were known to be outer membrane proteins and whose sequences had been reported earlier (Grodberg *et al*., 1988; McDonough and Falkow, 1989; Sodeinde and Goguen, 1989). The topology models shown in Figure 3 of I were based on identifying antiparallel β-strands in the predicted protein sequences, basically according to the criteria given by Koebnik *et al.* (2000) and Schulz (2000). Main criteria were a suitable length (between 6 and 25 residues), position of girdles of aromatic residues that can anchor the molecule in the membrane, and the presence of non-polar side-chains on protein surface within the membrane. Transmembrane β-strands do not contain cysteine residues, which also are lacking in all omptin sequences (see Figure 1G). The topology models of Pla and OmpT shown in Figure 3 of I indicated that the protein sequences have ten antiparallel β-strands connected by 20-35 residues long loops on the side facing outwards and by shorter, 5-8 residues long turns on the cytoplasmic side. These models were used as the basis in design of hybrid Pla-OmpT proteins as well as in production of anti-peptide antibodies specific for each loop of Pla, and the results obtained in these studies were in accordance with the predicted topology of Pla and OmpT (discussed in more detail below). During this study, we came aware of the topology models for OmpT reported by Kramer *et al.* (2000a,b), whose models are essentially similar to those of Pla and OmpT in Figure 3 of I.

The crystal structure of OmpT (Vandeputte-Rutten *et al*., 2001) confirmed its predicted β-barrel conformation. The peculiar properties of OmpT are its long, vase-shaped structure which makes it protrude 40 Å from the bacterial surface (see Figures 1A and 1B). The β-strands of OmpT have an average length of 23 residues, i.e. they are longer than what we predicted for OmpT and Pla in Figure 3 of I. The OmpT barrel is hollow, and the active site is within a large negatively charged groove. The coordinates of OmpT were used to model the structure of Pla and PgtE; these are shown in Figures 1C through 1F. The overall folding of the proteins remains the same, the active-site groove is conserved (modeled for Pla in Figure 1C), and the functionally important residues identified in this work (discussed in more detail in subsequent chapters) can be explained in accordance with the model structures.

The topology model shown in Figure 3 of I formed the basis for design of synthetic peptides that were used to obtain loop-specific antisera for Pla. The peptides were as follows: L1, amino acid residues 24-38; L2, residues 74-104; L3, residues 149-163; L4, residues 201-220; and L5, 249-278 (see Figure 1G). Anti-L2, anti-L3 and anti-L5 specifically reacted in indirect immunofluorescence staining (IF) with *E. coli* XL1 (pMRK1) but not with the host strain with the vector plasmid only (not shown), suggesting that these protein regions indeed are on the bacterial surface. The IF reaction was strongest with anti-L3, which may be explained by the large surface-exposed area of L3 (see Figure 1E). In contrast, very poor reactivity of XL1 (pMRK1) in IF was seen with anti-L1 and anti-L4 (not shown). In Western blotting, all anti-loop antisera specifically stained Pla as well as the correct Pla-OmpT hybrids described below, and their reactivities were strong (for an example on anti-L5, see Figure 5 of I), which suggested that the antisera indeed were specific for their corresponding loop structure of Pla. The failure of the anti-L1 and the anti-L4 to react in IF may result from the recognition of linear epitopes that are constrained and folded in the barrel and/or from the poor accessibility

of L1 and, in particular, of L4 (see Figures 1C-1E). It was however concluded that the topology models presented in Figure 3 of I as well as the three-dimensional models of Pla and PgtE in the supplementary material to III are reliable enough for the localization of active regions and residues in the omptins.

4.1.2. Omptin expression in recombinant *E. coli* (I, III)

The ORFs coding for Pla, PgtE and OmpT were cloned under an inducible *trc* promoter into pSE380, and the resulting plasmids pMRK1, pMRK2 and pMRK3 were transformed into *E. coli* XL1. After cultivation in inducing conditions, expression levels of the omptins by *E. coli* recombinant strains XL1(pMRK1), XL1(pMRK2) and XL1(pMRK3) were studied by Western blot analyses of purified cell envelope preparations and indirect immunofluorescence with primary antibodies raised in rabbits against the His_6-tag-omptin fusions. Western blotting revealed similar levels of expression of Pla, PgtE and OmpT by the recombinant strains (Figure 2; see also Figure 2 of I). Surface localization of Pla, OmpT and PgtE in the recombinant strains was studied by IF of intact cells and by heat modifiability of the omptins in cell envelope preparations. Anti-His_6-Pla and anti-His_6-OmpT antibodies specifically reacted with recombinant strains XL1(pMRK1) and XL1(pMRK2) (not shown). The anti-His_6-PgtE antibodies, on the other hand, had weaker affinity to PgtE in Western blot analysis and failed to react with XL1(pMRK3) cells. The β-barrel of outer membrane proteins is stable in the presence of SDS and denatures only after heat treatment. The unheated, folded form of the barrel has a higher mobility in SDS-PAGE gels than the heat denaturated form (Heller, 1978), and this heat modifiability has been used as a measure of proper folding and assembly of outer membrane proteins in the outer membrane (Kramer *et al*., 2000a,b). In Western blot analysis of cell envelopes of XL1(pMRK3), anti-His_6-tag-PgtE serum stained a peptide of apparent molecular weight (MW) of 27 kDa in the unheated sample (Figure 3, lane c of III), but a peptide of 34 kDa in the heat-denaturated sample (Figure 3, lane d of III); the calculated MW of PgtE is 32,8 kDa. OmpT from cell envelopes of XL1(pMRK2) was used as a positive control in heat modification (Kramer *et al*., 2000a,b) (data not shown). It was conluded that the omptins expressed in the XL1 recombinant strains were produced at comparable amounts and assembled into the outer membrane.

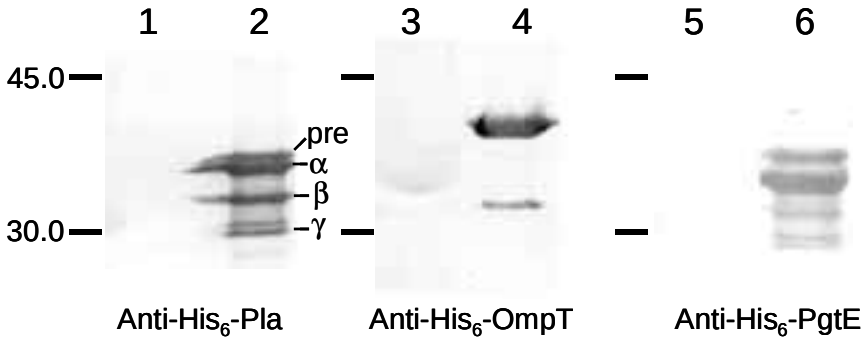

Figure 2. Comparison of the levels of expression of Pla, OmpT and PgtE in the *E. coli* XL1 host strain. Western blotting of cell envelope preparations from recombinant strains: XL1(pSE380) (lanes 1, 3, and 5); XL1(pMRK1) (*pla*+) lane 2; XL1(pMRK2) (*ompT*+) lane 4; and XL1(pMRK3) (*pgtE*+) lane 6. The primary antibodies and the different forms of Pla are indicated. The processed forms of PgtE i.e.the β-and γ-forms, are not visible in lane 6. Migration of molecular weight markers in kilodaltons is shown on the left.

In Western blotting of Pla in cell wall preparations from recombinant *E. coli*, several peptides were recognized by the anti-His_6-Pla antisera (Figure 2 of I). Such multiple omptin peptides have been described earlier, and in keeping with the nomenclature used earlier (Sodeinde and Goguen, 1988; Kutyrev *et al*., 1999), these peptides were named, in the order of decreasing MW, as pre-Pla (the nonprocessed form), α-Pla, β-Pla, and γ-Pla (Figure 2A of I). Pre-Pla is expected to have remained inside the cell, as inclusion bodies perhaps, and the α, the β, and the γ forms have been assumed to result from processing first to α-Pla and then to the other forms (Sodeinde and Goguen, 1988; 1989; Kutyrev *et al*., 1999). This processing was proposed to be an activation process, which was quite natural in the view of the proteolytic processing of eukaryotic Plg activators (reviewed in Rijken, 1995). Similar forms of Pla were detected when Pla was expressed in *ompT degP* mutant *E. coli* strain BL21 (I; not shown). As will be described below, the formation of the β-Pla was not seen when the proteolytic activity of Pla was inactivated, and it seems likely that β-Pla results from autoprocessing of α-Pla. In contrast, presence of γ-Pla was not dependent on the proteolytic activity of Pla (Table 1 of I), and it probably represents the mature form folded differently than α-Pla. Also OmpT showed two peptides, 39 kDa and 31 kDa in MW (Figure 2 of I), these has been proposed by Kramer *et al.* (2000b) to correspond mature and the autoprocessed forms of OmpT.

Also recombinant *E. coli* expressing *pgtE* showed the presence of several peptides specifically reacting with anti-His_6-PgtE antibodies (Figure 3B lane d of III). In an analogy to the Pla system, these were designated as α-PgtE, β-PgtE and γ-PgtE, with the MWs of 34kDa, 17 kDa, and 14 kDa, respectively. The PgtE forms and their possible autoprocessing were not studied in detail in this study, and hence their exact nature remains uncharacterised. However, it is noteworthy that the β and the γ forms of PgtE were not detectable in proteolytically deficient forms of PgtE (Figure 3B lanes f and l of III) which is in accordance with their resulting from autoprocessing and suggests that they both are cleavage products of PgtE. A major conclusion from the study of the various molecular forms of Pla was that prevention of the autoprocessing into β-Pla had no detectable effect on Plg activation by *E. coli* XL1 expressing the corresponding plasmids (Table 1 of I; Figure 5 of I). Thus Pla differs from the eukaryotic plasminogen activators, which are synthetized as proenzymes and are proteolytically activated into their active forms (reviewed in Rijken, 1995).

In summary, the predicted structures of Pla and PgtE are in accordance with the 10-stranded β-barrel recently reported for the crystal structure of OmpT (Vandeputte-Rutten *et al*., 2001). Both proteins seem to be autoprocessed, for Pla of *Y. pestis* this is not related to enzymatic activity, which makes a difference to eukaryotic Plg activators that are activated through proteolytic cleavage. Overall, the biological role, if any, of the autoprocessing event in omptin biosynthesis and activity remains open.

4.1.3. Serological crossreactivity of omptins (I)

We analysed the cross reactivity of omptins in Western blotting using the anti-His_6-omptin antisera and the anti-loop antisera obtained by immunizing with synthetic peptide mimicking the loops of Pla. No serological cross-reactivity in Western blotting was detected between Pla and OmpT (Figure 2A of I) or between PgtE and OmpT (Table 2). Instead, Pla and PgtE were cross reactive in Western blot analyses (data not shown). This was to be expected in view of the higher sequence identity between Pla and PgtE than between OmpT and Pla or PgtE and OmpT (Table 1). The five anti-loop antisera reacted well with Pla and stained all forms

(i.e.pre, α, β, or γ) of Pla molecule.The loop-specific sera did not react not at all with OmpT; with PgtE a positive reaction was seen only with anti-L5 (Table 2). The L5 peptide sequence used for immunizing is identical with the PgtE sequence at 22 of the 30 (73%) residues, which is less than in e.g. L4 (17/20; 85%) identical residues but more than e.g. in L1 (9/15; 60%) or L2 (20/31; 65%). Epitopes recognized by antibodies often contain charged side chains of amino acids. Charged residues in the loop regions of Pla and PgtE (see Figure 1G) are not conserved which may explain the observed lack of crossreactivity.

Table 2. Serological crossreaction of the omptins by Western blotting

	Reactivity with omptin		
Antiserum	Pla	OmpT	PgtE
Anti-His_6-Pla	+	-	+
Anti-His_6-OmpT	-	+	-
Anti-His_6-PgtE	+	-	+
Anti-L1	+	-	-
Anti-L2	+	-	-
Anti-L3	+	-	-
Anti-L4	+	-	-
Anti-L5	+	-	+

4.2. Structure-function relationships in omptins (I, III)

4.2.1. Proteolysis (I)

This study began by searching for protein regions of Pla that are important in Plg activation. A basis was the observation that, when expressed in recombinant *E. coli*, Pla and OmpT exhibited dramatically different levels of Plg activation (Figure 4 of I). This was not surprising in view of the existing literature on Plg activation by *Y. pestis* and *E. coli* (Leytus *et al*., 1981; Sodeinde and Goguen, 1989; Lundrigan and Webb, 1992). Another difference between the two omptins was that OmpT directly cleaved the chromogenic protease substrate Val-Leu-Lys-*p*-nitroaniline (S-2251), which was not cleaved by Pla expressed in *E. coli*. We tested various similar short, Arg-containing or Lys-containing chromogenic peptides but failed to find any that is degraded by Pla-expressing *E. coli* (not shown). On the other hand, proteolytic activity of OmpT can readily be studied with short peptides (Kramer *et al.*, 2000a,b; Dekker *et al.*, 2001), and our finding preliminarily suggests that OmpT is better suited to degrade small-molecular-weight substrates than Pla. This may be related to the differing biological functions of the two proteases, i.e. degradation of CAMPs (Stumpe *et al*., 1998) and nonfolded proteins (White *et al*., 1995) by OmpT or physiologically important proteins (such as Plg) by Pla.

We also observed that Pla-positive *Y. pestis* and recombinant *E. coli* inactivated and degraded α2AP (Figure 1A and C of I) which most likely is very important in creating the high proteolytic activity associated with plague (Sodeinde *et al*., 1992). The cleavage site in α2AP was not determined in this study, but the nearly complete degradation shown in Figure 1C of I and the finding that proteolytically negative mutants of Pla failed to inactivate α2AP (Table 1 of I), suggests that the inactivation is a result of proteolytic degradation of α2AP. OmpT was not able to cleave α2AP (Figures 2B and 4 of I).

The results above indicated that Pla and OmpT exhibit differing substrate specificities with Plg, α2AP, and S-2251. We next decided to identify the regions in Pla that are important for Plg activation and α2AP inactivation. This was initiated by constructing Pla-OmpT hybrids, where one or more loops were exchanged between the two proteins. The fusion sites were located in the conserved regions in the predicted membrane spanning β-strands, as we wanted to alter the membrane insertion of the hybrids as little as possible. The substitutions were analysed by Western blotting of cell envelope proteins using the anti-loop antisera. Each hybrid protein described in Figure 4 of I showed the expected reactivity with the anti-loop antisera; the loop specific sera recognized only those hybrids that carried the antigen sequence (for an example, see Figure 5 of I).

Analysis of Plg activation by the Pla-based hybrid proteins showed the importance of L4 and L5 in the process, substitution of L3 also had an effect on activation, whereas L1 and L2 could be substituted to the corresponding OmpT loops without any dramatic effect on Plg activation (Figure 4 of I). Further substitution of L4 showed that the C-terminal region of L4, i.e. the sequence ^{210}MRDLTFREKTSGSRYYGTVI^{229} was important for proteolytic activity. The importance of loop 4 was further indicated by the observation that the lack of the two L4-residues present in OmpT, D211 and P212, is essential for Plg activation by Pla (Table 1 of I). Similar reactivity of the hybrid proteins with α2AP was detected (Figure 4 of I), i.e. Hy12, Hy14, Hy1, and Hy17 were very poor in α2AP cleavage. None of the OmpT-based hybrids caused significant Plg activation, and Hy4 and Hy13 (with loops L1 and L4 exchanged) were nearly completely inable to cleave S-2251. Interestingly, Hy11 with L3 exchanged exhibited a low level of Plg activation, which indicated that L3 is important Plg recognition.

We next begun a more detailed substitution analysis of Pla to identify catalytic residues as well as residues affecting substrate specificity. At the time when this study was undertaken, omptins were regarded as serine proteases which have an Asp-Ser-His triad in the catalytic center. Our rational was that catalytic residues should be conserved in all omptins, and we substituted all six histidines in Pla as well as conserved serines and aspartates located in surface-exposed loops (see Figure 1G), and we also searched for local homologies to tPA and uPA sequences. A total of 25 substitution mutants was created and analysed for Plg activation, α2AP cleavage, processing (Table 1 of I), and, in the latter analysis, also for expression in the outer membrane.

Six substitutions, H101V, H208V, S99A, D84A, D86A, and D206A nearly completely prevented all three proteolytic activities (Table 1 of I). These residues all are oriented inwards in the upper part of the active-site groove in Pla (Figure 1C) and likely to make a contact with the substrates (i.e. Plg, α2AP, and Pla itself). Studies by Vandeputte-Rutten *et al.* (2001) and Kramer *et al*. (2000b, 2001) indicated that D83 and D85 as well as D210 and H212 are catalytic residues in OmpT (see Figure 1A), which is in a complete agreement with our substitution analysis of Pla proteolysis. In light of the hydrolysis mechanism proposed by Kramer *et al.* (2001), H101 and S99 of Pla most likely are involved in substrate binding. A few of the substitutions had more limited effects, i.e. they selectively affected only one the proteolytic functions studied in I. The substitution R211K at the tip of L4 (see Figure 1D) abolished α2AP cleavage and reduced Plg activation but did not affect the formation of β-Pla (Table 1 of I; Figure 5 of I). This residue most likely is important in recognition of α2AP and Plg by Pla.Conversely, the substitution of the inwards-oriented residues F215Y and E217S near L4 (Figure 1E) specifically decreased or abolished autoprocessing (Table 1 of I; Figure 5 of I). These residues are likely to be involved in self-recognition of Pla.

The proteolytically negative mutants of Pla did not form β-Pla, whereas γ-Pla was present (Figure 5 of I), which strongly suggested that β-Pla results from autoprocessing. We determined the site of autoprocessing in Pla. The N-terminal amino acid sequence of β-Pla equalled that of α-Pla, which suggested that Pla is processed at the C-terminus. The apparent MW of β-Pla suggested that it is ca. 30 amino acids shorter than α-Pla, and we substituted each basic residue at or near L5 (K218, K240, K249, K254, K262, and K280). Of these, the K262 derivative did not form β-Pla (Figure 5 of I), although it retained Plg activation at a level close to that shown by nonsubstituted Pla (Table 1 of I). The substitution derivative K262 also cleaved α2AP. It was concluded that K262 is the autoprocessing site in Pla. This residue is in L5 on top of the Pla-barrel (Figure 1E).

The results above indicated that surface-exposed residues, mainly in the loops of Pla, are critical for its ability to cleave Plg and α2AP. To test this hypothesis, we decided to convert the substrate specificity of OmpT into that of Pla (Figure 6 of I). The key elements in the conversion were the shortening of L4 by two residues, i.e. D214 and P215 (OmpT numbering, see Figure 1G) close to the catalytic Asp-His pair (residues 206 and 208 in Pla, see Figure 1C), the substitution K217R in L4 (residue R211 in Pla, Figures 1D and 1G), and shortening L3 by four amino acids to the size of the Pla L3 by substitution (Figure 1G). The resulting hybrid protein OmpT/ΔDP/K217R/L3 exhibited a remarkably efficient Plg activation (Figure 6A of I). The importance of these structural features are underscored by the finding that they are shared by PgtE (Figure 1G) that also is an efficient Plg activator (Figure 1 of III). As expected, further substitution of Pla loops into OmpT improved Plg activation as well as α2AP inactivation (Figure 6 of I). In particular, substitution of L5 from Pla into OmpT/ΔDP/K217R/L3 (Hy6) significantly improved the α2AP degradation (Figure 6C of I lanes 2 and 7) without improving the level of Plg activation (Figure 6A of 1). This suggests of the importance of L5 in recognition of α2AP.

In summary, this work gave evidence that omptins differ in substrate specificity and that sequence differences at surface loops of omptins are responsible for these differences. Gradual conversion of OmpT into Pla gives an example of how a housekeeping protease (OmpT) might have evolved into a powerful virulence factor by accumulation of subtle mutations at critical sites without changing the overall architecture of the protein fold. It appears that the size of the surface loops as well as the nature of the amino acids are important for the ability of Pla to recognize Plg and α2AP. OmpT has longer L3 and L4 than Pla or PgtE, and the active-site groove appears in OmpT to be less accessible for protein substrates that those in Pla and PgtE. OmpT, on the other hand, is able to cleave small-molecular weight substrates, which may reflect its function in degradation of CAMPs. Olsen and coworkers (2000) described a technique to isolate enzymes with novel substrate specificities from large libraries of protein variants. They developed the method with OmpT and searched for variants that would degrade novel small-molecular weight, fluorescent substrates. Olsen *et al*. (2000) isolated OmpT variants with improved cleavage of an Arg-Val sequence, and the altered residues were located in the crystal structure of OmpT either into loops or along the barrel. Comparison of the study of Olsen *et al.* (2000) to this thesis work is difficult, as the substrates are different in nature and the variants of Olsen *et al.* (2000) have multiple mutations in different parts of the OmpT molecule.

4.2.2. Rough LPS is critical for function of Pla and PgtE (III)

In vitro folding of OmpT into an enzymatically active form requires LPS (Kramer *et al*., 2000; 2002), and we found that renaturation of purified Pla into active form was dependent on rough LPS. His_6-Pla was isolated from recombinant *E. coli*, and reconstituted in the presence of the detergent N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulphonate ($DodMe_2NPrSO_3$) alone or in combination with Re or smooth LPS from *S. enterica* (Figure 2D of III). Plg activation was observed with Pla reconstituted with the Re LPS but not with the other two reconstituted forms. This finding is in accordance with those of Kramer *et al.* (2000) who, however, did not analyse the effect of smooth LPS on OmpT renaturation.

Y. pestis has evolved from *Y. pseudotuberculosis* O1b (Achtman *et al*., 1999: Skurnik *et al*., 2000), and this process has involved inactivation of several genes encoding proteins needed in O-antigen synthesis (Skurnik *et al.*, 2000; Prior *et al*., 2001). The O-antigen synthesis in *Y. pseudotuberculosis* is temperature-sensitive (Bengoechea *et al*., 1998), and we next expressed *pla* in *Y. pseudotuberculosis* Pa3606 cultured at 25 ^{o}C or, to suppress the O-antigen synthesis, at 37 ^{o}C as well as in *Y. pseudotuberculosis* PB1 and its isogenic rough Δ*wb* derivative cultivated at the both temperatures. The results showed that Plg activation was exhibited by *Y. pseudotuberculosis* carrying pMRK1 only when the bacteria were lacking an O-antigen (Figure 2A, B and C of III). No significant differences in cell wall localization or processing of Pla in the strains were observed (Figure 2A of III), which indicates that the O-antigen did not interfere with the transport of Pla into the outer membrane.

Plg activation by *S. enterica* has remained controversial (Sodeinde and Goguen, 1989; Lähteenmäki *et al*., 1995). In light of the results above and the efficiency of Plg activation by recombinant *E. coli* expressing *pgtE* (Sodeinde and Goguen, 1989), we reasoned that the failure to detect Plg activation by clinical isolates of *S. enterica* may result from its O-antigen. Indeed, efficient Plg activation was seen with the rough *S. enterica* strain SL1102 but not by the commonly used virulent, smooth strain 14028 (Figure 1A of III). In-frame deletion of *pgtE* abolished the Plg activation, and complementation of the mutation by pMRK3 restored the activity. Complementation of the *pgtE* deletion in 14028-1 supported a low level of Plg activation, which however remained clearly lower than what was seen with the rough strain SL1102-1(pMRK3) (Figure 1A of III). No significant differences in the levels of PgtE insertion in the cell wall preparations from the *pgtE*-positive strains were seen (Figure 1B of III), which indicated that the LPS type did not effect outer membrane location of PgtE.

We next determined the size of the O-antigen required to prevent Plg activation by PgtE. The plasmid pMRK3 was expressed in the well-characterized series of *S. enterica* strains (Schmidt and Lüderitz, 1969) possessing LPS molecules of different length (Figure 1C of III). The activity was seen in all rough strains, the semirough strain with one O-antigen unit was slightly decreased in activity, whereas the smooth strain did not exhibit Plg activation. Again, no significant differences in the membrane expression of PgtE were detected (Figure 1D of III). This is in accordance with the observation by Lugtenberg *et al.* (1976) that defects in the LPS core do not affect amount of OmpT in outer membranes of *E. coli*.

Taken together, these studies on PgtE and Pla expression were in accordance to each other and indicate that the Plg activator activities of Pla and PgtE are effective only in bacteria lacking an O-antigen. The successful reconstitution of Pla with rough LPS is in agreement with the results by Kramer *et al*. (2000; 2002) and indicate that the omptins require rough LPS for activity. Our observation that OmpT is able to cleave S-2251 also when expressed in

a smooth strain (I; not shown), indicates that the prevention of Plg activation by the O-antigen repeats probably results from a steric inhibition of the contact of omptins with a large-molecular-weight substrate, e.g. the Plg molecule. This hypothesis agrees well with the membrane localization and the crystal structure of OmpT (Vandeputte-Rutten *et al*., 2001) as well as with the predicted structures of Pla and PgtE (see supplementary material in III and Figures 1D-1F). Pla specifically potentiates spread of *Y. pestis* through tissue barriers. Oyston *et al*. (2003) found that expression of O-antigen in *Y. pestis* did not alter virulence in intravenously infected mice. However, the results are difficult to interprete as the peripheral infection route was not used and the gene cluster coding for O-antigen biosynthesis was introduced into *Y. pestis* in a vector incompatible with pPCP1 (Hu *et al.,* 1998).

4.2.3. The LPS-binding motif in PgtE (III)

The crystal structures of the FhuA-LPS complex and OmpT indicated the presence of a conserved LPS-binding motif that is predicted to bind to 4' phosphate in lipid A (Ferguson *et al*., 2000; Vandeputte-Rutten *et al*., 2001; Kramer *et al*., 2002). In the PgtE sequence, two residues of the motif, R138 and R171 are present (see Figure 1F). These residues are conserved in the OmpT and Pla sequences (Figure 1G), and they are predicted to form an electrostatic and a hydrogen bond interaction, respectively, with the lipid A 4' phosphate (Ferguson *et al*., 2000; Vandeputte-Rutten *et al*., 2001). To analyse whether these residues have an effect on enzymatic activity of omptins, R138 and R171 were substituted for glutamates separately and together, and the plasmids coding for the PgtE variants R138E (pMRK32), R171E (pMRK33) and R138E R171E (pMRK34) were used to complement Δ*pgtE* in the strain *S. enterica* SL1102-1. The effect of the substitutions on Plg activation and autoprocessing by PgtE were studied.

The single substitutions R138E and R171E did not affect Plg activation by PgtE, whereas the double substitution derivative R138E R171E PgtE was totally inactive (Figure 3A of III). Western blot analysis of cell envelope proteins from the recombinant strains SL1102-1(pMRK32) and SL1102-1 (pMRK33) showed that R138E PgtE and R171E PgtE were autoprocessed similarly to PgtE whereas the double substitution abolished autoprocessing (Figure 3B of III; lanes d, h, j and l). The folding and the assembly of β-barrel proteins in the outer membrane can be roughly assessed by analysing their heat-modifiable migration in SDS-PAGE (Heller, 1978; Kramer *et al*., 2002a; 2002b). Western blot analyses of cell envelope proteins from the recombinant strains showed that the migration of the three PgtE variants was heat-modifiable and similar to that of PgtE (Figure 3B of III; lanes c,g, i and k), which indicates that their ability to fold into the β-barrel conformation was not significantly affected. Further, the amounts of the proteins were similar in the cell wall preparations. These results indicate that the residues R138 and R171 are important for function of PgtE but not for its membrane transport or folding into β-barrel. We have not demonstrated the actual binding of PgtE to lipid A, but our hypothesis is that the residues R138 and R171 influence the enzymatic activity of PgtE by making a contact with the 4' phosphate of lipid A, as proposed for the corresponding residues in OmpT (Kramer *et al.*, 2000, 2002), and that this interaction is important for the correct conformation of the surface loops, L3 in particular.

In summary, the results above indicate that the omptins have a dual interaction with LPS. They need rough LPS for activity but are sterically inhibited by the O-antigen repeats present in most enterobacterial pathogens. The advantage of the rough LPS for *Y. pestis* has remained

unknown (Parkhill *et al.*, 2001), but our results indicate that lack of the O-antigen allows a full utilization of the proteolytic potential of Pla to enhance the spread of the bacterium into circulation. Our ongoing studies have indicated that the O-antigen also prevents inactivation of α2AP by Pla (K. Lähteenmäki, unpublished), which is needed for utilization of plasmin activity. On the other hand, it is surprising that *S. enterica* possesses a Plg activator which is cryptic in the smooth, virulent isolates. The O-chain length is influenced by the PhoPQ regulatory system (Guo *et al*., 1997; Baker *et al*., 1999) which responds to intracellular conditions (Groisman, 2001; Eriksson *et al*., 2003). The genes affecting the O-chain synthesis are downregulated in *S. enterica* living inside SCVs in mouse macrophages, whereas transcription of *pgtE* is upregulated (Eriksson *et al*., 2003). These findings suggest that the O-chain of intracellular *S. enterica* is short and that PgtE might be able to interact with large-molecular-weight substrates inside the macrophages. Overall, the role and function of PgtE in salmonellosis remain open.

4.2.4. Invasiveness and adhesiveness of omptins (II, III)

Y. pestis was recently noted to be able to invade epithelial cells, and its efficient invasiveness into epithelial HeLa-cells was associated with the presence of pPCP1 (Cowan *et al*., 2000), which suggested that Pla might have a role in the invasion. To confirm the role of Pla as an invasin of *Y. pestis* as well as to evaluate the activities of OmpT and PgtE in invasion, we tested the uptake of recombinant strains XL1(pMRK1), XL1(pMRK2) and XL1(pMRK3) into human endothelial cells using the standard gentamicin protection assay. In paper II, we used the ECV304 cell line (Takahashi *et al*., 1990) which has been widely used as a human endothelial cell line (e.g.Takahashi and Sawasaki, 1992 and references therein), but its endothelial nature has been recently questioned (Dirks *et al*., 1999; Brown *et al*., 2000). In paper III, we therefore also included in the assays the human HUV-EC-C (ATCC 1730-CRL) and primary endothelial (HUVEC) cells. These three human cells gave essentially similar results in regard of the role of omptins in the invasion; the ECV304 cells however supported a five-fold higher invasion rates (details not shown) and were therefore used as the main target cell. Approximately 1 % of the Pla expressing cells used for the infection invaded the ECV304 cells, whereas the levels with OmpT- and PgtE-expressing cells were 0.005% or less (Figure 1 of II; Figure 4 of III). The *E. coli* strain with vector plasmid alone did not invade cultured cells. The invasion did not involve the proteolytic activity of Pla, as similar invasion rates were observed with Pla and its nonproteolytic derivatives Pla S99A and Pla D206A (Figure 1 of II). Also, exogenously added Plg did not have significant effect on the invasiveness of any of the tested bacterial strains (II; details not shown).

Invasiveness of Pla expressing recombinant *E. coli* into ECV 304 cells varied from 1% to 3% depending on the host strain used and was significantly lower than the rate reported for the invasion of pPCP1- positive *Y. pestis* into epithelial cells. After one hour incubation, 30-50% of *Y. pestis* cells were internalised (Cowan *et al*., 2000). The apparent difference in the invasion rate most likely reflects the better intracellular survival or proliferation of *Y. pestis* compared to the K12 host strain used here.Our results and the findings that Pla-positive *Y. pestis* invades HeLa cells (Cowan *et al.*, 2000), suggests that Pla is able to mediate bacterial invasion into various cell types.

Pla has been reported to be an adhesin (Kienle *et al*., 1992; Lähteenmäki *et al*., 1998), and specific adhesion of the invading bacterium onto cellular receptors is the first step in the

invasion process. In this study we observed that the Pla-positive recombinant *E. coli* adhered to the intimate surroundings of the ECV304 cells (Figure 2A of II). We found that *E. coli* XL1 (pMRK1) adhered to ECM material secreted by the ECV304 cells (Figure 2B of II), which is in accordance with the previously reported binding of Pla to laminin and heparan sulfate of BMs (Lähteenmäki *et al*., 1998). OmpT- and PgtE-expressing recombinant *E. coli* showed much lower adhesiveness than Pla-expressing bacteria (Figure 4 of III), which indicates a correlation between the invasiveness and the adhesiveness of the omptins. We did not determine the apical/basolateral direction of the invasion, and it remains open whether adherence to laminin or heparan sulfate are directly involved in the invasion process by *Y. pestis*. HSPG has been shown to have a role in invasion of various bacteria into cultured cells (Rostrand and Esko, 1997; Hauck and Meyer, 2003). The apical route for *Y. pestis* invasion into endothelial cells seems unlikely, as the invasion is nearly completely inhibited by 5 % normal serum (Cowan *et al*., 2000; Ritva Virkola, unpublished). Pla-mediated invasion into human endothelial cells was also inhibited by the presence of smooth LPS on the host bacterium (Figure 4 of III), which is in accordance with the steric inhibition of Pla functions by the O-antigen repeats. PgtE seems less efficient an adhesin than Pla, and PgtE-mediated adhesiveness is unlikely to be significant for *S. enterica* because it was inhibited by the presence of O-antigen repeats (Figure 4 of III).

Invasion into and adhesion to non-phagocytic cells may help *Y. pestis* to penetrate tissue barriers or offer a protected niche against host defences. The pneumonic form of plague is transmitted via lung epithelium, and *Y. pestis* is known to cause invasive infections also via an intestinal route (Butler *et al*., 1982; Sebbane *et al*., 2001); the oral-faecal and respiratory routes may involve an intracellular stage. Overall, however, the importance of the intracellular survival in the pathogenesis of plague remains to be determined.

These studies show that Pla is an invasin and confirm its role as an adhesin with affinity to ECM components. Both properties were seen with proteolytically inactive forms of Pla, which indicates that Pla is a multifunctional protein with proteolytic, adhesive, and invasive properties.

5. CONCLUSIONS

Proteolytic activities in the human and the animal body are tightly regulated, and ca. 10 % of the protein mass in human serum consists of antiproteases (Travis and Salvesen, 1983). Free plasmin activity in circulation is rapidly inactivated by α2AP which binds to kringle domains of plasmin molecule. This inhibition by α2AP can be overcome by proteolytic inactivation of α2AP or by immobilizing plasmin on Plg/Pln receptors through the kringle domains of Plg /Pln. We observed that Pla, but not OmpT, inactivates α2AP through proteolytic cleavage. This most likely is important for the central roles of Pla and Plg in plague pathogenesis as it facilitates the uncontrolled use of plasmin activity to enhance bacterial spread across tissue barriers. It remains to be established whether the omptins cleave other important circulating antiproteases, such as α_2-macroglobulin which is abundant and inactivates plasmin in circulation when α2AP is depleted (Travis and Salvesen, 1983). Degradation of antiproteases by some other bacterial species than *Y. pestis* has been detected, these mainly are tissue destructive oral pathogens that degrade various host proteinase inhibitors (Carlsson *et al*., 1984; Nilsson *et al.,* 1985; Bedi and Williams, 1994; Grenier, 1996).

Pla of *Y. pestis* appears to be a multifunctional protease/adhesin/invasin. The multifunctional nature of bacterial surface proteases may be of more general occurrence, as it has been recently detected in e.g. the C5a peptidase of group B streptococci that binds to fibronectin and mediates invasion into human cultured cells (Cheng *et al*., 2002; Beckmann *et al*., 2002). Adhesiveness and invasiveness of Pla could be dissected in this study by demonstrating that nonproteolytic substitution derivatives of Pla retained the adhesive and invasive properties. At the present we do not know which structural features contribute to the efficient adhesiveness and invasiveness of Pla, as compared to the PgtE and OmpT. The relative importance of the various functions of Pla in the pathogenesis of plague need to be established. Adhesiveness to BM and laminin (Lähteenmäki *et al*., 1998; Paper II) probably potentiate tissue damage by localizing the Pla-generated plasmin onto a susceptible target, laminin of BMs (Salonen *et al*., 1984), which can enhance migration of *Y. pestis* through tissue barriers. The integrity of BMs is further weakened by plasmin-induced procollagenase activation. It is striking that the same features, i.e. Plg activation and adherence to BMs and laminin, are expressed by *Y. pestis* and metastatic tumor cells (Liotta *et al*., 1986). This indicates that the migration processes of a highly invasive prokaryote and invasive eukaryote cells share mechanistic features. The biological role of Pla-mediated invasion of host cells in plague pathogenesis, on the other hand, remains open at the present; similarly, its mechanisms and possible consequences for the target cell have not been experimentally addressed.

We observed that Pla, PgtE and OmpT exhibit significant functional differences, which are correlated with the disease spectrum of their host bacteria. These functional differences appear to be based on minor sequence variations in the surface-exposed loops of the omptins. This was shown by our successful conversion of OmpT into a Pla-like protease capable of cleaving Plg and α2AP. Such a conversion may exemplify how a housekeeping protease (OmpT) has evolved into an efficient virulence factor (Pla) that creates uncontrolled proteolysis by activating a host proteolytic cascade and by destroying its important control system. It is remarkable that *S. enterica* does not utilize these properties which remain cryptic in the presence of smooth LPS. *S. enterica* is an evolutionary "old" pathogen, i.e. ca. 100 million years in age, and displays a delicate relationship with the human host (for discussion, see Galan, 2001). The sophisticated relationship involves controlled, hierarchical expression of genes needed for intracellular survival of *S. enterica*, and, as this study suggests, a balance of surface structures, e.g. LPS and PgtE. In contrast, *Y. pestis* is a "young" bacterial species,

i.e. less than 20,000 years in age (Achtman *et al*., 1999), and perhaps under an evolution to become a less fatal pathogen and capable of long-term colonization of the human population. The results of this study indicate that the loss of the O-antigen, which lowers the virulence of other enterobacterial pathogens (al-Hendy *et al*., 1992; Zhang *et al*., 1997; Karlyshev *et al*., 2001 Mecsas *et al*., 2001; Najdenski *et al*., 2003), probably has been important in the evolution of the plague as it allows full use of the functional potential of Pla.

Due to high sequence identity, Pla has been proposed to have evolved from PgtE of *S. enterica* and been transferred by lateral gene transfer from *S. enterica* into *Y. pestis* (Sodeinde and Goguen, 1989). Our finding that the proteins are functionally close to each other, supports this hypothesis. It is interesting to note that structurally, the closest homolog to Pla is the omptin of the plant pathogen *E. pyrifoliae*, Pla endopeptidase A (McGhee *et al*., 2002). The functions of this omptin are not known, but its close identity of to Pla opens the intriguing possibility that it represent an intermediate between PgtE and Pla. The gene for Pla endopeptidase A is located on a plasmid that harbours also other genes that have homologs in *S. enterica* and *Y. pestis*, notably, some of these are transposases (McGhee *et al*., 2002). The omptin family offers an evolutionarily highly interesting group of related proteases which have evolved to fit the life style of their host organisms and exemplify how a backbone protein fold, the β-barrel, can be functionally modified through subtle changes at critical surface-exposed sites.

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