

Identification of *Legionella pneumophila rcp*, a *pagP*-Like Gene That Confers Resistance to Cationic Antimicrobial Peptides and Promotes Intracellular Infection

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In the course of characterizing a locus involved in heme utilization, we identified a *Legionella pneumophila* gene predicted to encode a protein with homology to the product of the *Salmonella enterica* serovar Typhimurium *pagP* gene. In *Salmonella*, *pagP* increases resistance to the bactericidal effects of cationic antimicrobial peptides (CAMPs). Mutants with insertions in the *L. pneumophila pagP*-like gene were generated and showed decreased resistance to different structural classes of CAMPs compared to the wild type; hence, this gene was designated *rcp* for resistance to cationic antimicrobial peptides. Furthermore, *Legionella* CAMP resistance was induced by growth in low-magnesium medium. To determine whether *rcp* had any role in intracellular survival, mutants were tested in the two most relevant host cells for Legionnaires' disease, i.e., amoebae and macrophages. These mutants exhibited a 1,000-fold-decreased recovery during a *Hartmannella vermiformis* coculture. Complementation of the infectivity defect could be achieved by introduction of a plasmid containing the intact *rcp* gene. Mutations in *rcp* consistently reduced both the numbers of bacteria recovered during intracellular infection and their cytopathic capacity for U937 macrophages. The *rcp* mutant was also more defective for lung colonization of A/J mice. Growth of *rcp* mutants in buffered yeast extract broth was identical to that of the wild type, indicating that the observed differences in numbers of bacteria recovered from host cells were not due to a generalized growth defect. However, in low-Mg²⁺ medium, the *rcp* mutant was impaired in stationary-phase survival. This is the first demonstration of a *pagP*-like gene, involved in resistance to CAMPs, being required for intracellular infection and virulence.

Legionella pneumophila is a facultative intracellular parasite of human monocytes, macrophages, and protozoa (48, 63, 69). In the natural environment, *Legionella* species occupy the niches of soil and water, either free-living, in biofilms, or, more commonly, residing within amoebal hosts (29, 69). Problems occur when legionellae enter and replicate in human-made water systems, from which inhalation of bacterium-laden aerosols leads to the establishment of a pneumonia referred to as Legionnaires' disease (28).

Pathogens, such as *L. pneumophila*, that adopt an intracellular lifestyle must possess multiple strategies to overcome or evade the defense system of the host cell. For example, mammalian macrophages and amoebae are well equipped to bring about the demise of engulfed bacteria via the action of both oxygen-independent and -dependent mechanisms (31, 40, 53). Oxygen-independent mechanisms include cationic antimicrobial peptides (CAMPs), a group of structurally diverse polypeptides that are thought to kill by membrane damage due to pore formation (41, 53). *L. pneumophila* overcomes much of the killing mechanisms of the host cell by initially residing in a phagosome with limited ability to fuse with lysosomes, the source of the majority of antibacterial substances within the phagocyte (13, 47, 57, 78, 79). However, several lines of evidence suggest that *L. pneumophila* is exposed to lysosomal factors and likely possesses resistance factors against at least

some of them. For example, some *L. pneumophila* mutants, which are readily delivered to a macrophage phagolysosome, do survive, albeit with little or no replication (9, 46, 56). Also, recent evidence suggests that phagolysosomal fusion does occur late in the intracellular infection cycle (75). Furthermore, extracellular *L. pneumophila* is inherently resistant to polymyxin B (PmB), a CAMP that is actually used for selection of *Legionella* spp. from clinical and environmental samples (23).

Although catalase/peroxidase and superoxide dismutase enzymes are known to promote *L. pneumophila* resistance to oxygen-dependent killing (2, 4, 74), the *Legionella* factors involved in CAMP resistance are completely unknown. Indeed, knowledge of bacterial CAMP resistance determinants is only beginning to emerge (7, 26, 67). The majority of the literature on CAMP resistance is from *Salmonella enterica* serovar Typhimurium, where several determinants have been identified, such as *pagP* (37–39, 81). This gene is transcriptionally activated by PhoPQ (hence the *pagP* gene designation as PhoP-activated gene P), a two-component regulator of genes involved in both enterocyte invasion and survival within macrophages (5, 39, 62). However, the importance of PagP in intracellular infections is not known (6).

Previous efforts by our group to isolate determinants important for iron acquisition and utilization in *L. pneumophila* led to the identification of a gene responsible for heme binding, *hbp* (65). Here, we demonstrate that in the *hbp* region of the *L. pneumophila* chromosome there is an open reading frame whose product has homology with PagP. The aims of our further investigations were to identify whether the *pagP*-like gene in *L. pneumophila* has a role in CAMP resistance and,

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TABLE 1. *Legionella* strains

Species	Strain	Serogroup	Source (reference)	
<i>L. pneumophila</i>	ATCC ^a BAA-74 (130b)	1	Clinical (25)	
	ATCC 33154	2	Clinical (59)	
	ATCC 33155	3	Clinical (59)	
	ATCC 33156	4	Clinical (59)	
	ATCC 33216	5	Clinical (35)	
	ATCC 33215	6	Clinical (60)	
	ATCC 33823	7	Clinical (10)	
	ATCC 35096	8	Clinical (12)	
	ATCC 43736	13	Clinical (55)	
	ATCC 43703	14	Clinical (8)	
	<i>L. birminghamensis</i>	ATCC 43702	1	Clinical (80)
	<i>L. erythra</i>	ATCC 35303	1	Environmental (14)
	<i>L. feeleii</i>	ATCC 35072	2	Clinical (77)
	<i>L. longbeachae</i>	ATCC 33462	1	Clinical (58)
<i>L. micdadei</i>	Stanford-R	1	Clinical (64)	
<i>L. parisiensis</i>	ATCC 35299	1	Clinical (14)	

^a ATCC, American Type Culture Collection.

more importantly, whether it has any relevance for intracellular infection and virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of *L. pneumophila* and other *Legionella* spp. used during these studies are listed in Table 1. *L. pneumophila* strain 130b (Wadsworth) was used for mutagenesis of the *Legionella pagP*-like gene and subsequently served as the wild-type control. A pBR322 plasmid library of 3- to 6-kb *Sau*3AI-restricted 130b genomic DNA was maintained in *Escherichia coli* K-12 strain HB101 (44). In the CAMP susceptibility assay, *Pseudomonas aeruginosa* strain PAK was used as a sensitive control (26). *L. pneumophila* strains were routinely grown on standard buffered charcoal-yeast extract (BCYE) agar for 3 days at 37°C (22). For selection of allelic exchange mutations, BCYE agar was supplemented with 5% (wt/vol) sucrose (54). For growth curves, *L. pneumophila* was grown either in buffered yeast extract broth or in chemically defined liquid medium (CDM) at 37°C with shaking at 250 rpm (Lab-Line Instruments model 3525) (54). The components of CDM have been described elsewhere (68); briefly, the base consisted of 3-(*N*-morpholino)propanesulfonic acid buffer, NaCl and KH₂PO₄, which was supplemented with reduced glutathione and a variety of amino acids and trace metals, including 0.7 mM MgSO₄. For CDM cultures, the starting inocula were from buffered yeast extract (BYE) cultures in the logarithmic phase of growth, i.e., with an optical density at 660 nm (OD₆₆₀) of 0.9 to 1.2. Bacteria were washed three times in CDM base before resuspension to an OD₆₆₀ of 0.15 in CDM containing various amounts of Mg²⁺. Luria-Bertani broth or agar was used for growth of *E. coli* and *P. aeruginosa* (70). Where appropriate, the medium was supplemented with the following antibiotics at final concentrations suitable for *L. pneumophila* (or *E. coli*): kanamycin (KAN), 25 µg/ml (50 µg/ml); and chloramphenicol (CHL), 3 or 6 µg/ml (30 µg/ml).

DNA sequence analysis. Double-stranded sequence was obtained from pEH12, a member of the pBR322 library containing *hbp* and surrounding genes, by the dideoxy chain termination method with ³⁵S-dATP and Sequenase (Amersham Life Sciences, Arlington Heights, Ill.) (65). Sequencing reactions were performed according to the manufacturer's recommendations using pUC-based subclones of pEH12, with vector-based primers. Nucleotide sequences were analyzed with PCGENE (IntelliGenetics), and homology searches were conducted through GenBank at the National Center for Biotechnology Information.

Mutation and complementation of an *L. pneumophila pagP*-like gene. A mutation in the cloned *pagP*-like gene of strain 130b was generated by insertion of a 1.1-kb KAN resistance cassette at the *Afl*III site of pEH12 (Fig. 1A), resulting in plasmid pBOC16. A 2.9-kb *Bam*HI-*Sac*I fragment from pBOC16 was cloned into pBOC20, resulting in pTA2 (Fig. 1A). Plasmid pBOC20 is based on a ColE1 replicon and facilitates allelic exchange in *L. pneumophila* by virtue of its *sacB* gene (54). Production of competent 130b cells and electroporation of pTA2 into *L. pneumophila* were achieved as previously described (54). Potential mutants were selected based on CHL sensitivity and KAN and sucrose resistance, which are indicative of the introduction of the mutated gene into the 130b chromosome

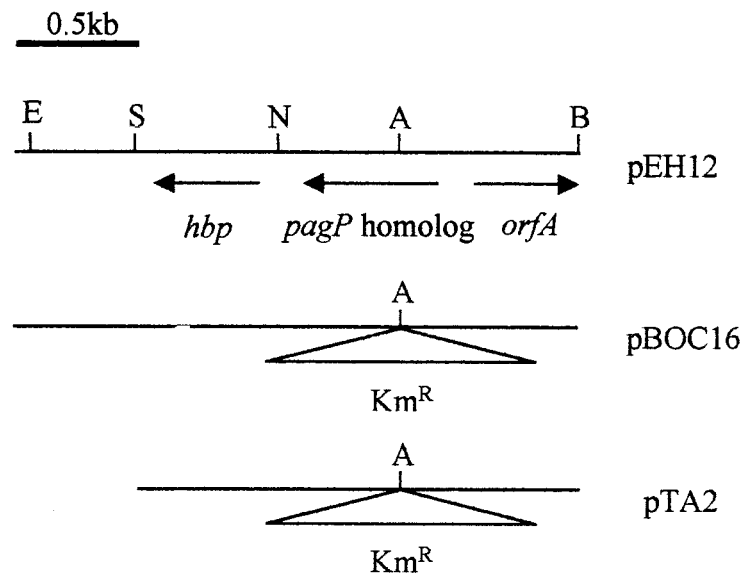
by homologous recombination. Verification of mutant genotypes was carried out by PCR and Southern hybridization (3, 54, 65, 70). A DNA fragment specific to the *pagP*-like gene encoded within 0.9 kb was generated by PCR using primers PAGP-F (5'-TGA TTC ATT GTC TGG CGA CC-3') and PAGP-R (5'-GCC AAG ATT ACA GCA CCG AT-3'). Primers were generated by the Northwestern University Biotechnology Center using an Applied Biosystems DNA synthesizer. Genomic DNA was isolated from *L. pneumophila* as described previously (24). For complementation analysis, the PCR product amplified using PAGP-F and PAGP-R was cloned into pGEMTEasy (Promega, Madison, Wis.) and then subcloned on a 0.9-kb *Not*I fragment into the low-copy-number CHL resistant pSU2719 (18), resulting in pS14. Another complementing plasmid, pS25, was constructed by cloning the 1.8-kb *Bam*HI-*Sac*I fragment containing the *pagP*-like gene from pEH12 into pSU2719.

CAMP microdilution susceptibility assay. C18G, a synthetic α-helical peptide based on the carboxy terminus of human platelet factor IV (21), and PmB (7,770 U/mg; U.S. Biochemicals; Swampscott, Mass.) were used to check the resistance of 130b and its mutants to CAMPs. To assess this resistance, a standard microdilution susceptibility assay, which uses minimal amounts of each CAMP, was performed (21, 36, 53, 73, 81). Bacteria grown in BYE, CDM, or CDM containing various amounts of Mg²⁺ were diluted to 5 × 10⁴ CFU/ml in 2× BYE. In triplicate wells of a 96-well microtiter tray, 50 µl of bacterial suspension was added to an equal volume of various concentrations of C18G or PmB (diluted in sterile distilled water from a fresh stock solution). Thus, the incubation medium throughout the CAMP resistance assays was BYE to prevent any observed differences in MIC being attributable to differences in growth potential. Following incubation at 37°C for 16 h (for *P. aeruginosa* PAK) or for 30 h (for *L. pneumophila* strains), the lowest concentration of C18G or PmB resulting in no visible growth was deemed to be the MIC (36). From wells where no growth was evident, four 10-µl samples were spotted onto BCYE agar (for *L. pneumophila*) or Luria-Bertani agar (for PAK) to assess the minimum bactericidal concentration (MBC) (36). To facilitate maximum availability of the peptides, all stages of the assay were performed using polypropylene plasticware, as the negatively charged surface of polystyrene plasticware has been shown to bind CAMPs (36).

Intracellular infection of macrophages and amoebae by *L. pneumophila*. Intracellular infection by *L. pneumophila* strains was assessed in human U937 cells (1, 54, 65, 66, 76) differentiated into macrophage-like cells by treatment with phorbol myristate acetate and in *Hartmannella vermiformis* amoebae (17, 19, 29, 54, 71). Assessment of the intracellular growth kinetics of *L. pneumophila* in U937 macrophages was performed as previously reported (54). Briefly, U937 cells were routinely cultivated in RPMI 1640 medium with L-glutamine (Gibco Life Technologies, Rockville, Md.) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, Utah) and 2.5 ml of amphotericin B (Fungizone; Gibco Life Technologies) in a 5% CO₂ incubator at 37°C. Using a multiplicity of infection (MOI) of 1, 10⁶ adherent U937 macrophages were infected with bacteria grown for 3 days on BCYE agar, as standardly performed (1, 3, 21, 56). After a 2-h incubation period to allow bacterial internalization, extracellular bacteria were removed by repeated washing, and then infected monolayers were incubated at 37°C in a 5% CO₂ incubator. At various times postinoculation, intracellular bacteria from triplicate wells per strain were released by lysis of the monolayer with 10 µl of 10% saponin (Sigma Chemical Co., St Louis, Mo.). For estimation of viable counts, serial 10-fold dilutions from triplicate wells for each strain were plated on BYCE agar. Initially, cocultures with *H. vermiformis* and *L. pneumophila* were performed as described previously (19). Briefly, in 24-well tissue culture trays, 10⁵ *H. vermiformis* trophozoites were infected with 10³ bacteria grown for 3 days on BCYE agar. At 24-h intervals, supernatant samples were taken from triplicate wells and viable counts of extracellular bacteria were estimated. Total numbers of bacteria were also determined in infected *H. vermiformis* monolayers by lysing amoebae with 10 µl of 10% saponin followed by vigorous pipetting at various time points and assessing viability on BCYE agar. *L. pneumophila* strains are unable to grow in the medium in which infections of macrophages or *H. vermiformis* were conducted; thus, increases in bacterial numbers reflect growth within the host cells (19, 66).

Macrophage cytopathicity assay. To determine whether the numbers of bacteria recovered from U937 cells were truly reflective of a diminished infection process, the viability of the macrophages was assessed by the ability of monolayers to reduce the dye alamarBlue (Biosource International, Camarillo, Calif.). Cytopathicity assays were performed as described previously (1, 3, 32, 50). Briefly, 10⁵ U937 macrophages were infected, as described above, at various MOIs. After 2 h, the infected monolayers were washed twice with RPMI to remove extracellular bacteria and then incubated at 37°C. At various time points, alamarBlue was added (1/11 volume in RPMI 1640 medium) to infected monolayers that had been previously washed twice, the monolayers were incubated at

A.



B.

<i>Lpn</i>	M - - K R L I S C L T I I C A L N R S A A A E T T S S P C S	28
<i>Sty</i>	M I I R K Y F L I I A L L - - L M P W L A I P S V S A A D K	28
<i>Eco</i>	M N V S K Y V A I F S F V - - F I Q L I S V G K V F A N A D	28
<i>Lpn</i>	R W I S L L K P V C Q R I H Q T W T E - G H D D M Y F S G Y	57
<i>Sty</i>	G G F N T F T - - - D N V A E T W R Q P E H Y D L Y V P A I	55
<i>Eco</i>	E W M T T F R - - - E N I A Q T W Q Q P E H Y D L Y I P A I	55
<i>Lpn</i>	A W H N R Y T Y R P E K I K S Y N E A A W G G G L G K S L F	87
<i>Sty</i>	T W H A R F A Y D K E K T D R Y N E R P W G V G F G Q S R W	85
<i>Eco</i>	T W H A R F A Y D K E K T D R Y N E R P W G G G F G L S R W	85
<i>Lpn</i>	D E K G N W H G L Y A I A F L D S H R H I E P A V G Y A Y L	117
<i>Sty</i>	D D K G N W H G L Y M M A F K D S F N K W E P I G G Y G W E	115
<i>Eco</i>	D E K G N W H G L Y A M A F K D S W N K W E P I A G Y G W E	115
<i>Lpn</i>	K T - - A S V N K D I K A G L G Y S V L V T S R V D Y D N V	145
<i>Sty</i>	K T W R P L E D D N F R L G L G F T A G V T A R D N W N Y I	145
<i>Eco</i>	S T W R P L A D	123
<i>Lpn</i>	P F P G A L P W V A L F Y K R T T V A A T Y I P G S A G A G	175
<i>Sty</i>	P I P V L L P L A S I G Y G P A T F Q M T Y I P G S Y N N G	175
<i>Eco</i>		123
<i>Lpn</i>	N V L Y I L G K I S L	186
<i>Sty</i>	N V Y F A W M R F Q F P	187
<i>Eco</i>		123

FIG. 1. Sequence analysis of the *L. pneumophila* *pagP*-like gene and PagP-like protein. (A) Sequence organization and restriction analysis of pEH12, pBOC16, and pTA2. For all plasmids, only the *L. pneumophila* strain 130b DNA fragment is depicted. Restriction sites are as follows: A, *Afl*II; B, *Bam*HI; E, *Eco*RI; N, *Nde*I; and S, *Sac*I. (B) Alignment (boxed regions) of PagP-like proteins in *L. pneumophila* (*Lpn*), *Salmonella* serovar Typhimurium (*Sty*) (GenBank accession number AF057021), and *E. coli* (*Eco*) (GenBank accession number AE000167). The Clustal method was used to generate alignment data (45).

37°C for 3 h, and fluorescence (at an excitation wavelength of 540 nm and an emission wavelength of 584) was assessed.

Pulmonary infection of A/J mice by *L. pneumophila*. The A/J mouse model mimics the acute *L. pneumophila*-induced pneumonia in humans (1, 15, 16, 20). The role of *rcp* in vivo was assessed using standard competition assays (30, 52, 61, 82). Six- to 8-week-old female mice (Jackson Laboratories, Bar Harbor, Maine) were anesthetized and then inoculated by intratracheal injection with a 25- μ l bacterial suspension in phosphate-buffered saline. Inocula consisted of 10^6 CFU of a ca. 1:1 ratio of 130b and NU260, which had been previously grown for 3 days on BCYE agar. At various time points, infected mice were sacrificed. Lungs were collected and disrupted by extrusion of the tissue through a fine-mesh grid into 10 ml of phosphate-buffered saline. Cells were lysed by incubation with 100 μ l of 10% saponin for 15 min at 37°C followed by vigorous pipetting. The numbers of viable bacteria and the ratio of 130b to NU260 were estimated by plating 10-fold serial dilutions on both plain and KAN-supplemented BCYE agar.

Detection of *pagP*-like sequences in other *Legionella* spp. and SG. Southern hybridization was carried out using *Bam*HI-restricted genomic DNAs from strains representing several *L. pneumophila* serogroups (SG) and a variety of *Legionella* spp. The digoxigenin nonradioactive labeling and detection system (Roche Molecular Biochemicals, Indianapolis, Ind.) was used. The probe was produced by PCR incorporation according to the manufacturer's recommendations using primers PAGP-F and PAGP-R and 130b genomic DNA as a template. High-stringency washes (0 to 10% base pair mismatch) were employed for hybridization of the probe to *L. pneumophila* DNAs (two washes with 2 \times SSC [1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate]-0.1% sodium dodecyl sulfate [SDS] at room temperature for 15 min and two washes with 0.1 \times SSC-0.1% SDS at 68°C for 15 min). Low-stringency washes (~30% base pair mismatch) were employed for hybridization of the probe to genomic DNAs from other *Legionella* spp. (two washes with 5 \times SSC-0.1% SDS at 50°C for 15 min).

Nucleotide sequence accession number. The GenBank accession number for the *L. pneumophila pagP*-like gene is AF348322.

RESULTS

Identification and mutation of an *L. pneumophila pagP*-like gene. Sequence analysis of the region surrounding *hbp* encoded on the 130b library clone pEH12 led to the discovery of a gene whose product has 42% identity and 57% similarity to PagP in *Salmonella* serovar Typhimurium (Fig. 1). The products of the *Salmonella pagP* gene and the homologous gene in *L. pneumophila* are both predicted to consist of 186 amino acid residues, providing substantiating evidence that these genes are related (Fig. 1B). The *L. pneumophila pagP*-like gene product also shows homology, i.e., 43% identity and 57% similarity, to the *crcA* (camphor resistance A) gene in *E. coli* K-12 (Fig. 1B). Initially, *crcA* was characterized as conferring camphor resistance when encoded on a high-copy-number plasmid in combination with *cspE* and *crcB* (49). However, recent evidence suggests that the product of *crcA* is functionally homologous to the *Salmonella* PagP, prompting an alternative designation from *CrcA* to the *E. coli* PagP (11). PagP and *CrcA* are the only proteins that show strong homology to the product of the *pagP*-like gene in *L. pneumophila* (data not shown). Upstream from the 559-bp *pagP*-like gene on pEH12 is an incomplete open reading frame, *orfA*, which is transcribed divergently from the *pagP*-like gene (Fig. 1B). Homology searches suggest that *orfA* encodes an amino acid permease (data not shown). The *pagP*-like gene and the downstream *hbp* are transcribed in the same orientation. Since Northern blot analysis indicates that transcription of *hbp* is monocistronic (65), the *pagP*-like gene and *hbp* are not thought to constitute an operon.

To be able to determine whether the *L. pneumophila pagP*-like gene has a role similar to that of its *Salmonella* counterpart, a mutation was introduced into the gene in virulent strain 130b. Mutants were isolated by allelic exchange, whereby the

chromosomal copy of the *pagP*-like gene was replaced by a mutant form containing a KAN insertion cassette at its *Afl*II restriction site, 100 bp from its putative translational start site (Fig. 1A). Two independent mutants whose CHL sensitive and KAN- and sucrose-resistant phenotypes were indicative of homologous recombination of the mutant gene into the chromosome were isolated from separate electroporation events. The mutants, designated NU260 and NU261, were verified by PCR and Southern hybridization to contain the 1.1-kb insertion in the *pagP*-like gene, using a probe specific to the *pagP*-like gene (data not shown). All further experiments described here were performed with both NU260 and NU261 with similar results, although, for clarity, only data from NU260 are presented in this paper. Thus, the phenotypes observed result directly or proximately from the mutation in the *pagP*-like gene and not from spontaneous second-site mutations. However, this does not rule out the possibility of the additional involvement of unlinked genes.

Extracellular growth of the *L. pneumophila pagP*-like mutant. The behavior of NU260 in liquid media was assessed to determine if loss of the *pagP*-like gene resulted in gross changes in extracellular growth. No differences were observed in the growth kinetics of the mutant compared to 130b in standard BYE and CDM broths (Fig. 2A and B). In *Salmonella*, the PhoPQ regulon senses Mg^{2+} cations, and bacterial growth in low- Mg^{2+} minimal medium increases the CAMP resistance generated by *pagP* (39, 72). Thus, we hypothesized that the *L. pneumophila* mutants might have reduced survival in low- Mg^{2+} broth. The growth maxima of wild type 130b were considerably reduced in standard CDM compared to BYE and decreased as the concentration of Mg^{2+} in CDM was lowered from 0.7 to 0.005 mM (Fig. 2), which would be predicted from the known metal requirements of *L. pneumophila* (68). No growth was observed in CDM lacking added Mg^{2+} (data not shown). More importantly, as the Mg^{2+} concentration of CDM was decreased, NU260 entered into stationary phase earlier than 130b, with an overall reduction in the growth maxima of the mutant, although the growth rate was unaffected (Fig. 2C and D and data not shown). Furthermore, bacterial viability, as judged by CFU/per milliliter normalized to OD_{660} , was decreased 20-fold in stationary-phase NU260 grown in 0.005 mM Mg^{2+} -CDM medium compared to 130b (data not shown). Thus, the *L. pneumophila pagP*-like gene appears to be involved in growth and/or survival in low- Mg^{2+} medium.

Resistance of *L. pneumophila* strains to CAMPs. Since PagP of *Salmonella* serovar Typhimurium and *E. coli* are involved in resistance to CAMPs, we sought to assess the relative resistance of NU260 to two CAMPs, the cyclical PmB and the α -helical C18G (Table 2). Initially, experiments determined that CAMP resistance was similar in both logarithmic and stationary phase (data not shown). Subsequent investigations were performed with logarithmic-phase bacteria to ensure maximum viability of the culture. After growth of 130b in BYE, the MIC and MBC of PmB were 12.5 and 25 to 50 μ g/ml, respectively (Table 2). The MIC of PmB for *P. aeruginosa* PAK was \leq 3.1 μ g/ml, a fourfold difference compared to that for 130b, in accordance with *Legionella* spp. being especially PmB resistant. The MIC and MBC of PmB for NU260 were 6.2 and 25 μ g/ml, respectively (Table 2), suggesting a role for the *pagP*-like gene in *L. pneumophila* resistance to CAMPs. The

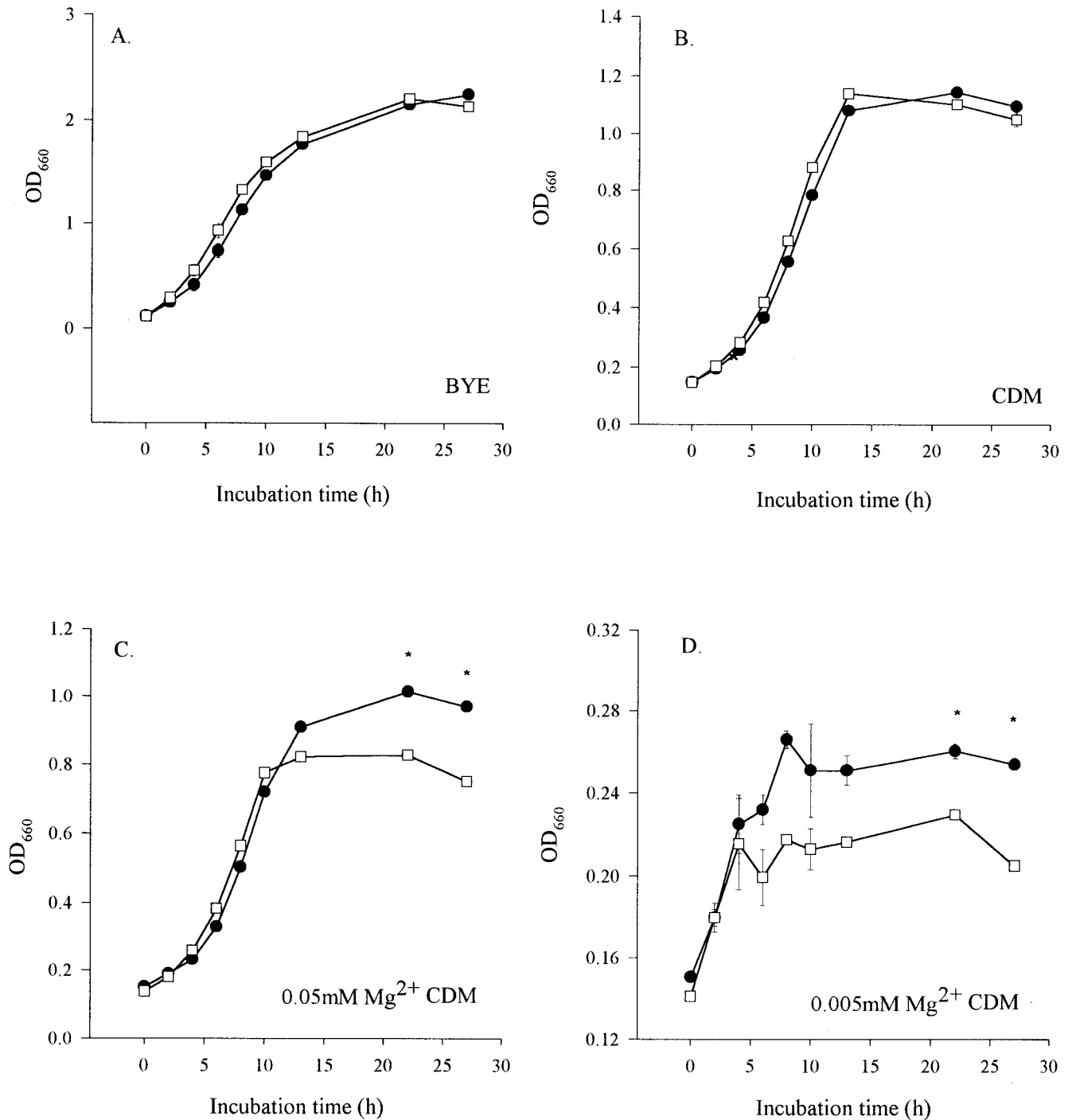


FIG. 2. Extracellular growth kinetics of *L. pneumophila* strains. Wild-type strain 130b (●) and mutant NU260 (□) were compared for growth in BYE (A), CDM (0.7 mM Mg²⁺) (B), and CDM with 0.05 mM (C), or 0.005 mM (D) Mg²⁺ cations. Growth was assessed by measuring the OD₆₆₀ of triplicate cultures at various times of incubation. Asterisks indicate significant differences ($P < 0.05$; Student's *t* test) between the 130b and NU260 cultures. Standard deviations are shown, but the error bars are too small to be observed in panels A to C. This experiment was performed in triplicate with comparable results, and representative data are presented here.

MIC for C18G of PAK was 32 to 64 $\mu\text{g/ml}$ following growth in BYE. However, *L. pneumophila* strain 130b appeared to be much more sensitive to the effects of this CAMP, with the C18G MIC and MBC being 16 and 32 $\mu\text{g/ml}$, respectively. The MIC and MBC of C18G for NU260 were 8 and 16 to 32 $\mu\text{g/ml}$,

respectively. The modest difference in both PmB and C18G resistance between 130b and NU260 was highly reproducible in independent experiments (Table 2). Taken together with the significant degree of sequence homology with PagP proteins in both *Salmonella* and *E. coli*, these data indicate that the *L.*

TABLE 2. CAMP susceptibilities of *L. pneumophila* strains^a

CAMP	Growth medium	Strain	MIC (μg/ml)	MBC (μg/ml)
PmB	BYE	130b	12.5	25–50
		NU260	6.2	25
	CDM	130b	3.1	25
		NU260	3.1	12.5–25
	0.05 mM Mg ²⁺ -CDM	130b	6.2–12.5	25
		NU260	6.2	12.5–25
	0.005 mM Mg ²⁺ -CDM	130b	12.5	25
		NU260	6.2	12.5–25
C18G	BYE	130b	16	32
		NU260	8	16–32
	CDM	130b	16	32
		NU260	4–8	8–16
	0.05 mM Mg ²⁺ -CDM	130b	16	32
		NU260	4	8–16
	0.005, mM Mg ²⁺ -CDM	130b	64	128
		NU260	16	16–32

^a The bacteria were grown in triplicate to mid-exponential phase in the indicated media and then, after dilution to 5×10^4 CFU/ml, were exposed to PmB or C18G. The incubation medium during all CAMP resistance assays was BYE. Similar MICs and MBCs were obtained in a replicate experiment (data not shown). A range of concentrations indicates the range of MICs of MBCs obtained from triplicate samples.

pneumophila counterpart of PagP plays a role in CAMP resistance. Hence, we designated the *L. pneumophila* *pagP*-like gene *rcp*, for resistance to cationic antimicrobial peptides. We avoided the *pag* (PhoP-activated gene) designation because PhoP has not yet been identified in *L. pneumophila* and the genetic regulation of *rcp* is wholly uncharacterized.

Given the reduced ability of the *L. pneumophila* *rcp* mutant to grow in low-Mg²⁺ medium (Fig. 2), we tested its relative resistance to C18G and PmB after growth in CDM containing various amounts of Mg²⁺. Growth in low-Mg²⁺ CDM increased the resistance of 130b toward both CAMPs (Table 2). The MIC of PmB increased from 3.1 μg/ml in standard CDM to 6.2 to 12.5 μg/ml as the Mg²⁺ content of CDM was lowered to 0.05 mM and finally to 12.5 μg/ml in 0.005 mM Mg²⁺-CDM. Using 0.005 mM Mg²⁺-CDM yielded the greatest CAMP resistance difference between 130b and NU260 but was also associated with poor growth, raising the possibility that complex physiological changes, other than Mg²⁺ depletion, could be responsible for the phenotype. However, reasonable growth was achieved using 0.05 mM Mg²⁺-CDM, which also resulted in increased PmB resistance in both 130b and NU260 compared to CDM (Table 2; Fig. 2C). Taken together, these results suggest that low Mg²⁺ does increase the PmB resistance of *L. pneumophila*. The MIC of C18G increased from 16 μg/ml in standard CDM to 64 μg/ml with 0.005 mM Mg²⁺ (Table 2). For NU260, the MIC of PmB increased from 3.1 μg/ml in

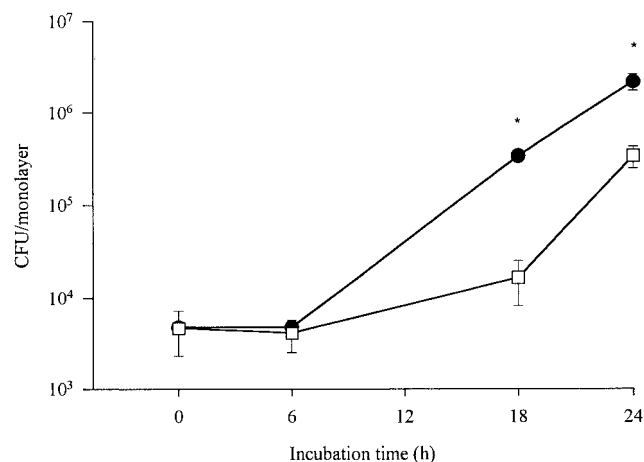


FIG. 3. Intracellular infection of human macrophages with *L. pneumophila* strains. U937 cell monolayers ($n = 3$) were infected at an MOI of 1 with strain 130b (●) and NU260 (□). Asterisks indicate significant differences ($P < 0.01$; Student's *t* test) in numbers of recovered bacteria between 130b and NU260. Comparable results were obtained in triplicate experiments, and the data presented here are from one representative experiment. Error bars indicate standard deviations.

standard CDM to 6.2 μg/ml in both 0.05 mM- and 0.005 mM Mg²⁺-CDM, and the MIC for C18G increased from between 4 and 8 μg/ml in standard CDM to 16 μg/ml in 0.005 mM-Mg²⁺ CDM (Table 2). Although CAMP resistance in both 130b and NU260 was modestly increased as the Mg²⁺ concentration in the CDM was lowered, there was a greater increase in the wild type, suggesting that *rcp* is partly responsible for the Mg²⁺-dependent CAMP resistance. In summary, these experiments represent the first identification of an *L. pneumophila* gene involved in CAMP resistance and the first demonstration of increased *Legionella* CAMP resistance due to low-Mg²⁺ conditions.

Intracellular infections by *L. pneumophila* strains. To assess the role of *rcp* in an intracellular environment, a phenotype not previously investigated for any *pagP*-like gene, U937 macrophages were infected with either 130b or NU260 and then numbers of bacteria were monitored over time. At 0 h, equivalent numbers of bacteria 130b and NU260 were recovered (Fig. 3). Thus, there was no obvious defect in the initial stages of infection, i.e., attachment and invasion. However, the mutant exhibited reduced recovery compared to the wild-type parent, by 1 log unit at 18 h postinoculation (p.i.) (Fig. 3). The difference in numbers of recovered bacteria diminished at later time points, until by 72 h similar numbers of mutant and wild-type bacteria were recovered (Fig. 3 and data not shown). No difference was noted in the abilities of NU260 and 130b to survive in the medium used to culture the macrophages (data not shown). Taken together, these data suggest that *rcp* promotes the ability of *L. pneumophila* to replicate and/or survive in macrophages. Since the PmB resistances of wild-type and mutant bacteria grown for 3 days on BCYE are comparable (data not shown), we suspect that the difference in numbers of recovered bacteria is due to intracellular induction of *rcp*-dependent CAMP resistance.

Since a low MOI with *L. pneumophila* ultimately leads to host cell death (1, 66), corroborating evidence of a role for *rcp*

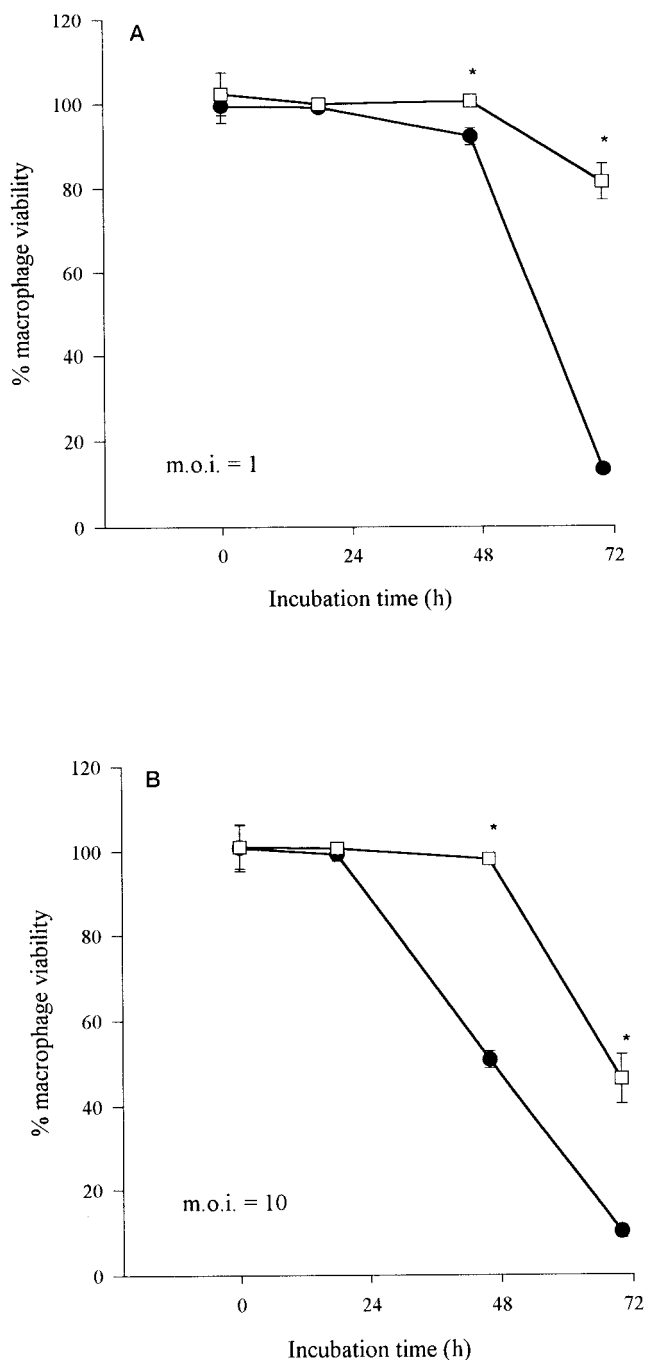


FIG. 4. Cytopathicity assay to assess the viability of *L. pneumophila*-infected human macrophages. U937 cell monolayers ($n = 6$) were infected at MOIs of 1 (A) and 10 (B) with wild-type strain 130b (●) and mutant NU260 (□). Macrophage viability is expressed as the percentage of alamarBlue dye reduction in infected compared to uninfected monolayers. Asterisks indicate significant differences in cytopathicity ($P < 0.0005$; Student's t test) between 130b and NU260. Comparable results were obtained from an additional experiment (data not shown). Error bars indicate standard deviations.

in macrophage infection was sought using a standard cytopathicity assay. Using the same MOI as during kinetic studies, the cytopathic effect of the wild type became apparent at 48 h p.i. and then proceeded to increase until by 72 h survival was only

~10% compared with uninfected cells (Fig. 4A). In contrast, the viability of macrophages infected with NU260 did not decrease until 72 h p.i., when survival was still ~80% compared to uninfected cells (Fig. 4A). A defect in cytopathicity was also observed using an MOI of 10; i.e., whereas 130b elicited 50% macrophage survival at 48 h, NU260 did so only after 72 h (Fig. 4B). Thus, *rcp* appears to be important for the ability of *L. pneumophila* to kill macrophages.

As amoebae are the natural host for *L. pneumophila* in the environment, intracellular growth of the mutant was tested in a coculture assay with *H. vermiformis*. This species of amoeba is known to support the growth of *L. pneumophila* and was isolated from a clinical case of legionellosis (29). Both the *rcp* mutant and 130b were recovered in similar numbers from the supernatants overlying the infected monolayers until 24 h p.i. (Fig. 5A). However, by 72 h p.i., the mutant exhibited a nearly 1,000-fold reduction in numbers compared with the wild type (Fig. 5A). By visual inspection, it was apparent that more cellular debris, indicative of cell lysis or necrosis, occurred in monolayers infected with 130b compared to NU260. When infected amoebal monolayers were lysed, the total numbers of bacteria were identical to that observed during coculture (data not shown and Fig. 5A). No difference in the abilities of the *rcp* mutant and 130b to survive in the assay media were noted (data not shown). Similar to the case with macrophages, the growth defect of the mutant during coculture with *H. vermiformis* could be due to a decreased ability to replicate and/or survive intracellularly.

Convincing evidence exists that the mutant's infectivity defects are associated with loss of *rcp* function. First, the infection phenotypes of two independent *rcp* mutants (NU260 and NU261) were identical (Fig. 3, 4, and 5 and data not shown). Second, *rcp* is not part of an operon, as the upstream gene is in the opposite orientation and the downstream *hbp* gene is monocistronic (Fig. 1A) (65). Third, a mutant bearing an insertion in *hbp* is not defective for intracellular infection of macrophages or amoebae (65). Finally, *trans* complementation of the amoebal infection defect was achieved with plasmids containing *rcp* (Fig. 5B), which rules out the possibility that these phenotypes are due to the effect of unlinked genes.

In vivo virulence studies in A/J mice. Due to the intracellular infectivity defect observed in the *rcp* mutant, in vivo virulence studies in A/J mice were undertaken (1, 15, 16, 20). Competition assays in which a mixture of 130b and NU260 in a ca. 1:1 ratio was inoculated by intratracheal injection into A/J mice were performed to assess the role of *rcp* in vivo. In competition assays, increases in the ratio of wild type to mutant over time reflect the ability of the wild type to outcompete the mutant. Following 24 h of incubation, the ratio of the wild type to the mutant recovered from infected lungs increased to 4 (Fig. 6). By 72 h, the ratio of wild type to mutant had further increased to 100 (Fig. 6). NU260 recovered from infected animals retained KAN resistance. Thus, we suspect that *rcp* promotes *L. pneumophila* virulence.

Detection of *pagP*-homologous sequences in *L. pneumophila* SG and other *Legionella* spp. Probes designed against the *rcp* gene of 130b were used to check for the presence of *pagP*-like sequences in other *L. pneumophila* SG and *Legionella* spp. Sequences homologous to the probe were observed in all SG tested, i.e., SG 2 to 8, 13, and 14 (Table 1). Under low-strin-

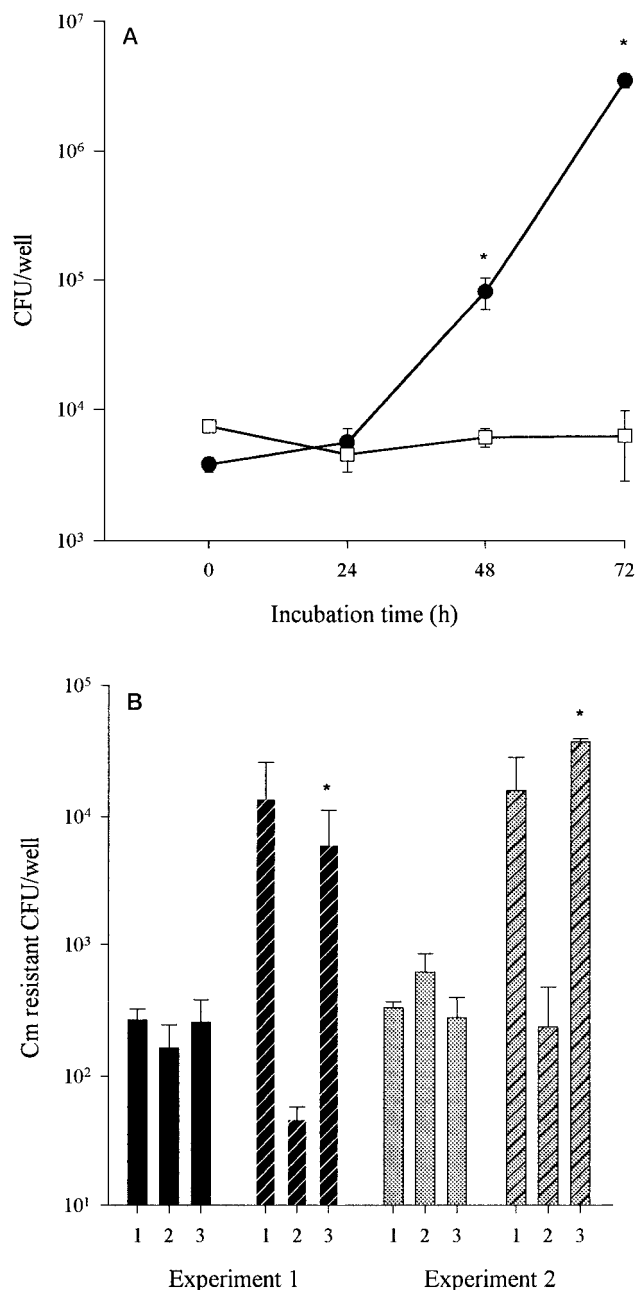


FIG. 5. Coculture of *L. pneumophila* strains with *H. vermiformis*. (A) Monolayers of amoebae ($n = 3$) were infected with wild-type strain 130b (●) and mutant NU260 (□) at an MOI of 0.01. Asterisks indicate significant differences ($P < 0.05$ at 48 h or $P < 0.005$ at 72 h; Student's t test) in numbers of recovered bacteria between 130b and NU260. The experiment was performed five times with comparable results, and representative data are presented here. (B) Genetic complementation of the *rcp* mutant's infectivity defect. Monolayers of amoebae were cocultured with either 130b containing the CHL resistance vector pSU2719 (bars 1), NU260 containing the vector (bars 2), or NU260 containing *rcp* cloned into pSU2719 (bars 3), and the numbers of recovered bacteria were determined at 0 h (solid bars) and 72h (hatched bars) p.i. In experiment 1 (black bars), the complementing plasmid was pS14. In experiment 2 (grey bars), which is representative of a replicate experiment, the complementing plasmid was pS25. Asterisks indicate significant differences ($P < 0.005$; Student's t test) in numbers of recovered bacteria between NU260 containing the vector and NU260 containing *rcp*. Error bars indicate standard deviations.

gency conditions (30% base pair mismatch), sequences homologous to the probe were observed in *L. birminghamensis*, *L. erythra*, *L. feeleii*, *L. longbeachae*, *L. micdadei*, and *L. parisiensis*.

DISCUSSION

In this study, we have identified a new *L. pneumophila* infectivity determinant, Rcp, a protein with homology to PagP of *Salmonella* serovar Typhimurium and *E. coli*. Sequences homologous to the *rcp* gene were detected in nine SG of *L. pneumophila* and in six *Legionella* spp., suggesting distribution of *rcp* throughout the genus. The loss of Rcp resulted in modestly decreased *Legionella* resistance to C18G, a CAMP based on human platelet-derived factor IV, and PmB, a bacterially derived CAMP. This result is entirely in keeping with the established link between PagP and CAMP resistance (39). Growth in low-Mg²⁺ medium, a condition known to induce *Salmonella* CAMP resistance, promoted *L. pneumophila* resistance to C18G and PmB (39). Although induction of CAMP resistance by low Mg²⁺ concentrations was evident in both the wild type and the *rcp* mutant, increased induction was demonstrated for 130b, especially against PmB. These results provide substantiating evidence that Rcp is, in part, responsible for resistance to CAMPs in *L. pneumophila*. Although *rcp* in *L. pneumophila* and *pagP* in *Salmonella* are genetically and functionally similar, differences between them are apparent. For example, in *Legionella* the *rcp* gene is involved in resistance to both C18G and PmB, whereas *pagP* mediates C18G resistance but not PmB resistance (39). The highly complex composition of CAMPs encountered by the organism in vivo is undefined, and therefore, it is possible that a larger difference could be obtained using a CAMP mixture more similar to that encountered in the intracellular niche occupied by legionellae. In *Salmonella* and *E. coli*, PagP functions as a palmitoyl transferase, able to modify the lipid A component of lipopolysaccharide by addition of the fatty acid palmitate (11, 39). The increased acylation is believed to promote resistance to CAMPs by decreasing membrane fluidity and preventing insertion of the peptide (39). Lipid A modifications which increase bacterial CAMP resistance are an emerging theme in gram-negative bacteria (26, 39). For example, in *P. aeruginosa*, the addition of palmitate to lipid A also increases CAMP resistance in a PhoP-dependent fashion in response to low Mg²⁺ concentrations (26). Based upon the strong sequence and functional homology with PagP of *S. enterica*, we strongly suspect that *L. pneumophila rcp* promotes similar lipid A modifications. Preliminary data suggest that NU260 contains lipopolysaccharide molecules with lower molecular masses (approximately 250 to 300 kDa) than 130b, in accordance with the expected loss of one fatty acid, as judged by electrophoretic separation (U. Zähringer, M. Robey, and N. P. Cianciotto, unpublished results). Detailed analysis to formally prove this hypothesis is currently being pursued. Since the *rcp* mutant was only partly defective in CAMP resistance, we suspect that additional factors are involved in *Legionella* resistance to antimicrobial peptides. In *Salmonella*, CAMP resistance is clearly promoted by several determinants, including the PhoPQ-regulated *pagP* gene. For example, PmrAB is a two-component regulator of PmB resistance which promotes aminoarabinose

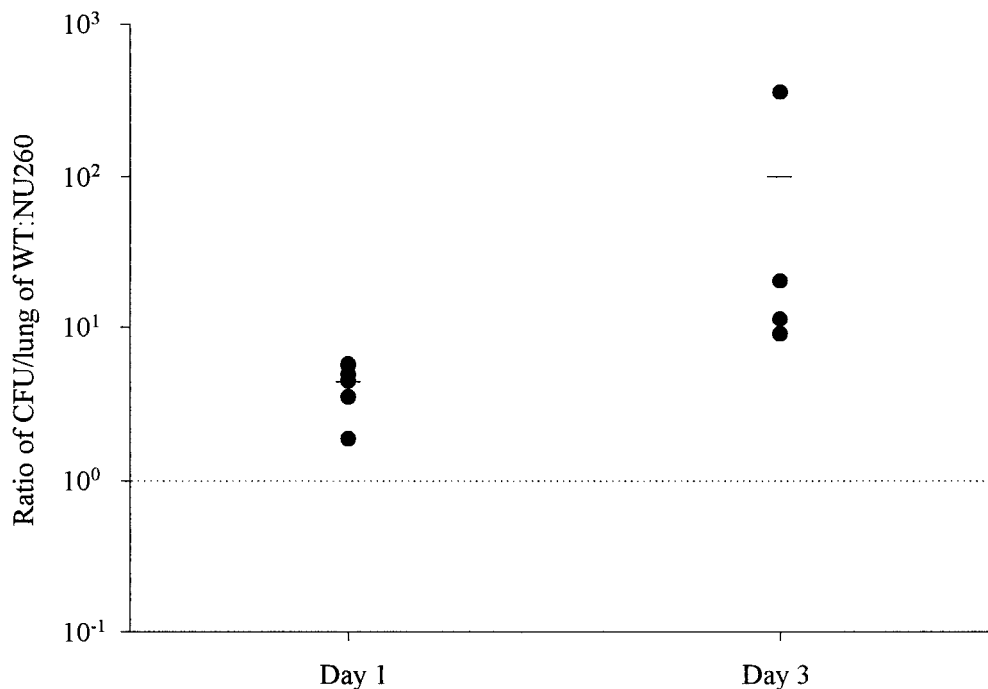


FIG. 6. In vivo competition assays with *L. pneumophila* 130b and NU260. The wild type (WT) and mutant were inoculated intratracheally into A/J mice ($n = 4$ to 6). The ratio of wild type to mutant in infected lungs was assessed by dividing the CFU per lung for the wild type by the CFU per lung for NU260. The values expressed are normalized to the deviation observed in the ratio of 130b to NU260 in the initial inoculum (i.e., 0.61).

additions to lipid A, PgtE is an outer membrane CAMP protease, and *waaP* is involved in lipopolysaccharide core modifications (37, 38, 81). Thus, in *L. pneumophila*, as in *Salmonella*, CAMP resistance appears to be multifactorial.

The growth of both the wild type and NU260 was similar in BYE or CDM, suggesting that mutation of *rcp* did not cause a generalized growth defect. However, inoculation into low-Mg²⁺ CDM resulted in lower total growth and decreased bacterial viability of the *rcp* mutant compared to wild-type 130b. These phenotypes were very similar to the kinetics and viability of *Salmonella* serovar Typhimurium *phoP*, *mgtA*, and *mgtCB* mutants in low-Mg²⁺ media (72). The *Salmonella* mutants are purported to have reduced growth in low-Mg²⁺ media due to the loss of the Mg²⁺ transporters (MgtA and MgtCB) or their activator (PhoP) (72). At present, there is no knowledge of *Legionella* magnesium transport. Thus, clarification of why an *rcp* mutant has extracellular growth similar to that of *Salmonella phoP*, *mgtA*, and *mgtCB* mutants requires detailed further characterization of *rcp*.

Here, we demonstrate, for the first time, that a *pagP*-like gene is important for intracellular growth and/or survival. Indeed, *rcp* promotes *L. pneumophila* growth in both *H. vermiformis* and U937 macrophages, representatives of the two most relevant host cells for *Legionella* infections. Preliminary data indicate that the *rcp* mutants are also defective for intracellular infection of peripheral blood monocytes (M. Robey and N. P. Cianciotto, unpublished results). Although other scenarios are possible, it is reasonable to postulate that the decreased infectivity of the *rcp* mutant is due, at least in part, to decreased CAMP resistance. In U937 macrophages, the difference between the numbers of intracellular bacteria for 130b and the

rcp mutant diminished over time. This suggests adaptation of the *rcp* mutants to their environment, possibly involving the induction of other CAMP resistance mechanisms. Interestingly, a typical cytopathic effect was not elicited by the mutant; i.e., the extent of cytopathicity did not closely correlate with numbers of recovered CFU (3, 32, 42, 50). Recently, mutants have been characterized which are defective for cytopathicity but have no intracellular growth defect and thus remain trapped inside the macrophage (1). However, to our knowledge, NU260 is the first example of an *L. pneumophila* mutant with an infectivity defect that ultimately achieves a wild-type level of replication in macrophages but lacks a full cytopathic effect. The intracellular infectivity defect of the *rcp* mutant was more pronounced in *H. vermiformis* than in U937 macrophages, suggesting greater importance for *rcp* in amoebal infections. It is possible that (i) amoebae contain more CAMPs than macrophages, (ii) amoebal CAMPs are more accessible to the *Legionella* intracellular environment, or (iii) amoebal CAMPs are more active against Rcp-induced resistance mechanisms. The notion that the protozoan environment is less permissive has been suggested by the behavior of other *Legionella* mutants that are more defective in amoebae than in macrophages (17, 27, 71).

Importantly, we have also shown that *rcp* is important for colonization of the mammalian lung, which is the first demonstration of a *pagP*-like gene being involved in virulence. The in vivo defect noted in NU260 could be due to changes in the ability of the bacterium to resist CAMPs intracellularly and/or extracellularly. In *Salmonella*, loss of *pagP* had no effect on virulence as judged by 50% lethal dose evaluation during intraperitoneal inoculation of BALB/c mice (6). It is possible

that the differences in the virulence of *pagP* in *Salmonella* and *rcp* in *L. pneumophila* reflect inherent differences in assay conditions (i.e., competition assay versus 50% lethal dose or use of BALB/c versus A/J mice). Alternatively, *pagP*-like genes may be more important for virulence in legionellosis.

The ability to sense and adapt to changing environmental conditions is highly advantageous to *L. pneumophila*, an organism that occupies diverse niches, from free-living in water to intracellular in protozoa or macrophages. As the PhoPQ regulon regulates *pagP* in *Salmonella*, the question arises whether *L. pneumophila rcp* is similarly regulated. The unfinished *L. pneumophila* genome database (www.genome3.cpmc.columbia.edu) contains an open reading frame the product of which has 37% identity and 60% similarity to PhoP in *Salmonella* serovar Typhimurium. However, the product of this open reading frame also appears to be homologous (44% identity and 61% similarity) to the *Salmonella* regulatory gene product PmrA, which is involved in PmB resistance. That *rcp* may be controlled by a PhoPQ-like regulator is suggested by the fact that upstream of the *rcp* putative transcription start site lie sequences homologous to a motif involved in PhoPQ-dependent Mg²⁺-responsive induction of transcription in *E. coli* (51). In *Salmonella*, Mg²⁺ is the signal for PhoPQ to transcriptionally activate genes involved in intramacrophage survival, leading to speculation that this cation is limiting within the phagosome (33, 34, 43). That an *L. pneumophila rcp* mutant shows decreased growth in low-Mg²⁺ media and decreased infectivity for both amoebae and macrophages suggests that Mg²⁺ limitation may be an environmental cue for *Legionella*. Thus, *Legionella*, like the successful intracellular pathogen *Salmonella*, may respond to its environment by activating various virulence determinants that facilitate survival and proliferation within the host. *Rcp* appears to be one such determinant, important for intracellular infection by mediating resistance to CAMPs.

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