The CRISPR-Associated Gene *cas2* of *Legionella pneumophila* Is Required for Intracellular Infection of Amoebae

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ABSTRACT **Recent studies have shown that the clustered regularly interspaced palindromic repeats (CRISPR) array and its associated (*cas*) genes can play a key role in bacterial immunity against phage and plasmids. Upon analysis of the *Legionella pneumophila* strain 130b chromosome, we detected a subtype II-B CRISPR-Cas locus that contains *cas9*, *cas1*, *cas2*, *cas4*, and an array with 60 repeats and 58 unique spacers. Reverse transcription (RT)-PCR analysis demonstrated that the entire CRISPR-Cas locus is expressed during 130b extracellular growth in both rich and minimal media as well as during intracellular infection of macrophages and aquatic amoebae. Quantitative reverse transcription-PCR (RT-PCR) further showed that the levels of *cas* transcripts, especially those of *cas1* and *cas2*, are elevated during intracellular growth relative to exponential-phase growth in broth. Mutants lacking components of the CRISPR-Cas locus were made and found to grow normally in broth and on agar media. *cas9*, *cas1*, *cas4*, and CRISPR array mutants also grew normally in macrophages and amoebae. However, *cas2* mutants, although they grew typically in macrophages, were significantly impaired for infection of both *Hartmannella* and *Acanthamoeba* species. A complemented *cas2* mutant infected the amoebae at wild-type levels, confirming that *cas2* is required for intracellular infection of these host cells.**

IMPORTANCE **Given that infection of amoebae is critical for *L. pneumophila* persistence in water systems, our data indicate that *cas2* has a role in the transmission of Legionnaires' disease. Because our experiments were done in the absence of added phage, plasmid, or nucleic acid, the event that is facilitated by Cas2 is uniquely distinct from current dogma concerning CRISPR-Cas function.**

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Clustered regularly interspaced palindromic repeat (CRISPR) arrays are found in ca. 40% of eubacteria and nearly all archaea (1–11). CRISPR arrays consist of palindromic repeats of 30 to 50 nt that are separated by unique spacers of 17 to 84 nt. Upstream of the array is an AT-rich leader that promotes transcription toward the repeats, and upstream of the leader are the CRISPR-associated sequence genes (*cas* genes) (2, 4, 12–14). Ten *cas* genes (*cas1* to *cas10*) have been defined, although *cas1* and *cas2* are the only ones that are conserved in all bacteria (3, 11, 15–18). Based on the number and arrangement of *cas* genes, CRISPR loci are classified into types I, II, and III, which are further divided into 10 subtypes (A, B, C, etc.) (3, 15). In various bacteria, CRISPR and *cas* genes (CRISPR-Cas) have been linked to phage and plasmid immunity. CRISPR-Cas has also recently been shown to be capable of interfering with DNA transformation (28). CRISPR-mediated immunity occurs in three steps: adaptation, expression/maturation, and interference (3, 11, 16, 29, 30). In the first step, arrays acquire new spacers from the invading phage or plasmid, with acquisition occurring at the 5′ end of the array (19, 31). In the next step, upon the introduction of phage or plasmid DNA or RNA, the CRISPR array is transcribed, and then the long transcript is processed into smaller "crRNAs" by one or more Cas proteins (12, 32–37). In the interference step, the foreign DNA or RNA is recognized and cleaved by a Cas/crRNA complex (12, 38–42). In some cases, the activity of purified Cas proteins has been determined (11, 15); e.g., Cas1 is a DNA endonuclease involved in incorporation of spacers (43, 44), Cas2 is a site-specific endoribonuclease that is also implicated in spacer selection and/or integration (11, 45), Cas4 is a RecB-like DNA exonuclease linked to spacer acquisition (46, 47), and Cas9 is important for the production of crRNAs and cleaving target DNA (3, 48, 49). Reviews have challenged the field to take a broader look at CRISPR-Cas loci and examine their function in more bacteria (3, 6, 11, 17, 29, 50). Among eubacteria, ca. 12 types been experimentally examined for the physiologic role of the CRISPR-Cas locus (12, 19, 21, 22, 25–27, 34, 45, 48, 50–54), yet >400 bacteria have the locus (3). Also, ca. 98% of spacers do not have matches in GenBank, indicating that the origin of most spacers is unknown (46, 55). Finally, Takeuchi et al. have argued that Cas1 and Cas2 likely have roles that are distinct from and additional to their roles in phage and plasmid immunity (56). It was in this context that we embarked on the study of CRISPR-Cas in *Legionella pneumophila*.

L. pneumophila is a Gram-negative bacterium that causes a pneumonia known as Legionnaires' disease (57, 58) that is of increasing incidence in the United States and elsewhere (59). Humans contract *L. pneumophila* by inhaling contaminated droplets

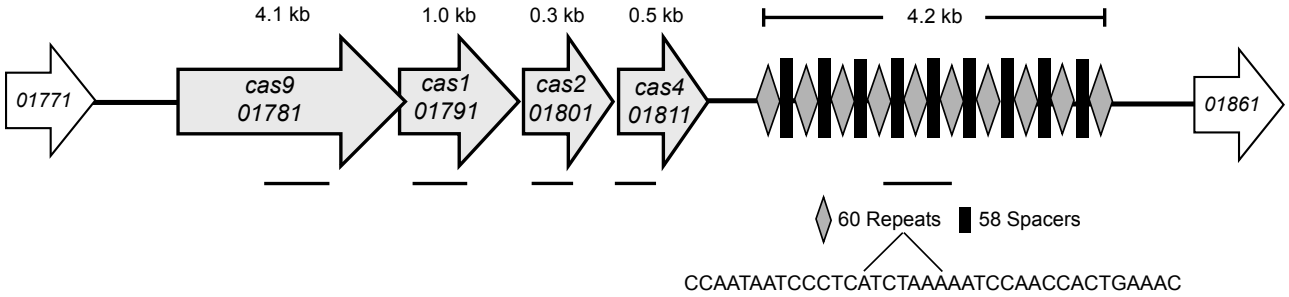

FIG 1 The CRISPR-Cas locus of L. *pneumophila* strain 130b. Horizontal arrows denote the locations and orientations of *cas9*, *cas1*, *cas2*, and *cas4*, which are designated in the 130b genome as lpw_01781, lpw_01791, lpw_01801, and lpw_01811. Above the arrows, the sizes of the genes are indicated. There is a 3-bp overlap between *cas9* and *cas1*, a 7-bp gap between *cas1* and *cas2*, and a 60-bp gap between *cas2* and *cas4*. Located 216 bp downstream of the genes is a 4.2-kb CRISPR array which consists of 60 identical 37-bp direct repeats (grey diamonds) separated by 58 spacers that vary in size between 34 and 38 bp (black bars). The sequence of the repeats is indicated below the map. The CRISPR-Cas locus is bounded on one side by lpw_01771 and on the other by lpw_01861. There are 476 bp between the end of lpw_01771 and the start of *cas9* and 427 bp between the end of the CRISPR array and lpw_01861. The thin lines underneath signify the approximate sizes and locations of transcripts identified by RT-PCR.

that originate from aerosol-generating devices (58). In its aquatic habitats, *L. pneumophila* survives planktonically, as an intracellular parasite of protozoa, and in multiorganism biofilms (60–62). However, the major replicative niche of *L. pneumophila* is in amoebae (58, 61–63), with *Acanthamoeba* and *Hartmannella* species being the most critical hosts (60, 62–64). In lungs, *L. pneumophila* proliferates in macrophages (65, 66). Most sequenced strains have CRISPR arrays and *cas* genes (67, 68), and epidemiological studies indicate that a CRISPR-Cas locus is usually present in strains linked to disease (69). Previously examined *L. pneumophila* CRISPR spacers lack similarity to plasmid, phage, and other sequences in the database (68). No experiments on the role of CRISPR-Cas in *L. pneumophila* have been reported. We now demonstrate, among other things, that *L. pneumophila cas* genes are expressed upon intracellular infection and that the *cas2* gene is required for infection of multiple amoebal hosts.

RESULTS

Detection of a CRISPR-Cas locus in *L. pneumophila* strain 130b. Utilizing CRISPR Finder (70), we identified a single CRISPR-Cas locus in the chromosome of the virulent *L. pneumophila* strain 130b. The 130b CRISPR-Cas locus consisted of four *cas* genes (i.e., *cas9*, *cas1*, *cas2*, and *cas4*) and a downstream CRISPR array (Fig. 1). The positioning of the four *cas* genes suggested that they constitute an operon. Based upon the most recent CRISPR-Cas classification scheme, the 130b locus belongs to subtype II-B (3, 11, 15). A similar subtype II-B CRISPR-Cas locus was found in the chromosome and on a plasmid in *L. pneumophila* strain Paris, another clinical isolate belonging to serogroup 1 (67, 68). The amino acid sequences of the four 130b Cas proteins were 99 to 100% identical to those of the Paris Cas proteins. The direct repeats in the 130b CRISPR array were identical to the repeats in the Paris CRISPR array. However, the spacer sequences in strain 130b were different from those of strain Paris as well as being unlike any other sequences in the GenBank database. The 130b CRISPR-Cas locus was bounded by genes encoding hypothetical proteins (Fig. 1) and occupied a position in the bacterial chromosome location that was different that of the Paris chromosomal CRISPR array. To our knowledge, no members of CRISPR-Cas subtype II-B have been studied experimentally; the closest subtype to be examined is subtype II-A, which includes the loci of *Streptococcus* species (3, 15, 48, 71).

Expression of the CRISPR-Cas locus in *L. pneumophila* grown extracellularly. To begin to determine if the CRISPR-Cas locus is expressed by *L. pneumophila* grown under standard laboratory conditions, we grew strain 130b in buffered yeast extract (BYE) broth at 37°C (Fig. 2A) and then performed reverse transcription-PCR (RT-PCR) using primer pairs that are specific for each of the *cas* genes (Fig. 1). Transcripts corresponding to *cas9*, *cas1*, *cas2*, and *cas4* were detected during exponential, early stationary, and late stationary phases (Fig. 2A). Additional RT-PCR analysis confirmed that the four *cas* genes are, as predicted, transcriptionally linked (see Fig. S1 in the supplemental material). *cas* genes were also expressed during growth at lower temperatures (Fig. 2B) as well as in a chemically defined medium (Fig. 2C). Pre-crRNA was also detected during growth in BYE broth at 25°C, 30°C, and 37°C and in cultures in chemically defined medium (CDM) (Fig. 2A to C). These data confirmed that all *cas* genes and pre-crRNA are expressed when *L. pneumophila* grows extracellularly. Using qRT-PCR, we next determined that the *cas* gene transcripts are more abundant in early stationary-phase cultures than they are in exponential-phase cultures (Fig. 2D). The *cas2* gene was the most highly upregulated gene, displaying an approximately 75-fold-higher level of transcripts during stationary phase, compared to 30- to 45-fold elevations for *cas9*, *cas1*, and *cas4*. This type of expression profile, i.e., heightened expression during stationary phase, is reminiscent of the hyperexpression of infectious traits (72). This correlation suggested that the CRISPR-Cas locus might be relevant during intracellular infection by *L. pneumophila*.

Expression of the CRISPR-Cas locus in *L. pneumophila* grown intracellularly. To determine if the *L. pneumophila* CRISPR-Cas locus is expressed during intracellular growth in macrophages, we infected human U937 cells with strain 130b (Fig. 3A) and then performed RT-PCR analysis on intracellular bacteria at 12, 18, 24, and 48 h after inoculation of the monolayer. These initial experiments demonstrated that *cas9*, *cas1*, *cas2*, *cas4*, and pre-crRNA are all expressed during growth in the macrophage cell line (Fig. 3B). That the transcripts were detected at 12 h and 18 h indicated that the CRISPR-Cas locus is expressed during the initial rounds of intracellular growth prior to lysis of the spent host cells. qRT-PCR analysis further demonstrated that the four *cas* genes are more highly expressed during intracellular infection

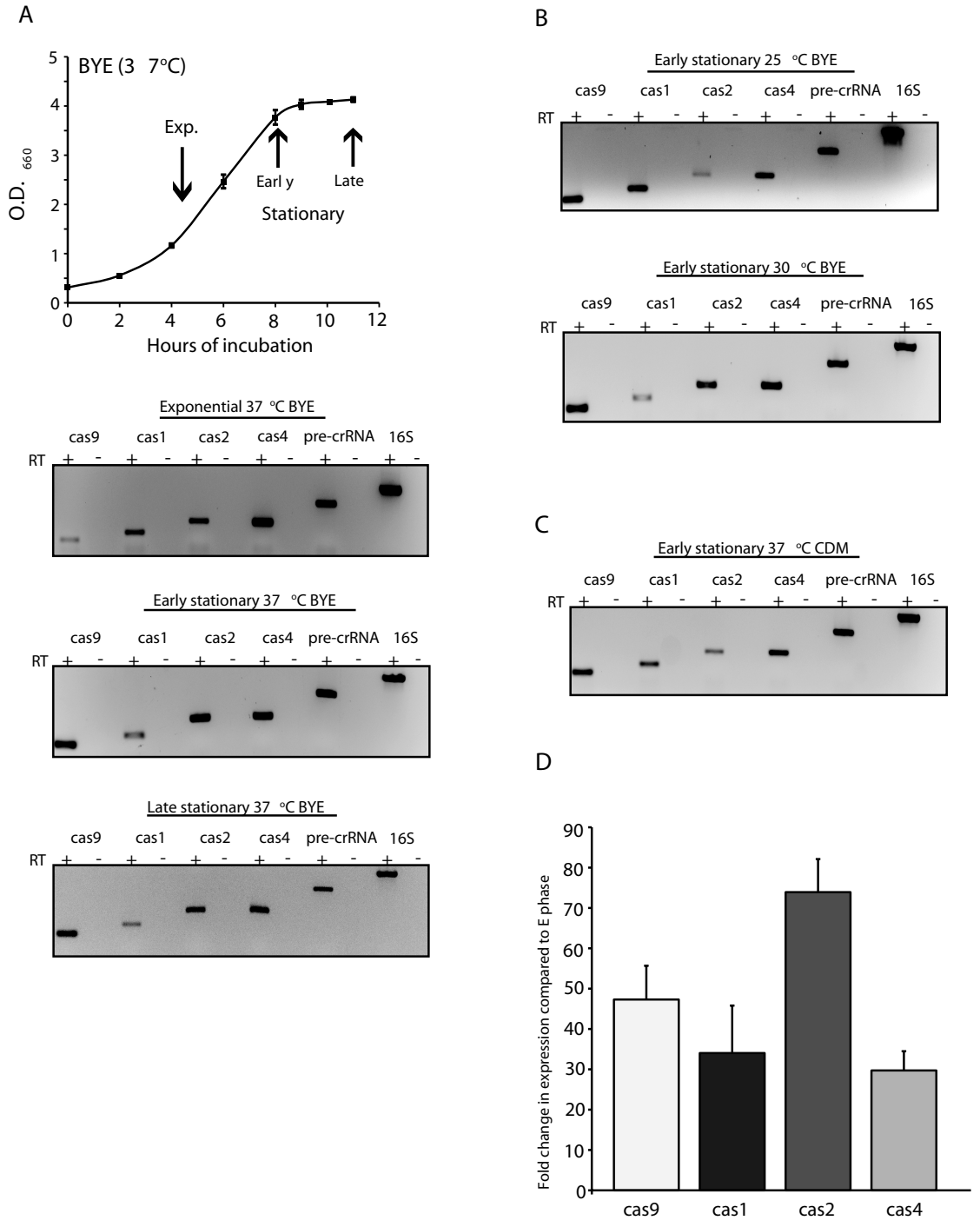

FIG 2 Expression of the *L. pneumophila* CRISPR-Cas locus during extracellular growth. (A, top panel) Growth of 130b in BYE broth. Strain 130b was inoculated into BYE broth and then incubated at 37°C. At various times postinoculation, the extent of growth was monitored spectrophotometrically. Data are means and standard deviations for triplicate cultures. The arrows indicate the points when samples were taken from exponential, early stationary, and late stationary phases for RNA extraction. (A, bottom panels) Expression of the CRISPR-Cas locus during exponential, early stationary, and late stationary phases in BYE broth at 37°C. RNA samples were analyzed by RT-PCR utilizing primers specific for *cas9*, *cas1*, *cas2*, *cas4*, or pre-crRNA. The amplicons obtained were resolved on agarose and visualized by ethidium bromide. As a control, amplification of 16S rRNA was included. That the PCR products obtained were from mRNA was confirmed by the lack of product obtained when the PCR mixture did not include RT. That the mRNAs were of the expected size was confirmed by comparing them to products obtained using genomic DNA. (B) Expression of the 130b CRISPR-Cas locus during early stationary phase in BYE broth at 25°C and 30°C, using the methods used for panel A. (C) Expression of 130b CRISPR-Cas during early stationary phase in CDM broth at 37°C, using the methods used for panel A. (D) The expression of *L. pneumophila* cas genes during early stationary phase compared to that during log phase. After 130b was grown in BYE broth at 37°C and bacterial RNA was obtained at exponential and early stationary phases, qRT-PCR and gene-specific primers were used to assess the fold change in *cas* gene expression during early stationary phase compared to exponential (E) phase. The data are means and standard deviations obtained from triplicate cultures or RNA samples. All of the increases in *cas* gene expression during the stationary phase were statistically significant, with the expression of *cas2* being significantly higher than that of the other three genes ($P < 0.05$; Student's *t* test). All results are representative of three independent experiments.

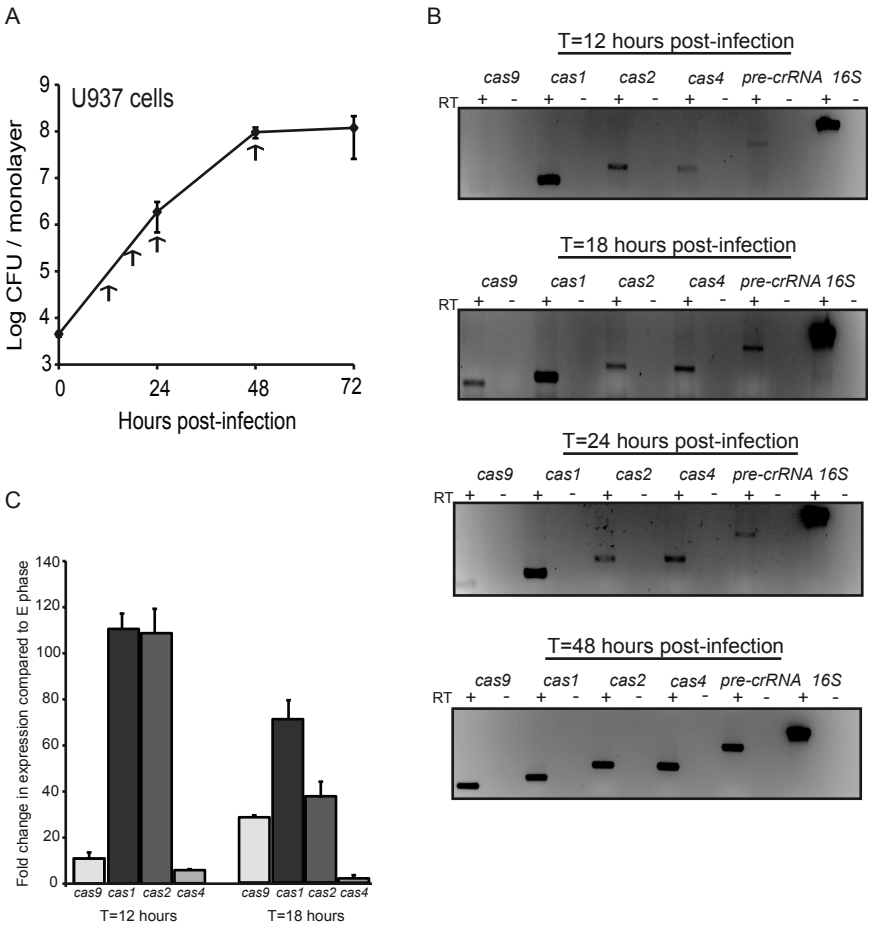

FIG 3 Expression of the *L. pneumophila* CRISPR-Cas locus upon intracellular infection of macrophages. (A) Intracellular growth of 130b in macrophages. U937 cells were infected with 130b, and then at the indicated times, the numbers of CFU in the infected monolayers were determined. Data are means and standard deviations from three infected monolayers. Arrows indicate when samples were taken from the monolayers for RNA extraction. (B) Expression of the CRISPR-Cas locus at 12, 18, 24, and 48 h after inoculation. RNAs obtained from intracellular bacteria were analyzed by RT-PCR using primers specific for *cas9*, *cas1*, *cas2*, *cas4*, or pre-crRNA. That the PCR products obtained resulted from mRNA was confirmed by the lack of product obtained when the PCR did not use RT. (C) Expression of *L. pneumophila cas* genes during growth in macrophages compared to the log phase of extracellular growth. After 130b was grown in U937 cells at 37°C and bacterial RNA obtained at 12 and 18 h, qRT-PCR, and gene-specific primers were used to assess the fold change in *cas* gene expression during intracellular infection compared to the exponential (E) phase in BYE broth at 37°C. Data are means and standard deviations obtained from triplicate monolayers or RNA samples. All increases in *cas* gene expression during intracellular infection were statistically significant, with the expression of *cas1* and *cas2* being significantly higher than that of the other two *cas* genes at 12 h and the expression of *cas1* remaining higher at 18 h ($P < 0.05$; Student's *t* test). All results are representative of three independent experiments.

(at 12 and 18 h) than they are during exponential growth in broth (Fig. 3C). *cas1* and *cas2* displayed the greatest elevation in gene expression during intracellular infection, with ca. 110-fold higher levels of mRNA at 12 h postinoculation and 40- to 70-fold higher levels at 18 h. Considering both time points, *cas9* transcripts were elevated 10- to 30-fold and *cas4* 5- to 10-fold. To determine the CRISPR-Cas expression pattern in legionellae infecting a protozoan, we infected *Acanthamoeba castellanii* amoebae with 130b (Fig. 4A) and did additional RT-PCR analysis. Standard RT-PCR confirmed that *cas9*, *cas1*, *cas2*, *cas4*, and pre-crRNA are expressed during growth in acanthamoebae (see Fig. S2 in the supplemental material). Upon qRT-PCR analysis, the *cas* genes were again expressed at higher levels during intracellular infection than during broth growth (Fig. 4B). *cas1* had the most notable increase in expression, exhibiting a ca. 100-fold increase in transcript levels. *cas2* and *cas9* transcripts were elevated ca. 25- and 50-fold, respectively, whereas *cas4* transcripts were ca. 5-fold higher. These data confirmed that the CRISPR-Cas locus is expressed during infection of amoebae and macrophages, with the *cas1* and *cas2* genes exhibiting the most dramatic increases in expression when intracellular legionellae and extracellular legionellae are compared.

Isolation of *L. pneumophila* CRISPR-Cas mutants. In order to determine if the CRISPR-Cas locus is needed for *L. pneumophila* growth, we generated a panel of 130b mutants specifically lacking *cas1*, *cas2*, *cas4*, *cas9*, or the CRISPR array. RT-PCR analysis determined that the mutation in each *cas* gene did not abolish the expression of the downstream *cas* gene(s) (data not shown). All of the mutants grew normally in BYE broth (see Fig. S3 in the supplemental material), indicating that the mutants do not have a generalized growth defect and that the Cas locus, though expressed, is not required for extracellular growth. The mutants exhibited typical colony morphology when grown on BCYE agar as well as normal shape and swimming motility (data not shown). They also behaved like the wild type did in terms of sliding motility, surfactant production, and secretion (see Fig. S4 in the supplemental material).

Intracellular infection by *L. pneumophila* CRISPR-Cas mutants. To begin to determine the importance of CRISPR-Cas in infection, we assessed the relative ability of the mutants to grow in U937 cell macrophages. All of the mutants grew as well as the wild type did (see Fig. S5A to E in the supplemental material), indicating that *cas1*, *cas2*, *cas4*, *cas9*, and the CRISPR array are not required for optimal infection of macrophages. In support of this conclusion, all of the CRISPR-Cas mutants grew normally within bone marrow-derived (BMD) macrophages obtained from A/J mice (see Fig. S5F in the supplemental material). Turning to a protozoan model of intracellular infection, we observed that the *cas1*, *cas4*, *cas9*, and CRISPR array mutants grew normally in *A. castellanii*, indicating that Cas1, Cas4, Cas9, and the array are also not required for infection of protozoa (Fig. 5A, B, D, and E). In marked contrast, *cas2* mutant NU411, although it grew normally in broth and in macrophages, exhibited significantly reduced recovery upon infection of the acanthamoebae (Fig. 5C). Indeed, at 48 h postinoc-

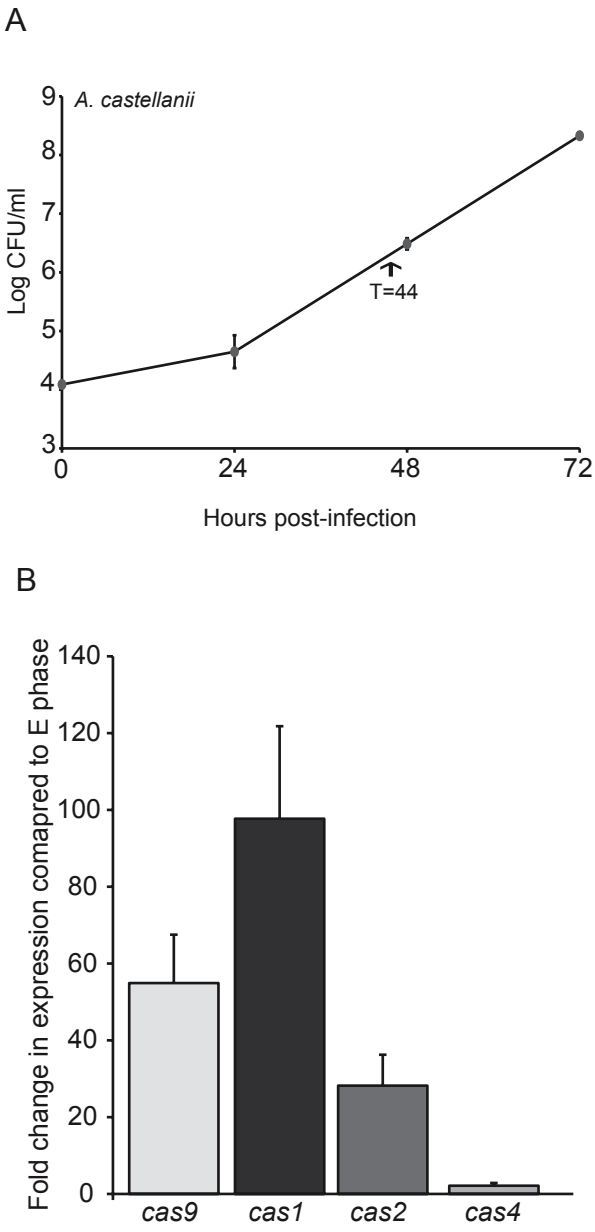

FIG 4 Expression of the *L. pneumophila* CRISPR-Cas locus during intracellular infection of *A. castellanii*. (A) Intracellular growth of 130b in *A. castellanii*. Amoebae were infected with 130b, and then at the indicated times, CFU in the cultures were determined. Data are means and standard deviations from four infected wells. The arrow indicates when samples were obtained for RNA extraction. (B) Expression of *cas* genes during intracellular growth in amoebae compared to the exponential phase of extracellular growth. After 130b was grown in amoebae at 37°C and RNA was obtained at 44 h postinoculation, qRT-PCR assessed the fold change in *cas* gene expression during infection compared to exponential (E) phase in BYE broth at 37°C. Data are means and standard deviations for four samples. All increases in expression during infection were significant, with the expression of *cas1* being significantly higher than that of the other genes ($P < 0.05$; Student's *t* test). Data are representative of three experiments.

ulation, the *cas2* mutant-infected amoebal cultures contained about 55-fold fewer bacteria, and at 72 h, the *cas2* mutant's defect increased to approximately 1,000-fold. The *cas2* mutant did not exhibit reduced survivability when incubated in the assay medium alone (data not shown), indicating that its reduced recovery from infected monolayers is due to impaired intracellular infection. Because a second, independently derived *cas2* mutant (NU412) displayed the same defect (Fig. 6A), the reduced infectivity that we observed was likely due to the mutation of *cas2* versus a spontaneous second-site mutation(s). Given that a mutation in *cas4*, the gene directly downstream of *cas2*, did not alter *L. pneumophila* infection of the amoebae (Fig. 6D), the reduced infectivity of the *cas2* mutants was not due to a polar effect. Complementation of the mutant phenotype occurred when intact *cas2* was introduced on a plasmid (Fig. 6B), confirming that the *cas2* gene is required for optimal intracellular infection of *A. castellanii*. Because *L. pneumophila* is known to infect a variety of amoebae, we assessed the ability of the *cas2* mutant and its complement to infect *H. vermiformis*. The *cas2* mutant NU411, but not its complement, exhibited a reduced ability to infect the hartmannellae, displaying approximately 6-fold- and 20-fold-reduced recovery at 48 h and 72 h postinoculation, respectively (Fig. 6C). As a step toward possibly explaining the role of Cas2 in amoebae, we assessed the *cas2* mutants' sensitivity to DNA-damaging agents, because other Cas2 proteins are known to be nucleases (45, 73) and other bacterial Cas proteins have been implicated in resistance to DNA damage (74). However, the *cas2* mutants did not show increased sensitivity to UV (see Fig. S6 in the supplemental material). Moreover, NU411 and NU412 did not display heightened sensitivity to mitomycin C or nalidixic acid; i.e., for both 130b and the mutants, the MIC for mitomycin C was 2.5 μg/ml, and that for nalidixic acid was 12.5 μg/ml. In sum, our infection data demonstrated that the *cas2* gene and, by inference, the Cas2 protein are required for *L. pneumophila* intracellular infection of multiple amoebae.

DISCUSSION

Arguably, one of the major findings in microbial genetics in recent years is the discovery of the CRISPR-Cas system and the characterization of its role in immunity against phage and plasmids. However, the possibility that a CRISPR-Cas system might do more than provide immunity to invading or transforming nucleic acid has only been hinted at. Here, we have demonstrated that the entire (subtype II-B) CRISPR-Cas locus of *L. pneumophila* is expressed under a wide variety of conditions, including extracellular replication in both rich and minimal media incubated at temperatures ranging from 25 to 37°C as well as intracellular multiplication in both mammalian macrophages and multiple aquatic amoebae. Importantly, qRT-PCR further documented that the levels of *cas* gene transcripts, especially those encoding Cas1 and Cas2, are appreciably greater during intracellular growth as well as in the late stationary phase of broth culture, which is known for the expression of infective traits. Even more significantly, we have found that the *cas2* gene (and, by inference, the Cas2 protein) is required for the ability of *L. pneumophila* to optimally infect multiple types of amoebae. Because infection of protozoa is critical for *L. pneumophila* persistence in and transmission from man-made water systems, Cas2 must also have a significant role in the genesis of Legionnaires' disease. For several reasons, we posit that the event that is mediated or facilitated by *L. pneumophila* Cas2 is entirely distinct from the current dogma concerning CRISPR-Cas function. First, although plasmids occur in some *L. pneumophila* strains and a preliminary study gives evidence for phage that can infect *L. pneumophila* (75, 76), our experiments were done in the

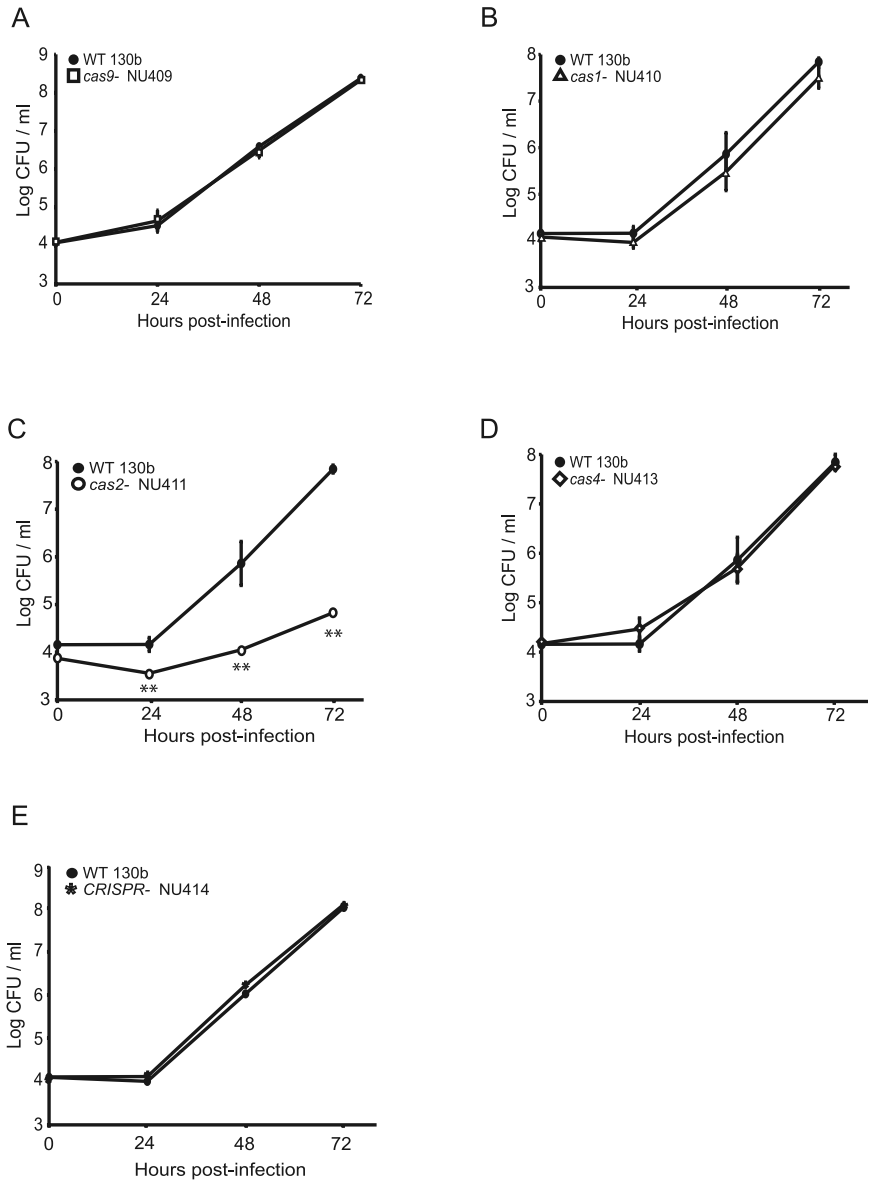

FIG 5 Intracellular growth of the wild type and *cas* mutants in *A. castellanii*. (A to E) Cultures of *A. castellanii* were infected with wild-type (WT) 130b, the *cas9* mutant NU409, the *cas1* mutant NU410, the *cas2* mutant NU411, the *cas4* mutant NU413, or the CRISPR array mutant NU414, and then at the indicated times, the numbers of CFU in the infected cultures were determined by plating. Data are means and standard deviations for four infected wells. Asterisks in panel C indicate points where the recovery of NU411 was significantly less than that of the wild type ($P < 0.05$; Student's *t* test). Each panel is representative of at least three experiments.

absence of any added phage, plasmid, or nucleic acid. Second, only Cas2, not any other component of the *L. pneumophila* CRISPR-Cas locus, was required for infection. Our data add significantly to a growing set of observations that point toward there being other roles for the CRISPR-Cas locus. For example, *Escherichia coli* strains with deletions of *cas1* or the array have increased sensitivity to DNA-damaging agents, suggesting that some components of the CRISPR-Cas system have a function in DNA repair (74). Moreover, envelope stress, in the absence of phage or plasmid, can activate transcription of *E. coli* CRISPR-Cas (77, 78). Compatible with these studies in *E. coli*, UV irradiation and osmotic stress increase *cas* gene transcription in *Thermoproteus tenax* (79). Finally, a CRISPR-Cas locus has been implicated in differentiation events in *Myxococcus xanthus*, the modulation of histidyl-tRNA pools in *Pelobacter carbinolicus*, and phage-dependent inhibition of biofilm formation by *Pseudomonas aeruginosa* (50, 52, 80–82). Our data are the first documentation of a novel role for *cas2* or any component of a CRISPR-Cas locus in an infection event.

Members of the Cas2 family are small proteins (80 to 120 amino acids) that contain a ferredoxin fold that is found in RNA-binding proteins and an N-terminal β-strand followed by a polar amino acid, most often an aspartate or asparagine (45, 46, 83, 84). Cas2 proteins exist as homodimers (45). Crystal structures are known for six Cas2 proteins, including 3 from archaea (*Sulfolobus solfataricus* [2 paralogs] and *Pyrococcus furiosus*) and 3 from bacteria (*Bacillus halodurans*, *Thermus thermophilus*, and *Desulfovibrio vulgaris*) (11, 73, 84). The Cas2 proteins of 2 bacteria (*Thermotoga maritime* and *Nitrosomonas europaea*) and 3 archaea (*S. solfataricus*, *Archaeoglobus fulgidus*, and *Methanobacterium thermoautotrophicum*) have been shown to have endoribonuclease activity, cleaving single-stranded RNA preferentially within U-rich regions (45). Based on predicted structure and alignment to characterized Cas2 proteins, the Cas2 proteins from other eubacteria and archaea have been considered to be RNases (15, 46), although there is a recent report ascribing DNase activity to the Cas2 protein of *B. halodurans* (73). Using standard secondary-structure prediction programs, we determined that *L. pneumophila* Cas2 has both the N-terminal β-strand followed by aspartate and a ferredoxin fold analogous to those in *B. halodurans*, *D. vulgaris*, *P. furiosus*, *S. solfataricus*, and *T. thermophilus* (see Fig. S7 in the supplemental material). The 130b protein also has tyrosine, aspartic acids, and phenylalanine residues that are like those that were defined in *S. solfataricus* as being key for catalytic activity (45) (see Fig. S7). Thus, *L. pneumophila* Cas2 is probably an RNase or alternately a DNase. Because deletion of the entire CRISPR array did not decrease infectivity in the way that loss of *cas2* did, it is unlikely that the processing of pre-crRNA by Cas2 is the critical event in infection of amoebae. Rather, it is conceivable that RNase activity modulates the level(s) or configuration(s) of another regulatory RNA(s) and/or mRNA(s) that influences or encodes factors needed for infection. In light of *in vitro* observations made with other bacteria, noted

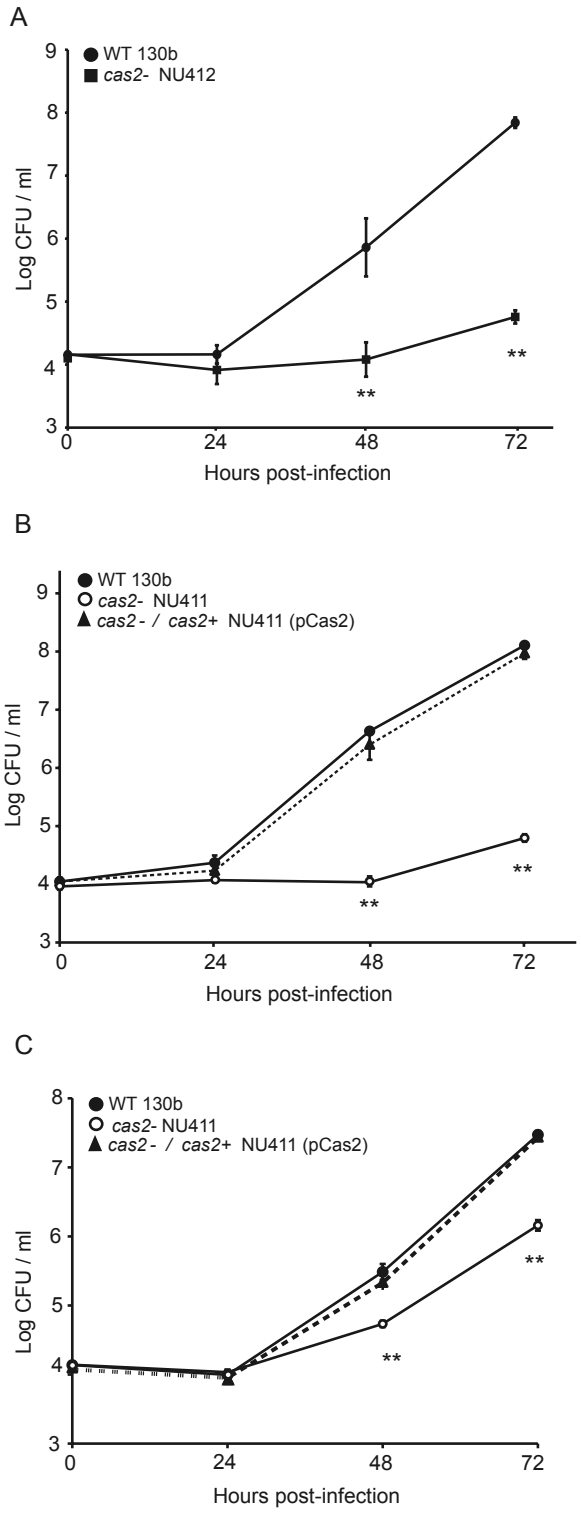

FIG 6 Intracellular growth of the wild type, *cas2* mutants, and a complemented *cas2* mutant in *A. castellanii* and *H. vermiformis*. (A to C) Monolayers of *A. castellanii* (A and B) and *H. vermiformis* (C) were infected with WT 130b, *cas2* mutant NU412, *cas2* mutant NU411, or complemented *cas2* mutant NU411 (pCas2), and at the indicated times, the numbers of CFU in the cultures were determined. Data are means and standard deviations for 4 infected monolayers. Asterisks indicate when the recovery of NU411 and NU412 was significantly less than that of WT and the complemented mutant ($P < 0.05$; Student's *t* test). Each panel is representative of three independent experiments.

above, perhaps *L. pneumophila* Cas2 modulates RNAs that influence bacterial resistance to oxidative stress or other damaging agents that are present during infection of an amoebal host. That a bacterial cytoplasmic RNase could have this sort of effect is evident in the literature; e.g., the CvfA protein of *Streptococcus pyogenes* is an RNase with endonuclease activity that positively regulates virulence determinants (85). Clearly, further experimentation will need to be done in order to discern if *L. pneumophila* Cas2 is in fact an RNase (or DNase) and then ascertain if and how such a nuclease is necessary for optimal intracellular infection of host cells.

Given that the CRISPR-Cas locus of *L. pneumophila* 130b is essentially the same as that of *L. pneumophila* Paris, with the Cas2 proteins sharing 100% identity (see Fig. S7 in the supplemental material), our observations concerning the hyperexpression of CRISPR-Cas and the importance of Cas2 likely have implications for strain Paris as well as the many virulent serogroup 1 strains that are related to 130b and Paris (69, 86). Compatible with this hypothesis, a supplemental table included as part of a microarray study indicated that *cas1*, *cas2*, *cas4*, and *cas9* of Paris are expressed during infection of *A. castellanii* (87). Because some strains, including Philadelphia-1 and Corby, do not carry a CRISPR-Cas locus (67, 68), Cas2 must not be an absolute requirement for intracellular infection by all strains *of L. pneumophila*. That strains can differ in terms of individual virulence determinants is not unheard of; e.g., there is variability among the effectors that are secreted via the type IV secretion system (57, 86). Finally, our results may also have implications for other CRISPR-Cas-containing bacteria, including other pathogens. For example, the subtype II-B locus of 130b is similar to an uncharacterized locus in *Francisella tularensis* strains (e.g., NCBI YP 898402.1 to 898405.1), with the predicted proteins sharing 42% amino acid identity (*E* value $= 8 \times 10^{-12}$); *Francisella*, like *Legionella*, can grow in amoebae (88).

In conclusion, we have demonstrated, among other things, that the *L. pneumophila* CRISPR-Cas locus is expressed during intracellular growth and that the *cas2* gene in particular is required for infection of multiple types of amoebae. In addition to revealing the importance of a CRISPR-Cas component in the natural history and pathogenesis of Legionnaires' disease, these results clearly indicate that *L. pneumophila cas2* has a role that is unique from the prevailing view of CRISPR-Cas function. Our findings with *Legionella* further suggest that CRISPR-Cas loci present in other bacteria and archaea may have physiologically relevant functions that are unrelated to phage and plasmid immunity or DNA transformation and other forms of horizontal gene transfer.

MATERIALS AND METHODS

Bacterial strains, media, and extracellular growth assessments. *L. pneumophila* serogroup-1 strain 130b (ATCC BAA-74) served as our wild type (89). Mutants that were isolated in this study are listed in Table 1. Legionellae were grown at 37°C on buffered charcoal yeast extract (BCYE) agar, which, when appropriate, contained chloramphenicol at 3 μg/ml, kanamycin at 25 μg/ml, or gentamicin at 2.5 μg/ml (89). *Escherichia coli* strain DH5α (Invitrogen, Carlsbad, CA) was the host for recombinant plasmids and was grown in Luria-Bertani medium. Unless otherwise noted, chemicals were from Sigma-Aldrich (St. Louis, MO). To monitor extracellular growth of *L. pneumophila* strains as well isolate RNA, legionellae were inoculated into buffered yeast extract (BYE) or chemically defined medium (CDM) broth and then incubated with shaking (90). The optical densities of cultures were determined at 660 nm (Beckman Coulter, Indi-

anapolis, IN). Microscopy was used to observe shape and swimming motility. Sliding motility and surfactant production were monitored as previously described (89).

DNA and protein sequence analysis. DNA was isolated from *L. pneumophila* as described before (89). Primers used for sequencing and/or PCR were obtained from Integrated DNA Technologies (Coralville, IA). Primer sequences are listed in Table S1 in the supplemental material. DNA sequences were analyzed using Lasergene (DNASTAR, Madison, WI), and protein alignments were done using the Clustal method. The CRISPRFinder software available at http://crispr.u-psud.fr/Server/ (70) was used to both identify the CRISPR-Cas locus (http://www.ncbi.nlm.nih.gov/nuccore/FR687201.1) and to analyze the individual spacers and repeats. Other BLAST homology searches were done through the National Center for Biotechnology Information (NCBI) and the other *L. pneumophila* databases at http://genolist.pasteur.fr/LegioList/. To obtain secondary structures, we used the I-TASSER and Phyre servers (90, 91, 92).

RT-PCR analysis. To monitor *L. pneumophila* transcription, reverse transcription-PCR (RT-PCR) was done essentially as described before (90). RNA was isolated from BYE and CDM cultures by using the RNA STAT-60 reagent (Tel-Test, Friendswood, TX) and following the manufacturer's instructions, with the exception that glycogen and sodium acetate were added during precipitation (93). To isolate bacterial RNA from infected host cells, U937 cells and *Acanthamoeba castellanii* were infected as described below. The monolayer was lysed with 50% RNA Protect (Qiagen, Valencia, CA)-1% saponin, and RNA was extracted using RNA STAT-60. RNA samples were treated with DNase I (Life Technologies, Carlsbad, CA), extracted using acid-phenol-chloroform, and precipitated with sodium acetate-ethanol (94). cDNA was synthesized in a 20-μl reaction mixture containing 1 μg of RNA, a 1 μM concentration of the gene-specific primer Cas9-4RT-R116 or 0.12 μg of random primers, and the following items obtained from Life Technologies: 1× first strand buffer, a 2 mM concentration of each deoxynucleoside triphosphate (dNTP), 10 mM dithiothreitol (DTT), 40 U RNaseOut, and 200 U of SuperScript III reverse transcriptase. Primers CAS1-F692 and CAS1-R869 were used to examine transcription of *cas1*, CAS2-F50 and CAS2-R293 were used for *cas2*, CAS4-F36 and CAS4-R279 were used for *cas4*, CAS9-F3047 and CAS9-R3188 were used for *cas9*, and CRISPRII-FL1 and CRISPRII-R5′.S5 were used for pre-crRNA transcripts. As a control, 16S rRNA gene transcription was assessed using primers 16S rRNA-F and 16S rRNA-R. In order to assess cotranscription of *cas9* and *cas1*, primers CAS9-F3047 and CAS1-R282 were used. For monitoring cotranscription of *cas1* and *cas2*, we used CAS1-F692 and CAS2-R293, and for *cas2* and *cas4*, CAS2-F50 and CAS4-R279. Endpoint PCRs were separated by electrophoresis and detected with ethidium bromide.

Quantitative RT-PCR. To precisely determine levels of RNA, quantitative RT-PCR (qRT-PCR) was done. The cDNA sample was diluted 1:20, and a 5-μl aliquot was added to a 25-μl reaction mixture, which included 12.5 μl of SYBR green master mix (Bio-Rad, Hercules, CA) and a 0.5 μM concentrated of each primer. The primer pairs used were as follows; CAS9-F3047 and CAS9-R3188 for *cas9*, Cas1-F and CAS1-R for *cas1*, CAS2-F and CAS2-R for *cas2*, and CAS4-F and CAS4-R for *cas4*. Using the CFX96 Touch real-time PCR detection system (Bio-Rad), the reaction cycle was 95°C for 3 min, 95°C for 10 s, and 60°C for 30 s for 40 cycles. For each assessment, three biological replicates were obtained, with each one being tested in triplicate. To quantitate products, standard curves using genomic DNA were generated for each primer set. *L. pneumophila* lpw_00031 (i.e., *gyrB*) and lpw_16991 were used as reference genes to normalize gene expression, as previously done (94–96). The primer pair for *gyrB* was GYRB-F and GYRB-R, and that for lpw_16991 was LPW16991-F and LPW16991-R. The level of gene expression was assessed by determining the cycle at which the amplification curve crossed the detection threshold, and the relative change in gene expression was calculated using the $2^{\Delta\Delta CT}$ method (97).

Mutant construction and genetic complementation. To generate *L. pneumophila* mutants lacking either *cas9*, *cas1*, *cas2*, *cas4*, or the CRISPR array, we performed variations on allelic exchange (89, 90). To obtain a *cas9* (lpw_01781) mutant, the 5′ and 3′ ends of the gene were separately amplified from 130b DNA using primers CAS9-F1 and CAS9-R1SmaI and primers CAS9-F2SmaI and CAS9-R2, respectively. The generated fragments were ligated into pGEM-T Easy (Promega, Madison, WI), and the resulting plasmids were digested with SmaI and SpeI. Finally, a trimolecular ligation was done, placing a kanamycin resistance cassette (Km^r), obtained from pMB2190 (90), between the beginning and end of *cas9*. The plasmid obtained, pGEM-cas9-Km, had a 4.1-kb deletion in the center of *cas9*. Mutated *cas9* was introduced into the chromosome of 130b by transformation (90) of pGEM-cas9-Km, and the mutant genotype was confirmed by PCR using primers CAS9-F19 and CAS9-R4079. Utilizing primers CAS1-F1 and CAS1-R1StuI and CAS1-F2StuI and CAS1-R2, a similar allelic exchange procedure was used to mutate *cas1* (lpw_01791). In this case, the two initial plasmids were digested with StuI and SphI, and the trimolecular ligation inserted a gentamicin resistance cassette (Gm^r), obtained from pX1918-GT (90), in *cas1*. The final plasmid, pGEM-cas1-Gm, had an 800-bp deletion in *cas1*. Following transformation of pGEM-cas1-Gm into 130b, the genotype was confirmed by PCR using CAS1-F1 and CAS1-R2. To mutate *cas2* (lpw_01801), CAS2-F1–CAS2-R1StuI and CAS2-F2StuI–CAS2-R2 were used, and the trimolecular ligation placed Km^r into *cas2*. The final plasmid made, pGEM-cas2-Km, had a 200-bp deletion in *cas2*. After transformation of pGEM-cas2-Km into 130b, the mutant was confirmed with primers CAS2-F1 and CAS2-R2. To obtain a *cas4* (lpw_01811) mutant, allelic exchange was again used but with primer pairs CAS4-F1 and CAS4-R1StuI and CAS4-F2StuI and CAS4-R2. The initial pGEM-based plasmids were digested with StuI and SpeI, and the trimolecular ligation inserted Gm^r between the beginning and end of *cas4*. The final plasmid, pGEM-cas4-Gm, had a 350-bp deletion in *cas4*, and a mutated gene was confirmed using CAS4-F1 and CAS4-R2. To obtain a mutant that lacked the entire CRISPR array, the 5′ and 3′ ends of the array were separately amplified from 130b DNA using CRISPR-F1 and CRISPR-R1SmaI and CRISPR-F2SmaI and CRISPR-R2, respectively. The generated fragments was ligated into pGEM-T Easy, and the resulting plasmids were digested with SmaI and SpeI. Lastly, a trimolecular ligation was done by placing Km^r between the beginning and end of the array. The plasmid obtained, pGEM-CRISPR-Km, contained a 4.2-kb deletion. The mutated array was introduced into 130b by transformation, and the genotype was verified by PCR using primers CRISPR-DIAGF and CRISPR-DAIGR. To generate a plasmid for complementation of the *cas2* mutant, intact *cas2* (but no other gene) was amplified from 130b DNA using CAS2-SphI-F and CAS2-SacI-R. After the product was digested with SphI and SacI, the *cas2*-containing fragment was cloned into pMMB2002 (90), and the new pCas2 was electroporated (90) into mutant NU411.

TABLE 1 *L. pneumophila* strains used in this study

Strain(s)	Description	Reference or source
130b	Clinical isolate	89
NU409	*cas9* mutant of strain 130b	This study
NU410	*cas1* mutant of strain 130b	This study
NU411, NU412	*cas2* mutants of strain 130b	This study
NU413	*cas4* mutant of strain 130b	This study
NU414	CRISPR mutant of strain 130b	This study

Intracellular infection assays. *Hartmannella vermiformis* (ATCC 50237) and *A. castellanii* (ATCC 30234) were infected with *L. pneumophila* as described before (89). To assess *L. pneumophila* growth in mammalian cells, we infected human U937 cells (ATCC CRL-1593.2) and bone marrow-derived (BMD) macrophages obtained from mice (98). To prepare the murine macrophages, progenitor cells were extracted from the femurs of A/J mice (The Jackson Laboratory, Bar Harbor, ME), laid down in plates, and incubated in RPMI medium with 20% fetal bovine serum (FBS), 30% L-cell supernatant, 1% penicillin-streptomycin, and 1 μg/ml Fungizone. On day 3, the cultures were given fresh medium. On day 7, cultures were washed with phosphate-buffered saline (PBS) and incu-

bated at 4°C until the adherent cells detached. Recovered macrophages were used to form monolayers of 2.5×10^5 cells, and after 3 days in RPMI with 10% FBS and 10% L-cell supernatant, monolayers were infected with bacteria as before (98).

Assays for bacterial sensitivity to DNA-damage. To judge sensitivity to UV light, overnight BYE cultures of wild-type and mutant bacteria were diluted in BYE broth to an optical density at 660 nm (OD_{660}) equal to 0.3, and then 10-fold serial dilutions were made in PBS. Analogous to previous studies (99–102), we spotted 10-μl aliquots of the suspensions onto BCYE agar, and then the plates were exposed to various doses of 254-nm UV light (Stratalinker; Stratagene, Santa Clara, CA). To assess sensitivity to chemical damaging agents, we determined the MICs of mitomycin C and nalidixic acid according to a standard protocol using broth microdilutions (103–105). All sensitivity assays were performed on three independent occasions.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00074-13/-/DCSupplemental.

Figure S1, EPS file, 2.7 MB.
Figure S2, EPS file, 2.2 MB.
Figure S3, EPS file, 0.4 MB.
Figure S4, EPS file, 19.6 MB.
Figure S5, EPS file, 0.5 MB.
Figure S6, EPS file, 19.6 MB.
Figure S7, EPS file, 0.9 MB.
Table S1, DOCX file, 0.1 MB.

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