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Legionella pneumophila Entry Gene *rtxA* Is Involved in Virulence

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Successful parasitism of host cells by intracellular pathogens involves adherence, entry, survival, intracellular replication, and cell-to-cell spread. Our laboratory has been examining the role of early events, adherence and entry, in the pathogenesis of the facultative intracellular pathogen *Legionella pneumophila*. Currently, the mechanisms used by *L. pneumophila* to gain access to the intracellular environment are not well understood. We have recently isolated three loci, designated *enh1*, *enh2*, and *enh3*, that are involved in the ability of *L. pneumophila* to enter host cells. One of the genes present in the *enh1* locus, *rtxA*, is homologous to repeats in structural toxin genes (RTX) found in many bacterial pathogens. RTX proteins from other bacterial species are commonly cytotoxic, and some of them have been shown to bind to β_2 integrin receptors. In the current study, we demonstrate that the *L. pneumophila rtxA* gene is involved in adherence, cytotoxicity, and pore formation in addition to its role in entry. Furthermore, an *rtxA* mutant does not replicate as well as wild-type *L. pneumophila* in monocytes and is less virulent in mice. Thus, we conclude that the entry gene *rtxA* is an important virulence determinant in *L. pneumophila* and is likely to be critical for the production of Legionnaires' disease in humans.

Legionella pneumophila is an intracellular pathogen that causes Legionnaires' disease in humans, a potentially lethal pneumonia. The ability of *L. pneumophila* to enter, survive, and replicate in monocytic cells is essential for pathogenesis. Differences in the mechanisms used to enter monocytes correlate with subsequent intracellular survival and replication (13). In addition, it has recently been shown that the bacterial entry mechanism and/or factors expressed very early after entry alter intracellular trafficking (52). *L. pneumophila* has been shown to enter monocytes by an unusual mechanism, coiling phagocytosis (28), in addition to the conventional phagocytic mechanism observed in most other bacterial species. Although coiling phagocytosis also occurs in spirochetes (12, 47, 48), the bacterial factors and host cell components involved are not known. Complement (31, 40, 45) and antibody (30, 31) opsonization have effects on adherence of *L. pneumophila* to monocytes. In addition, growth conditions (14) and opsonization with complement (13) or antibodies (28) have been shown to affect the frequencies of coiling and conventional phagocytosis. However, both complement (13) and antibody (28) opsonization results in higher frequencies of conventional phagocytosis. Furthermore, conventional phagocytosis correlates with lower replication rates of *L. pneumophila* in monocytes (13). These data suggest that further study of the mechanisms of nonopsonic phagocytosis by monocytes is critical to obtaining a better understanding of *L. pneumophila* pathogenesis.

Our laboratory has recently identified three chromosomal loci, designated *enh1*, *enh2*, and *enh3*, that affect nonopsonic entry of *L. pneumophila* into monocytes (15). These loci are different from the loci that encode the type IV pilus (58) and major outer membrane protein (38) previously shown to play roles in nonopsonic adherence to and entry into monocytes, respectively. One of the genes present in the *enh1* locus, designated *rtxA*, encodes a "repeats in structural toxin" (RTX). A cytotoxic activity previously observed in *L. pneumophila* (32) displayed characteristics reminiscent of RTX proteins, including a bacterial surface-associated cytotoxic activity. Since RTX proteins from *Bordetella* (24) are involved in adherence to host cells and colonization of host epithelium, the *rtxA* gene is a likely candidate for an *L. pneumophila* entry gene. RTX proteins from several other species, including *Actinobacillus actinomycetemcomitans* (39) and *Escherichia coli* (8), have the ability to bind specifically to host cells. Adherence of RTX proteins to host cells is thought to be mediated by β_2 integrins (1, 39). Since complement receptors are β_2 integrins, these data fit well with studies demonstrating that anti-complement receptor antibodies inhibit adherence to and entry into monocytes by *L. pneumophila* (40, 45), though these studies were done in the presence of complement.

In order to better characterize the role of *rtxA* in adherence and pathogenesis, we characterized the phenotype of an *L. pneumophila* strain containing an in-frame deletion in this gene. The resulting mutant strain displayed significantly reduced adherence, cytotoxicity, pore formation, intracellular replication, and virulence in mice compared to wild-type *L. pneumophila*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *L. pneumophila* strain used for these studies was the streptomycin-resistant variant (43) of *L. pneumophila* serogroup 1 strain AA100 (20). This strain has been shown to be virulent in both in vitro and in vivo models of infection (43) and was passaged no more than twice in the laboratory before use in these studies. The *L. pneumophila rtxA* in-frame deletion mutant strain Ψlp24 (Δ*rtxA*) has been described previously (15). *L. pneumophila* strains were grown on BCYE agar (19) for 3 days at 37°C in 5% CO_2 as described previously (13). The *E. coli* K-12 strain Ψec47, used for

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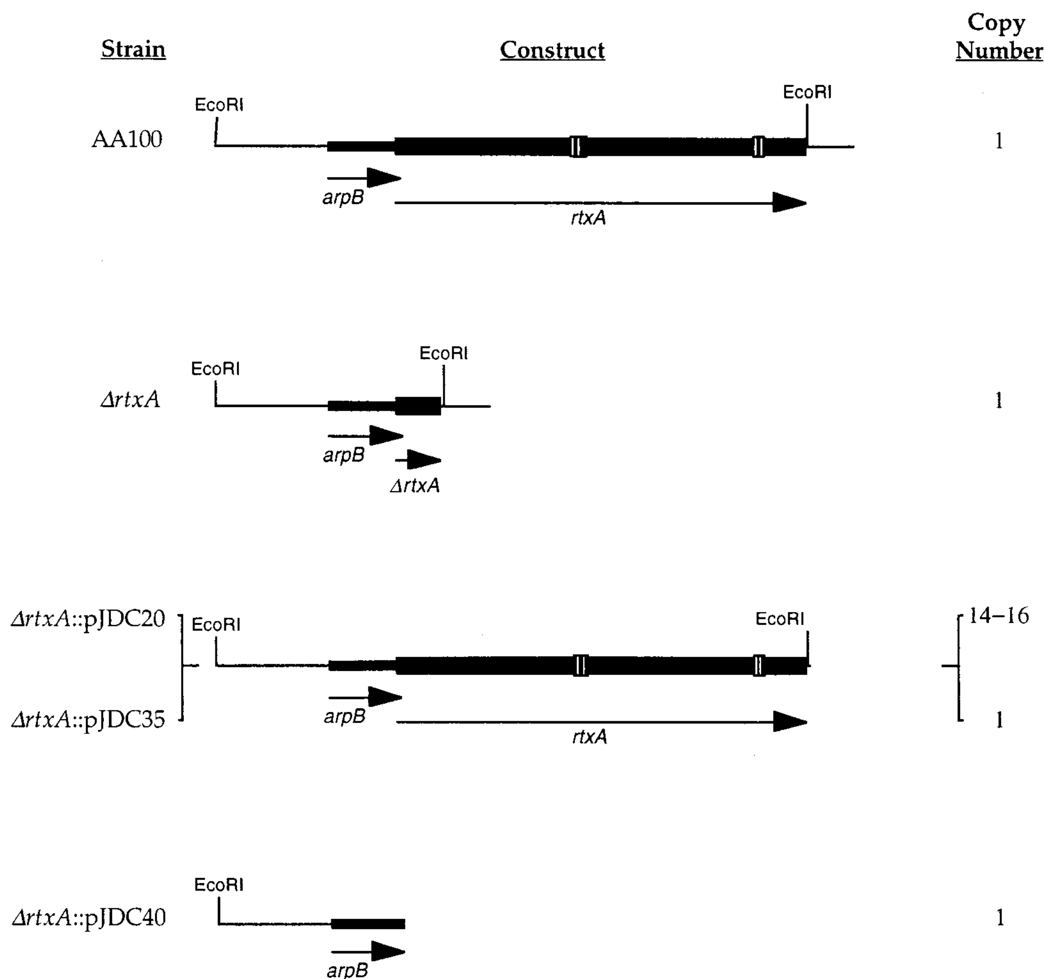

FIG. 1. Structure of the *rtxA* region, Δ*rtxA* mutant, and complementing constructs. All constructs are in single copy in the *L. pneumophila* chromosome except pJDC20, which is present in the low-copy-number (26) plasmid pYUB289 (3, 15). The Δ*rtxA* mutant carries an in-frame deletion in the *rtxA* gene, producing a 130-amino-acid protein product, consisting of 6 amino acids from the amino terminus and 124 amino acids from the carboxy terminus. Gene designations are below the arrows on the constructs illustrating the direction of transcription of the genes. Open boxes in AA100 indicate the positions of the 9-amino-acid repeat sequences characteristic of RTX proteins.

propagation of R6K *ori* plasmids (XL1-Blue [Stratagene] lysogenized with λ*pir*), was grown in Lennox broth (Difco Laboratories) at 37°C. When necessary, kanamycin (Sigma) was added at a concentration of 25 μg/ml, NaCl was added at 5 mg/ml, and sucrose was added at 50 mg/ml to bacterial growth media.

Cell culture. HEp-2 cells (ATCC CCL23), established from a human epidermoid carcinoma, were grown in RPMI 1640 plus 5% heat-inactivated fetal calf serum (Gibco). THP-1 (ATCC TIB202) and U-937 cells (ATCC CRL1593.2), both human monocytic cell lines, were grown in RPMI 1640 plus 10% heat-inactivated fetal calf serum. RAW264.7 (ATCC TIB71) and J774A.1 (ATCC TIB67), both murine cell lines, were grown in Dulbecco's modified Eagle's medium plus 10% heat-inactivated fetal calf serum.

Molecular techniques and plasmid construction. Previous studies in our laboratory demonstrated that the *rtxA* gene affects entry into epithelial cells (Hep-2) and monocytes (THP-1) (15). In the current study, the wild-type *L. pneumophila* strain AA100, the Δ*rtxA* mutant, and complementing strains were examined for other phenotypic characteristics that may help to determine whether *rtxA* plays a role in virulence. The structure of the in-frame deletion in *rtxA* and the constructs used for complementation are shown in Fig. 1. Both single-copy and multicopy complementation constructs were used to control for enhanced expression of *rtxA* due to copy number effects. To ensure physiologically normal RtxA levels for complementation, we expressed *rtxA* from its endogenous promoter in the same position as it is found in the *L. pneumophila* chromosome. Since sequence analysis of the *rtxA* region suggests that the gene is the second in an operon of two genes (15), we utilized complementing constructs that contain the entire operon and putative promoter (pJDC20 and pJDC35). In order to determine whether complementation is solely due to the presence of the *rtxA* gene on this construct, an identical construct without *rtxA* was also used (pJDC40). The combination of these constructs allows definitive demonstration of the activity of the *rtxA* gene under conditions that are as close as possible to those that naturally occur in the *L. pneumophila* chromosome.

DNA manipulations were carried out essentially as described previously (53). The construction of the Δ*rtxA* mutant has been described previously (15). The complementation plasmid pJDC20 (Fig. 1) carries the *Eco*RI fragment that contains only the *enh1* locus with *rtxA* and putative promoter region (15). The *L. pneumophila* suicide plasmid pJDC35 (Fig. 1) was constructed by insertion of this same *Eco*RI fragment into the *Eco*RI site of pJDC15 (15). The *L. pneumophila* suicide plasmid pJDC40 (Fig. 1) was constructed by digestion of pJDC35 with *Swa*I and *Eco*RV followed by self-ligation. Both pJDC35 and pJDC40 were propagated in Ψec47 prior to transformation into *L. pneumophila*. These plasmids can only be maintained by integration into the *L. pneumophila* chromosome via homologous recombination. The presence of the appropriate integrated plasmid was confirmed by Southern and PCR analysis of chromosomal DNA from the resulting strains (data not shown) as described previously (15).

Phenotypic characterization of strains. The presence of pili and flagella on the Δ*rtx* mutant and wild-type *L. pneumophila* strains was assessed by transmission electron microscopy of negatively stained specimens as described previously (15). Ultrastructural morphology of these strains was examined as described previously (14, 15). Motility was assessed using microscopy (15). Sodium sensitivity

was measured by plating dilutions of each strain on BCYE agar and BCYE agar plus 100 mM NaCl and determining colony-forming units (CFU) as described previously (11). The level of sodium sensitivity was expressed as a percentage of the titer in the presence of sodium and compared to that of the wild type. Osmotic sensitivity was measured as described previously (11). Complement sensitivity was examined by incubating 10^8 CFU of each strain in RPMI containing 50% complete or heat-inactivated human serum for 10 min at 37°C, followed by plating dilutions on BCYE agar to determine CFU. A particular strain was considered sensitive to complement if it displayed a significant decrease in CFU in the presence of complete serum compared to heat-inactivated serum or prior to incubation with serum. Conjugation frequencies were determined essentially as described previously (56) except that pJDC1 (15) was used as the donor plasmid and a naturally arising rifampin-resistant AA100 strain was used as the recipient. Growth rate in laboratory media was determined as described previously (11, 15).

Adherence assays. Adherence assays were carried out by the immediate assay method described previously (13, 14). HEp-2 cells were seeded in 24-well tissue culture dishes (Falcon) at a concentration of 1.5×10^6 cells/well and allowed to adhere overnight at 37°C. After adding the bacteria, the medium was gently mixed by rocking back and forth, immediately washed five times with phosphate-buffered saline (PBS) to remove nonadherent bacteria, and then lysed by incubation for 10 min in 1 ml of water followed by vigorous pipetting. Although we tested multiple multiplicities of infection (MOIs) (1, 10, and 100) in these experiments, all data shown are for an MOI of 10. Within this range, the MOI did not significantly affect the data obtained. In the case of THP-1 cells, the assays were carried out in suspension. This requires that the cells be pelleted by centrifugation at $100 \times g$ for 1 min before each change of solution. After lysis, the number of cell-associated bacteria was determined by plating for CFU on BCYE. Adherence assays on formaldehyde-fixed cells (23) were carried out in the same manner except that the cells were fixed in 3.7% formaldehyde for 10 min, washed three times with PBS, and suspended in RPMI prior to addition of the bacteria. For formaldehyde-fixed cells, the bacteria were coincubated with the cells for 30 min (THP-1 cells) or 90 min (Hep-2 cells). Adherence levels were determined by calculating the percentage of the inoculum that became cell associated over the course of the assay [i.e., % adherence = 100 × (CFU cell associated/CFU in inoculum)]. For the wild-type bacterial strain AA100, adherence averaged approximately 0.004% to THP-1 cells and 0.06% to Hep-2 cells under these assay conditions in both formaldehyde-fixed and untreated cells. In order to correct for variation in levels of uptake between experiments, adherence is reported relative to AA100 (i.e., relative adherence = % adherence of test strain/% adherence of AA100).

Intracellular growth assays. The 48-h growth assays were carried out in a manner similar to that described elsewhere (67). The THP-1 cells used for growth assays were seeded into 24-well tissue culture dishes at 1.5×10^6 cells/well in RPMI plus 10% serum and activated with gamma interferon and lipopolysaccharide (LPS; Difco; *E. coli* O127:B8) as described previously (13). THP-1 cells treated with gamma interferon and LPS were used in these assays because in our previous studies they were found to be more sensitive to differences in the levels of *L. pneumophila* virulence than resting monocytes (13). However, activated THP-1 cells were only used for intracellular growth assays because their increased constitutive phagocytic activity makes them a poor choice for use in adherence and entry assays, as illustrated by an increase in the uptake of noninvasive *E. coli* strain HB101 compared to invasive *L. pneumophila* strains in adherent monocytic cells (14). For 48-h growth assays, bacteria were added to the cells and incubated at 37°C for 1 h, washed multiple times with warm PBS, and suspended in fresh medium for 48 h before lysis with water. Detailed growth assays were carried out as described previously (13). In these assays the bacteria were incubated with the host cells for 5 min and then treated in the same manner as for the 48-h assays with lysis at various times after washing. Dilutions of the resulting lysates were plated on BCYE agar to determine CFU immediately after the washes and at each time point. All assays were carried out using an MOI of 10. Growth is reported as the mean number of CFU present in triplicate samples at various times divided by the number of CFU present immediately after washing (mean CFU T_x/T_0). Under these experimental conditions, AA100 regularly displays between 500 and 1,000 CFU/ml in detailed growth assays and between 10,000 and 50,000 CFU/ml in 48-h growth assays at T_0.

Cytotoxicity and pore formation assays. The standard lactate dehydrogenase release cytotoxicity assay (5, 9) was used in these studies. 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 MOIs of 500, 250, 100, and 10 in a final volume of 100 μl for each assay using 2×10^4 THP-1 or 2.5×10^3 Hep-2 cells. Appropriate numbers of cells for CytoTox96 assays were determined as suggested by the manufacturer (Promega). The cells were incubated with the bacteria for 4 h at 37°C with 5% CO_2. Cytotoxicity readings were taken using an enzyme-linked immunosorbent assay plate reader at 450 nm. Percent 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 exactly as described previously (37, 67) using THP-1, U-937, RAW264.7, J774A.1, and Hep-2 cells. Stained coverslips were examined using a Nikon TE300 inverted microscope with fluorescein isothiocyanate and tetramethyl rhodamine isothiocyanate filters. Dual images of multiple fields were captured using an Optronics charge-coupled device video camera and analyzed as described previously (67). 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 are stained with acridine orange since, unlike ethidium bromide, acridine orange readily crosses the membranes of eukaryotic cells.

Mouse infections and examination for pathology. In order to examine the virulence of the different *L. pneumophila* strains in mice, we used methods described previously (6, 10, 13). A/J mice were infected by intratracheal inoculation with 10^6 bacteria. The mice were harvested 1, 4, 24, and 48 h after infection, and bacteria in the lungs were quantitated as described previously (6, 10, 13). Data represent the mean CFU and standard deviation per gram of lung from 12 mice in each experimental group. All preparations were suspended in PBS prior to inoculation.

Histopathology examination was conducted essentially as described previously (10, 43). Lungs were fixed by immersion in 10% neutral phosphate-buffered formalin, processed routinely, embedded in paraffin, cut at 5 μm, and stained with hematoxylin and eosin. *L. pneumophila* in sections was detected through the use of Warthin-Starry silver stain (36, 43). Each section was assigned a number code to allow blinded examination by light microscopy.

Statistical analyses. All in vitro experiments were carried out in triplicate and repeated three times. The experiments in vivo were carried out using 12 mice per experimental group. The significance of the results was analyzed using analysis of variance. Values of P of <0.05 were considered significant.

RESULTS

***rtxA* affects adherence to monocytes and epithelial cells.** The entry mechanism used by *L. pneumophila* may be the result of interaction of the host cell with the bacteria at the level of adherence, entry, or a combination of these two events. The *rtxA* gene has previously been shown to have a twofold effect on entry into monocytes (THP-1) and epithelial (HEp-2) cells, but adherence and other phenotypic characteristics potentially related to virulence have not been examined (15). The sodium, osmotic, and complement sensitivity of the Δ*rtxA* mutant was not significantly different from that of wild-type *L. pneumophila* (data not shown). Furthermore, there were no differences in the presence of pili, presence of flagella, ultrastructure, conjugation frequency, or motility of the Δ*rtxA* mutant compared to wild-type bacteria (data not shown). The lack of any other obvious phenotype in vitro suggests that the primary defect in the Δ*rtxA* mutant is in its ability to enter host cells.

In order to ascertain the role of adherence in *rtxA*-mediated entry, we examined the adherence of the *rtxA* mutant to epithelial and monocytic cells (Fig. 2). Adherence to both cell types was reduced by approximately 50% in the *rtxA* mutant compared to wild-type *L. pneumophila*. In contrast, levels of adherence similar to wild-type levels were observed in the *rtxA* mutant carrying a complementing construct containing the complete *rtxA* gene. However, the *rtxA* mutation could not be complemented by the same construct containing the putative promoter region and complete *arpB* gene in the absence of *rtxA*. All strains were tested for sensitivity to assay conditions such as osmotic lysis, culture medium, and serum. No signifi-

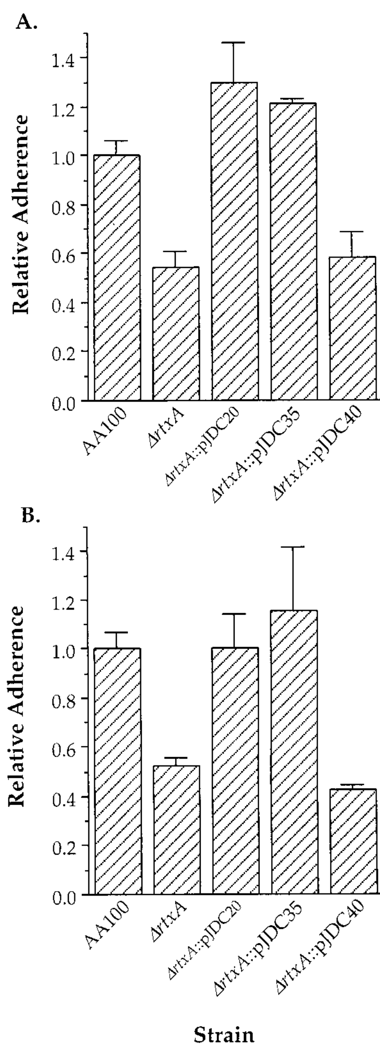

FIG. 2. Ability of AA100, the Δ*rtxA* mutant (Δ*rtxA*), and complemented clones to adhere to HEp-2 epithelial cells (A) and THP-1 monocytic cells (B). Data points and error bars represent the means of triplicate samples from a representative experiment and their standard deviations, respectively. All experiments were performed at least three times.

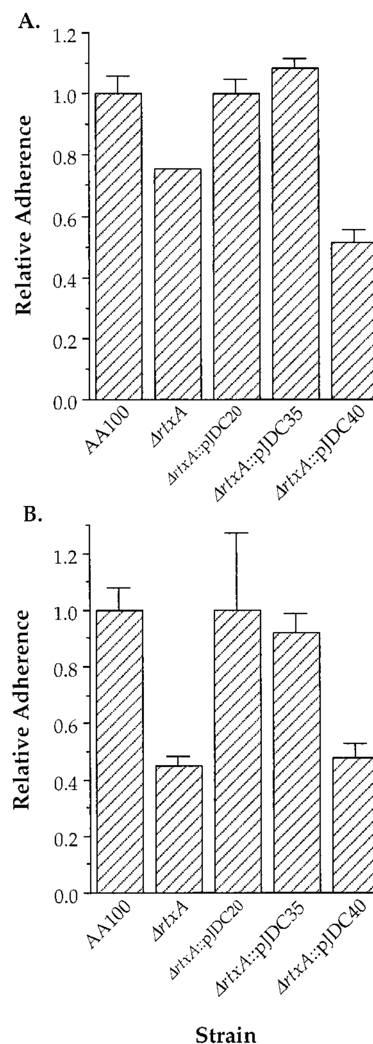

FIG. 3. Ability of AA100, the Δ*rtxA* mutant (Δ*rtxA*), and complemented clones to adhere to formaldehyde-fixed HEp-2 epithelial cells (A) and THP-1 monocytic cells (B). Data points and error bars represent the means of triplicate samples from a representative experiment and their standard deviations, respectively. All experiments were performed at least three times.

cant differences were observed between the Δ*rtxA* and wild-type strains. These data suggests that the *rtxA* gene is involved in adherence of *L. pneumophila* to epithelial and monocytic cells.

Although adherence assays are carried out quickly to prevent the possibility of intracellular killing by the host cell, we initially felt that it was possible that some portion of the difference in adherence observed is due to effects on survival after internalization. In order to test this possibility, we examined the adherence of the *rtxA* mutant to formaldehyde-fixed epithelial and monocytic cells (Fig. 3), in which internalization cannot occur. Similar differences were observed between the *rtxA* mutant and wild-type *L. pneumophila* strains. Both assay methods result in nearly all bacteria remaining extracellular (99.7 to 99.9%), where they are killed by gentamicin (data not shown). Thus, both assay methods are sufficient to allow evaluation of the role of *rtxA* in adherence, and killing subsequent to uptake does not contribute significantly to the data obtained. This information suggests that the preferred assay for adherence of *L. pneumophila* would be the immediate assay, since it results in nearly all bacteria remaining extracellular and is unlikely to have aberrant affects on the host cell.

rtxA **affects cytotoxicity and pore formation caused by *L. pneumophila*.** One common characteristic of RTX proteins from other bacterial species is their involvement in pore-forming cytotoxicity for eukaryotic cells (62). Genes involved in pore-forming cytotoxicity have recently been associated with the ability of *L. pneumophila* to replicate intracellularly (37, 61). Thus, the *rtxA* gene is a potential mediator of the cytotoxicity and/or pore formation associated with *L. pneumophila* infection. When we examined the role of *rtxA* in cytotoxicity, we found that the Δ*rtxA* mutant displayed less cytotoxicity for monocytic cells than the wild type (Fig. 4). The level of cytotoxicity observed in wild-type *L. pneumophila* (~35%) was less

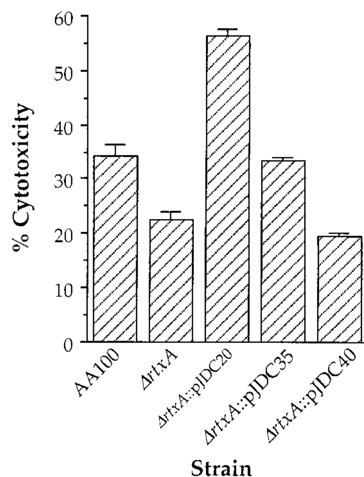

FIG. 4. Cytotoxicity of AA100, the Δ*rtxA* mutant (Δ*rtxA*), and Δ*rtxA* transformed with pJDC20, pJDC35, and pJDC40 for human monocytic cell line THP-1. Data points and error bars represent the means of triplicate samples from a representative experiment and their standard deviations, respectively. All experiments were performed at least three times.

than that observed previously (~65%) using a similar assay (37). These differences are likely due to differences in the cell lines (bone marrow-derived murine macrophages versus THP-1 human monocytes) and bacterial strains (Lp02 versus AA100) used. No cytotoxicity was observed for the epithelial cell line HEp-2 with the wild-type or *rtxA* mutant strain (data not shown). These data suggest that the cytotoxicity of *L. pneumophila* is at least somewhat specific for monocytes. However, the fact that *rtxA* affects cytotoxicity is not necessarily directly related to the pore formation previously observed during *L. pneumophila* infection of monocytes.

In order to determine the role of *rtxA* in pore formation, we compared the pore-forming ability of wild-type *L. pneumophila* with that of the *rtxA* mutant and complemented clones in four different monocytic cell lines (Fig. 5). We utilized both human and murine cells for these assays to determine whether the pore-forming activity was species specific, as is sometimes observed with RTX proteins from other bacterial species (4, 25, 50, 57, 59, 60). Our data indicate that *rtxA* is involved in a pore-forming activity that occurs in both murine and human monocytes. Although the level of pore formation varies in different cell types, pore formation is consistently reduced in the *rtxA* mutant compared to the wild type and correlates with increased bacteria-per-cell ratios. The smallest difference is observed in RAW264.7 cells, a mouse macrophage cell line, though this difference is still significant ($P = 0.043$). No pore formation was observed in HEp-2 cells with wild-type *L. pneumophila* or the *rtxA* mutant (data not shown). These data indicate that the *L. pneumophila rtxA* gene is involved in a cytotoxic and pore-forming activity affecting both human and murine monocytic cells but not HEp-2 cells.

Optimal intracellular survival and replication require *rtxA*. Since pore formation is thought to be involved in the intracellular survival of *L. pneumophila* (37, 61), the activities that are associated with *rtxA* may also affect intracellular viability. In order to elucidate whether *rtxA* plays an important role early during intracellular infection, we compared the ability of the Δ*rtxA* mutant to survive and replicate in monocytes. Although the Δ*rtxA* mutant replicates like the wild type in BYE broth (data not shown), growth in monocytes is significantly lower during the first 48 h of growth (Fig. 6). In order to examine the intracellular viability of *L. pneumophila* at very early time points during intracellular growth, we used a 5-min coincubation with host cells. This procedure allowed detailed examination of the kinetics of intracellular growth from 5 min to 48 h (Fig. 7). These data demonstrate that the Δ*rtxA* mutant appears to be killed more efficiently in monocytes during the first 2.5 h after uptake. The apparent difference between the fold increase observed in Fig. 6 and 7 is due to the different time zero used (1 h as opposed to 5 min) along with the rapid intracellular killing observed at early time points during intracellular growth. Taking these two factors into consideration, the data are consistent with AA100 increasing from approximately 0.1 to 6 (60-fold) and Δ*rtxA* increasing from approximately 0.05 to 2 (40-fold) over 48 h. The early intracellular killing of the Δ*rtxA* mutant suggests that the effects of *rtxA* on entry affect intracellular viability. This early defect in intracellular survival leads to continuously lower intracellular replication, even at time points as late as 48 h.

***rtxA* affects virulence.** Since the differences between the Δ*rtxA* and wild-type strains are relatively small in these in vitro assays, we wished to determine whether these small differences in phenotype significantly affect the ability of *L. pneumophila* to cause disease. In order to elucidate whether the phenotypic effects observed in vitro correlate with changes in virulence, we compared the ability of the wild-type, Δ*rtxA* mutant, and complemented strains to infect mice (Table 1). Although the initial of bacteria found in the lung 1 h after infection is similar for all strains, the CFU for the Δ*rtxA* mutant decrease over time. By 24 h after infection, there is a 10-fold difference in CFU between the Δ*rtxA* mutant and the wild type, whereas there was no significant difference between the single-copy complemented strain (Δ*rtxA*::pJDC35) and the wild type at any time point. This corresponds well with our observation that the Δ*rtxA* mutant displays reduced intracellular survival in monocytes. Interestingly, the CFU for the Δ*rtxA* mutant containing the multicopy plasmid pJDC20 increase more quickly than for the wild type, suggesting that the increased copy number of this region enhances the ability of *L. pneumophila* to survive and/or replicate in mouse lungs.

Throughout the course of these experiments, the animals were monitored for signs of disease. At 1 h after infection, no mice displayed any adverse symptoms. However, by 48 h all of the mice infected with the wild type (AA100) and Δ*rtxA*::pJDC20 mutant and the majority of the mice infected with the Δ*rtxA*::pJDC35 mutant displayed malaise and ruffled fur, whereas only one mouse infected with the Δ*rtxA* mutant showed malaise. Histopathologic examination of lungs from mice infected with these strains (Fig. 8) confirmed the disease state of the mice in each group and showed characteristics similar to those found in previous studies on *L. pneumophila* infections in mice (10). Lung tissue from mice infected with wild-type AA100, Δ*rtxA*::pJDC20, and Δ*rtxA*::pJDC35 strains displayed lesions consisting of lobular areas of parenchymal consolidation characterized by severe suppurative inflammation together with peribronchial and perivascular interstitial edema. Infiltration

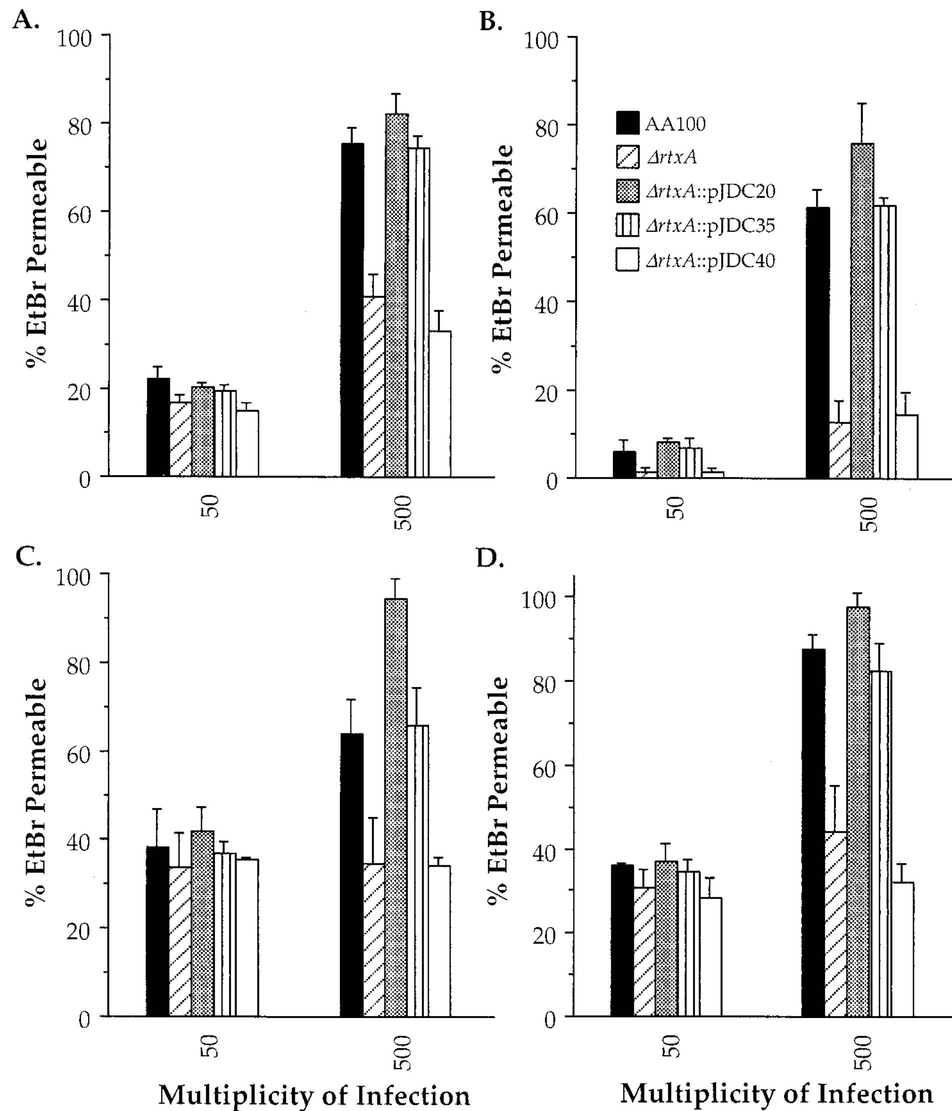

FIG. 5. Pore formation by AA100, the Δ*rtxA* mutant (Δ*rtxA*), and Δ*rtxA* transformed with pJDC20, pJDC35, and pJDC40 for THP-1 (A), U-937 (B), RAW264.7 (C), and J774.1 (D) cells. Data points and error bars represent the means of triplicate samples from a representative experiment and their standard deviations, respectively. All experiments were performed at least three times. EtBr, ethidium bromide.

by mixed inflammatory cells, primarily polymorphonuclear neutrophils, was also observed. Large clusters of leukocytic exudate mixed with necrotic cellular debris and red blood cells are present in bronchiolar lumena and extend to the surrounding alveolar air spaces. However, the respiratory mucosa remain intact. Silver stain sections from mice infected with the wild-type strain display small clusters of apparently intracellular rod-shaped organisms in mononuclear inflammatory cells present within affected alveolar lumena (Fig. 8). In contrast, lung tissues from mice infected with the Δ*rtxA* and Δ*rtxA*::pJDC40 mutant are negative for lesions, and bacteria are often extracellular and less abundant throughout the sections. These data indicate that the *rtxA* gene is a key virulence determinant and that the mechanism of entry used is likely to be critical to *L. pneumophila* pathogenesis.

DISCUSSION

Monocytes utilize a number of relatively nonspecific mechanisms to phagocytose particles, including LPS- (54), surfactant- (51), Fc- (7, 18, 29, 44), complement- (18, 45, 51, 55), and mannose-mediated (33) mechanisms. In addition, pathogens can trigger specific mechanisms to enter monocytes (16, 46, 49). However, little is known about the effects of different entry mechanisms on subsequent intracellular viability of pathogens. We are interested in determining the effects of different entry mechanisms on the pathogenesis of *L. pneumophila*. Through the identification of the *L. pneumophila* genes involved in entry and characterization of their role in the establishment of a preferred intracellular niche and production of disease, we hope to improve our understanding of the importance of different entry mechanisms. This information is likely to lead to

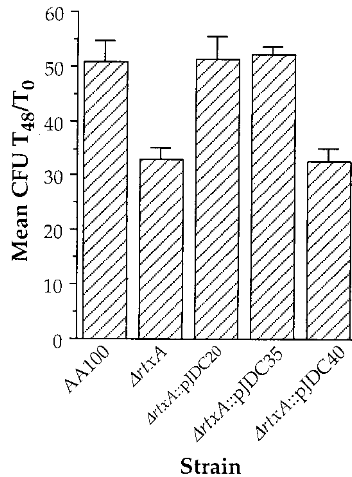

FIG. 6. Growth of AA100, the Δ*rtxA* mutant (Δ*rtxA*), and Δ*rtxA* transformed with pJDC20, pJDC35, and pJDC40 in THP-1 cells over 48 h. Data points and error bars represent the mean number of CFU present at 48 h/number of CFU present at time zero (mean CFU T_x/T_0) of triplicate samples from a representative experiment and their standard deviations, respectively. All experiments were performed at least three times.

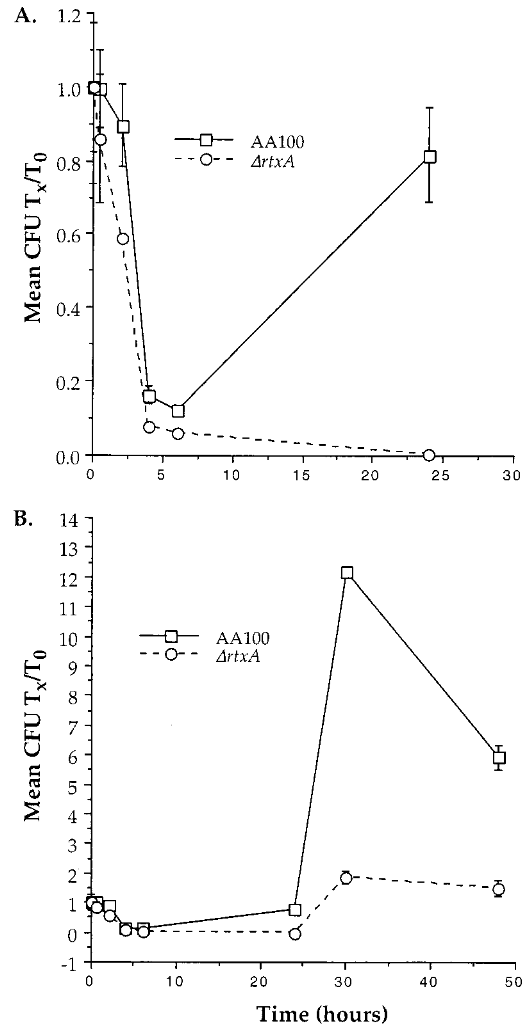

FIG. 7. Growth of AA100 and the Δ*rtxA* mutant (Δ*rtxA*) in THP-1 monocytic cells during the first 24 (A) and 48 (B) h after entry. Data points and error bars represent the means of triplicate samples from a representative experiment and their standard deviations, respectively. Many of the error bars are not visible because they overlap the symbols. All experiments were performed at least three times.

novel methods for prevention of the disease process prior to invasion, the first step in pathogenesis, before an infection can become well established. The *rtxA* gene was initially identified because of its role in entry (15). However, in the current study we demonstrate that this gene also affects a number of other phenotypic characteristics potentially associated with pathogenesis, including virulence in mice. Further studies are necessary to determine whether the observed phenotypic effects on adherence, entry, cytotoxicity, pore formation, intracellular growth, and virulence are due to the direct involvement of *rtxA* or indirect effects on other bacterial factors.

Although the *rtxA* gene affects adherence to epithelial cells, it is more critical for adherence to monocytes. This observation may provide some insight into the potential host cell receptors involved. The β_2 integrin receptor has been shown to be a receptor for RTX proteins from other bacteria (1, 39). Thus, if RtxA binds to a similar receptor, our results may be explained by the fact that epithelial cells normally express much lower levels of β_2 integrins than monocytic cells (21, 42). Although the current study does not examine the receptors involved, this model fits well with previous data demonstrating a role for complement receptors in adherence and entry (40, 45). The absence of observable pore formation and cytotoxicity in HEp-2 cells may also be due to the potential involvement of β_2 integrins in these events. It is intriguing to speculate that the lack of *rtxA* cytotoxicity for epithelial cells may help to explain the histopathological observation of the maintenance of an intact respiratory mucosa in the presence of a severe inflammatory response. This pathologic observation is consistent with previous studies on *L. pneumophila* infections in guinea pigs (34, 64). However, many additional experiments are necessary to clearly demonstrate that the RtxA is involved in a mechanism of entry that occurs via β_2 integrins. The Δ*rtxA* mutant isolated in the current studies should greatly facilitate further research into the role of host cell receptors and signaling pathways in *L. pneumophila* adherence and entry mechanisms.

It is possible that all of the other phenotypic characteristics that are associated with the Δ*rtxA* mutation are related to adherence and/or entry. Hypothetically, the mechanism of entry triggered by *rtxA* could result in signaling events that affect intracellular trafficking. These effects may be responsible for the ability of *L. pneumophila* to inhibit lysosomal fusion (27, 52). Thus, the role of *rtxA* in intracellular survival may be explained through effects on trafficking. Examination of the intracellular trafficking of the Δ*rtxA* mutant after uptake into monocytes should allow us to obtain a better understanding of the role of the *rtxA* gene in pathogenesis. It has been shown that *L. pneumophila* replicates primarily within monocytes during disease (17, 22, 41, 65). Although the phenotypic effects of Δ*rtxA* in vitro were relatively small, the effects in vivo were

TABLE 1. Replication of *L. pneumophila* in lungs after intratracheal inoculation[a]

Time after infection (h)	Mean bacteria (CFU/g of lung) ± SD				
	AA100	Δ*rtxA*	Δ*rtxA*::pJDC20	Δ*rtxA*::pJDC35	Δ*rtxA*::pJDC40
1	$4.1 (\pm 0.3) \times 10^4$	$4.9 (\pm 0.3) \times 10^4$	$8.4 (\pm 0.2) \times 10^4$	$2.4 (\pm 0.3) \times 10^4$	$3.5 (\pm 0.2) \times 10^4$
4	$6.5 (\pm 0.3) \times 10^4$	$6.1 (\pm 0.2) \times 10^4$	$1.9 (\pm 0.2) \times 10^{5*}$	$5.9 (\pm 0.3) \times 10^4$	$3.1 (\pm 0.2) \times 10^4$
24	$8.3 (\pm 0.4) \times 10^4$	$5.8 (\pm 0.3) \times 10^{3*}$	$2.6 (\pm 0.4) \times 10^{6*}$	$6.4 (\pm 0.3) \times 10^4$	$8.4 (\pm 0.3) \times 10^{3*}$
48	$1.9 (\pm 0.2) \times 10^5$	$1.6 (\pm 0.2) \times 10^{2*}$	$1.2 (\pm 0.3) \times 10^{7*}$	$8.1 (\pm 0.3) \times 10^4$	$1.2 (\pm 0.4) \times 10^{3*}$

[a] An inoculum of 10^6 bacteria was used for all experimental groups. Data represent the means ± standard deviations of duplicate platings from 12 mice. *, significantly different ($P < 0.05$) from wild-type *L. pneumophila* (AA100).

quite obvious. These data suggest that subtle defects in the ability of *L. pneumophila* to enter, survive, and replicate in monocytes may dramatically affect the ability to survive in vivo. This is not surprising, considering that all of the components of the host immune system are available in vivo to combat infections. This phenomenon is particularly likely in immune cells such as monocytes, in which proper lymphokine modulation is important for the prevention of intracellular infections. In the absence of a complete understanding of the factors involved in the proper modulation of the bactericidal activity of monocytes, we cannot duplicate these conditions in vitro. These data suggest that it is important to carefully examine potential virulence determinants in vitro for subtle defects and underscore the importance of virulence studies in animal models, where the selection for optimal pathogen-host cell interactions may be more stringent.

The effects of *rtxA* on adherence may be directly responsible for the defect in the persistence of the Δ*rtxA* mutant in mouse lungs. However, it is equally possible that the *rtxA* gene has dual functions, adherence and pore formation, both of which may be important for the pathogenesis of *L. pneumophila*. Proper intracellular trafficking, cytotoxicity, and prevention of lysosomal fusion by *L. pneumophila* are thought to be due to a pore-forming activity involving a type IV secretion apparatus (37, 52, 61, 63). Since RTX proteins are known to cause pore formation in host cells (62), it is possible that the *rtxA* gene product is responsible for this activity. Our observation of a pore-forming activity that requires the presence of the *rtxA* gene supports this hypothesis. However, it is unlikely that *rtxA* is solely responsible for the cytotoxicity associated with the *dot/icm* complex, since an *rtxA* mutation only partially reduces cytotoxicity (~37% reduction), whereas *dot/icm* mutations more significantly reduce cytotoxicity (~62% reduction) (37). Furthermore, additional cytotoxic (2, 35) and hemolytic (35, 66) proteins are known to be produced by *L. pneumophila*. It should be possible to construct a conditional mutant to modulate the *rtxA* phenotype in order to determine whether this gene has dual functions or whether its effects on entry are sufficient to cause the other phenotypic effects observed. However, the *rtxA* gene is clearly involved in adherence to and entry into monocytes and is critical for the ability of *L. pneumophila* to survive and replicate in vivo.

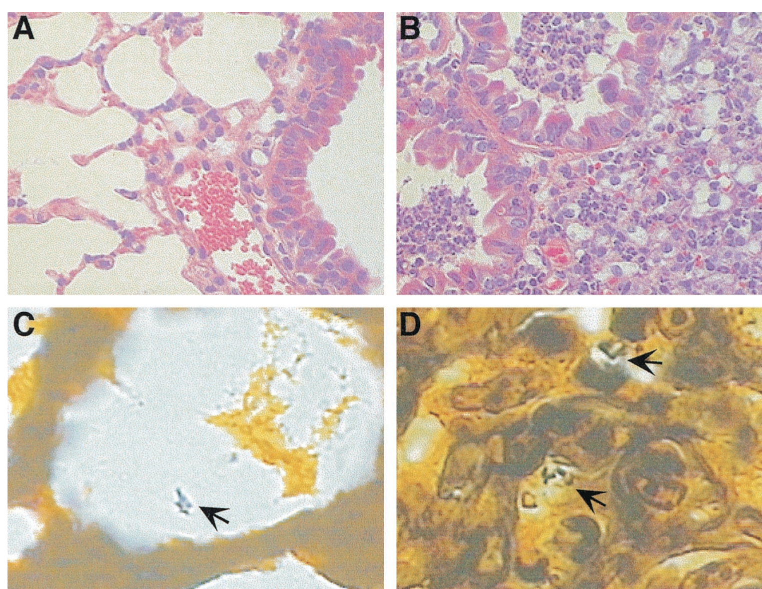

FIG. 8. Histopathologic examination of mouse lungs 48 h after infection with wild-type (B and D) and the *rtxA* mutant (A and C) *L. pneumophila* strains. Lung sections were stained with hematoxylin and eosin (A and B) or Warthin-Starry silver stain (C and D). (B) Characteristic example of a mouse lung infected with wild-type *L. pneumophila*, displaying severe peribronchial pneumonia with leukocytic exudate, necrotic debris, and red blood cells within two small bronchioles. (A) In striking contrast, lungs infected with the Δ*rtxA* mutant displayed characteristics similar to those of normal healthy mouse lungs, with clear bronchioles and alveoli showing very little or no inflammatory infiltrate. Silver-stained sections (C and D) allowed visualization of bacteria (arrows). Magnification: (A and B) ×400, (C and D) ×1,000.

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