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 have 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...
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 GenBank database. The 130b CRISPR-Cas genes were different from those of strain Paris as well as being unlike any Paris CRISPR array. However, the spacer sequences in strain 130b repeats in the 130b CRISPR array were identical to the repeats in the 130b 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.

**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.

**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.
(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 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-
Increased to approximately 1,000-fold. The about 55-fold fewer bacteria, and at 72 h, the population, 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...
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 *Mycobacterium xanthus* pools in *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 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 *Deinococcus vulgaris*) (11, 73, 84). The Cas2 proteins of 2 bacteria (*Thermotoga maritima* 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...
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 NU411, 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.
**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 (Corvalle, 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 BVD 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, which included 1 μg of each primer. The primer pairs used were as follows; cas1-F50 and CAS1-R293, and for cas2, CAS2-F50 and CAS2-R293, and for cas4, CAS4-F36 and CAS4-R279. Endpoint PCRs were separately amplified from 130b DNA using CRISPR-F1 and CRISPR-R1SmaI and CRISPR-F2 and CRISPR-R2, respectively. The generated fragments were ligated into pGEM-T Easy, and the resulting plasmids were digested with SmaI and SpeI. Lastly, a trimolecular ligation generated fragments was ligated into pGEM-T Easy, and the resulting plasmids were digested with SmaI and SpeI. Finally, a trimo-locular ligation was done, placing a kanamycin resistance cassette (Km’), 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-F1 and CAS9-R4079. Utilizing primers CAS1-F1 and CAS1-R1StuI and CAS4-F2Stul 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 Stul and Sphl, and the trimo-locular ligation inserted a gentamicin resistance cassette (Gmr’), 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-F2Stul–CAS2-R2 were used, and the trimo-locular ligation placed Km’ 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-F2Stul and CAS4-R2. The initial pGEM-based plasmids were digested with Stul and SpeI, and the trimo-locular ligation inserted Gm’ 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-R1StuI and CRISPR-F2Stul and CRISPR-R2, respectively. The generated fragments were ligated into pGEM-T Easy, and the resulting plasmids were digested with Smal and SpeI. Lastly, a trimo-locular ligation was done by placing Km’ 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-DIAF and CRISPR-DAIg. 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 SpI and SacI, the cas2-containing fragment was cloned into pMMB202 (90), and the new pCas2 was electroporated (90) into mutant NU411."

**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-

**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         |
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.

**REFERENCES**

1. Grissa I, Vergnaud G, Pourcel C. 2007. The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. BMC Bioinformatics 8:172.
2. Jansen R, Embden JD, Gaastra W, Schouls LM. 2002. Identification of genes that are associated with DNA repeats in prokaryotes. Mol. Microbiol. 43:1565–1575.
3. Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Makarova KS, Aravind L, Wolf YI, Koonin EV, van der Oost J. 2011. Unification of cas candidates for adaptive immunity in bacteria and archaea. Nat. Rev. Genet. 11:181–190.
4. Terns MP, Terns RM. 2011. CRISPR-based adaptive immune systems. Curr. Opin. Microbiol. 14:321–327.
5. Haft DH, Selengut J, Mongodin EF, Nelson KE. 2005. A guild of 45 CRISPR-associated (cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. PLoS Comput. Biol. 1:e60. http://dx.doi.org/10.1371/journal.pcbi.0010060.
6. Barrangou R, Fremeaux B, Deveau H, Charpentier S, Clermont O, Rocha EP, Denamur E, Jeltsch A, Koonin EV. 2011. The CRISPR/Cas system provides acquired resistance against viruses in prokaryotes. Science 334:1843–1846.
7. Garneau JE, Dupuis ME, Villion M, Romero DA, Barrangou R, Boyaval P, Moineau S, Romero DA, Horvath P. 2007. CRISPR provides acquired resistance against viruses in prokaryotes. Science 315:1709–1712.
8. Edgar R, Qimron U. 2010. The Escherichia coli CRISPR system protects from a λ lysogenization, lysogens, and prophage induction. J. Bacteriol. 192:6291–6294.
9. Marraffini LA, Sontheimer EJ. 2008. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. Science 322:1843–1845.
10. Garneau JE, Dupuis ME, Villion M, Romero DA, Barrangou R, Boyaval P, Fremeaux B, Deveau H, Charpentier S, Clermont O, Rocha EP, Denamur E, Jeltsch A, Koonin EV. 2011. The CRISPR/Cas system provides acquired resistance against viruses in prokaryotes. Science 315:1709–1712.
11. Edgar R, Qimron U. 2010. The Escherichia coli CRISPR system protects from a λ lysogenization, lysogens, and prophage induction. J. Bacteriol. 192:6291–6294.
12. Lopez-Sanchez MJ, Sauvage E, Da Cunha V, Clermont D, Ratsima H, Lopez-Sanchez MJ, Sauvage E, Da Cunha V, Clermont D, Ratsima H, Nam KH, Haitjema C, Liu X, Ding F, Wang H, DeLisa MP, Ke A. 2010. The CRISPR/Cas adaptive immune system of Pseudomonas aeruginosa mediates resistance to naturally occurring and engineered phages. J. Bacteriol. 192:5728–5738.
13. Westra ER, Swarts DC, Staal KS, Jere MM, Brouns SJ, van der Oost J. 2012. The CRISPR/Cas adaptive immune system of Pseudomonas aeruginosa mediates resistance to naturally occurring and engineered phages. J. Bacteriol. 192:5728–5738.
14. Louwen R, Brouns SJ, van der Oost J, Westra ER. 2013. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. Science 322:1843–1845.
15. Garneau JE, Dupuis ME, Villion M, Romero DA, Barrangou R, Boyaval P, Fremeaux B, Deveau H, Charpentier S, Clermont O, Rocha EP, Denamur E, Jeltsch A, Koonin EV. 2011. The CRISPR/Cas system provides acquired resistance against viruses in prokaryotes. Science 315:1709–1712.
16. Edgar R, Qimron U. 2010. The Escherichia coli CRISPR system protects from a λ lysogenization, lysogens, and prophage induction. J. Bacteriol. 192:6291–6294.
17. Lopez-Sanchez MJ, Sauvage E, Da Cunha V, Clermont D, Ratsima H, Lopez-Sanchez MJ, Sauvage E, Da Cunha V, Clermont D, Ratsima H, Nam KH, Haitjema C, Liu X, Ding F, Wang H, DeLisa MP, Ke A. 2010. The CRISPR/Cas adaptive immune system of Pseudomonas aeruginosa mediates resistance to naturally occurring and engineered phages. J. Bacteriol. 192:5728–5738.
18. Westra ER, Swarts DC, Staal KS, Jere MM, Brouns SJ, van der Oost J. 2012. The CRISPR/Cas adaptive immune system of Pseudomonas aeruginosa mediates resistance to naturally occurring and engineered phages. J. Bacteriol. 192:5728–5738.
19. Louwen R, Brouns SJ, van der Oost J, Westra ER. 2013. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. Science 322:1843–1845.
20. Garneau JE, Dupuis ME, Villion M, Romero DA, Barrangou R, Boyaval P, Fremeaux B, Deveau H, Charpentier S, Clermont O, Rocha EP, Denamur E, Jeltsch A, Koonin EV. 2011. The CRISPR/Cas system provides acquired resistance against viruses in prokaryotes. Science 315:1709–1712.
21. Edgar R, Qimron U. 2010. The Escherichia coli CRISPR system protects from a λ lysogenization, lysogens, and prophage induction. J. Bacteriol. 192:6291–6294.
22. Lopez-Sanchez MJ, Sauvage E, Da Cunha V, Clermont D, Ratsima H, Lopez-Sanchez MJ, Sauvage E, Da Cunha V, Clermont D, Ratsima H, Nam KH, Haitjema C, Liu X, Ding F, Wang H, DeLisa MP, Ke A. 2010. The CRISPR/Cas adaptive immune system of Pseudomonas aeruginosa mediates resistance to naturally occurring and engineered phages. J. Bacteriol. 192:5728–5738.
23. Westra ER, Swarts DC, Staal KS, Jere MM, Brouns SJ, van der Oost J. 2012. The CRISPR/Cas adaptive immune system of Pseudomonas aeruginosa mediates resistance to naturally occurring and engineered phages. J. Bacteriol. 192:5728–5738.
24. Louwen R, Brouns SJ, van der Oost J, Westra ER. 2013. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. Science 322:1843–1845.
25. Garneau JE, Dupuis ME, Villion M, Romero DA, Barrangou R, Boyaval P, Fremeaux B, Deveau H, Charpentier S, Clermont O, Rocha EP, Denamur E, Jeltsch A, Koonin EV. 2011. The CRISPR/Cas system provides acquired resistance against viruses in prokaryotes. Science 315:1709–1712.
26. Edgar R, Qimron U. 2010. The Escherichia coli CRISPR system protects from a λ lysogenization, lysogens, and prophage induction. J. Bacteriol. 192:6291–6294.
27. Lopez-Sanchez MJ, Sauvage E, Da Cunha V, Clermont D, Ratsima H, Lopez-Sanchez MJ, Sauvage E, Da Cunha V, Clermont D, Ratsima H, Nam KH, Haitjema C, Liu X, Ding F, Wang H, DeLisa MP, Ke A. 2010. The CRISPR/Cas adaptive immune system of Pseudomonas aeruginosa mediates resistance to naturally occurring and engineered phages. J. Bacteriol. 192:5728–5738.
28. Westra ER, Swarts DC, Staal KS, Jere MM, Brouns SJ, van der Oost J. 2012. The CRISPR/Cas adaptive immune system of Pseudomonas aeruginosa mediates resistance to naturally occurring and engineered phages. J. Bacteriol. 192:5728–5738.
29. Louwen R, Brouns SJ, van der Oost J, Westra ER. 2013. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. Science 322:1843–1845.
30. van der Oost J, Brouns SJ. 2009. RNAi: prokaryotes get in on the act. Cell 139:863–865.
31. Tyson GW, Banfield JF. 2008. Rapidly evolving CRISPRs implicated in acquired resistance of microorganisms to viruses. Environ. Microbiol. 10:200–207.
32. Hale C, Kleppe K, Terns RM, Terns MP. 2008. Prokaryotic silencing (psi)RNAs in Prococcus furiosus. RNA 14:2572–2579.
33. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
34. Semenova E, Nagornykh M, Pyatnitskiy M, Artamonova II, Severinov Wiedenheft B, van Duijn E, Bultema JB, Waghmare SP, Zhou K, Hatoum-Aslan A, Maniv I, Marraffini LA.
35. Haurwitz RE, Jinek M, Wiedenheft B, Zhou K, Doudna JA.
36. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
37. Semenova E, Nagornykh M, Pyatnitskiy M, Artamonova II, Severinov Wiedenheft B, van Duijn E, Bultema JB, Waghmare SP, Zhou K, Hatoum-Aslan A, Maniv I, Marraffini LA.
38. Haurwitz RE, Jinek M, Wiedenheft B, Zhou K, Doudna JA.
39. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
40. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
41. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
42. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
43. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
44. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
45. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
46. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
47. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
48. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
49. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
50. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
51. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
52. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
53. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
54. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
55. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
56. Lillestøl RK, Shah SA, Brügger K, Redder P, Phan H, Christiansen J, Garrett RA. 2009. CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. Mol. Microbiol. 72:259–272.
regularly interspaced short palindromic repeats (CRISPR)-associated Cas2 protein. J. Biol. Chem. 287:35943–35952.
74. Babu M, Beloglozava N, Flick R, Graham C, Skarina T, Nocek B, Gagarinova A, Pogoutse O, Brown G, Binkowski A, Phanse S, Joachimiak A, Koonin EV, Savchenko A, Emili A, Greenblatt J, Edwards AM, Yakunin AF. 2011. A dual function of the CRISPR-Cas system in bacterial antivirus immunity and DNA repair. Mol. Microbiol. 79:484–502.
75. Lammertyn E, Vande Voorde J, Meyen E, Maes L, Mast J, Anné J. 2008. Evidence for the presence of Legionella bacteriophages in environmental water samples. Microb. Ecol. 56:191–197.
76. Gomez-Valero L, Rusniok C, Buchrieser C. 2009. Legionella pneumophila: population genetics, phylogeny and genomics. Infect. Genet. Evol. 9:727–739.
77. Perez-Rodriguez R, Hsiao C, Huang Q, Nam KH, Bernardis S, Knauf M, Lovley DR. 2011. Envelope stress is a trigger of CRISPR RNA-mediated DNA silencing in *Escherichia coli*. Mol. Microbiol. 79:584–599.
78. Raivio T. 2011. Identifying your enemies—could envelope stress trigger microbial immunity? Mol. Microbiol. 79:557–561.
79. Plagens A, Tijen B, Hagemann A, Randau L, Hensel R. 2012. Characterization of the CRISPR/Cas subtype I-A system of the hyperthermophilic crenarchaeon *Thermoproteus tenax*. J. Bacteriol. 194:2491–2500.
80. Palmer KL, Whiteley M. 2011. DM53-42: the secret to CRISPR-dependent biofilm inhibition in *Pseudomonas aeruginosa*. J. Bacteriol. 193:3431–3432.
81. Viswanathan P, Murphy K, Julien B, Garza AG, Kroos L. 2007. Regulation of dev, an operon that includes genes essential for *T. tenax* development and CRISPR-associated genes and repeats. J. Bacteriol. 189:3738–3750.
82. Aklujkar M, Lovley DR. 2010. Interference with histidyl-tRNA synthetase by a CRISPR spacer sequence as a factor in the evolution of *Thermoproteus tenax*. BMC Evol. Biol. 10:230.
83. Maris C, Dominguez C, Allain FH. 2005. The RNA recognition motif, a plastic RNA-binding platform to regulate post-transcriptional gene expression. FEBS J 272:2118–2131.
84. Samai P, Smith P, Shuman S. 2010. Structure of a CRISPR-associated protein Cas2 from *Desulfovibrio vulgaris*. Acta Crystallogr. 66:1552–1556.
85. Kang SO, Caparon MG, Cho KH. 2010. Virulence gene regulation by CviA, a putative Rnase: the CviA-eno1ase complex in *Streptococcus pyogenes* links nutritional stress, growth-phase control, and virulence gene expression. Infect. Immun. 78:2754–2767.
86. Schroder GN, Petty NK, Mousnier A, Harding CR, Vogrin AJ, Wei B, Fry NK, Harrison TG, Newton HJ, Thomson NR, Beaton SA, Dougan G, Hartland EL, Frankel G. 2010. Legionella pneumophila strain 130b possesses a unique combination of type IV secretion systems and novel Dot/Icm secretion system effectors. J. Bacteriol. 192:6001–6016.
87. Brüggemann H, Hageman A, Jules M, Sismeiro O, Dillies MA, Gouyette C, Kunst F, Steinert M, Heuner K, Coppée JY, Buchrieser C. 2006. Virulence strategies for infecting phagocytes deduced from the in vivo transcriptional program of *Legionella pneumophila*. Cell. Microbiol. 8:1228–1240.
88. Santic M, Ozanic M, Semic V, Pavokovic G, Mrvcic V, Kvaik YA. 2011. Intravascular proliferation of *F. novicida* within *H. vermiformis*. Front Microbiol. 2:78.
89. Stewart CR, Burnside DM, Cianciotto NP. 2011. The surfactant of *Legionella pneumophila* is secreted in a TolC-dependent manner and is antagonistic toward other *Legionella* species. J. Bacteriol. 193:5971–5984.
90. Chatfield CH, Mulhern BJ, Burnside DM, Cianciotto NP. 2011. *Legionella pneumophila* LbtU acts as a novel, TonB-independent receptor for the lejobactin siderophore. J. Bacteriol. 193:1563–1575.
91. Bennetts-Lovey RM, Herbert AD, Sternberg MJ, Kelley LA. 2008. Exploring the extremes of sequence/structure space with ensemble fold recognition in the program Phyre. Proteins 70:611–625.
92. Zhang Y. 2008. i-TASSER server for protein 3D structure prediction. BMC Bioinformatics 9:40.
93. Koo JT, Allee NY, Schiano CA, Nalini WW. 2011. Global discovery of small RNAs in *Yersinia pseudotuberculosis* identifies *Yersinia*-specific small, noncoding RNAs required for virulence. Proc. Natl. Acad. Sci. U. S. A. 108:709–717.
94. Faucher SP, Mueller CA, Shuman HA. 2011. *Legionella pneumophila* transcriptome during intracellular multiplication in human macrophages. Front Microbiol. 2:60.
95. Broich M, Rydzewski K, McNealy TL, Marre R, Fieger A. 2006. The global regulatory proteins LetA and RpoS control phospholipase A, lysophospholipase A, acyltransferase, and other hydrolytic activities of *Legionella pneumophila* JR32. J. Bacteriol. 188:1218–1226.
96. Doleans-Jordheim A, Akermi M, Ginevra C, Cazalet C, Kay E, Schneider D, Buchrieser C, Atlan D, Vandenesch F, Etienne J, Jarraud S. 2006. Growth-phase-dependent mobility of the lvh-encoding region in *Legionella pneumophila* strain Paris. Microbiology 152:3561–3568.
97. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–408.
98. McCoy-Simandle K, Stewart CR, Dao J, Debroy S, Rossier O, Bryce PJ, Cianciotto NP. 2011. *Legionella pneumophila* type II secretion dampens the cytokine response of infected macrophages and epithelia. Infect. Immun. 79:1984–1997.
99. Charpentier X, Kay E, Schneider D, Shuman HA. 2011. Antibiotics and UV radiation induce competence for natural transformation in *Legionella pneumophila*. J. Bacteriol. 193:1114–1121.
100. Knudson GB. 1985. Photoreactivation of UV-irradiated *Legionella pneumophila* and other Legionella species. Appl. Environ. Microbiol. 49:975–980.
101. Courcelle J, Crowley DJ, Hanawalt PC. 1999. Recovery of DNA replication in UV-irradiated *Escherichia coli* requires both excision repair and recF protein function. J. Bacteriol. 181:916–922.
102. Al-Deib AA, Mahdi AA, Lloyd RG. 1996. Modulation of recombination and DNA repair by the recG and PriA helicases of *Escherichia coli K-12*. J. Bacteriol. 178:6782–6789.
103. Yoshida S, Mizuguchi Y. 1984. Antibiotic susceptibility of *Legionella pneumophila* Philadelphia-1 in cultured guinea-pig peritoneal macrophages. J. Gen. Microbiol. 130:901–906.
104. Robey M, O’Connell W, Cianciotto NP. 2001. Identification of *Legionella pneumophila* rcp, a pagP-like gene that confers resistance to cationic antimicrobial peptides and promotes intracellular infection. Infect. Immun. 69:4276–4286.
105. Hazan R, Sat B, Engelberg-Kulka H. 2004. *Escherichia coli* nspE2-mediated cell death is triggered by various stressful conditions. J. Bacteriol. 186:3663–3669.