RESEARCH ARTICLE

csrR, a Paralog and Direct Target of CsrA, Promotes *Legionella pneumophila* Resilience in Water

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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 (*lpg1593*) 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 *lpg1593* 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*.**

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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-

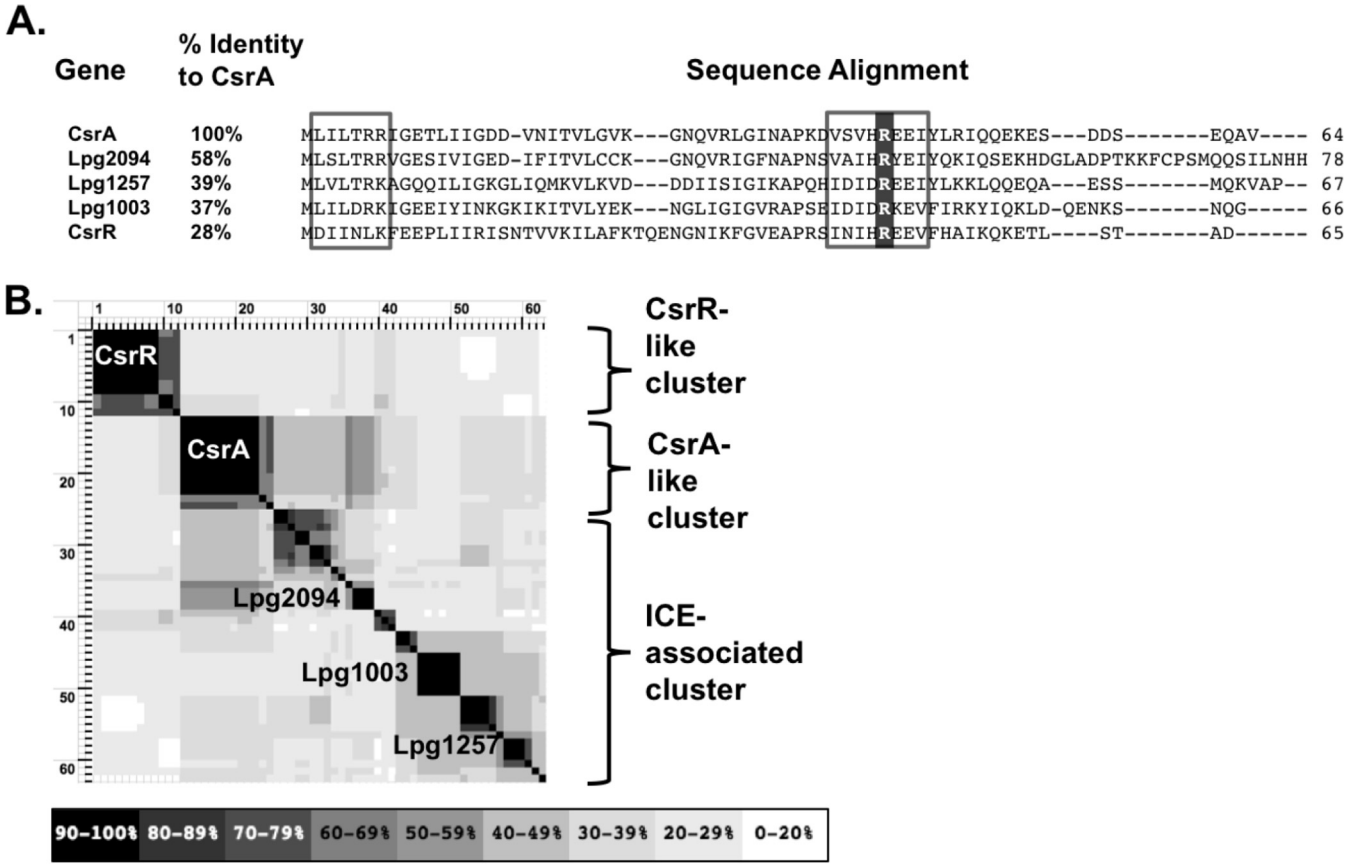

FIG 1 CsrA paralogs in 12 strains of legionellae identified *in silico*. (A) Alignment of the five CsrA-like proteins in *L. pneumophila* Philadelphia-1, with the essential arginine residue highlighted and the two RNA binding pockets outlined (1). (B) Sequence identity of the 63 CsrA-like proteins found in 12 strains of legionellae. Darker shading indicates greater percent identity. Each of the five CsrA proteins encoded by *L. pneumophila* Philadelphia-1 is marked, and clusters are named for two core and the remaining ICE-associated genes.

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 *c*arbon *s*torage *r*egulator 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 *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 (WT) *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*

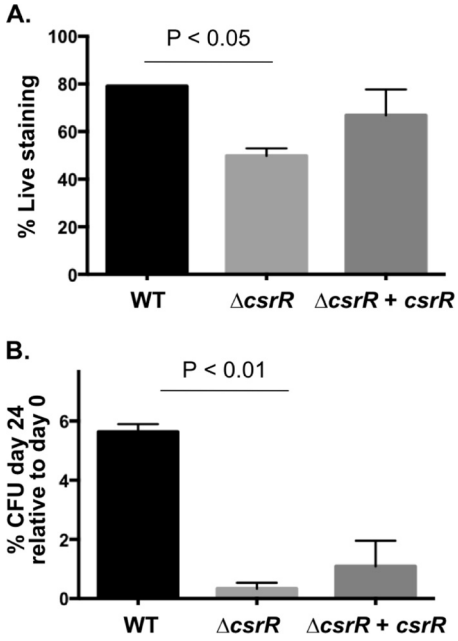

FIG 2 *csrR* enhances survival of *L. pneumophila* in water. After incubation for 24 days in 45°C tap water, survival of the wild-type strain (MB1368; black), Δ*csrR* deletion mutants (MB1370 and MB1371; light gray), and merodiploid strains (MB1373 and MB1374; dark gray) was assessed microscopically by LIVE/DEAD fluorescence staining (A) and by enumerating CFU (B). Error bars represent standard deviations calculated from the results determined for duplicate samples from two independent isolates of each genotype analyzed in parallel. Significance was calculated using a Student's *t* test. Differences reported for the wild-type and Δ*csrR* strains are representative of the results of multiple independent experiments.

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,

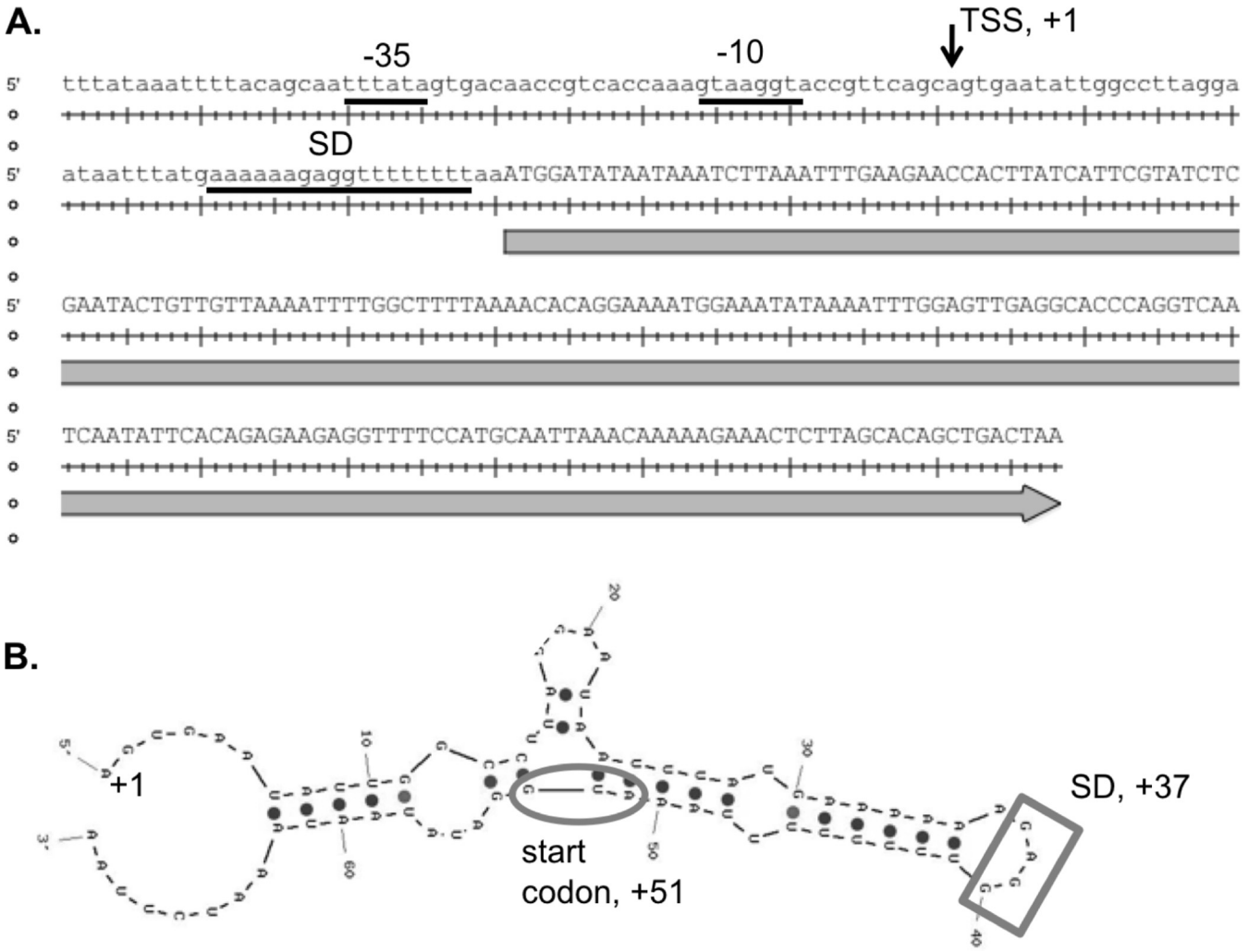

FIG 3 A putative CsrA binding site on the *csrR* RNA overlaps with a ribosome binding site motif. (A) The *csrR* transcriptional start site (TSS, +1; black arrow) was mapped by 5′ RACE. Likely −10 and −35 promoter elements are underlined and labeled; a putative Shine-Dalgarno (SD) sequence and the associated 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).

CsrA protein bound tightly to *csrR* RNA, with a calculated dissociation constant of 32 ± 9 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-

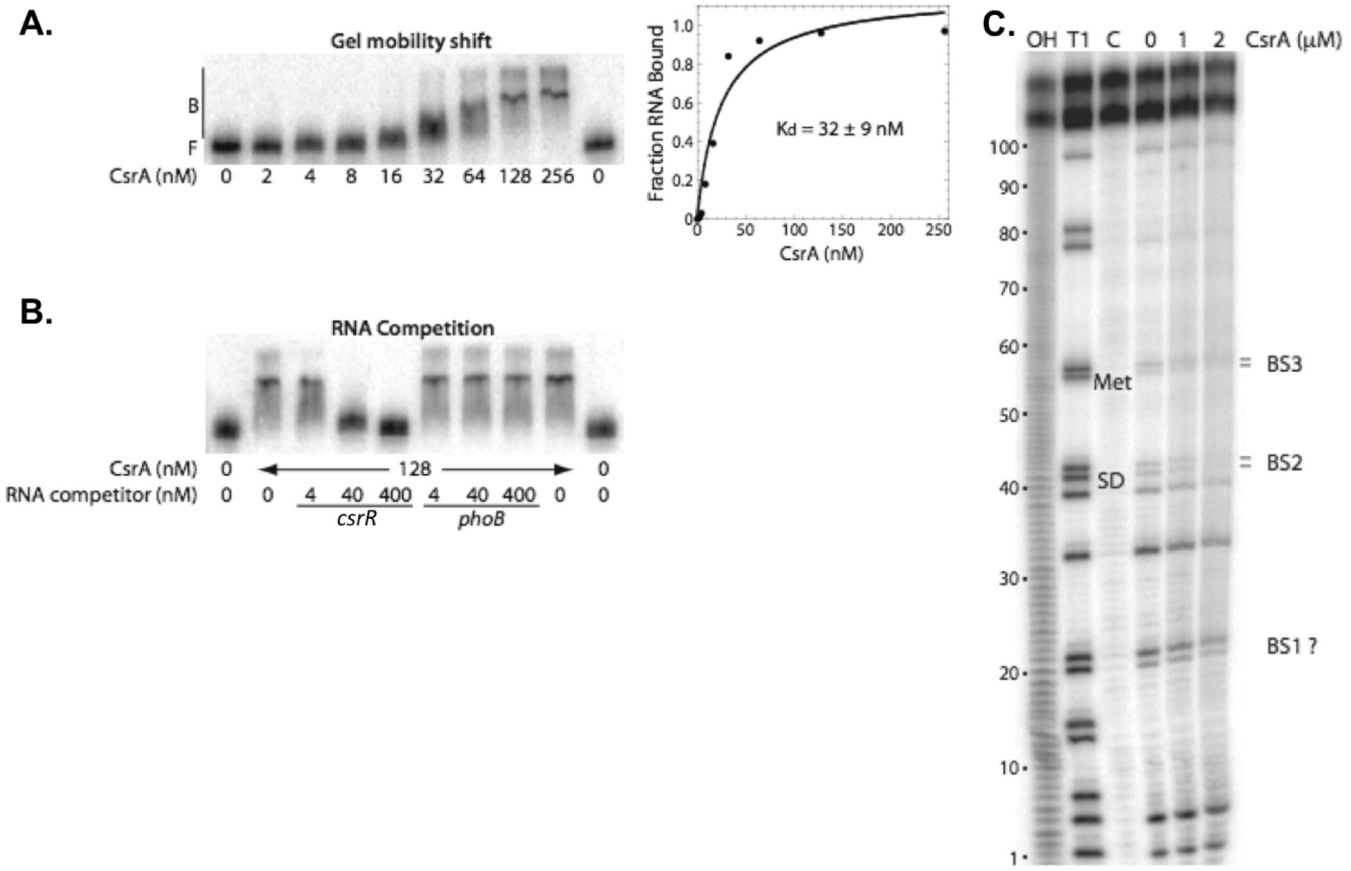

FIG 4 CsrA binds tightly and specifically to *csrR* RNA. (A) 5′-end-labeled *csrR* RNA (0.1 nM) was incubated with CsrA protein at the indicated concentration (nM), and the complexes were separated by gel electrophoresis. Positions of bound (marked B) and free (marked F) RNA species are shown. The binding 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).

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

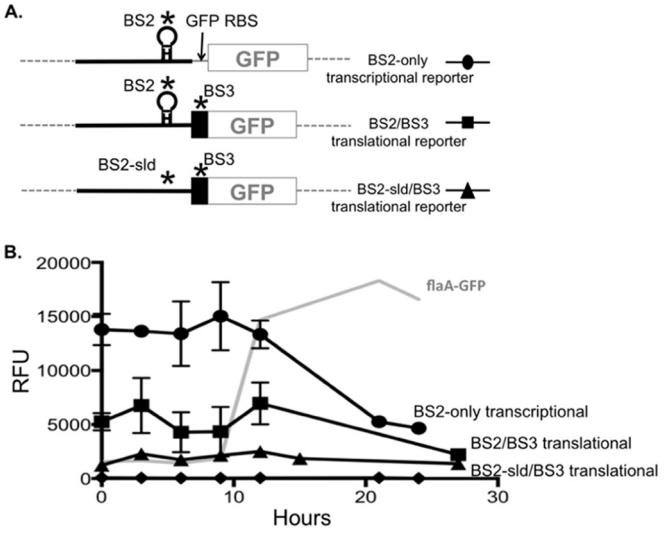

FIG 5 BS3 mediates repression of *csrR*-GFP reporters. (A) Schematics of *csrR*-GFP reporters to analyze *csrR* regulation. The BS2-only transcriptional reporter (MB1375; circles) encodes ~300 bp 5′ to the translational start sequence, including the *csrR* SD sequence, which overlaps the BS2 CsrA binding site, and the *gfp* ribosome binding site (RBS). In the BS2/BS3 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.

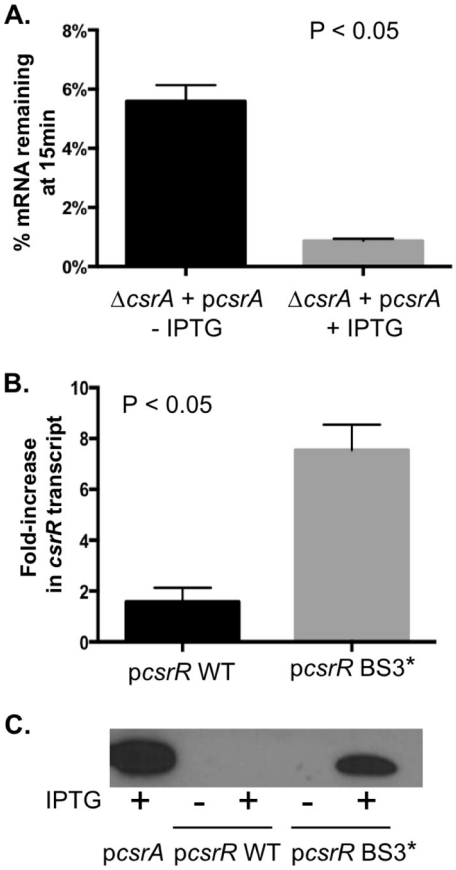

FIG 6 CsrA regulates *csrR* expression *in vivo*. (A) To analyze the impact of CsrA protein on *csrR* RNA stability, a conditional *csrA* mutant (MB464) was cultured without (black) or with (gray) IPTG to the E phase, rifampin was added to inhibit new transcription, and RNA was isolated 0 and 15 min 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.

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*, *csrR* RNA is destabilized by a mechanism that requires its BS3 binding site for the CsrA repressor protein.

Finally, we tested the prediction that, by decreasing *csrR* transcript stability, CsrA repressor binding reduces CsrR protein levels. For this purpose, we performed Western analysis on the strains that encode His-tagged CsrR on mRNA that either contained or lacked the BS3 RNA binding site for CsrA protein. CsrR protein was detected only in *L. pneumophila* that carried the *csrR* allele that lacked the BS3 binding site for CsrA (Fig. 6C). Therefore, CsrR protein expression is inhibited by the BS3 sequence positioned at the start of the *csrR* opening reading frame, most likely by direct binding of *csrR* mRNA by the CsrA repressor protein (Fig. 3 to 5).

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* here (Fig. 1; see also Table S3 in the supplemental material). Four genetic observations establish that CsrR is not redundant with CsrA. First, a *csrA* deletion mutant cannot replicate despite the presence of chromosomal *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

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 posttranscriptional 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 *csrR* genes, indicating that the BS3 motif is selected for and retained specifically, likely to conserve the CsrA-mediated repression identified here.

CsrA binds at BS3 in the coding region of the *csrR* transcript (Fig. 4), an uncommon, though not unprecedented, location (11, 12). A second binding site, BS2, was predicted bioinformatically and verified by RNA footprinting (Fig. 4). Nevertheless, in our reporter assays, BS2 was not necessary for inhibition. Instead, we observed that mutation of the bases surrounding BS2, which likely disrupt the stem-loop formation that is favorable for CsrA binding, actually decreased expression of the reporter (Fig. 5), indicating that BS2 (or at least the stem-loop) may have a stabilizing effect. Alternatively, disruption of the stem-loop may inhibit GFP expression by a CsrA-independent mechanism or it may enhance rather than disrupt binding by CsrA. More-detailed biochemical studies can investigate whether CsrA alternately stabilizes or destabilizes the *csrR* transcript depending on whether it binds at BS2 or BS3, respectively. By analogy to the *E. coli* molybdenum cofactor system (44), perhaps a conditional riboswitch governs CsrA binding site selection to ensure *csrR* expression during prolonged starvation.

At the amino acid level, CsrR is only 28% identical to canonical CsrA. Despite significant genetic drift, CsrR retains many of the residues necessary for RNA binding by *E. coli* CsrA (1) (Fig. 1; see also Table S2 in the supplemental material). Likewise, among the homologues of CsrA encoded by many different species of bacteria, the RNA binding pockets are largely conserved, whereas the rest of the coding region varies considerably (1), as do the targets of regulation. For example, CsrA proteins repress motility of *B. subtilis* by binding directly to the flagellar subunit mRNA (*hag*) but induce motility of *E. coli* by binding to and stabilizing mRNA of the flagellar regulator *flhDC* (45–47). Evidently, *L. pneumophila* CsrR is under selective pressure to retain RNA binding function. The significant drift in the residues outside the two binding pockets defined by Mercante et al. (1) likely confers CsrR specificity for a distinct regulon.

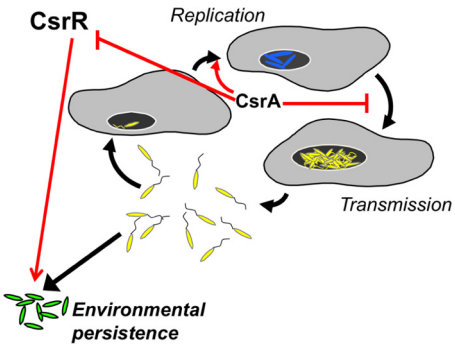

FIG 7 Model of CsrA and CsrR regulation of the *L. pneumophila* life cycle. Within host cells, CsrA induces transition into the replicative phase (blue bacteria) and represses both transmissive traits and *csrR* translation. When nutrients become limiting, CsrA repression is relieved, transcription of *csrR* declines, and *L. pneumophila* differentiates to the transmissive phase (yellow 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

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 cysteine (Fisher), and 135 μg/ml ferric nitrate (J. T. Baker) (51). Thymidine was omitted when culturing 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 (OD_{600}) of 0.05 to 0.2 and then cultured to the E phase (OD_{600} of 1.0 to 2.0) or PE phase (OD_{600} 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 overhang 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 restriction site of pZL790 (54) to create vector pZL790-MCS. Next, a kanamycin resistance cassette flanked by FLP recombination target (FRT) sites was amplified from pKD4 (52) with primers Sc3 and Sc4 and cloned into the SacI 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 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 a 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 MB110, 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, MB1373, and MB1374 were analyzed to avoid thymidineless 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 OD_{600} 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 in 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.

RNA 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 UNAFold 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 ZA33 and ZA34, ligated into plasmid p206gent, and transformed into *E. coli* Rosetta(DE3) pLysS. The strain was cultured in Terrific Broth media containing 20 μg/ml chloramphenicol and 10 μg/ml gentamicin at 37°C to an OD_{600} of ~0.5, supplemented with 500 μM IPTG, and cultured for 16 h at 22°C. Cells were then harvested by centrifugation and flash frozen with liquid N_2. Protein was purified using a 5-ml Hi-Trap metal affinity column (GE Healthcare) following the manufacturer's instructions. Protein was loaded in a His buffer (25 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole) and eluted using an imidazole gradient of 20 to 300 mM. Protein-containing fractions were then diluted 1:6 in the elution buffer (due to low solubility) and dialyzed overnight at 4°C in freezing buffer (10 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 100 mM KCl, 10% glycerol).

pYH215 construction. A *csrR* fragment (−168 to +103) was amplified from Lp02 genomic DNA using primers HY2 and HY4 and cloned into the EcoRI and HindIII sites of the pTZ18U polylinker (Stratagene).

Gel mobility shift assay. RNA was synthesized using a 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-^{32}P]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 $MgCl_2$, 100 mM KCl, 200 ng/μl yeast RNA, 0.2 mg/ml bovine serum albumin (BSA), 7.5% glycerol, 20 mM dithiothreitol (DTT), 0.1 nM *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 0.5× 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 (K_d) of 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, ligated directly 5′ of GFPmut3 in vector pBH6119, 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 pBH6119 using primers ZA72 and ZA84 via the SOE PCR strategy using primers ZA49 and ZA84. This product was then ligated into pBH6119, 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 thymidines in the DNA sequence (resulting in uracils in the RNA). To do so, the mutation was inserted into two overlapping PCR products using p*csrR*-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 pBH6119, 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 OD_{600} of 0.1. Aliquots were collected at the times shown and normalized to an OD_{600} of 1, and their fluorescence was quantified. Strain MB355, containing p*flaA*-GFP, served as a marker of the entry into the PE phase, and strain BH006 carrying pBH6119, encoding GFP but no promoter, was the negative control (39). Growth levels of all strains were similar, as assessed by OD_{600} 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, included 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 IPTG. These samples were then cultured to an OD_{600} of ~1 and aliquots collected for RNA isolation. Next, 100 μg/ml rifampin was added and cultures were incubated for 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 ($\Delta\Delta C_T$) analysis.

Western analysis. Strains MB1378 and MB1379 were inoculated from colonies and cultured to the E phase, diluted to an OD_{600} 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 OD_{600} 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% bromophenol 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 Kaleidoscope 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 (Fisher) staining was performed on a duplicate membrane. CsrR was detected via the 6×His epitope that was inserted in frame at its carboxy terminus using a 1:5,000 dilution of anti-His (C-terminal)-horseradish peroxidase (HRP) antibody (Novex; Life Technologies) and SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). A His-tagged derivative of CsrA paralog Lpg2094 was the positive control.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00595-15/-/DCSupplemental.

Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.1 MB.
Table S4, DOCX file, 0.1 MB.

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