Critical to microbial versatility is the capacity to express the cohort of genes that increase fitness in different environments. Legionella pneumophila occupies extensive ecological space that includes diverse protists, pond water, engineered water systems, and mammalian lung macrophages. One mechanism that equips this opportunistic pathogen to adapt to fluctuating conditions is a switch between replicative and transmissive cell types that is controlled by the broadly conserved regulatory protein CsrA. A striking feature of the legionellae surveyed is that each of 14 strains encodes 4 to 7 csrA-like genes, candidate regulators of distinct fitness traits. Here we focus on the one csrA paralog (lpq1593) that, like the canonical csrA, is conserved in all 14 strains surveyed. Phenotypic analysis revealed that long-term survival in tap water is promoted by the lpq1593 locus, which we name csrR (for “CsrA-similar protein for resilience”). As predicted by its GGA motif, csrR mRNA was bound directly by the canonical CsrA protein, as judged by electromobility shift and RNA-footprinting assays. Furthermore, CsrA repressed translation of csrR mRNA in vivo, as determined by analysis of csrR-gfp reporters, csrR mRNA stability in the presence and absence of csrA expression, and mutation of the CsrA binding site identified on the csrR mRNA. Thus, CsrA not only governs the transition from replication to transmission but also represses translation of its paralog csrR when nutrients are available. We propose that, during prolonged starvation, relief of CsrA repression permits CsrR protein to coordinate L. pneumophila’s switch to a cell type that is resilient in water supplies.

Many Gram-negative and Gram-positive bacteria rely on a CsrA protein to regulate gene expression (1–3). Some of these highly conserved and well-studied RNA binding proteins repress translation by binding to the 5′ untranslated region (5′-UTR) of target mRNAs and preventing ribosome access to the Shine-Dalgarno (SD) sequence. Several species of bacteria, including Escherichia coli (4–7), Pseudomonas aeruginosa (8), Bacillus subtilis (9), Salmonella enterica serovar Typhimurium (10), and Legionella pneumophila (11), practice this mode of posttranscriptional regulation. CsrA binding can also regulate mRNA translation or stability by other mechanisms (3). For example, the E. coli CsrA protein binds in the 5′ coding region of the sdiA transcript to repress its translation (12).

In the Gram-negative, aquatic bacterium L. pneumophila, CsrA regulates a key intracellular life cycle switch within host amoeba or mammalian macrophages (13–15). L. pneumophila differentiates from a replicative form—capable of multiplying to high numbers within a host cell when nutrients are abundant—to a transmissive form—equipped to lyse out of and infect new host cells (16). CsrA is essential for transmissive-phase cells to convert to the replicative phase, and it actively represses transmissive phenotypes when nutrients are available to support growth (11, 13, 15, 17). When nutrients become limiting, the LetA/LetS two-component system activates transcription of two noncoding RNAs, rsmY and rsmZ, that directly bind and sequester CsrA, thus relieving its repression of transmission traits, including expression of dot and icm substrates (11, 17–20).

L. pneumophila can also persist in diverse freshwater reservoirs, ranging from ponds to engineered water systems and water-cooling towers (21–25). When machinery vaporizes water con-

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**ABSTRACT** Critical to microbial versatility is the capacity to express the cohort of genes that increase fitness in different environments. Legionella pneumophila occupies extensive ecological space that includes diverse protists, pond water, engineered water systems, and mammalian lung macrophages. One mechanism that equips this opportunistic pathogen to adapt to fluctuating conditions is a switch between replicative and transmissive cell types that is controlled by the broadly conserved regulatory protein CsrA. A striking feature of the legionellae surveyed is that each of 14 strains encodes 4 to 7 csrA-like genes, candidate regulators of distinct fitness traits. Here we focus on the one csrA paralog (lpq1593) that, like the canonical csrA, is conserved in all 14 strains surveyed. Phenotypic analysis revealed that long-term survival in tap water is promoted by the lpq1593 locus, which we name csrR (for “CsrA-similar protein for resilience”). As predicted by its GGA motif, csrR mRNA was bound directly by the canonical CsrA protein, as judged by electromobility shift and RNA-footprinting assays. Furthermore, CsrA repressed translation of csrR mRNA in vivo, as determined by analysis of csrR-gfp reporters, csrR mRNA stability in the presence and absence of csrA expression, and mutation of the CsrA binding site identified on the csrR mRNA. Thus, CsrA not only governs the transition from replication to transmission but also represses translation of its paralog csrR when nutrients are available. We propose that, during prolonged starvation, relief of CsrA repression permits CsrR protein to coordinate L. pneumophila’s switch to a cell type that is resilient in water supplies.

**IMPORTANCE** Persistence of L. pneumophila in water systems is a public health risk, and yet there is little understanding of the genetic determinants that equip this opportunistic pathogen to adapt to and survive in natural or engineered water systems. A potent regulator of this pathogen’s intracellular life cycle is CsrA, a protein widely distributed among bacterial species that is understood quite well. Our finding that every sequenced L. pneumophila strain carries several csrA paralogs—including two common to all isolates—indicates that the legionellae exploit CsrA regulatory switches for multiple purposes. Our discovery that one paralog, CsrR, is a target of CsrA that enhances survival in water is an important step toward understanding colonization of the engineered environment by pathogenic L. pneumophila.
taminated with *L. pneumophila*, the bacteria can gain access to the human lung. Remarkably, strategies that evolved to promote persistence within phagocytic amoebae also equip the pathogen to survive and replicate in alveolar macrophages (22, 26). Therefore, understanding environmental survival and persistence is critical to designing strategies to reduce the risks *L. pneumophila* poses to human health.

Although distinct extracellular forms of *L. pneumophila*, including biofilm communities, mature intracellular forms (MIFs), and viable but nonculturable cells (VBNCs), have been described previously (27–31), the mechanisms that dictate the switch into these environmental forms are not yet known. By applying phenotypic, biochemical, and molecular assays, we identified in the *L. pneumophila* core genome a dual-CsrA system whose integrated design is predicted to promote reciprocal expression of distinct cell types.

**RESULTS**

**Identification of 71 csrA-like genes in *L. pneumophila* species.**

Initial genome sequencing projects revealed three csrA-like genes in the carbon storage regulator family (32). By performing a bioinformatic search of the *L. pneumophila* Philadelphia-1 genome, we identified five csrA-like genes, including the well-characterized canonical csrA (Fig. 1A) (13). The amino acid sequences of the four paralogs exhibit 28% to 58% identity and 59% to 80% similarity. The amino acid residues determined to be critical for CsrA function in *E. coli* are retained in CsrA proteins across myriad genera surveyed, including the canonical *L. pneumophila* CsrA protein (1). By aligning the amino acid sequences of the csrA paralogs of *L. pneumophila* Philadelphia-1, we confirmed that several key residues, including the arginine residue that is essential for the RNA binding activity, are retained in all five (1) (Fig. 1A). Based on the conserved amino acid sequence, the five *L. pneumophila* Philadelphia-1 loci are likely valid csrA paralogs.

To determine if the Philadelphia-1 strain is unique in encoding multiple csrA paralogs, the in silico search was expanded to include 13 more fully sequenced strains of legionellae: 8 additional independent isolates of *L. pneumophila*, 2 laboratory strains derived from Philadelphia-1, 2 strains of *L. longbeachiae*, and 1 strain of *L. drancourtii* (see Table S1 in the supplemental material). Altogether, 71 csrA-like genes were identified, and each retained several residues predicted to be key for RNA binding (1). In particular, the arginine residue critical for RNA binding was conserved in 70 of the 71 proteins; in the one exception, another cationic residue, lysine, was in place of the arginine (see Table S2). Each of the 14 surveyed strains contained between 4 and 7 csrA-like genes. Without exception, all 14 encoded not only the canonical csrA gene but also a copy of a previously uncharacterized paralog, *lpg1593*, which we name here csrR. Accordingly, both the csrA and
**csrR** genes appear to belong to the core genome of *Legionella* species. In stark contrast, the remainder of the *csrA*-like genes are located within previously characterized or putative integrative conjugative elements (ICEs), large genomic islands known or predicted to transfer horizontally between strains of legionellae (see Table S3) (33–35).

To compare the sequences of the 63 *Legionella* *csrA* genes found in the 12 strains, excluding the two laboratory-derived strains Lp02 and JR32, we generated a heat map that displays the amino acid similarity of each protein to each of the other paralogs. By this approach, we recognized three clusters: two that correspond to the presumptive core CsrA and CsrR proteins and a third that encompasses a more diverse group of ICE-related CsrA-like proteins (Fig. 1B). Because *csrR* is encoded by all surveyed strains, is highly conserved, and retains key RNA binding residues, we hypothesized that this gene would regulate traits fundamental to *L. pneumophila*’s life style, as CsrA does.

*L. pneumophila* requires the conserved *csrR* locus to persist under nutrient-poor conditions. Since *csrR* is a paralog of the essential regulator *csrA*, we first tested whether the two genes regulate the same developmental stage. To do so, we either deleted or induced expression of the *csrR* gene and then assayed phenotypes known to be regulated by the canonical *csrA* gene (13). Analyzed under standard laboratory culture conditions (37°C in rich ACES-buffered yeast extract with thymidine [AYET] medium), neither loss nor gain of *csrR* function altered expression of any of *L. pneumophila* replication-phase or transmission-phase traits tested. These included, for broth cultures, growth, pigmentation, motility, and sensitivity to sodium, heat, and osmotic stress, as well as infection and intracellular growth in primary mouse macrophages and amoebae (data not shown) (13). Since *csrR* appeared unlikely to regulate traits that are hallmarks of the commonly studied replicative and transmissive cell types, we next considered conditions that *L. pneumophila* likely encounters in the environment. Indeed, a dual-CsrA system (RsmA/RsmF) equips *P. aeruginosa* to modulate biofilm formation (36).

The legionellae naturally thrive in freshwater and soil habitats, and *L. pneumophila* can survive for long periods in water in a VBNC state (29, 30). Therefore, we tested whether *csrR* coordinates differentiation to a resilient environmental cell type. Employing the method of Garduño and colleagues (30), we inoculated autoclaved tap water at 45°C with wild-type (*W*) *L. pneumophila*, a *csrR* mutant, or a *csrR* mutant strain in which a wild-type *csrR* allele had been integrated into the chromosome. After 24 days, we quantified bacterial viability by fluorescence microscopy using a LIVE/DEAD BacLight stain and culturability on rich nutrient agar (37). The wild-type culture retained 79% viability, which was significantly higher than the mutant culture’s 50% viability (*P* value = 0.049; Fig. 2A). Likewise, the level of colony forming units (CFU) recovered at 24 days relative to day 0 was significantly higher for the wild-type culture than for the mutant culture (5.6% of WT, 0.3% of mutant; *P* value = 0.0027) (Fig. 2B). Although the merodiploid mutant strain exhibited an intermediate level of viability as measured by microscopy assay (Fig. 2A) (*P* > 0.05 compared either to the wild type or to the mutant), genetic complementation was incomplete. Two control experiments indicated that the lack of full complementation was likely due to poor expression of *csrR*. First, quantitative reverse transcriptase PCR (qRT-PCR) revealed that the merodiploid strain contained approximately 5% of the wild-type levels of *csrR* RNA (data not shown). Second, we quantified reduced viability relative to wild-type *L. pneumophila* by both microscopy and CFU assays after isolating and analyzing nine independent *csrR* mutant strains constructed by three different strategies.

**csrR** mRNA contains multiple CsrA binding sites. Because knowledge of a protein’s regulation can provide insight into its function, we next analyzed the upstream sequences of *csrR*. Bioinformatic analysis identified motifs typical of CsrA binding sites in the 5′ noncoding region of *csrR* (11). As a first step to analyze *csrR* regulation, we mapped by 5′ rapid amplification of cDNA ends (5′-RACE) its transcriptional start site to −52 relative to the translational start (data not shown). Three independent sequencing events revealed that the transcript starting with the −52 start site was predominant, although rare transcripts started at −53 or −54. On the basis of the data indicating the predominant species, we made several predictions about the *csrR* mRNA. Putative −10 and −35 sites as well as the likely SD sequence approximately 10 bases 5′ of the translational start codon were identified (Fig. 3A). RNA-fold software predicted a secondary structure for the *csrR* transcript in which the SD sequence is positioned in a loop extending from a long stem (Fig. 3B), a common binding motif for CsrA protein (38). Accordingly, we postulated that the *csrR* transcript is bound and its stability regulated by CsrA.

To test directly whether CsrA protein binds to *csrR* mRNA, we performed an *in vitro* assay. Purified CsrA protein was incubated with 5′-end-labeled *csrR* RNA, and then binding was analyzed by gel electromobility shift assay. As predicted by the *in silico* analysis,
CsrA protein bound tightly to csrR RNA, with a calculated dissociation constant of 32 \pm 9 \text{nM} (Fig. 4A). This physical interaction was specific, since incubation with an excess of unlabeled csrR RNA effectively competed with CsrA protein binding to labeled csrR RNA (Fig. 4B). In contrast, incubation with even higher concentrations of a heterologous competitor RNA, E. coli phoB, did not inhibit CsrA binding to csrR. Therefore, CsrA protein binds csrR RNA tightly and specifically in vitro.

To verify our observation that CsrA protein binds to the csrR transcript at the predicted stem-loop structure surrounding the SD sequence, we performed RNA footprinting experiments. Interestingly, although CsrA did bind at the predicted SD site (“BS2” in Fig. 4C), the protein also protected a GGA motif within the first two codons of the csrR coding region (“BS3” in Fig. 4C). Although binding in the 5’ untranslated region of its target gene is typical, in some cases CsrA does bind in coding regions (12). The RNA footprinting assay also revealed a potential third binding site (BS1); however, because this third site was protected only weakly, we focused next on the stronger BS2 and BS3 binding sites.

**Transcriptional and translational fusions reveal a contribution of BS3 to csrR regulation.** To analyze in live cells whether either or both BS2 and BS3 binding sites contribute to csrR post-transcriptional regulation, we constructed a series of green fluorescent protein (GFP) reporters (Fig. 5A). For the BS2-only transcriptional reporter, gfp expression is driven by a sequence corresponding to approximately 300 bp immediately 5’ of the csrR coding region that includes BS2. Although this reporter contains the csrR SD sequence, its gfp SD sequence likely controls translation; thus, the BS2-only construct is referred to as a transcriptional reporter. The BS2/BS3 translational reporter includes both the BS2 and BS3 directly 5’ and in frame with gfp coding sequences, eliminating the gfp SD sequence and including the first 5 codons of csrR. For the BS2 stem-loop-disrupted (BS2-sld)/BS3 reporter, the BS2 stem-loop structure was disrupted on the translational re-

**FIG 3** A putative CsrA binding site on the csrR RNA overlaps with a ribosome binding site motif. (A) The csrR transcriptional start site (TSS, +1; black arrow) was mapped by 5’ RACE. Likely −10 and −35 promoter elements are underlined and labeled; a putative Shine-Dalgarno (SD) sequence and the associated flanking bases predicted to form a stem-loop structure recognized by CsrA and located 10 bases 5’ of the translational start are underlined; and the csrR open reading frame is marked with a gray arrow and capitalized letters. (B) The csrR RNA encodes a putative SD (+37) sequence located on a stem-loop and an ATG start codon (+51) positioned at the base of two stem-loops, as predicted by the IDT UNAFold algorithm. CsrA protein is predicted to bind the csrR RNA SD sequence (boxed).
porter by changing the six consecutive adenines immediately 5’ of the SD sequence to uracils, thereby preventing stem formation with the eight uracils 3’ of the SD sequence.

*L. pneumophila* strains carrying each reporter were cultured in broth, and then aliquots were assessed for GFP fluorescence. As a marker for the growth phase, we also analyzed a reporter of the *flaA* flagellin gene, which is strongly induced in post-exponential (PE) phase (39). The BS2-only reporter generated a high level of fluorescence in exponential (E) phase (~15,000 relative fluorescence units [RFU]) that then decreased (to ~5,000 RFU) in PE phase (Fig. 5B), the period when transmission traits are induced (16). In comparison to the BS2-driven expression, in E phase, the BS2/BS3 and the BS2-sld/BS3 translational reporters demonstrated 3-fold and 6-fold less fluorescence, respectively (Fig. 5B).

Therefore, the BS3 site appears to destabilize the mRNA and/or inhibit translation of the transcript. In addition, on the basis of the reduced fluorescence seen when the stem-loop was disrupted, the BS2 site may somehow stabilize the mRNA.

**Mutation of the CsrA binding site relieves CsrA repression in vivo.** To assess whether CsrA protein destabilizes the *csrR* transcript in *vivo*, we utilized a previously characterized conditional *csrA* mutant (13). In this strain, the chromosomal *csrA* locus is deleted, and a plasmid carries a functional copy of the gene under IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible control. Thus, we measured the impact of CsrA protein on *csrR* transcript levels by culturing cells in the presence and absence of IPTG.

To determine whether CsrA alters the stability of *csrR* transcripts, the conditional *csrA* mutant was cultured with or without IPTG for 9 h, which corresponded to E phase, a period when *csrR* promoter activity is high (Fig. 5B) (40). Next, transcription was inhibited by treatment with rifampin. RNA was isolated 0 and 15 min later, and then the relative levels of *csrR* mRNA were quantified by qRT-PCR. There was an inverse correlation between the presence of CsrA protein and *csrR* mRNA levels (Fig. 6A), indicating that the presence of CsrA decreased *csrR* transcript stability. A similar pattern has been reported for other CsrA-mRNA interactions (41, 42).

We then tested in *vivo* whether CsrA destabilization of *csrR* transcript depends on its BS3 motif (Fig. 4 and 5). To do so, we performed qRT-PCR on wild-type *L. pneumophila* strains that carried plasmids in which an IPTG-inducible promoter drives either a wild-type or BS3 mutant *csrR* allele that also encodes a 6-histidine epitope. To ensure the presence of active CsrA, RNA was isolated from strains cultured with or without IPTG for 9 h in

FIG 4 CsrA binds tightly and specifically to *csrR* RNA. (A) 5’-end-labeled *csrR* RNA (0.1 nM) was incubated with CsrA protein at the indicated concentration (nM), and the complexes were separated by gel electrophoresis. Positions of bound (marked B) and free (marked F) RNA species are shown. The binding constant (K_d) was calculated from the binding curve shown at right. (B) Labeled *csrR* RNA (0.1 nM) was incubated with CsrA protein in the absence or presence of unlabeled specific (*csrR*) or nonspecific (*E. coli* *phoB*) competitor RNA at the concentrations indicated. (C) 5’-End-labeled *csrR* RNA was treated with RNase T_1 in the presence of CsrA protein at the concentration indicated. Also shown are partial alkaline hydrolysis (column OH) and RNase T_1 digestion (column T1) ladders, as well as a control sample that lacked RNase T_1 treatment (column C). Labels indicate residues for which CsrA protein reduced RNase T_1 cleavage (BS1 to BS3), the *csrR* Shine-Dalgarno (SD) sequence, and the translation initiation codon (Met). Numbering is with respect to the start site of *csrR* transcription (+1).
E phase. Induction of the BS3 mutant generated >8-fold more csrR transcript than the corresponding uninduced control, whereas the wild-type allele showed a <2-fold increase (P value = 0.0327; Fig. 6B). Thus, in cultures of wild-type L. pneumophila where the wild-type allele showed a csrR transcript than the corresponding uninduced control, the wild-type allele showed a csrR transcript than the corresponding uninduced control.

DISCUSSION

In nature, legionellae must adapt to the stresses of the intracellular environment of professional phagocytes and nutrient-poor aqueous habitats (22, 26–28, 30, 43). L. pneumophila relies on the CsrA RNA binding protein to regulate directly and indirectly the transition between the replicative and transmissive phases of its pathogenic life cycle (11, 13, 15, 17). Here we demonstrate that L. pneumophila survival during aquatic stress is enhanced by the locus encoding the CsrA-like protein CsrR, a broadly conserved factor whose translation is directly repressed by CsrA.

Of the numerous csrA paralogs encoded by Legionella species, only two are completely conserved in all surveyed strains: canonical csrA (13) and the gene we name csrR (13). Second, inducing expression of csrR fails to complement the csrA growth defect (data not shown). Third, neither loss nor gain of csrR function alters expression of the panel of traits.

FIG 5 BS3 mediates repression of csrR-GFP reporters. (A) Schematics of csrR-GFP reporters to analyze csrR regulation. The BS2-only transcriptional reporter (MB1375; circles) encodes ~300 bp 5′ to the translational start sequence, including the csrR SD sequence, which overlaps the BS2 CsrA binding site, and the gfp ribosome binding site (RB5). In the BS2/BS translational reporter (MB1376; squares) the gfp RBS is replaced by the first five codons of csrR, which encode the BS3 CsrA binding site. The BS2-sld/BS3 translational reporter (MB1377; triangles) was constructed by eliminating the stem-loop base pairing to disrupt the BS2 CsrA binding site on the dual reporter. (B) E-phase cultures of L. pneumophila carrying the reporter indicated were incubated for 24 to 28 h, and GFP fluorescence was quantified at the times indicated and expressed in relative fluorescence units (RFU). Reference strains were L. pneumophila carrying the empty gfp vector (diamonds) or flaA-GFP reporter (gray line) as an indicator of the transition into PE phase. Shown are means ± standard deviations calculated from the results determined from triplicate samples; error bars for the stem-loop mutant are too small to be depicted. Similar patterns were observed in at least two other experiments.

FIG 6 CsrA regulates csrR expression in vivo. (A) To analyze the impact of CsrA protein on csrR RNA stability, a conditional csrA mutant (MB464) was cultured without (black) or with (gray) IPTG to the E phase, rifampin was added to inhibit new transcription, and RNA was isolated 0 and 15 min later. Error bars indicate standard deviations of the results from duplicate samples, and significance was calculated using a Student’s t test. Results are representative of two independent experiments. (B) csrR transcript stability was assessed via qRT-PCR of E-phase L. pneumophila cultured without or with IPTG to induce expression of either the WT strain (MB1378, black) or BS3 mutant csrR (MB1379, gray). Data shown represent the fold increase in the transcript level in the induced strains relative to their uninduced controls. Error bars indicate standard deviations of the results from duplicate samples, and significance was calculated using a Student’s t test. Results are representative of three independent experiments. (C) The strains whose results are shown in panel B were cultured into E phase with or without IPTG, and then protein levels were analyzed by Western assay using anti-His antibody. L. pneumophila encoding His-tagged CsrA on a plasmid served as the positive control. Data are representative of the results of four independent experiments done with two independent isolates of each strain.
regulated by CsrA (data not shown). Instead, the csrR locus enhances *L. pneumophila* persistence in 45°C tap water (Fig. 2), a condition that induces resilient VBNC cells (30). Together, our initial phenotypic analyses predict that CsrR regulates environmental resilience of *L. pneumophila* whereas CsrA controls the intracellular replication-transmission cycle (13).

In addition, CsrA represses CsrR protein expression by a post-transcriptional mechanism. CsrA directly binds *csrR* mRNA in vitro (Fig. 4); *in vivo*, CsrA destabilizes *csrR* mRNA (Fig. 5 and 6A) and prevents CsrR protein expression (Fig. 6B). CsrA binding is mediated by a GGA motif in the second codon of *csrR* (Fig. 4C), and mutation of this motif relieves posttranscriptional repression (Fig. 6B). CsrA’s direct repression of *csrR* ensures reciprocal expression of this dual-CsrA system, a design that creates two mutually exclusive regulons for *L. pneumophila*.

CsrA represses CsrR protein expression by binding directly to its mRNA at BS3, a site comprised of a GGA motif within the first two codons of the *csrR*-coding region (Fig. 3). It is noteworthy that BS3 overlaps the codon for an aspartic acid residue in the second amino acid position, a striking deviation from the ubiquitous leucine residue found not only in 55 of the 57 non-CsrR CsrA paralogs encoded in the *Legionella* pan-genome but also in every CsrA homologue of over 30 surveyed species of Gram-negative and -positive bacteria (see Table S2 in the supplemental material) (1). This leucine-to-aspartic acid replacement is perfectly conserved in all 14 surveyed *L. pneumophila* CsrA homologues of CsrA encoded by many different species of bacteria. After prolonged exposure to nutrient-poor water, CsrR is activated and promotes conversion to a resilient cell type (green bacteria).

Our discovery that CsrR is posttranscriptionally repressed by CsrA (Fig. 3 to 5) may explain why its deletion conferred no discernible phenotype under standard laboratory conditions, which are repressive. Likewise, inducing transcription of a plasmid-borne *csrR* allele in cells cultured in rich media may be inconsequential because *csrR* mRNAs are bound and degraded by CsrA before they can be translated (Fig. 3 to 5). However, inducing CsrR protein expression from a *csrR* allele that lacks the CsrA BS3 binding site (Fig. 5) was also not sufficient to trigger *L. pneumophila* differentiation into a resilient, nonreplicative cell type. In rich AYET broth, bacterial growth, pigmentation, and motility were not affected by ectopic CsrR protein expression (data not shown). It is possible that the mRNA targets of CsrR are not expressed under the luxuriant culture conditions used, negating the effect of accumulated CsrR protein. We speculate that *L. pneumophila* differentiation into an environmental, resilient cell type requires additional factors to be expressed and/or CsrA repression of other determinants to be relieved.

Although the pathway that triggers CsrA derepression of CsrR expression remains to be discovered, the results reported here suggest an expanded model of the *Legionella* life cycle (Fig. 7). When a phagocytic cell is encountered, the transmissive form of the bacterium infects it and establishes a protective vacuole (16, 48). The CsrA repressor then equips the intracellular bacterium to differentiate into a replicative form and multiply to high numbers (13). In replication-phase cells, CsrA actively represses not only transmissive traits but also accumulation of CsrR, a regulator of environmental persistence (11, 13, 15, 17) (Fig. 2 to 5). When nutrients are exhausted within the host cell, LetA and LetS induce expression of the noncoding RNAs RsmY and RsmZ to relieve CsrA repression of transmissive traits (11, 19, 40, 49, 50). At the same time, *csrR* transcription declines (Fig. 5). As a consequence, the cells transition back to the motile, infectious transmissive form that can spread efficiently from one phagocytic cell to another. However, if *L. pneumophila* does not readily encounter another host cell and remains in a nutrient-poor extracellular environment for a prolonged period, we propose that, by mechanisms defined by Mercante et al. (1) likely confers CsrR specificity for a distinct regulon.
that remain to be discovered, CsrR is activated and promotes the pathogen’s long-term survival (Fig. 2).

We favor a model in which the mechanism to relieve CsrA repression of transmissive traits is distinct from its derepression of CsrR protein expression. As nutrients are consumed by replicating bacteria, CsrA repression of virulence traits is relieved at least in part by two noncoding RNAs, RsmY and RsmZ (17, 19, 49). In contrast, csrR transcript—but not protein—levels are elevated in the replicative phase, as shown by our reporter assay (Fig. 5), qRT-PCR (data not shown), Western analysis (Fig. 6), and deep sequencing of RNA pools (40). The high activity of the csrR promoter in the replicative phase indicates that the L. pneumophila is spring-loaded to respond to abrupt stress, an advantage should the bacteria lack the time or resources needed to complete their natural transition into the transmissive phase. We therefore postulate that some dedicated and yet unknown mechanism relieves CsrA repression of CsrR protein expression. For example, when conditions require rapid deployment of CsrR, replicating cells could induce expression of a direct inhibitor of CsrA binding, or the conditions could facilitate a change in the secondary structure of the csrR mRNA that promotes its stabilization by binding of CsrA to BS2 over BS3.

Having two functional csrA-like genes in their core genome is an unusual feature of the legionellae. We propose that duplication and genetic drift of the gene endowed these environmental intracellular pathogens with a new regulatory protein that retained the mRNA binding function of CsrA but acquired a new cohort of targets. This evolutionary sequence may have bestowed on the legionellae the capacity to alternate between a facultative intracellular life cycle and a resilient state that can withstand a broad range of environmental conditions. Packaged within this new regulatory csrR gene is an on/off switch: a CsrA binding site in its coding region. We propose that this parsimonious design equips L. pneumophila’s two CsrA paralogs to mediate reciprocal expression of distinct regulons under appropriate conditions.

**MATERIALS AND METHODS**

**Bacterial strains, culture conditions, and reagents.** Strains, plasmids, and primers used in this study are listed in Table S4 in the supplemental material. L. pneumophila Philadelphia-1 laboratory-derived strain Lp02, a thymidine auxotroph, was cultured at 37°C in AYE broth and on ACES-buffered charcoal (Fisher)-yeast extract (Becton, Dickinson) (CYE) agar supplemented with 100 μg/ml thymidine (Sigma), 400 μg/ml cytochrome (Fisher), and 135 μg/ml ferric nitrate (J. T. Baker) (51); Thymidine was used when cultivating thymidine prototroph strains. When necessary for antibiotic selection of mutants or plasmids, media were supplemented with kanamycin (Sigma) (10 μg/ml); chloramphenicol (Fisher) (5 μg/ml); or gentamicin (Gibco) (10 μg/ml). Where indicated, gene expression was induced by adding IPTG (Gold Biotechnology) to reach a final concentration of 200 μM. For all experiments, colonies were first inoculated into broth, incubated overnight, and diluted to an optical density at 600 nm (OD600) of 0.05 to 0.2 and then cultured to the E phase (OD600 of 1.0 to 2.0) or PE phase (OD600 of 3.7 to 4.0), as indicated.

**csrA gene identification and heat map generation.** To identify the csrA-like genes in the Legionella pan-genome (see Table S1 in the supplemental material), the amino acid sequence of CsrA from L. pneumophila Philadelphia-1 was submitted to BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The heat map was generated by first entering the percent amino acid identity of each protein pair into an Excel spreadsheet and then assigning a grayscale to the values. The proteins were then manually clustered by similarity. The order of the proteins from top to bottom (and left to right) is listed in Table S2.

**csrR deletion and merodiploid.** A chloramphenicol resistance cassette was amplified from pKD3 (52) using primers ZA44 and ZA45. Flanking homology to ~500 bp 5’ and 3’ of csrR was generated from Lp02 genomic DNA using primers ZA37 and ZA43 and primers ZA46 and ZA38, respectively. The three PCR products were then joined by splicing by overlap extension (SOE) PCR using outermost primers ZA37 and ZA38 to generate the csrR::camR allele used to replace the csrR gene by natural transformation (53), producing strain MB1369.

To insert a wild-type csrR locus into a neutral site on the chromosome of the csrR mutant, the csrR gene and its native promoter were integrated into the 154-bp intergenic region between lpg2528 and lpg2529 (54). To do so, we first constructed plasmid pSU-pZLkm. The multicloning site of pSU2719 (55) was amplified using primers SC1 and SC2 and cloned into the SalI site of pZL790-MCS, creating vector pZL790-MCS-Km. Then, the lpg2528-MCS-Km-lpg2529 fragment from pZL790-MCS-Km was amplified with primers SC5 and SC6 and ligated into pSU2719 at the Nco1 and HindIII sites, after the HindIII site was site was made blunt with Klenow fragment, producing vector pSU-pZLkm. csrR with its native promoter (~300 bp 5′ of the translational start) was amplified from Lp02 genomic DNA with primers ZA49 and ZA60 and ligated into the EcoRI site of pSU-pZLkm. This plasmid was then integrated by natural transformation into the 154-bp intergenic region between lpg2528 and lpg2529 in csrR mutant MB1369 to generate strain MB1372. Merodiploid mutants were verified by growth on CYETCam+kan and by PCR.

**Restoration of thymidine prototrophy.** L. pneumophila is naturally a thymidine prototroph, but the Lp02 laboratory strain was made thymidine auxotroph in 1993 to exploit “thymineless death” as a strategy to identify intracellular growth mutants (56). To avoid the potentially confounding phenotype of thymineless death under conditions of prolonged starvation, thymidine prototrophy was restored to strains MB1101, MB1369, and MB1372 by replacing by natural transformation their mutant thyA allele with the wild-type thyA gene from L. pneumophila Philadelphia-1 encoded on pJB3395 as described previously (53), generating strains MB1368, MB1370, MB1371, MB1373, and MB1374.

**Water incubation and survival.** Thymidine prototroph strains MB1368, MB1370, MB1371, and MB1374 were analyzed to avoid thymineless death during the prolonged incubation. The wild type and two independent isolates of each mutant and merodiploid strain were cultured in AYET medium to the E phase, diluted to an OD600 of 0.2, and then cultured to the PE phase. The cultures were then washed twice by suspension in 50 ml of autoclaved tap water and centrifugation at 4,500 g for 15 min. Cells were then resuspended in 10 ml of autoclaved tap water in 15-ml conical tubes with the caps loosely affixed to allow air exchange and incubated statically at a 45°C incubator. The viability of duplicate samples was assessed by LIVE/DEAD BacLight (Life Technologies) staining. A 165-μl volume of culture was mixed with 165 μl of autoclaved tap water, 1 μl of each stain was added, and the samples were incubated at room temperature in the dark for 15 min. Aliquots (10 μl) were mounted on slides pretreated for 5 min with 5 μl poly-L-lysine, and 100 bacteria were scored for each replicate of each strain. Culturability of duplicate samples was quantified by plating 10-fold serial dilutions in autoclaved tap water on CYE agar.

**DNA isolation.** For all experiments analyzing RNA, 2 ml of liquid culture was pelleted by centrifugation at 10,000 × g for 5 min at 4°C, the cells were resuspended in 1 ml of Trizol reagent (Life Technologies), and RNA was isolated following the manufacturer’s protocol. Residual DNA was degraded using a Turbo DNA-free kit (Life Technologies) following the manufacturer’s protocol, and RNA was stored at −20°C.

**5’-RACE and RNA fold prediction.** 5’-RACE was performed using a ligation-anchored PCR strategy described previously (57). Briefly, RNA was isolated as previously described, and first-strand cDNA synthesis was performed using SuperScript II reverse transcriptase (Life Technologies).
the manufacturer’s protocol, and ZA54 as the csrR gene-specific primer. The ZA80 adaptor oligonucleotide, with 5’ phosphorylation for the ligation and a 3’ amino modifier to prevent its ligation, was ligated to cDNA using T4 RNA ligase (NEB) following the manufacturer’s protocol. PCR amplification was performed using ZA81 and ZA54 with platinum Taq DNA polymerase (Invitrogen), and ligation into pGEM T-easy (Promega) was performed following the manufacturer’s protocols. Sequencing from the pGEM plasmid was performed on three independent clones using primer SL3. mRNA fold prediction was done using the IDT DNA UNiAFold tool, inputting the first 70 bp of the csrR mRNA sequence by using the experimentally determined transcriptional start.

CsrA Protein purification. csrA with a His-tagged epitope was amplified from Lp02 genomic DNA using primers HY2 and HY4 and cloned into the EcoRI and HindIII sites of the pTIZ18U poly linker (Stratagene).

Gel mobility shift assay. RNA was synthesized using the MEGAScript kit (Life Technologies) and a PCR fragment containing a T7 promoter derived from primer HY1. A DNA fragment containing csrR sequence from +3 to +84 relative to the start of transcription was used as the template. Gel-purified RNA was 5’ end labeled with [γ-32P]ATP. RNA suspended in Tris-EDTA (TE) buffer was heated to 90°C for 1 min followed by slow cooling to room temperature. Binding reaction mixtures (10 μl) contained 10 mM Tris- HCl (pH 7.5), 10 mM MgCl2, 100 mM KCl, 200 ng/μl yeast RNA, 0.2 mg/ml bovine serum albumin (BSA), 7.5% glycerol, 20 mM diethiothreitol (DTT), 0.1 μM csrR RNA, CsrA-H6 (various concentrations), and 0.1 mg/ml xylene cyanol. Competition assay mixtures also contained unlabeled competitor RNA. To allow CsrA-RNA complex formation, reaction mixtures were incubated for 30 min at 37°C. Samples were then fractionated through native 15% polyacrylamide gels using 0.5X Tris-borate-EDTA (TBE) running buffer. Radioactive bands were visualized with a phosphorimager and quantified using ImageQuant 5.2 software. The apparent equilibrium binding constant (Kd) of the CsrA-csrR RNA interaction was calculated as described previously (58).

RNA footprint assay. RNA was synthesized using a MEGAScript kit and a PCR fragment containing a T7 promoter derived from primer HY3. The template was a DNA fragment containing csrR sequence extending from 32 to +84 relative to the start of transcription. Binding reactions (10 μl) containing 5 nM csrR RNA and the indicated concentrations of CsrA-H6 were otherwise identical to those in the gel shift assay. After the initial binding reaction, 0.075 U RNase T1 (Roche) was added to the reaction mixtures, and incubation continued for 15 min at 37°C. Reactions were terminated by addition of 10 μl of gel loading buffer, and reaction mixtures were placed on ice. Partial alkaline hydrolysis and RNase T1 digestion ladders of each transcript were prepared as described previously (59). After samples were fractionated through the use of standard 6% polyacrylamide sequencing gels, radioactive bands were visualized with a phosphorimager.

Construction and assays of GFP reporters. To construct the csrR-GFP BS2-only transcriptional reporter, ~300 bp immediately 5’ of the csrR coding region was amplified from Lp02 genomic DNA using primers ZA49 and ZA50, digested directly 5’ of GFPmut3 in vector pH6119, a derivative of pJB98 that encodes a promoterless GFPmut3 locus (39), and then electroporated into Lp02 to generate strain MB1375. For the csrR-GFP BS2/BS3 translational reporter, the sequence incorporated into the BS2 reporter was extended to include the first 5 codons of csrR. After PCR amplification using primers ZA49 and ZA73, the fragment was fused directly 5’ and in frame with the second codon of GFPmut3 amplified from pH6119 using primers ZA72 and ZA84 via the SOE PCR strategy using primers ZA49 and ZA84. This product was then ligated into pH6119, replacing the promoterless GFPmut3, and electroporated into Lp02 to generate strain MB1376. To construct the csrR-GFP BS2-sld/BS3 translational reporter, the stem-loop structure of BS2 predicted to favor CsrA binding was disrupted by changing the six consecutive adenines immediately 5’ of the SD sequence to thymines in the DNA sequence (resulting in uracils in the RNA). To do so, the mutation was inserted into two overlapping PCR products using pcrR-GFP_BS2/BS3 as the template, primer pair ZA49 and ZA75, and primer pair ZA74 and ZA84. The two products were then joined via SOE PCR using primers ZA49 and ZA84 to generate the BS2 mutant allele, which was then ligated into pH6119, replacing the promoterless GFPmut3, and electroporated into Lp02 to produce strain MB1377. For fluorescence experiments, colonies of MB1375 to MB1377 were cultured in broth to the E phase and then diluted to an OD600 of 0.1. Aliquots were collected at the times shown and normalized to an OD600 of 1, and their fluorescence was quantified. Strain MB355, containing pRfAA-GFP, served as a marker of the entry into the PE phase, and strain BH006 carrying pH6119, encoding GFP but no promoter, was the negative control (39). Growth levels of all strains were similar, as assessed by OD600 readings throughout the experiment (data not shown).

Inducible CsrR constructs. Wild-type csrR was amplified from Lp02 genomic DNA using primers ZA59 and ZA60 and the PCR product ligated into p206cam (60). This plasmid was electroporated into Lp02 to generate strain MB1378.

The BS3 mutant allele of csrR was constructed by SOE PCR. Two PCR products were generated. Product 1 started ~500 bp 5’ of the transcriptional start generated by forward primer ZA37 and included a GAT-to-TTG mutation in the second codon of csrR that was inserted using reverse primer ZA83. Product 2 encoded the csrR coding region, including the GAT-to-TTG mutation in the second codon that was inserted by the forward primer ZA82, and extended ~500 bp 3’ of the coding region by using reverse primer ZA38. One microliter of each of these products then served as the template in a SOE PCR using outermost primers ZA37 and ZA38. Next, this PCR product containing the BS3 mutation was used as the template to amplify a His-tagged version of only the coding region, using primers ZA59 and ZA60. Finally, the product was ligated into p206cam. This plasmid was electroporated into Lp02 to generate strain MB1379.

Rifampin and RNA stability experiment. The conditional csrA mutant strain MB464 was cultured on CYE agar supplemented with 200 μM IPTG and then inoculated into AYET medium containing 200 μM IPTG. After culture to the E phase, cells were collected by centrifugation, resuspended in AYET medium without IPTG, and then divided between two tubes that contained or lacked 200 μM rifampin. These samples were then collected for RNA isolation. After 15 min before a second aliquot was collected for RNA isolation.

qRT-PCR. cDNA was generated using 1 μl of isolated RNA in a 20-μl reaction mixture with an iScript cDNA synthesis kit from Bio-Rad following the manufacturer’s protocol. A 1:50 dilution of cDNA served as the template for qRT-PCR in 20-μl reaction mixtures using iQ SYBR green Supermix (Bio-Rad). Primers ZA53 and ZA54 were used to assess csrR mRNA levels, which were normalized to the internal control 16S rRNA amplified using primers SL1 and SL2 and threshold cycle (ΔΔCt) analysis.

Western analysis. Strains MB1378 and MB1379 were inoculated from colonies and cultured to the E phase, diluted to an OD600 of 0.05, and divided into two tubes containing or lacking 200 μM IPTG. After incubation for ~15 h to the E phase, cultures were normalized to an OD600 of 10.
by centrifugation of aliquots at 13,000 rpm for 2 min, and then cells were resuspended in 100 μl of Laemmli buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.005% bromphenol blue, 62.5 mM Tris-HCl, pH 6.8). Samples were then lysed by boiling for 5 min and debris pelleted at 13,000 rpm for 2 min. Proteins were separated on a 12% mini-Protean TGX precast gel (Bio-Rad); a Precision Plus Kaledioscope protein standard ladder (Bio-Rad) was used as a size marker and to verify transfer. To determine relative loading levels and transfer of each sample, Ponceau S staining was performed on a duplicate membrane. CarR was determined via the flagellin protein (hag) by blocking ribosome binding. Mol Microbiol 64:1605–1620. http://dx.doi.org/10.1111/j.1365-2958.2007.05765.x.

Jonas K, Edwards AN, Ahmad I, Romeo T, Römling U, Melefors O. 2010. Complex regulatory network encompassing the Csr, c-di-GMP and motility systems of Salmonella Typhimurium. Environ Microbiol 12:524–540. http://dx.doi.org/10.1111/j.1462-2920.2009.02097.x.

Matsui O, Zuzman T, Rosen M, Zuges Z, Segal G. 2014. Identification of Legionella pneumophila effectors regulated by the LetAS-RsmYZ-CsrA regulatory cascade, many of which modulate vesicular trafficking. J Bacteriol 196:681–692. http://dx.doi.org/10.1128/JB.01175-13.

Yakhnin H, Baker CS, Bereznik I, Evangelista MA, Rassin A, Romeo T, Babitzke P. 2011. CsrA represses translation of sdiA, which encodes the N-acylhomoserine-l-tactone receptor of Escherichia coli, by binding exclusively within the coding region of sdiA. J Bacteriol 193:6162–6170. http://dx.doi.org/10.1128/JB.00595-11.

Molofsky AB, Swanston MS. 2003. Legionella pneumophila CsrA is a pivotal repressor of transmission traits and activator of replication. Mol Microbiol 50:445–461. http://dx.doi.org/10.1046/j.1365-2958.2003.07306.x.

Ohno A, Kato N, Sakamoto R, Kimura S, Yamaguchi K. 2008. Temperature-dependent parasitic relationship between Legionella pneumophila and a free-living amoeba (Acanthamoeba castellanii). Appl Environ Microbiol 74:4585–4588. http://dx.doi.org/10.1128/AEM.00083-08.

Forsbach-Birk V, McNealy T, Shi C, Lynch D, Marre R. 2004. Reduced expression of the global regulator protein CsrA in Legionella pneumophila affects virulence-associated regulators and growth in Acanthamoeba castellanii. Int J Med Microbiol 294:15–25. http://dx.doi.org/10.1016/j.ijmm.2003.12.003.

Byrne B, Swanston MS. 1998. Expression of Legionella pneumophila virulence traits in response to growth conditions. Infect Immun 66:3029–3034.

Rasis M, Segal G. 2009. The LetA-RsmYZ-CsrA regulatory cascade, together with RpoS and PrmA, post-transcriptionally regulates stationary phase activation of Legionella pneumophila Lcm/Dot effectors. Mol Microbiol 72:995–1010. http://dx.doi.org/10.1111/j.1365-2958.2009.06705.x.

Edwards RL, Dalebroux ZD, Swanston MS. 2009. Legionella pneumophila couples fatty acid flux to microbial differentiation and virulence. Mol Microbiol 71:1190–1204. http://dx.doi.org/10.1111/j.1365-2958.2009.06593.x.

Sahr T, Brüggemann H, Jules M, Lomma M, Albert-Weissenberger C, Sahr T, Brüggemann H, Jules M, Lomma M, Albert-Weissenberger C, Brüggemann H, Jules M, Lomma M, Albert-Weissenberger C. 2010. Complex regulatory network encompassing the Csr, c-di-GMP and motility systems of Salmonella Typhimurium. Environ Microbiol 12:524–540. http://dx.doi.org/10.1111/j.1462-2920.2009.02097.x.

Hammber BK, Tateda ES, Swanston MS. 2002. A two-component regulator induces the transmission phenotype of stationary-phase Legionella pneumophila. Mol Microbiol 44:107–118. http://dx.doi.org/10.1046/j.1365-2958.2002.02084.x.

Rowbotham TJ. 1980. Preliminary report on the pathogenicity of Legionella pneumophila for freshwater and soil amoeba. J Clin Pathol 33:1179–1183. http://dx.doi.org/10.1136/jcp.33.12.1179.

Fields BS. 1996. The molecular ecology of legionellae. Trends Microbiol 4:286–290. http://dx.doi.org/10.1016/0966-842X(96)10041-X.

Cohn PD, Gleason JA, Rudowski E, Tsai SM, Genese CA, Fagliano JA. 2009. Temperature-dependent parasitic relationship between Legionella pneumophila and a free-living amoeba (Acanthamoeba castellanii). Appl Environ Microbiol 74:741–762. http://dx.doi.org/10.1128/AEM.00083-08.

Ozawa K, Shigemura K, Abe Y, Jikimoto T, Yoshida H, Fujisawa M, Arakawa S. 2014. A case of nosocomial Legionella pneumiaeae associated
with a contaminated hospital cooling tower. J Infect Chemother 20: 68–70. http://dx.doi.org/10.1016/j.jiac.2013.07.007.

26. Horwitz MA, Silverstein SC. 1980. Legionnaires’ disease bacterium (Legionella pneumophila) multiplesc intracellularly in human monocytes. J Clin Invest 66:441–450. http://dx.doi.org/10.1172/JCI109874.

27. Murga R, Forster TS, Brown E, Pruckler JM, Fields BS, Donlan RM. 2001. Role of biofilms in the survival of Legionella pneumophila in a model potable-water system. Microbiology 147:3121–3126.

28. Declerck P, Behets J, Margineanu A, van Hoef V, De Keersmaecker B, Olлевier F. 2009. Replication of Legionella pneumophila in biofilms of water distribution pipes. Microbiol Res 164:593–603. http://dx.doi.org/10.1016/j.mib.2007.06.001.

29. Steinitz M, Emody I, Amann R, Hacker J. 1997. Resuscitation of viable but nonculturable Legionella pneumophila Philadelphia JR32 by Acantamoeba castellanii. Appl Environ Microbiol 63:2047–2053.

30. Al-Bana BH, Haddad MT, Gardner RA. 2014. Stationary phase and mature infectious forms of Legionella pneumophila produce distinct viable but non-culturable cells. Environ Microbiol 16:382–395. http://dx.doi.org/10.1111/1462-2920.12219.

31. Gardner RA, Gardner E, Hiltz M, Hoffman PS. 2002. Intracellular growth of Legionella pneumophila gives rise to a differentiated form similar to stationary-phase forms. Infect Immun 70b:6273–6283. http://dx.doi.org/10.1128/IAI.70b.6273–6283.2002.

32. Chien M, Morozova I, Shi S, Sheng H, Chen J, Gomez SM, Asamani G, Hill K, Nuara J, Feder M, Riner J, Greenberg JJ, Shestenko V, Park SH, Zhao B, Kropf-Stephenson E, Edwards JR, Pampou S, Georgiou A, Chou IC, Iannuccilli W, Ulz ME, Kim DH, Gerrie-Sameth A, Goldbercy C, Morozov P, Fischer SG, Segal G, Qu X, Rzhefsky A, Zhang P, Cayanis E, De Jong PJ, Ju J, Kalachikov S, Shuman HA, Russo JS. 2004. The genomic sequence of the accidental pathogen Legionella pneumophila. Science 305:1966–1968. http://dx.doi.org/10.1126/science.1103049.

33. Dolánés-Jordheim A, Akermi M, Ginevra C, Cazalet C, Kay E, Schneider C, Buchrieser D, Buchrieser C, Atlan D, Vandenesch F, Etienne J, Jarraud S. 2010. The genomic sequence of Legionella pneumophila strain Paris. Microbiology 156:7369–7378. http://dx.doi.org/10.1099/mic.0.032927-0.

34. Flynn KJ, Swanson MS. 2014. Integrative conjugal element ICE-box confers oxidative stress resistance to Legionella pneumophila in vitro and in macrophages. mBio 5:e01091-14. http://dx.doi.org/10.1128/mBio.01091-14.

35. Wee BA, Woolfit M, Beatson SA, Petty NK. 2013. A distinct and divergent lineage of genomic island-associated type IV secretion systems in Legionella. PLoS One 8:e82221. http://dx.doi.org/10.1371/journal.pone.0082221.

36. Marden JN, Díaz MR, Walton WG, Gode CJ, Betts L, Urbanowski ML, Redinbo MR, Yah TL, Wolfgang MC. 2013. An unusual CSR family member operates in series with RsmA to amplify posttranscriptional regulation via a cofactor-responsive mRNA. Mol Microbiol 87:383–398. http://dx.doi.org/10.1111/mmi.12136.

37. Edwards RL, Jules M, Sahr T, Buchrieser C, Swanson MS. 2010. The Legionella pneumophila LetA/LetS two-component system exhibits rhoeost-like behavior. Infect Immun 78:2571–2583. http://dx.doi.org/10.1128/IAI.01107-09.

38. Dalebroux ZD, Yagi BF, Sahr T, Buchrieser C, Swanson MS. 2010. Distinct roles of pGpp and DksA in Legionella pneumophila differentiation. Mol Microbiol 76:220–219. http://dx.doi.org/10.1111/j.1365-2958.2010.07094.x.

39. Feeley JC, Gibson RJ, Gorman GW, Langford NC, Rasheed JK, Mackel DC, Baine WB. 1979. Charcoal-yeast extract agar: primary isolation medium for Legionella pneumophila. J Clin Microbiol 10:437–441.

40. Danskeno KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in Escherichia coli-K12 using PCR products. Proc Natl Acad Sci U S A 97:6640–6645. http://dx.doi.org/10.1073/pnas.97.12.6640.

41. Sexton JA, Vogel JP. 2004. Regulation of hypercompetence in Legionella pneumophila. J Bacteriol 186:3814–3825. http://dx.doi.org/10.1128/JB.186.12.3814-3825.2004.

42. Liu Y, Gao P, Banga S, Luo QZ. 2008. An in vivo gene deletion system for determining temporal requirement of bacterial virulence factors. Proc Natl Acad Sci U S A 105:9385–9390. http://dx.doi.org/10.1073/pnas.080105105.

43. Martinez E, Bartolomé B, de la Cruz F. 2005. The Dot/lcm transporter of Legionella pneumophila: a minimal system for Legionella pneumophila. J Bacteriol 187:3877–3886. http://dx.doi.org/10.1128/JB.187.12.3877-3886.2005.

44. Berger KH, Isberg RR. 1992. Two distinct defects in intracellular growth complemented by a single genetic locus in Legionella pneumophila. Mol Microbiol 7:19–19. http://dx.doi.org/10.1111/j.1365-2958.1993.tb01092.x.

45. Trott AB, McHeyzer-Williams MG, Pulendran B, Nossal GJ. 1992. Ligation-anchored PCR: a simple amplification technique with single-sided specificity. Proc Natl Acad Sci U S A 90:3953–3959. http://dx.doi.org/10.1073/pnas.90.8.3953.

46. Bivalacqua J, Ribeiro A. 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene 99:379–47. http://dx.doi.org/10.1016/0378-1119(91)90007-X.