***In vitro* secretion kinetics of proteins from *Legionella pneumophila* in comparison to proteins from non-*pneumophila* species**

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It has been shown that the loss of PilD, a prepilin peptidase necessary for type IV pilus biogenesis and establishment of the type II secretion apparatus is associated with loss of virulence in *Legionella pneumophila*. *L. pneumophila* is the species most frequently associated with Legionnaires' disease, but virulence factors unique to this species are not known, so the secretion kinetics of several *pilD*-dependent enzyme activities, including protease, acid phosphatase, phospholipase A (PLA) and lysophospholipase A (LPLA), of *L. pneumophila* and non-*pneumophila* species were compared during growth in BYE broth. Enzyme activity appeared during mid-exponential growth phase and reached maximal levels on entry into stationary growth phase. None of the enzyme activities were unique to *L. pneumophila* and it did not exclusively secrete the highest amounts of the hydrolytic proteins. However, the timing of PLA and LPLA secretion in *L. pneumophila* differed compared to other species. PLA activity was secreted prior to LPLA activity in *L. pneumophila*, which may lead to an accumulation of the cytotoxic agent lysophosphatidylcholine (LPC). In addition to *L. pneumophila*, several other *Legionella* species, including *Legionella steigerwaltii* and *Legionella gormanii*, were able to enrich for LPC due to a very potent PLA activity accompanied by only moderate LPLA activity. These species, in contrast to *L. pneumophila*, have not been shown to multiply within monocytic host cells. Thus none of the secreted enzymic activities investigated were unique to *L. pneumophila*, nor were they secreted at high concentrations. However, the timing of PLA and LPLA secretion may contribute to pathogenicity.

Keywords: virulence, exotoxins, intracellular bacteria, phospholipase A, lysophospholipase A

INTRODUCTION

The genus *Legionella* contains more than 40 species. *Legionella pneumophila* is the species most frequently isolated from patients suffering from Legionnaires' disease. However, virulence factors unique to *L. pneumophila* that contribute to its importance are not known. Recently, it was shown that the loss of the

prepilin peptidase (PilD), a key enzyme in type IV pilus biogenesis and in the establishment of the type II protein secretion apparatus in *L. pneumophila*, leads to a decrease in virulence (Liles *et al.*, 1999; Aragon *et al.*, 2000).

Enzyme activities of *L. pneumophila* secreted via the type II protein secretion pathway include protease, acid

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Abbreviations: CS, culture supernatant; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; FFA, free fatty acid; LPC, lysophosphatidylcholine; LPLA, lysophospholipase A; MPLPC, monopalmitoyllysophosphatidylcholine; PLA, phospholipase A; p-NPP, *p*-nitrophenylphosphate.

This work was in part presented as a poster and published as an abstract at the 5th International Conference on *Legionella*.

Fig. 1. Growth of different *Legionella* species in BYE broth and secreted activities of PLA (release of FFA from DPPG and DPPC), LPLA (release of FFA from MPLPC), acid phosphatase (release of p-NP), and proteinase (hydrolysis of azocasein) in

Table 1. *Legionella* strains used for production of culture supernatants

Abbreviations: h, human isolate; e, environmental isolate; ATCC, American Type Culture Collection; CDC, Centers for Disease Control, strains were kindly provided by Barry Fields.

Species	Serogroup/ Subtype	Origin	Source	No. passages on BCYE α agar	Abbreviation
<i>L. pneumophila</i>	1 (Wadsworth) strain 130b	h	Nicholas P. Cianciotto, NorthWestern University Medical School, Chicago, USA	<3	Lp1-130b
<i>L. pneumophila</i>	1 (Philadelphia-1)	h	CDC stock 5	Numerous	Lp1-CDC
<i>L. pneumophila</i>	1 (Pontiac-1)	h	Gotthardt Ruckdeschel, University of Munich, Germany	<3	Lp1P-c
<i>L. dumoffii</i>		e	CDC F1407	<3	Ld-CDC
<i>L. gormanii</i>		e	CDC F462	<3	Lg-CDC
<i>L. longbeachae</i>	1	e	ATCC 33462	Unknown	Ll-ATCC
<i>L. micdadei</i>		e	CDC F976	<3	Lm-CDC
<i>L. steigerwaltii</i>		e	CDC 464	<3	Ls-CDC

phosphatase, lipase-like activity, nuclease, and both phospholipase A (PLA) and lysophospholipase A (LPLA) activities (Liles *et al.*, 1999; Hales & Shuman, 1999; Aragon *et al.*, 2000; Flieger *et al.*, 2001; Rossier & Cianciotto, 2001).

The well-described zinc metalloproteinase of *L. pneumophila* has been characterized as one of the virulence factors of the bacterium. It has been shown to be responsible for some of the lethal effects of the bacterium in guinea pigs, but is not essential for entry, survival or growth in *Acanthamoeba* or macrophages (Moffat *et al.*, 1994; Szeto & Shuman, 1990). The ability of the protease to degrade interleukin-2 and TNF- α , and to cleave CD4, suggests that it may contribute to the pathogenesis of Legionnaires' disease by interfering with immune responses (Mintz *et al.*, 1993; Hell *et al.*, 1993). Immunogold labelling using anti-protease antibody showed that the enzyme localizes within phagosomes and is distributed throughout the macrophage (Rechnitzer *et al.*, 1992).

Another enzyme, the major acid phosphatase of *L. pneumophila*, has been shown to be unnecessary for entry and multiplication in macrophages and *Hartmannella vermiformis* (Aragon *et al.*, 2001). Besides the major acid phosphatase, a minor tartrate-resistant phosphatase has also been found to be secreted by the bacterium, but its importance in virulence has not been evaluated (Aragon *et al.*, 2001).

Destruction of phospholipids by bacterial phospholipases and the subsequent change of membrane constituents, which can lead to cell damage, are thought to

be a major virulence mechanism in infection. Enzymes such as PLA and LPLA hydrolyse phospholipids and have been shown to be secreted by *L. pneumophila* (Flieger *et al.*, 2000a, 2001). *L. pneumophila* cells and their culture supernatants (CSs) can cleave lung surfactant phospholipids and generate free fatty acids (FFAs) and cytotoxic lysophosphatidylcholine (LPC) (Flieger *et al.*, 2000a, b).

Despite efforts to characterize possible virulence determinants, it is uncertain why *L. pneumophila* is the species most frequently isolated from patients suffering from Legionnaires' disease. In addition, it has not been established whether some of the secreted enzymes or their levels of activity are unique to *L. pneumophila*. Therefore, we aimed to characterize the secretion kinetics of putative virulence factors of *L. pneumophila* and to compare the kinetics to those of non-*pneumophila* species during late log and early stationary phases of growth, when *L. pneumophila* switches to a virulent phenotype (Byrne & Swanson, 1998) and secretes maximal activities of PLA, protease and acid phosphatase activities (Byrne & Swanson, 1998; Flieger *et al.*, 2000a; Aragon *et al.*, 2000).

METHODS

Reagents. BYE supplement for *Legionella* BYE broth was obtained from Oxoid. Yeast extract for *Legionella* BYE broth, ingredients for BCYE α agar, sodium azide, Tris/HCl, methanol, silica gel TLC plates and chloroform were purchased from Merck. Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), monopalmitoyllysophosphatidylcholine (MPLPC), MES, Triton X-100,

the CS. Data shown are from three experiments. Maximal standard deviations: growth, ± 0.04 ; PLA (DPPG), ± 0.07 except Ls-CDC at 14 h, ± 0.16 ; PLA (DPPC), ± 0.04 ; LPLA (MPLPC), ± 0.08 ; protease, ± 0.02 ; acid phosphatase, ± 0.03 except Ld-CDC at 18 h, ± 0.08 and Lg-CDC at 20 h, ± 0.12 . ■, Growth; ▲, PLA (DPPG); ×, PLA (DPPC); ✕, LPLA (MPLPC); ○, protease; ◆, acid phosphatase. (a) Lp1-CDC (*L. pneumophila*), (b) Lp1P-c (*L. pneumophila*), (c) Lp1-130b (*L. pneumophila*), (d) Ll-ATCC (*L. longbeachae*), (e) Lg-CDC (*L. gormanii*), (f) Ld-CDC (*L. dumoffii*), (g) Lm-CDC (*L. micdadei*), (h) Ls-CDC (*L. steigerwaltii*).

p-nitrophenylphosphate (p-NPP, ammonium salt), *p*-nitrophenol, calcium chloride, 1,2-dipalmitoylglycerol and palmitic acid were acquired from Sigma-Aldrich.

SDS-PAGE buffer strips, 12.5% homogeneous SDS-PAGE ready gels and silver staining kits for protein were obtained from Amersham Pharmacia Biotech. Low molecular mass standards for SDS-PAGE were purchased from Bio-Rad.

Bacteria. *Legionella* strains used for this investigation are listed in Table 1.

Preparation of CSs. Lp1-130b, Lp1-CDC, Lp1P-c, Ld-CDC, Lg-CDC, Ll-ATCC, Lm-CDC and Ls-CDC were grown on BCYE α agar at 37 °C in 3% CO₂ for 3 d (Pasculle *et al.*, 1980). Colonies were resuspended in 20 ml BYE broth (Baine, 1988) and adjusted to OD₅₇₈ 0.2. Bacteria were cultured at 37 °C in 3% CO₂ with vigorous shaking. CSs were obtained after 0, 8, 10, 12, 14, 16, 18, 20, 22 and 36 h incubation by centrifugation of samples at 5000 *g* for 5 min; 3 mM sodium azide was added to prevent bacterial growth. CSs were concentrated tenfold by 2-propanol precipitation. Two sample volumes of 2-propanol were added, the mixture was incubated for 10 min at -20 °C and centrifuged at 5000 *g* for 10 min. The pellet was dissolved in 20 mM Tris/HCl, pH 7.5.

SDS-PAGE. This was performed under reducing conditions (Laemmli, 1970). The molecular masses of the protein bands were determined using a low molecular mass calibration kit from Bio-Rad. Ten microlitre samples of the concentrated and reduced CSs were loaded onto the gels. Proteins were visualized using a silver staining kit for proteins (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Detection of enzyme activities. Hydrolysis of p-NPP by CSs was detected by incubating 20 mM p-NPP, 6 mM NaN₃, 40 mM CaCl₂, 1% (v/v) Triton X-100 in 100 mM MES, pH 6.0 and an equal volume of CS at 37 °C with continuous agitation. The absorbance was determined after 2 h at 410 nm.

Hydrolysis of azocasein was detected by incubating 100 μ l 2% (w/v) azocasein in 20 mM Tris/HCl, pH 7.2, with 50 μ l CS at 37 °C for 1 h with continuous agitation and then the mixture was treated as described by Prestidge *et al.* (1971).

PLA and LPLA activities were detected by incubating different phospholipids with CSs in a mixture containing 6 mM lipid substrate (DPPG, DPPC or MPLPC), 3 mM NaN₃, 0.5% (v/v) Triton X-100 and 20 mM Tris/HCl, pH 7.2. The lipid substrates were vortexed for 15 min and sonicated three times for 1 min at a power setting of 5 (B12-Sonifier; Branson Sonic Power). All reactions were performed for 5 h at 37 °C with continuous agitation. FFAs were detected using the NEFA-C-Kit (Wako Chemicals), according to the manufacturer's instructions. Data were expressed as the difference between the amount of FFA in the samples and in the negative control (BYE broth). Additionally, lipids from incubation of CSs with DPPC were extracted according to Bligh & Dyer (1959) and generation of the reaction product LPC was analysed by TLC.

TLC. Lipids were separated using the solvent mixture chloroform:methanol:water (65:25:4 by vol.). For visualization of the lipids, silica plates were sprayed with the reagent described by Touchstone *et al.* (1983).

RESULTS

Secreted enzyme activity pattern of different *Legionella* species

Protease, acid phosphatase, PLA and LPLA activities were secreted by all the *Legionella* species studied, except *Legionella longbeachae* and *Legionella micdadei*, which lacked most of the activities tested (Fig. 1). When present, we found that PLA, protease and acid phosphatase activities appeared during mid-exponential growth phase, reaching maximum levels on entry into stationary phase.

Only the timing of maximal LPLA activity seemed to vary between the different *Legionella* species. LPLA activity reached maximal levels at the same time as all other enzymic activities in *Legionella dumoffii*, later than all other enzymic activities in *L. pneumophila*, and prior to all other enzymic activities in *Legionella gormanii* and *Legionella steigerwaltii* (Fig. 1).

Different *Legionella* species secreted differing amounts of each of the enzyme activities, but different isolates of *L. pneumophila* resembled each other in their secreted hydrolytic activity. In contrast to its frequent association with disease, apparently *L. pneumophila* is not the species which generally produced the highest amounts of the tested enzymic activities (Fig. 1).

L. pneumophila and *L. gormanii* secreted the highest amounts of protease (Fig. 1). *L. gormanii*, *L. dumoffii* and *L. steigerwaltii* secreted the highest amounts of phosphatase (Fig. 1). *L. gormanii*, *L. steigerwaltii* and *L. pneumophila* exhibited the highest PLA activity and both *L. gormanii* and one of the *L. pneumophila* strains had the most prominent LPLA activity (Fig. 1).

When the timing of secretion of PLA and LPLA was examined it was apparent that PLA activity reached its peak prior to LPLA only in *L. pneumophila*, whereas non-*pneumophila* species secreted maximal LPLA activity simultaneously with or prior to PLA activity (Fig. 1). Since PLA generates LPC and LPLA degrades LPC, the ratio and the timing of secretion of both activities may be important for the generation and enrichment of LPC, which has several functions that may contribute to pathogenesis.

Generation of LPC

To estimate the amount of LPC generated from CSs of different *Legionella* species, we incubated supernatants with DPPC and analysed the lipids by TLC. CSs from the exponential growth phase of both *L. pneumophila* and *L. steigerwaltii* generated considerable amounts of LPC (Fig. 2). LPC was also produced by *L. gormanii* but not before the bacteria entered stationary phase. Probably due to their low PLA activity, *L. dumoffii* and *L. micdadei* did not enrich for LPC. The accumulation of LPC by *L. steigerwaltii* and *L. gormanii* can be explained by their very high PLA activity and more moderate LPLA activity. LPLA activity is high in comparison to

Fig. 2. TLC of lipid extracts from DPPC incubations with CS from different *Legionella* species. St, standard phospholipid; 0, 8, 10, 14, 18, 20, 22 and 36, incubation time (h) of bacteria in BYE broth. A representative experiment is shown.

PLA activity in *L. pneumophila* (see Fig. 1b). However, *L. pneumophila* might be able to generate LPC because secretion of maximal PLA precedes secretion of maximal LPLA.

Protein secretion pattern of different *Legionella* species

To learn more about proteins present in the culture supernatants at different times of growth, we compared SDS-PAGE protein patterns of different *Legionella* species. As with the enzyme activities, the protein patterns of distinct *Legionella* species showed considerable variation (Fig. 3). However, the patterns of proteins secreted by the different isolates of *L. pneumophila* resembled each other, with major protein bands of 34, 38, 41, 67 and 80 kDa. The 41 kDa protein band observed in *L. pneumophila* has been shown to be the zinc metalloproteinase by amino acid sequencing (Flieger *et al.*, 2001). Species like *L. micdadei* and *L. longbeachae*, which had the lowest enzyme activities, secreted large amounts of protein and *L. steigerwaltii*, which had high enzyme activities, secreted small amounts of protein into culture supernatants (Fig. 2).

DISCUSSION

Secreted enzyme activities seem to be important for many bacteria in the process of host-cell occupation and exploitation. For example, the secreted elastase of *Pseudomonas aeruginosa* cleaves IgG into fragments that inhibit bacterial uptake (Bainbridge & Fick, 1989) and its haemolytic phospholipase C suppresses respiratory burst activity in neutrophils (Terada *et al.*, 1999). A plasmid-encoded tyrosine phosphatase secreted by *Yersinia pseudotuberculosis* dephosphorylates activated host-cell tyrosine kinases (Bliska *et al.*, 1992) and the phosphatidylinositol-specific phospholipase C from *Listeria monocytogenes* mediates the escape of bacteria from the phagosome (Camilli *et al.*, 1993).

L. pneumophila becomes infectious, cytotoxic, sodium sensitive, motile and capable of evading macrophage lysosomes in response to starvation (Byrne & Swanson, 1998). *In vitro* this occurs when bacteria exit the exponential growth phase (Byrne & Swanson, 1998). We found the highest levels of secreted protease, acid phosphatase, PLA and LPLA activities in several *Legionella* species as they entered stationary phase, suggesting a possible role for these activities in the switch to a virulent phenotype. In accordance with previous studies, we failed to detect extracellular protease activity in *L. longbeachae* and *L. micdadei* (McIntyre *et al.*, 1991; Berdal, 1983). As six discrete protease activities have been recovered from CSs of *L. pneumophila* by ion-exchange chromatography in previous experiments, legionellae might secrete other proteolytic enzymes besides the zinc metalloproteinase (Conlan *et al.*, 1986).

PLA activity was detected in culture supernatants from both *L. pneumophila* and a variety of non-*pneumophila* species, but not in those from *L. longbeachae* or *L. micdadei*. This has already been noted for two different isolates of *L. micdadei* (Flieger *et al.*, 2000a). However, in previous experiments, PLA activity was found in CSs of another isolate of *L. longbeachae* which, in contrast

Fig. 3. SDS-PAGE of concentrated CS of different *Legionella* species. St, molecular mass standard; 0, 8, 10, 12, 14, 16, 18, 20, 22 and 36, incubation time (h) of bacteria in BYE broth. The 34, 38, 41, 67 and 80 kDa proteins of Lp1-CDC are indicated by arrows. A representative experiment is shown.

to the isolate tested in the present studies, had been passaged less than three times on BCYE α agar (Flieger *et al.*, 2000a). Attenuation of *Legionella* species by multiple passages on artificial media (Nagl *et al.*, 2000) may be related to the reduction of PLA secretion, although differences in PLA secretion by different isolates of *L. steigerwaltii* have been described and might also occur in other species (Flieger *et al.*, 2000a).

Previously, it was not known whether non-*pneumophila* species export acid phosphatase or LPLA activity. Like the other activities examined, their secretion was not restricted to *L. pneumophila*. Acid phosphatase and LPLA activity were found in all *Legionella* species tested, except in *L. longbeachae* and *L. micdadei*. The absence of the type II secreted activities in *L. micdadei* suggests that this species does not possess the genes for the enzymes, is not able to express these genes or lacks the secretion apparatus. Since PLA activity has been found in another isolate of *L. longbeachae* (Flieger *et al.*, 2000a), the *L. longbeachae* strain investigated in this study might not express the genes for the secretion system or the secretion system may be inactivated by the artificial growth conditions.

Aside from *L. pneumophila*, only certain non-*pneumophila* species (*L. steigerwaltii* and *L. gormanii*) exhibiting PLA activity were able to accumulate LPC when their CSs were incubated with DPPC. Enrichment of LPC occurred when PLA activity was high in comparison to LPLA activity and/or when LPLA was secreted in a delayed manner. LPC is known to contribute to induction of inflammation, impairment of lung function, cytotoxicity, pore formation and signal transduction (Aronson & Johns, 1977; Dennis, 1997; Flieger *et al.*, 2000b; Holm *et al.*, 1991; Kume *et al.*, 1992; Lindahl *et al.*, 1986; Niewoehner *et al.*, 1987; Prokazova *et al.*, 1998; Weltzien, 1979). Accumulation of LPC could therefore trigger important mechanisms in the development of Legionnaires' disease.

In the search for reasons for the more frequent association of *L. pneumophila* with Legionnaires' disease compared to non-*pneumophila* species, another observation has to be considered. In this and another recent study it has been shown that PLA secretion commences during the mid-exponential growth phase and peaks upon entry into stationary phase (Flieger *et al.*, 2000a). Since *L. pneumophila* is the only species examined that is known to replicate efficiently in macrophages, the non-*pneumophila* species investigated might not reach mid-log growth phase in their host (Neumeister *et al.*, 1997). Therefore it is conceivable that *L. steigerwaltii* and *L. gormanii* may not secrete PLA and accordingly may not produce dangerous amounts of LPC *in vivo*. Thus, future experiments should include the detection of enzyme secretion within the specialized *Legionella* phagosome.

In conclusion, we have shown that the secreted protease, acid phosphatase, PLA and LPLA activities were neither unique nor extraordinarily high in *L. pneumophila*. However, a different pattern of secretion of PLA and

LPLA was found that was unique to *L. pneumophila*, and this is likely to be important in the timing of LPC enrichment.

ACKNOWLEDGEMENTS

We acknowledge Markus Koerber and Marianne Robey for helpful suggestions. We thank Barry Fields, Nicholas P. Cianciotto and Gotthardt Ruckdeschel for providing *Legionella* strains.

Antje Flieger was supported by grants of Boehringer-Ingelheim-Pharma-KG (Biberach, Germany), Boehringer-Ingelheim-Foundation (Mainz, Germany) and fortune Fund (179-1 and 179-2, University Hospital of Tübingen, Germany). Shimei Gong was supported by fortune Fund (305-1 and 305-2, University Hospital of Tübingen, Germany).

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Received 23 February 2001; revised 31 May 2001; accepted 6 July 2001.