Molecular and functional characterization of type I signal peptidase from *Legionella pneumophila*

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*Legionella pneumophila* is a facultative intracellular Gram-negative rod-shaped bacterium that has become an important cause of both community-acquired and nosocomial pneumonia. Numerous studies concerning the unravelling of the virulence mechanism of this important pathogen have been initiated. As evidence is now accumulating for the involvement of protein secretion systems in bacterial virulence in general, the type I signal peptidase (LepB) of *L. pneumophila* was of particular interest. This endopeptidase plays an essential role in the processing of preproteins carrying a typical amino-terminal signal peptide, upon translocation across the cytoplasmic membrane. This paper reports the cloning and the transcriptional analysis of the *L. pneumophila* lepB gene encoding the type I signal peptidase (SPase). Reverse transcription PCR experiments showed clear *lepB* expression when *L. pneumophila* was grown both in culture medium, and also intracellularly in *Acanthamoeba castellanii*, a natural eukaryotic host of *L. pneumophila*. In addition, LepB was shown to be encoded by a polycistronic mRNA transcript together with two other proteins, i.e. a LepA homologue and a ribonuclease III homologue. SPase activity of the LepB protein was demonstrated by *in vivo* complementation analysis in a temperature-sensitive *Escherichia coli lepB* mutant. Protein sequence and predicted membrane topology were compared to those of leader peptidases of other Gram-negative human pathogens. Most strikingly, a strictly conserved methionine residue in the substrate binding pocket was replaced by a leucine residue, which might influence substrate recognition. Finally it was shown by *in vivo* experiments that *L. pneumophila* LepB is a target for (S,S,6S)-6-[(R)-acetoxyethyl]-penem-3-carboxylate, a specific inhibitor of type I SPases.

INTRODUCTION

*Legionella pneumophila*, the causative agent of Legionnaires’ disease in man, is a Gram-negative facultative intracellular parasite of monocytes and alveolar macrophages. In the environment, *L. pneumophila* inhabits fresh water, where it can reside and proliferate within various free-living protozoa, or is present in a biofilm-associated state. Human infection can occur following inhalation of small aerosolized droplets containing *Legionella* cells. Since the initial isolation of *L. pneumophila* in 1976, significant progress has been made in identifying virulence determinants for entry and intracellular multiplication in eukaryotic hosts. As in other Gram-negative pathogens, these virulence factors are usually proteins and are generally either secreted into the extracellular environment or attached to the cell surface (Finlay & Falkow, 1997). Although the secreted proteins are numerous and diverse, only a few pathways exist by which these proteins are transported from the bacterial cytoplasm to the extracellular environment (reviewed by Lee & Schneewind, 2001). For *L. pneumophila*, the presence of type II and type IV secretion systems has been described. The first type IV apparatus, encoded by the *icm/dot* genes (Segal & Shuman, 1998; Segal *et al.*, 1998), was shown to be required for export of virulence factors that play a role in evasion of the endocytic maturation pathway upon infection and in the recruitment of secretory vesicles to *L. pneumophila* containing phagosomes. A second type IV secretion system, with strong homology to the Agrobacterium tumefaciens Vir system for T-DNA transfer and the system for secretion of *Bordetella pertussis* toxin (reviewed by Christie, 2001), was described (Segal *et al.*, 1999). This system, encoded by *lvh* (*Legionella vir homologues*) genes, was, however, found to be dispensable for intracellular growth in macrophages and *Acanthamoeba castellanii*. A set of genes (called *isp*) encodes products with

†These two authors contributed equally to the work described in this paper and to the writing of the paper.

**Abbreviation:** SPase, signal peptidase.

The GenBank accession number for the sequence of the *L. pneumophila* lepB gene is AJ808705.

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homology to proteins of the type II secretion pathway in other Gram-negative bacteria (Hales & Shuman, 1999). Another study on type II secretion demonstrated the presence of a pilBCD locus, later shown to be involved in formation of type IV pili and the assembly of a type II protein secretion system (Liles et al., 1999; Rossier et al., 2004). Mutagenesis of lsp genes and the prepilin peptidase gene pilD revealed that type II secretion promotes the ability to infect both protozoan and macrophage hosts and to grow in the mammalian lung (Hales & Shuman, 1999; Liles et al., 1999; Rossier et al., 2004). In L. pneumophila, different proteins were shown to depend on the type II secretion machinery for transport across the outer membrane. These include a zinc metalloprotease, lipases, phospholipase A, acid phosphatase, RNase and an esterase (Liles et al., 1999; Aragon et al., 2000, 2001, 2002). In general, proteins secreted by this type II pathway follow an initial Sec-dependent step for export across the inner membrane. The crossing of the outer membrane involves the formation of a pore, by a set of additional inner- and outer-membrane proteins (Sandkvist, 2001). The exported proteins are synthesized as preproteins, carrying a typical amino-terminal signal sequence, involved in targeting the precursors to and passage through the inner membrane. The signal peptide consists of three distinct domains, of which the carboxy-terminal C-domain allows sequence-specific cleavage by the type I signal peptidase (SPase), a membrane-bound endopeptidase. Statistical analysis of the amino acid residues constituting the SPase recognition site has led to the so-called (−3,−1) rule, which states that the residues at the −3 and −1 positions, relative to the mature part, must be small and neutral residues with a strong preference for alanine (von Heijne, 1983). Therefore, the consensus SPase recognition site is often designated Ala-X-Ala. The type I SPase plays a key role in the secretion process by removing the signal peptide and, in this way, releasing the mature protein. Type I SPases have been identified in eubacteria, archaea, the thylakoid membrane of chloroplasts, the inner membrane of mitochondria and the endoplasmic reticulum of yeast and higher eukaryotes (Paetzel et al., 2002). So far, the leader peptidase (LepB) of the Gram-negative Escherichia coli is the best characterized SPase (for a review see Paetzel et al., 2000). Although many Gram-negative bacteria have only one SPase for preprotein processing, multiple type I SPases can be present.

Type I SPases belong to a novel group of serine proteases and have been classified into the evolutionary clan of serine proteases SF, which utilize a Ser-Lys or Ser-Asp catalytic dyad rather than the more common Ser-Asp dyad mechanism (Black, 1993; Paetzel et al., 1998). Type I SPases can be irreversibly inhibited by certain penem compounds which are responsible for the acylation of the serine residue in the active site (Black & Bruton, 1998; Paetzel et al., 1998). SPase activity was found to be essential for cell viability, defining type I SPases as potential targets for the development of novel antibacterial agents.

We report here on the cloning and transcriptional analysis of the L. pneumophila lepB gene encoding the SPase I. In addition, functional activity of the encoded protein was demonstrated by complementation analysis in an E. coli SPase mutant. Finally, the effect of a penem derivative on growth of L. pneumophila and an E. coli temperature-sensitive SPase mutant expressing L. pneumophila lepB was evaluated.

### METHODS

**Strains, media and growth conditions.** L. pneumophila (ATCC 33152) was cultured on buffered charcoal yeast extract (BCYE) agar plates or in buffered yeast extract broth (BYE) (Edelstein, 1981) supplemented with 2-oxoglutarate, l-cysteine and ferric pyrophosphate, at 37°C and 5% CO₂. E. coli strains were grown in Luria–Bertani medium (Miller, 1972), if necessary supplemented with ampicillin (50 μg ml⁻¹) or chloramphenicol (25 μg ml⁻¹). For functional complementation analysis, the E. coli strain IT89, containing a temperature-sensitive mutation in lepB, was used (Inada et al., 1989); this strain was grown at 27°C. Acanthamoeba castellanii (ATCC 30234) was cultured in Acanthamoeba medium (Moffat & Tompkins, 1992) at room temperature.

**DNA manipulations.** All DNA manipulations, including purification, restriction digestion, ligation, agarose gel electrophoresis, PCR amplification and transformation of E. coli cells, were performed using standard techniques (Sambrook et al., 1989). Restriction endonucleases and other DNA-modifying enzymes were purchased from Roche Diagnostics and Invitrogen-Life Technologies. Oligonucleotides (Table 1) were obtained from Eurogentec. Nucleotide sequence analysis was performed on ALFexpress (Amersham Biosciences)

### Table 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’–3’)</th>
<th>Feature</th>
</tr>
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<tbody>
<tr>
<td>LspF</td>
<td>taacatgtgaatttctgtaatattg</td>
<td>Ndel</td>
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| LspR | tggctctcgaagttctctctcgcctctcagcttcctgtgattctctgacattctctgacattctcctctgcctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattctctgacattc...
using Cy-labelled oligonucleotides. Genomic DNA of *Legionella* strains was extracted with the Wizard Genomic DNA Purification Kit (Promega).

**Plasmid constructions.** The *L. pneumophila lepB* gene was isolated by PCR using the primers LspF and LspR, from chromosomal DNA (100 ng) as template. To enable final detection of the encoded protein at a later stage, a sequence encoding the c-Myc tag was introduced into the LspR primer. To facilitate cloning, LspF and LspR primers were designed with Ndel and BamHI restriction sites, respectively. PCR was performed on a Mini Cycler PTC-150 (MJ Research) using SuperTaq polymerase (HT Biotechnology) under the following conditions: an initial denaturation step of 3 min at 95 °C, 30 cycles of (30 s at 95 °C, 45 s at 50 °C, 1 min at 72 °C), and a final extension for 5 min at 72 °C. The resulting 0·8 kb PCR fragment was cloned into pGEM-T Easy (Promega) and the DNA sequence was determined. Subsequently, the *lepB* gene was cloned as an Ndel–BamHI restriction fragment behind the lac promoter and a suitable ribosome-binding site (RBS) in pEX50 (Geukens et al., 2001), resulting in pEXLepLpn. Additionally, the same restriction fragment containing the *lepB* gene was inserted behind the lac promoter in pBCN, which is a pBS–KS + derivative with a Ndel site introduced at the start codon of the lacZα gene, resulting in pBCNLepLpn.

**Southern blot hybridization.** Genomic DNA from *L. pneumophila* was digested with specific restriction enzymes (EcoRV, EcoRI and HindIII) and separated by agarose gel electrophoresis. DNA fragments were subsequently transferred to Hybond-N membranes (Amersham) using a vacuum blot device (VacuGene, Amersham Biosciences). A *lepB*-specific DNA probe was generated by labelling the PCR fragment obtained with primers LspF and LspR with digoxigenin (DIG) according to the instructions of the manufacturer (Roche Diagnostics). The UV cross-linked DNA fragments were hybridized with the *lepB*-specific probe as described previously (Engler-Blum et al., 1993). Hybridization signals were detected using 0·25 mM CDP-Star (Roche Diagnostics) according to the method of Hoelte et al. (1995).

**RNA isolation and RT-PCR experiments.** Total RNA was isolated from *L. pneumophila* cultures grown to exponential and stationary phase using the RNaseasy mini kit (Qiagen). For this purpose, the cells from 3 ml of culture were collected by centrifugation, the RNAs were fixed using the RNA Protect kit (Qiagen) and the samples frozen at −20 °C until immediately prior to use. To investigate whether the *L. pneumophila lepB* gene was also expressed when the cells were grown intracellularly, total RNA was isolated from *L. pneumophila* grown within *A. castellanii*. For these experiments, *A. castellanii*, adherently grown in a 24-well plate, was infected with *L. pneumophila* cells at an m.o.i. of 1. Infection was allowed to proceed for 2 h at 37 °C and then gentamicin (100 µg ml−1) was added for 1 h to kill the bacteria present in the extracellular environment; this represents the initial time point (0 h). At 2 h and 4 h post-infection, culture supernatants from 12 wells were collected and the bacteria were released from the amoebae by hypotonic lysis with ice-cold distilled water. After mixing the released bacteria with those present in the cell culture supernatant, bacteria were collected and RNA was isolated as described above. As *L. pneumophila* does not replicate in cell culture medium, the bacteria present in the samples result from intracellular growth and subsequent host cell lysis. After isolation, a control for the quality of the RNA samples was carried out by 1·3% agarose-formaldehyde gel electrophoresis followed by ethidium bromide staining. RT-PCR experiments were carried out using the various primers given in Table 1 (see also Fig. 3) by means of the Access RT-PCR kit (Promega). Initial synthesis of the cDNA strand and subsequent amplification were carried out starting from 0·5 µg total RNA using the following cycling conditions: 45 min at 48 °C, 3 min at 95 °C, 40 cycles of (30 s at 95 °C, 45 s at 50 °C, 2 min at 72 °C), 5 min at 72 °C. The same reactions were also performed on RNA treated with RNase to check for the possible presence of traces of DNA in the samples.

**Protein analysis, SDS-PAGE and immunodetection.** Expression of the LepB protein was monitored by Western blotting (12·5% SDS-PAGE) and immunodetection with antibodies directed against the c-Myc tag of the proteins in combination with a suitable secondary alkaline-phosphatase-conjugated antibody (Sigma). Proteins were visualized using the chromogenic substrate solution NBT/BCIP (Roche Diagnostics).

**Computer-aided analysis.** Nucleotide and amino acid sequences were analysed using Vector NTI Suite (Informax) and NCBI BLAST search analysis (Altschul et al., 1997). Amino acid sequence alignment was done using the Vector NTI Suite software and topology prediction was performed using HHMTOP (Tusnady & Simon, 1998).

**Complementation analysis of the *L. pneumophila lepB* gene.** The complementation assay protocol was similar to those described previously (Inada et al., 1989; Cregg et al., 1996; Chu et al., 2002). *E. coli* strain IT89 (temperature-sensitive *lepB* mutant) was transformed with either pEXLepLpn, pBCNLepLpn or control plasmids PEX50 or pBCN. Single transformants were grown overnight in LB medium at 27 °C. After dilution to an OD 540 of 0·2, cultures were grown at the non-permissive temperature 42 °C in the absence of IPTG and the OD 540 was followed as a function of time.

In addition, growth of *E. coli* IT89(pEXLepLpn) at 42 °C was monitored in the presence of 1 mM or 2 mM IPTG to stimulate expression of the *L. pneumophila lepB* gene.

**In vivo analysis of the inhibitory effect of (SS,6S)-6-[(R)-acetoxyethyl]-penem-3-carboxylic acid on LepB.** Overnight cultures of *E. coli* IT89(pEXLepLpn) and *L. pneumophila* were diluted to an OD 540 of 0·02 and 0·2, respectively (in triplicate). To one of the cultures 100 µM (SS,6S)-6-[(R)-acetoxyethyl]-penem-3-carboxylic acid (dissolved in DMSO) was added. As a control, an equal amount of DMSO was added to one of the cultures to investigate the effect of this solution on both bacterial strains. No DMSO or inhibitor was added to the third culture. The cultures were then incubated at 42 °C [*E. coli* IT89(pEXLepLpn)] or 37 °C (*L. pneumophila*). Growth of the different cultures was compared during exponential growth [OD 540 0·5 for *E. coli* IT89(pEXLepLpn) without DMSO or inhibitor added; OD 540 1·5 for *L. pneumophila* without DMSO or inhibitor] and post-exponential growth [OD 540 0·8 for *E. coli* IT89(pEXLepLpn) without DMSO or inhibitor; OD 540 4·0 for *L. pneumophila* without DMSO or inhibitor].

**RESULTS AND DISCUSSION**

**Cloning and sequence analysis of the *L. pneumophila lepB* gene**

In order to determine whether *L. pneumophila* possesses one or more type I signal peptidase genes, a BLAST (tBlastn) search for genes encoding homologues of the *E. coli* leader peptidase (LepB) was carried out on the (as yet incomplete) *L. pneumophila* genome sequence (available at http://genome3.cpmc.columbia.edu/~legion/). This screening revealed one, not yet annotated, ORF of 756 bp. This gene was cloned by PCR from *L. pneumophila* genomic DNA as described in Methods and the DNA sequence of the genome bank could be confirmed. Using Southern blot hybridization of *L. pneumophila* genomic DNA with a
DIG-labelled lepB-specific probe, the presence of only one SPase gene could be demonstrated (Fig. 1). The isolated L. pneumophila gene (GenBank/EMBL accession no. AJ608705) was predicted to code for a SPase protein (251 aa with a predicted molecular mass of 28.8 kDa) according to its overall homology with SPase proteins of other bacteria.

In Fig. 2(a), an alignment of the deduced amino acid sequence of L. pneumophila LepB with SPase I proteins of other important Gram-negative human pathogens is shown. Overall sequence similarity with these SPases is 40–65%. In particular, five regions of high conservation were identified; these regions, designated boxes A, B, C, D and E, are conserved in all type I SPases (Dalbey et al., 1997) and contain all the residues important for activity. The deduced amino acid sequence of L. pneumophila lepB is most similar to that of Pseudomonas aeruginosa. In both proteins, the region between boxes D and E is typically shorter compared to E. coli-like SPases.

Among the conserved domains, box A is a part of an amino-terminal transmembrane segment. Conserved box B contains the catalytic Ser (Ser90 in E. coli numbering) and is characterized by the consensus motif [IPSGSMxPTLx]. The consensus sequence is read with the bold upper-case character meaning absolutely conserved, the upper case character meaning conserved and ‘x’ meaning not conserved. In L. pneumophila LepB, the Met in this consensus motif is replaced by Leu. Note that in all naturally occurring SPase I enzymes described so far, this Met was strictly conserved. Site-directed mutagenesis of Bacillus subtilis SipS showed that the corresponding Met (Met44 in B. subtilis numbering) could be replaced by Ala without losing activity (van Dijl et al., 1995), but until now, there has been no report on the natural occurrence of other residues than Met at this position. From the E. coli SPase structure, it was concluded that Met91 is part of the S1 substrate-binding pocket and its replacement by a larger Leu residue in L. pneumophila LepB can therefore influence the substrate specificity of the SPase. Box C has conserved amino acids [RGDIVYFxxP]. The second catalytic residue Lys or His (Lys145 in E. coli numbering) is situated in box D, which has the consensus motif [YIKRxGxPGDxV]. Box E is the largest conserved domain with the following motif: [VPxGxFxMGDNRDNSxDSR]. It is noteworthy that the L. pneumophila LepB protein is small compared to other SPases from Gram-negative pathogens (251 aa versus 324 aa for E. coli LepB). The topology of type I SPases from Gram-negative bacteria can vary significantly. The large catalytic periplasmic domain is anchored to the cytoplasmic membrane by 1–3 amino-terminal transmembrane segments. Furthermore, an additional anchor may be present at the carboxy-terminus of the enzyme. Hydrophobicity analysis of the L. pneumophila LepB amino acid sequence suggested two hydrophobic segments (aa 1–21 and aa 43–63) to act as transmembrane anchors. The predicted topology (Fig. 2b) is therefore similar to that of E. coli LepB.

Transcriptional analysis of the L. pneumophila lepB gene

In the first instance, the organization of the genes in the neighbourhood of the lepB gene was analysed (Fig. 3a). The lepB gene is situated in the middle of two other putative genes that are transcribed in the same direction. Upstream of lepB, a gene encoding a homologue of GTP-binding LepA proteins was found, while downstream of lepB, a gene encoding a ribonuclease III (Rnc) homologue is present. The intergenic region between the lepA and lepB gene is 72 bp, while that between the SPase gene and the putative rnc gene is 390 bp. The predicted ORFs located further upstream and downstream are transcribed in the opposite direction and encode proteins with homology to lytic murein transglycosylases and an ATP-binding protein of an ABC transporter, respectively.

To investigate whether L. pneumophila expresses the lepB gene and whether the three unidirectionally described genes constitute an operon, RT-PCR experiments on total RNA were carried out (Fig. 3b). Total RNA was isolated either from L. pneumophila grown to exponential and stationary phase in liquid culture, or from L. pneumophila grown intracellularly in A. castellanii. RT-PCR with primers LspF and LspR, both binding to the lepB gene, clearly showed the presence of a 9.8 kb transcript in the case of L. pneumophila grown in liquid culture, as well as in L. pneumophila cells arising from infection of, and intracellular multiplication within, A. castellanii. Similar results were obtained with RNA isolated from exponential- or stationary-phase cultures (data not shown). RT-PCR on RNase-treated samples, included to detect traces of DNA in the samples, did not result in a band on agarose gel, indicating the absence of DNA and hence confirming a specific RNA-dependent amplification. In addition, the presence of a...
Fig. 2. (a) Amino acid sequence alignment of *L. pneumophila* (Lpn) LepB with that of other Gram-negative pathogens. Type I SPases used in this alignment are those of *E. coli* (Eco), *Shigella flexneri* (Sfl), *Salmonella enterica* serovar Typhi (Sen), *Yersinia pestis* (Ype), *Vibrio cholerae* (Vch), *Pseudomonas aeruginosa* (Pae), *Haemophilus influenzae* (Hin), *Helicobacter pylori* (Hpy), *Neisseria meningitidis* (Nme) and *Rickettsia prowazekii* (Rpr). All these sequences are available at www.tigr.org. Conserved domains (boxes) A, B, C, D and E are indicated. The similarity with *L. pneumophila* LepB was determined using VectorNTIsuite. (b) Membrane topology of *L. pneumophila* LepB predicted using the HMMTOP software. The membrane anchors are shaded in grey and conserved domains A, B, C, D and E are indicated. N, amino-terminus; C, carboxy-terminus; In, the cytoplasmic side of the inner membrane; Out, the periplasmic side of the inner membrane; CM, the cytoplasmic or inner membrane.
transcript of 1.7 kb comprising the three genes could be demonstrated, indicating that the three genes are cotranscribed. The lower intensity of the 1.7 kb fragment compared to that of the smaller fragments could be an indication of a post-transcriptional cleavage of the longer message but might also be the consequence of a lower stability of the mRNA, or even of a less efficient PCR amplification. Based on these results and taking into account that the more upstream located gene is transcribed in the opposite direction, we assume the presence of a promoter upstream of the \( \text{lepA} \) gene. Based on database analysis, the \( \text{lepA--lepB--rnc} \) cluster, with highly variable intergenic distances, can also be found in the genomes of other Gram-negative human pathogens, such as enteropathogenic \( \text{E. coli} \), \( \text{Vibriocholerae} \), \( \text{Yersinia pestis} \), \( \text{Shigella flexneri} \) and \( \text{Salmonella typhimurium} \) genomes. For most of the pathogens, however, information on transcriptional organization is not available. In contrast to \( \text{L. pneumophila} \), where \( \text{lepB} \) and \( \text{rnc} \) are part of the same operon (\( \text{lepA--lepB--rnc} \)), for \( \text{E. coli} \) it was shown that the \( \text{lepB} \) gene is situated in an operon structure together with the upstream \( \text{lepA} \) gene (March & Inouye, 1985) and this \( \text{lep} \) operon is followed immediately by the \( \text{rnc} \) operon. Recently, it was shown that in the case of the obligate intracellular pathogen \( \text{Rickettsia rickettsii} \), the \( \text{lepB} \) gene is cotranscribed with the \( \text{secF} \) gene (encoding a membrane protein involved in protein translocation across the inner membrane), the \( \text{nuoF} \) gene (encoding a putative NADH dehydrogenase I chain F), and the \( \text{rnc} \) gene in the \( \text{secF--nuoF--lepB--rnc} \) cluster (Rahman et al., 2003). In \( \text{Rhodobacter capsulatus} \) no \( \text{lepA} \) is present but the \( \text{lepB} \) gene forms an operon with the downstream genes, \( \text{rnc} \) and \( \text{era} \) (encoding a GTP-binding protein) (Rauhut et al., 1996). From these examples it is clear that, although homologous genes are often present in the \( \text{lep} \) region, the transcriptional organization can vary considerably between different organisms. A tripartite operon structure consisting of \( \text{lepA--lepB--rnc} \) as observed in \( \text{L. pneumophila} \) is unique so far.

### Expression and functional analysis of the \( \text{L. pneumophila lepB} \) gene in \( \text{E. coli} \)

Assessment of in vivo activity of \( \text{L. pneumophila} \) LepB depended on complementation of the temperature-sensitive LepB mutant of \( \text{E. coli} \) IT89 (Inada et al., 1989). \( \text{E. coli} \) IT89 contains an amber mutation in the \( \text{lepB} \) gene at codon 39, which gives a temperature-sensitive phenotype (Cregg et al., 1996). The strain shows normal growth at 27°C, but growth is dramatically affected at the non-permissive temperature (42°C). This temperature-sensitive mutation can be complemented by a plasmid carrying...
a functional lepB gene. This assay has already been used to demonstrate type I SPase activity for SPase genes of Salmonella typhimurium (van Dijl et al., 1990), Bradyrhizobium japonicum (Bairl & Müller, 1998; Müller et al., 1995), Staphylococcus aureus (Cregg et al., 1996), Streptococcus pneumoniae (Zhang et al., 1997), Streptomyces lividans (Parro et al., 1999), Bacillus amyloliquefaciens (Chu et al., 2002), and Rickettsia rickettsii and Rickettsia typhi (Rahman et al., 2003).

The L. pneumophila lepB gene was cloned on a low-copy-number (pEX50) and a high-copy-number (pBCN) plasmid to evaluate the effect of overexpression of L. pneumophila LepB on its complementation capacity. The pBCN plasmid has a 10 times higher copy number than pEX50. Growth of E. coli IT89 harbouring pEXLepLpn, pBCNLepLpn and the respective control plasmids pEX50 and pBCN at 42 °C was monitored in the absence of IPTG. Fig. 4(a) clearly shows that L. pneumophila LepB supported growth at the non-permissive temperature, demonstrating that L. pneumophila LepB correctly inserts into the E. coli inner membrane and that it can process all E. coli proteins necessary for cell viability. The growth rate of E. coli IT89(pBCNLepLpn) was significantly retarded compared to that of E. coli IT89(pEXLepLpn). This observation, together with the finding that addition of 1 mM and 2 mM IPTG to E. coli IT89(pEXLepLpn) resulted in a decrease of growth rate, suggests that overexpression of the L. pneumophila lepB gene results in lethality.

Expression of L. pneumophila LepB in E. coli was analysed using Western blotting. A protein of approximately 28 kDa was detected in the E. coli IT89 strains harbouring pEXLepLpn or pBCNLepLpn, which was not present in the control (Fig. 4b). The production of L. pneumophila LepB in E. coli IT89(pBCNLepLpn) was significantly higher compared to its production in E. coli IT89(pEXLepLpn); this most likely results from the difference in copy number of the expression vector. No significant increase in L. pneumophila LepB production was detected in E. coli IT89(pEXLepLpn) upon addition of IPTG as monitored by Western blotting (Fig. 4b); however, a negative effect on the growth rate was observed (Fig. 4a). From this we conclude that the higher the production of L. pneumophila LepB was, the more the growth rate of the respective E. coli IT89 strain was retarded, which is in agreement with the hypothesis of overexpression lethality. Similar effects on the viability of E. coli were also observed when the SPases from B. amyloliquefaciens were overexpressed (Chu et al., 2002).

**Effect of a penem derivative on the activity of L. pneumophila LepB**

To evaluate L. pneumophila LepB as a target for novel antibiotics, growth of E. coli IT89(pEXLepLpn) and L. pneumophila was monitored in the presence and absence of (5S, 6S)-6-[(R)-acetoxyethyl]-penem-3-carboxylate, a specific SPase inhibitor (Allsop et al., 1995; Black & Bruton 1998; Paetzel et al., 1998). As a control, the effect of adding DMSO, used to dissolve the penem compound, was included. Fig. 5 shows that addition of 100 μM (5S, 6S)-6-[(R)-acetoxyethyl]-penem-3-carboxylate resulted in a clear reduction (14–18 %) of the growth of E. coli IT89(pEXLepLpn) cells at 42 °C compared to the control with only DMSO added. A clearly retarded growth (18–25 %) was also observed when L. pneumophila was grown in BYE medium containing 100 μM of the penem derivative. In this case, no effect due to the addition of DMSO was observed. For both strains, reduction of the growth was observed for exponentially-growing cells as

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**Fig. 4.** (a) Growth curves of E. coli IT89 transformants containing the appropriate plasmids. Cultures grown overnight at 27 °C were diluted to OD₅₄₀ 0.02 and incubated at 42 °C in the absence of IPTG; the OD₅₄₀ was monitored as a function of time. For E. coli IT89(pEXLepLpn), growth was also monitored in the presence of 1 mM and 2 mM IPTG. All points are the mean of at least two independent experiments. (b) Expression analysis of c-Myc tagged L. pneumophila LepB in E. coli IT89. Proteins present in total cell lysates of E. coli IT89 cultures grown to OD₅₄₀ 0.3 were separated on a 12-5 % SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-c-Myc antibodies. Lane 1, E. coli IT89(pEX50LepLpn) grown at 42 °C; lane 2, E. coli IT89(pEX50LepLpn) grown at 42 °C in the presence of 2 mM IPTG; lane 3, E. coli IT89(pEX50) grown at 27 °C; lane 4, E. coli IT89(pBCNLepLpn) grown at 42 °C.
In summary, we have cloned and characterized the functional type I signal peptidase gene of the human pathogen \textit{L. pneumophila}. Further experiments will reveal the extent to which this key enzyme is important in the secretion of established virulence factors and to what degree it may be useful, from a therapeutic point of view, as a target for novel antibiotic compounds.

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