Role of the *Legionella pneumophila* rtxA gene in amoebae

Suat L. G. Cirillo, Ling Yan, Michael Littman, Mustapha M. Samrakandi and Jeffrey D. Cirillo

Author for correspondence: Jeffrey D. Cirillo. Tel: +1 402 472 8587. Fax: +1 402 472 9690.
e-mail: jcirillo1@unl.edu

Department of Veterinary and Biomedical Sciences, University of Nebraska, Lincoln, 203 VBS, Fair and East Campus Loop, Lincoln, NE 68583, USA

***Legionella pneumophila* infects humans, causing Legionnaires' disease, from aerosols generated by domestic and environmental water sources. In aquatic environments *L. pneumophila* is thought to replicate primarily in protozoa. A 'repeats in structural toxin' (RTX) gene, *rtxA*, from *L. pneumophila* was identified recently that plays a role in entry and replication in human macrophages and also has the ability to infect mice. However, the role of this gene in the interaction of *L. pneumophila* with environmental protozoa and its distribution in different *Legionella* species has not been examined. Southern analyses demonstrated that *rtxA* is present in all *L. pneumophila* isolates tested and correlates with species that have been shown to cause disease in humans. To evaluate the importance of *rtxA* in the interaction with protozoa a series of studies was carried out in an environmental host for *L. pneumophila*, *Acanthamoeba castellanii*. The *L. pneumophila* *rtxA* gene plays a role in both adherence and entry into *A. castellanii* similar to that observed in human monocytic cells. Furthermore, it was found that *rtxA* is involved in intracellular survival and trafficking. In addition to demonstrating involvement of *rtxA* in the interaction of *L. pneumophila* with host cells, these data support a role for this gene both during disease in humans and in environmental reservoirs.**

Keywords: phagocytosis, lysosomes, amoeba, pathogenesis, pneumonia

INTRODUCTION

Legionnaires' disease, a potentially lethal pneumonia, is the result of the ability of *Legionella* to enter, survive and replicate in macrophages (Davis *et al.*, 1983; Nash *et al.*, 1984; Winn & Myerowitz, 1981). There are not any known animal reservoirs for *L. pneumophila*; however, protozoa in the environment provide an important environmental source of the bacteria that infect humans (Anand *et al.*, 1983; Henke & Seidel, 1986; Rowbotham, 1980, 1986). Since person to person transmission is not thought to occur (Yu *et al.*, 1983), it appears that the natural hosts for *L. pneumophila* are these aquatic protozoa. In addition, the interaction of *L. pneumophila* with protozoa in the environment appears to enhance the ability to infect mammalian cells (Cirillo *et al.*, 1994) and cause disease in animals (Brieland *et al.*, 1996, 1997a, b; Cirillo *et al.*, 1999). Thus, it is likely that the virulence mechanisms used by *L. pneumophila* to

cause disease in humans evolved for optimal parasitism of protozoa.

Acanthamoeba castellanii, one potential environmental host for *L. pneumophila* (Rowbotham, 1980), undergoes a respiratory burst (Davies *et al.*, 1991), produces oxygen radicals (Davies & Edwards, 1991), expresses cell surface receptors (Allen & Dawidowicz, 1990a) and has phagocytic mechanisms (Allen & Dawidowicz, 1990b; Brown *et al.*, 1975; Lock *et al.*, 1987) similar to human macrophages. Furthermore, the mechanisms that provide a selective advantage in amoebae and macrophages are likely to be similar, since they enter by coiling phagocytosis (Bozue & Johnson, 1996; Horwitz, 1984), inhibit lysosomal fusion (Bozue & Johnson, 1996; Horwitz & Maxfield, 1984) and localize to a rough endoplasmic reticulum-lined compartment (Abu Kwaik, 1996; B. S. Fields *et al.*, 1986; Horwitz, 1983; Newsome *et al.*, 1985; Swanson & Isberg, 1995) in both cells. Certainly, a large number of potential virulence factors have been identified that affect the ability of *L. pneumophila* to survive in macrophages and amoebae (Cianciotto & Fields, 1992; Gao *et al.*, 1997; Pruckler *et al.*

Abbreviations: LY, lucifer yellow; RhR, rhodamine red; RTX, repeats in structural toxin.

al., 1995; Segal & Shuman, 1999). Although there are at least 42 *Legionella* species, approximately 90% of Legionnaires' disease cases in humans is caused by *Legionella pneumophila* (Marston *et al.*, 1994, 1993; Reingold *et al.*, 1984). Only 18 of the remaining species are infrequently associated with disease and the rest are primarily found in aquatic environments (Morris *et al.*, 1979; Rowbotham, 1993). Even in these aquatic environments, *L. pneumophila* is the predominant species isolated (Fliermans *et al.*, 1981).

These data suggest that *L. pneumophila* differs from other *Legionella* species in a manner that provides a selective advantage in both humans and the environment. Recently, our laboratory has identified three *L. pneumophila* loci that are involved in entry into human epithelial cells and macrophages (Cirillo *et al.*, 2000). A gene present in one of these loci, *rtxA*, encodes a 'repeats in structural toxin' (RTX). RTX proteins are a large family of pore-forming cytolysins present in a number of different bacterial pathogens (Welch, 1991). The *L. pneumophila rtxA* gene has previously been shown to play a role in entry and replication in macrophages and virulence in mice (Cirillo *et al.*, 2001). In the current study, we examined the distribution of the *rtxA* gene in different isolates of *Legionella*, including both environmental and clinical samples. Although the presence of *rtxA* correlates with the ability to cause disease in humans we found that it also affects the ability of *L. pneumophila* to infect, traffic and survive in amoebae. These data indicate that *rtxA* may affect both the severity of disease in humans and the ability of *L. pneumophila* to persist in environmental reservoirs.

METHODS

Bacterial strains and growth conditions. The *L. pneumophila* strains used for these studies were a streptomycin-resistant variant of AA100 transformed with the kanamycin-resistant vector pYUB289 (Cirillo *et al.*, 2000) to control for effects of antibiotic resistance, the same strain containing an in-frame deletion of *rtxA* and transformed with pJDC35 ($\Delta rtxA::pJDC35$) for complementation or pJDC40 ($\Delta rtxA::pJDC40$) as a vector control, as well as environmental and clinical isolates as listed in Table 1. The *rtxA* mutant is an in-frame deletion that includes only the amino-terminal 6 aa and carboxy-terminal 124 aa of RtxA (Cirillo *et al.*, 2001). This mutation can be complemented by transformation of the mutant with the wild-type *rtxA* gene (Cirillo *et al.*, 2001). As described previously (Cirillo *et al.*, 2001), pJDC35 is an integrating vector containing the complete *rtxA* gene and pJDC40 is the same vector with *rtxA* deleted. Strain ψ lp55 is an AA100 clone that has been passed 14 times in the laboratory until it is sodium-resistant and unable to replicate in monocytic cells (data not shown). All *L. pneumophila* strains other than ψ lp55, including the environmental and clinical isolates, were passaged no more than twice in our laboratory before use in these studies to prevent loss of virulence. *L. pneumophila* was grown on BCYE agar (Edelstein, 1981) for 3 days at 37 °C in 5% CO₂. When necessary, kanamycin (Sigma) was added at a concentration of 25 µg ml⁻¹ to bacterial growth medium.

Amoeba. The amoeba *A. castellanii* ATCC 30234 was grown in PYG broth in 75 cm² tissue culture flasks in the dark at 23 °C (Cirillo *et al.*, 1994; Moffat & Tompkins, 1992).

Amoeba cells were brought into suspension by rapping the flask sharply and the number of viable cells was determined as described by Moffat & Tompkins (1992).

Entry and adherence assays. Entry assays were carried out as described previously (Cirillo *et al.*, 1994). Amoebae were seeded in 24-well tissue culture dishes (Falcon) at a concentration of 1.5×10^5 cells per well and allowed to adhere overnight at 23 °C in the dark. The cells were then washed with Ac buffer (HS) (Moffat & Tompkins, 1992), 1 ml HS added and equilibrated at 37 °C for 1 h. The bacteria to be assayed were suspended and diluted in HS and added to the cells at an m.o.i. of 10. After the bacteria were allowed to interact with the cells for 30 min at 37 °C the wells were then washed with HS and incubated in HS plus 100 µg gentamicin ml⁻¹ for 2 h. After antibiotic treatment the cells were washed with HS, then lysed by incubation for 10 min in 1 ml water followed by vigorous pipetting. After lysis, the number of intracellular bacteria was determined by plating for c.f.u. on BCYE. Entry levels were determined by calculating the percentage of the inoculum that became gentamicin-resistant over the course of the assay (i.e. entry = c.f.u. gentamicin-resistant/c.f.u. inoculum). To correct for variation in levels of uptake between experiments, entry is reported relative to AA100 [i.e. percentage entry = (entry test strain/entry AA100) × 100]. Adhesion was tested in a similar manner to that for entry, except that bacteria were added to the cells, mixed and immediately washed three times to remove non-adherent bacteria prior to lysis.

Intracellular growth assays. Intracellular growth assays were carried out as described previously (Cirillo *et al.*, 1997). Bacteria were added to a monolayer of 1.5×10^5 amoebae in 24-well tissue culture dishes at an m.o.i. of 0.5 and incubated at 37 °C for 30 min. The cells were washed three times with HS, incubated in HS plus 100 µg gentamicin ml⁻¹ for 2 h, placed in fresh HS and lysed at various time points with water. Survival is expressed as the percentage of c.f.u. present at each time point as compared to time zero (2.5 h).

Frequencies of lysosomal fusion. Lysosomes were stained and the frequency of fusion with the bacterial phagosome was determined by transmission electron microscopy, as described previously (Cirillo *et al.*, 1997). After pre-labelling the amoebae with thorium dioxide (Polysciences) they were infected with the bacteria for 30 min. The samples were then fixed and prepared for electron microscopy at various time points as described previously (Bowers & Korn, 1968; Cirillo *et al.*, 1997; Niszl & Markus, 1989).

Lysosomes were stained for fluorescence microscopy with lucifer yellow (LY) as described by Bizal *et al.* (1991), Heinzen *et al.* (1996) and Swanson (1989). After pre-labelling the amoebae with 1 mg LY ml⁻¹ for 2 h the cells were washed with HS and incubated in HS for 1 h at 37 °C. *L. pneumophila* cells were pre-stained with rhodamine red (RhR) prior to infection in a similar manner to that previously described using fluorescein (Francis *et al.*, 1993; Wiater *et al.*, 1998). Basically, the bacteria were suspended in PBS plus 5 µg RhR ml⁻¹, incubated at 37 °C for 30 min and washed five times to remove unbound RhR. The RhR staining procedure did not affect bacterial viability or intracellular trafficking. Viability of the labelled bacteria was confirmed using the LIVE-DEAD assay (Molecular Probes) and by plating dilutions for c.f.u. on BCYE agar (Difco). The LY-labelled amoebae were infected with the bacteria for 30 min, washed with HS and incubated in HS for various periods of time. At each time point the monolayers were fixed in 4% (v/v) paraformaldehyde for 30 min and examined on a Nikon TE300 inverted microscope

Table 1. Characteristics of *Legionella* strains

Strain (isolate)	<i>rtxA</i> *	Disease†	Reference
<i>L. pneumophila</i> sg 1 (130b)	+	+	Engleberg <i>et al.</i> (1984)
<i>L. pneumophila</i> sg 1 (Allentown I) ATCC 43106	+	+	Joly <i>et al.</i> (1986)
<i>L. pneumophila</i> sg 1 (Camperdown 1) ATCC 43113	+	+	Joly <i>et al.</i> (1986)
<i>L. pneumophila</i> sg 1 (Benidorm 030 E) ATCC 43108	+	+	Joly <i>et al.</i> (1986)
<i>L. pneumophila</i> sg 1 (OLDA) ATCC 43109	+	+	Joly <i>et al.</i> (1986)
<i>L. pneumophila</i> sg 7 (Chicago 8) ATCC 33823	+	+	Bibb <i>et al.</i> (1983)
<i>L. anisa</i> (CH-47-C1) ATCC 35290	—	—	Gorman <i>et al.</i> (1985)
<i>L. brunensis</i> (441-1) ATCC 43878	—	—	Wilkinson <i>et al.</i> (1988)
<i>L. feeleyi</i> sg 1 (WO-44C) ATCC 35072	+	+	Herwaldt <i>et al.</i> (1984)
<i>L. longbeachae</i> sg 2 (Tucker 1) ATCC 33484	—	+	Bibb <i>et al.</i> (1981)
<i>L. gormanii</i> (86A5796) ATCC 43769	—	—	Morris <i>et al.</i> (1980)
<i>L. moravica</i> (316-86) ATCC 43877	—	—	Wilkinson <i>et al.</i> (1988)
<i>L. dumoffii</i> (NY 23) ATCC 33279	—	—	Brenner <i>et al.</i> (1980)
<i>L. saintelensis</i> (MSH-4) ATCC 35248	—	—	Campbell <i>et al.</i> (1984)

* The presence of the *rtxA* gene in the chromosome, as demonstrated by Southern analysis, is indicated by + and the absence by —.

† Strains that were directly isolated from diseased human lungs or strongly linked to disease (data exist showing that this isolate is the same as one directly isolated from diseased lungs) are indicated by + and environmental isolates that are not associated or only weakly (no data exist showing that this isolate is the same as one that causes disease) or rarely associated with disease in humans by —.

with fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate filters. Dual images were captured using an Optronics charge-coupled device video camera for subsequent manual quantification of lysosomal fusion frequencies. Images were merged and adjusted for brightness and contrast using Adobe Photoshop version 5.5.

Cytotoxicity and pore formation. The standard lactate dehydrogenase (LDH) release cytotoxicity assay (Behl *et al.*, 1994; Brander *et al.*, 1993) was used in these studies as described previously (Cirillo *et al.*, 2001). The procedure used was essentially as recommended by the manufacturer of the CytoTox96 Non-Radioactive Cytotoxicity Assay system (Promega). Serial dilutions were made of each bacterial strain at m.o.i.s of 500, 250, 100 and 10 in a final volume of 100 µl for each assay using 2×10^4 amoebae. Appropriate numbers of cells for CytoTox96 assays were determined as suggested by the manufacturer (Promega). As a positive control for 100% cytotoxicity and the utility of this assay in amoebae, the cells were lysed with 9% (v/v) Triton X-100 (Promega). The cells were incubated with the bacteria for 4 h at 37 °C with 5% (v/v) CO₂. Cytotoxicity readings were taken using an ELISA plate reader at 450 nm. Percentage cytotoxicity was calculated as recommended by the manufacturer and corrected for small differences in the inocula used.

Formation of pores in host cells was assayed by ethidium bromide and acridine orange staining in exactly the same manner as described previously (Cirillo *et al.*, 2001; Kirby *et al.*, 1998; Zuckman *et al.*, 1999) using *A. castellanii* cells. Stained coverslips were examined using a Nikon TE300 inverted microscope with fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate filters. Dual images of multiple fields were captured using an Optronics charge-coupled device video camera and analysed in the same manner as described by Zuckman *et al.* (1999). Pore formation is expressed as the percentage of acridine-orange-stained cells that also stain with ethidium bromide resulting from incorporation of this dye into chromosomal DNA due to

increased permeability of the host cell. All cells were stained with acridine orange since, unlike ethidium bromide, acridine orange readily crosses membranes of eukaryotic cells. As a positive control, amoebae were first fixed with formaldehyde and then permeabilized with methanol prior to staining. Under these conditions, all amoebae stained well with both ethidium bromide and acridine orange.

Southern analyses and colony hybridization. Probes were labelled by PCR with digoxigenin using the methods suggested by the manufacturer of the Genius System (Boehringer Mannheim). Membranes were prepared by the suggested methods for both colony hybridization and Southern analysis. Hybridization and washes were carried out at several different stringencies to ensure that the data obtained were not due to sequence divergence between *Legionella* species. Experiments generating the data shown were carried out under low stringency hybridization conditions. The probe for the *rtxA* gene was prepared by PCR using oligo 1 (5'-CTGATGCTGCTACGGAACAC-3') and oligo 2 (5'-CCG-CAGTCATTACACCTGCG-3'). This resulted in the production of a 542 bp fragment within the *rtxA* gene. The probe for the *mip* gene was also prepared by PCR using oligo 3 (5'-GCAGCTGTTATGGGGCTTGC-3') and oligo 4 (5'-GGC-AATACAACAACGCCTGG-3'), producing a 352 bp fragment within the *mip* gene.

Statistical analyses. All experiments were carried out in triplicate and repeated at least twice. Significance of the results was analysed using ANOVA. Values of $P < 0.05$ were considered significant.

RESULTS

Correlation of *rtxA* with disease

The *rtxA* gene has previously been associated with the ability of *Legionella* to cause disease in mice (Cirillo *et al.*, 2001). However, it remains unclear whether *rtxA* is

Fig. 1. Southern analysis of different *Legionella* isolates. All lanes are *EcoRI* digests of total chromosomal DNA probed with a 542 bp internal fragment of the *Legionella rtxA* gene. This blot was hybridized under low stringency conditions. The positions of λ phage DNA size standards cut with *HindIII* are shown on the left. *Lp*, *Legionella pneumophila*.

of particular importance for pathogenesis in humans or is required for survival and replication of *Legionella* in the environment. Predominance in the environment is likely to correlate with the ability of different *Legionella* to interact positively with water-borne protozoa, including amoebae. To better evaluate the importance of the *rtxA* gene in human disease and survival in the environment, we examined whether there is a correlation between the presence of this gene and the strength of association of different *Legionella* isolates with human disease. The presence of the *rtxA* gene in 14 *Legionella* isolates was determined by Southern analysis (Fig. 1). The approximately 5 kbp fragment visible in *L. pneumophila* Allentown and Benidorm corresponds to the size of fragment observed in AA100 (data not shown). Under the same hybridization conditions, a probe from the *mip* gene hybridizes to chromosomal DNA from all of these species, suggesting that sequence divergence alone is not responsible for the inability to detect *rtxA* (data not shown). Differences in signal intensity by Southern analysis were observed in species other than *L. pneumophila*, suggesting that, as expected due to their more distant relationship to AA100, greater DNA sequence divergence exists within the *rtxA* gene in these species. Since nearly all *Legionella* species are thought to be able to cause disease at some frequency, we used stringent criteria to evaluate whether the isolates tested are strongly or weakly associated with disease in our studies. Thus, if an isolate was directly obtained from post-mortem tissue or sputum from a clinically ill individual or there was documented evidence that it is the same as that causing disease, the isolate was considered strongly associated with disease. If, however, the isolate was obtained from an environmental source, even if it was the same species as that observed to cause

Fig. 2. The ability of the wild-type *L. pneumophila* strain AA100, $\Delta rtxA$ mutant ($\Delta rtxA::pJDC40$) and complemented clone ($\Delta rtxA::pJDC35$) to adhere to (a) and enter into (b) *Acanthamoeba castellanii*. Data points and error bars represent the means of triplicate samples and their standard deviations, respectively. The adherence and entry levels of wild-type *L. pneumophila* strain AA100 are arbitrarily set to 100%. All experiments were performed at least three times.

disease in humans, or the species of the isolate only rarely causes disease in humans, we considered the isolate to be only weakly associated with human disease. Using these criteria, eight of the isolates examined had a strong association with disease and, of these, seven (88%) also had the *rtxA* gene (Table 1). Furthermore, none of the isolates that are only weakly or rarely associated with disease in humans has the *rtxA* gene. Thus, the presence of the *rtxA* gene correlates ($P < 0.01$) with the ability of *Legionella* to cause disease in humans.

***L. pneumophila* does not cause cytotoxicity or pore formation in amoebae**

The prevalence of certain *Legionella* strains in human disease may be the result of higher virulence, a selective advantage in the environment or a combination of these features. As a measure of the importance of *rtxA* in the

Fig. 3. Growth of wild-type *L. pneumophila* strain AA100 (□), $\Delta rtxA$ mutant ($\Delta rtxA::pJDC40$, ○) and complemented clone ($\Delta rtxA::pJDC35$, △) in amoebae. All data are expressed relative to time zero ($t_0=2.5$ h). * ($P<0.05$) and ** ($P<0.01$) indicate data points significantly different from wild-type. Data points and error bars represent the means of quadruplicate samples and their standard deviations, respectively. All experiments were performed at least three times.

Fig. 4. Fusion of lysosomes containing thorium (electron-dense granules) with the *L. pneumophila* strain AA100 (A, C) and $\Delta rtxA$ mutant (B, D) vacuole 120 min after entry. Examples of unfused vacuoles are shown for the wild-type (A) and *rtxA* mutant (B). Fused vacuoles are infrequently observed for the wild-type strain AA100 (C), whereas approximately half of the *rtxA* mutant vacuoles contain thorium (D). Bar, 1 μ m.

survival of *L. pneumophila* in the environment, we examined the effects of this gene on the interaction of *L. pneumophila* with *A. castellanii*. An in-frame deletion

Table 2. Fusion of *Legionella* vacuole with lysosomes by electron microscopy

Time after entry (min)	Percentage fusion*		
	AA100	$\Delta rtxA::pJDC40$	$\Delta rtxA::pJDC35$
15	27.9 $\pm$ 2.4	32.8 $\pm$ 1.8	25.1 $\pm$ 1.6
60	24.2 $\pm$ 1.3	45.1 $\pm$ 0.8†	23.9 $\pm$ 1.2
120	21.2 $\pm$ 2.4	54.7 $\pm$ 2.0†	20.5 $\pm$ 2.1

* Percentage of wild-type (AA100), $\Delta rtxA$ mutant ($\Delta rtxA::pJDC40$) and complemented mutant ($\Delta rtxA::pJDC35$) *L. pneumophila* vacuoles containing thorium. Results are the mean values $\pm$ SE for three counts of vacuoles within at least 50 amoebae on different sections of the same preparation.

† Significantly different ($P<0.01$) from the result for wild-type *L. pneumophila*.

of the *L. pneumophila rtxA* gene transformed with a single-copy construct that complements the *rtxA* mutation (pJDC35) or the same construct without *rtxA* (pJDC40) were used for these studies. All of the strains used in the current study, as well as their phenotypes in mammalian cells, have been described previously (Cirillo *et al.*, 2001). In contrast to previous results with murine and human macrophages (Cirillo *et al.*, 2001; Kirby *et al.*, 1998), no cytotoxicity or pore formation was observed after incubation of wild-type *L. pneumophila* or the *rtxA* mutant with *A. castellanii* (data not shown). To ensure that the conditions of the experiment were not responsible for this negative result, we examined bacteria to host-cell ratios as high as 1000:1, as well as co-incubation periods from 1 to 6 h. None of these conditions resulted in significant cytotoxicity or pore formation (data not shown). In addition, the positive controls of Triton-X-100-killed amoebae (cytotoxicity) and permeabilization with methanol after fixation followed by staining for fluorescent microscopy (pore formation) demonstrated that these assay methods work well in amoebae (data not shown). Thus, *L. pneumophila* does not cause cytotoxicity or pore formation in *A. castellanii*.

rtxA affects adherence and entry

Since the *L. pneumophila rtxA* gene plays a role in entry into human cells (Cirillo *et al.*, 2001, 2000), we examined its role in adherence and entry into *A. castellanii* (Fig. 2). The *L. pneumophila rtxA* mutation reduced adherence ($P<0.03$) and entry ($P<0.02$) to amoebae by approximately 50% compared to wild-type. Levels of adherence that were not significantly different than wild-type *L. pneumophila* were obtained in the *rtxA* mutant carrying a complementing construct, pJDC35. All strains were tested for sensitivity to assay conditions such as osmotic lysis, culture medium and serum. No significant differences in sensitivity to these conditions were observed with the strains used. These data suggest that, similar to its role in mammalian cells, *rtxA* is

Fig. 5. Fusion of lysosomes (green) containing LY with the *L. pneumophila* (red) vacuoles for strains AA100 (A–F), Δ *rtxA* (G–L) and the avirulent ψ lp55 (M–R). All panels shown are 150 min after infection of the amoebae. Split and merged (Lp + LY) images are shown using RhR and LY filter sets. The red colour indicates RhR-stained bacteria, which appear as rods (A, D, G, J) or, when they are presumably partially degraded, as diffuse red-staining regions (M, P). The green colour indicates lysosomes stained with LY. When the bacterial vacuole co-localizes with lysosomes, a yellow colour results in the merged images (F, L, O, R). Fused vacuoles are infrequently observed for the wild-type strain AA100 (F), whereas approximately half of the *rtxA* mutant (L) and the majority of the ψ lp55 (O, R) vacuoles co-localize with LY. Bar, 10 μ m.

involved in adherence to and entry into amoebae in environmental reservoirs.

rtxA plays a role in intracellular survival

Since *rtxA* plays a role in entry by *L. pneumophila* into amoebae it may also impact subsequent intracellular events. To evaluate this possibility, we compared the ability of the *rtxA* mutant to survive and replicate in amoebae with wild-type *L. pneumophila* (Fig. 3). The *rtxA* mutant did not survive as well as wild-type

($P < 0.01$) for the majority of time points through the first 32 h after entry. Interestingly, the *rtxA* mutant complemented with a plasmid-borne copy of the gene displayed enhanced intracellular survival. Since *rtxA* does not affect growth in laboratory medium (Cirillo *et al.*, 2001), its role in survival is specific to the intracellular growth environment. The *rtxA* mutant was killed (80%) much more efficiently than wild-type (62%) during the first 6 h in amoebae. The subsequent rate of intracellular replication was the same as wild-type, with the *rtxA* mutant increasing 50-fold and wild-type 45-fold by 32 h.

Table 3. Fusion of *Legionella* vacuole with lysosomes by fluorescent microscopy

Time after entry (min)	Percentage fusion*			
	AA100	$\Delta rtxA::pJDC40$	$\Delta rtxA::pJDC35$	$\psi lp55$
30	30.0 $\pm$ 1.2	35.8 $\pm$ 2.4†	28.7 $\pm$ 0.7	46.6 $\pm$ 1.4‡
90	22.8 $\pm$ 0.3	43.0 $\pm$ 1.2‡	22.9 $\pm$ 1.0	78.6 $\pm$ 1.2‡
150	24.0 $\pm$ 1.0	43.7 $\pm$ 0.9‡	23.3 $\pm$ 0.9	78.1 $\pm$ 3.0‡

* Percentage of wild-type (AA100), $\Delta rtxA$ mutant ($\Delta rtxA::pJDC40$), complemented mutant ($\Delta rtxA::pJDC35$) and avirulent mutant ($\psi lp55$) *L. pneumophila* vacuoles containing LY. Results are the mean values $\pm$ SE for three counts of vacuoles within at least 50 amoebae from different samples.

† Significantly different ($P < 0.05$) from the result for wild-type *L. pneumophila*.

‡ Significantly different ($P < 0.01$) from the result for wild-type *L. pneumophila*.

These data suggest that *rtxA* is primarily involved in the early steps of the interaction of *L. pneumophila* with amoebae.

Role of *rtxA* in intracellular trafficking

One potential mechanism by which a gene that affects entry might impact initial intracellular survival would be through changes in trafficking of the bacterial vacuole resulting from signal transduction during phagocytosis. *L. pneumophila* normally has the ability to inhibit lysosomal fusion in macrophages (Horwitz & Maxfield, 1984) and amoebae (Bozue & Johnson, 1996). We examined the frequencies of lysosomal fusion with the wild-type and *rtxA* mutant bacterial vacuole by electron microscopy using thorium dioxide (Fig. 4), an electron-dense colloidal marker that is retained within secondary lysosomes (Armstrong & Hart, 1971). Vacuoles containing the *rtxA* mutant fused with lysosomes at a significantly higher rate ($P < 0.01$) than wild-type *L. pneumophila* (Table 2). Although there was no significant difference in the frequencies of fusion between the *rtxA* mutant and wild-type at 15 min, the difference was nearly twofold by 1 h and more than twofold by 2 h after entry.

To confirm this result using a different method we prelabelled the amoebal lysosomes with LY. This marker has been used extensively to label secondary lysosomes in macrophages and other eukaryotic cells (Bizal *et al.*, 1991; Heinzen *et al.*, 1996; McClure & Schiller, 1996; Straub *et al.*, 1997; Swanson, 1989). This label allowed clear differentiation between fused and unfused bacterial vacuoles (Fig. 5). In addition, similar frequencies of lysosomal fusion were obtained for wild-type *L. pneumophila* to those by electron microscopy (Table 3). The laboratory-passaged avirulent AA100 mutant $\psi lp55$ displayed the expected high frequencies of lysosomal fusion (78%) observed previously for similar mutants (Roy *et al.*, 1998; Swanson & Isberg, 1996). We found that, even as early as 30 min after entry, the *rtxA* mutant fused with LY-labelled lysosomes at a significantly higher level ($P < 0.05$) than wild-type *L. pneu-*

mophila. The difference in the frequencies of lysosomal fusion between the *rtxA* mutant and wild-type was much more pronounced by 90 min ($P < 0.01$). These data confirm that the *L. pneumophila rtxA* gene plays a role in intracellular trafficking in amoebae.

DISCUSSION

We have found that the *L. pneumophila rtxA* gene is not present in all *Legionella* species and that its presence correlates with the ability to cause disease in humans. This observation, along with previous studies demonstrating that this gene plays an important role in virulence (Cirillo *et al.*, 2001), has helped to fulfil 'molecular Koch's postulates' for *rtxA*, supporting the conclusion that this gene plays a role in disease (Falkow, 1988). There are three criteria required to fulfil these postulates. First, specific inactivation of the *rtxA* gene leads to a measurable loss of virulence in mice (Cirillo *et al.*, 2001). Second, complementation of the mutated *rtxA* gene leads to restoration of virulence in mice (Cirillo *et al.*, 2001). Third, the *rtxA* gene is associated with pathogenic members of the genus, as demonstrated in the current study. This represents an important step in our understanding of the molecular aspects of Legionnaires' disease. However, although *rtxA* fits the criteria for an important role in pathogenesis, the primary function(s) of this gene both during disease and in the environment are not fully understood.

Similar to data obtained in mammalian cells (Cirillo *et al.*, 2001, 2000), the *rtxA* gene affects adherence and entry into amoebae. The mechanism by which *rtxA* affects adherence and entry in mammalian cells is not known, though it may be the result of the ability of RTX proteins to bind to β_2 integrin receptors (Ambagala *et al.*, 1999; Lally *et al.*, 1997). Although receptors analogous to integrins have not yet been identified in *Acanthamoeba*, they have been identified in the amoebae *Hartmannella* (Venkataraman *et al.*, 1997) and *Entamoeba* (Adams *et al.*, 1993; Vines *et al.*, 1998) as well as in coral and sponges (Brower *et al.*, 1997). These observations suggest that integrin receptors are highly

conserved throughout all eukaryotic cells. This conclusion is supported by evidence that the *Entamoeba* receptor shares an immunologically cross-reactive epitope with human β_2 integrins (Adams *et al.*, 1993) and contains a β_2 integrin motif in its cytoplasmic domain (Vines *et al.*, 1998). Thus, it remains possible that a similar receptor is available for binding to *L. pneumophila* via RtxA in both macrophages and amoebae. Considering that a similar receptor may be utilized, it is surprising that no pore formation or cytotoxicity is observed in *Acanthamoeba*, in contrast to macrophages. Since environmental amoebae are most likely the natural host for *L. pneumophila*, these data suggest that pore formation and cytotoxicity are not the primary roles of *rtxA* in bacterial–host-cell interactions. Several other potential roles in pathogenesis have been suggested for RTX proteins from other bacteria, including colonization (Goodwin & Weiss, 1990), immune modulation (Bhakdi *et al.*, 1990; Scheffer *et al.*, 1985) and inhibition of superoxide production (Keane *et al.*, 1987). Therefore, closer examination of the role of the *L. pneumophila* *rtxA* gene in adherence, entry and intracellular survival are likely to provide a better understanding of its primary role in Legionnaires' disease and may lead to insight into alternate functions of RTX proteins from other bacteria.

It is possible that the effects of the *rtxA* mutant on intracellular survival are directly related to the role of *rtxA* in adherence or entry, though the defect in trafficking is less pronounced than the salt-resistant mutant ψ lp55. This concept would suggest that proper intracellular trafficking of *L. pneumophila*, and thereby survival, are the result of signalling events that occur during uptake. However, in our own and other studies (Joshi *et al.*, 2001; Roy *et al.*, 1998), little difference has been found between the trafficking of *L. pneumophila* mutants and wild-type during the first 5–10 min following entry. If signalling during uptake is involved, it has long-lasting effects upon trafficking of the bacterial phagosome. It is equally possible that RtxA is a multi-functional protein, binding a host-cell receptor affecting adherence and entry, as well as subsequently inhibiting lysosomal fusion. There are two primary mechanisms by which RtxA might inhibit lysosomal fusion independent of events associated with entry. First, it may be that the formation of pores caused by RtxA in the vacuole itself prevents proper docking of lysosomes, preventing fusion. Second, an as yet unidentified activity of RtxA modifies the vacuole or other host-cell component that is involved in trafficking. It remains to be determined whether RtxA is one of the proteins secreted by the *dot/icm* secretion apparatus previously implicated in the intracellular trafficking of *L. pneumophila* (Berger *et al.*, 1994; Swanson & Isberg, 1996). Based on the frequencies of lysosomal fusion observed (40–50% with the *rtxA* mutant and 80–90% with a *dotA* mutant), there must be additional factors involved in the inhibition of lysosomal fusion other than RtxA. Our studies were carried out in amoebae rather than the human or murine monocytes used for the evaluation of *dotA*

mutants. However, similar results were obtained by our laboratory for frequencies of lysosomal fusion using the *rtxA* mutant in murine macrophages (L. Yan & J. D. Cirillo, unpublished observations). The fact that other factors are likely to be involved in the prevention of lysosomal fusion by *L. pneumophila* suggests the importance of further studies in this area.

The *rtxA* gene plays a role in the ability of *L. pneumophila* to survive in *A. castellanii*. The impact of the *rtxA* mutation on intracellular survival is, however, relatively small. One possible explanation for this observation might be that the wild-type bacteria, when grown under standard laboratory conditions, do not express *rtxA* optimally and thus the absence of *rtxA* only results in a limited impact on survival. The fact that complementation of the *rtxA* mutant with a plasmid enhances intracellular survival supports this conclusion, since these bacteria are likely to express the *rtxA* gene at higher levels than wild-type due to copy number effects. Since several of the species that did not have this gene according to Southern analysis can still cause disease in animals and the *rtxA* mutant still replicates in human macrophages, albeit poorly (Cirillo *et al.*, 2001), the *rtxA* gene is probably not absolutely required for pathogenesis. It is also likely that, similar to other intracellular pathogens, including *Salmonella*, *Shigella* and *Listeria*, the ability of *L. pneumophila* to survive and replicate intracellularly is multi-factorial. In the case of *Salmonella*, nearly 3% of the genome is thought to be required for the ability to survive intracellularly (P. I. Fields *et al.*, 1986). Application of this model to *L. pneumophila* would suggest that between 100 and 150 genes could be involved in the ability to survive and replicate in macrophages. Thus, one would expect that few of these genes would completely debilitate *L. pneumophila* intracellularly. Mutations in most genes, other than those required for metabolism and biosynthesis, would result in only partial and potentially subtle intracellular defects.

These observations suggest that careful evaluation of potential virulence determinants using both laboratory and animal models of infection in both susceptible and resistant hosts is necessary to allow identification of the important factors involved in pathogenesis by *L. pneumophila*. The Δ *rtxA* mutant should greatly facilitate further investigation of the host-cell receptors, signalling pathways and intracellular trafficking mechanisms of *L. pneumophila*. Further studies are needed, in particular to determine whether the mechanism of entry involving RtxA is responsible for subsequent intracellular events or whether RtxA is a multi-functional protein having the ability to bind host-cell receptors as well as independently preventing lysosomal fusion. In addition to its role in virulence in mammals (Cirillo *et al.*, 2001), the *rtxA* gene plays an important role in the ability of *L. pneumophila* to infect amoebae. These observations suggest that the *rtxA* gene is at least partially responsible for the high percentage of Legionnaires' pneumonias caused by and the predominance in environmental

isolates of *L. pneumophila*, as compared to other *Legionella* species.

ACKNOWLEDGEMENTS

This work was supported by grant AI40165 from the National Institutes of Health.

REFERENCES

- Abu Kwaik, Y. (1996). The phagosome containing *Legionella pneumophila* within the protozoan *Hartmannella vermiformis* is surrounded by the rough endoplasmic reticulum. *Appl Environ Microbiol* **62**, 2022–2028.
- Adams, S. A., Robson, S. C., Gathiram, V., Jackson, T. F., Pillay, T. S., Kirsch, R. E. & Makgoba, M. W. (1993). Immunological similarity between the 170 kD amoebic adherence glycoprotein and human beta 2 integrins. *Lancet* **341**, 17–19.
- Allen, P. G. & Dawidowicz, E. A. (1990a). Phagocytosis in *Acanthamoeba*: I. A mannose receptor is responsible for the binding and phagocytosis of yeast. *J Cell Physiol* **145**, 508–513.
- Allen, P. G. & Dawidowicz, E. A. (1990b). Phagocytosis in *Acanthamoeba*: II. Soluble and insoluble mannose-rich ligands stimulate phosphoinositide metabolism. *J Cell Physiol* **145**, 514–521.
- Ambagala, T. C., Ambagala, A. P. N. & Srikumaran, S. (1999). The leukotoxin of *Pasteurella haemolytica* binds to $\beta 2$ integrins on bovine leukocytes. *FEMS Microbiol Lett* **179**, 161–167.
- Anand, C. M., Skinner, A. R., Malic, A. & Kurtz, J. B. (1983). Interaction of *L. pneumophila* and a free living amoeba (*Acanthamoeba palestinensis*). *J Hyg* **91**, 167–178.
- Armstrong, J. A. & Hart, P. D. A. (1971). Response of cultured macrophages to *Mycobacterium tuberculosis* with observations on fusion of lysosomes with phagosomes. *J Exp Med* **134**, 713–740.
- Behl, C., Davis, J. B., Lesley, R. & Schubert, D. (1994). Hydrogen peroxide mediates amyloid beta protein toxicity. *Cell* **77**, 817–827.
- Berger, K. H., Merriam, J. J. & Isberg, R. R. (1994). Altered intracellular targeting properties associated with mutations in the *Legionella pneumophila* *dotA* gene. *Mol Microbiol* **14**, 809–822.
- Bhakdi, S., Muhly, M., Korom, S. & Schmidt, G. (1990). Effects of *Escherichia coli* hemolysin on human monocytes. Cytocidal action and stimulation of interleukin 1 release. *J Clin Invest* **85**, 1746–1753.
- Bibb, W. F., Sorg, R. J., Thomason, B. M., Hicklin, M. D., Steigerwalt, A. G., Brenner, D. J. & Wulf, M. R. (1981). Recognition of a second serogroup of *Legionella longbeachae*. *J Clin Microbiol* **14**, 674–677.
- Bibb, W. F., Arnow, P. M., Dellinger, D. L. & Perryman, S. R. (1983). Isolation and characterization of a seventh serogroup of *Legionella pneumophila*. *J Clin Microbiol* **17**, 346–348.
- Bizal, C. L., Butler, J. P., Feldman, H. A. & Valberg, P. A. (1991). Kinetics of phagocytosis and phagosome–lysosome fusion in hamster lung and peritoneal macrophages. *J Leukoc Biol* **50**, 229–239.
- Bowers, B. & Korn, E. D. (1968). The fine structure of *Acanthamoeba castellanii*. I. The trophozoite. *J Cell Biol* **39**, 95–111.
- Bozue, J. A. & Johnson, W. (1996). Interaction of *Legionella pneumophila* with *Acanthamoeba castellanii*: Uptake by coiling phagocytosis and inhibition of phagosome–lysosome fusion. *Infect Immun* **64**, 668–673.
- Brander, C., Wyss-Coray, T., Mauri, D., Bettens, F. & Pichler, W. J. (1993). Carrier-mediated uptake and presentation of a major histocompatibility complex class I-restricted peptide. *Eur J Immunol* **23**, 3217–3223.
- Brenner, D. J., Steigerwalt, A. G., Gorman, G. W. & 8 other authors (1980). *Legionella bozemanii* sp. nov. and *Legionella dumoffii* sp. nov.: classification of two additional species of *Legionella* associated with human pneumonia. *Curr Microbiol* **4**, 111–116.
- Brieland, J., McClain, M., Heath, L., Chrisp, C., Huffnagle, G., LeGendre, M., Hurley, M., Fantone, J. & Engleberg, C. (1996). Coinoculation with *Hartmannella vermiformis* enhances replicative *Legionella pneumophila* lung infection in a murine model of Legionnaires' disease. *Infect Immun* **64**, 2449–2456.
- Brieland, J., McClain, M., LeGendre, M. & Engleberg, C. (1997a). Intrapulmonary *Hartmannella vermiformis*: a potential niche for *Legionella pneumophila* replication in a murine model of legionellosis. *Infect Immun* **65**, 4892–4896.
- Brieland, J. K., Fantone, J. C., Remick, D. G., LeGendre, M., McClain, M. & Engleberg, N. C. (1997b). The role of *Legionella pneumophila*-infected *Hartmannella vermiformis* as an infectious particle in a murine model of Legionnaires' disease. *Infect Immun* **65**, 5330–5333.
- Brower, D. L., Brower, S. M., Hayward, D. C. & Ball, E. E. (1997). Molecular evolution of integrins: Genes encoding integrin β subunits from a coral and a sponge. *Proc Natl Acad Sci USA* **94**, 9182–9187.
- Brown, R. C., Bass, H. & Coombs, J. P. (1975). Carbohydrate binding proteins involved in phagocytosis by *Acanthamoeba*. *Nature* **254**, 434–435.
- Campbell, J., Bibb, W. F., Lambert, M. A., Eng, S., Steigerwalt, A. G., Allard, J., Moss, C. W. & Brenner, D. J. (1984). *Legionella sainthelensi*: a new species of *Legionella* isolated from water near Mt. St. Helens. *Appl Environ Microbiol* **47**, 369–373.
- Cianciotto, N. P. & Fields, B. S. (1992). *Legionella pneumophila* *mip* gene potentiates intracellular infection of protozoa and human macrophages. *Proc Natl Acad Sci USA* **89**, 5188–5191.
- Cirillo, J. D., Falkow, S. & Tompkins, L. S. (1994). Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infect Immun* **62**, 3254–3261.
- Cirillo, J. D., Falkow, S., Tompkins, L. S. & Bermudez, L. E. (1997). Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. *Infect Immun* **65**, 3759–3767.
- Cirillo, J. D., Cirillo, S. L. G., Yan, L., Bermudez, L. E., Falkow, S. & Tompkins, L. S. (1999). Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*. *Infect Immun* **67**, 4427–4434.
- Cirillo, S. L. G., Lum, J. & Cirillo, J. D. (2000). Identification of novel loci involved in entry by *Legionella pneumophila*. *Microbiology* **146**, 1345–1359.
- Cirillo, S. L. G., Bermudez, L. E., El-Etr, S. H., Duhamel, G. E. & Cirillo, J. D. (2001). *Legionella pneumophila* entry gene *rtxA* is involved in virulence. *Infect Immun* **69**, 508–517.
- Davies, B. & Edwards, S. W. (1991). Chemiluminescence and superoxide production in *Acanthamoeba castellanii*: free radicals generated during oxidative stress. *J Gen Microbiol* **137**, 1021–1027.
- Davies, B., Chattings, L. S. & Edwards, S. W. (1991). Superoxide generation during phagocytosis by *Acanthamoeba castellanii*: similarities to the respiratory burst of immune phagocytes. *J Gen Microbiol* **137**, 705–710.
- Davis, G. S., Winn, W. C., Jr., Gump, D. W. & Beaty, H. N. (1983).

The kinetics of early inflammatory events during experimental pneumonia due to *Legionella pneumophila* in guinea pigs. *J Infect Dis* 148, 823–825.

Edelstein, P. H. (1981). Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. *J Clin Microbiol* 14, 298–303.

Engleberg, N. C., Drutz, D. J. & Eisenstein, B. I. (1984). Cloning and expression of *Legionella pneumophila* antigens in *Escherichia coli*. *Infect Immun* 44, 222–227.

Falkow, S. (1988). Molecular Koch's postulates applied to microbial pathogenicity. *Rev Infect Dis* 10, S274–S276.

Fields, B. S., Barbaree, J. M., Shotts, E. B., Jr., Feeley, J. C., Morrill, W. E., Sanden, G. N. & Dykstra, M. J. (1986). Comparison of guinea pig and protozoan models for determining virulence of *Legionella* species. *Infect Immun* 53, 553–559.

Fields, P. I., Swanson, R. V., Haidaris, C. G. & Heffron, F. (1986). Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci USA* 83, 5189–5193.

Fliermans, C. B., Cherry, W. B., Orrison, L. H., Smith, S. J., Tison, D. L. & Pope, D. H. (1981). Ecological distribution of *Legionella pneumophila*. *Appl Environ Microbiol* 41, 9–16.

Francis, C. L., Ryan, T. A., Jones, B. D., Smith, S. J. & Falkow, S. (1993). Ruffles induced by *Salmonella* and other stimuli direct macropinocytosis of bacteria. *Nature* 364, 639–642.

Gao, L. Y., Harb, O. S. & Abu Kwaik, Y. (1997). Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa. *Infect Immun* 65, 4738–4746.

Goodwin, M. S. & Weiss, A. A. (1990). Adenylate cyclase toxin is critical for colonization and pertussis toxin is critical for lethal infection by *Bordetella pertussis* in infant mice. *Infect Immun* 58, 3445–3447.

Gorman, G. W., Feeley, J. C., Steigerwalt, A., Edelstein, P. H., Moss, C. W. & Brenner, D. J. (1985). *Legionella anisa*: a new species of *Legionella* isolated from potable waters and a cooling tower. *Appl Environ Microbiol* 49, 305–309.

Heinzen, R. A., Scidmore, M. A. & Rockey, D. D. (1996). Differential interaction with endocytic and exocytic pathways distinguish parasitophorous vacuoles of *Coxiella burnettii* and *Chlamydia trachomatis*. *Infect Immun* 64, 796–809.

Henke, M. & Seidel, K. M. (1986). Association between *Legionella pneumophila* and amoebae in water. *Isr J Med Sci* 22, 690–695.

Herwaldt, L. A., Gorman, G. W., McGrath, T. & 12 other authors (1984). A new *Legionella* species, *Legionella feeleyi* species nova, causes Pontiac fever in an automobile plant. *Ann Intern Med* 100, 333–338.

Horwitz, M. A. (1983). Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila*) in human monocytes. *J Exp Med* 158, 1319–1331.

Horwitz, M. A. (1984). Phagocytosis of the Legionnaires' disease bacterium (*Legionella pneumophila*) occurs by a novel mechanism: engulfment within a pseudopod coil. *Cell* 36, 27–33.

Horwitz, M. A. & Maxfield, F. R. (1984). *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. *J Cell Biol* 99, 1936–1943.

Joly, J. R., McKinney, R. M., Tobin, J. O., Bibb, W. F., Watkins, I. D. & Ramsay, D. (1986). Development of a standardized sub-grouping scheme for *Legionella pneumophila* serogroup 1 using monoclonal antibodies. *J Clin Microbiol* 23, 768–771.

Joshi, A. D., Sturgill-Koszycki, S. & Swanson, M. S. (2001).

Evidence that Dot-dependent and -independent factors isolate the *Legionella pneumophila* phagosome from the endocytic network in mouse macrophages. *Cell Microbiol* 3, 99–114.

Keane, W. F., Welch, R., Gekker, G. & Peterson, P. K. (1987). Mechanism of *Escherichia coli* alpha-hemolysin-induced injury to isolated renal tubular cells. *Am J Pathol* 126, 350–357.

Kirby, J. E., Vogel, J. P., Andrews, H. L. & Isberg, I. I. (1998). Evidence for pore forming ability by *Legionella pneumophila*. *Mol Microbiol* 27, 323–336.

Lally, E. T., Kieba, I. R., Sato, A. & 8 other authors (1997). RTX toxins recognize a $\beta 2$ integrin on the surface of human target cells. *J Biol Chem* 272, 30463–30469.

Lock, R., Öhman, L. & Dahlgren, C. (1987). Phagocytic recognition mechanisms in human granulocytes and *Acanthamoeba castellanii* using type 1 fimbriated *Escherichia coli* as phagocytic prey. *FEMS Microbiol Lett* 44, 135–140.

Marston, B. J., Plouffe, J. F., Breiman, R. F. & 9 other authors (1993). Preliminary findings of a community-based pneumonia incidence study. In *Legionella Current Status and Emerging Perspectives*, pp. 36–37. Edited by J. M. Barbaree, R. F. Breiman & A. P. Dufour. Washington, DC: American Society for Microbiology.

Marston, B. J., Lipman, H. B. & Breiman, R. F. (1994). Surveillance for Legionnaires' disease. *Arch Intern Med* 154, 2417–2422.

McClure, C. D. & Schiller, N. L. (1996). Inhibition of macrophage phagocytosis by *Pseudomonas aeruginosa* rhamnolipids *in vitro* and *in vivo*. *Curr Microbiol* 33, 109–117.

Moffat, J. F. & Tompkins, L. S. (1992). A quantitative model of intracellular growth of *Legionella pneumophila* in *Acanthamoeba castellanii*. *Infect Immun* 60, 296–301.

Morris, G. K., Patton, C. M., Feeley, J. C. & 7 other authors (1979). Isolation of Legionnaires' disease bacterium from environmental samples. *Ann Intern Med* 90, 664–666.

Morris, G. K., Steigerwalt, A., Feeley, J. C., Wong, E. S., Martin, W. T., Patton, C. M. & Brenner, D. J. (1980). *Legionella gormanii* sp. nov. *J Clin Microbiol* 12, 718–721.

Nash, T. W., Libby, D. M. & Horwitz, M. A. (1984). Interaction between the Legionnaires' disease bacterium (*Legionella pneumophila*) and human alveolar macrophages. *J Clin Invest* 74, 771–782.

Newsome, A. L., Baker, R. L., Miller, R. D. & Arnold, R. D. (1985). Interactions between *Naegleria fowleri* and *Legionella pneumophila*. *Infect Immun* 50, 449–452.

Niszl, I. A. & Markus, M. B. (1989). Processing of free-living amoebae for transmission electron microscopy. *Stain Tech* 64, 259–260.

Pruckler, J. M., Benson, R. F., Moyenuddin, M., Martin, W. T. & Fields, B. S. (1995). Association of flagellum expression and intracellular growth of *Legionella pneumophila*. *Infect Immun* 63, 4928–4932.

Reingold, A. L., Thomason, B. M., Brake, B. J., Thacker, L., Wilkinson, H. W. & Kuritsky, J. N. (1984). *Legionella* pneumonia in the United States: the distribution of serogroups and species causing human illness. *J Infect Dis* 149, 819.

Rowbotham, T. J. (1980). Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoeba. *J Clin Pathol* 33, 1179–1183.

Rowbotham, T. J. (1986). Current views on the relationships between amoebae, legionellae and man. *Isr J Med Sci* 22, 678–689.

Rowbotham, T. J. (1993). *Legionella*-like amoebal pathogens. In *Legionella: Current Status and Emerging Perspectives*, pp.

137–140. Edited by J. M. Barbaree, R. F. Breiman & A. P. Dufour. Washington, DC: ASM Press.

Roy, C. R., Berger, K. H. & Isberg, R. R. (1998). *Legionella pneumophila* DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. *Mol Microbiol* **28**, 663–674.

Scheffer, J., Konig, W., Hacker, J. & Goebel, W. (1985). Bacterial adherence and hemolysin production from *Escherichia coli* induces histamine and leukotriene release from various cells. *Infect Immun* **50**, 271–278.

Segal, G. & Shuman, H. A. (1999). *Legionella pneumophila* utilizes the same genes to multiply within *Acanthamoeba castellanii* and human macrophages. *Infect Immun* **67**, 2117–2124.

Straub, M., Bredschneider, M. & Thumm, M. (1997). AUT3, a serine/threonine kinase gene, is essential for autophagocytosis in *Saccharomyces cerevisiae*. *J Bacteriol* **179**, 3875–3883.

Swanson, J. (1989). Fluorescent labeling of endocytic compartments. *Methods Cell Biol* **29**, 137–151.

Swanson, M. S. & Isberg, R. R. (1995). Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. *Infect Immun* **63**, 3609–3620.

Swanson, M. S. & Isberg, R. R. (1996). Identification of *Legionella pneumophila* mutants that have aberrant intracellular fates. *Infect Immun* **64**, 2585–2594.

Venkataraman, C., Haack, B. J., Bondada, S. & Abu Kwaik, Y. (1997). Identification of a Gal/GalNAc lectin in the protozoan *Hartmannella vermiformis* as a potential receptor for attachment and invasion by the Legionnaires' disease bacterium, *Legionella pneumophila*. *J Exp Med* **186**, 537–547.

Vines, R. R., Ramakrishnan, G., Rogers, J. B., Lockhart, L. A., Mann, B. J. & Petri, W. A., Jr. (1998). Regulation of adherence and virulence by the *Entamoeba histolytica* lectin cytoplasmic domain, which contains a β_2 integrin motif. *Mol Biol Cell* **9**, 2069–2079.

Welch, R. A. (1991). Pore-forming cytolysin of Gram-negative bacteria. *Mol Microbiol* **5**, 521–528.

Wiater, L. A., Dunn, K., Maxfield, F. R. & Shuman, H. A. (1998). Early events in phagosome establishment are required for intracellular survival of *Legionella pneumophila*. *Infect Immun* **66**, 4450–4460.

Wilkinson, H. W., Drasar, V., Thacker, W. L., Benson, R. F., Schindler, J., Potuznikova, B., Mayberry, W. R. & Brenner, D. J. (1988). *Legionella moravica* sp. nov. and *Legionella brunensis* sp. nov. isolated from cooling-tower water. *Ann Inst Pasteur Microbiol* **139**, 393–402.

Winn, W. C., Jr & Myerowitz, R. L. (1981). The pathology of *Legionella* pneumonias. A review of 74 cases and the literature. *Human Pathol* **12**, 401–422.

Yu, V. L., Zuravleff, J. J., Gavlik, L. & Magnussen, M. H. (1983). Lack of evidence for person-to-person transmission of Legionnaires' disease. *J Infect Dis* **147**, 362.

Zuckman, D. M., Hung, J. B. & Roy, C. R. (1999). Pore-forming activity is not sufficient for *Legionella pneumophila* phagosome trafficking and intracellular growth. *Mol Microbiol* **32**, 990–1001.

Received 5 November 2001; revised 18 December 2001; accepted 6 February 2002.