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Characterization of legiolysin (*lly*), responsible for haemolytic activity, colour production and fluorescence of *Legionella pneumophila*

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Summary

A genomic library of *Legionella pneumophila*, the causative agent of Legionnaires' disease in humans was constructed in *Escherichia coli* K12 and the recombinant clones were tested for haemolysis and other phenotypic properties. Seven clones were identified which were able to confer haemolysis of human, sheep, and canine erythrocytes but which were unable to mediate proteolytic activities or cytotoxic effects on CHO- or Vero cells. Clones that exhibited this haemolytic property were also able to produce a brown colour and a yellow-green fluorescence activity detected on M9 plates containing tyrosine. The genetic determinant encoding these properties, termed legiolysin (*lly*) was mapped by Tn*1000* mutagenesis and by subcloning experiments. Southern hybridization with an *lly*-specific gene probe showed that this determinant is part of the genome of *L. pneumophila* but is not identical to a protease gene of *L. pneumophila* which also mediates haemolysis. Minicell analysis of *lly*-specific plasmids exhibited a protein of 39 kDa. Polyclonal antibodies generated against a LacZ–Lly hybrid protein also recognized a 39 kDa protein produced either by the recombinant legiolysin-positive *E. coli* K12 clones or by *L. pneumophila* wild-type strains.

Introduction

Legionella pneumophila is a Gram-negative, rod-shaped bacterium and is the causative agent of Legionnaires' disease (McDade *et al.*, 1977; Winn, 1988; Horwitz, 1988). The organism is a facultative intracellular parasite and is able to survive and multiply in human monocytes and alveolar macrophages (Horwitz, 1983; 1984). *L. pneumophila* inhabits aquatic biotops and hot water systems, where the bacteria are able to live and multiply within protozoan organisms such as environmental amoebae (Winn, 1988).

The cytopathology of destroyed lung tissue infected by specimens of *L. pneumophila* suggests an involvement of cytotoxins in the pathogenic processes of Legionnaires' disease (Williams *et al.*, 1987; Winn, 1988). Several cytotoxic or haemolytic factors produced by *L. pneumophila* have been described in the last decade (Baine *et al.*, 1979; Friedman *et al.*, 1980; Thorpe and Miller, 1981; Berdal *et al.*, 1982; Baine, 1984) but so far only a protease that exhibits haemolytic activity has been characterized accurately and studied at the molecular level (Dreyfus and Iglewski, 1986; Quinn and Tompkins, 1989; Keen and Hoffman, 1989; Black *et al.*, 1990). It was also shown that *Legionella* strains exhibit other properties such as production of catalase, superoxide dismutase and peroxidase, extracellular and intracellular fluorescence activities or production of a brown melanin-like pigment (Baine and Rasheed, 1979; Vickers and Yu, 1984; Pine *et al.*, 1984; Berg *et al.*, 1985). The role of these properties in the pathogenesis of *Legionella* or in its ability to survive in aquatic biotops remains to be established.

Here we describe the cloning of a DNA fragment from the chromosome of *L. pneumophila* which is able to confer haemolysis and which is responsible for the production of a brown pigment-like colour and fluorescence activity but does not exhibit any proteolytic or cytolytic activities. Furthermore, our studies show that the corresponding gene product, a 39 kDa protein termed legiolysin (Lly), is produced by *L. pneumophila* wild-type strains and is not identical to the haemolytic protease described recently.

Results

Detection of haemolysis of L. pneumophila *strains and recombinant* Escherichia coli *K12 clones*

As recently shown (Keen and Hoffmann, 1989; Bornstein *et al.*, 1988), *L. pneumophila* isolates confer haemolysis on blood agar plates. Strains Philadelphia I and 685 produce lysis zones on plates containing canine, human, or sheep erythrocytes, respectively (see Fig. 1). In order to clone the determinants responsible for the haemolytic phenotypes

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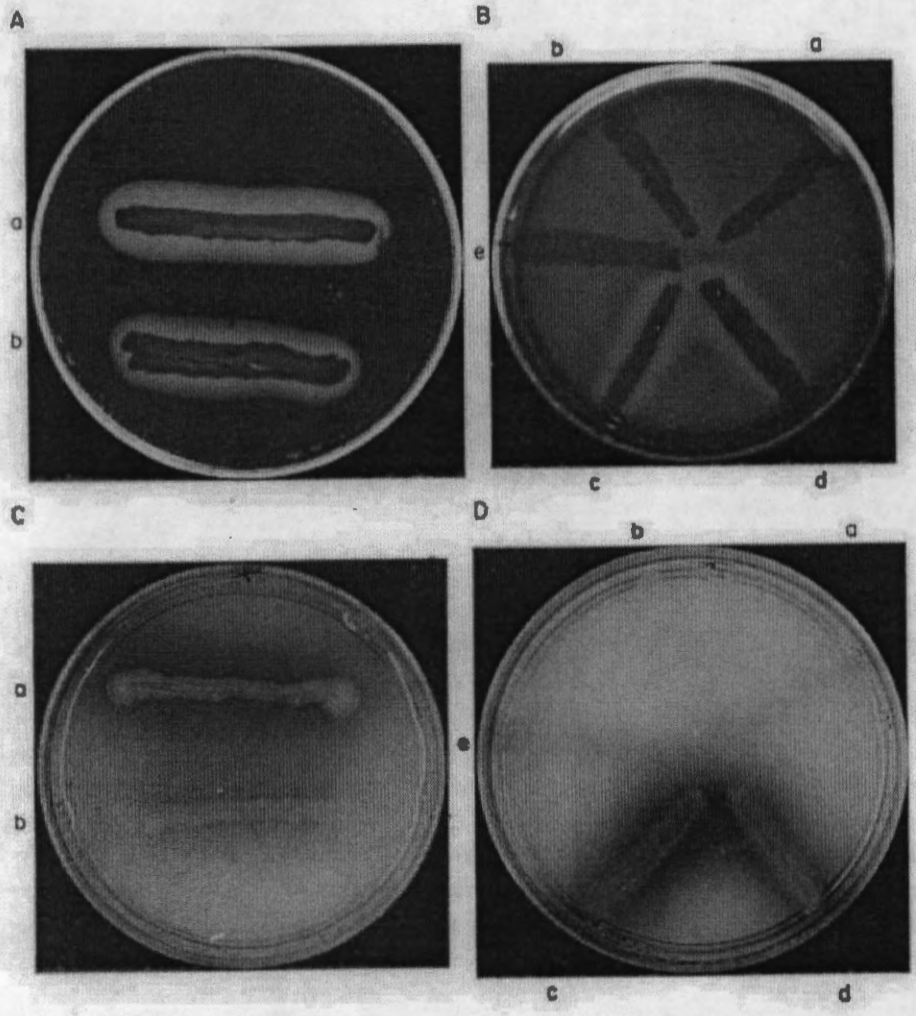

Fig. 1. Haemolysis (plates A and B) and colour production (plates C and D) of *L. pneumophila* strains and recombinant *E. coli* clones carrying *L. pneumophila* insert DNA fragments. The clones were tested on plates with human erythrocytes and on agar plates containing 4 mM tyrosine. The following strains are indicated: A, C (a), *L. pneumophila* Phil I; A, C (b), *L. pneumophila* 685; B, D (a), HB101(pBLL4133); B, D (b), HB101(pBLL2745); B, D (c), HB101(pBLL426); B, D (d), HB101(pBLL3148); B, D (e), HB101(pLAFR2) (control).

of *Legionella* we constructed a genomic library. For this, chromosomal DNA was isolated from *L. pneumophila* strain Philadelphia I. *Sau*3A DNA fragments of nearly 20 kb were ligated into the vector molecule pLAFR2 (Knapp and Mekalanos, 1988) and, following packaging and transduction, 2885 recombinant *E. coli* K12 clones were isolated. The recombinant *E. coli* clones were first screened on canine blood agar plates. After two days, 15 clones displayed a zone of haemolysis around the colonies. Seven of these clones were also able to lyse human and sheep erythrocytes, while the remaining eight clones conferred the haemolytic phenotype only on plates containing canine erythrocytes (see Fig. 1 and Table 1).

Properties of haemolytic E. coli *K12 clones*

Legionella strains are able to produce a protease and also

Table 1. Phenotypic properties of *L. pneumophila* strains and *E. coli* K12 clones carrying *Legionella*-specific DNA fragments.

Strain	Haemolysis of Erythrocytes			Colour production[a]	Fluorescence[b]	Proteolysis	Cell Cytotoxicity	
	Human	Canine	Sheep				CHO	Vero
L. pneumophila Phil. I	+	++	++	++	+	++	++	++
L. pneumophila 685	+	++	++	++	+	++	++	++
HB101(pLAFR2)	–	–	–	–	–	–	–	–
HB101(pBLL426)	++	++	++	++	++	–	–	–
HB101(pBLL3148)	++	++	++	++	++	–	–	–
HB101(pBLL3209)	++	++	++	++	++	–	–	–
HB101(pBLL4133)	–	++	–	–	–	+	++	++
HB101(pBLL2745)	–	++	–	–	–	+	++	++
HB101(pBLL3725)	–	++	–	–	–	+	++	++

a. Tested on plates containing 4 mM tyrosine.
b. Tested in the dark with a long-wave u.v. lamp.

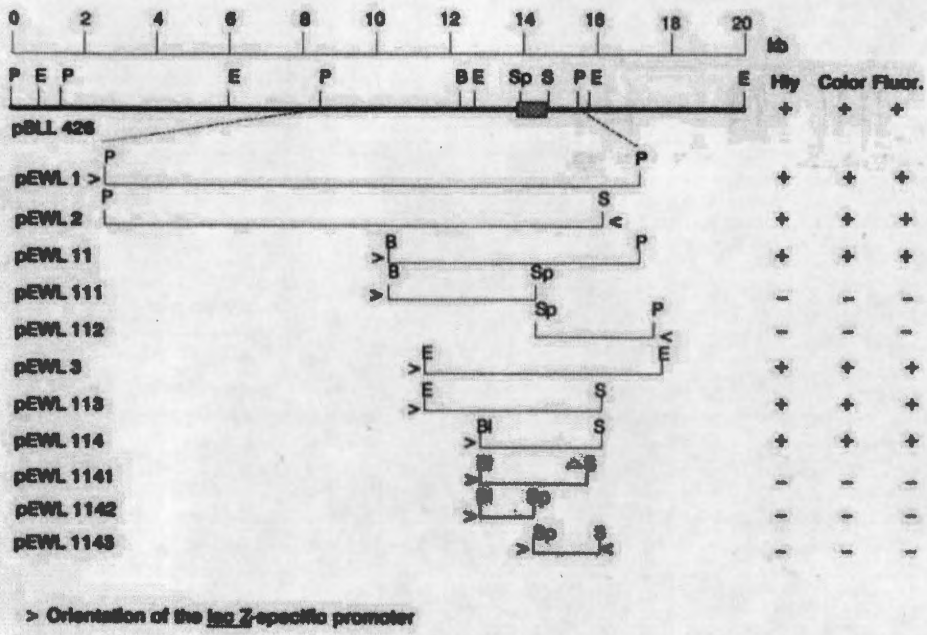

Fig. 2. Physical map of the *L. pneumophila*-specific insert of cosmid pBLL426, conferring haemolysis, colour production and fluorescence. Subclones and deletion mutants which were derived from plasmid pEWL1 are given. Haemolysin production (Hly), colour production and fluorescence are indicated by + signs. The hatched box represents the location of the *lly*-specific determinant. *Sph*I sites were only mapped for the DNA fragment at map position 13–15 kb. Abbreviations: B, *Bam*HI; BI, *Bal*I; E, *Eco*RI; P, *Pst*I; S, *Sma*I; Sp, *Sph*I.

cause browning of the medium and exhibit fluorescence activity (Baine and Rasheed, 1979; Vickers and Yu, 1984; Dreyfus and Iglewski, 1986). In addition, cytotoxic effects were seen following incubation of various cell lines with extracts or supernatants of *Legionella* (Quinn *et al.*, 1989; see also Table 1). In order to detect one of these properties in haemolytic *E. coli* K12 clones, they were initially cultivated on L-agar plates containing 1.5% skim milk. The eight clones which only exhibited a canine-specific haemolytic activity showed proteolytic properties as well. Cell extracts of these clones, but not of the haemolytic clones with specificities to human and sheep erythrocytes, were also cytotoxic for Vero cells and CHO cells (see Table 1). In contrast, the other seven clones were negative for proteolytic and cytotoxic properties but were able to confer production of a melanin-like brown pigment after cultivation on M9-agar plates with tyrosine (see Fig. 1). In addition, these clones showed yellow-green fluorescence activity under long-wave u.v. light (see Table 1). We suggest, therefore, that two different types of haemolytic clones were isolated: one has proteolytic and cytotoxic activities, and the other mediates browning of the medium and also has fluorescence activity.

Subcloning of the gene coding for the haemolytic, colour-producing, fluorescence-positive phenotype

DNA was isolated from the haemolysin-positive, colour-producing, fluorescence-positive cosmid pBLL426, and a restriction map was established (Fig. 2). Different DNA fragments were ligated into the vector pUC18 (Norrander *et al.*, 1983). The subclones were analysed for the three phenotypes. A 7.0 kb *Pst*I fragment located in pEWL1 conferred these properties. The determinant responsible for the three phenotypes seems to be transcribed from its own promoter, since the properties could be detected after subcloning of the fragment in both orientations (data not shown). In order to localize the gene on the 3.4 kb *Bam*HI/*Pst*I insert in plasmid pEWL11 a set of deletion mutants was constructed (Fig. 2). The data demonstrate that the gene must be located between co-ordinates 13 and 15 in Fig. 2, next to the *Sph*I site at position 14. A *Bal*I–*Sma*I fragment of 1.6 kb on plasmid pEWL114 was able to mediate the three phenotypes as the smallest insert. Plasmid pEWL1141, which resulted from exonuclease III digestion of pEWL114, conferred negative phenotypes.

Transposon mapping of the gene coding for the three phenotypes

A complementary approach to mapping of the determinant was attempted by using insertional inactivation of the haemolysin-, colour- and fluorescence-specific gene with transposon Tn*1000* (Guyer, 1978). For mutagenesis, the 3.3 kb *Eco*RI fragment of plasmid pEWL3 (see Fig. 2) was ligated into vector pBR328 (Soberon *et al.*, 1980), resulting in plasmid pEWL4. Insertion mutants in pEWL4 were screened on blood agar plates as well as M9 plates with tyrosine and the locations of insertions were determined by suitable digestion of the recombinant DNAs. A set of representative insertional mutants are given in Fig. 3. All 31 Tn*1000* insertions obtained that resulted in negative phenotypes were located between the *Bal*I site and the *Sma*I site next to the *Sph*I site at map position 14 kb. These data lend further weight to the idea that the gene is located in this particular DNA region. Mutations located outside this region were haemolytic and still produced the

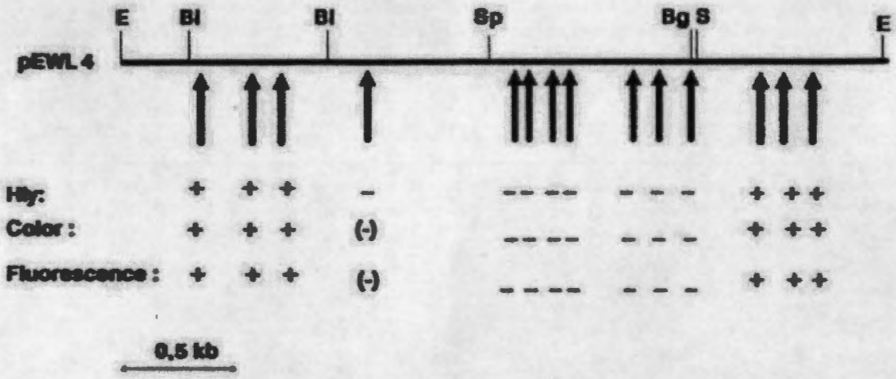

Fig. 3. Tn*1000* insertions of plasmid pEWL4. Insertion points are marked by an arrow; +, haemolysin (Hly), colour production and fluorescence; −, no haemolysin, no colour production and fluorescence; (−), no haemolysin, weak colour production and fluorescence. Abbreviations: E, *Eco*RI; BI, *Bal*I; Sp, *Sph*I; Bg, *Bgl*I; S, *Sma*I.

pigment-like colour and showed fluorescence activity. With the exception of one mutant, which was non-haemolytic but showed very weak browning and fluorescence, all the haemolysin-negative mutants also failed in their ability to mediate colour production and to produce fluorescence activity.

Comparison of legiolysin and protease determinants

DNA was isolated from plasmids pEWL113 (see Fig. 2) and pBLL4133 (Table 1). In addition, chromosomal DNA was isolated from *L. pneumophila* strain Philadelphia I. These DNAs were cleaved with *Eco*RI–*Sma*I, *Bgl*II–*Sal*I, and with *Hin*dIII, respectively, and were hybridized with the radioactively labelled 2.4kb *Eco*RI–*Sma*I fragment of pEWL113. As indicated in Fig. 4 no homology was found between the specific DNA probe and the protease-encoding plasmid DNA, pBLL4133, but a strong signal representing a *Hin*dIII fragment of 10kb was observed after hybridization with the *Legionella*-specific DNA. These data confirm that the gene responsible for haemolysis, colour production and fluorescence is not identical to the protease-specific determinant of *L. pneumophila* described recently (Quinn and Tompkins, 1989) and is originated from the *Legionella* genome. The corresponding determinant was termed legiolysin (*lly*).

Detection of the legiolysin protein in minicells

In order to analyse the protein encoded by the legiolysin-specific gene, minicells were isolated from *E. coli* strain P678-54 carrying plasmids pEWL114, which conferred the three phenotypes, and pEWL1141, which represents a deletion mutant of pEWL114 (see Fig. 2). As indicated in Fig. 5 (lane C), minicells harbouring plasmid pEWL114 expressed a protein of about 39kDa. The vector plasmid pUC18 and the legiolysin-negative deletion mutant clone P678-54 (pEWL1141) (lanes B and D) did not produce this protein. We therefore concluded, following sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS–PAGE) analysis, that the legiolysin gene *lly* codes for a protein with a molecular mass of 39kDa.

Western blot analysis with antiserum directed against a LacZ–Lly fusion protein

Vectors pSEM1–3 (Knapp *et al.*, 1990) were used to construct fusions between the proximal (5′) part of the *lacZ* gene coding for the enzyme β-galactosidase and *lly*-specific sequences. Consequently, a *Sph*I–*Sph*I fragment of plasmid pEWL114, with one *Sph*I site located in the *lly*-specific insert (see Fig. 2) and the other *Sph*I site being part of the polylinker of vector pUC18, was ligated into the polylinker of plasmid pSEM-2 (Fig. 6). A fusion protein was encoded by the resulting clone, W3110-(pAAD114) with a molecular mass of 90kDa following induction with isopropyl-β-D-thiogalactopyranoside (IPTG). Owing to the large amount of fusion protein, inclusion bodies were formed which facilitated the isolation of the protein from SDS–PAGE (data not shown; see

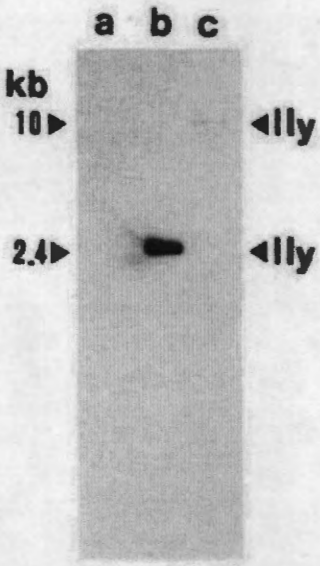

Fig. 4. Southern hybridization of *Bgl*II–*Sal*I-cleaved plasmid DNA of pBLL4133 (protease production, lane a), of *Eco*RI–*Sma*I-cleaved DNA of pEWL113 (legiolysin production, lane b) and of *Hin*dIII-cleaved chromosomal DNA of *L. pneumophila* Phil. I (lane c). The DNAs were hybridized with the 2.4kb *Eco*RI–*Sma*I fragment of pEWL113 which was labelled by the random priming procedure. As size marker (λ)-DNA cleaved with *Hin*dIII was used. DNA sizes are given in kb.

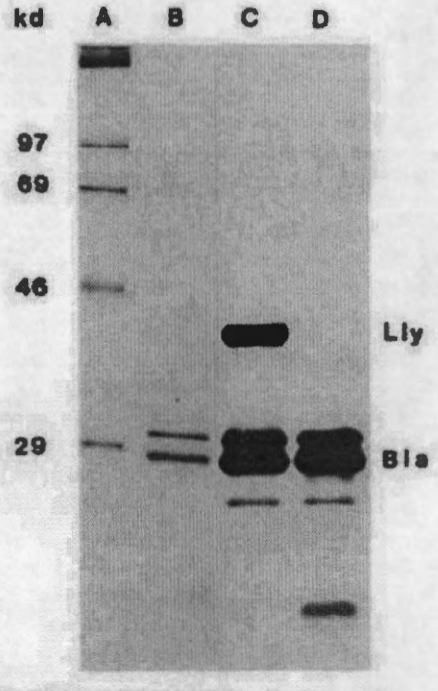

Fig. 5. Sodium dodecyl sulphate/polyacrylamide gel electrophoresis pattern of ^{35}S-labelled proteins from minicells containing plasmids: marker (lane A); pUC18 (lane B), pEWL114 (Lly$^+$, lane C), and pEWL1141 (lane D).

Knapp *et al.*, 1990). These protein preparations were used for immunization of rabbits. Antisera generated against the LacZ–Lly fusion protein were used in Western blotting experiments.

As indicated in Fig. 7, the antiserum was specific for a protein of about 50 kDa which represents the *N*-terminal half of LacZ in strain W3110(pSEM-2) and for the LacZ–Lly fusion protein of 90 kDa produced by W3110(pAAD114) (lanes A and B). In addition, a 39 kDa protein in strain P678-54(pEWL114) was recognized by the antiserum. The molecular weight of this protein is identical to that of the plasmid-encoded protein detected in minicells (see above). No signals were observed in a Western blot with the proteolytic clone HB101(pBLL4133) (lane D). In *L. pneumophila* wild-type strains a protein of the same size can be detected in Western blots using the LacZ–Lly-specific antiserum (Fig. 7, lanes E and F). It is concluded, therefore, that the legiolysin responsible for haemolysis, colour production and fluorescence activity represents a protein of 39 kDa, produced either by recombinant *E. coli* K12 clones or by *L. pneumophila* wild-type strains.

Discussion

In recent years, cytotoxic, haemolytic and proteolytic activities as well as phospholipase C production have been ascribed to *L. pneumophila*, the causative agent of Legionnaires' disease (Baine *et al.*, 1979; Friedman *et al.*, 1980; Thorpe and Miller, 1981; Berdal *et al.*, 1982; Baine, 1984; Bornstein *et al.*, 1988; Belyi *et al.*, 1989). In addition, *L. pneumophila* exhibits several properties such as colour production, fluorescence or the secretion of various enzymes (Pine *et al.*, 1984; Vickers and Yu, 1984; Winns, 1988). Of these factors, only a zinc-metalloprotease which also confers haemolysis was cloned and studied intensively (Dreyfus and Iglewski, 1986; Quinn and Tompkins, 1989; Quinn *et al.*, 1989; Black *et al.*, 1990). It was demonstrated recently that this protease is not required for intracellular growth in cell cultures or for *in vivo* virulence of *L. pneumophila* in a guinea-pig model (Szeto and Shuman, 1990; Blander *et al.*, 1990).

By screening a *L. pneumophila*-specific genomic library in suitable phenotypic tests and cytotoxicity assays it became evident that in addition to the protease, another type of *Legionella*-specific haemolysin exists which was termed legiolysin (Lly). Clones coding for legiolysin mediated lysis of canine, human, and sheep erythrocytes. The new haemolysin also conferred colour production of recombinant *E. coli* strains. This phenomenon, also termed 'browning' or production of 'melanin-like pigmentation' was previously described by several authors (Baine and Rasheed, 1979; Pine *et al.*, 1984; Berg *et al.*, 1985) for *L. pneumophila* and other species of the Legionellaceae following cultivation on plates containing tyrosine. Colour production was used, therefore, as a taxonomic marker in differentiation tests. As a third phenotype, the legiolysin-specific clones produced yellow-green fluorescence activity which was also described for *Legionella* strains several years ago (Vickers and Yu, 1984). It is interesting to note that one DNA sequence, coding for a protein of 39 kDa, is responsible for the three different phenotypic properties.

Different methods have been used to show that the

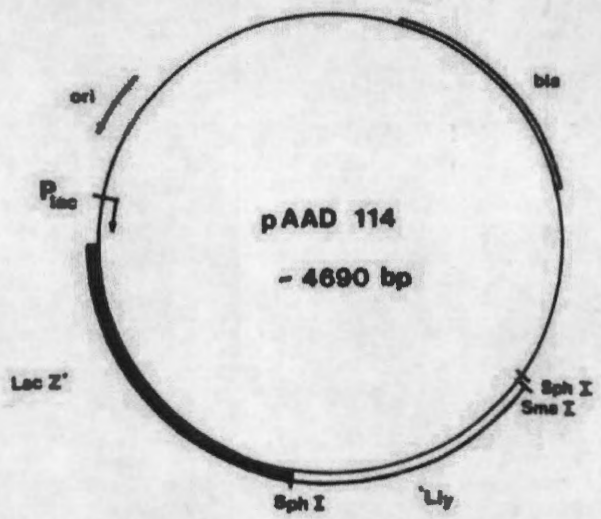

Fig. 6. Genetic map of the plasmid pAAD114 producing LacZ–Lly fusion proteins. Cloning sites are indicated.

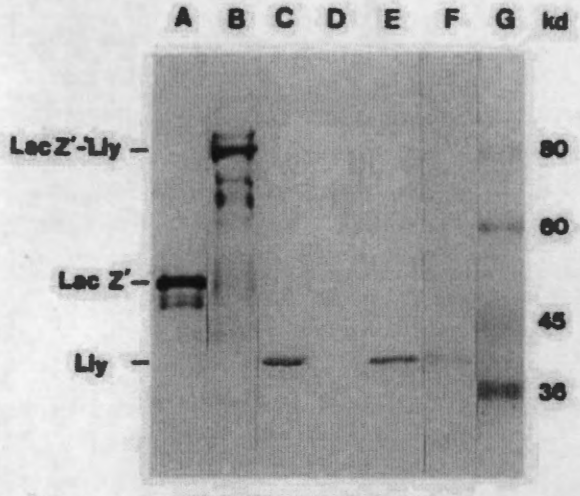

Fig. 7. Western blots of cell extracts from different strains and clones using Lly-specific antiserum. Lane A: W3110(pSEM-2, control); lane B: W3110(pAAD114); lane C: P678-54(pEWL114); lane D: HB101(pBLL4133); lane E: *L. pneumophila* Phil. I; lane F: *L. pneumophila* 685; lane G: marker.

legiolysin is not identical to the haemolytic protease. The restriction map of the corresponding DNA provides a strong argument for non-identity of the *lly* gene to the already known protease determinant, termed *pro* or *msp* (see Fig. 2). In addition, the phenotypic tests, Southern hybridization data (Fig. 4), and Western blots using monospecific polyclonal antibodies directed against the cloned LacZ–Lly hybrid protein confirm that the legiolysin gene is different from the zinc-metalloprotease determinant. However, data concerning the isolation of a mutant carrying an insertion in the chromosome of a *L. pneumophila* strain which results in a protease-negative, non-haemolytic phenotype (suggesting that the metalloprotease represents the only haemolytic substance produced by *L. pneumophila* (Keen and Hoffmann, 1989)), contrast with the results presented here. One reason for the discrepancy may be that legiolysin is not detectable with the blood-agar plates used by the authors. In addition, one cannot exclude the possibility that the mutation which abolished protease-induced haemolysis also affected the production of legiolysin in *L. pneumophila*. It can be speculated that a regulatory element located next to the protease locus may influence expression of the *lly* gene. Strong linkage of virulence-associated genes and regulatory loci has been described recently for various species like *Bordetella pertussis*, *Staphylococcus aureus*, *Escherichia coli* and others (Reiman *et al.*, 1989; Roy *et al.*, 1989; Janzon and Arvidson, 1990; Caron *et al.*, 1989). Yet another possibility is that production of the protease is a prerequisite for expression of legiolysin in *L. pneumophila*.

Clinical observations and histological studies suggest an involvement of toxins in the pathogenesis of Legionnaires' disease (Friedman *et al.*, 1980; Winn, 1988). Toxic substances like legiolysin may contribute to tissue damage or may attack polymorphonuclear leucocytes. Furthermore, toxins seem to be necessary for production of a necrotic reaction in the lung tissue of infected patients. Marker exchange experiments with the cloned legiolysin-specific gene will be useful for determining a putative role for legiolysin in Legionnaires' disease.

Experimental procedures

Media, enzymes and chemicals

Legionella strains were cultivated on CYE agar plates at 37°C in a 5% CO_2 atmosphere for 48 h prior to use. *E. coli* K12 strains were grown in L-broth. Colour production (browning) and fluorescence of *L. pneumophila* was tested on BYE agar plates containing 4 mM tyrosine. *E. coli* K12 clones were assayed on M9 plates containing 4 mM tyrosine (Vickers and Yu, 1984). Blood plates and skim milk plates for testing of haemolysis and proteolysis, respectively, were prepared as described (Knapp *et al.*, 1984; Quinn and Tompkins, 1989). Radiochemicals were purchased from NEN Research Products. Restriction enzymes, exonuclease III, S1 Nuclease, Klenow Fragment and T4 ligase were obtained from Pharmacia, Biolabs, and Gibco BRL. All other chemicals were obtained from Merck, Difco, BRL, Oxoid, Roth, and Serva. Antibiotics were a gift from Serva or Sigma.

Bacterial strains and plasmids

The genomic library was constructed from *L. pneumophila* strain Philadelphia I (McDade *et al.*, 1977). *L. pneumophila* strain 685 represents an environmental isolate which was used for phenotypic characterization. Tn*1000* (γδ) mutagenesis was performed with strain SBC 53 (F::Tn*1000*, *thyA*, *deoC*, *rpsL*) (Guyer, 1978). Minicell experiments were done with *E. coli* P678-54 (Noegel *et al.*, 1979). For cloning experiments the *E. coli* K12 strains HB101, JM109, and W3110 were used. Plasmid pLAFR2 is a cosmid vector described previously (Friedman *et al.*, 1982; Knapp and Mekalanos, 1988). For subcloning, vectors pBR322, pBR328 (Bolivar *et al.*, 1977; Soberon *et al.*, 1980), and pUC18/19 (Norrander *et al.*, 1983) were used. The LacZ–Lly fusion proteins were constructed with the pSEM-vector system and expressed following induction with IPTG (Knapp *et al.*, 1990). Cosmids and subclones are indicated in Table 1, Fig. 2 and Fig. 6. Antibiotics were used at concentration of 100 μg ml^{-1} (ampicillin) and 15 μg ml^{-1} (tetracycline).

Cosmid cloning procedure

Legionella-specific chromosomal DNA was isolated as described (Knapp *et al.*, 1984; Ott *et al.*, 1988). Large (20 kb) chromosomal *Sau*3A fragments were ligated into the *Bam*HI site of cosmid pLAFR2 and transduced into HB101 with the help of the cosmid packaging system (Hohn and Collins, 1980).

Recombinant DNA techniques

Plasmid DNA was isolated as described previously (Grinstedt *et al.*, 1978; Birnboim and Doly, 1979). For restriction enzyme

analysis, DNA was treated with appropriate enzymes and the resulting fragments were separated by gel electrophoresis on 0.8–1.0% agarose gels (Knapp *et al.*, 1984). DNA fragments were isolated from the agarose gel by the freeze-squeeze method (Thuring *et al.*, 1975). For cloning, DNA fragments were ligated into suitable vector molecules after heat-inactivation of the restriction endonucleases at 65°C for 10 min (Sambrook *et al.*, 1989). Digestion of DNAs with exonuclease III was done according to Henikoff (1987). *E. coli* K12 strains were transformed by the $CaCl_2$ method (Lederberg and Cohen, 1974).

Tn1000 (γδ) transposon mutagenesis

Tn*1000* insertions in the legiolysin (*lly*) gene were isolated following mobilization of *lly*-specific plasmid pEWL4 by the F-factor which occurs by Tn*1000*-mediated cointegration of the two plasmids. Resolvation of the cointegrate in recipient cells results in *lly*::Tn*1000* insertion derivatives, which were analysed by suitable digestion (Guyer, 1978).

Gene probes and radioactive labelling

As a legiolysin (*lly*)-specific gene probe the 2.4 kb *Eco*RI/*Sma*I fragment of pEWL113 (see Fig. 2) was used. After elution from agarose gels the DNA fragment was labelled by the method of Feinberg and Vogelstein (1983) with a random priming kit purchased from Boehringer.

Southern hybridization

The transfer of DNA from agarose gels to nitrocellulose paper, and the washing and autoradiography of the filters were performed as described earlier (Southern, 1975). The filters were hybridized in 50% formamide for 24 h at 42°C. Stringent conditions were used for the washing procedure (Sambrook *et al.*, 1989).

Detection of haemolysis, protease activity, colour production and fluorescence

Haemolysin production of *L. pneumophila* wild-type and of recombinant *E. coli* K12 clones was detected on agar plates containing 5% erythrocytes from either humans, dogs or sheep after cultivation for 2 d at 37°C. Protease activity was detected using L-broth containing 1.5% skim milk. Colour production (browning) of strains and clones was assayed after cultivation on agar plates with 4 mM tyrosine. A long-wave u.v.-lamp was used in the dark to detect yellow-green fluorescence excreted into the medium.

Analysis of protein synthesis in minicells

Analysis of plasmid-encoded proteins in minicells was performed as described previously (Noegel *et al.*, 1979).

Construction of LacZ–Lly fusion proteins and preparation of Lly-specific antibodies

A suitable *lly*-specific DNA fragment containing the distal part of the *lly* gene starting at the *Sph*I site was ligated into the vector pSEM-2 (Knapp *et al.*, 1990). The LacZ–Lly fusion protein of 90 kDa was isolated following induction with IPTG from SDS–PAGE and injected into rabbits together with complete Freund's adjuvant.

Western blots

Western blots with Lly-specific antiserum were performed according to the method of Towbin *et al.* (1979).

Cytotoxicity assays

CHO- and Vero cells were cultured in RPMI 1640 or Dulbecco modified Eagle medium (DMEM), respectively. Each was supplemented with 5% fetal calf serum and 200 mM glutamine. Cytotoxicity was determined by incubating confluent monolayers together with bacterial cell extracts, which were obtained from stationary cultures. For this purpose bacteria were harvested by centrifugation and, after removal of the supernatants they were suspended in saline and sonicated. Serial dilutions of sterile filtered extracts were applied in a 96-well microtitre plate. Cytopathic effects were evaluated 24 h after exposure of the cells to the bacterial extracts, as described (Guerrant *et al.*, 1974; Mahajan and Rodgers, 1990; Bouzari and Varghese, 1990).

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