

The LetE Protein Enhances Expression of Multiple LetA/LetS-Dependent Transmission Traits by *Legionella pneumophila*

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Legionella pneumophila colonizes freshwater amoebae and can also replicate within alveolar macrophages. When their nutrient supply is exhausted, replicating bacteria become cytotoxic, motile, and infectious, which is thought to promote transmission to a new amoeba. The differentiation of *L. pneumophila* is coordinated by the sigma factors RpoS and FliA and the two-component regulator LetA/LetS and is enhanced by the *letE* locus. Here we demonstrate that *letE* promotes motility by increasing expression of the flagellin gene *flaA* but has little impact on the transcription of *fliA*, the flagellar sigma factor gene. In addition to promoting motility, *letE* induces the characteristic shape, pigment, and heat resistance of stationary-phase *L. pneumophila*. To gain insight into how *letE* promotes the expression of the transmission phenotype, we designed molecular genetic experiments to discriminate between the following three models: *letE* mutations are polar on *milX*; *letE* encodes a small novel protein; or, by analogy to *csrB*, *letE* encodes a regulatory RNA that sequesters CsrA to relieve repression. We report that *letE* encodes an activator protein, as it does not complement an *Escherichia coli* *csrB* mutant, it directs the synthesis of an ~12-kDa polypeptide, and a *letE* nonsense mutation eliminates function. A monocistronic *letE* RNA is abundant during the exponential phase, and its decay during the stationary phase requires RpoS and LetA/LetS. We also discuss how the LetE protein may interact with LetA/LetS and CsrA to enhance *L. pneumophila* differentiation to a transmissible form.

Normally found in fresh water as a parasite of amoebae, *Legionella pneumophila* can also infect human alveolar macrophages and cause a severe pneumonia, Legionnaires' disease. As one approach to identifying virulence factors that are required for phagocyte infection, analyses of the regulatory circuit that controls the differentiation of replicating *L. pneumophila* to a transmissible form have been conducted. From genetic screens, isogenic mutant analyses, and overexpression studies of *L. pneumophila* cultured in broth or in macrophages, the following model for the genetic control of its life cycle has been constructed. By a stringent response-like mechanism, replicating *L. pneumophila* organisms respond to low amino acid levels by synthesizing the second messenger (p)ppGpp (17). In response to this or some other signal (40), the sigma factors RpoS and FliA induce the transcription of genes of the transmission regulon (4, 5, 14, 23), while the two-component regulator LetA/LetS and the *letE* locus cooperate to overcome posttranscriptional repression by CsrA (14, 18, 29, 31). As a result, replicating bacteria within phagocyte vacuoles can respond to stress, including amino acid starvation, by rapidly converting to a resilient cytotoxic, motile, and infectious form.

Genetic and biochemical studies of a variety of gram-negative bacteria support a paradigm in which the LetA/LetS family of two-component regulators activates gene expression indirectly by counteracting the repressor of translation known as CsrA or RsmA (reviewed in references 21 and 32). In *Esche-*

richia coli, CsrA binds to particular mRNAs at a consensus sequence encompassing the ribosome binding site (RBS), destabilizing the mRNAs and preventing their translation (6). CsrA is antagonized by an untranslated regulatory RNA, known as *csrB* or *rsmB*, which contains multiple repeats of a ribosome binding site-like sequence that presumably bind CsrA and sequester it from mRNAs (32). LetA/LetS orthologues activate *csrB* expression, thereby promoting the translation of transcripts bound by CsrA (1, 20). For example, the overproduction of *csrB* by *Salmonella enterica* serovar Typhimurium bypasses its requirement for the LetA orthologue BarA, as judged by its more efficient invasion of cultured epithelial cells (2).

This broadly conserved mechanism of posttranscriptional regulation also appears to govern the cellular differentiation of *L. pneumophila*. When conditions are favorable for replication, a CsrA homologue of *L. pneumophila* represses transmission traits (14, 31). When conditions deteriorate, LetA/LetS functions to relieve CsrA repression, since the expression by *letA* mutants of an entire panel of transmission phenotypes is restored when CsrA is genetically inactivated (31). Accordingly, *L. pneumophila* LetA/LetS is predicted to induce the expression of a *csrB* regulatory RNA that alleviates CsrA binding to mRNAs, but the putative *csrB* homologue has not been identified.

The magnitude of the LetA/LetS induction of *L. pneumophila* macrophage infection, flagellar motility, and cytotoxicity is enhanced ~50% by the *letE* locus by a mechanism that has not been defined (18). Defined by four transposon insertions, the ~0.4-kb *letE* locus (GenBank accession no. AY135376) is not predicted to encode a protein according to the GLIMMER algorithm (Columbia Genome Center Legionella Genome Proj-

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ect [<http://genome3.cpmc.columbia.edu/~legion>]). The LaserGene Map Draw program identifies a 372-bp open reading frame (ORF), but its predicted 123-amino-acid polypeptide lacks significant homology to known proteins (18). Thus, *letE* mutant phenotypes may be caused by a disruption of this coding sequence, as the *letE-81*, *-108*, and *-80* mutations map within the predicted ORF and the fourth insertion mutation (*letE-121*) lies upstream, perhaps in its promoter. Alternatively, *letE* transposon insertions may be polar on *milX*, which is a locus ~1 kb downstream that is required for intracellular replication (J. D. Sauer and M. Swanson, unpublished data) and is predicted to encode a membrane transporter protein similar to MilA (32% amino acid identity and 51% similarity), a factor that also contributes in some way to the intracellular replication of *L. pneumophila* (18, 19). The available phenotypic data are also consistent with a third model stating that *letE* encodes a regulatory RNA analogous to *csrB* (18).

To ascertain whether the *letE* locus enhances *L. pneumophila* post-exponential-phase (PE-phase) gene expression by encoding a regulatory RNA, by acting in *cis* on *milX*, or by encoding an activator protein, we performed a series of genetic and molecular tests. The transcription of two genes of the flagellar cascade by wild-type and *letE* mutant cells was also analyzed. The data demonstrate that the LetE protein enhances multiple traits that are characteristic of stationary-phase *L. pneumophila*, including a coccoid shape, pigmentation, motility, heat resistance, and the efficient infection of macrophages. We will also discuss how LetE and LetA/LetS may cooperate to counteract posttranscriptional repression by CsrA.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. *L. pneumophila* Lp02, a virulent thymine auxotroph (7), and MB419 (18), a *letE-121* mutant, were cultured in *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES; Sigma)-buffered yeast extract broth (AYE) supplemented with 100 μ g of thymidine per ml at 37°C to the exponential (E; optical density at 600 nm [OD₆₀₀] of 0.8 to 1.2) or the PE (OD₆₀₀ of 3.1 to 3.6) phase. Similar results were obtained for at least one other *letE* allele in assays of motility, cytotoxicity, infectivity, and growth in macrophages (data not shown). Bacteria were plated on ACES-buffered charcoal-yeast extract-agar supplemented with 100 μ g of thymidine per ml (CYET). When indicated, gentamicin was added to a final concentration of 10 μ g per ml. *E. coli* strains MG1655, TR1-5 MG1655 (*csrA::kanR*), and RG1-B MG1655 (*csrB::cam*) were grown on Kornberg medium (15).

Plasmids encoding inducible *letE* and *milX* genes were constructed as follows. The 3.6-kb *letE-milX* locus was amplified by a PCR using primers LetE1 and LetE2 (18). The PCR fragment was purified (Qiaquick PCR purification kit; Qiagen) and ligated into pGEM-T Easy (Promega). The resulting plasmid, pMB436, was digested with EcoRI, producing a 1-kb fragment containing *letE* and a 2.6-kb fragment containing *milX*. These fragments were gel purified (Qiaquick gel purification kit; Qiagen) and ligated into pMMB-Gent Δ mob digested with EcoRI and treated with shrimp alkaline phosphatase (Roche). The resulting plasmids were screened for the presence and orientation of the insert. The plasmids pLetE and pMilX contained the genes of interest and any upstream, native promoter sequences colinear with the pTac promoter of pMMB-Gent Δ mobA.

Bacterial cell morphology. Phase-contrast images of *L. pneumophila* were captured at a \times 1,000 magnification with a Zeiss Axioplan 2 microscope and a Spot digital camera. To measure the cells, we analyzed the images with the NIH Image software program (<http://rsb.info.nih.gov/ni-image/>). Images were processed for a reduction of the background, converted to threshold mode, and measured with the Wand auto-measure function. For each image, all single bacteria were selected manually and the major axis of the bacterial rods was calculated by the software. To minimize errors in calculating bacterial lengths, we omitted clusters of two or more cells from the analysis.

Pigment production. Pigment accumulation was measured as described previously (40). The density of overnight E-phase broth cultures was quantified by measuring the OD₆₀₀, and then aliquots were diluted to an OD of ~0.1 and

cultured overnight to an OD of ~2.5. At 4-h intervals, samples were removed and centrifuged for 10 min at 16,000 \times g; supernatants were transferred to cuvettes, and cell pellets were resuspended in the same volume of phosphate-buffered saline (PBS). Pigment accumulation in the supernatants was quantified by measuring the OD₅₅₀, and the bacterial density was determined by measuring the OD₆₀₀ of a 1:10 dilution of the cell suspension. To determine viability at the last time point, we diluted the bacterial suspensions in PBS and plated them on CYET with gentamicin.

Stress resistance. The bacterial viability after a 1-h incubation under either low-pH (citric acid, pH 3), hyperosmolar (5 M NaCl), oxidative (10 mM H₂O₂), or high-heat (57°C) conditions was measured as previously described (16, 17).

Infectivity. Infectivity, a measure of the number of viable adherent and intracellular bacteria, was measured as previously described (10), with slight modifications. Briefly, PE-phase bacteria were diluted in RPMI plus 10% fetal bovine serum (Invitrogen), and samples of each inoculum were plated for counts of CFU. For each bacterial strain, triplicate monolayers of 2.5 \times 10⁵ murine bone marrow-derived macrophages were infected with ~5 \times 10⁴ bacteria for 2 h at 37°C. For the removal of the majority of the extracellular bacteria, the monolayers were then washed with RPMI prior to being lysed by trituration with cold PBS, diluted in AYE, and plated on CYET for counts of bacterial CFU.

Complementation of *E. coli* glycogen accumulation. *E. coli* strains MG1655, TR1-5 MG1655 (*csrA::kanR*), and RG1-B MG1655 (*csrB::cam*) were transformed by electroporation with pLetE or the control vector pMMB-Gent Δ mob. Transformants were isolated, colony purified, and cultured overnight on Kornberg medium containing 200 μ M IPTG (isopropyl- β -D-thiogalactopyranoside). Intracellular glycogen accumulation was visualized with iodine vapor by inverting plates over iodine crystals (15).

Nucleotide sequence analysis. The RNA secondary structure was predicted with both Lasergene Genequest, using Vienna RNA folding parameters with default settings, and mfold (<http://bioinfo.rpi.edu/applications/mfold/>), using the following parameters: window, 7; maximum folds, 50; maximum base pairs, 20 (30, 39). Protein folding was predicted by using the predict protein server (34). The prediction of transmembrane domains was done by using the dense alignment surface method (11) and a Kyte-Doolittle hydrophobicity plot (25).

Site-directed mutagenesis. Single nucleotide substitutions were introduced into the *letE* ORF in pLetE by use of a QuikChange XL site-directed mutagenesis kit (Stratagene). The plasmid pLetE_{STOP} was constructed by switching codon 7 from a leucine-encoding codon to a stop codon by changing TTA to TAA with primers LetE UAA21A and LetE UAA21B. The mutagenesis control plasmid pLetE_{SIL} was made by altering codon 7 from TTA to CTA, a trinucleotide that also encodes leucine, with primers LetE SilA and LetE SilB (Table 1). Plasmid DNA synthesis, the digestion of template DNA, and transformation were performed as directed by the manufacturer. The fidelity of mutagenesis was confirmed by DNA sequencing of the *letE* locus with primers LetETn1 and LetETn2 (University of Michigan Sequencing Core Facility).

Northern blot analysis. The expression of *letE* RNA by wild-type strain Lp02 was compared to that of the following mutants: *letA-22*, *letS-36*, *letE-121* (18), and *spoS* null (5). For each strain, the densities of overnight cultures were quantified by measuring the OD₆₀₀, and then aliquots were diluted to an OD₆₀₀ of 0.3 or 0.03 and incubated for an additional 16 h to produce PE- and E-phase cultures, respectively. Bacteria from 4.5 ml of E-phase cultures and 1.5 ml of PE-phase cultures were collected by centrifugation and resuspended in 1 ml of Trizol reagent, and then the RNAs were purified according to the manufacturer's instructions (Invitrogen). RNA samples were quantified by measuring the OD₂₆₀ and then samples of 10 μ g of total RNA were electrophoresed in a 1.2% formaldehyde gel. The gel was prepared, electrophoresed, and transferred to BrightStar BioDetect nylon membranes as described for NorthernMax kits (Ambion, Inc.). Biotin-labeled, single-stranded DNA probes were synthesized with the Strip EZ PCR kit (Ambion, Inc.). The probe template was synthesized by a PCR using primers LetETn1 and LetETn2, which are specific for the region of *letE* transposon insertions (Table 1). Ten nanograms of the resulting fragment was used as a template for a linear PCR using LetETn2 and 200 nM biotin 14-ATP (Invitrogen). Membranes were hybridized by using NorthernMax Ultra-hyb and the *letE* probe at a final concentration of ~0.7 ng/ml. Probes were quantified and the *letE* mRNA was subsequently detected by using a BrightStar BioDetect kit (Ambion, Inc.) and Kodak ML chemiluminescence film. The size of the *letE* transcript was determined by comparing the migration of the major *letE* band with a gel mobility plot generated from RNA molecular weight markers (RNA molecular weight marker III; Roche Molecular Biochemicals). Probes were stripped by incubating the blot in probe degradation buffer for 10 min at room temperature and then for 10 min in a 68°C water bath, and finally they were washed in blot reconstitution buffer for 10 min at 68°C (Strip EZ PCR kit; Ambion, Inc.). The analysis of *fliA* and *flaA* expression in wild-type and *letE-121*

TABLE 1. Strains, primers, and plasmids used for this study

Strain, primer, or plasmid	Relevant characteristics	Reference or source
Strains		
<i>E. coli</i>		
MG1655		32
TR1-5 MG1655	<i>csrA::kanR</i>	15
RG1-B MG1655	<i>csrB::cam</i>	5
JM109	F' <i>traD36 proA⁺ proB⁺ lacI^q lacZΔM15/recA1 endA1 gyrA96 (Nal^r) thi hsdR17 supE44 relA1 Δ(lac-proAB) mcrA</i>	Lab collection
<i>L. pneumophila</i>		
Lp02	Str ^r Thy ⁻ HsdR ⁻	7
MB 379	Lp02 <i>rpoS::kan</i>	5
MB 414	Lp02 <i>letA-22</i>	18
MB 417	Lp02 <i>letS-36</i>	18
MB 419	Lp02 <i>letE-121</i>	18
MB 447	Lp02 <i>letE-121</i> containing pMMB-GentΔmob	This work
MB 448	Lp02 <i>letE-121</i> containing pLetE	This work
MB 449	Lp02 <i>letE-121</i> containing pLetE _{STOP}	This work
MB 450	Lp02 <i>letE-121</i> containing pLetE _{SIL}	This work
MB 451	Lp02 containing pMMB-GentΔmob	This work
MB 452	Lp02 containing pLetE	This work
Primers		
LetE1	5'-ATG GAA GGT TGG TTA ATG GTT GAA-3'	18
LetE2	5'-TTC CCA TGC CAT AAT ATC CAC CTA-3'	18
LetE UAA21A	5'-CAT CAT GAA CGA TAC AAC ATC ATA ATT ATC ACA TCT TAG ATT ACG-3'	This work
LetE UAA21B	5'-CGT AAT CTA AGA TGT GAT AAT TAT GAT GTT GTA TCG TTC ATG ATG-3'	This work
LetE SilA	5'-CAT CAT GAA CGA TAC AAC ATC ACT ATT ATC ACA TCT TAG ATT ACG-3'	This work
LetE SilB	5'-CGT AAT CTA AGA TGT GAT AAT AGT GAT GTT GTA TCG TTC ATG ATG-3'	This work
LetETn1	5'-TAC ATG CAC TAA AAA GCG GTT CTG-3'	This work
LetETn2 ^a	5'-TGA TCA TGC TAC GAG CTC AAG TAA-3'	This work
MilX ORF1	5'-AGG CCC CTG TTT CAT TTT TAG TGA-3'	This work
MilX ORF2 ^a	5'-ATA TGC CGG ACC TAT TGC CTC TC-3'	This work
Plasmids		
pGEMT-Easy	Multiple cloning site within coding region of β-lactamase α-fragment linearized with single-T overhangs, Amp ^r	Promega
pMB436	pGEMT-Easy containing 3.6-kb <i>letE-milX</i> locus PCR fragment	This work
pMMB-GentΔmob	pMMB67EH with fragment containing Gm ^r , digested with AgeI and ligated to remove <i>mobA</i> ATG site	17
pLetE	pMMB-GentΔmob containing the <i>letE</i> locus in a 1 kb EcoRI fragment	This work
pMilX	pMMB-GentΔmob containing the <i>milX</i> locus in a 2.6-kb EcoRI fragment	This work
pLetE _{STOP}	pLetE with a TTA→TAA point mutation in <i>letE</i> codon 7, creating a stop codon	This work
pLetE _{SIL}	pLetE with a TTA→CTA point mutation in <i>letE</i> codon 7, creating a silent mutation (both encode leucine)	This work

^a Used for synthesis of single-stranded DNA probe.

strains was performed as previously described (4). Samples for time course experiments were collected as previously described (4) and processed as described above.

Protein analysis. To analyze *letE*-dependent protein synthesis, we prepared total cell extracts from three *L. pneumophila* strains: wild-type Lp02 transformed with vector pMMB-GentΔ*mobA* (MB451), the *letE-121* mutant transformed with vector pMMB-GentΔ*mobA* (MB447), and the *letE-121* mutant transformed with the complementing plasmid pLetE (MB448). E-phase broth cultures were diluted into AYE containing 2 mM IPTG and then were cultured to the PE phase (OD₆₀₀ of 3.6 to 3.8). Aliquots of 3 × 10⁹ bacteria were collected by centrifugation and then stored at -20°C until they were used. For the preparation of protein extracts, samples were resuspended in 30 μl of water, diluted with Tricine sample buffer (Bio-Rad) (final concentrations of 200 mM Tris-HCl [pH 6.8], 40% glycerol, 2% sodium dodecyl sulfate [SDS], 0.04% Coomassie blue G-250, and 2% β-mercaptoethanol), heated for 3 min in a boiling water bath, and then cleared by centrifugation. The equivalent of 3 × 10⁷ bacteria per lane was separated in a precast Tris-Tricine-16.5% acrylamide gel (Bio-Rad). The molecular weight of the *letE* product was estimated by comparing its migration to that of the Benchmark prestained protein size standard (Bio-Rad). After fixation for 30 min in 40% methanol and 10% acetic acid, the proteins were stained with a Coomassie blue G-250 solution (Bio-Rad).

RESULTS

***letE* enhances *flaA* expression in PE phase.** To construct a flagellum as it enters the PE phase, *L. pneumophila* expresses *flaA*, encoding the flagellar sigma factor, and then *flaA*, encod-

ing flagellin (14, 22). Consistent with their reciprocal control of the transmission phenotype, LetA/LetS and RpoS induce the expression of both *fliA* and *flaA* RNAs (4, 5, 18, 29), whereas CsrA represses *L. pneumophila* motility by decreasing the quantities of both transcripts (14, 31). The *letE* locus was originally identified in a genetic screen as a positive regulator of *flaA*: *letE* mutants carrying a *flaA-gfp* transcriptional fusion formed colonies that were a lighter green than those of the corresponding wild-type strain (18).

To investigate the mechanism of regulation by *letE*, we evaluated the expression levels of *fliA* and *flaA* in wild-type and *letE* mutant bacteria by Northern blot analysis. In wild-type *L. pneumophila*, the *fliA* transcript of ~750 nucleotides was in good agreement with the predicted 716-bp ORF (GenBank accession no. X98892); the origin of the smaller ~200-nucleotide species is not known. Wild-type cells expressed a *flaA* RNA of ~1,500 nucleotides, of sufficient length to encode the 1,428-bp ORF (GenBank accession no. X83232) (Fig. 1). Relative to PE-phase wild-type cultures, *letE* mutants contained slightly less *fliA* RNA. In comparison, the effect of *letE* mutations on *flaA* expression was more severe: *flaA* transcripts were abundant in wild-type PE-phase cultures but scarce in *letE* mutant cells (Fig. 1). Therefore, the primary effect of *letE* on

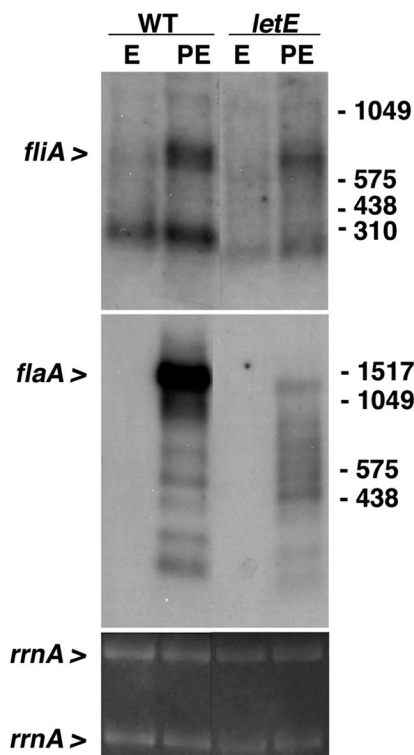


FIG. 1. LetE enhances *flaA* expression in PE phase. A Northern blot analysis was performed with 10 μ g each of total RNAs collected from wild-type strain Lp02 and the *letE-121* mutant strain MB419 cultured to the E (OD_{600} of 0.8 to 1.2) and PE (OD_{600} of 3.1 to 3.6) phase. The hybridization pattern shown is representative of results obtained from two independent sets of RNA samples. Equal loading of the samples is demonstrated by ethidium bromide staining of *rrnA* in the agarose gel prior to transfer. An expanded version of this figure appears in a separate article (4).

L. pneumophila motility appears to enhance either the production or stability of *flaA* RNA.

A *letE* mutant is defective for coccoid cell shape, motility, and pigmentation. To gain insight into the full scope of traits regulated by *letE*, we compared the morphologies of a *letE* mutant and wild-type *L. pneumophila*. The *letE-121* mutant strain MB419 (18) and the wild-type strain Lp02 were cultured in broth to PE phase, and then wet mounts of each culture were observed by phase-contrast microscopy. Unlike wild-type cells, the mutant cells retained the elongated shape that is more typical of the replicative form of *L. pneumophila* (14, 17, 31), often appearing as doublets (Fig. 2A versus B). Furthermore, <10% of the *letE-121* cells were motile, and these bacteria tumbled in a manner unlike the directed movement of wild-type cells (Table 2; data not shown). The failure of PE-phase *letE* mutants to differentiate into a coccoid, motile form is characteristic of *L. pneumophila* cells that either lack the LetA activator or carry multiple copies of the wild-type locus encoding the CsrA repressor (14, 31).

L. pneumophila produces a brown pigment that accumulates in the broth of PE-phase cultures (13); this pathway is also induced by LetA and repressed by CsrA (14, 31). To investigate whether *letE* also regulates pigment production, we collected samples of broth cultures at the late E phase and at 4-h

intervals thereafter and then assayed them for pigment accumulation (OD_{550}) and optical density (OD_{600}) (Fig. 3). Pigment accumulation in both wild-type and *letE* cultures began when the cultures reached an OD_{600} of ~ 4 . However, *letE* cells produced 50% less pigment than wild-type cells (Fig. 3A; compare open diamonds with open triangles). During the course of the experiment, we also observed that the OD_{600} of *letE* mutants dropped to ~ 2.5 (Fig. 3B). The decreased OD_{600} of *letE* cultures did not indicate cell lysis, as no corresponding change in CFU occurred (data not shown). Consistently, the decline in OD_{600} coincided with the onset of pigment production, but whether this reflects a defect in either pigment production or secretion is not known. The phenotypic data indicate that *letE* induces the characteristic shape, motility, and pigmentation of PE-phase *L. pneumophila*, all of which are induced by LetA and repressed by CsrA (14, 18, 31).

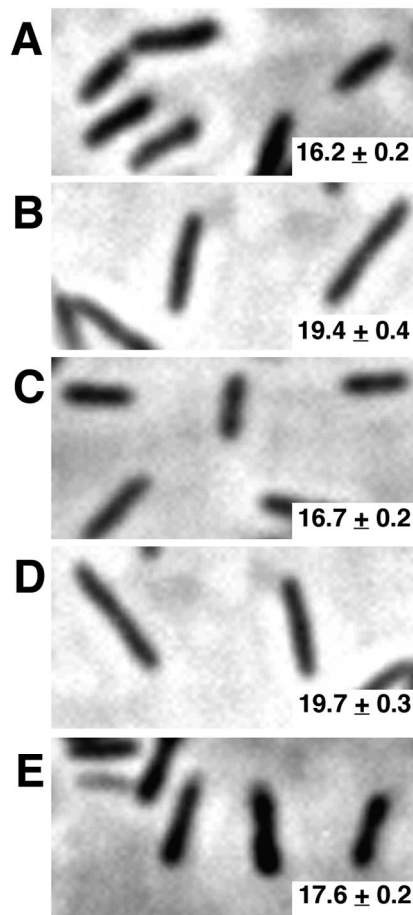


FIG. 2. *letE* is required for coccoid cell morphology in PE phase. Phase-contrast images of PE-phase *L. pneumophila* cells magnified by a factor of 1,000 are shown. (A) Coccoid wild-type Lp02 containing pMMB-Gent Δ mob (MB451); (B) elongated *letE* mutants containing pMMB-Gent Δ mob (MB447); (C) *letE* mutants containing pLetE (MB448) and resembling the wild type; (D) *letE* mutants containing pLetE_{STOP} (MB449) (elongated, resembling the *letE* mutants in panel B); (E) *letE* mutants containing pLetE_{SIL} (MB450) and resembling the wild type. Cell lengths are shown as mean numbers of pixels \pm standard errors. For each strain, at least 275 bacteria were analyzed. The lengths shown are representative of two independent experiments.

TABLE 2. Summary of complementation studies of PE-phase *letE* mutants^a

Strain	Presence of phenotype			
	Motility ^b	Coccoid ^c	Pigment ^d	Sodium sensitivity ^e
WT/vector	+	+	+	+
<i>letE</i> /vector	-	-	-	-
<i>letE</i> /pLetE	+	+	+	-
<i>letE</i> /pLetE _{SIL}	+	+	+	ND
<i>letE</i> /pLetE _{STOP}	-	-	-	ND
<i>letE</i> /pMilX	-	-	ND	-
WT/p-CsrA ^f	-	-	-	+

^a Phenotypes were scored relative to a wild-type PE-phase positive control (+) and a *letE* PE-phase negative control (-). All assays were performed at least twice, with similar results.

^b Cultures in which > 50% of the bacteria exhibited directed movement when observed by phase-contrast microscopy were scored as positive; negative cultures were typically 5 to 10% motile.

^c For the scoring of morphology, phase-contrast images of samples were compared to the positive (Fig. 2A) and negative (Fig. 2B) reference strains.

^d Cultures whose supernatants had an OD₅₅₀ of >0.4 were scored as positive (Fig. 3B); negative cultures typically had an OD₅₅₀ of ~0.2. ND, not done.

^e Calculated by the formula (CFU/ml on CYET plus 100 mM NaCl)/(CFU/ml on CYET) × 100. The plating efficiency of positive control samples was <0.3% and that of negative control cultures was >30%. ND, not done.

^f Phenotypic data for *L. pneumophila* cells containing multiple copies of the wild-type *csrA* locus (WT/p-CsrA [14, 31]) are shown for comparison.

L. pneumophila requires *letE* to resist heat in PE phase. Coincident with the expression of virulence phenotypes, *L. pneumophila* becomes resistant to multiple stresses, including high levels of osmolarity, heat, acidity, and oxidation (16). Contrary to prevailing models for the regulation of stationary-phase physiology by other gram-negative bacteria (28), PE-phase *L. pneumophila* does not require RpoS to resist extreme conditions (5, 16). Instead, LetA/LetS induces resistance to oxidative, osmotic, heat, and acid stress in PE-phase *L. pneumophila* (29, 31). To determine whether the *letE* product cooperates with LetA/LetS to supplant RpoS as a regulator of PE-phase stress resistance, we exposed *letE* mutant and wild-type bacteria to acidity (citric acid, pH 3), hyperosmolarity (5 M NaCl), oxidation (10 mM H₂O₂), or heat (57°C) for 1 h and compared their survival rates. A PE-phase *letE* mutant was as tolerant as the wild type to a low pH, hyperosmolarity, and oxidative stress (Fig. 4A and data not shown). However, PE-phase *letE* mutants were exquisitely sensitive to heat, as evidenced by the recovery of ~6,000-fold fewer mutant CFU of the mutant than of the wild type (Fig. 4B). Therefore, PE-phase *L. pneumophila* requires *letE* to tolerate heat, but not other environmental stresses.

The *letE* locus is transcribed in E phase as an approximately 500-base monocistronic RNA. The *letE* locus, as defined by four insertion mutations (18), is located ~1 kb upstream from *milX*, a gene that *L. pneumophila* requires for its replication in macrophages (J. D. Sauer and M. Swanson, unpublished data) and that bears sequence similarity to the infectivity gene *milA* (19). To investigate whether transposon insertions in *letE* are polar on *milX*, we first performed a Northern blot analysis of RNA samples collected from E- and PE-phase broth cultures. A probe specific for the *letE* locus hybridized to a transcript that was present in E-phase wild-type bacteria but absent from *letE* mutants (Fig. 5A). The RNA was approximately 500 bases in length, which is in agreement with the ~400-bp region

defined by the four *letE* mutations and sufficient to encode the 372-bp putative ORF, but not the 3' *milX* locus (GenBank accession no. AY135376) (18). When these and similar RNA samples were assayed with a *milX* probe, no transcripts were detected for either growth phase (data not shown). Given the ~500-nucleotide length of the abundant *letE* transcript, its absence in *letE-121* mutant bacteria, the ~1-kb separation between the *letE* and *milX* loci, and the lack of detectable *milX* transcripts in either wild-type or *letE-121* mutant cells, it appears unlikely that *letE* is cotranscribed with *milX* or that *mariner* transposon mutations in *letE* are polar on *milX*.

Expression of *letE* is dependent on growth phase. The *letE* transcripts were abundant in E-phase bacteria but were rare in PE-phase cells (Fig. 5). To test whether either LetA/LetS or RpoS induces the transmission phenotype in part by regulating *letE* expression, we compared the amounts of *letE* RNA in E-

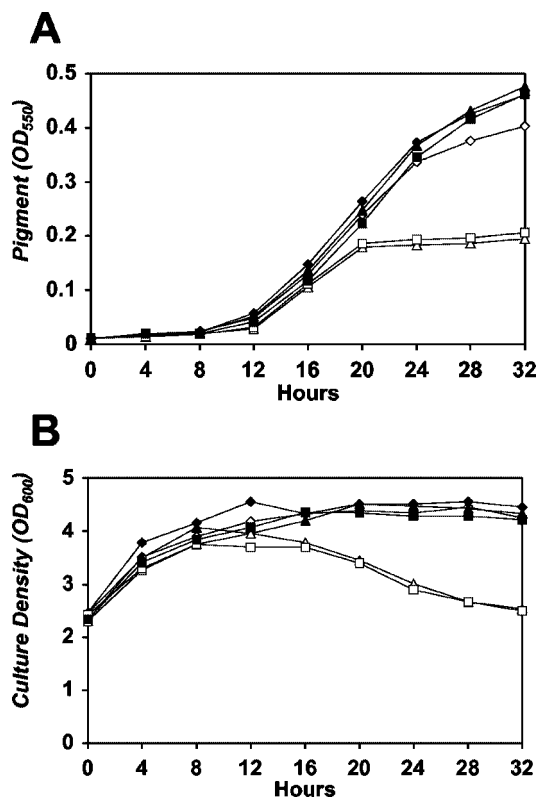


FIG. 3. *letE* is required for wild-type pigment levels and maintenance of OD₆₀₀ in PE phase. (A) Pigment production measured first during late E phase (OD₆₀₀ of 2.5) and at 4-h intervals thereafter. At the times shown, samples of wild-type Lp02 containing pMMB-GentΔmob (MB451; open diamonds), wild-type Lp02 containing pLetE (MB452; closed diamonds), the *letE-121* mutant containing pMMB-GentΔmob (MB447; open triangles), the *letE-121* mutant containing pLetE (MB448; closed triangles), the *letE-121* mutant containing pLetE_{STOP} (MB449; open squares), and the *letE-121* mutant containing pLetE_{SIL} (MB450; closed squares) were collected by centrifugation at 16,000 × g, and then the pigment accumulation in supernatants was measured as the OD₅₅₀. The results shown are representative of three independent experiments. (B) OD₆₀₀ values of the culture samples used for panel A. At the times shown, bacteria collected by centrifugation as described above were resuspended to their original volume and diluted 1:10, and then the OD₆₀₀ was measured. The results are representative of three independent experiments.

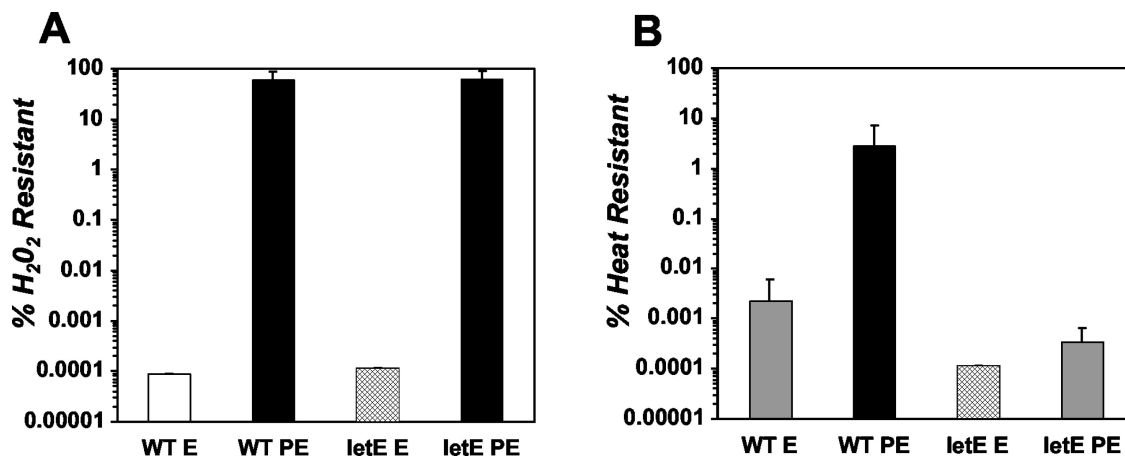


FIG. 4. *letE* is required for heat resistance, but not H₂O₂ resistance, in PE phase. (A) Broth cultures of wild-type strain Lp02 (WT) and *letE-121* mutant strain MB419 (*letE*) were grown to E and PE phase, and then duplicate samples of each culture were collected by centrifugation and resuspended in either 1× M63 salts or 10 mM H₂O₂. Samples were incubated for 1 h at 37°C, washed, diluted, and plated for counts of CFU. The percent H₂O₂ resistance was calculated by the formula % resistance = (CFU_{H₂O₂}/CFU_{M63}) × 100. Black bars represent the mean values from three independent experiments; error bars represent standard deviations. For all three experiments, after treatment with 10 mM H₂O₂, the WT E-phase and *letE* E-phase CFU were below the limit of detection, a value represented by the white and gray bars, respectively. (B) Duplicate samples of each culture were collected by centrifugation, resuspended in 1× M63 salts, and then incubated for 1 h at 37 or 57°C. Samples were washed in 1× M63 salts, diluted, and plated for counts of CFU. The percent heat resistance was calculated by the formula % resistance = (CFU_{37°C}/CFU_{57°C}) × 100. Black bars represent the mean values calculated from three independent experiments; error bars represent standard deviations. For each of the three *letE* E-phase samples, the CFU was below the limit of detection, which is represented by the hatched bar. For two of the three WT E-phase samples and one of the three *letE* PE-phase samples, the CFU was below the limit of detection; for these samples, the maximum possible value was determined and incorporated into the calculated mean, a value represented by the gray bars. Therefore, the calculated means for the wild-type E-phase and *letE* mutant PE-phase cultures are overestimates of the actual mean heat resistance levels.

and PE-phase wild-type, *letA*, *letS*, and *rpoS* cultures (Fig. 5A). In the E phase, the expression of *letE* was completely independent of RpoS and largely independent of LetA/LetS, as mutations in the two-component regulator genes had a modest effect on *letE* transcript levels. In contrast, the down-regulation of the ~500-base *letE* RNA by PE-phase *L. pneumophila* was strongly affected by each of the regulatory mutations (Fig. 5A). In the PE phase, *letA* and *letS* mutants contained *letE* RNA molecules that were smaller than the wild-type transcript. More striking was the accumulation by *rpoS* mutants of a large pool of RNA molecules of a range of low molecular weights that hybridized to the *letE* probe. The small *letE* RNA species could result either from initiation at different sites or from posttranscriptional processing; in either case, since most of the small RNAs were <300 bases long, they could not encode the entire 372-bp ORF. The results of the Northern blot analysis indicated that *L. pneumophila* expresses an ~500-base monocistronic *letE* RNA in the E phase; in the PE phase, LetA/LetS and RpoS regulate its decay. Its scarcity in the PE phase is inconsistent with the idea that *letE* encodes a regulatory RNA that sequesters CsrA to relieve repression of the transmission phenotype (20).

The *letE* locus is sufficient to restore PE-phase phenotypes to *letE* mutants. To test whether *letE* mutant phenotypes are caused by polar effects on *milX*, we defined the genomic region that confers *letE* activity by using genetic complementation tests. Either the control vector, pMMB-GentΔmob, or pLetE, which bears the putative *letE* ORF but not *milX*, was transformed into *letE-121* mutant MB419, and then the ability of each strain to infect bone marrow-derived macrophages was compared to that of the wild type (Fig. 6). After a 2-h incubation, only 13% of the PE-phase *letE* mutant inoculum was

viable and cell associated, whereas 29% of the PE-phase mutants that carried pLetE were infectious, which is similar to the efficiency of 36% of the PE-phase wild type. The complementation of infectivity was statistically significant according to the Student *t* test ($P < 0.05$). Furthermore, PE-phase cultures of the mutant strain carrying pLetE developed the shape (Fig. 2) and motility (Table 2) that are characteristic of wild-type *L. pneumophila*, produced wild-type quantities of pigment (Fig. 3A), and maintained their OD₆₀₀ value (Fig. 3B). In contrast, the *milX* locus carried by plasmid pMilX failed to complement the motility defect (Table 2) and exacerbated the cytotoxicity defect of *letE* mutants, reducing it from ~50% to only 25% of wild-type levels (data not shown). Neither pMilX nor pLetE restored sodium sensitivity to *letE* mutant cultures (Table 2). The sodium resistance of *letE* mutants is unlikely to be caused by a second site mutation, since both the original *letE-121* isolate and the backcrossed mutant strain (MB419) are sodium resistant (18). The capacity to complement five of six *letE* defects in *trans* demonstrates that the *letE* locus encodes an activity that enhances the expression of several traits that are characteristic of PE-phase *L. pneumophila*.

***letE* does not complement an *E. coli* *csrB* mutant.** Several of the phenotypes conferred by *letE* mutations are also caused by multiple copies of the wild-type *csrA* locus (14). Initially identified by its nucleotide sequence similarity, the *L. pneumophila* *csrA* gene was shown to complement an *E. coli* *csrA* mutant (14). To investigate whether *letE* impinges upon the CsrA regulatory pathway, we transformed plasmid pLetE into isogenic wild-type and *csrB* mutant *E. coli* cells (kindly provided by Tony Romeo) and then assessed their ability to accumulate glycogen by staining colonies with iodine vapor (27, 33). When *letE* expression from the P_{tac} promoter was induced by IPTG,

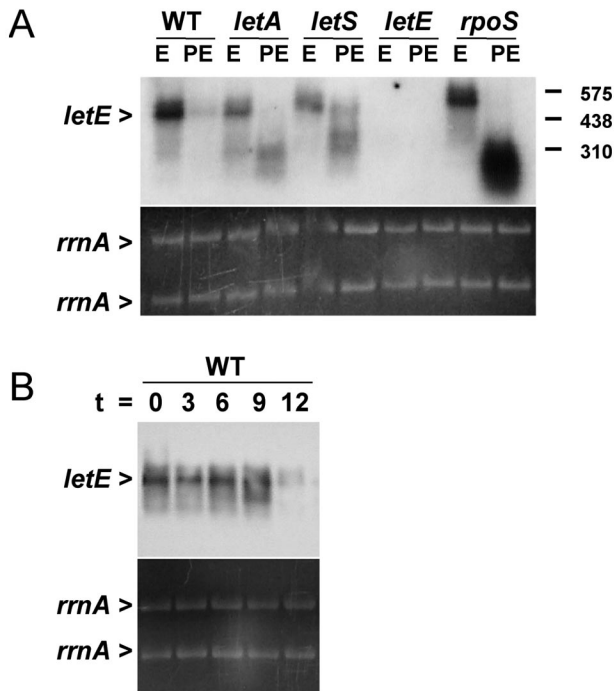


FIG. 5. A monocistronic *letE* RNA is expressed by E-phase *L. pneumophila*. (A) Northern blot analysis performed with a *letE*-specific probe on 10 μ g of total RNA from an E- or PE-phase culture of wild-type Lp02 (WT) or *letA*-22 (MB414), *letS*-36 (MB417), *letE*-121 (MB419), or *rpoS* null (MB379) mutant bacteria. The hybridization patterns shown are representative of results obtained from three independent sets of RNA samples. Equal loading of the samples is demonstrated as described for Fig. 1. The positions of RNA size standards are indicated to the right. (B) Northern blot analysis of 10- μ g samples of total RNA harvested from wild-type *L. pneumophila* Lp02 at 3-h intervals from E phase (OD₆₀₀ of ~0.6) to PE phase (OD₆₀₀ of ~4), using a *letE* probe with the same sequence as that used for panel A. The temporal expression patterns shown are representative of two independent experiments. Equal sample loading is demonstrated as described for Fig. 1.

the *letE* locus did not complement the *csrB* mutant. Therefore, if *letE* encodes a regulatory RNA, it cannot replace *E. coli* *csrB*.

The *letE* locus directs the synthesis of a 12-kDa protein. To investigate whether the *letE* locus directs the synthesis of a protein, we compared cell extracts prepared from *L. pneumophila* strains that harbored zero, one, or multiple copies of the *letE* locus after separation by SDS-polyacrylamide gel electrophoresis. Wild-type *L. pneumophila* produced a protein species of ~12 kDa that was not apparent in *letE*-121 mutants but that was restored by the transformation of *letE* mutant cells with pLetE (Fig. 7). The electrophoretic mobility of the *letE*-dependent protein was in agreement with the ~14-kDa size predicted from its nucleotide sequence (GenBank accession no. AY135376); nevertheless, an understanding of whether this species is the LetE protein or a target of *letE* regulation requires more specific molecular analysis.

As an independent test of whether *letE* acts as a regulatory RNA or a protein, we constructed two separate mutations in pLetE and then tested their effects on its complementation activity. Codon 7 of the predicted *letE* ORF was converted by site-directed mutagenesis from TTA to TAA, a stop codon, creating pLetE_{STOP}. Although the nucleotides of codon 7 were

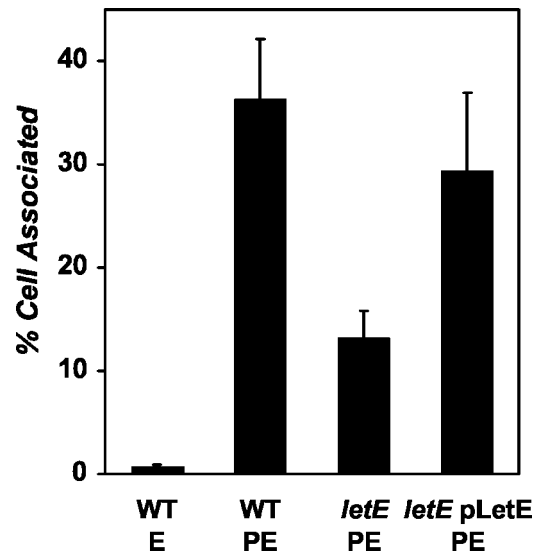


FIG. 6. *L. pneumophila* requires the *letE* ORF to infect macrophages efficiently. The percentage of each *L. pneumophila* strain that was cell associated after a 2-h infection of 2.5×10^5 macrophages is shown. Macrophages were infected at a multiplicity of infection of 0.2 with the following strains: PE-phase wild-type Lp02 containing pMMB-Gent Δ mob (MB451), E-phase wild-type Lp02 containing pMMB-Gent Δ mob (MB451), PE-phase *letE*-121 mutant containing pMMB-Gent Δ mob (MB447), and PE-phase *letE*-121 mutant containing pLetE (MB448). The results shown are the means from triplicate samples of infected macrophages; error bars represent the standard deviations.

not predicted by either the mfold or Lasergene Genequest algorithm to contribute to the RNA secondary structure of this region, as a control we introduced a different mutation that would alter the sequence of an RNA but not a protein at the

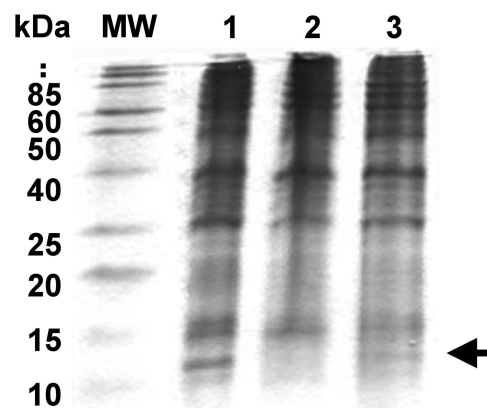


FIG. 7. *letE* directs the synthesis of an ~12-kDa protein. Total cell extracts prepared from PE-phase cultures of wild-type *L. pneumophila* Lp02 transformed with vector pMMB-Gent Δ mob (MB451; lane 1), the *letE*-121 mutant strain transformed with vector pMMB-Gent Δ mob (MB447; lane 2), or the *letE*-121 mutant strain transformed with complementing plasmid pLetE (MB448; lane 3) were separated by SDS-polyacrylamide gel electrophoresis in a Tris-Tricine-16.5% acrylamide gel and then were stained with Coomassie blue. The arrow indicates the position of the predicted LetE protein. Approximate molecular masses of the size standards are shown; the positions of the 120- and 190-kDa species are indicated with dots. Similar results were obtained in two other independent experiments.

same position. Codon 7 was changed from TTA to CTA, which is a silent mutation because both sequences encode leucine. If LetE functions as a protein, the resulting plasmid, pLetE_{SIL}, was predicted to complement a *letE* mutant, whereas pLetE_{STOP} was not.

A series of complementation tests indicated clearly that LetE functions as an activator protein. Most striking was the failure of pLetE_{STOP} to complement pigment production by the *letE* mutant (Fig. 3A, open squares), whereas pLetE_{SIL} restored its pigment production to wild-type levels (Fig. 3A, closed squares). In addition, pLetE_{STOP} did not prevent the OD₆₀₀ decline of *letE* mutant cultures, which caused a decrease in the OD₆₀₀ from 3.76 to 2.76 over a period of 24 h. In contrast, cultures of *letE* bacteria carrying pLetE_{SIL} maintained an OD₆₀₀ of ~4.2 (Fig. 3B). The complementation of cell morphology and motility also required the intact protein coding sequence (Fig. 2; Table 2). Taken together, the molecular, biochemical, and genetic data indicate that the 372-bp *letE* ORF encodes a protein that cooperates with LetA/LetS to mediate the differentiation of replicating *L. pneumophila* cells to a form that is suitable for transmission between host phagocytes.

DISCUSSION

In *L. pneumophila*, the LetE protein amplifies the expression of several stationary-phase traits that are thought to promote bacterial transmission from one amoebae to another (36). In addition to the coccoid morphology, pigment production, and heat tolerance that are characteristic of PE-phase bacteria, *letE* enhances *L. pneumophila* cytotoxicity, motility, and infection of macrophages. Each of the traits affected by *letE* mutation is activated by the LetA/LetS two-component regulatory system (18) and repressed by CsrA (31), a protein that binds particular mRNAs and inhibits their translation (6, 32). Accordingly, the phenotypic data predict that the LetE protein either augments LetA/LetS activity or inhibits the CsrA function directly or indirectly.

The transmission regulon activated by LetA/LetS and LetE is thought to promote the fitness of planktonic *L. pneumophila* cells during their travel between host amoebae. During stationary phase, many *Legionella* spp. secrete a brown pigment that confers protection from light (35). Pigment production requires at least two distinct genetic loci, named *pig* and *lly*. The *lly* gene product, legiolsin, catalyzes the formation of homogentisic acid, which likely polymerizes to form a melanin-like pigment (37, 38). Although not required for intracellular growth (35), pigment accumulation and the efficiency of macrophage infection by *L. pneumophila* are correlated genetically. For both traits, the wild type and *rpoS* mutants exhibit high levels of activity (16), *letE* mutant bacteria are intermediate (Fig. 3 and 6), and *letA/letS* mutants are as defective as E-phase *L. pneumophila* (31). It remains to be determined whether pigment accumulation contributes to *L. pneumophila* fitness by scavenging reactive oxygen species or by protecting planktonic bacteria in their natural reservoir from light or if it instead indicates a degradation of the aromatic amino acids that power transmission to a new host. In any case, pigment production can be regarded as a reliable marker of the *L. pneumophila* transmissive phase.

LetE also potentiates structural changes in PE-phase *L.*

pneumophila, as judged by alterations in the cell shape (Fig. 2) and the maintenance of the OD of broth cultures (Fig. 3). Starved *E. coli* adopt a coccoid shape through reductive division which requires RpoS-dependent genes such as *bolA* (9, 26). Wild-type and *letE* mutant PE-phase cultures retain roughly constant CFU, but wild-type cells differentiate to form short rods while *letE* mutants continue to elongate in the PE phase, a phenomenon that may affect their OD in culture. LetE, together with LetA/LetS, influences multiple stationary-phase phenotypes, indicating that the expression of the transmission phenotype is tightly coupled to a broad physiological adaptation to starvation.

Studies of broth cultures that model the *L. pneumophila* life cycle indicate that a stringent response-like mechanism coordinates the transformation of replicating bacteria to a form that can be efficiently transmitted to a new phagocyte (5, 17). The differentiation of intracellular *L. pneumophila* is postulated to be triggered by (p)ppGpp (17), an alarmone synthesized by RelA and also by SpoT which in some manner activates the LetA/LetS and LetE regulon (18). Although *L. pneumophila* requires neither RelA nor LetE for its replication in phagocytes, *relA* and *letE* mutants do exhibit similar quantitative defects in *flaA* expression, motility, and pigment production in the PE phase (Fig. 1 and 3) (18, 40). In particular, it appears that only some of the *letE* mutant cells sense or reach the threshold that triggers differentiation to the transmission phenotype. When cultured in broth, PE-phase *letE* mutants contain very little *flaA* RNA (Fig. 1), but they do harbor a small number of motile bacteria (Table 2). Accordingly, in some manner, LetE enhances either the amplitude or the fidelity of the signaling cascade initiated by (p)ppGpp in response to stress.

In numerous species of gram-negative bacteria, homologues of LetA/LetS activate gene expression indirectly by a posttranscriptional mechanism. Members of this family of two-component regulators induce the expression of the *csrB* regulatory RNA, which sequesters the CsrA repressor, permitting the translation of target mRNAs (20). However, unlike *csrB*, *L. pneumophila* LetE appears to function as a protein, not an RNA. The wild-type locus did not complement an *E. coli csrB* mutant, the cloned gene directed the synthesis of an ~12-kDa protein (Fig. 7), and a *letE* nonsense mutation eliminated functioning, whereas a mutation in the same position that maintained the protein sequence did not (Fig. 2 and 3; Table 2). Also, a *letE* mutation did not affect the flagellar regulon in the manner reported for *csrA* overexpression: *letE* mutations greatly reduced the amount of *flaA* RNA (Fig. 1), whereas multiple copies of *csrA* repressed both *flaA* and *fliA* (14). Therefore, the LetE protein enhances the expression of the transmission phenotype by a LetA/LetS-dependent mechanism that remains to be discovered.

A computer analysis of the LetE amino acid sequence provided few clues to its biochemical activity. The LetE polypeptide is predicted to contain 123 amino acids and to have a molecular mass of 14 kDa and an isoelectric point of 4.1. Two folding algorithms (the PredictProtein and Garnier-Robson algorithms) indicated that LetE assembles into five alpha-helical regions; the fifth helix, encompassing residues 99 to 116, is predicted to contain a transmembrane domain based on its hydrophobicity and a comparison to known membrane pro-

teins (by a Kyte-Doolittle hydrophobicity plot [Lasergene Pro-
tean] and a DAS transmembrane prediction profile). Standard
BLAST searches found no homology (defined as an E value of
<1) to any dynamically translated microbial genomes in Gen-
Bank; likewise, a search of all GenBank proteins yielded no
entries with an E value of <0.4. A reiterative PSI-BLAST anal-
ysis (3) of the LetE predicted protein sequence did reveal a
limited similarity to proteins whose biochemical activities differ
but which each interact with tRNAs, including some peptidyl-
tRNA hydrolases, aminoacyl-tRNA synthetases, and retroviral
reverse transcriptases (A. Molofsky, unpublished observations).
Whether LetE interacts with tRNA to augment the expression
of the transmission phenotype requires further study.

Certain aspects of the regulation of the *L. pneumophila*
transmission phenotype parallel the regulation of the *E. coli*
universal stress protein A, which coordinates tolerance to a
wide variety of harsh conditions. During periods of starvation,
repression of the *uspA* promoter by an active FadR protein can
be overridden when the alarmone ppGpp accumulates (24). By
this “emergency derepression” mechanism, cells can activate
critical metabolic pathways despite a paucity of resources.
Transcripts of *letE* are abundant during the E phase in broth,
and their production does not require LetA, LetS, or RpoS
(Fig. 5A). Therefore, a pool of *letE* RNA is likely available to
intracellularly replicating *L. pneumophila*. Should conditions
suddenly deteriorate, the LetE protein could quickly respond
to the ppGpp alarm by overriding CsrA-mediated repression,
thereby promoting transmission to a new host.

A number of observations suggest that a posttranscriptional
mechanism contributes to the induction of the *L. pneumophila*
transmission phenotype. First, in a variety of other gram-neg-
ative bacteria, homologues of LetA/LetS activate gene expres-
sion indirectly by counteracting the effects of CsrA, a repressor
of translation (8, 12, 20, 21). Secondly, in PE-phase *L. pneu-
mophila*, LetA/LetS and RpoS may mediate the decay of *letE*
RNA (Fig. 5A). Likewise, short *rpoS* RNA species also accu-
mulate in PE-phase *letA*, *letS*, and *letE* mutants (4). Differen-
tiation from the replicative to the transmissive form is a com-
prehensive physiological change that must occur rapidly in the
face of declining precursors for gene expression. For a mini-
mization of this energetic challenge, the induction of transmis-
sion loci could be coupled to the decay of replicative gene
transcripts. According to this model, LetE would amplify the
developmental switch by stabilizing LetA/LetS-dependent
transmission transcripts and destabilizing LetA/LetS-independ-
ent transcripts, including *letE* itself.

L. pneumophila alternately replicates within potentially
deadly phagocytes and travels between cells through nutrient-
sparse freshwater. Its biphasic life cycle demands rapid and
extensive adaptations to stress and starvation through efficient
selective gene expression. For example, when nutrients are
depleted within the replicative vacuole, a strong, immediate
induction of cytotoxicity may mediate the lysis of host mem-
branes and bacterial egress (10). Additional experiments can
determine how LetE functions as one member of a multicom-
ponent sensor and response mechanism that coordinates the
conversion of *L. pneumophila* from the replicative to the trans-
missive form.

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