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Utilization of Similar Mechanisms by *Legionella pneumophila* To Parasitize Two Evolutionarily Distant Host Cells, Mammalian Macrophages and Protozoa

LIAN-YONG GAO, OMAR S. HARB, AND YOUSEF ABU KWAIK*

Department of Microbiology and Immunology, University of Kentucky Chandler Medical Center, Lexington, Kentucky 40536-0084

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The Legionnaires' disease bacterium, *Legionella pneumophila*, is an intracellular pathogen of humans that is amplified in the environment by intracellular multiplication within protozoa. Within both evolutionarily distant hosts, the bacterium multiplies in a rough endoplasmic reticulum-surrounded phagosome that is retarded from maturation through the endosomal-lysosomal degradation pathway. To gain an understanding of the mechanisms utilized by *L. pneumophila* to invade and replicate within two evolutionarily distant hosts, we isolated a collection of 89 mini-Tn*10*::kan insertion mutants that exhibited defects in cytotoxicity, intracellular survival, and replication within both U937 macrophage-like cells and *Acanthamoeba polyphaga*. Interestingly, the patterns of defects in intracellular survival and replication of the mutants within both host cells were highly similar, and thus we designated the defective loci in these mutants *pmi* (for protozoan and macrophage infectivity loci). On the basis of their ability to attach to host cells and their growth kinetics during the intracellular infection, the mutants were grouped into five groups. Groups 1 and 2 included 41 mutants that were severely defective in intracellular survival and were completely or substantially killed during the first 4 h of infection in both host cells. Three members of group 1 were severely defective in attachment to both U937 cells and *A. polyphaga*, and another four mutants of group 1 exhibited severe defects in attachment to *A. polyphaga* but only a mild reduction in their attachment to U937 cells. Four members of groups 1 and 2 were serum sensitive. Intracellular replication of mutants of the other three groups was less defective than that of mutants of groups 1 and 2, and their growth kinetics within both host cells were similar. The mutants were tested for several other phenotypes in vitro, revealing that 14 of the *pmi* mutants were resistant to NaCl, 3 had insertions in *dot* or *icm*, 3 were aflagellar, 12 were highly intolerant to a hyperosmotic medium, and one failed to grow in a minimal medium. Our data indicated that similar mechanisms are utilized by *L. pneumophila* to replicate within two evolutionarily distant hosts. Although some mechanisms of attachment to both host cells were similar, other distinct mechanisms were utilized by *L. pneumophila* to attach to *A. polyphaga*. Our data supported the hypothesis that preadaptation of *L. pneumophila* to infection of protozoa may play a major role in its ability to replicate within mammalian cells and cause Legionnaires' disease.

Along with *Streptococcus pneumoniae* and *Haemophilus influenzae*, the Legionnaires' disease bacterium, *Legionella pneumophila*, is one of the most common etiologic agents of bacterial pneumonia (12, 26, 43, 63). Upon transmission to humans through environmentally generated aerosols, the bacteria invade and replicate within alveolar macrophages and epithelial cells (21, 33–35, 37, 38, 46, 49, 50).

In the aquatic environment, *L. pneumophila* is a parasite of at least 13 species of amoebae and ciliated protozoa (for a review, see reference 28). In many outbreaks of Legionnaires' disease, the bacteria and protozoa have been isolated from the same water source, and the isolated protozoa have been shown to support intracellular multiplication of the Legionnaires' disease isolate (9, 17, 29, 41). In addition, in many confirmed cases of Legionnaires' disease, the bacterium could be isolated only by its capacity to multiply within protozoa (7, 18, 30, 58). Interestingly, it has recently been shown that nonculturable *L. pneumophila* can be "resuscitated" by intracellular replication within protozoa, indicating that the intracellular environment within protozoa is more favorable for growth than is rich medium in vitro (56). Intracellular replication within protozoa causes an increase in the resistance of *L. pneumophila* to harsh environmental conditions, which may allow the bacteria to survive extracellularly for long periods in the environment (5, 10, 11). Moreover, intracellular multiplication within protozoa enhances the infectivity of *L. pneumophila* for human-derived cells (22). These observations may explain the infective-dose paradox of Legionnaires' disease: no person-to-person transmission occurs, and transmission occurs despite the presence of a low concentration of *L. pneumophila* in the aerosol source (14, 24, 48).

The hallmark of Legionnaires' disease is the intracellular survival and replication of *L. pneumophila* within mammalian macrophages in a rough endoplasmic reticulum (RER)-surrounded phagosome whose maturation through the endosomal-lysosomal degradation pathway is inhibited (23, 33, 34, 59). Interestingly, ultrastructural characterization of the intracellular infection of protozoa by *L. pneumophila* showed that it is indistinguishable from that of human cells (1, 33, 34, 59). Within protozoa, this bacterium also multiplies within an RER-surrounded phagosome that does not fuse with lysosomes (1, 16). In contrast, mechanisms of attachment and uptake of *L. pneumophila* are different for the two evolutionarily distant hosts (4, 40, 50). Attachment to and invasion of the protozoan host *Hartmanella vermiformis* by *L. pneumophila*

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Kentucky Chandler Medical Center, Lexington, KY 40536-0084. Phone: (606) 323-3873. Fax: (606) 257-8994. E-mail: yabukw@pop.uky.edu.

are mediated by attachment to a 170-kDa Gal or GalNAc lectin receptor present on the surfaces of protozoa (32, 61). Moreover, bacterial attachment and invasion are associated with tyrosine dephosphorylation of multiple *H. vermiformis* proteins, including the 170-kDa Gal/GalNAc lectin receptor (32, 61). Attachment of *L. pneumophila* to mammalian cells has been shown to be mediated by complement and non-complement receptors (31, 39, 50).

A few mutants of *L. pneumophila* that are partially defective in replication within mammalian cells have been tested in protozoan cells and have been found to exhibit similar phenotypes (6, 19, 21, 53). On the basis of these limited observations, it has been hypothesized that the mechanisms utilized by this pathogen to parasitize two evolutionarily distant hosts may be similar yet unknown (1, 19, 28).

To test this hypothesis comprehensively and to start characterizing these mechanisms, we identified 89 mini-Tn*10*::kan insertion mutants that manifested defects in their cytotoxicity to, adherence to, survival in, and replication within the cells of both hosts. Interestingly, the defects in kinetics of intracellular replication were remarkably similar in both hosts. Our data showed that similar mechanisms are utilized by *L. pneumophila* to replicate within two evolutionarily distant hosts. The data showed that although some mechanisms of attachment to the cells of both hosts were similar, other, distinct mechanisms are utilized by *L. pneumophila* for attachment to *Acanthamoeba polyphaga*.

MATERIALS AND METHODS

Bacterial strains and vectors. The virulent AA100 strain of *L. pneumophila* has been described previously (4). *L. pneumophila* strains were grown on buffered charcoal-yeast extract (BCYE) agar plates or in buffered yeast extract (BYE) broth. *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used for the majority of cloning experiments. The plasmid pUC-4K was purchased from Pharmacia (Piscataway, N.J.) and was the source of the kanamycin resistance gene used as a probe for Southern hybridization. The plasmid pCDP05, a chloramphenicol-resistant plasmid that contains miniTn*10*::kan and the *sacB* gene from *Bacillus subtilis*, was a kind gift from N. Cianciotto (Northwestern University, Chicago, Ill.) (51). The *sacB* gene encodes levansucrase and is lethal to *L. pneumophila* grown in the presence of sucrose (20). The plasmid pAM10, which contains a 12-kb *Eco*RI insert encompassing both the *dot* and *icm* loci, was kindly provided by H. A. Shuman (Columbia University, New York, N.Y.) (42).

Transposon mutagenesis and construction of a bank of mutants. Electroporation of the plasmid pCDP05 into the *L. pneumophila* wild-type strain AA100 was performed under previously described optimal conditions (51). Following electroporation, bacteria were incubated in BYE broth for 2 h at 37°C and then plated onto BCYE agar plates containing 20 μg of kanamycin per ml and 6% sucrose. For each electroporation, 200 to 300 colonies were isolated for measurements of their infectivities to U937 cells and *A. polyphaga*.

DNA manipulations. Chromosomal DNA preparations, transfections, and restriction enzyme digestions were performed as described elsewhere, unless otherwise specified (55). Restriction enzymes were obtained from Bethesda Research Laboratories.

Plasmid DNA preparations were performed with the Qiagen (Chatsworth, Calif.) plasmid kit according to the manufacturer's recommendations. Transformations were carried out by electroporation, using a Gene Pulser, as recommended by the manufacturer (Bio-Rad, Hercules, Calif.). Purification of DNA fragments from agarose gels for Southern hybridization was done with a Qiaex kit, according to the manufacturer's recommendations (Qiagen Inc.). Transfer of DNA from agarose gels onto membranes, fluorescein labeling of DNA probes, hybridizations, and detection were performed as described before (3).

Tissue culture and protozoan culture. Macrophage-like U937 cells were maintained at 37°C and 5% CO_2 in RPMI 1640 tissue culture medium (BioWhittaker, Inc., Walkersville, Md.) supplemented with 10% heat-inactivated fetal calf serum (Sigma Chemical Co., St. Louis, Mo.). Prior to infection, the cells were differentiated in 96-well tissue culture plates for 48 h, using phorbol 12-myristate 13-acetate as described previously (2). Differentiated cells are nonreplicative, adherent, macrophage-like cells. Monolayers were washed three times with the tissue culture medium prior to infection. For infection of monolayers, *L. pneumophila* grown for 20 h (late log phase) at 37°C in BYE broth were resuspended in RPMI 1640. The infection was carried out as described for each experiment.

Axenic *A. polyphaga* was obtained from B. S. Fields (Centers for Disease Control and Prevention, Atlanta, Ga.) and cultured as adherent cells in PYG medium (16). Infections were performed in 96-well tissue culture plates with *Acanthamoeba* buffer (47) as described for each experiment.

Transmission electron microscopy. *A. polyphaga* monolayers were infected with *L. pneumophila* at a multiplicity of infection (MOI) of 10 for 1 h; this was followed by extensive washing to remove extracellular bacteria with tissue culture medium. At several time intervals, infected monolayers were fixed for 1 h on ice with 3.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and then for 1 h on ice with 1% OsO_4 in 0.1 M phosphate buffer. Dehydration was accomplished by serial exposure to ethanol (50 to 100%), for 10 min each, at room temperature. The cells were embedded in Eponate 12 resin according to the manufacturer's recommendations (Ted Pella, Redding, Calif.). Ultrathin sections were stained with uranyl acetate followed by lead citrate and examined under a Hitachi H-7000/STEM electron microscope at 75 kV.

Cytotoxicity of *L. pneumophila* mutants to U937 cells and *A. polyphaga*. *L. pneumophila* strains were grown in BYE for 20 h in 48-well plates. Infection of phorbol 12-myristate 13-acetate-differentiated U937 monolayers with *L. pneumophila* strains was performed in triplicate in 96-well plates containing 10^5 cells/well at an MOI of 5. The infected monolayers were incubated at 37°C for 38 h. For measurements of the number of viable cells remaining in the monolayer, the monolayers were treated with 10% Alamar blue dye, as recommended by the manufacturer (Alamar Bioscience Inc., Sacramento, Calif.). Measurements of optical density were performed at a wavelength of 570 nm and corrected for background at 600 nm with a Molecular Devices (Sunnyvale, Calif.) microplate reader. The relative degree of macrophage cytotoxicity was expressed as the percentage of the ratio of the optical density value of an infected monolayer to that of an uninfected one. Cytotoxicity assays using MOIs of 5 to 20 did not result in differences in cytotoxicity levels (data not shown); thus, only the cytotoxicity data from assays performed at MOIs of 5 are reported.

Infection of *A. polyphaga* with *L. pneumophila* strains was performed in triplicate in 96-well tissue culture plates with 4×10^4 cells/well at an MOI of 20. After 1 h of coincubation, monolayers were washed three times with PYG medium and then incubated at 37°C for 30 h. The viability of *A. polyphaga* was determined by trypan blue dye exclusion assay. The relative degree of cytotoxicity to *A. polyphaga* was expressed as described above for the U937 cells.

Attachment of *L. pneumophila* mutants to U937 cells and *A. polyphaga*. *A. polyphaga* or differentiated U937 cells in 96-well plates were infected with *L. pneumophila* in triplicate at an MOI of 10. The plates were spun at $1,000 \times g$ for 5 min followed by incubation for 30 min at 37°C. At the end of this infection period, monolayers were washed three times with tissue culture medium to remove nonadherent bacteria. The time point at the end of the final wash was the initial time point (T_0). Monolayers were subsequently lysed either hypotonically (for U937 cells) or with a mild detergent (0.05% Triton X-100) (for *A. polyphaga*). Aliquots were diluted immediately and plated on BCYE agar plates for enumeration of intracellular bacteria. This treatment did not have a significant effect on the viability of the bacteria (data not shown).

To distinguish between defects in attachment and those of invasion or early intracellular killing, we performed identical attachment assays using U937 cell monolayers treated with cytochalasin D (1 μg/ml) and *A. polyphaga* treated with 10 mM methylamine to prevent uptake of the attached bacteria by both host cells (40). In these experiments, monolayers were pretreated with the corresponding inhibitor for 30 min. After the infection, the monolayers were washed extensively with medium containing the corresponding inhibitor and subsequently lysed, as described above. The number of attached bacteria was subsequently determined after growth on BCYE agar plates. To ensure that the inhibitors were effective in inhibition of uptake, control monolayers infected in the presence of the inhibitor were treated with gentamicin to kill extracellular bacteria. This treatment caused complete sterilization of the infected U937 cell monolayers (data not shown). Methylamine inhibited uptake by *A. polyphaga* by approximately 98% (data not shown).

Growth kinetics of *L. pneumophila* mutants in U937 cells and in *A. polyphaga*. To determine the number of intracellular bacteria at several time intervals, infections were performed exactly as described above for the attachment assays. At the end of the infection period, the monolayers were washed three times with the respective culture medium and then incubated for 1 h at 37°C in the presence of gentamicin (50 μg/ml) to kill extracellular bacteria. At the end of each time interval, monolayers were lysed as described above. Aliquots were diluted immediately and plated on BCYE agar plates for enumeration of intracellular bacteria.

Colony immunoblotting. Colony blots were probed with a rabbit polyclonal antiserum (kindly provided by J. Pruckler and B. Field, Centers for Disease Control and Prevention) that was raised against *L. pneumophila* flagella (53). Colony immunoblotting was performed exactly as described previously, with strain AA100A as a negative control (6).

Human serum sensitivity assays. *L. pneumophila* strains were grown in BYE for 20 h in 48-well plates. Bacteria were diluted to approximately 10^6 CFU/ml, mixed with an equal volume of tissue culture medium or normal human serum, and incubated for 1 h at 37°C. The number of viable bacteria was determined following growth of diluted aliquots on BCYE plates. Three different nonimmune human sera were tested.

Screening mutants for defects in iron acquisition and assimilation. We used the same strategy employed by Pope et al. (52) to screen for sensitivity of the mutants to the iron chelator ethylenediamine di(*O*-hydroxyphenylacetic acid)

(EDDHA) and for resistance to the antibiotic streptonigrin, whose lethal effects require high levels of intracellular iron (52). Strains were grown on BCYE agar plates, supplemented with EDDHA or streptonigrin, exactly as described previously (52).

Resistance of the mutants to NaCl. Bacteria were grown in BYE broth for 20 h. Dilutions were plated on BCYE plates in the presence or absence of 0.6% NaCl. The fold increase in efficiency of plating on NaCl-containing plates was calculated as the ratio of the plating efficiency in the presence of NaCl to that in its absence. An increase in resistance to NaCl of at least 100-fold was considered as NaCl-resistant phenotype.

RESULTS

Use of *A. polyphaga* as a model to study parasitism of protozoa by *L. pneumophila*. Studies of cytotoxicity and intracellular growth kinetics of *L. pneumophila* in *A. polyphaga* showed that strain AA100 was cytotoxic and that it replicated within *A. polyphaga* (see below). Transmission electron microscopy also confirmed the ability of *L. pneumophila* to form an RER-surrounded phagosome within *A. polyphaga* at 4 h postinfection and the subsequent intracellular replication within the phagosome (Fig. 1). These observations showed that the intracellular infection of *A. polyphaga* was similar to that of mammalian or other protozoan cells (1, 28, 33, 34, 59). These data showed that *L. pneumophila* replicated efficiently within an RER-surrounded phagosome in *A. polyphaga.*

Isolation of *L. pneumophila* mutants defective in cytotoxicity and intracellular replication within human-derived macrophages and *A. polyphaga*. To identify *L. pneumophila* genetic loci involved in cytotoxicity and intracellular replication within both human macrophages and protozoa, a bank of mini-Tn*10* insertion mutants was constructed. A total of 5,280 insertion mutants from 21 independent transpositions were isolated. To test for cytotoxicity of the mutants, macrophages and *A. polyphaga* were infected by the mutants or the wild-type AA100 strain of *L. pneumophila*, and cytotoxicity was expressed as the percentage of cytotoxicity to the infected monolayers compared to that to the uninfected ones. To identify mutants that were defective in both host cells, our strategy was to isolate mutants that exhibited at least a 50% reduction in their cytotoxicities to U937 cells (compared to the wild-type strain), had reduced cytotoxicity to *A. polyphaga*, and exhibited defects in intracellular replication within the cells of both hosts. In the original screening, we isolated 625 defective mutants. Upon subsequent testing, the phenotypes of many of the mutants were not reproducible, particularly the partially and mildly defective ones. Eighty-nine mutants were isolated on the basis of their reproducible defective phenotypes (performed three to five times independently) in both *A. polyphaga* and U937 macrophage-like cells (Fig. 2). In general, most of the mutants were more cytotoxic to *A. polyphaga* than to U937 cells (Fig. 2). Compared to the wild-type strain, the cytotoxicities of these mutants were 0 to 49.8% for U937 cells and 0 to 78% for *A. polyphaga*. Among the 89 mutants, 41 were completely or severely defective in cytotoxicity to both host cells, as indicated by the similarity in the viability of the infected monolayers to that of uninfected ones (Fig. 2). Interestingly, but for unknown reasons, mutants belonging to groups 4 and 5 were in general more cytotoxic to *A. polyphaga* than to U937 cells.

Southern blot analysis of *Eco*RI-digested genomic DNA of each of the 89 mutants probed with the kanamycin resistance cassette showed that all of them contained a single copy of the mini-Tn*10*::kan insertion (data not shown). Importantly, insertions were random and distinct, which was consistent with previous observations (51–53).

Characteristics of attachment of mutants to and intracellular survival and replication of mutants within U937 cells and *A. polyphaga*. We examined the intracellular survival and growth kinetics of the mutants within the U937 cells and *A. polyphaga*. These growth kinetics studies were performed three to five times independently for each of the mutants. The initial time point (T_0) represents the time at the end of the 30-min infection period, after the unattached bacteria have been washed off, and thus represents cell-associated bacteria (attached and intracellular). Many of the mutants showed a reduction in cell-associated numbers for one or both of the two hosts' cells, compared to those of the wild-type strain (see below). To distinguish attachment from invasion and immediate killing after entry, we examined the number of attached bacteria during inhibition of uptake by U937 cells or by *A. polyphaga* (see Materials and Methods). Nine mutants were defective in attachment, and they are described below.

To examine the number of intracellular bacteria at several time intervals, extracellular bacteria were killed at the end of the 30-min infection period by treatment with gentamicin, and the number of intracellular bacteria was determined (see Materials and Methods). Since the maximum numbers of bacteria were achieved at 20 h postinfection for *A. polyphaga* and at 48 h postinfection for U937 cells (data not shown), determinations at time points beyond the corresponding periods were performed, but they are not shown. None of the mutants was sensitive to components of the tissue culture medium, and there were no detectable differences in their growth in BYE compared to that of the wild-type strain after 20 h of incubation (data not shown).

The mutants showed a diverse spectrum of defects in their attachment, intracellular survival, and replication, indicative of their distinct defective phenotypes conferred by defects in different genetic loci. Interestingly, the defects in growth kinetics of the mutants in both host cells were remarkably similar (Fig. 3 and data not shown). To simplify the representation of these data, we grouped the 89 mutants into five phenotypic groups on the basis of their defective characteristics of attachment and intracellular growth kinetics (Fig. 2). The growth kinetics of a representative mutant from each group are shown in Fig. 3. In general, the relative degree of defect in cytotoxicity correlated with the relative degree of defect in intracellular survival and replication (Fig. 2 and 3). For example, mutants that were severely defective in cytotoxicity (groups 1 and 2) were severely defective in intracellular survival and replication. It is important to note that there were variations in the severity of the defect within each group, but the general trends of the defect in intracellular growth kinetics were similar within each group. The characteristics of these five phenotypic groups are described below.

Group 1 included seven mutants that exhibited defects in attachment. These mutants were completely or substantially killed within the first 4 h of infection within both host cells (Fig. 3). Three of these mutants (GG104, GB112, and GP65) were severely defective in attachment to both macrophages and *Acanthamoeba* cells (Fig. 4). The other four mutants (GF162, GO128, GQ262, and GM128) were severely defective in attachment to *A. polyphaga* but exhibited mild reductions in their capacities to attach to U937 cells (Fig. 4). In addition, all seven of these mutants were completely defective in their cytotoxicities to U937 cells and *A. polyphaga* (Fig. 2).

Group 2 consisted of 34 members (Fig. 2) that were killed within the first 4 h to a degree similar to that of group 1 mutants. Group 2 members differed from those of group 1 in their wild-type attachment phenotype (data not shown). Studies of the intracellular growth kinetics of this group of mutants showed that they were completely or severely defective in intracellular replication within both macrophages and *A. polyphaga* (Fig. 3).

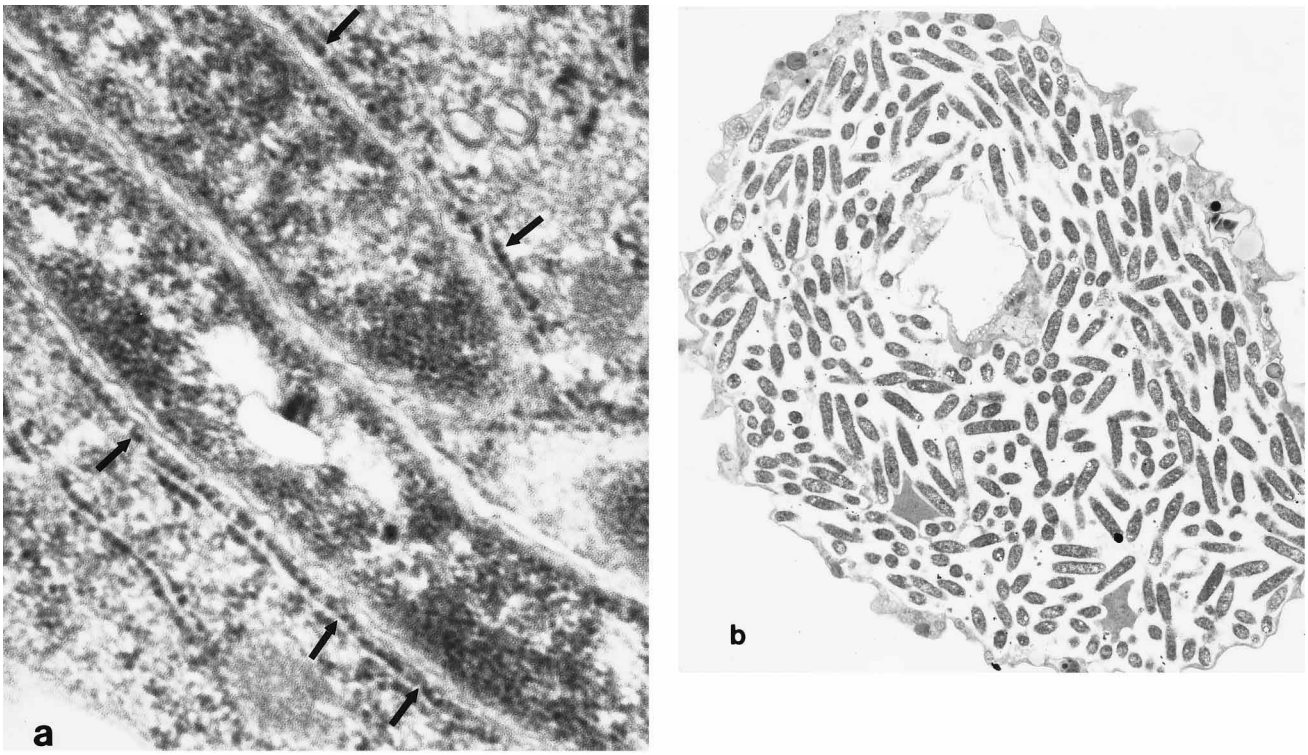

FIG. 1. Transmission electron micrographs of *A. polyphaga*, infected by *L. pneumophila* AA100, at 4 h (a) and 18 h (b) postinfection. The arrows indicate the RER-surrounded phagosome. Magnifications, ×40,000 (a) and ×5,000 (b).

Group 3 contained four mutants (Fig. 2). The intracellular growth kinetics of this group was characterized by approximately a 2- to 10-fold reduction in their intracellular numbers during the first 4 h of the infection in both host cells compared to the wild-type strain. After 4 h, some of the mutants maintained relatively constant intracellular numbers while others showed very limited intracellular replication (one or two generations). By 48 h after infection, the number of intracellular mutant bacteria was at least a 1,000-fold less than that of the wild-type strain (Fig. 3).

Studies of the intracellular growth kinetics of mutants in group 4 (eight mutants) within both host cells showed that the cells either had a normal or an approximately 10-fold lower number of intracellular bacteria within the first 4 h of infection. Subsequent intracellular growth was partially defective, reaching a level that was approximately 100-fold less than that of the wild-type strain.

Group 5 had 36 members with the least-defective phenotype. Studies of the intracellular growth kinetics of this group showed that they were indistinguishable from the wild-type strain during the early stages of the intracellular infection but that their numbers were approximately 10-fold lower than those of strain AA100 by 48 or 72 h after infection of U937 cells or *A. polyphaga* (Fig. 3). Two mutants in this group (GM224 and GT251) were defective in attachment to both U937 cells and *A. polyphaga* (Fig. 4).

These data showed that the defective phenotypes for cytotoxicity, intracellular survival, and growth kinetics of the mutants were highly similar in both host cells. Therefore, we designated the defective genetic loci in the 89 mutants *pmi* (for protozoan and macrophage infectivity loci).

Resistance of *L. pneumophila* mutants to NaCl. It has been shown that NaCl-resistant strains of *L. pneumophila* are defective with regard to their intracellular survival in mammalian and protozoan cell lines and are also attenuated in animal models, but the relationship of the NaCl resistance to attenuation is not known (4, 36, 42, 44, 54, 60). Moreover, most attenuated strains of *L. pneumophila* isolated by other investigators are resistant to NaCl (54, 60). We examined the relationship between NaCl resistance and the defective phenotype of the 89 *pmi* mutants. In contrast to other reports, in this study only 14 of the 89 *pmi* mutants exhibited increased resistance to NaCl, which resulted in between a 100- and a 300-fold increase (compared to the wild-type strain) in their plating efficiency on 0.6% NaCl-containing plates (Fig. 2 and 5) (54, 60). Interestingly, 12 of the NaCl-resistant strains were members of the most defective group (group 2), while the other two mutants (GO38 and GT94) belong to groups 3 and 4, respectively. These data showed that 75 of 89 attenuated *pmi* mutants, including many of the most defective strains (of groups 1 and 2), were not NaCl resistant, indicating that the two phenotypic traits were distinct.

Interestingly, 12 of the *pmi* mutants were hypersensitive to NaCl (Fig. 2 and 6). Compared to the wild-type strain, these mutants manifested 100- to 1,200-fold reductions in their plating efficiencies on 0.6% NaCl-containing BCYE plates. These data showed that these mutants were intolerant to hyperosmotic stress conditions.

Serum sensitivity. All 89 *pmi* mutants were tested for their susceptibilities to three different nonimmune human sera. Our data showed that four *pmi* mutants were sensitive to all three sera tested (Fig. 2). There was at least a 90% loss of viability for each of these mutants following 1 h of incubation in 50% human serum (Fig. 7). All the other mutants were indistinguishable from the wild-type strain in their resistances to all of the sera tested.

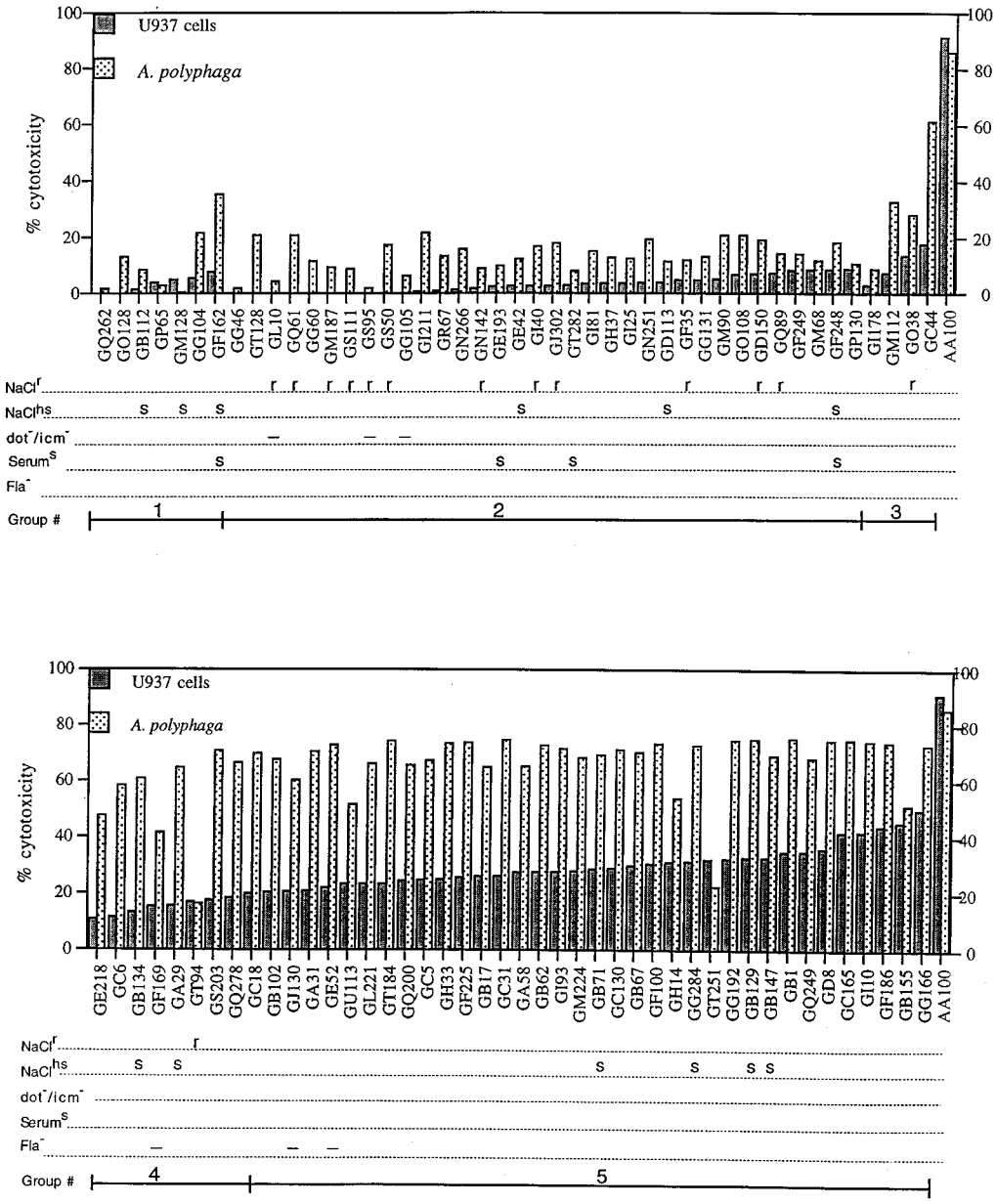

FIG. 2. Cytotoxicities of the *L. pneumophila* mutants to U937 macrophage-like cells and *A. polyphaga*, and comparison to the wild-type strain AA100. The data are representative of three to five independent experiments, performed in triplicate. The five phenotypic groups and a summary of some of the in vitro phenotypes of the mutants are shown underneath the figure. Abbreviations: r, resistant; s, sensitive; $NaCl^r$, resistant to NaCl; $NaCl^{hs}$, hypersensitive to NaCl; $serum^s$, sensitive to serum; Fla^-, aflagellar mutant; dot^-/icm^-, mutation in *dot* or *icm* loci.

Since serum sensitivity of gram-negative bacteria is in general mediated by complement, the four *pmi* mutants were examined for their sensitivities to heat-inactivated serum (in which the complement was inactivated). Our data showed that the four *pmi* mutants were resistant to heat-inactivated serum. These data indicated that the sensitivity of these mutants to serum was mediated by complement.

Flagellum-defective (Fla^-) mutants. Flagellum expression has been shown to be associated with the ability of *L. pneumophila* to survive intracellularly (53). We examined flagellar expression by all the 89 *pmi* mutants in a colony immunoblot assay, using as a probe a rabbit antiserum raised against *L. pneumophila* flagella (53). Three mutants (strain GF169 of group 4 and strains GJ130 and GE52 of group 5) were defective in flagellum expression (Fla^-) (Fig. 2).

Identification of *dot* or *icm* mutants among the *pmi* mutants. The two loci *dot* and *icm* have been shown to be crucial for intracellular survival of *L. pneumophila* in macrophages, but the role of these loci in the intracellular infection of protozoa is not known (13, 42). Southern blots of *Eco*RI-digested genomic DNA from all the mutants, probed with a 12-kb *Eco*RI fragment containing both *dot* and *icm*, showed that only three strains had an insertion in one of these loci (Fig. 2 and data not shown). Consistent with previous reports, the three *dot-icm* mutants were among the most defective mutants (group 2). These data showed that *dot* and *icm* also play a

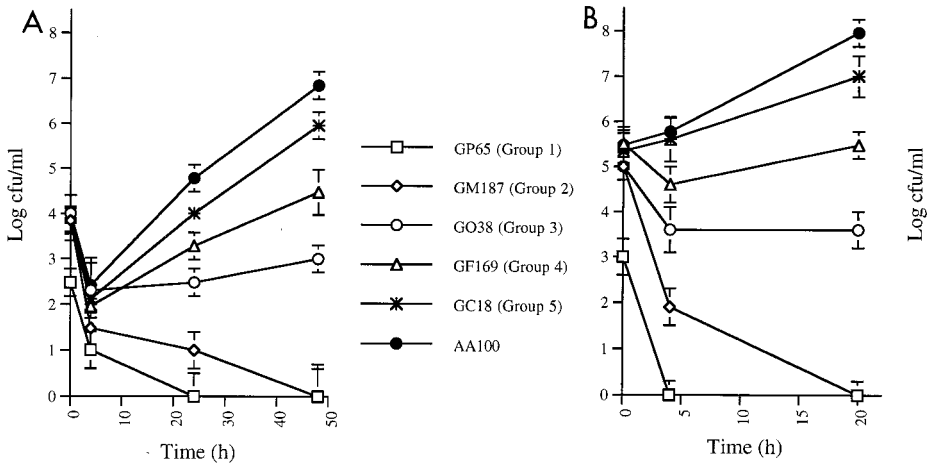

FIG. 3. Growth kinetics of a representative strain from each of the five phenotypic mutant groups within U937 cells (A) and *A. polyphaga* (B). The initial time points (T_0) represent the numbers of cell-associated bacteria (attached and intracellular) after 30 min of infection. Subsequent time points represent numbers of intracellular bacteria at the corresponding times postinfection. Studies performed at 48 and 72 h postinfection for *A. polyphaga* and at 72 h postinfection for U937 cells did not reveal any increase in the number of bacteria (data not shown). The data are representative of three to five independent experiments, performed in triplicate, and error bars represent standard deviations.

major role in intracellular survival of *L. pneumophila* in protozoa.

Growth of the *pmi* mutants in minimal medium. We examined whether some of the *pmi* mutants were auxotrophs. Growth of the mutants in CAA minimal medium (45) was examined. Only one mutant, GQ278 (group 4), failed to grow in this medium. This medium is known to be deficient in tryptophan and thymidine (45), but supplementation of the minimal medium with these two compounds did not allow growth of this mutant. These data suggested that the mutant was an auxotroph for a nutrient other than tryptophan or thymidine. This mutant was mildly defective in intracellular replication in the cells of both hosts.

Iron acquisition and assimilation by the *pmi* mutants. Pope et al. showed that 6 of 17 *L. pneumophila* mutants that were defective in iron acquisition and assimilation were also defective for intracellular replication within U937 cells (52). We used the same strategy employed by these investigators to screen for sensitivity of the 89 mutants to the iron chelator EDDHA and for resistance to the antibiotic streptonigrin (52). Although in the original examination one of the *pmi* mutants (strain GS203) exhibited reduced growth in the presence of EDDHA, its phenotype was irreproducible upon four indepen-

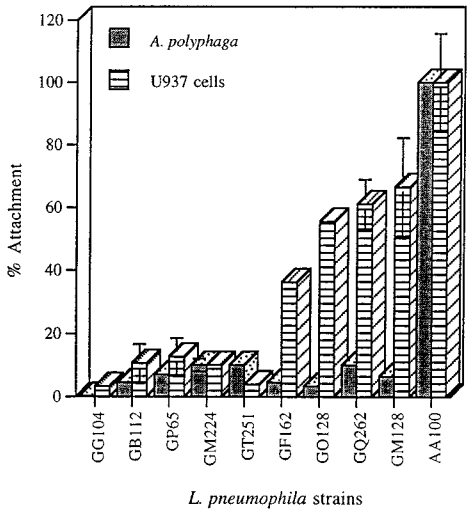

FIG. 4. Levels of attachment of *L. pneumophila pmi* mutants to U937 macrophages and *A. polyphaga*. The numbers of bacteria (log CFU) represent the percentages of attached bacteria compared to that for the wild-type strain AA100 in the presence of an inhibitor of uptake in both host cells. All strains belong to group 1 except strains GM224 and GT251, which belong to group 5 (see Fig. 2). Values are the means of triplicate measurements, and the error bars represent standard deviations. Some of the error bars cannot be seen due to their small values. All other *pmi* mutants were not significantly distinguishable from AA100 with regard to attachment to both host cells (data not shown).

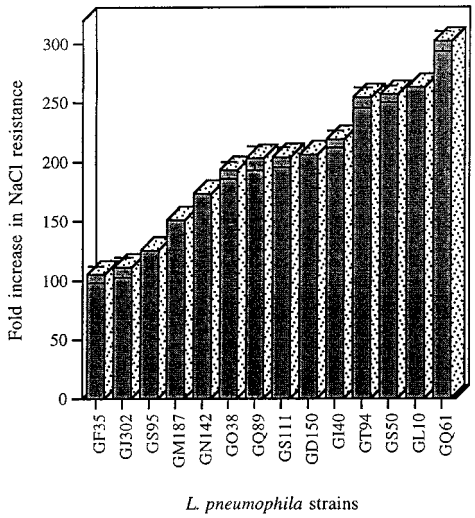

FIG. 5. Degree of resistance of *pmi* mutants of *L. pneumophila* to NaCl. Data are presented as fold increases in plating efficiency on BCYE plates supplemented with 0.6% NaCl compared to that of the wild-type strain AA100. Values are the means of triplicate measurements, and the error bars represent standard deviations. Some of the error bars cannot be seen due to their small values. All strains belong to group 2 except strains GO38 and GT94, which belong to groups 3 and 4, respectively.

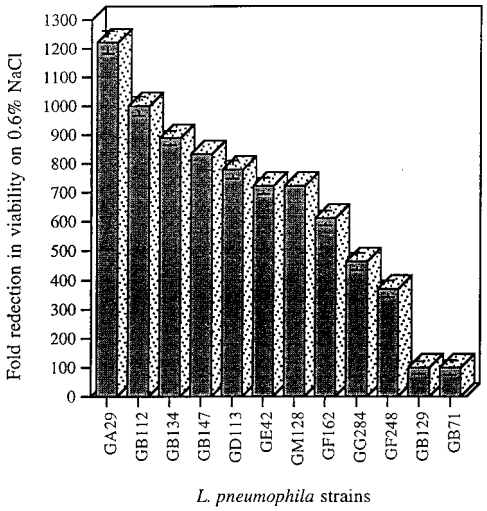

FIG. 6. Susceptibility of the NaCl-hypersensitive *pmi* mutants of *L. pneumophila* to 0.6% M NaCl. Data are presented as fold reductions in viability upon plating on NaCl-BCYE plates compared to that of strain AA100. These mutants were scattered among all of the groups (see Fig. 2). Values are the means of triplicate measurements, and the error bars represent standard deviations. Some of the error bars cannot be seen due to their small values.

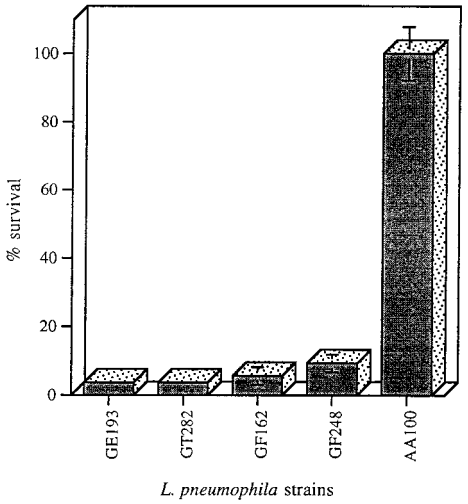

FIG. 7. Serum sensitivity of *pmi* mutants. Data are presented as the percentage of mutant bacteria surviving after 1 h of incubation at 37°C in 50% normal human serum compared to that of the wild-type strain AA100. Similar results were obtained with two other normal human sera (data not shown). Mutants GE193, GT282, and GF248 belong to group 2, while mutant GF162 belongs to group 1 (see Fig. 2). Values are the means of triplicate measurements, and the error bars represent standard deviations. Some of the error bars cannot be seen due to their small values. All other mutants were not significantly distinguishable from the wild-type strain with regard to resistance to normal human sera (data not shown).

dent subsequent testings. This phenomenon has also been encountered by other investigators (18a).

DISCUSSION

Although the mechanisms of uptake of *L. pneumophila* by mammalian and protozoan cells are different (4, 32, 40, 61), the similarity in the intracellular infections of macrophages and protozoa by *L. pneumophila* is quite remarkable (1, 16, 33, 34, 59). Within the cells of both of these evolutionarily distant hosts, the bacterium multiplies within a phagosome that does not fuse with lysosomes and is surrounded by the RER. A few mutants of *L. pneumophila* that are partially defective in intracellular replication within mammalian cells have been tested in protozoa, and they have been found to exhibit a similar phenotype (6, 19, 21, 53). These limited observations suggest that *L. pneumophila* may utilize similar, yet unknown, mechanisms to survive and replicate within both of these evolutionarily distant hosts. To test this hypothesis comprehensively and to start characterizing these mechanisms, we identified 89 mutants that exhibited similar phenotypes of defects in cytotoxicity, attachment, and intracellular replication within both macrophages and protozoa. The similarity in the patterns of growth kinetics of this collection of defective mutants within both hosts' cells indicated that similar mechanisms are utilized by *L. pneumophila* to replicate within mammalian and protozoan cells. Since our screening strategy selected for mutants defective in both hosts' cells, our data did not exclude the possibility that certain mechanisms may be utilized by *L. pneumophila* within one host but not the other.

The wide range of phenotypic defects in the survival and growth kinetics of the mutants within both hosts' cells are indicative of the genetic complexity manifested by *L. pneumophila* to survive and replicate within host cells. It is likely that some of the mutants have insertions within the same locus but at different locations. Nevertheless, future studies to characterize the insertions and determine the function of the proteins encoded by the *pmi* loci will allow determination of the mechanisms utilized by this pathogen to adapt to and replicate within the intracellular environment of mammalian and protozoan cells.

We have recently shown that *L. pneumophila* expresses multiple, morphologically distinct pili, and we characterized a genetic locus involved in expression of one type of pilus on the surface of *L. pneumophila* (57). Although expression of the respective pili by an isogenic mutant resulted in reduced adherence to epithelial cells, the pilin mutant was not defective in intracellular replication in different mammalian and protozoan host cells (57). Thus, none of the *pmi* mutants fails to express the respective pili. Three of the *pmi* mutants exhibited severe defects in attachment to both macrophages and *Acanthamoeba* cells, indicating that there are similar mechanisms involved in attachment to the cells of both hosts. On the other hand, another four mutants were severely defective in attachment to *A. polyphaga* but exhibited only mild reductions in their capacities to attach to macrophages. These data indicated that although certain mechanisms utilized by *L. pneumophila* to attach to both hosts' cells were similar, some other mechanisms of attachment were unique to protozoa. These observations are consistent with other reports of different mechanisms of uptake by the two hosts (4, 40). These observations have been substantiated by recent evidence from our laboratory which showed that attachment to and invasion of the protozoan host *H. vermiformis* by *L. pneumophila* is mediated by attachment to a 170-kDa Gal/GalNAc lectin receptor on the surfaces of protozoa (32, 61). Moreover, bacterial attachment and invasion are associated with tyrosine dephosphorylation of multiple host cell proteins, including the 170-kDa Gal/GalNAc lectin receptor (32, 61). Attachment of *L. pneumophila* to mammalian cells has been shown to be mediated by complement and noncomplement receptors (31, 39, 50).

All seven mutants of group 1 were defective in attachment and were also severely defective in intracellular survival and replication. None of these mutants was resistant to NaCl. We speculate that due to the defective ligands in these mutants,

they attached to alternate receptors on the host cell, which subsequently altered their fate within the host cell. These speculations may be supported by the fact that coating of *L. pneumophila* or *Toxoplasma gondii* with antibody to alter the mode of uptake and allow it to occur through the Fc receptor results in subsequent killing of these pathogens (8, 39). On the other hand, two mutants (of group 5) that were severely defective in attachment were mildly defective in intracellular replication. These data may suggest that the two mutants attached less efficiently than the wild-type strain to the same host cell receptor or that they attached to a different receptor that did not influence their subsequent fate within the host cell.

Four of the *pmi* mutants (of groups 1 and 2) were sensitive to normal human serum. All four mutants were severely defective in their survival and intracellular replication within both host cells, and none of them was NaCl resistant. Interestingly, one of these mutants (GF162, a member of group 1) was also severely defective in attachment to *A. polyphaga* and exhibited a reduction in attachment to U937 cells. These serum-sensitive mutants were most probably defective in outer membrane components, which rendered them susceptible to serum and defective in intracellular survival within the two hosts. These data indicated that the defective outer membrane components in these mutants played major roles in attachment of the bacteria to the host cell and their survival within it. Future work will determine whether these components are outer membrane proteins or lipopolysaccharides.

The relationship of the NaCl resistance of *L. pneumophila* to attenuation is not understood. However, in contrast to previous observations that the majority of mutants of *L. pneumophila* are NaCl resistant, most of the *pmi* mutants are not (36, 42, 44, 54, 60). Only 14 of 89 mutants were highly resistant to NaCl, indicating that NaCl resistance and the defect in intracellular survival and replication of *L. pneumophila* are two independent phenomena (54, 60). Our data caution that isolation of defective mutants of *L. pneumophila* solely on the basis of their NaCl-resistant phenotype may not yield a random and comprehensive representation of the loci required for intracellular replication (62).

Three of the *pmi* mutants were defective in flagellar expression. The variability in the degree of defects in intracellular replication of these aflagellar mutants confirmed previous observations that expression of other virulence-related genes was affected by the loss of flagellar expression (53). Our data showed that Fla^- mutants were among the least-defective mutants (groups 4 and 5), further indicating that flagella play a minor role, if any, in intracellular survival and replication. These observations are supported by the fact that 3 of 10 Fla^- mutant isolates had a wild-type intracellular replication phenotype (27). Moreover, Fla^- mutants are virulent in guinea pigs (25). It has recently been hypothesized that expression of flagella by *L. pneumophila* laboratory isolates can be an indirect measurement of their ability to survive and multiply intracellularly (15). Our data showed that flagella cannot be used as an indicator of virulence of genetically constructed mutants of *L. pneumophila*. However, our data did not exclude the possibility that flagella may be a reliable indicator of virulence of *L. pneumophila* laboratory strains, since the loss of flagella by the passaged strains may be associated with a defect or loss of other genetic loci required for intracellular survival and replication (15).

In summary, we isolated 89 mutants of *L. pneumophila* that exhibited similar defects of cytotoxicity and intracellular replication in mammalian and protozoan cells. We conclude that there are many similar mechanisms utilized by *L. pneumophila* to replicate within two evolutionarily distant hosts. However, we cannot exclude the possibility that there are additional loci required for survival within one host but not the other. Some of the mutants were defective in attachment to both host cells, serum sensitive, NaCl hypersensitive, or auxotrophic. Characterization of the role of the defective loci in intracellular infection should yield some interesting information about the mechanisms utilized by *L. pneumophila* to manipulate two evolutionarily distant hosts.

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