The Pla surface protease/adhesin of *Yersinia pestis* mediates bacterial invasion into human endothelial cells

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Abstract The plasminogen activator Pla of *Yersinia pestis* belongs to the omptin family of enterobacterial surface proteases and is responsible for the highly efficient invasion of the plague bacterium from the subcutaneous infection site into the circulation. *Y. pestis* has been reported to invade human epithelial cells. Here, we investigated the role of Pla in bacterial invasion into human endothelial cells. Expression of Pla in recombinant *Escherichia coli* XL1(pMRK1) enhanced bacterial invasion into ECV304 cells. The invasiveness was not affected by substitution mutation at the residues S99 or D206 that are needed for the proteolytic activity of Pla. Pla-expressing bacteria adhered to the extracellular matrix of ECV304 cells. Only weak adhesion and poor invasion were seen with the recombinant *E. coli* XL1(pMRK2), which expresses the omptin homolog from *E. coli*. The results identify Pla as an invasion protein of *Y. pestis* and show that the invasive function does not involve the proteolytic activity of Pla. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Pla; Plague; Invasion; Endothelial cell; *Yersinia*

1. Introduction

Plague remains one of the most feared infectious diseases in humans. The etiological agent of the disease, *Yersinia pestis*, is disseminated by fleas and infects both humans and rodents. *Y. pestis* is transmitted from rodents to humans by a flea bite and rapidly invades from the subcutaneous infection site into the lymphatic system and circulation, to produce the systemic and often fatal disease (reviewed in [1]). The bacterium is highly invasive and has caused three pandemic waves of plague with millions of deaths. *Y. pestis* still persists endemically in rodent populations in many countries and occasionally causes local outbreaks. The plague epidemics are driven by the dynamics of the disease in the rodent population [2,3].

Genetic analyses of *Y. pestis* populations have revealed that the species is highly uniform and has evolved from the intestinal pathogen *Yersinia pseudotuberculosis* serotype O1:b only 1500–20 000 years ago [4,5]. In view of the highly invasive character of *Y. pestis*, it is surprising that *Y. pestis* lacks the invasin and YadA proteins that promote invasion and virulence of *Y. pseudotuberculosis* and *Yersinia enterocolitica*. The *inv* gene of *Y. pestis* is inactivated through insertion of an IS*200*-like element [6] and the *yadA* adhesin gene due to a

frameshift mutation [7]. On the other hand, *Y. pestis* has two plasmids that are absent in other yersiniae: the 100-kb plasmid pMT1 (also known as pFra) proposed to contribute to the survival of the bacteria in the flea [8], and the 9.5-kb plasmid pPCP1 (also known as pPst), which is responsible for the invasive character of plague in the mammalian host [9]. After subcutaneous administration of *Y. pestis* in mice, pPCP1 potentiates the spread of bacteria into the circulation [9]. It was recently demonstrated that pPCP1 also enhances invasion of *Y. pestis* into human epithelial cells [10].

The plasmid pPCP1 encodes three proteins: pesticin, pesticin immunity protein and the surface protein Pla. Of these, Pla is required for the migration of *Y. pestis* from the subcutaneous infection site into the circulation [11]. Pla appears to be a multifunctional protein. It belongs to the omptin family of enterobacterial surface proteins [12] and derives its name from the fact that it can activate the mammalian plasma proenzyme plasminogen into plasmin [13]. Bacterium-induced formation of plasmin potentiates degradation of fibrin [13] and extracellular matrices [14] and promotes plague infection in vivo [15]. Pla also cleaves the complement C3 component [11], modifies bacterium-produced Yops (*Yersinia* outer proteins) [16,17], and proteolytically inactivates α_2-antiplasmin [18]. α_2-Antiplasmin is the major inhibitor of plasmin, and its cleavage probably promotes uncontrolled proteolysis. In addition to proteolytic functions, Pla has been found to mediate bacterial adhesion to eukaryotic cells and extracellular matrices, especially to laminin [14,19].

Pla is an outer membrane protein, and we recently reported a β-barrel topology model for the protein and identified proteolytically important residues in it [18]. The predicted structure of Pla is highly similar to that of its homolog in *Escherichia coli*, OmpT. Pla and OmpT share 48% sequence identity and have a similar β-barrel topology with 10 transmembrane β-strands and five surface-exposed loops [18,20,21]. However, they have differences in their substrate specificity, as OmpT activates plasminogen only weakly and does not cleave α_2-antiplasmin [18]. OmpT has been found to degrade denatured bacterial proteins [22] and to cleave antimicrobial peptides in the urine [23]. In this report, we show that expression of Pla mediates invasion of recombinant *E. coli* into human endothelial cells, whereas expression of OmpT confers no invasiveness. We also show that the invasion does not involve the proteolytic activity of Pla.

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2. Materials and methods

2.1. Cell culture, bacterial strains, and plasmids

The spontaneously immortalized human umbilical vein endothelial cell line ECV304 [24] was maintained in Medium 199 (Gibco Life Technologies) supplemented with 10% fetal calf serum and 2 mM L-glutamine. The cells were split twice weekly and, for invasion assays, grown on 24-well plates (Nunc, Denmark) for 3 or 4 days. For adhesion assays, the cells were grown on diagnostic slides (Diagnostica, Germany) for 2 days (adhesion onto cells) or for 4 days (adhesion to the extracellular matrix). The plasmids pMRK1 encoding *Y. pestis* Pla, pMRK2 encoding *E. coli* OmpT and pMRK1(S99A) and pMRK1(D206A) were expressed in *E. coli* XL1 and have been described in detail [18]. Bacterial cultivation and induction of Pla and OmpT with isopropyl β-D-thiogalactopyranoside (IPTG; Promega) were performed as in [18], except that before invasion assays bacteria were grown to the mid-exponential phase in the presence of 5 μM IPTG.

2.2. Invasion assay

A standard gentamicin protection assay [25] was used to study bacterial invasion into ECV304 cells. Briefly, ECV304 cells were washed with phosphate-buffered saline, pH 7.1 (PBS) and fresh Medium 199 was added. No fetal calf serum was added to the medium, since it has been found to prevent *Y. pestis* invasion [10]. Bacteria (10^5 cfu) were added and gently centrifuged ($128 \times g$, 10 min) onto the cells to facilitate association between bacteria and the cells. Cells were incubated for 2 h at 37°C with 5% CO_2, and after washing and killing of extracellular bacteria with gentamicin (100 μg/ml), the cells were lysed with 0.2% Triton X-100. The number of intracellular bacteria was determined by viable counting.

2.3. Adhesion assays

Bacteria (5×10^8/ml in Medium 199) were incubated on ECV304 cells for 2 h at 37°C with 5% CO_2. After washing six times with PBS, the slides were fixed with methanol for 10 min, dried, and stained with Giemsa stain. Bacterial adherence on the cells was examined with an Olympus BX50 microscope (Hamburg, Germany) and the images were digitally recorded using the Image-Pro Plus program (Media Cybernetics, Silver Spring, USA). To quantitate bacterial adhesion to the extracellular matrix, the endothelial cells were detached from the slides by detergent treatment [26] and adherence of the bacteria (10^8/ml, 5×10^8/ml and 10^9/ml in PBS) on the remaining matrix was defined as described [14,27]. The number of bacteria in 20 randomly chosen microscopic fields was determined.

3. Results

3.1. Y. pestis Pla mediates bacterial invasion into ECV304 human endothelial cells independently of its proteolytic activity

Invasiveness of recombinant *E. coli* expressing Pla and OmpT was studied by incubating bacteria on ECV304 cells. Extracellular bacteria were killed with gentamicin, and the number of intracellular bacteria was determined by viable counting. *E. coli* XL1(pMRK1) expressing Pla invaded into ECV304 cells (Fig. 1). The amount of bacteria released after cell lysis was ca. 1% of the original bacterial inoculum in the wells. The *E. coli* XL1 host strain carrying the vector plasmid pSE380 alone did not invade ECV304 cells, indicating that expression of Pla was required for invasion. In contrast to Pla-expressing bacteria, invasion of *E. coli* XL1(pMRK2) expressing OmpT was negligible, only 0.005% of the bacteria were internalized (Fig. 1). Bacterial growth in the assay wells before gentamicin treatment was determined, and no significant differences between the strains were noted. All assay wells were done in triplicate and the assay was repeated four times with essentially similar results; a representative example is shown in Fig. 1.
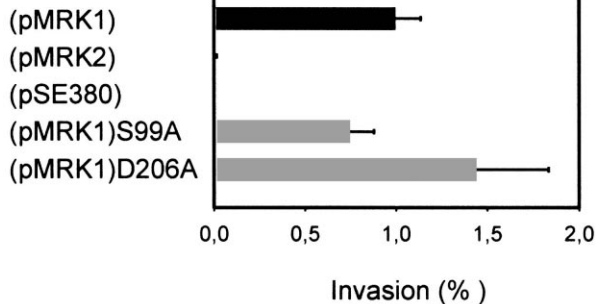

Fig. 1. Invasion into ECV304 human endothelial cells of recombinant *E. coli* XL1 expressing Pla (pMRK1), OmpT (pMRK2), or mutated Pla devoid of proteolytic activity ((pMRK1)S99A and (pMRK1)D206A). Invasion rate of the host strain *E. coli* XL1(pSE380) is also shown. Invasion was measured as the percentage of inoculated bacteria surviving gentamicin treatment. Note the efficient invasion mediated by Pla and its derivatives, and the poor invasion mediated by OmpT.

Substitution mutations S99A and D206A abolish the proteolytic activity of Pla [18]. To find out whether the proteolytic activity is required for invasion, we measured invasion of recombinant bacteria carrying these substitutions. Invasion rates of *E. coli* XL1(pMRK1)S99A and *E. coli* XL1(pMRK1)D206A were close to those seen with the bacteria expressing wild-type Pla (Fig. 1). This suggests that the proteolytic activity of Pla is not required for invasion. Also, extragenously added plasminogen had no significant effect on the invasiveness of any of the strains (data not shown).

3.2. Adhesion of the bacteria to ECV304 cells and extracellular matrix

Pla has earlier been found to mediate bacterial adhesion to epithelial cells and extracellular matrices [14,19]. In this work, we compared adhesion of Pla and OmpT to ECV304 endothelial cells. *E. coli* XL1(pMRK1) expressing Pla adhered efficiently to ECV304 cells. Adhesion was most prominent in the intimate surroundings of the endothelial cells (Fig. 2A). The proteolytically inactive mutants S99A and D206A mediated as efficient adhesion as the wild-type Pla (data not shown). In contrast, *E. coli* XL1(pMRK2) expressing OmpT adhered only weakly to ECV304 cells (Fig. 2A).

As the adherent bacteria seemed to localize mainly on the surroundings of the cells, we measured bacterial adhesion to the extracellular matrix secreted by the cells. The endothelial cells were detached by detergent treatment, and the bacteria were incubated on the remaining matrix. Efficient adhesion by *E. coli* XL1(pMRK1), *E. coli* XL1(pMRK1)S99A and *E. coli* XL1(pMRK1)D206A was noted (Fig. 2B). Adherence of *E. coli* XL1(pMRK2) to the matrix was about four to five times weaker than that of *E. coli* XL1(pMRK1), however, it showed a weakly higher adherence than the *E. coli* XL1(pSE380) host strain (Fig. 2B). None of the strains adhered to the control surface coated with bovine serum albumin. These results indicate that Pla is a more efficient adhesin than OmpT.

4. Discussion

Migration through tissue barriers is a major task for a bacterial pathogen in its dissemination within the host body. The
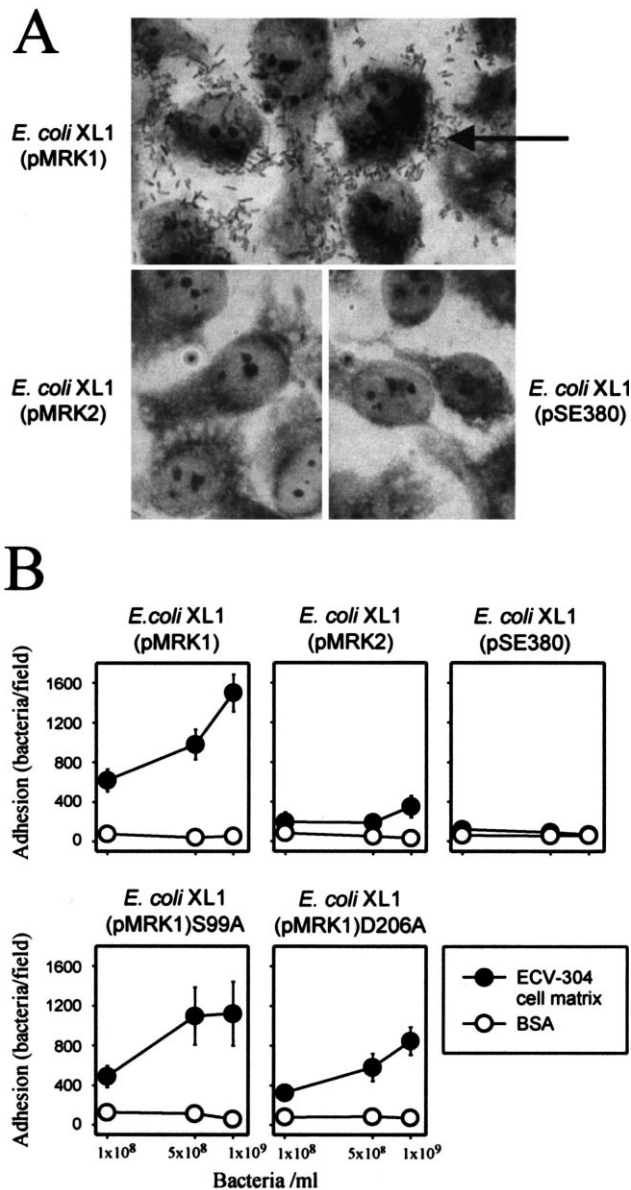
Fig. 2. A: Adhesion of Pla-expressing *E. coli* XL1(pMRK1), OmpT-expressing *E. coli* XL1(pMRK2), and *E. coli* XL1(pSE380) to ECV304 cells. Pla-expressing bacteria (arrow) adhere mainly onto the intimate surroundings of the cells. OmpT-expressing bacteria and the host strain with the vector plasmid adhere only weakly to ECV304 cells. B: Adhesion to ECV304 cell matrix of Pla-expressing *E. coli* XL1(pMRK1), OmpT-expressing *E. coli* XL1(pMRK2), as well as *E. coli* XL1(pMRK1)S99A and *E. coli* XL1(pMRK1)D206A, which express proteolytically inactive Pla. Pla-expressing bacteria adhere efficiently to the matrix, whereas adherence of OmpT-expressing bacteria is weak. None of the strains adheres to the control surface coated with bovine serum albumin (BSA).

process consists of two parts: (i) migration through extracellular matrices beneath cells and (ii) passing through the cellular layers themselves. *Y. pestis* is a remarkably invasive bacterium, which during the plague infection migrates from the subcutaneous infection site into the lymphatic and blood vessels, and from there, into the liver and spleen. Other internal organs may also become colonized, and ultimately, bacteria colonize the lungs, which leads to the most contagious phase of the disease with direct air-borne transmission to other susceptible mammals [1]. Such an efficient dissemination

obviously involves bacterial migration through several epithelial and endothelial layers.

Pathogenic bacteria can penetrate epithelial cell layers paracellularly (between the cells), transcellularly (by invading a cell) or by facilitating transport inside a circulating phagocytic cell, such as a monocyte [28]. Many pathogenic bacteria, including *Y. enterocolitica* and *Y. pseudotuberculosis*, have been demonstrated to invade eukaryotic cells in vitro [28,29]. Earlier reports suggested that *Y. pestis* does not invade non-phagocytic eukaryotic cells in vitro [30,31], however, it has been found to survive and multiply inside professional phagocytes [32,33]. The work by Cowan et al. [10] indicated that *Y. pestis* invades HeLa human epithelioid cells in a highly efficient manner. Invasion is detected only in the absence of fetal calf serum, which probably partly explains earlier findings of non-invasiveness. Cowan et al. [10] found that the 9.5-kb plasmid pPCP1 was the major bacterial factor contributing to the invasion. Our results indicate that the Pla surface protein, a product of pPCP1, promotes bacterial invasion into human endothelial cells.

Pla-mediated invasion seems not to be dependent on the proteolytic activity of Pla, as recombinant bacteria with the protease-negative mutant proteins S99A and D206A were essentially as invasive as bacteria expressing wild-type Pla. Addition of plasminogen and, thus, generation of plasmin had no effect on invasion, also suggesting that factors other than proteolytic activity are involved in the observed invasiveness. We have earlier shown that Pla is also an adhesin which mediates bacterial binding to the extracellular matrix of lung epithelial cells [14]. In this work, Pla was found to mediate bacterial adhesion also to the matrix of ECV304 endothelial cells. Interestingly, we found that OmpT-mediated adherence to ECV304 cells and extracellular matrix is much weaker than Pla-mediated adhesion. Furthermore, OmpT does not promote bacterial invasion into ECV304 cells. The predicted secondary structures of OmpT and Pla are very similar, however, the two omptins have profound differences in their proteolytic activities towards physiological targets [18]. This work indicates that Pla and OmpT differ also in their ability to adhere and invade into ECV304 cells. The differences in the reported functions between Pla and OmpT reflect their virulence roles. OmpT has been proposed to have a role in urinary tract infections [34], but it is not associated with invasive infections, whereas Pla is a well-established virulence factor of the highly invasive *Y. pestis*. Several functions that are likely to enhance migration of *Y. pestis* through the extracellular matrix have been proposed for Pla [14,18]. Our present results suggest that Pla is also involved in bacterial penetration through the endothelial cells and may promote bacterial migration via a transcellular route.

It seems likely that there is a link between Pla-mediated adhesion and invasion, but additional, yet unknown factors in Pla may also affect invasion. Adhesion to the extracellular matrix has been proposed to have a role in invasion of *Shigella flexneri*, since contact with extracellular matrix proteins promotes the release of bacterial Ipa proteins that mediate *Shigella* invasion [35]. The invasion rate of our Pla-expressing recombinant *E. coli* is considerably lower than that observed by Cowan et al. [10] for the pPCP1-positive *Y. pestis*; invasion of *Y. pestis* was extremely efficient, after 1 h incubation 30–50% of the bacteria were internalized into HeLa cells. The difference may result from poor growth of K-12 *E. coli* within

the endothelial cells, and also may indicate that other factors in *Y. pestis*, possibly in combination with Pla, also play a role in invasion. Cowan et al. [10] estimated that 5–10% of the invasiveness of *Y. pestis* was due to other factors than the presence of pPCP1 plasmid. Although other, yet unidentified factors may contribute to the invasiveness of *Y. pestis*, our finding that Pla by itself renders a non-invasive *E. coli* K-12 strain invasive suggests that Pla has a significant role in the invasion process.

In *Y. pseudotuberculosis* and *Y. enterocolitica* the surface proteins invasin and YadA play central roles in adhesion and invasion. Invasin promotes invasion by binding to β1-integrins on the cell surface, and YadA binds to various cell surface molecules and extracellular matrix components [29,36]. Pla is known to bind to laminin on extracellular matrices [14], but it is not yet known how the contact to host cell surfaces is mediated in *Y. pestis*. *Y. pestis* evolved from *Y. pseudotuberculosis* only shortly before the first plague pandemic [4]. Evolution has resulted in loss of the invasin and YadA functions but, on the other hand, in gain of Pla, which seems to have evolved to a remarkably multifunctional virulence factor. Further work is required to solve the actual roles of the various functions found in vitro for Pla. So far, plasminogen activation has been shown to be involved in *Y. pestis* infection in vivo [15], but results from infection studies also suggest that it is not the sole effect mediated by Pla [11,15]. Current evidence suggests that Pla is a unique surface molecule that provides the plague bacterium with a combination of proteolytic, adhesive, and invasive functions.

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