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A *Legionella pneumophila* Gene Encoding a Species-Specific Surface Protein Potentiates Initiation of Intracellular Infection

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To investigate the pathogenesis of Legionnaires disease at a molecular level, we mutated by directed allelic exchange a gene encoding a *Legionella pneumophila*-specific 24,000-dalton (Da) surface protein. Southern hybridization and immunoblot analyses demonstrated that the predicted DNA rearrangement occurred in *L. pneumophila* with a specific loss of 24-kDa antigen expression. Compared with its isogenic parent, the mutant was significantly impaired in its ability to infect transformed U937 cells, a human macrophagelike cell line; i.e., the bacterial inoculum of the mutant strain that was required to initiate infection of the macrophage monolayer was ca. 80-fold greater than that of the isogenic parent strain. The mutant strain regained full infectivity on reintroduction of a cloned 24-kDa protein gene, indicating that the reduced infectivity was due specifically to the mutation in that gene. Compared with the parent strain, the mutant strain was recovered at titers that were ca. 10-fold lower shortly after infection, but it exhibited a similar intracellular growth rate over the next 40 h, indicating that the mutant was defective in its ability to initiate macrophage infection rather than in its ability to replicate intracellularly. When opsonized, the mutant strain was still significantly less infectious than the parent strain, despite equivalent macrophage association, suggesting that the mutant was not merely missing a ligand for macrophage attachment. The mutant also exhibited reduced infectivity in explanted human alveolar macrophages, demonstrating the relevance of the U937 cell model for analyzing this mutant phenotype. These results represent the first identification of a cloned *L. pneumophila* gene that is necessary for optimal intracellular infection; we designate this gene *mip*, for macrophage infectivity potentiator.

A variety of serious human infections are caused by bacterial pathogens that flourish in the intracellular environment of the macrophage. *Legionella pneumophila*, the causative agent of Legionnaires disease, is such a pathogen, and its capacity to produce this disease is thought to be directly dependent on its ability to parasitize human alveolar macrophages (31). In attempting to identify bacterial factors responsible for this parasitism, which is still poorly understood at the molecular level, several investigators have used biochemical analyses to identify components of *L. pneumophila* that may be virulence factors (1, 4, 12, 13).

To initiate a genetic analysis of intracellular parasitism, we and others have cloned and expressed several *L. pneumophila* antigens in *Escherichia coli* (9, 15). However, since intracellular parasitism is presumed to be a complex and multifactorial process, one would not expect these cloned genes, even those that encode an *L. pneumophila* virulence factor, to confer a pathogenic phenotype on *E. coli*. Therefore, to identify a factor with a definitive role in intracellular infection, we recently developed a system for genetic manipulation in a clinical isolate of *L. pneumophila*, which includes efficient conjugal DNA transfer (8) and site-specific mutagenesis by allelic exchange (3). In addition, our laboratory has developed an in vitro model for *L. pneumophila* intracellular infection in which we used monolayers of transformed U937 cells, a human macrophagelike cell line (25, 28). This system is capable of distinguishing strains of *L. pneumophila* that are virulent in guinea pigs from derivatives that have lost their virulence after prolonged passage on agar. To confirm that our methods of genetic manipulation do not influence *L. pneumophila* infectivity in this model system, we also demonstrated that the introduction of foreign DNA by allelic exchange into a neutral site on the *L. pneumophila* chromosome did not itself interfere with the ability of the altered strain to infect transformed U937 cells (3).

To identify targets for mutagenesis, we cloned genes encoding surface protein antigens specific to the pathogenic *L. pneumophila* species (6, 9–11, 24), reasoning that among the factors necessary for macrophage infection some would have these characteristics. Based solely on these immunochemical data (i.e., no functional information), we chose the gene encoding a 24,000-dalton (Da) *L. pneumophila*-specific antigen as a target for a site-specific mutation. This protein is distinct from the major outer membrane protein of *L. pneumophila*, which is a porin with a molecular size of 24 to 29 kDa (7, 13). In this study we determined that the gene encoding the 24-kDa antigen is necessary for optimal intracellular infectivity, since a strain containing a site-specific mutation in this gene has a significantly reduced ability to infect both U937 cells and human alveolar macrophages but regains full infectivity on reintroduction of the wild-type 24-kDa protein gene.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *L. pneumophila* AA103 is a spontaneous nalidixic acid-resistant and streptomycin-resistant (Str^r) derivative of clinical isolate strain 130b (Los Angeles), serogroup 1 (3, 9). Strain AA103 expresses the *L. pneumophila*-specific 24-kDa antigen. *E. coli* HB101

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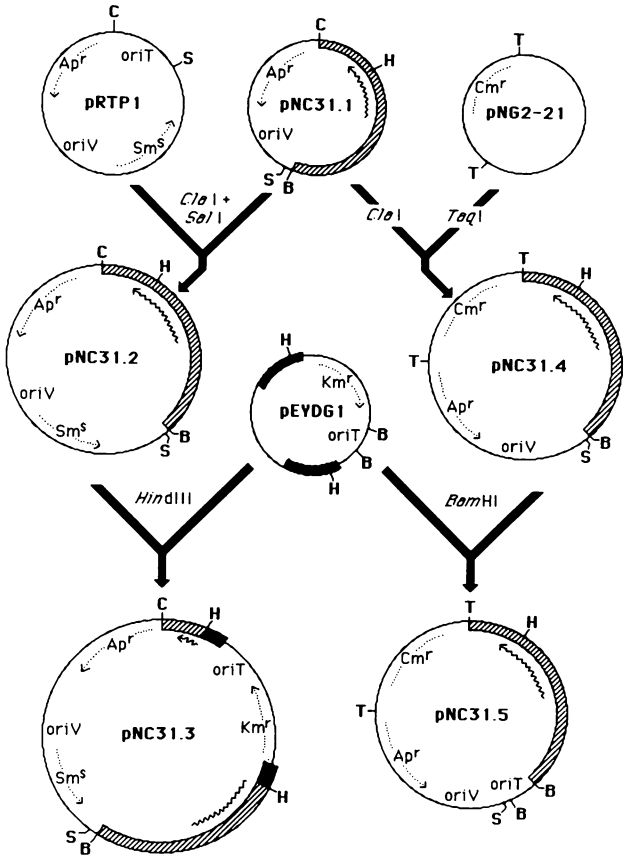

FIG. 1. Construction of plasmids pNC31.3 and pNC31.5. The segment of the *L. pneumophila* chromosome contributed by pNC31.1 (▨) is as indicated. The transpositionally defective remnants of IS*50* from pEYDG1 (■) are also indicated. The 24-kDa protein gene is indicated by the wavy, solid arrow. Restriction enzyme sites are abbreviated as follows: B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; S, *Sal*I; T, *Taq*I.

served both as a host for recombinant plasmids and as a donor strain in conjugal matings.

The 24-kDa protein gene was first identified in our original clone bank on a plasmid designated pSMJ31 (9). In preparation for the experiments presented here, a ca. 4.5-kilobase (kb) *Bam*HI-*Cla*I fragment from pSMJ31 containing the entire 24-kDa protein gene was ligated with the *Bam*HI-*Cla*I fragment of pBR322 containing the ColE1 origin of replication (*oriV*) and the ampicillin resistance gene (Ap^r) to yield pNC31.1 (Fig. 1). Plasmid pEYDG1 served as the source of the origin of transfer of the conjugative plasmid RK2 (*oriT*) (32). The *oriT* site in pEYDG1 is cloned within a functional copy of Tn*5* and inactivates the streptomycin resistance gene (22) but not the kanamycin resistance gene (Km^r) of Tn*5*. Plasmid pRK212.1 is an IncP helper plasmid (32) that was used to mobilize *oriT*-containing ColE1 replicons into *L. pneumophila*. Plasmid pRTP1 served as the source of the counterselectable *rpsL* (Sm^s) allele of *E. coli* (27). The *rpsL* allele encodes the wild-type ribosomal protein S12 and, when provided in *trans*, renders Str^r strains of *E. coli* streptomycin sensitive (Sm^s) (5). Plasmid pNG2-21 was the source of the chloramphenicol resistance gene (Cm^r) of pACYC194 (T. Serwold-Davis, personal communication).

L. pneumophila strains were grown on buffered charcoal-yeast extract (BCYE) agar for 48 to 72 h at 37°C (9). The following antimicrobial agents were added to the indicated concentrations: nalidixic acid, 25 μg/ml; kanamycin, 25 μg/ml; streptomycin, 300 μg/ml; chloramphenicol, 5 μg/ml. *E. coli* strains were maintained on L agar containing 50 μg of kanamycin per ml, 30 μg of chloramphenicol per ml, or 50 μg of ampicillin per ml.

Bacterial conjugations and allelic exchange mutagenesis. ColE1 replicons containing *oriT* sites and selectable kanamycin resistance markers were introduced into *L. pneumophila* by triparental matings as described previously (8). The transfer frequency of *oriT*-containing ColE1 replicons into strain AA103 was 10^{-2} to 10^{-3} per recipient (3).

The procedure for allelic exchange mutagenesis in *L. pneumophila* with ColE1 vectors containing the counterselectable *rpsL* allele was previously described in detail (3). We demonstrated that the *rpsL* allele, when provided in *trans*, rendered Str^r strains of *L. pneumophila* Sm^s. The frequency of allelic exchange was estimated to be 10^{-5} per transconjugant.

DNA isolation and molecular genetic techniques. Whole-cell DNA was extracted from strains of *L. pneumophila* as described previously (9). Plasmid DNAs were isolated from *E. coli* and *L. pneumophila* by the alkaline lysis method (2). Molecular cloning procedures have been described previously (9). Southern hybridizations were performed under conditions of high stringency by standard procedures (20).

Immunoassays. Protein extracts of *L. pneumophila* were prepared as described previously (24) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5%). Immunoblot analysis and in situ immunoassays were performed as described previously (3, 9). Monoclonal antibodies (MAbs) directed against the lipopolysaccharide of serogroup 1 (MAb 1C5) and the 24-kDa surface-exposed protein of *L. pneumophila* (MAb 12F4) were isolated in our laboratory (7, 25), as was rabbit anti-*L. pneumophila* serum.

The U937 cell model of intracellular macrophage infection. U937 cells are a human histiocytic lymphoma cell line, which, when treated with phorbol esters, differentiate into nondividing, glass-adherent cells with the phenotypic characteristics of macrophages (28). Details describing the infection of U937 cells by *L. pneumophila* were presented previously (25). For the experiments described here, U937 cell infection was established by similar methods, and then the procedures were modified, depending on whether infectivity, intracellular growth and survival, or cytopathogenicity was being measured. In all experiments, monolayers containing approximately 1×10^5 to 2×10^5 transformed U937 cells were prepared in Dulbecco modified Eagle medium (DMEM) in 96-well microtiter plates. After separate inoculation of replicate monolayers ($n = 8$) with test strains suspended in 100 μl of DMEM, the monolayers were incubated for 2 h to permit bacterial contact and uptake by the monolayers and were then washed and treated with DMEM containing 50 μg of gentamicin per ml for 2 h to kill adherent extracellular bacteria. After antibiotic treatment, the monolayers were washed three times with DMEM to remove the gentamicin and were then incubated with DMEM containing 10% fetal bovine serum at 37°C. It was often noted that as many as 10 to 25% of the macrophages in the monolayer were washed away after this inoculation process was completed.

To assay the relative infectivity of strains of *L. pneumophila* for U937 cells, the 50% infective dose (ID_{50}) was determined. Eight replicate monolayers were inoculated separately with each of the 10-fold dilutions of each strain (range of inocula, 10^2 to 10^8 bacteria per monolayer), treated

with gentamicin as described above, incubated for 3 days, and then lysed by the addition of 0.1% Triton X-100 to release any viable intracellular bacteria into the supernatant. One-tenth of the total volume in each well (i.e., 10 μl) was spotted onto BCYE agar, and after 48 to 72 h of incubation, each spot was examined for colonies (CFU) of *L. pneumophila*. Any growth within a spot represented the product of intracellular growth, survival, or both since *L. pneumophila* does not replicate extracellularly in DMEM. The proportion of spots that contained growth was determined for each of the inocula. The ID_{50} was defined as the minimum inoculum size that yielded intracellular *L. pneumophila* in 50% of the inoculated monolayers (i.e., CFUs in four of eight spots) and was calculated by the method of Reed and Muench (26).

The infection of U937 cells with preopsonized *L. pneumophila* was performed as described above with the addition of a preincubation step. Basically, ca. 10^9 CFU was incubated in DMEM in the presence of heat-inactivated, hyperimmune rabbit antiserum or MAb 1C5 (0.01 to 0.1 μg/ml) for 40 min at 37°C with rotation. The preopsonized bacteria were then serially diluted in DMEM (10-fold dilutions), and ID_{50} experiments were performed as described above, with unopsonized bacteria used as controls. Binding of the antibodies to the bacteria was quantitated by a kinetic enzyme-linked immunosorbent assay (30). The isogenic pair of strains examined in this study bound similar amounts of MAb 1C5; however, as expected, the strain lacking the 24-kDa surface antigen bound slightly less antibody after incubation with the hyperimmune serum (data not shown). Cellular uptake of the opsonized bacteria was confirmed by immunofluorescence and was found to be similar for the two strains. When inoculated with 10^8 CFU of opsonized *L. pneumophila*, >95% of U937 cells contained intracellular bacteria (>20 bacteria per cell).

To assay intracellular growth, survival, or both, the monolayers (n = 8) were inoculated separately with each of 10-fold dilutions of each strain (range, 10^5 to 10^8 CFU per set of eight monolayers), incubated for various times after gentamicin treatment (range, 0 to 72 h), and lysed. Tenfold serial dilutions of the supernatant were made in DMEM and then spotted onto BCYE agar. After 48 to 72 h of incubation, the number of CFU per spot was determined, and the number of CFU per monolayer was calculated. Quantitative culturing of the monolayer shortly after inoculation (i.e., 0 to 1 h after gentamicin treatment) was a reflection of the extent of uptake and intracellular survival, since intracellular replication did not begin before approximately 3 h after gentamicin treatment. Because of the low amounts of gentamicin that may have remained in the well after washing, it is very possible that the CFU recovered shortly after inoculation is an underestimate of the absolute numbers of intracellular bacteria.

The cytopathic effect (CPE) of *L. pneumophila* was determined by assaying the number of viable U937 cells remaining in the monolayer after infection with each strain (inocula, 10^6 CFU per monolayer) (25). The vital stain tetrazolium salt 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, Mo.) was used to distinguish between live and dead U937 cells. At 0, 24, 48, 72, and 96 h after gentamicin treatment, 5 μl of a stock solution of MTT (5 mg/ml) was added to the monolayers. After 4 h of incubation, the supernatants were expelled, and 0.1 ml of acid-isopropanol was added to each well. After thorough mixing, the optical density was read on an automatic enzyme-linked immunosorbent assay reader (EAR 400 AT; SLT Labinstruments USA, Ronkonkoma, N.Y.) at an optical density of 550 nm. Under the conditions used in these experiments, there was a linear relationship between the optical density at 550 nm and the number of viable U937 cells. Control experiments determined that no CPE occurred during the 2-h inoculation period (data not shown).

Infection of human alveolar macrophages. Human alveolar macrophages (AMs) were obtained from healthy volunteers as described previously (29), but with several modifications. Bronchoalveolar lavage was performed by using a fiberoptic bronchoscope. Preprocedure medication was 0.8 mg of atropine given intramuscularly. Topical anesthesia was achieved by using 0.5% lidocaine applied locally in the nose and posterior pharynx. The bronchoscope was wedged into a subsegmental bronchus of the lingula or right middle lobe, and 30-ml samples of sterile saline were injected and aspirated until 120 ml of saline was delivered. Bronchoalveolar washes were passed through gauze to remove particulate matter and excess mucus and were centrifuged; the sedimented cells were washed twice in Hanks balanced salt solution without Ca^{2+} and Mg^{2+}. Cells were then counted in a hemacytometer, and cytocentrifuge preparations were made to determine the cellular composition. AMs were identified by morphology on Wright-stained cytocentrifuge preparations. The bronchoalveolar cells of normal volunteers consisted of >90% AMs. The cells were suspended in DMEM containing penicillin (100 U/ml) and streptomycin (100 μg/ml) to a concentration of 5×10^6 cells per ml. The cell suspension (200 μl) was placed in flat-bottom wells of a 96-well microtiter plate (10^5 cells per well). The cells were allowed to adhere for 1 h and were then washed three times with DMEM. The cells were then incubated in DMEM containing 10% normal human serum for 36 h prior to infection.

The procedure for infecting AMs with *L. pneumophila* was essentially the same as that described above for U937 cell infection. Replicate monolayers (n = 4) were inoculated with ca. 4×10^6 CFU of each test strain per monolayer. At various times after gentamicin treatment, the number of viable bacteria per monolayer was quantitated by culturing 10-fold serial dilutions of the lysed monolayer supernatants.

RESULTS

Construction of a plasmid for the site-specific mutagenesis of the 24-kDa protein gene in *L. pneumophila*. The exact location and restriction map of the 24-kDa protein gene was determined by fine-structure mapping and DNA sequence analysis of subclones of pSMJ31 (7). As a site for insertional inactivation of the 24-kDa protein gene, we chose a *Hin*dIII site which was located within and near the 3′ end of the gene because preliminary experiments determined that a 4-base-pair insertion in that site removed antigen expression in *E. coli*. To construct pNC31.3 (Fig. 1), the plasmid used in the allelic exchange experiment, a *Cla*I-*Sal*I fragment from pNC31.1 containing the 24-kDa protein gene was ligated with a *Cla*I-*Sal*I fragment from pRTP1 containing *oriV*, Ap^r, and the counterselectable *rpsL* allele (Sm^s) to give pNC31.2. To introduce simultaneously a stable mutation and a rescuable marker into the 24-kDa protein gene sequence, a 4-kb *Hin*dIII fragment from pEYDG1 containing the selectable Km^r marker, but not the transposition functions, of Tn*5* was then inserted into the unique *Hin*dIII site of pNC31.2. The 4-kb insert DNA also provided an *oriT* site which mediated the transfer of pNC31.3 into *L. pneumophila* in triparental matings. Expression of the 24-kDa protein in *E. coli* was verified by immunoanalysis by using MAb 12F4. As ex-

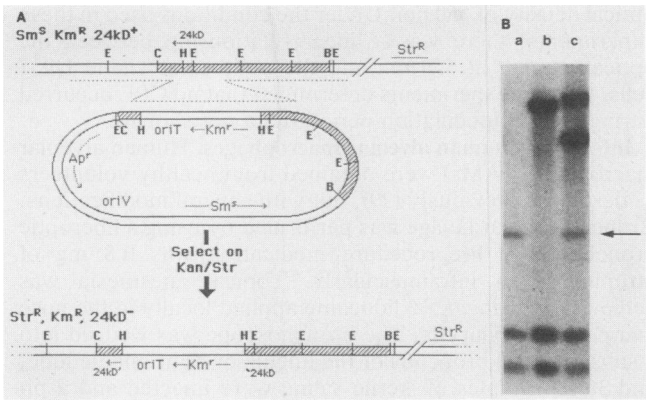

FIG. 2. Mutagenesis of the 24-kDa protein gene of *L. pneumophila*. (A) Diagram of the mutagenesis procedure. The top half depicts a pNC31.3-containing *L. pneumophila* transconjugant. A restriction map of the chromosomal region carrying the 24-kDa protein gene is shown as a double line. A map of pNC31.3 is indicated below, and the dotted line indicates a possible double crossover event between this plasmid and the chromosome. The predicted result of this recombination, with the mutated 24-kDa protein gene replacing the wild-type gene, is shown at the bottom. The locations of *Eco*RI (E) sites are indicated, and all other notations are the same as those given in the legend to Fig. 1. (B) Demonstration of allelic exchange by Southern hybridization analysis. DNAs from three strains were digested with *Eco*RI, electrophoresed in 0.8% agarose, and probed with ^{32}P-labeled pNC31.3. Lanes: a, strain AA103; b, strain AA105 which had undergone allelic exchange; c, strain AA103(pNC31.3). Note that the 1.15- and 1.1-kb fragments which hybridized to the probe appear as a doublet in the autoradiograph. The arrow indicates the 3-kb fragment.

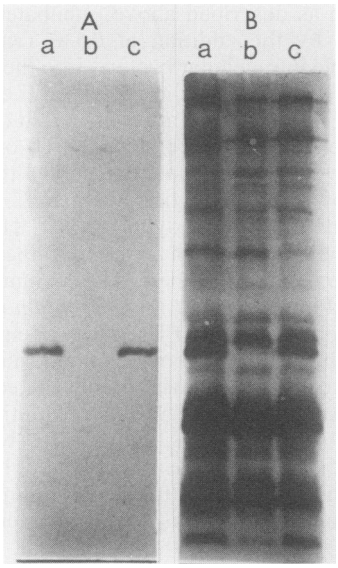

FIG. 3. Immunoblot analysis of *L. pneumophila* strains. (A) Immunoblot using anti-24-kDa protein MAb 12F4. (B) Immunoblot using anti-*L. pneumophila* serum. Lanes: a, strain AA103; b, strain AA105; c, strain AA103(pNC31.3). Aside from the conspicuous absence of the 24-kDa antigen in lanes b, the slight differences between the samples in panel B represent normal, lane-to-lane variability of banding patterns.

pected, clones containing pNC31.2 expressed the antigen, whereas those containing pNC31.3 did not (data not shown).

Construction of a *L. pneumophila* mutant that lacks the 24-kDa antigen. To introduce pNC31.3 into *L. pneumophila*, strains AA103, HB101(pNC31.3), and HB101(pRK212.1) were mated; and the conjugation mixture was plated on BCYE-nalidixic acid-kanamycin-streptomycin. Km^r Str^r colonies were obtained at a frequency of approximately 10^{-6} per recipient. To identify a clone that had completed the allelic exchange process (Fig. 2A), genomic DNA was isolated from 10 Km^r Str^r strains, digested with *Eco*RI, and examined by Southern hybridization analysis (Fig. 2B). There were four *Eco*RI fragments (approximately 3.0, 1.5, 1.15, and 1.1 kb in size) in *L. pneumophila* AA103 that hybridized to the probe (Fig. 2B, lane a). The model of recombination predicted that the 3-kb fragment (Fig. 2B, arrow) containing the *Hin*dIII site within the 24-kDa protein gene should be lost during allelic exchange (Fig. 2A). The two largest *Eco*RI fragments of pNC31.3 (approximately 7.6 and 5.0 kb in size) would also be absent in a strain that had undergone exchange and lost the plasmid, whereas the 1.5- and 1.1-kb fragments would be retained (Fig. 2B, lane c). One of the strains examined, designated strain AA105, lost the chromosomal and plasmid bands predicted by the model and acquired a new *Eco*RI fragment (approximately 7 kb in size) in their place (Fig. 2B, lane b). Southern hybridization analysis of the DNAs that were digested with a variety of other restriction enzymes confirmed that strain AA105 underwent allelic exchange (data not shown).

To confirm that allelic exchange resulted in the loss of 24-kDa antigen expression in *L. pneumophila*, strain AA105 was examined by immunoblot analysis (Fig. 3). Immunoblots with either MAb 12F4 or anti-*L. pneumophila* serum confirmed that strain AA105 did not express the 24-kDa protein antigen or any cross-reactive polypeptides. In addition, strain AA105 did not appear to exhibit any other significant alteration in antigen profile (Fig. 3B). Silver stain and immunoblot analyses of two-dimensional sodium dodecyl sulfate-polyacrylamide gels confirmed that there was no detectable difference in protein expression between strains AA103 and AA105, other than the loss of the 24-kDa antigen (7).

Effect of the mutation in the 24-kDa protein gene on infectivity of *L. pneumophila* for U937 cells. To determine whether the 24-kDa protein gene is necessary for intracellular infection of macrophages, we compared the ability of strains AA103 and AA105 to infect U937 cells by culturing inoculated monolayers for *L. pneumophila* after 3 days of incubation. The relative infectivity was determined from the inoculum required to infect 50% of the monolayers (ID_{50}) (Table 1). In each of 15 experiments (results of 5 of which are presented in Table 1 and 2 of which are presented in Table 3), the mutant exhibited an ID_{50} that was significantly higher than that of its parent ($P < 0.001$). The mean difference in the log ID_{50} was 1.9 log units or ca. 80-fold (standard deviation, 0.53 log units). This difference in infectivity was maintained after passage of the two strains through U937 cell monolayers. A ca. 2-log-unit difference in infectivity was also obtained if the infection was done in the absence of gentamicin. Moreover, the effect was observed in the absence of Triton X-100 (see CPE experiment below). Finally, the isogenic pair had similar survivabilities in DMEM at 37°C (data not shown). Taken together, these control experiments indicate that the reduced infectivity of the mutant is not simply due to an increased sensitivity to the chemical substances used in the assay.

To determine whether the reduced infectivity of strain AA105 was due specifically to the mutation in the 24-kDa

TABLE 1. Infectivity of *L. pneumophila* strains in U937 cells

Expt[a]	Log ID_{50}[b]		ΔLog ID_{50}[c]
	AA103	AA105	
1	4.85 ± 0.31	7.60 ± 0.31	2.75
2	4.38 ± 0.32	6.43 ± 0.23	2.05
3	4.42 ± 0.31	6.08 ± 0.32	1.66
4	3.23 ± 0.31	4.79 ± 0.29	1.56
5	3.05 ± 0.34	4.81 ± 0.30	1.76

[a] Differences in log ID_{50} of a given strain in different experiments were caused by day-to-day variations in the number of U937 cells per monolayer. Therefore, log ID_{50}s can only be compared within a single experiment. In all experiments, the ID_{50}s for the two strains were significantly different ($P < 0.001$; Student's t test).

[b] ID_{50} is expressed as a log value ± the standard deviation expressed as a log value.

[c] Δlog ID_{50} = log ID_{50} of strain AA105 − log ID_{50} of strain AA103. The mean Δlog ID_{50} of the five experiments in this table and the two experiments in Table 3 was 1.90 (95% confidence limits, 0.94 to 2.96 log units). Eight additional experiments were performed but were excluded from the statistical analysis because the endpoint for one of the ID_{50}s was beyond the range of inocula tested. The Δlog ID_{50}s for these experiments were >2.33, >2.24, >1.63, >0.49, <1.06, >1.00, >1.14, and >0.5.

protein gene, we complemented the defect by reintroducing an intact copy of the gene into the mutant strain. Toward that end, plasmid pNC31.5 was constructed (Fig. 1) and mobilized into strain AA105 in a triparental mating, and transconjugants were selected on BCYE-kanamycin-chloramphenicol. Upon introduction of pNC31.5 into strain AA105, antigen expression was restored (data not shown), and in five separate experiments, increased infectivity in the U937 system was observed ($P < 0.001$); e.g., AA105 (pNC31.5) tested in parallel with the isogenic pair in experiment 5 in Table 1 had a log ID_{50} equal to 3.83 ± 0.33. Another *L. pneumophila* mutant that was constructed by allelic exchange mutagenesis but that contained the DNA insertion at a locus distinct from that of the 24-kDa protein gene was indistinguishable from the parent strain AA103 in terms of U937 cell infectivity (3). These results indicate that the loss of infectivity exhibited by the mutant strain is due specifically to the mutation in the 24-kDa protein gene and that this gene is necessary for optimal intracellular infection of U937 cells by *L. pneumophila*.

Effect of the mutation in the 24-kDa protein gene on the ability of *L. pneumophila* to initiate infection. The high ID_{50} of AA105 relative to that of AA103 can be explained by a reduced ability of the mutant strain either to initiate infection of the macrophage or to replicate intracellularly within the macrophage. To test the former possibility, we infected U937 cell monolayers separately with strains AA103 or AA105, but harvested intracellular bacteria 0 to 1 h after gentamicin treatment, i.e., before the onset of bacterial replication (Table 2). In over 10 separate experiments (7 of which are presented in Table 2), we recovered ca. 5- to 20-fold fewer intracellular bacteria from monolayers infected with AA105 than from those infected with an equivalent number of strain AA103 bacteria. The phenotype of the mutant was again complemented by an intact 24-kDa protein gene (Table 2). These data suggest that the mutant has a defect that is apparent in the earliest stages of infection. Moreover, this defect alone could account for the difference in the ID_{50} observed at 3 days postinoculation, since multiple rounds of reinfection occurred in the monolayer over the 3-day incubation period.

The reduced recovery of strain AA105 shortly after infection could have been due to a defect either in the uptake

TABLE 2. Recovery of *L. pneumophila* strains from U937 cells shortly after infection[a]

Expt[b]	Proportion of the following inoculum recovered[c]		
	AA103	AA105	AA105 (pNC31.5)
1	13.3 ± 5.4[d]	1.9 ± 1.1	
2	36 ± 15	4.3 ± 3.3	
3	40 ± 12	4.7 ± 2.3	15 ± 10[e]
4	43 ± 21	8.6 ± 5.7	36 ± 14
5	265 ± 140	50 ± 15	
6	300 ± 100	16 ± 4.9	140 ± 100
7	760 ± 150	140 ± 70	

[a] The numbers of intracellular bacteria were determined at 0 to 1 h after gentamicin treatment. At that time the bacteria and the U937 cells were in contact for ca. 4 to 5 h (see text).

[b] Differences in the recovery of a given strain in different experiments were caused by a variation in the number of macrophages per monolayer and the size of the bacterial inoculum. Therefore, the recovery of the test strains can only be compared within a single experiment.

[c] Values are (average CFU per well/CFU in inoculum) × 10^7 ± standard deviation. In each experiment similar inocula of each strain were used. In experiments 1 to 4 and experiments 5 to 7, the inocula were within the ranges of 10^7 to 10^8 CFU and 10^6 to 10^7 CFU, respectively.

[d] $P < 0.001$ with respect to strain AA105 for all experiments except those indicated by *e*.

[e] $P < 0.02$ with respect to strain AA105.

stage of infection, i.e., attachment to or penetration into the macrophage, or in resistance to killing mechanisms which operate immediately after phagocytosis. To begin to distinguish between these two possibilities, we compared the infectivities of antibody-opsonized bacteria, reasoning that if the mutation removed a ligand for uptake, opsonization would bypass the defect and equalize the infectivities of the parent and mutant strains. We have previously determined that opsonization with antibodies significantly increases the infectivity of *L. pneumophila* for U937 cells by increasing uptake (E. Pearlman, N. C. Engleberg, and B. I. Eisenstein, manuscript in preparation). Opsonization with either hyperimmune antiserum or MAb 1C5 enhanced infectivity (i.e., lowered the ID_{50}) of strains AA103 and AA105 to similar extents such that the difference in their ID_{50}s was not reduced (Table 3). These results suggest that the mutant did not simply lose a ligand for macrophage attachment, but rather, that the defect was at a later step in the initiation of intracellular infection.

Effect of the mutation in the 24-kDa protein gene on the intracellular replication of *L. pneumophila*. To examine the ability of the mutant to replicate intracellularly, we infected U937 monolayers separately with strains AA103 or AA105 and assayed the monolayers for changes in the number of

TABLE 3. Infectivity of opsonized *L. pneumophila* strains in U937 cells

Expt	Opsonin	Log ID_{50}		ΔLog ID_{50}[a]
		AA103	AA105	
1	None	5.23 ± 0.26	6.40 ± 0.30	1.17
	Anti-*L. pneumophila*[b]	<3.90[c]	5.60	>1.70
2	None	3.34 ± 0.30	5.70 ± 0.31	2.36
	MAb 1C5	1.60 ± 0.41	4.23 ± 0.28	2.63

[a] See footnote *c* in Table 1.

[b] Heat-inactivated, hyperimmune serum.

[c] The endpoint was not defined.

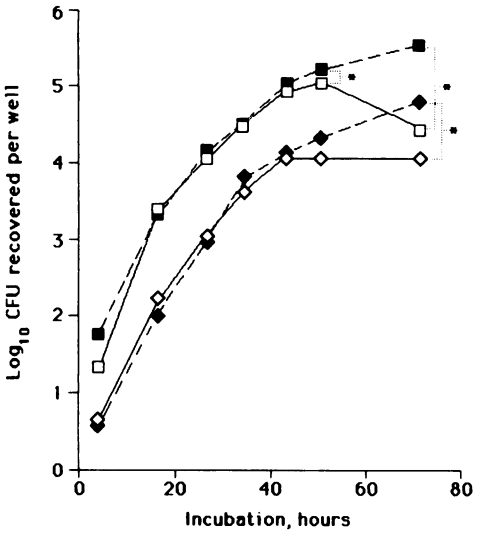

FIG. 4. Intracellular replication of *L. pneumophila* strains in U937 cells. Monolayers were infected separately with strain AA103 or AA105, and after various incubation periods the numbers of intracellular *L. pneumophila* were determined in eight replicate monolayers. The strains and the inoculum used for each experiment are indicated as follows: strain AA103 with an inoculum of 2.3×10^5 (◆) or 2.3×10^6 (■); strain AA105 with an inoculum of 4.2×10^6 (◇) or 4.2×10^7 (□). Note that time zero on the *x* axis corresponds to the time when the monolayer was first inoculated; i.e., the 4-h time point corresponds to 0 h after gentamicin treatment. Although the calculated standard deviations were excluded for the sake of clarity, the mean CFU recovered from monolayers that contained comparable numbers of parent and mutant bacteria at the first time point became significantly different after ca. 40 h (the asterisks indicate $P < 0.05$).

intracellular bacteria over a 3-day period (Fig. 4). When monolayers were inoculated with comparable numbers of the two strains, monolayers infected with strain AA105 yielded approximately 10-fold fewer intracellular bacteria for the first 40 h after inoculation and nearly 100-fold fewer bacteria at 3 days, supporting the results in Tables 1 to 3. When monolayers were inoculated with 10-fold more mutant bacteria, so that there would be comparable numbers of intracellular parent and mutant bacteria at the earliest time point, strains AA103 and AA105 exhibited very similar growth kinetics for the first 40 h. These data indicate that the mutation in the 24-kDa protein gene does not produce a detectable difference in intracellular growth. The fact that the parent and mutant strains had different intracellular growth curves after 40 h postinoculation may reflect the reduced ability of the mutant to initiate subsequent rounds of cellular infection in the monolayer.

We have previously determined that after 72 h of intracellular growth of *L. pneumophila* there is a detectable reduction in the viability of the U937 cell monolayer (CPE) (25). Moreover, the magnitude of the CPE correlates with the extent of bacterial replication within the monolayer. Therefore, to obtain an independent measurement of the relative infectivity of strain AA105, we inoculated U937 monolayers with ca. 10^6 CFU of each strain and then assayed the number of viable U937 cells remaining in the monolayer after various periods of incubation (Fig. 5). A reduced CPE for AA105 relative to that for AA103 was detected by 3 days postinoculation ($P < 0.05$). This difference in CPE was compatible with the ca. 10-fold fewer CFU recovered from such monolayers infected with AA105. This mutant phenotype was complemented by reintroduction of an intact copy of the 24-kDa protein gene (Fig. 5).

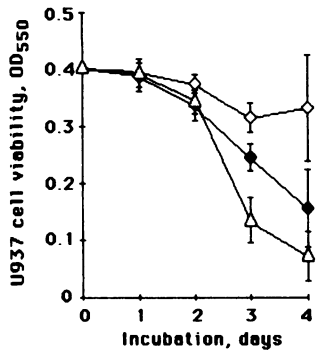

FIG. 5. CPE of *L. pneumophila* strains on U937 cell viability. Replicate monolayers were inoculated with 10^6 bacteria. After various periods of incubation, the number of viable U937 cells remaining in the monolayer was determined by staining the cells with MTT and measuring the optical density. Strains included AA103 (◆), AA105 (◇), and AA105(pNC31.5) (△). OD_{550}, Optical density at 550 nm.

Intracellular infection of human alveolar macrophages with strains of *L. pneumophila*. To extrapolate the results obtained with the U937 cell system to a more physiologic cell type, we compared the infectivities of these strains for human AMs (Fig. 6). Again, significantly fewer numbers of strain AA105 were recovered from AMs shortly after inoculation, and there were ca. 1.5 log fewer bacteria recovered by 24 h ($P < 0.001$). In marked contrast, strain AA105(pNC31.5) was as infective for AMs as was strain AA103. These data further validate the U937 cell system as a model for the study of macrophage infectivity and confirm that the mutant lacking the 24-kDa antigen is defective in its ability to infect U937 cells and human macrophages.

DISCUSSION

Using molecular genetic techniques, we constructed an *L. pneumophila* mutant that was defective in the expression of a 24-kDa species-specific surface protein. The mutant strain was impaired in its ability to infect U937 cells and human alveolar macrophages, but regained its infectivity on reintroduction of an intact 24-kDa gene, indicating that the 24-kDa

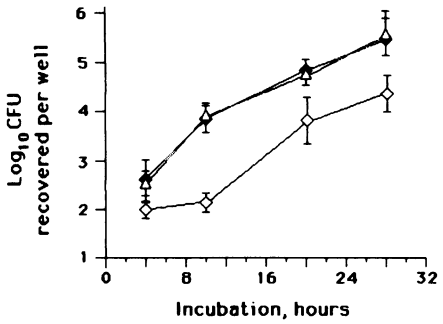

FIG. 6. Infection of explanted human AMs with strains of *L. pneumophila*. AM monolayers ($n = 4$) were inoculated with *L. pneumophila* strains (4×10^6 CFU per well), incubated, and then quantitatively cultured at various time intervals. Strains included AA103 (◆), AA105 (◇), and AA105(pNC31.5) (△).

protein gene is necessary for the full infectivity of *L. pneumophila*. We designated the 24-kDa protein gene as *mip* for macrophage infectivity potentiator.

The nature of the mutation introduced into the *mip* gene and the complementation of that mutation warrant several comments. First, the mutation was stable. Therefore, the interpretation of the macrophage infection experiments was not complicated by the possibility of strain reversion. Second, the mutation in the *mip* gene resulted in a loss of detectable 24-kDa antigen expression. Although truncated proteins or a fusion protein could have been produced as a result of the insertion mutation, none were detected. These cross-reactive proteins, if produced at all, were presumably degraded rapidly or were present in very low amounts. Third, although it is possible that the insertion in the *mip* gene could have a polar effect on a downstream gene that is necessary for macrophage infectivity (a gene that is also carried on pNC31.5), this is unlikely since DNA sequence analysis of the *mip* region of the chromosome indicated that there is a putative, factor-independent transcription termination site immediately after the *mip*-coding region, suggesting that the *mip* mRNA is monocistronic (7). In addition, the first 300 of 500 base pairs following the *mip* gene on pNC31.5 were sequenced, and there were no apparent open reading frames or initiation codons in this region. Fourth, in some experiments it appeared that the defect in strain AA105 was not fully complemented by pNC31.5. This phenomenon could be due to slight differences in the level of expression of the *mip* gene when it was present on a plasmid as compared with when it was present on the chromosome. In addition, ColE1 replicons such as pNC31.5 are unstable in *L. pneumophila* and can readily integrate into the chromosome since strain AA103 is Rec^+ (3, 8). Therefore, the amount of *mip* expression in a given inoculum of AA105(pNC31.5) might have varied from day to day as the state of the plasmid varied within the bacterial population.

The mutant analyzed here is the first of a new class of *L. pneumophila* mutants. All previously isolated avirulent mutants of *L. pneumophila* have been shown to be unable to replicate intracellularly (17, 21, 25). Two such mutants were derived by passing a virulent strain on artificial medium and may contain multiple mutations (17, 25). A third mutant deficient in intracellular replication was derived by chemical mutagenesis and was a thymidine auxotroph (21). In contrast to these mutants, strain AA105 was defective in its ability to initiate macrophage infection rather than in its ability to replicate intracellularly and contained a mutation in a gene with an identified product.

We performed an initial characterization of the cellular events that determine the infectivity step(s) that was altered in the mutant. We found that there was a reduced recovery at early time points. The infectivity experiments done with antibody-opsonized bacteria suggest, although do not prove, that the mutation in the *mip* gene does not simply remove a ligand that is involved in the uptake stage of infection. It has recently been demonstrated that the infectivity of *L. pneumophila* for monocytes (23) and U937 cells (E. Pearlman, personal observations) is enhanced in the presence of serum, i.e., complement. It will be interesting to compare the infectivities of the parent and mutant under these conditions of uptake. Nevertheless, results of preliminary experiments done by fluorescence microscopy suggest that the unopsonized *mip* mutant is not impaired in its ability to bind and enter U937 cells (unpublished data). These data suggest that the mutant has reduced survivability immediately after uptake into the macrophage. It has been demonstrated that *L. pneumophila* resists intracellular killing after uptake by inhibiting the oxidative burst (19), the acidification of the phagosome (18), and phagosome-lysosome fusion (16). The mutant could be defective in one or more of these activities or in other cellular processes which are not as well characterized. The magnitude of the reduction in infectivity (i.e., a ca. 80-fold reduction in infectivity and not an all-or-nothing effect) is compatible with this type of defect because bacterial functions involved in the initiation of intracellular infection may be redundant or overlapping and because the defensive mechanisms operating within the phagocyte are not 100% efficient.

Circumstantial evidence suggests that the 24-kDa protein is itself involved in the infection process either directly or proximately. First, the mutation in the *mip* gene did not result in a detectable change in a variety of specific bacterial phenotypes, other than the loss of the 24-kDa antigen; e.g., the parent and the mutant strains had comparable growth rates on artificial medium, similar colonial and cellular morphology and motility, and a similar binding capacity to a variety of antibodies and complement (data not shown). Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis indicated that the *mip* mutant did not have a detectable change in protein expression other than the loss of the 24-kDa protein (7). Second, the mutation appeared to have an effect on an early step in the infection process rather than a generalized effect on intracellular infection. Third, the 24-kDa protein had characteristics that were compatible with the type of change in infectivity that occurred in the mutant; the protein was surface-expressed, highly basic (pI 9.8), and unique to the species *L. pneumophila* (6, 7, 9–11, 24). Cationic polypeptides are known to enhance membrane interactions, and cationic lysosomotropic agents such as NH_4Cl and various amines can raise the intralysosomal pH and inhibit phagosome-lysosome fusion (14). We are in the process of determining the precise function of the 24-kDa protein in the intracellular life cycle of *L. pneumophila*. In addition, the methods used to establish that the *mip* gene is necessary for full infectivity of human macrophages can now be applied to examine the importance of genes encoding other *Legionella*-specific surface proteins.

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