Legionella pneumophila exhibits plasminogen activator activity

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Based on their localization at the boundary of the bacterial cell and its environment, outer-membrane proteins (Omps) are important determinants for interaction of bacteria with their host cell. Therefore, they can be considered as important determinants for virulence. Looking for *Legionella pneumophila* Omps potentially involved in virulence, we identified a gene encoding a homologue of the plasminogen activator (Pla) of *Yersinia pestis*. Pla belongs to the class of omptins, a family of surface proteases/adhesins that exhibit different virulence-associated functions. In this report we describe the cloning and identification of the plasminogen activator homologue Lpa of *L. pneumophila* and demonstrate its outer-membrane localization.

Transcriptional analysis of the Lpa region revealed expression of the gene in both exponential and stationary growth phase and showed that transcription of the *lpa* gene is directed by its own promoter. We also show, to our knowledge for the first time, that *L. pneumophila* has the capacity to convert plasminogen into plasmin by the action of the outer-membrane Lpa protein.

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INTRODUCTION

Legionella pneumophila is a Gram-negative, facultative intracellular human pathogen found worldwide in fresh-water environments, where it replicates in various protozoa. Intracellular growth enhances the environmental survival capability and the virulence of the bacteria. People typically become infected with *L. pneumophila* upon inhalation of contaminated aerosols generated by man-made devices such as whirlpools, cooling towers and air-conditioning systems. If the host immune response is impaired, adhesion to and infection of alveolar macrophages can lead to Legionnaires' disease, a life-threatening pneumonia. Since its discovery in 1976, but particularly in the past 10 years, our understanding of the pathogenesis of *L. pneumophila* has grown tremendously. Both the unravelling of the biphasic life cycle of *Legionella*, in which two clearly distinct phenotypes can be differentiated (Molofsky & Swanson, 2004; Molmeret *et al.*, 2005; Brüggemann *et al.*, 2006; Hilbi *et al.*, 2007), together with the identification of a great number of virulence factors, form the basis of the progress made (Albert-Weissenberger *et al.*, 2007). In recent years the number of reported cases of legionellosis has risen dramatically, resulting in increased attention to control measurements to prevent infections by this pathogen and to the identification and mechanism of action of additional virulence factors. A variety of *L. pneumophila* surface components and secreted

proteins have been shown to be implicated in pathogenesis. In several bacterial pathogens, functional characterization of outer-membrane proteins (Omps) has revealed diverse roles for these molecules, including iron uptake, anti-microbial peptide and multi-drug resistance, bile resistance, pore formation, adhesion, invasion, bacteriophage adsorption and serum resistance. Although significant advances have been made regarding the unravelling of the structure and function of Omps, the number of Omps that have been characterized represents only a small portion of the total number of Omps revealed by bacterial genome sequences (Lin *et al.*, 2002).

In the search for *L. pneumophila* genes encoding putative Omps, and more precisely Omps that might be involved in bacterial virulence, we identified a gene encoding a homologue of the plasminogen activator protein (Pla) of *Yersinia pestis*. The latter protein belongs to the family of the omptins, which is a class of surface proteases/adhesins found in the outer membrane of enterobacterial pathogens.

Although the omptin homologues show high sequence identity and a conserved β -barrel, they are multifunctional and exhibit different virulence-associated functions. Pla of *Y. pestis* contributes to the virulence of this pathogen by several mechanisms. The protein is involved in uncontrolled plasmin activity by efficient conversion of human proenzyme plasminogen (Plg) to plasmin, inactivation of the plasmin inhibitor α_2 -antiplasmin (α_2 -AP), and binding to laminin, which localizes the plasmin activity onto basement membranes. These properties are important for the bacterial invasiveness in plague (Sodeinde *et al.*, 1992)

Abbreviations: α_2 AP, α_2 -antiplasmin; Lpa, *Legionella* plasminogen activator; MOMP, major outer-membrane protein; Omps, outer-membrane proteins; Pla, plasminogen activator; Plg, plasminogen.

and enhance migration of *Y. pestis* through tissue barriers (Kukkonen & Korhonen, 2004). PgtE, the homologue of Pla in *Salmonella enterica*, is significantly less efficient in plasminogen activation than Pla (Kukkonen *et al.*, 2004), but inactivates the plasmin inhibitor α_2 AP more efficiently (Lähteenmäki *et al.*, 2005). This discrepancy may reflect differences in the mechanisms of infection exerted by these two pathogens. The generation of plasmin is indispensable for the predominantly extracellular *Y. pestis* to proteolyse tissues in order to disseminate all over the human body. Since spread of *S. enterica* occurs predominantly by means of macrophages, Lähteenmäki *et al.* (2005) hypothesize that PgtE-mediated inactivation of α_2 AP serves to protect the plasmin generated by both the bacteria and the macrophages. Based on these results obtained for other bacterial pathogens, we hypothesized that the *L. pneumophila* omptin homologue could play an important role in the virulence of this pathogen as well.

In this study, we cloned the gene encoding the *L. pneumophila* plasminogen activator homologue and demonstrated, to our knowledge for the first time, that *L. pneumophila* possesses a surface protease that exhibits plasminogen activator activity.

METHODS

Strains, plasmids and cultivation of bacteria and cells. Strains used in this study are listed in Table 1. *Legionella* strains were grown at 37 °C on buffered charcoal yeast extract (BCYE) agar plates or in

buffered yeast extract broth (BYE) supplemented with α -ketoglutarate, L-cysteine and ferric pyrophosphate (Edelstein, 1981). When necessary, kanamycin (10 μ g ml⁻¹) or chloramphenicol (5–12 μ g ml⁻¹) was added. *Escherichia coli* TG1 (Sambrook *et al.*, 1989) was used as host for cloning purposes and *E. coli* BL21* and *E. coli* BL21(DE3)pLys were used for the expression of recombinant proteins (Studier & Moffatt, 1986). Cultures were grown in Luria–Bertani medium (Miller, 1972) and if necessary supplemented with ampicillin (50 μ g ml⁻¹) or chloramphenicol (50 μ g ml⁻¹). *Acanthamoeba castellanii* (ATCC 30234) was cultured in PYG712 medium at room temperature.

DNA manipulations and cloning strategies. All DNA manipulations used in this work were performed using standard techniques unless specified otherwise. The plasmids and primers used in this study are given in Tables 1 and 2, respectively. Standard PCR reactions were carried out using SuperTaq (HT Biotechnology).

The *L. pneumophila lpa* gene was isolated by PCR using the primers LpaF and LpaR, from chromosomal DNA (100 ng) as template. The resulting 0.92 kb PCR fragment was cloned into pGEM-T Easy (Promega). Subsequently, the *lpa* gene was cloned downstream of the IPTG-inducible *tac* promoter in pMMBN. The pMMBN vector is a derivative of pMMB207 (Morales *et al.*, 1991) with an extra *NdeI* site introduced at the start codon of the *lacZx* gene and with part of the *mobA* gene deleted by *AgeI* digestion and religation. In this way, the plasmid pMMBNlpa was obtained.

Using the primers PromlpaF and LpaR the *lpa* gene with its promoter region was amplified by PCR from chromosomal DNA (100 ng) as template. The resulting 1.2 kb PCR fragment was cloned into pGEM-T Easy (Promega) and subsequently as a *SacI*–*SacII* restriction fragment cloned into pBCKS(+). In this way, the plasmid pBCpromlpa containing the gene together with its putative promoter region was obtained.

Table 1. Strains and plasmids

Name	Relevant characteristics*	Reference or source
Strains		
<i>L. pneumophila</i>		
Philadelphia	<i>L. pneumophila</i> serogroup 1	ATCC 33152
Philadelphia Δ lpa	Philadelphia <i>lpa</i> ::Km	This study
Corby	<i>L. pneumophila</i> serogroup 1	Lück <i>et al.</i> (2001)
Corby LPS mutant	Spontaneous LPS mutant from <i>L. pneumophila</i> sg 1 strain Corby	Lück <i>et al.</i> (2001)
<i>E. coli</i>		
TG1	Host for cloning purposes	Studier & Moffatt (1986)
BL21*	Host for overexpression	Novagen
BL21(DE3)pLys	Host for overexpression	Novagen
<i>A. castellanii</i>		ATCC 30234
Plasmids		
pGEM-T Easy	Used for cloning of PCR-amplified DNA fragments, MCS, Ap ^r , <i>lacZx</i>	Promega
pGEMlpa	pGEM-T Easy derivative containing the <i>lpa</i> fragment	This study
pGEMpromlpa	pGEM-T Easy derivative containing the <i>promlpa</i> fragment	This study
pGEMlpaHis	pGEM-T Easy derivative containing the (His) ₁₀ <i>lpa</i> fragment	This study
pMMB207	<i>E. coli</i> – <i>Legionella</i> shuttle vector, <i>lacZx</i> , Cm ^r	Morales <i>et al.</i> (1991)
pMMBN	pMMB207 derivative with an extra <i>NdeI</i> site and part of the <i>mobA</i> gene deleted	De Buck <i>et al.</i> (2007)
pMMBNlpa	pMMBN derivative containing the <i>lpa</i> fragment of pGEMlpa	This study
pBCKS(+)	MCS, Cm ^r , <i>lacZx</i>	Stratagene
pBCpromlpa	pBCKS(+) derivative containing the <i>promlpa</i> fragment of pGEMpromlpa	This study
pETlpaHis	pET3a derivative containing the (His) ₁₀ <i>lpa</i> fragment of pGEMlpaHis	This study

*Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; MCS, multiple cloning site.

Table 2. Primers

Restriction endonuclease cleavage sites are underlined. The (His)₁₀-encoding sequence is in italic.

Name	Sequence (5'–3')	Feature
LpaF	tacatatgaaaataaaatagttttgttgc	<i>NdeI</i>
PromlpaF	ggttttgcctattgtcc	
LpaR	tagaattcttaaaaaagatactgtaaacc	<i>EcoRI</i>
LpaFHis	tacatatgcaccatcaccatcatcatcaccgcggattacgaatttaattg	<i>NdeI</i> , (His) ₁₀ -tag

For the construction of an *L. pneumophila* Philadelphia mutant lacking the *lpa* gene, the chromosomal *lpa* gene was substituted by a kanamycin-resistance gene by means of double homologous recombination as described for other *L. pneumophila* genes (De Buck *et al.*, 2005). To make sure that the observed effects after deletion of the *lpa* gene could be specifically ascribed to the deletion of the *lpa* gene, we constructed a complemented *L. pneumophila* *lpa* deletion strain by introducing pMMBNlpa into the mutant strain. A second complemented strain with *lpa* under transcriptional control of its own promoter was constructed by introducing pBCpromlpa into the mutant strain.

To construct pETlpaHis, the *lpa* gene was amplified by PCR using LpaFHis and LpaR. The forward primer was designed to add ten histidines at the N-terminal end of the mature protein. After cloning into pGEM-T Easy, the fragment was digested with *NdeI* and ligated into pET3a.

RNA isolation and reverse transcriptase (RT)-PCR. Total RNA from exponentially growing and stationary-phase *L. pneumophila* cells and from *L. pneumophila* intracellularly grown in *A. castellanii* was isolated using the RNeasy mini kit (Qiagen) as described previously (De Buck *et al.*, 2004). Samples used for RT-PCR were additionally treated with DNase I (Qiagen) (final concentration 0.5 units μl^{-1}) to remove any residual chromosomal DNA. RT-PCR was performed with 100 ng of total RNA using the Access RT-PCR System (Promega). To investigate the presence of an operon structure encompassing the *lpa* gene, the primers PromlpaF and LpaR were used.

Cell fractionation. For the preparation of protein fractions containing either extracellular proteins, total cell proteins, soluble, inner- or outer-membrane proteins, a 100 ml culture was grown overnight starting from a 1 ml overnight-grown preculture. The culture was centrifuged (5000 g, 15 min) and the supernatant obtained was filter-sterilized using a 0.2 μm filter. Proteins present in the supernatant were concentrated by TCA precipitation (20% TCA) for 1 h on ice. After centrifugation (10 000 g, 15 min) the extracellular protein fraction was washed with acetone and dried. The remaining cell pellet was resuspended in 15 ml lysis buffer (10 mM Tris, 10 mM EDTA, pH 7.5) and subsequently lysed in a French pressure cell. To obtain the total cell protein fraction, the cell debris was removed by centrifugation (20 min, 12 000 g). Membrane proteins and the soluble protein fraction were obtained following centrifugation of the cell lysate for 2 h at 100 000 g. The soluble protein fraction is located in the supernatant, whereas the membranes with the membrane proteins are situated in the sediment. To set the inner-membrane proteins free from the membranes, the sediment was resuspended in 1.5 ml 10 mM Tris/HCl (pH 7.5) containing 1.5% Sarkosyl and incubated for 30 min at room temperature, and subsequently recentrifuged for 2 h at 100 000 g. The supernatant, containing the inner-membrane proteins, was collected. The pellet, containing the outer-membrane proteins, was resuspended in 500 μl 10 mM Tris/HCl (pH 7.5) and 10 mM EDTA, containing 1% Triton X-100.

SDS-PAGE and Western blotting. (His)₁₀-Lpa was isolated from the cytoplasm of *E. coli* BL21(DE3)pLys/pETLpaHis under denaturing conditions as described in Qia Expressionist (Qiagen). To obtain extra pure protein for immunization purposes, purified fractions were additionally separated by SDS-PAGE and subsequently the Lpa protein was electro-eluted from the gels. After dialysis of the purified fractions against PBS, 150 μg of the (His)₁₀-Lpa protein was used for immunization of a Dutch White rabbit, in order to obtain Lpa-specific antibodies.

Expression of Lpa was monitored by Western blotting (12.5% SDS-PAGE) and immunodetection with Lpa-specific antibodies and in a second step with alkaline-phosphatase-conjugated anti-rabbit antibodies (Sigma). Visualization of the proteins was done using the chromogenic substrate solution NBT/BCIP (Roche Diagnostics).

As a control for the protein fractionation, detection of *L. pneumophila* DnaK, a cytoplasmic protein, LepB (signal peptidase), an inner-membrane protein, and MOMP (major outer-membrane protein), an outer-membrane protein, was performed with antibodies against *E. coli* DnaK, LepB and MOMP, respectively.

Plasminogen activation tests. Measurement of plasminogen activation was performed as described previously (Kukkonen *et al.*, 2001), by incubating 8×10^7 bacteria, 4 μg human Glu-Plg (American Diagnostica) and the chromogenic plasmin substrate S-2251 (0.45 mM Val-Leu-Lys-p-nitroaniline dihydrochloride, Chromogenix) in a total volume of 200 μl at 37 °C. Plasmin activity was measured in the Infinite 200 microplate reader (Tecan) at 405 nm as the breakdown of the chromogenic substrate as a function of time. Activity of inner- and outer-membrane protein fractions was measured by the same test as described above, but instead of bacteria, 20 μl of these fractions (see preparation above) was used in the samples.

Amoebae plate test. Growth of *L. pneumophila* in the presence of *A. castellanii* was analysed as described by Albers *et al.* (2005). Briefly, 1.5 ml *A. castellanii* (2.6×10^6 ml⁻¹) was spread on BCYE agar plates, allowed to dry for 1–2 h in a laminar-flow hood and left overnight at room temperature. Stationary-phase bacterial cultures were adjusted to an identical OD₆₀₀ and series of tenfold dilutions in BYE were prepared; 3 μl of stationary culture and of each dilution was spotted onto the BCYE agar plate and the plates were incubated for 5 days at 37 °C.

RESULTS AND DISCUSSION

Cloning and analysis of the *lpa* gene of *L. pneumophila*

In order to identify novel virulence factors of *L. pneumophila*, the genome sequence of *L. pneumophila* Philadelphia was screened for the presence of genes

encoding proteins that are associated with the bacterial outer membrane using the Psortb algorithm. One of the genes resulting from this search (lpg2387) was predicted to encode a plasminogen activator homologue. For this reason we named the gene *lpa* (*Legionella* plasminogen activator). Homologues in the Paris and Lens strain are lpp2452 and lpl2311, respectively. The gene was cloned by PCR from *L. pneumophila* Philadelphia genomic DNA and the DNA sequence present in the genome bank could be confirmed. The cloned fragment encodes a protein of 308 amino acids with a calculated molecular mass of 35.3 kDa and a predicted pI of 6.0. Analysis of the predicted protein sequence with SignalP confirmed the presence of a typical signal peptide at the N terminus, with the most likely cleavage site between the amino acids at positions 23 and 24.

The protein sequence of *L. pneumophila* Lpa was compared to the omptin sequences of other Gram-negative human pathogens: *E. coli*, *Y. pestis* and *S. enterica* serovar Typhimurium (Fig. 1). Overall identity of Lpa with these proteins is respectively 39, 49 and 47%. The structure of the best-studied member of the omptin family, OmpT, elucidated by Vandeputte-Rutten *et al.* (2001), appears to consist of a β -barrel with ten antiparallel β -strands connected by four short periplasmic turns and five surface-exposed loops. The extracellular loops of OmpT are indicated in Fig. 1.

Although the omptins are structurally very similar and the catalytically important residues (Vandeputte-Rutten *et al.*, 2001; Kramer *et al.*, 2000, 2001) are conserved (Fig. 1), they have clearly different substrates (Kukkonen & Korhonen, 2004). It is assumed that this substrate specificity is dependent on slight differences in the amino acid sequence in the regions important for substrate recognition in the extracellular loops of the protein. There are three important structural features to convert the substrate specificity of OmpT into that of Pla and as a result change it into a protein with enhanced plasminogen activator activity (Kukkonen *et al.*, 2001). The three key elements are (i) shortening of L3 by four residues to the size of the Pla L3, (ii) deletion of two amino acids in L4, i.e. Asp²¹³ and Pro²¹⁴, and (iii) substitution of residue Lys²¹⁶ by Arg in L4. These typical plasminogen activator-related structural features are also present in the *L. pneumophila* protein, leading to the hypothesis that this protein exhibits plasminogen activator activity.

Transcriptional analysis of the *L. pneumophila* *lpa* gene

On the *L. pneumophila* Philadelphia chromosome the *lpa* gene is situated between a putative ORF with unknown function (lpg2386) and a gene encoding an amino acid permease (lpg2388) (Fig. 2a). The latter is oriented in the same direction as *lpa* with an intergenic distance of 130 bp. RT-PCR experiments on total *L. pneumophila* RNA were performed to see whether the *lpa* gene is effectively expressed under the experimental conditions used and to determine if

it is cotranscribed with the upstream ORF. RT-PCR with *lpa*-specific primers clearly showed the presence of a 0.92 kb transcript in the case of *L. pneumophila* grown to exponential and stationary phase, confirming *lpa* expression in both phases (Fig. 2b). The *lpa* gene is also expressed in intracellularly grown bacteria (Fig. 2b). Furthermore, no transcript that encompasses the intergenic region could be obtained, indicating that transcription of the *lpa* gene is directed by its own promoter. RT-PCR on RNase-treated samples was negative, confirming a specific, DNA-independent amplification.

Cellular localization of Lpa

Based on homology with other omptin proteins and on an *in silico* prediction, we assumed that the *L. pneumophila* Lpa protein is located in the outer membrane of the bacterial cells. In order to confirm this localization, different protein fractions were studied for the presence of Lpa. We first started with the analysis of total cell lysates of *L. pneumophila*. However, after SDS-PAGE of total cell proteins and detection with Lpa-specific antibodies, Lpa could only be detected in tiny amounts (Fig. 3). When the same analysis was performed with total cell proteins of *L. pneumophila*/pMMBNlpa, overexpressing the *lpa* gene upon IPTG induction, a clear band at a molecular mass of 33 kDa, corresponding to the predicted molecular mass of 32.8 kDa of the mature form of Lpa, could be observed (Fig. 3).

Separation of the different cellular fractions by SDS-PAGE, followed by immunodetection with Lpa-specific antibodies, revealed no immunoreactive protein band in the extracellular, soluble and inner-membrane protein fraction, while a clear band was observed in the outer-membrane protein fraction (Fig. 3). As a control for a correct cellular fractionation, detection of *L. pneumophila* DnaK, a cytoplasmic protein, LepB (signal peptidase), an inner-membrane protein, and MOMP, an outer-membrane protein, was performed. The control proteins were found to be present in the expected fractions (results not shown). Based on these experiments we could conclude that Lpa is an outer-membrane protein.

Functional analysis of Lpa

We studied the capacity of Lpa to convert plasminogen to plasmin with plasminogen activator tests on cells and on inner- and outer-membrane protein fractions. As stated above, the capacity to convert plasminogen into plasmin is very different for each of the characterized omptins. For example, the Pla protein of *Y. pestis* shows a more efficient activation of Plg than OmpT of *E. coli* and PgtE of *S. enterica* (Kukkonen *et al.*, 2004). In order to assess the plasminogen activation ability of the Lpa protein, its activity was monitored as a function of time for *L. pneumophila* cells and *L. pneumophila*/pMMBNlpa cells with and without induction with IPTG. For a similar experiment in *E. coli*, the OmpT-deficient *E. coli* BL21* was

Fig. 1. Alignment of the amino acid sequence of the mature part of *L. pneumophila* Lpa with known omptins of other Gram-negative pathogens: Pla of *Y. pestis*, OmpT of *E. coli* and PgtE of *S. enterica*. Sequences were aligned with CLUSTAL W. Conserved residues are shaded and identical residues are marked by an asterisk. Catalytically important residues are indicated in bold and extracellular loops determined for OmpT are indicated by lines above the sequences (Vandeputte-Rutten *et al.*, 2001).

used as host strain in order to minimize interference of residual chromosomally encoded plasminogen activator activity. Results of both tests are given in Fig. 4.

For *L. pneumophila*, no activation of plasminogen could be detected under non-overexpressing conditions (i.e. *L. pneumophila* cells without the overexpression vector and cells with this vector but without IPTG induction). However, induction of Lpa expression by 50 μ M IPTG revealed the plasmin-specific conversion of the chromogenic substrate S-2251. For *E. coli* BL21*/pMMBNlpa, plasminogen activation could be observed with and without induction of expression with 50 μ M IPTG. *E. coli* BL21* was, as expected, not able to convert Plg into plasmin. In the absence of Plg, no cleavage of S-2251 was observed, confirming the specific plasminogen activation capacity of Lpa.

Although *L. pneumophila* wild-type cells showed no detectable activation of plasminogen (Fig. 4a), plasminogen

activation activity by an *L. pneumophila* wild-type outer-membrane preparation, as shown in Fig. 4(c), starts to increase from about 10 h of incubation, corresponding to the large amount of Lpa protein found in the outer membrane following immunoblotting and detection with Lpa-specific antibodies (Fig. 3). As expected, no activity was detected in the inner-membrane protein fraction of *L. pneumophila* wild-type (results not shown), which confirms the outer-membrane localization of Lpa. The outer-membrane protein fraction of *L. pneumophila* Δ lpa has lost the ability to activate plasminogen. Providing the *lpa* gene *in trans* on a plasmid complements this defect as can be clearly seen in Fig. 4(c). Maximum plasminogen activation by the outer-membrane fraction of the complemented strain upon induction with 50 μ M IPTG is reached within 5–7 h incubation. Also, the outer-membrane protein fraction of the *lpa*-overexpressing strain *L. pneumophila*/pMMBNlpa showed maximum activity after 5–7 h upon induction with 50 μ M IPTG.

Fig. 2. Gene organization and transcriptional analysis of the *lpa* region of *L. pneumophila*. (a) Schematic representation of the gene region of *lpa*. (b) Agarose gel electrophoresis of RT-PCR products with the indicated primer pairs. Template RNA was isolated from *L. pneumophila* grown to exponential (lanes 2 and 3) and stationary (lanes 4, 5 and 8) phase and from *L. pneumophila* grown intracellularly in *A. castellanii* (lanes 6 and 7). Control reactions were performed without reverse transcriptase to confirm the absence of DNA contamination (lanes 3, 5 and 7). Smart ladder in lane 1.

Influence of O-antigen on Lpa activity

The fact that Lpa activity of the *L. pneumophila* cells did not exceed background levels is remarkable, as transcriptional analysis revealed *lpa* expression during both the exponential and stationary growth phase (Fig. 2b). A Western blot experiment of total *L. pneumophila* using Lpa-specific antibodies showed expression as well, albeit at a very low level (Fig. 3). *L. pneumophila* might need specific conditions for an increased production of Lpa such as intracellular growth in protozoa or macrophages, as has been shown for the production of PgtE in *S. enterica* (Lähteenmäki *et al.*, 2005). As well as the possibility that plasminogen activator activity did not exceed background levels due to a low *in vitro* expression level, it is possible that the protein is not fully active under the laboratory conditions used and that it has specific requirements with respect to, for example, the lipopolysaccharide (LPS) structure or composition. Kramer *et al.* (2002) showed that reconstitution of an enzymically active, purified OmpT was only achieved after addition of LPS. LPS was shown to be required for obtaining a native conformation of the protein by inducing subtle conformational changes in the surface-exposed loops. Otherwise, the *L. pneumophila* Lpa protein might be sterically hindered by long O-polysaccharides present in smooth-type LPS, as was shown

Fig. 3. Cellular localization of the *L. pneumophila* Lpa protein. SDS-PAGE gel electrophoresis and immunoblotting with anti-Lpa antibodies: 1, total cell lysate *L. pneumophila*; 2, total cell lysate *L. pneumophila*/pMMBNlpa (induced with 50 µM IPTG); 3, extra-cellular fraction *L. pneumophila*; 4, soluble protein fraction *L. pneumophila*; 5, inner-membrane protein fraction *L. pneumophila*; 6, outer-membrane protein fraction *L. pneumophila*.

for PgtE of *S. enterica* (Kukkonen *et al.*, 2004). To get an idea about the influence of the length of the O-polysaccharides on the activity of Lpa, plasminogen activation was studied in a modified LPS environment. To simulate a rough *Legionella* LPS background, an LPS mutant from *L. pneumophila* serogroup 1 strain Corby (Lück *et al.*, 2001) was used. This LPS mutant has lost reactivity with monoclonal antibody 3/1 and fails to produce high-molecular-mass long-chain O-polysaccharides. Whereas the wild-type *L. pneumophila* Corby strain produces long-chain O-polysaccharides (40–75 repeating units), the LPS mutant strain produces O-polysaccharides of highly reduced chain length of 7–15 repeating units (Lück *et al.*, 2001). Plasminogen activation by these two strains was monitored as a function of time (Fig. 5). Although the plasminogen activation is lower compared to that of the induced *L. pneumophila* and *E. coli* Lpa-overexpressing strain, a clear difference in activity can be seen between the wild-type Corby strain and the isogenic LPS mutant strain. The LPS mutant strain shows a more than threefold increase in the plasminogen activation capacity. This experiment indicates that, like OmpT of *E. coli*, Pla of *Y. pestis* and PgtE of *S. enterica*, Lpa of *L. pneumophila* might also be sterically hindered by O-antigen repeats in smooth LPS.

Amoebae plate test

Most omptins have a clear role in virulence. To analyse the possible role of Lpa in the virulence, *L. pneumophila* wild-type, the *lpa* mutant strain and the complemented strain were grown on BCYE agar plates in the presence or, as a control, in the absence of *A. castellanii* in the amoebae plate test (Albers *et al.*, 2005). As can be seen in Fig. 6, wild-type *L. pneumophila* was able to grow in the presence of amoebae up to a dilution of 10^{-6} . The *lpa* mutant strain formed robust colonies until a dilution of 10^{-2} , but failed to grow from dilution 10^{-6} , showing a partial growth defect in the presence of *A. castellanii*. Supplying *lpa* on a plasmid restored growth to the wild-type level. In the absence of *A. castellanii* all strains grew equally well.

Fig. 4. Plasminogen activation as measured by the conversion of S-2251 in the presence of Glu-Plg by: (a) *L. pneumophila* (◆); *L. pneumophila*/pMMBNlpa both non-induced (△) and induced with 50 μM IPTG (▲); (b) *E. coli* BL21* (◆); *E. coli* BL21*/pMMBNlpa both non-induced (△) and induced with 50 μM IPTG (▲); (c) outer-membrane fractions of *L. pneumophila* (◆), *L. pneumophila* Δlpa (□), *L. pneumophila* Δlpa/pMMBNlpa (50 μM IPTG) (○) and *L. pneumophila*/pMMBNlpa (50 μM IPTG) (▲). Conversion of the substrate is given as a function of time.

Conclusion

An important prerequisite in the pathogenesis of various invasive bacterial infections is the ability of the infecting micro-organism to adhere to host tissues. Subsequent degradation of the epithelial barrier and basement membranes depends on bacterial proteases and renders penetration of the bacteria through the basement membrane and the extracellular matrix possible. For some pathogenic bacteria such as streptococci and certain *Enterobacteriaceae*, plasminogen activation has been found to play a key role in activating a proteolytic cascade that leads to damage of the tissue barriers.

With respect to the pathogenesis of *L. pneumophila*, research has focused almost exclusively on the interaction of the bacteria with the macrophage host. Nevertheless, besides a possible role for Lpa in the adhesion to the host cell and in intracellular replication, one might hypothesize a role in later stages of infection as well. These include penetration of the alveolar epithelial barrier and basement membrane and dissemination of the bacteria in the lung tissue, causing alveolar damage and bacterial spread. Very recently a new peptidyl prolyl *cis/trans* isomerase (PPIase)-dependent mechanism for bacterial tissue

invasion as well as a model for alveolar damage and bacterial spread was proposed (Wagner *et al.*, 2007). The virulence protein Mip binds collagen and renders the extracellular matrix protease sensitive in a PPIase-dependent way. An as yet not further defined serine protease

Fig. 5. Plasminogen activation as a function of time as measured by the conversion of S-2251 in the presence of Glu-Plg by *L. pneumophila* Corby WT (◆) and *L. pneumophila* Corby LPS mutant (□).

Fig. 6. Growth of wild-type *L. pneumophila*, the *lpa* mutant strain *L. pneumophila* Δlpa and the complemented strain *L. pneumophila* $\Delta lpa/pBCpromlpa$ in the absence (upper photo) or presence (lower photo) of *A. castellanii* in the amoebae plate test.

would contribute to the perforation of the basal membrane allowing bacterial transmigration. We hypothesize that Lpa might fulfil the role of this additional protease. We report here, to our knowledge for the first time, that *L. pneumophila* produces a protein with the capacity to convert plasminogen to plasmin. Whether this protein is important for the virulence of *L. pneumophila* will be the issue of further research.

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