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The prepilin peptidase is required for protein secretion by and the virulence of the intracellular pathogen *Legionella pneumophila*

Mark R. Liles,[1] Paul H. Edelstein[2] and Nicholas P. Cianciotto[1]*

[1]*Department of Microbiology-Immunology, Northwestern University, 303 E. Chicago Ave., Chicago, IL 60611, USA.*
[2]*Departments of Pathology and Laboratory Medicine, The University of Pennsylvania, Philadelphia, PA 19104, USA.*

Summary

Prepilin peptidases cleave, among other substrates, the leader sequences from prepilin-like proteins that are required for type II protein secretion in Gram-negative bacteria. To begin to assess the importance of type II secretion for the virulence of an intracellular pathogen, we examined the effect of inactivating the prepilin peptidase (*pilD*) gene of *Legionella pneumophila*. Although the *pilD* mutant and its parent grew similarly in bacteriological media, they did differ in colony attributes and recoverability from late stationary phase. Moreover, at least three proteins were absent from the mutant's supernatant, indicating that PilD is necessary for the secretion of *Legionella* proteins. The absence of both the major secreted protein and a haemolytic activity from the mutant signalled that the *L. pneumophila* zinc metalloprotease is excreted via type II secretion. Most interestingly, the *pilD* mutant was greatly impaired in its ability to grow within *Hartmannella vermiformis* amoebae and the human macrophage-like U937 cells. As reintroduction of *pilD* into the mutant restored infectivity and as a mutant lacking type IV pilin replicated like wild type, these data suggested that the intracellular growth of *L. pneumophila* is promoted by proteins secreted via a type II pathway. Intratracheal inoculation of guinea pigs revealed that the LD_{50} for the *pilD* mutant is at least 100-fold greater than that for its parent, and the culturing of bacteria from infected animals showed a rapid clearance of the mutant from the lungs. This is the first study to indicate a role for PilD and type II secretion in intracellular parasitism.

Received 14 August, 1998; revised 22 October, 1998; accepted 30 October, 1998. *For correspondence. E-mail n-cianciotto@nwu.edu; Tel. (+1) 312 503 0385; Fax (+1) 312 503 1339.

Introduction

In recent years, much attention has been directed towards understanding protein secretion by bacteria (Pugsley, 1993). This effort has been driven, in large part, by the intriguing ways in which protein secretion pathways promote the virulence of animal and plant pathogens (Hueck, 1998). The secretion mechanisms of Gram-negative bacteria have been divided into four types, i.e. types I, II, III and IV (Salmond and Reeves, 1993; Finlay and Falkow, 1997). The type I pathway (referred to as type IV in early reviews) is composed of three proteins that form a channel for the transport of proteins directly from the cytoplasm to the exterior (Wandersman, 1992; Salmond and Reeves, 1993; Hueck, 1998). Virulence determinants that are handled via this secretion pathway include the haemolysin of *Escherichia coli*, the adenylate cyclase of *Bordetella pertussis* and the alkaline protease of *Pseudomonas aeruginosa* (Finlay and Falkow, 1997). In type II secretion, exoproteins are transported across the inner membrane in *sec*-dependent fashion, and then the mature proteins cross the outer membrane through the combined action of some 14 proteins (Wandersman, 1992; Pugsley, 1993; Salmond and Reeves, 1993; Pugsley *et al*., 1997; Russel, 1998). Virulence factors that depend upon type II secretion include the exotoxin A and phospholipase C of *P. aeruginosa*, the haemolysin and protease of *Aeromonas hydrophila* and the enterotoxin and protease of *Vibrio cholerae* (Bally *et al*., 1991; Strom *et al*., 1991; Pepe *et al*., 1996; Finlay and Falkow, 1997). Type III secretion involves approximately 20 proteins that carry exoproteins directly from the cytoplasm to the cell surface, from where they are then injected into an animal or plant cell upon contact (Salmond and Reeves, 1993; Finlay and Falkow, 1997; Hueck, 1998). The Yops of *Yersinia enterocolitica* and invasins of *Shigella flexneri* and *Salmonella typhimurium* are virulence factors secreted via a type III system (Anderson and Schneewind, 1997; Finlay and Falkow, 1997). In the type IV system (referred to as type I in early reviews), *sec*-dependent transport of proteins across the inner membrane is followed by self-directed passage through the outer membrane (Wandersman, 1992; Finlay and Falkow, 1997; Hueck, 1998). Factors that complete their own export are the vacuolating cytotoxin of *Helicobacter pylori*, the IgA protease of *Neisseria gonorrhoeae* and SepA of *S. flexneri*.

It has been suggested recently that a fifth secretion system exists, dictating the conjugal transfer of plasmids in *Agrobacterium tumefaciens* and the release of pertussis toxin by *B. pertussis* (Winans *et al*., 1996; Hueck, 1998). The *dotIicm* loci of *Legionella pneumophila* may encode a related pathway that is required for the infection of macrophages (Segal *et al*., 1998; Vogel *et al*., 1998). Curiously, of these secretion pathways, only type II has not been identified previously in an intracellular pathogen. Our recent discovery of a locus in *L. pneumophila* that, in other Gram-negative bacteria, is required for type II secretion suggested that this secretory mechanism might promote intracellular parasitism as well (Liles *et al*., 1998).

L. pneumophila exists primarily as a parasite of protozoa, replicating within amoebae and ciliates in freshwater habitats (Fields, 1996). However, the inhalation of contaminated water droplets can lead to a potentially fatal pneumonia known as Legionnaires' disease, an infection characterized by the proliferation of *L. pneumophila* within alveolar macrophages (McDade *et al*., 1977; Horwitz and Silverstein, 1980; Dowling *et al*., 1992). In both protozoa and macrophages, *L. pneumophila* multiplies within a ribosome-studded phagosome that does not fuse with endosomes or lysosomes (Horwitz, 1983; Abu Kwaik, 1996; Fields, 1996). The factors that enable *L. pneumophila* to subvert the host's bactericidal mechanisms and to access nutrients have been sought by numerous investigators (Dowling *et al*., 1992). Recently, while characterizing a locus involved in iron acquisition, we discovered an operon (*pilBCD*) encoding analogues of proteins involved in the biogenesis of pili and type II secretion in other Gram-negative bacteria (Liles *et al*., 1998). The PilB- and PilC-like proteins are required for the translocation of type IV prepilin across the inner membrane, at which point the prepilin peptidase (PilD) cleaves the hydrophobic leader sequence, thereby releasing the mature pilin monomer into the periplasm (Strom and Lory, 1993). The pilin subunits are then assembled into the pilus structure via a dedicated outer membrane apparatus (Bally *et al*., 1992; Strom and Lory, 1993). PilB and PilC are thought to be strictly involved in type IV pilus biogenesis (Nunn *et al*., 1990; Turner *et al*., 1993). In contrast, PilD can have as many as five prepilin-like substrates that are directly required for type II secretion across the outer membrane (Nunn and Lory, 1992; Bleves *et al*., 1998). Therefore, in many extracellular pathogens, the prepilin leader peptidase is required for the assembly of both a type IV pilus and a type II secretory apparatus (Kaufman *et al*., 1991; Nunn and Lory, 1992; Freitag *et al*., 1995; Pepe *et al*., 1996). Stone and Abu Kwaik (1998) recently discovered the *L. pneumophila* type IV pilin gene ($pilE_L$) and determined that a $pilE_L$ mutation eliminates the production of type IV pili and modestly reduces adherence to host cells, but does not impair intracellular growth. To begin to determine whether a type II system promotes intracellular parasitism, we constructed and characterized an *L. pneumophila pilD* mutant. This mutant was substantially impaired in its ability to replicate within amoebae and human macrophages, indicating, for the first time, that factors secreted via a type II apparatus contribute to the virulence of an intracellular pathogen.

Results

Construction, piliation and colony morphology of an L. pneumophila pilD *mutant*

To help to determine whether *L. pneumophila* expresses a type II secretion system and, if so, what the importance of this protein export mechanism is to the virulence of the organism, a *pilD* mutant of strain 130b was constructed and characterized. The *pilD* locus was inactivated by the insertion of a kan^R gene 559 bp past the gene's translational start. After electroporation of the allelic exchange plasmid pMRL13 into strain 130b, Kan^R/Suc^R colonies were easily isolated at a frequency of 10^{-7}. Southern hybridization of *Hin*dIII-digested DNA revealed that six out of six Kan^R clones had undergone the predicted chromosomal rearrangement, i.e. 1.9 kb and 2.7 kb hybridizing fragments were observed for each clone in contrast to the 3.5 kb hybridizing fragment for strain 130b (data not shown). The Kan^R clone chosen for further analysis was designated NU243.

To verify that the *pilD* mutation abolished type IV pilus production, electron microscopy was conducted on 130b and NU243 grown on BCYE agar. As greater piliation had been observed at 30°C than at 37°C (Liles *et al*., 1998), we tested the strains for pilus formation at both temperatures. This analysis revealed the complete loss of piliation for NU243 under all growth conditions, whereas 130b exhibited pili as before (Fig. 1). While not unexpected, the observation that NU243 lacked pili was the first experimental evidence that *L. pneumophila pilD* encodes a protein with functional homology to other prepilin peptidases.

Interestingly, upon the initial isolation of the Kan^R colonies and with further handling of NU243, the *pilD* mutants were observed to have a distinct colony difference from wild type when grown on BCYE agar (Fig. 2). The *pilD*-negative colonies exhibited a flatter shape and a darker grey colour, although their size appeared to be similar to those of 130b. This result suggested that the *pilD* mutation resulted in alterations of the *L. pneumophila* surface. As *pilD* is the last gene in an operon (Liles *et al*., 1998), it was likely that the NU243 phenotypes were caused solely by the loss of PilD function. To confirm this hypothesis, we examined NU243 expressing pMRL13, which contains the intact *pilD*. NU243 (pMRL13) exhibited pili and wild-type colony morphology, whereas NU243 carrying the control vector pBBR1MCS retained the mutant colony morphology and lacked pili (Figs 1 and 2), indicating that the loss of *pilD*

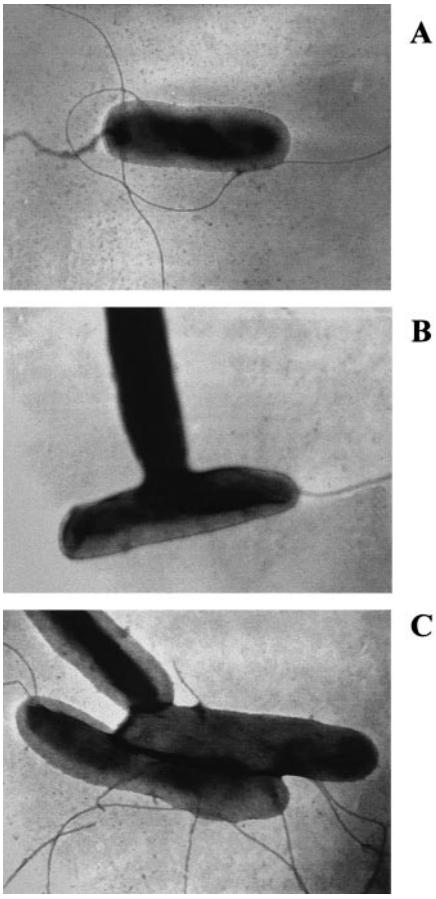

Fig. 1. Piliation state of *L. pneumophila* strains. Bacteria representing strains 130b (A), NU243 (B) and NU243(pMRL13) (C) grown on BCYE agar at 30°C were stained with phosphotungstic acid and examined by transmission electron microscopy. The greater degree of piliation in (C) is probably caused by the multiple copies of *pilD* in NU243(pMRL13). Note the significantly larger diameters of flagella in (A) and (B) in comparison with the thinner pili in (A) and (C). All electron micrographs are at an original magnification of ×27 000.

is responsible for these phenotypical changes. As the loss of type IV pili can induce changes in the colony morphology of other Gram-negative bacteria (e.g. pilin mutants of *N. gonorrhoeae* are flatter and less reflective) (Freitag *et al*., 1995), we re-examined the colonies of the *L. pneumophila pilE*$_L$ mutant. Supporting the observation of Stone and Abu Kwaik (1998), we did not see any colony differences resulting from the absence of pilin (data not shown). Thus, the unique colony morphology of the *pilD* mutant is probably attributable to a deficiency in type II protein secretion.

Extracellular growth of the L. pneumophila pilD *mutant*

The initial observations of growth on BCYE agar indicated that NU243 grew at a rate similar to wild-type *L. pneumophila*. To study the extracellular growth of this mutant in more detail, we examined the abilities of strains 130b and NU243 to grow and survive in BYE broth. In four separate experiments, the 37°C growth of NU243 resembled that of 130b (Fig. 3A). To confirm this, the number of colony-forming units (cfu) was assessed at different times after inoculation. No differences in cfu were observed from the early logarithmic phase (i.e. culture $OD_{660} = 0.3$) to the 24 h, mid-stationary phase (i.e. $OD_{660} = 2.0$). However, there was a fourfold decrease in the cfu of NU243 relative to 130b at 48 h and a sevenfold difference in cfu by 72 h, despite the fact that the late stationary phase cultures from the two strains shared OD_{660} values of 1.4–1.5. The number of bacterial particles and the degree of filamentation was the same for both strains at 72 h after inoculation (data not shown), indicating that the moderate decrease in the cfu of the *pilD* mutant resulted from a loss in recoverability of NU243 during late stationary phase. On numerous occasions, a five- to 10-fold reduction in recoverable NU243 was also observed when bacteria were examined after 72 h growth on BCYE agar.

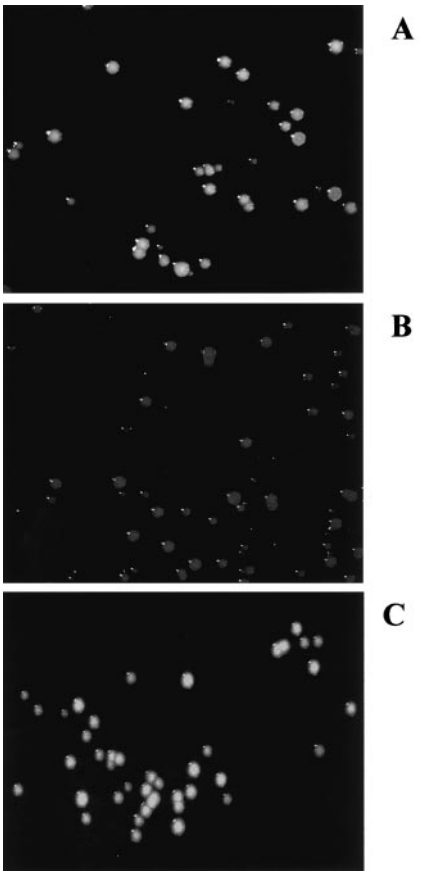

Fig. 2. Colony morphology of strains 130b and NU243. After 3 days of growth at 37°C on BCYE agar, images of 130b (A), NU243 (B) and NU243(pMRL13) (C) colonies were recorded. Colonies of NU243(pBBR1MCS) appeared as did those of NU243. The differences in colonial growth that were associated with loss of *pilD* were also seen after growth at 30°C.

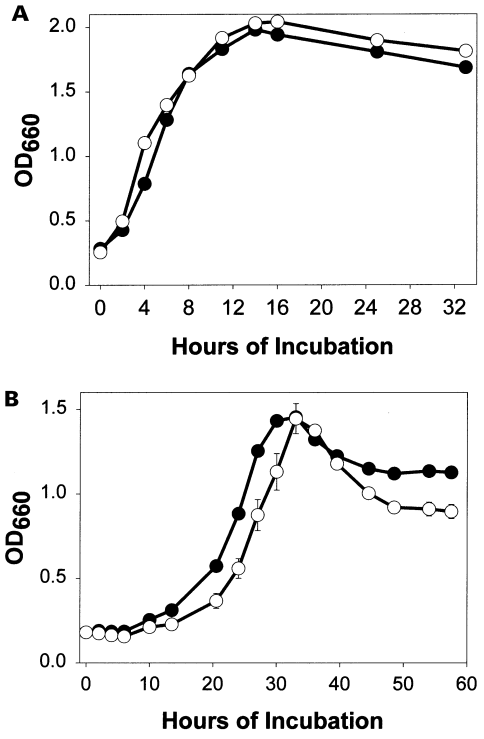

Fig. 3. Growth of wild type and a *pilD* mutant in BYE broth. Strains were grown overnight in BYE at 37°C and then subcultured into fresh BYE and incubated with shaking at either 37°C (A) or 30°C (B). The growth of strain 130b (filled circles) and NU243 (open circles) was quantified by measuring the OD_{660} at various times. Each time point represents the mean ± standard error of three replicate cultures. Error bars are not visible at many time points because of their small values. The growth observed at 37°C was as seen before with strain 130b and others, but the growth at 30°C has, to our knowledge, not been reported before. The difference in OD between 30°C cultures was significant after 45 h ($P < 0.05$; Student's *t*-test). The slightly longer lag period of NU243 in (B) was not observed in subsequent experiments.

As previous data indicated that transcription of the *pilBCD* operon increased at 30°C relative to 37°C (Liles *et al*., 1998), the extracellular growth of these two strains in BYE was compared at this lower temperature (Fig. 3B). Although the lag and logarithmic phases of growth were similar for strains 130b and NU243 at 30°C, a decrease in the optical density of the NU243 culture was observed during stationary phase on five separate occasions (Fig. 3B). A reduction in the cfu of NU243 relative to 130b occurred beginning in the stationary phase, with a fourfold difference in cfu evident at 34 h after inoculation and an eightfold difference in cfu observed at 50 h. Again, no difference was observed in numbers of bacterial particles or degree of filamentation between strains 130b and NU243 (data not shown).

These experiments indicated that the *pilD* mutant is fully competent for replication in an extracellular growth medium, yet its recoverability during the late stationary phase of growth is impaired compared with wild-type *L. pneumophila*. NU243 expressing pMRL13 grew like strain 130b and did not exhibit the moderate decrease in the numbers of recoverable bacteria at either 30°C or 37°C (data not shown), indicating that this phenotype results from the loss of PilD. To determine whether the absence of type IV pili contributes to late stationary phase recoverability, the $pilE_L$ mutant was tested for extracellular growth. The growth of the $pilE_L$ mutant was the same as strain 130b at 30°C and 37°C, without a drop in OD_{660} or cfu ml^{-1} during stationary phase (data not shown). Thus, this evidence suggests that proteins exported by a type II machinery contribute to *L. pneumophila* recoverability during the late stationary phase of growth in a complex extracellular medium.

Protein secretion by the L. pneumophila pilD *mutant*

If *L. pneumophila* possesses a type II secretion system, then a *pilD* mutation should result in the absence of some proteins in the bacterial supernatant. To test this hypothesis, supernatants from BYE cultures grown at 30°C and 37°C were examined for proteins secreted by strains 130b and NU243. The protein profiles of the two strains were very different in logarithmic and stationary phase growth. During early and late log phases, all seven or eight proteins that were apparent in the 130b supernatants were absent in the NU243 supernatants (Fig. 4, left). Likewise, several prominent protein bands were lacking in the stationary supernatants of the mutant (Fig. 4, right). Curiously, a number of protein species that were present in the mutant stationary samples were absent from wild-type samples, suggesting that NU243 might secrete 'new' proteins in an attempt to compensate for the loss of *pilD*-dependent exoproteins. Alternately, this observation could simply be an early manifestation of the reduced viability of mutant cultures (see above). Nevertheless, this series of experiments indicated that the *L. pneumophila pilD* mutant is defective for the secretion of a variety of proteins.

The *Legionella* species are known to secrete several proteins during growth in extracellular media at 37°C, including an acid phosphatase, a phospholipase C, a lipase and several proteases (Baine, 1985; Dowling, 1992). The major secretory protein of *L. pneumophila* is a zinc metalloprotease, which migrates as a 38 kDa band in SDS–polyacrylamide gels (Conlan *et al*., 1988; Keen and Hoffman, 1989; Black *et al*., 1990; Szeto and Shuman, 1990). Interestingly, this protein appeared to be absent from the mutant's supernatant (Fig. 4). As this protease confers a β-haemolytic phenotype on *L. pneumophila* (Keen and Hoffman, 1989; Quinn and Tompkins, 1989), strains 130b and NU243 were tested for their abilities to lyse red blood

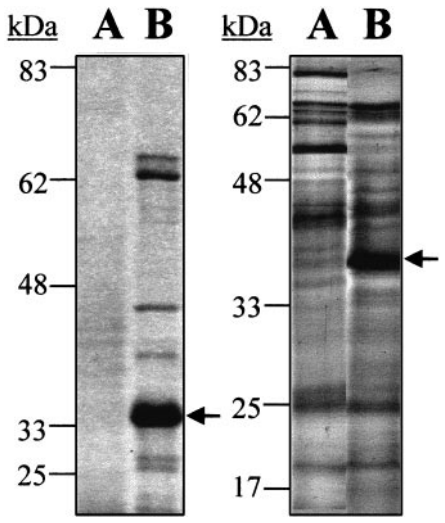

Fig. 4. Protein profiles of 130b and NU243 supernatants. Strains NU243 (A lanes) and 130b (B lanes) were grown to either late logarithmic (i.e. $OD_{660} = 1.2–1.4$)(left) or stationary phase (i.e. $OD_{660} = 1.8–1.9$)(right) in BYE at 37°C, and the proteins present in the supernatants were electrophoresed through a 12.5% (left) or 15% (right) SDS–polyacrylamide gel and subsequently stained with Coomassie blue. The major secretory protein is denoted by an arrow. The migration of molecular weight standards is indicated to the left of the respective lanes. The pattern depicted on the left was also obtained when bacteria were grown at 37°C to early log phase (i.e. $OD_{660} = 0.4$). The loss of proteins from the *pilD* mutant supernatant was also observed when bacteria were grown at 30°C.

cells. As predicted, the *pilD* mutant did not exhibit haemolysis when grown on BYE blood plates at 37°C (data not shown), suggesting that the zinc metalloprotease is exported via the type II machinery. In contrast, NU243 remained positive for acid phosphatase activity (data not shown), demonstrating that only some *L. pneumophila* secreted proteins require the prepilin peptidase for their export. In summary, the loss of both supernatant proteins and haemolytic activity demonstrates that the *pilD* mutant is defective for protein secretion. Given precedent, we strongly suspect that this phenotype is caused by the loss of type II protein secretion.

Intracellular growth of the L. pneumophila pilD *mutant*

To begin to assess the contribution of the type II secretion pathway to the intracellular replication of *L. pneumophila*, human macrophages and freshwater amoebae were infected with the wild-type and *pilD* mutant. U937 cell monolayers were first inoculated with strains 130b and NU243 grown to mid-log phase at 37°C in BYE to ensure similar numbers of viable, infecting bacteria. Interestingly, NU243 exhibited only an 85-fold increase in cfu over 72 h compared with the typical 24 000-fold growth of strain 130b (Fig. 5). These data indicate that the *L. pneumophila pilD* mutant has a severe impairment in its replication within human macrophages. In three additional experiments, in which the bacterial inocula were derived from BCYE agar, a similar impairment of NU243's intracellular replication was observed, with only 80-, 83- and 125-fold increases in cfu observed over 72 h. Complementation of this intracellular growth defect by pMRL13 was also observed (data not shown). Inoculation of macrophage monolayers with a low multiplicity of infection (MOI) of *L. pneumophila* eventually results in death of the host cells (Pearlman *et al*., 1988). Indeed, the viability of U937 cell monolayers infected with strain 130b was abolished by 72 h after infection compared with an uninfected control (Fig. 6). However, no decrease in cell viability was observed for U937 cells infected with the *pilD* mutant (Fig. 6). Therefore, the reduced intracellular replication of NU243 was, as expected, associated with a loss of cytopathic effect.

To test whether PilD promotes intracellular replication within an amoebal host, NU243 and its 130b control were co-cultured with *H. vermiformis*. In four separate experiments, the kinetics of growth for NU243 revealed only an approximately 10-fold cfu increase over 5 days, compared with a $\geq$ 1000-fold increase for strain 130b (Fig. 7). This growth defect of NU243 was complemented by pMRL13, confirming that the *pilD* mutation attenuated the intracellular replication of *L. pneumophila*, whether in a human macrophage or in a protozoan host (Fig. 7).

The defect in intracellular growth of the *pilD* mutant could have been the consequence of a loss of type IV pili and/or type II protein secretion. However, an earlier study reported that the pilin-negative derivative of strain 130b was fully competent for replication within U937 cells (Stone and Abu Kwaik, 1998), an observation that we have confirmed (data not shown). In addition, it has been

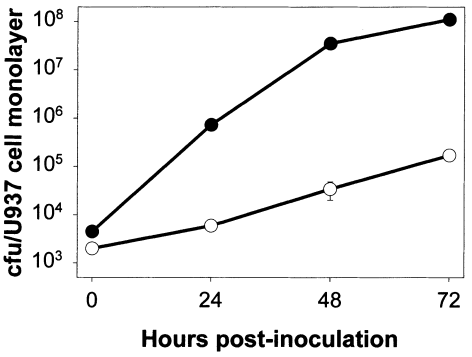

Fig. 5. Growth of strains 130b and NU243 within U937 cells. Macrophage monolayers ($n = 3$) were infected with log phase strain 130b (filled circles) and NU243 (open circles). Bacterial cfu per monolayer were quantified at 0, 24, 48 and 72 h after inoculation. Each data point represents the mean ± standard error for three independent monolayers. The small difference in cfu recovery at $t = 0$ reflects, at least in part, the slightly more wild-type bacteria that were inoculated into the tissue culture wells. Significant differences in recovery were observed at $t = 24$, 48 and 72 h ($P < 0.01$; Student's *t*-test).

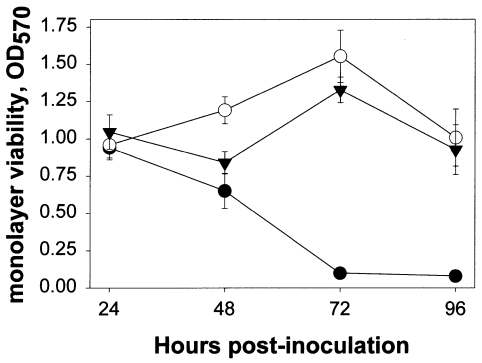

Fig. 6. Cytopathic effect of strains 130b and NU243 on U937 cells. Replicate monolayers ($n = 6$) were either not infected (filled triangles) or were infected with 8×10^6 cfu of strain 130b (filled circles) or 3×10^6 cfu of strain NU243 (open circles). After various periods of incubation, the viability of host cells was measured by their ability to convert MTT to formazan. Each point represents the mean OD_{540}, and vertical bars indicate standard deviations. Differences in cytopathic effect between strains was significant at 72 h and 96 h after inoculation ($P < 0.0001$, Student's *t*-test). These differences were observed in two additional experiments.

demonstrated that the pilin mutant could multiply like wild type within the amoeba *Acanthamoeba castellanii* (Stone and Abu Kwaik, 1998). Finally, we observed that the *pilE*$_L$ mutant did not show any impairment in intracellular growth in *H. vermiformis* (Fig. 7). Thus, the intracellular growth defect of the *pilD* mutant is probably attributable to a lack of type II secretion.

Pulmonary infection of guinea pigs with the L. pneumophila pilD *mutant*

The reduced ability of the *pilD* mutant to infect macrophages indicates that type II secretion is a virulence factor for *L. pneumophila*. To gain support for this hypothesis, we assessed the ability of NU243 to cause pneumonia in guinea pigs following intratracheal inoculation. To obtain a qualitative assessment of the virulence of the *pilD* mutant first, groups of four guinea pigs were inoculated with differing doses of NU243 or its wild-type parent and then observed for signs of infection. Despite receiving inocula as high as 10^8 cfu, none of the guinea pigs infected with the mutant died, and none had weight loss or respiratory distress. In contrast, strain 130b killed three out of four guinea pigs infected with 6×10^6 cfu. Therefore, the *pilD* mutant was substantially attenuated in its ability to cause disease.

To gain a more quantitative assessment of this attenuated phenotype, guinea pigs were inoculated with strains 130b and NU243, and the *L. pneumophila* concentrations in the lungs and spleens were determined on days 1–6 after infection (Fig. 8A). Although 100-fold greater numbers of NU243 than of 130b were inoculated into the animals, *L. pneumophila* lung concentrations were 50-fold lower in the NU243-infected than in the 130b-infected guinea pigs after 1 day (Fig. 8A). Furthermore, the number of viable *pilD* mutants per lung never increased but rather dropped over 1000-fold to less than 10^3 cfu per lung by 6 days after infection (Fig. 8A). In contrast, lung concentrations of 130b bacteria increased over time, reaching a maximum of over 10^{10} cfu per lung by 2 days after infection (Fig. 8A). Histological examination of lungs revealed a greater degree of exudation in animals infected with the *pilD* mutant than with 130b on day 3. By day 6, there was resolution of this lung consolidation in the *pilD* group, but a high degree of consolidation in the 130b group (Fig. 9). As systemic bacterial dissemination occurs in this experimental model (Edelstein *et al*., 1984; Reinhardt *et al*., 1987), we determined *L. pneumophila* spleen concentrations of the infected animals (Fig. 8B). There was a marked difference in the spread of bacteria to the guinea pig spleen, with strain 130b reaching a maximum of 2×10^6 cfu per spleen by day 2. In contrast, the spleen concentration of NU243 never exceeded 8×10^2 cfu per spleen and, in 10 of the 19 animals challenged with this bacterial strain, no *L. pneumophila* bacteria were detected in the spleens (Fig. 8B). As would be expected in guinea pigs exposed to virulent *L. pneumophila* (Edelstein *et al*., 1984), animals infected with strain 130b had an average weight loss of 18% by day 4 after infection, in contrast to a 5% weight gain in the NU243 group. Animals in both experimental groups had a significant increase in body temperature in comparison with the baseline value on days 1–4 after infection

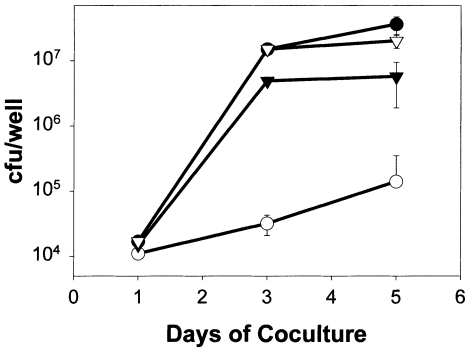

Fig. 7. Growth of NU243 within *Hartmannella vermiformis*. Wells ($n = 4$) containing amoebae were infected with 5×10^5 cfu of strain 130b (pBBR1MCS) (filled circles), 4×10^5 cfu of NU243 (pBBR1MCS) (open circles), 7×10^5 cfu of NU243 (pMRL13) (filled triangles) or 5×10^5 cfu of the *pilE*$_L$ mutant (open triangles). Bacterial cfu per well were quantified at 1, 3 and 5 days after inoculation. Each data point represents the mean ± standard error for three independent co-cultures. Significant differences were observed between NU243 expressing the control vector and wild type and complemented NU243 at days 3 and 5 ($P < 0.05$; Student's *t*-test). The intracellular replication of either 130b or NU243 expressing pBBR1MCS was the same as strains not expressing this vector.

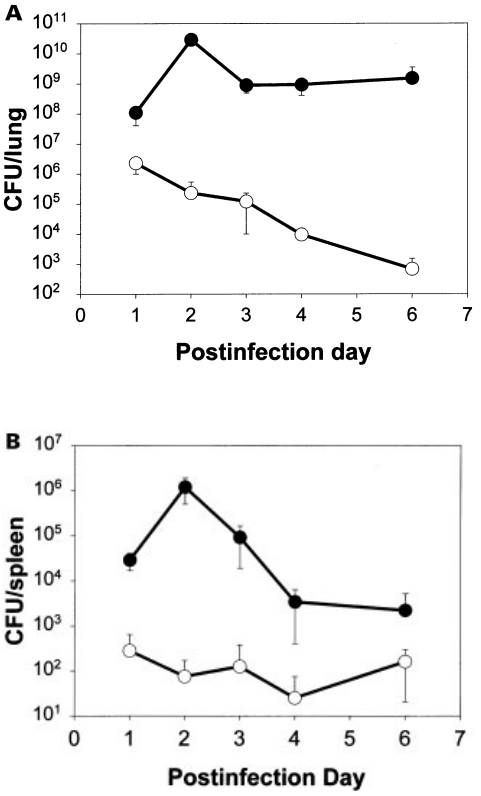

Fig. 8. Replication of legionellae within guinea pig lung and spleen. Strains 130b (8×10^5 cfu) (filled circles) or NU243 (8×10^7 cfu) (open circles) were inoculated intratracheally into guinea pigs, and the total number of bacterial cfu per lung (A) and spleen (B) were quantified at various times after inoculation. Each data point represents the mean ± standard error for four animals, with the exception of day 4 ($n=2$ for 130b) and day 6 ($n=2$ for 130b; $n=3$ for NU243). After the first day, the average splenic cfu of the mutant appeared at or below the level of detection (i.e. 100 cfu), because culturing of some of the infected animal spleens did not yield any bacteria. At every time point, there was a statistically significant difference between the number of 130b cfu and the number of NU243 cfu ($P<0.05$; Welch's *t*-test approximation).

($P<0.0001$–0.02, depending on the day of comparison, by Student's *t*-test). However, animals that received strain 130b had significantly greater body temperatures than did those receiving NU243 ($P<0.0001$, by Student's *t*-test) on days 1–4 after infection. These data demonstrate conclusively an attenuation of the virulence of the *pilD* mutant in the guinea pig model of Legionnaires' disease.

Discussion

The loss of *L. pneumophila* PilD resulted in a defect in protein secretion that was distinct from a simple loss of type IV pilus biogenesis. Likewise, *Klebsiella oxytoca*, *P. aeruginosa*, *V. cholerae* and *A. hydrophila* mutants lacking the prepilin peptidase are deficient in protein secretion (Pugsley and Reyss, 1990; Kaufman *et al*., 1991; Nunn and Lory, 1992; Pepe *et al*., 1996). In the last three cases, one of the absent exoproteins was a protease, as was observed for the *L. pneumophila* mutant (Kaufman *et al*., 1991; Nunn and Lory, 1992; Pepe *et al*., 1996). In the other bacteria, the peptidase mutations are believed to prevent processing of prepilin-like proteins (e.g. XcpT, U, V, W and X in *P. aeruginosa*) that are directly involved in type II secretion (Nunn and Lory, 1992). Indeed, mutations in the *xcp* genes result in a loss of protein secretion, without a concomitant change in piliation (Tommassen *et al*., 1992). Although we aimed to identify the Xcp-like components in *L. pneumophila*, our functional data already strongly suggest that this organism has a type II protein secretion system. The *Legionella pilD* mutant had a unique colony morphology, intimating that *L. pneumophila* secretes a protein(s) via the type II pathway that modifies its surface characteristics. Although it has been suggested that the type II exoproteins of *V. cholerae* include outer membrane proteins (Sandkvist *et al*., 1997), none of the other prepilin peptidase mutants exhibit differences in colony morphology that are not attributable to the loss of type IV pili (Kaufman *et al*., 1991; Nunn and Lory, 1992; Freitag *et al*., 1995; Pepe *et al*., 1996). The *L. pneumophila pilD* mutant had a reduced recoverability during the late stationary phase of extracellular growth, an observation that reflects either a true reduction in bacterial viability or a diminution in the number of culturable bacteria. The existence of viable, non-culturable legionellae has been observed, but their nature is essentially unexplained (Hay *et al*., 1995). We hypothesize that the reduced recoverability of NU243 is related to its loss of type II secretion, e.g. the absence of a protease or other excreted enzymes that

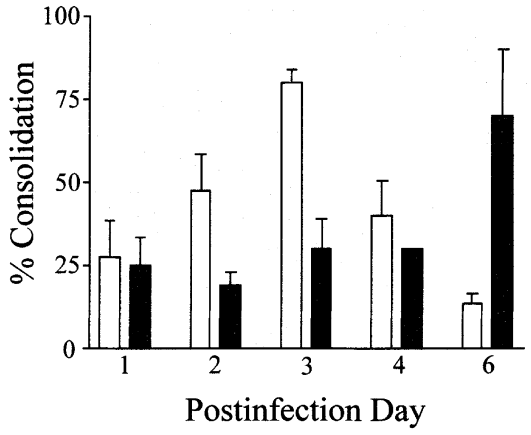

Fig. 9. Consolidation within infected guinea pig lungs. Lungs infected with strains 130b (dark boxes) or NU243 (light boxes) were fixed and stained with haematoxylin and eosin. The percentage exudation was estimated as a percentage of the total area of the section. Significant increases in consolidation were observed for lungs infected with NU243 relative to wild type at days 3 and 6 after inoculation ($P<0.05$; Welch's *t*-test approximation).

contribute to nutrient acquisition could alter the phenotype of late stationary phase cultures. Alternatively, the modest drop in mutant recovery could have been caused by an accumulation of prepilin-like proteins or type II exoproteins in the inner membrane or periplasmic space. However, abnormal protein accumulation has not been shown to be toxic to any of the other prepilin peptidase mutants (Kaufman *et al*., 1991; Nunn and Lory, 1992; Freitag *et al*., 1995; Pepe *et al*., 1996).

Our data indicate, for the first time, that a prepilin peptidase and type II protein secretion function in an intracellular parasite. This observation is made even more interesting because of the greatly reduced intracellular replication of the *pilD* mutant within the evolutionarily divergent macrophage and amoeba hosts. Because the pilin mutant was fully competent for growth within host cells, the defective intracellular infectivity of NU243 can be attributed to its alteration in protein secretion. As mutants specifically deficient in zinc metalloprotease expression replicate like wild type in macrophages and amoebae (Szeto and Shuman, 1990), the attenuated phenotype of NU243 cannot be a result of its loss of one proteolytic activity. The intracellular growth kinetics of the *pilD* mutant indicates that type II secreted proteins are most important to events that occur relatively late in the infective process. For example, as monolayers infected with NU243 and wild-type bacteria yielded similar numbers of cfu at the earliest of time points, we suspect that type II secretion is not significantly involved in entry and immediate intracellular survival. On the other hand, type II exoproteins could have major roles in intracellular nutrient acquisition or host cell lysis. It is possible, although unlikely, as noted above, that the reduced intracellular growth of the mutant results from an accumulation of prepilin-like proteins or type II exoproteins in the bacterial envelope. While the type II secreted factors that are crucial for the intracellular replication of *L. pneumophila* will be the focus of our future research efforts, it will be important for others to ascertain whether additional intracellular parasites require type II secreted proteins.

The contribution of *L. pneumophila* PilD to *in vivo* pathogenesis is certainly significant, considering the sharply attenuated virulence of NU243 within guinea pigs. This attenuation is undoubtedly caused, at least in part, by the mutant's defect in intracellular replication. Like the previous observations of extracellular growth, this is the first occasion that a prepilin peptidase mutant has been tested in an animal model. Interestingly, the *pilD* mutant appeared to be more attenuated *in vivo* than it did *in vitro*. There are several conceivable explanations for this result. First, the loss of factors that provide resistance to complement or polymorphonuclear leucocytes could have reduced the extracellular survival of the mutant in the mammalian lung. Secondly, the loss of type IV pili might have reduced the adherence of the *pilD* mutant to the alveolar epithelium, a process that has been suggested to promote *L. pneumophila* virulence (Mody *et al*., 1993; Cianciotto *et al*., 1995; Stone and Abu Kwaik, 1998). Thirdly, the loss of the zinc metalloprotease would be expected to lead to partial attenuation of the pulmonary infection (Moffat *et al*., 1994). Finally, the *pilD* mutant may be hypersensitive to the enhanced bacteriocidal activity that exists within activated alveolar macrophages. Regardless, these data indicate that factors secreted by the *L. pneumophila* type II secretion system are not only promoters of extracellular survivability and intracellular growth but also key potentiators of virulence.

Experimental procedures

Bacterial strains and media

The *L. pneumophila pilD* mutant as well as the cloned *Legionella pilD* gene was derived from serogroup 1 strain 130b (Wadsworth) (Hickey and Cianciotto, 1997). The $pilE_L$ mutant, also a derivative of strain 130b, was obtained from B. Stone and Y. Abu Kwaik (Stone and Abu Kwaik, 1998). The wild-type strain was maintained in a virulent state by passage through a guinea pig and stored as a spleen homogenate at −70°C. Before experimentation, *L. pneumophila* was generally cultured on buffered charcoal–yeast extract (BCYE) agar for 3 days at 37°C (Edelstein, 1981). When appropriate, 3 μg ml^{-1} chloramphenicol (Chl), 25 μg ml^{-1} kanamycin (Kan) or 5% (w/v) sucrose (Suc) was added to the medium. To assess *L. pneumophila* extracellular growth kinetics, bacteria grown overnight in buffered yeast extract (BYE) were subcultured into 2.5 ml of BYE to an optical density at 660 nm (OD_{660}) of approximately 0.1. Cultures were then incubated in an orbital incubator-shaker (LabLine Instruments) set at 250 r.p.m. and either 30°C or 37°C. Growth was assessed at various time intervals by recording the OD_{660} and by plating onto BCYE agar. Additionally, the number of bacterial particles was determined with a Petroff–Hauser counter (C. A. Hausser and Son), using crystal violet as the stain.

E. coli strain XL1 Blue (Stratagene) served as the host for recombinant plasmids (Yang, 1992). Plasmid DNAs were obtained from *E. coli* by the alkaline lysis method and were introduced by transformation into $CaCl_2$-treated *E. coli* (Ausubel *et al*., 1987). *E. coli* were grown on Luria–Bertani agar (Ausubel *et al*., 1987), which contained Kan (50 μg ml^{-1}) or Chl (30 μg ml^{-1}) when appropriate.

Directed mutagenesis of L. pneumophila pilD

The chromosomally encoded *pilD* gene was insertionally inactivated by allelic exchange (O'Connell *et al*., 1995). First, a Tn*903*-derived kanamycin resistance gene (kan^R) was isolated as an *Acc*I fragment from pMB2190 (Grindley and Joyce, 1980) and ligated into the single *Nar*I site of the cloned *pilD* on pML219 (Liles *et al*., 1998). Subsequently, a 9 kb *Sph*I fragment containing *pilD:*kan^R plus surrounding *L. pneumophila* DNA was ligated into the *Sph*I site of the counterselectable pBOC20, generating pMRL11. The suicide vector pBOC20 contains a Chl resistance marker and *sacB*, which can counterselect for plasmid maintenance by encoding Suc sensitivity

(O'Connell *et al*., 1995). Next, pMRL11 was electroporated into strain 130b, and transformants that may have undergone the desired double cross-over event were selected on BCYE containing Kan and Suc (Hickey and Cianciotto, 1997). Finally, genomic DNA was isolated from doubly resistant strains exhibiting Chl sensitivity (Hickey and Cianciotto, 1997), and allelic exchange was verified by Southern hybridization analysis using the Genius system kit (Boehringer Mannheim) and a *pilD*-specific probe. To help to generate the probe, primers F3 (5′-GGATTGGTGGTGGGTATG) and R34 (5′-CAAGAGCTAATGGCCAGAAG) were used to polymerase chain reaction (PCR) amplify *pilD* from strain 130b DNA (Liles *et al*., 1998). The reaction contained 100 ng of genomic DNA, with 3 mM $MgCl_2$, 25 pM each primer, 0.2 mM dNTPs and 1 unit of *Taq* polymerase (Gibco BRL) and was conducted with 45 s melting at 94°C, followed by 45 s annealing at 50°C and 90 s elongation at 72°C for 30 cycles.

Trans-complementation of the L. pneumophila pilD *mutation*

To help to determine whether the mutant's phenotypes were caused by the loss of PilD and not a second site mutation, a *pilD*-containing plasmid was constructed. First, the 1.3 kb *pilD*-specific PCR product (see above) was cloned into the vector pCR2.1 (Clontech Laboratories). The *pilD* gene was then removed as a *Kpn*I/*Xba*I fragment and ligated into the broad-host-range vector pBBR1MCS (Kovach *et al*., 1995), generating pMRL13. The orientation of *pilD* in pMRL13 was in the same direction as *lacZ* transcription, permitting expression of the gene in the absence of the *pilBCD* promoter. The *pilD* mutant was electroporated with pMRL13 or pBBR1MCS as a control, and colonies were selected on BCYE containing Chl. A Southern blot of *Hin*dIII-digested DNA isolated from transformants was hybridized with the *pilD* probe to verify the presence of pMRL13 and its mutated *pilD*. Passage of NU243 (pMRL13) on BCYE agar without antibiotic selection resulted in plasmid loss. Thus, NU243 (pMRL13), NU243 (pBBR1MCS) and 130b (pBBR1MCS) were always grown on media containing Chl.

Electron microscopic examination of L. pneumophila

Pili on the surface of *L. pneumophila* were visualized as before (Liles *et al*., 1998). Strains were grown on BCYE agar, and then isolated colonies were suspended in PBS and adhered to Formvar-coated copper grids (Ladd Industries). Bacteria adherent to the grid were stained using 10 μl of 1% phosphotungstic acid for 2 min and visualized on a Jeol JEM-100 CxII transmission electron microscope at 60 kV.

SDS–PAGE analysis of proteins in L. pneumophila *supernatants*

Excreted proteins of *L. pneumophila* were analysed essentially as described previously (Keen and Hoffman, 1989). Bacteria were grown for up to 18 h at 30°C or 37°C in 2 l flasks containing 250 ml of aerated BYE. Supernatants were isolated by centrifugation of the cultures at 12 000 × *g* at 4°C, followed by filtration through 0.2 μm membranes. The samples were concentrated to 10 ml with a 1 kDa (YM1 membrane) ultrafiltration cell (Millipore), and then the concentrates were dialysed against three 1 l volumes of 50 mM Tris buffer containing 5 mM EDTA (pH 7.8). The amount of protein in each sample was determined by the Lowry assay (Ausubel *et al*., 1987). Samples containing equivalent amounts of protein in SDS–loading buffer were then boiled for 5 min before separation on either a 12.5% or a 15% SDS–polyacrylamide gel run at 25 V for 15 h at 15°C (Ausubel *et al*., 1987). After electrophoresis, protein bands were stained with Coomassie blue for visualization and compared with molecular weight standards (New England Biolabs) (Ausubel *et al*., 1987).

Detection of L. pneumophila *enzymatic activities*

To observe the haemolytic activity resulting from the zinc metalloprotease of *L. pneumophila* (Keen and Hoffman, 1989), strains were grown on BYE agar supplemented with 5% defibrinated sheep erythrocytes and examined daily for evidence of β-haemolysis. Acid phosphatase activity was detected by lifting *L. pneumophila* colonies from BCYE agar on a nitrocellulose filter onto an indicator plate containing 0.8% agarose and 40 μg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate (pH 5.0) (Albano *et al*., 1992), with a positive reaction generating a blue colour. Liquid phosphatase assays were conducted on concentrated bacterial supernatants using the Sigma 104 phosphatase substrate (Albano *et al*., 1992).

Intracellular infection of U937 cells and amoebae by L. pneumophila

Human U937 cells, which differentiate into adherent, macrophage-like cells after treatment with phorbol esters, have been used by many investigators to study the intracellular replication of *L. pneumophila* (Pearlman *et al*., 1988; Cianciotto *et al*., 1990; Fields *et al*., 1990; Hacker *et al*., 1991; Berger and Isberg, 1993). U937 cell monolayers were prepared and infected as described previously (Cianciotto *et al*., 1990; Pearlman *et al*., 1988). The number of viable bacteria added to the macrophage monolayers was determined by plating inocula on BCYE agar. After inoculation with approximately 10^6 cfu, replicate monolayers ($n = 3$) consisting of 10^6 U937 cells were incubated for 2 h to permit bacterial uptake and then washed to remove unattached bacteria. The infected monolayers were then incubated at 37°C in RPMI-1640 medium supplemented with heat-inactivated 5% fetal bovine serum (FBS; Gibco BRL). After incubation for 0–72 h, the monolayers were lysed in 0.1% saponin (Sigma), an agent that acts upon cholesterol and hence does not alter the viability of bacteria (Leung *et al*., 1997). Finally, 10-fold serial dilutions of the lysates were plated on BCYE agar. The resulting cfu were used to calculate the corresponding numbers of bacteria per monolayer. As *L. pneumophila* does not replicate in the tissue culture medium, all cfu recovered represent bacteria that have replicated intracellularly. For the assessment of the cytopathic effect of strains for U937 cells, infected monolayers ($n = 6$) were treated with the vital stain 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide as described previously (Pearlman *et al*., 1988). To each well was added 5 μl of 5 mg ml^{-1} of the stain and, after 4 h of incubation, the formazan product

was dissolved in acid isopropanol. The OD_{570} of individual wells was then determined.

To assess the ability of legionellae to grow within protozoa, *Hartmannella vermiformis* was used as a host, as performed by various investigators (Fields *et al*., 1986; Cianciotto and Fields, 1992; Venkataraman *et al*., 1997). The co-culture was carried out as described previously (Cianciotto and Fields, 1992). Briefly, approximately 10^5 amoebae were suspended in 2 ml of axenic medium 1034 without FBS that was diluted 1:1 with Puck's saline and added to replicate wells ($n = 4$) in a 24-well tissue culture plate. Next, bacteria suspended in assay medium were added at a final concentration of 10^5 cfu ml^{-1}, and the co-cultures were incubated for 5 days at 35°C. Numbers of bacteria in each well were determined at various time points by plating dilutions on BCYE agar. As the assay medium does not support the growth or survival of *L. pneumophila*, the cfu represent bacteria that have replicated within the amoebae.

Experimental infection of guinea pigs by L. pneumophila

To assess the virulence of *L. pneumophila* strains, guinea pigs were infected intratracheally with the bacteria as described previously (Winn *et al*., 1982; Edelstein *et al*., 1984; Cianciotto *et al*., 1990); this mode of infection produces bilateral pneumonia similar to that seen in immunocompromised patients with Legionnaires' disease (Winn *et al*., 1982; Edelstein *et al*., 1984). Inocula for infection were prepared by first culturing the bacteria on BCYE agar, then in BYE broth, at 37°C in a shaking incubator (Edelstein *et al*., 1984). Mid-log phase cultures (10^8 cfu ml^{-1}) were used. The bacteria were then suspended at the desired concentrations in sterile water for injection. Bacteria were injected into the surgically exposed tracheas in 0.3 ml volumes using a syringe and needle. The bacterial inoculum delivered was determined by quantitative plating of the inoculum onto BCYE agar.

Two animal studies were performed, i.e. an infectious dose titration study and a bacterial clearance study. Both studies used Hartley male guinea pigs weighing 250–300 g. For the titration study, animals in randomly assigned groups of four each were infected with either 1×10^6, 1×10^7 or 1×10^8 cfu of NU243. The animals were observed clinically for 7 days, with daily measurements of weight and temperature for the first 5 days. A previous study using the same methods showed that the parent strain 130b strain killed three out of four animals given 6×10^6 cfu by 4 days after infection, and no animals given 1×10^5 cfu (unpublished data). The bacterial clearance study was performed by infecting 16 guinea pigs with 8×10^5 cfu of 130b and 19 animals with 8×10^7 cfu of NU243. The group assignments were random. The body weight and rectal temperature of each animal were measured daily. Four animals in each group were sacrificed daily for 3 days, starting 1 day after infection. Animals were killed in numerical order to avoid bias resulting from degree of illness. Two animals receiving 130b were killed on day 4 and two on day 6; the respective numbers for NU243 were four and three. The right lower lobe of the lung and the spleen were harvested aseptically, and their ground homogenates were cultured quantitatively on BCYE agar. The lower limit of detection of bacteria in the organs was about 100 cfu per organ. The remaining lungs were fixed in cold (4°C) 10% buffered formalin for 1 week before dissection (Edelstein *et al*., 1984). Each day, one set of animal tissues from NU243 recipients was also cultured quantitatively on BCYE agar containing Kan to look for revertants. Histology was performed on the fixed lungs of all animals, which were coded to obscure animal group. Three or four of these sections were stained per lung with haematoxylin and eosin and examined microscopically at 25× magnification. The average amount of consolidation in the sections was estimated as a percentage of the total area of the section (Edelstein *et al*., 1984). The code of treatment group identity was broken only after all results had been recorded.

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