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Intracellular Survival and Expression of Virulence Determinants of Legionella pneumophila

Summary: *Legionella pneumophila*, the causative agent of Legionnaires' disease is able to live and multiply within macrophages as well as within protozoan organisms. *Legionella* strains inhibit phagosome-lysosome fusion and phagosome acidification. By using two different cell culture systems, one derived from human macrophages and the other from human embryo lung fibroblastic cells, it is demonstrated that *Legionella* strains lose their virulence following cultivation in the laboratory. In order to study the mechanisms involved in intracellular survival of *Legionella* a genomic library of strain *Legionella pneumophila* Philadelphia I was established in *Escherichia coli* K-12. By cosmid cloning technique we were able to clone five putative virulence factors, two of which exhibit hemolytic activities and three of which represent membrane-associated proteins of 19, 26 and 60 kilodalton. One of the hemolytic proteins, termed legiolysin, represents a new toxin which specifically lyses human erythrocytes. The other hemolysin exhibits proteolytic properties in addition and is cytolytic for Vero and CHO cells. Further studies will be necessary to determine the exact role of the cloned proteins in the pathogenesis of *Legionella*.

Zusammenfassung: *Intrazelluläres Überleben und Expression der Virulenzdeterminanten von Legionella pneumophila. L. pneumophila*, der Erreger der Legionärskrankheit, hat die Fähigkeit, sowohl in Makrophagen als auch in Protozoen zu leben und sich dort zu vermehren. Legionellen inhibieren die Fusion von Phagosom und Lysosom und hemmen die Ansäuerung des Phagosoms. Mit Hilfe von zwei unterschiedlichen Zellkultur-Systemen konnte gezeigt werden, daß *Legionella*-Stämme ihre Virulenz nach Laborpassage verlieren. Um die Mechanismen zu studieren, die für das intrazelluläre Überleben von Legionellen verantwortlich sind, haben wir eine Genbank des *Legionella pneumophila*-Stammes Philadelphia I in *Escherichia coli* K-12 angelegt. Mit Hilfe der Cosmid-Klonierungstechnik war es möglich, fünf putative Virulenzfaktoren zu klonieren. Zwei von diesen Faktoren haben hämolytische Eigenschaften und drei sind Membran-assoziierte Proteine mit Molekulargewichten von 19, 26 und 60 kilodalton. Eines der hämolytischen Proteine, das Legiolysin, lysiert spezifisch humane Erythrozyten. Das zweite Hämolysin zeigt zusätzlich proteolytische Eigenschaften und schädigt sowohl Vero- als auch CHO-Zellen. Weitere Studien sind notwendig, um die Rolle der klonierten Proteine in der Pathogenese von *Legionella* exakt zu bestimmen.

Introduction

Legionella pneumophila is a gram-negative, aerobic inhabitant of freshwater and soil environments. It is also a facultative intracellular pathogen that invades and grows in human alveolar macrophages leading to acute bronchopneumonia referred to as legionellosis [1,2]. In addition, *L. pneumophila* isolates are able to live and multiply within protozoan organisms such as environmental amoebae [3]. As analyzed by *Horwitz* and co-workers, live *Legionella* strains enter eukaryotic cells by a common mechanism, termed coiled phagocytosis; furthermore they induce the formation of a novel ribosome-lined phagosome, inhibit phagosome-lysosome fusion and inhibit phagosome acidification [4,5]. The bacterial factors responsible for intracellular survival of legionellae are still unknown. Genetic and cell biological techniques, however, may offer new possibilities to determine the *Legionella* specific virulence factors.

Intracellular Growth of Legionella

In order to study the factors and conditions which influence the ability of *Legionella* strains to survive in eukaryotic cells, different cell culture systems were established [6–8]. The macrophage-like cell line U937 which was derived from human histiocytic cells and HL60, which originate in human peripheral blood cells, are widely used for such studies. In addition, *Legionella* strains are able to survive and multiply in human embryo lung fibroblastic cells termed MRC5 and MRC9.

In experiments shown in Figure 1 cells of *L. pneumophila* strain Philadelphia I were grown together with U937 cells. Extracellular bacteria were killed by the presence of 40 mg/l in the culture medium gentamicin. Therefore colonies which appear on plates following lysis of the eukaryotic cells must result from the intracellular bacterial pool of *Legionella*. As indicated in Figure 1, *L. pneumophila* strain Philadelphia I is able to multiply within U937 cells measured over a period of two days. Many colony forming units (cfu) were newly generated in the host cells using bacteria which are subcultivated on agar plates several times. Following passage of the *Legionella* strain in MRC9 cells the number of bacteria reisolated from U937 cell cultures increased by a factor of more than ten. These data clearly show that *Legionella* strains lose their infectivity following cultivation on synthetic medium and are able to regain their virulence following passage in MRC9 cells.

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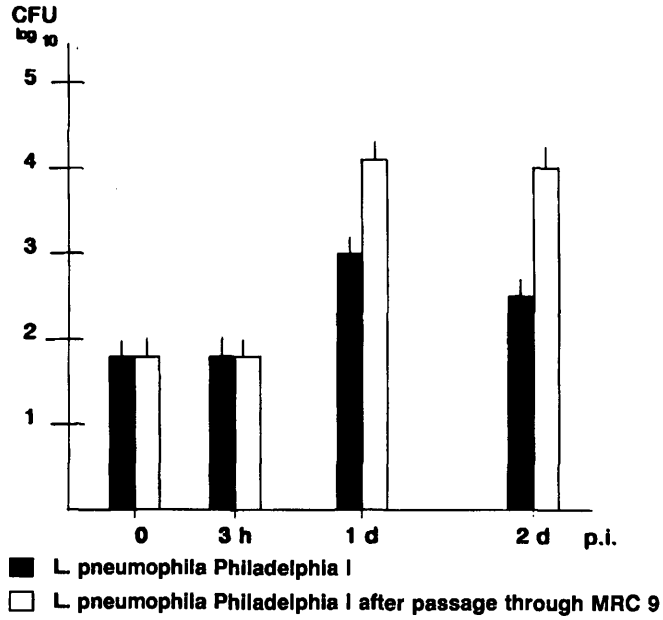

Figure 1: Intracellular growth kinetics of *Legionella pneumophila* Philadelphia I in U 937 cells before and after passage through MRC9 cells. In a 24-well microtiter plate 10^5 U937 cells, pretreated with TPA (12-o-tetra decanoylphorbol-13-acetate, 10^{-8} M) were incubated together with 10^6 *L. pneumophila* bacteria. After allowing entry for 2 h, extracellular bacteria were killed by adding 40 mg/l gentamicin [6]. Survival and replication of *L. pneumophila* was measured by determining colony forming units (cfu) after 0/3 h, 1 d, and 2 d. Passaging through MRC9 cells was performed as described by *Wong* et al. [8].

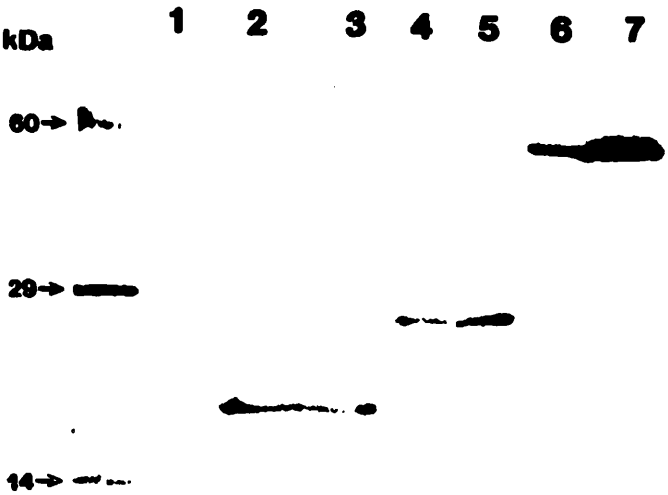

Figure 2: Western blot analysis of whole cell extracts of recombinant *Escherichia coli* K-12 clones, derived from a genomic library of *Legionella pneumophila* Philadelphia I using antiserum prepared against whole *L. pneumophila*, Philadelphia I bacteria. Strains are as follows: HB101 (pLAFR2) (Lane 1); HB101 (pBLL2511) (Lane 2); HB101 (pBLL2819) (Lane 3); HB101 (pBLL3039) (Lane 4); HB101 (pBLL2544) (Lane 5); HB101 (pBLL1920) (Lane 6); HB101 (pBLL1941) (Lane 7). Molecular weights of standard proteins are indicated.

In order to compare bacteria with high virulence to those with low potential of intracellular growth, the proteins of either variant were analysed in two dimensional SDS PAGE gels. Various proteins appear, which are synthesized by the virulent strain but are absent on the gel prepared from the non-virulent variant. In particular one abundant gene product of about 120 kilodalton seems to represent a virulence-specific *Legionella* protein (*Chirinos, Rdest* and *Hacker*, unpublished results). Gene products like the 120 kd protein may belong to a set of factors necessary for intracellular survival of *L. pneumophila* which has to be analysed in future studies.

Expression of Outer Membrane Proteins

To determine the interaction of *Legionella* and eukaryotic cells we and others have studied the envelope of this bacterium [9-11]. The major outer membrane protein (MOMP) of *L. pneumophila*, a cationic selective porin of 29 kilodalton (kd) represents the main protein component of the outer membrane. As the C3 acceptor molecule, it plays a role in the uptake of *Legionella* by phagocytic cells [12].

In order to clone the major outer membrane protein and other membrane-associated proteins of putative medical importance we have constructed an *Escherichia coli* K-12 genomic library. Following *Sau*3A digestion of genomic *Legionella* DNA, subsequent ligation of 20 kilobases (kb) DNA fragments into the *Bam*HI site of the vector pLAFR2 and *in vitro* phage packaging, about 2850 *E. coli* K-12 clones carrying *Legionella*- specific insert DNAs were selected. Using *Legionella*-specific antisera 76 (2.6%) *E. coli* K-12 clones were identified which expressed *Legionella* antigens. As indicated in Figure 2 which shows a Western blot experiment, the antigens were characterized as proteins of 19 kd, 26 kd and 60 kd.

In cooperation with *R. Benz* (Würzburg) we were able to show that the 19 kd protein is located in the outer membrane of both *Legionella* and the recombinant *E. coli* clones [13]. The 60 kd protein which shares some features with heat shock proteins of the *gro*EL family [14] seems to be associated with the cytoplasmic membrane of *Legionella*.

The 26 kd protein also represents a membrane-associated protein. In order to show whether this particular protein is identical with the MOMP described above, or with the *mip* gene product described by *Engleberg* and coworkers [15] a 26-mer oligonucleotide probe with *mip* specific sequences was synthesized. With the help of DNA-DNA hybridization studies we were able to demonstrate that all the clones which express *Legionella*-specific proteins of 26 kd carry *mip*-specific sequences. The *mip* protein (macrophage infectivity potentiator) is involved in the initiation of macrophage infection rather than in the ability of *Legionella* to replicate intracellularly [16]. It is interesting to note that all the 76 *E. coli* K-12 clones which produce *Legionella*-specific proteins express one of the three proteins

Table 1: *Escherichia coli* K-12 clones expressing *Legionella*-specific membrane-associated proteins.

Cosmid	*Legionella*-specific antigen	Localization
pLAFR2 (Tc^r)	–	
pBLL2511	Protein 19kd[1)]	Outer membrane
pBLL2819	Protein 19kd[1)]	Outer membrane
pBLL3039	Protein 26kd[2)]	Outer membrane-associated
pBLL2544	Protein 26kd[2)]	Outer membrane-associated
pBLL1941	Protein 60kd[2)]	Inner membrane-associated
pBLL1920	Protein 60kd[2)]	Inner membrane-associated

[1)] The proteins can be detected in cell lysates.
[2)] The proteins can be detected in the supernatant and in cell lysates.

mentioned, but none was able to produce the MOMP protein of 29 kd. These data are in agreement with those of other groups [13–17] who also failed in their attempts to clone and express the *Legionella* MOMP protein in *E. coli* K-12. The characteristics of the *Legionella* proteins expressed by *E. coli* K-12 strains are summarized in Table 1.

Expression of Toxins

The cytopathology of lungs infected by *L. pneumophila* suggests involvement of extracellular proteolytic enzymes or toxins in the pathogenesis of *Legionella* [2,18]. In order to select cloned toxin determinants of *Legionella* the recombinant *E. coli* K-12 clones were screened on blood agar plates containing human and canine erythrocytes. In addition, the clones were cultivated on LB plates with 1% skim milk to screen for proteolytic properties. After two days eleven clones showed a zone of hemolysis around the colonies after growing on blood agar plates with human erythrocytes, respectively. In addition four *E. coli* clones were selected, which exhibit proteolytic properties shown by lysis zones on skim milk plates and a hemolytic phenotype on canine blood plates only (see Table 2). These data together with Southern hybridization studies strongly argue for the presence of two different hemolysins produced by *Legionella*; one of these also mediates a proteolytic phenotype.

The protease described above is identical with the *msp* (major secretory protein) gene product, a zinc metallo-protease which has been characterized as a protein of 38 kd. This protein is not required for intracellular growth [19]. The second hemolysin, termed legiolysin (*lly*) which has not been described until now, was subcloned into the vector molecule pUC19 on a 1.1 kb *BalI*/*SmaI* DNA fragment. Two proteins, one of these has a molecular weight of 30 kd, seem to be necessary for the production and transport of legiolysin [20,21]. In order to correlate the production of the two hemolysins with cytotoxic properties, the recombinant *E. coli* clones which produced hemolysin were tested in quantitative assays with Vero cells and Chinese hamster ovary (CHO) cells. As shown in Table 2, the *msp* gene product is cytotoxic for both cell types while the Lly protein does not influence growth and morphology of Vero and CHO cells.

Conclusion

We have described the cloning and characterization of five *Legionella*-specific gene products in *E. coli* K-12, two of which exhibit hemolytic activities and three are associated with the inner or outer membranes of *Legionella*. The exact role of these gene products in intracellular growth of *Legionella* remains to be established. In particular, it will be interesting to determine their contribution to the process of blocking of the phagosome-lysosome fusion and to inhibition of acidification of the cell vacuole.

It was shown recently that other intracellular pathogens exhibit similar functions as *Legionella* during infection of eukaryotic cells [22]. Thus the phagosomes infected with *Toxoplasma gondii* and *Chlamydia psittaci* share some of the morphological features of the *Legionella* phagosome [23]. All three organisms are able to inhibit phagosome-lysosome fusion. In addition, *T. gondii* has been reported to share the capacity of inhibiting phagosome acidification with *L. pneumophila*. Complement receptors are also involved in phagocytosis of *Mycobacterium tuberculosis*, an

Table 2: Properties of *Escherichia coli* K-12 clones expressing *Legionella*-specific hemolysins.

Cosmid	Hemolysis[1)] Human erythrocytes	Hemolysis[1)] Canine erythrocytes	Damage[2)] Vero cells	Damage[2)] CHO cells	Proteolysis[3)]
pLAFR2	–	–	–	–	–
pBLL426	+	+	–	–	–
pBLL3148	+	+	–	–	–
pBLL3209	+	+	–	–	–
pBLL4132	–	+	+	+	+
pBLL2745	–	+	+	+	+

[1)] Hemolysis was detected 48 h after cultivation.
[2)] Damage was detected 24 h after incubation.
[3)] Proteolysis was detected 24 h after cultivation.

organism which is also able to inhibit phagosome-lysosome fusion. Future genetic analysis of the putative virulence factors of these microorganisms will help to answer the question, whether all these intracellular pathogens do not only share similar functions but also express gene products with similar or identical characteristics.

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