ArgR-Regulated Genes Are Derepressed in the Legionella-Containing Vacuole[▽][†]

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Legionella pneumophila is an intracellular pathogen that infects protozoa in aquatic environments and when inhaled by susceptible human hosts replicates in alveolar macrophages and can result in the often fatal pneumonia called Legionnaires' disease. The ability of *L. pneumophila* to replicate within host cells requires the establishment of a specialized compartment that evades normal phagolysosome fusion called the *Legionella*-containing vacuole (LCV). Elucidation of the biochemical composition of the LCV and the identification of the regulatory signals sensed during intracellular replication are inherently challenging. L-Arginine is a critical nutrient in the metabolism of both prokaryotic and eukaryotic organisms. We showed that the *L. pneumophila* arginine repressor homolog, ArgR, is required for maximal intracellular growth in the unicellular host *Acanthamoeba castellanii*. In this study, we present evidence that the concentration of L-arginine in the LCV is sensed by ArgR to produce an intracellular transcriptional response. We characterized the *L. pneumophila* ArgR regulon by global gene expression analysis, identified genes highly affected by ArgR, showed that ArgR repression is dependent upon the presence of L-arginine, and demonstrated that ArgR-regulated genes are derepressed during intracellular growth. Additional targets of ArgR that may account for the *argR* mutant's intracellular multiplication defect are discussed. These results suggest that L-arginine availability functions as a regulatory signal during *Legionella* intracellular growth.

Legionella pneumophila is a Gram-negative gammaproteobacterial species with a remarkable capacity for robust growth in eukaryotic host cells (24, 35, 58). In both natural and man-made aquatic systems, *L. pneumophila* replicates within a wide variety of unicellular protozoa (23). Inhalation of aerosolized water contaminated with *Legionella* species, often from showers or whirlpool baths, can result in infection of human alveolar macrophages (60, 74). In susceptible individuals, this infection can lead to the development of a potentially fatal form of pneumonia called Legionnaires' disease (35, 58, 77).

Replication of *Legionella* in both unicellular protozoa and human alveolar macrophages occurs through a series of ordered events that begins with phagocytosis (32). Following uptake, *L. pneumophila* abrogates normal host vesicular trafficking to prevent the phagosome from acidifying and fusing with lysosomes (33, 34). Subsequent steps of phagosome maturation include interactions with host cell organelles such as mitochondria and endoplasmic reticulum, which will ultimately decorate the vacuole (1, 32). At approximately 8 h after uptake, the phagosome has developed into a *Legionella*-containing vacuole (LCV) that supports robust bacterial replication (32). Between 18 and 24 h postinfection, vacuoles filled with *L. pneumophila* are released and able to infect the next round of host cells (32, 73). This process requires the Icm/Dot type IVB secretion system (TFBSS), which is essential for the evasion of

lysosomal fusion with the *Legionella*-containing phagosome and prevention of vacuole acidification (66, 70, 72). The Icm/Dot TFBSS is able to translocate >150 protein substrates into the host cytoplasm (12, 16, 20, 21, 61, 63). Icm/Dot-translocated substrates can alter host cell functions in a variety of ways that are predicted to promote bacterial intracellular survival (16, 37, 54, 61).

The ordered nature of the *Legionella* infection suggests that it is a highly regulated process, likely to require the ongoing detection of, and response to, specific environmental signals (29, 36, 59). Although the two-component systems CpxR/A, LetA/S, and PmrA/B and the global regulator σ^S have been shown to affect *L. pneumophila* intracellular multiplication, the signals that they respond to intracellularly are unknown (2, 4, 25, 30, 36, 51). Both LetA/S and σ^S accumulate in response to ppGpp during growth in rich medium in a process that affects the accumulation of the small regulatory RNAs RsmY and RsmZ (6, 36, 59), but it is not known if this also occurs during intracellular growth. Determining the internal composition of the LCV and identifying signaling molecules required for intracellular growth are inherently challenging.

Recently we reported that the σ^S-regulated gene *lpg0490*, a homolog of the arginine repressor, *argR*, is required for maximal intracellular multiplication of *L. pneumophila* within the protozoan host *Acanthamoeba castellanii* but not in the stabilized macrophage cell line THP-1 (36). The ArgR protein has been characterized in other bacteria as a repressor of arginine biosynthetic genes, which are typically distributed throughout the genome and required for the synthesis of the amino acid L-arginine from L-glutamate (19, 46, 52). This set of genes is commonly referred to as the ArgR regulon (46). In multiple bacterial genera, it has been demonstrated that ArgR mono-

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mers oligomerize to form homohexamers (28, 39, 62). The ArgR hexamers are allosterically activated by bound L-arginine to form a transcriptional repressor that binds to well-conserved DNA operator sites (28, 39, 78). Thus, in other bacteria ArgR is a direct sensor of L-arginine availability that represses the transcription of its target genes when arginine biosynthesis is not required.

Legionella species are arginine auxotrophs because they lack genes encoding the enzymes that carry out the preliminary steps of the biosynthetic conversion of L-glutamate into L-arginine (26). Legionellae are, however, capable of synthesizing L-arginine from compounds that occur later in this biosynthetic pathway such as L-ornithine and citrulline (26). Amino acid metabolism is of central importance in *Legionella* biology because amino acids can be utilized as its sole source of carbon and nitrogen and meet most of its energy needs (81).

Based on the function of ArgR in other bacteria and the intracellular replication defect of an *L. pneumophila* Δ*argR* mutant, we hypothesized that L-arginine availability is a regulatory signal affecting gene expression in the LCV. In order to understand how arginine availability affects gene expression during intracellular growth, we studied the global gene expression profile of an Δ*argR* mutant and used it to define the *L. pneumophila* ArgR regulon. Using a novel dual-fluorescence transcriptional reporter system, the regulation of genes controlled by ArgR and L-arginine was analyzed in chemically defined medium (CDM) and during intracellular growth. This system, in conjunction with quantitative PCR (qPCR) estimates of mRNA abundance from intracellularly growing bacteria, allowed us to demonstrate that several *L. pneumophila* genes whose transcription is regulated by ArgR and L-arginine availability are derepressed during intracellular growth. These results contribute to understanding how nutrient availability can affect gene expression during *L. pneumophila* intracellular growth.

MATERIALS AND METHODS

Bacterial strains and mutants. The bacterial strains used in this study are listed in Table 1. Media and antibiotics were used as previously described (15). The *L. pneumophila* strains used in this study were JR32, a streptomycin-resistant, restriction-negative mutant of *L. pneumophila* Philadelphia-1 (68); LELA3118, an isogenic *dotA* (*lpg2686*) derivative of JR32 (68); KS79, an isogenic Δ*comR* (*lpg2717*) derivative of JR32 that renders the bacteria genetically competent for DNA uptake (20); and GAH280, an isogenic Δ*argR* (*lpg0490*) derivative of KS79 (therefore Δ*comR* Δ*argR*) (36). KS79 (Δ*comR*) is the isogenic parent of all strains used in this study and is referred to as the wild type (WT) in all experiments. Mutants made in this study were produced by creating allelic exchange fragments using *long flanking homology* (LFH)-PCR as described previously (36). The oligonucleotides used in mutant formation are lpg0493 P1 (GGCCAGGATGACGTTGCGATTGCC), lpg0493 P2 (CCCGGCCAAGC TAAAAAACACGGATCCCGATGCGTTAATGACTTGCAGC), lpg0493 P3 (GCTCGATGAGTTTTTCTAAGGATCCGCGATTGTTGGTTGTAAAGCA AGG), lpg0493 P4 (GCTGCGTCACATCAATGAATGCATCC), lpg0495 P1 (CCACTAATTGCTCAAAAGCTTGTTGAAATTGC), lpg0495 P2 (CCCGGC CAAGCTAAAAAACACGGATCCCCTGACTGCCATTAATGTCCTGGTC), lpg0495 P3 (GATGCTCGATGAGTTTTTCTAAGGATCCGCAGGAGGATA AAGAAGGATTATTTGATACG), and lpg0495 P4 (GCCTCCTTAACAATA GCCTCATTAGGC).

Growth of bacterial strains and preparation of media. Chemically defined medium (CDM) was prepared as previously described (85); however no L-arginine was added to stock solutions (CDM with no L-arginine). For CDM growth and transcription assays, bacteria were grown to the mid-exponential phase in AYE complex growth medium [*N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract], washed three times in CDM with no

L-arginine, and resuspended to an optical density (OD) of 0.10 for time zero measurement in CDM containing the desired concentration of L-arginine or its biosynthetic precursors. The standard final concentration of L-arginine or its biosynthetic precursors for experiments conducted in this study was 1.0 mg/ml. Absorbance (optical density at 600 nm [OD$_{600}$]) and fluorescence measurements were conducted in a TECAN Infinite M200 plate reader in triplicate. Growth experiments were performed three times, and the data shown are from one representative experiment. The data points reflect the average value of three technical replicates. Isolation of exponentially and postexponentially growing *L. pneumophila* for microarray analysis from complex medium AYE was performed as described previously (36) on triplicate samples.

Plasmid construction. Plasmids pGAH140 and pGAH141, which contain the promoter fragments *P491* and *P491ΔO*, respectively, were constructed from the restriction digest of PCR fragments bearing the restriction sites XmaI and KpnI cloned into digested pXDC94 vector (for the map, see Fig. 8A). The oligonucleotides used in reporter construction are p0491AFw (TATCCCGGGGATAGC AAGACTGTTTGCATTACCAGG), p0491ARv (TATGGTACCATCGTTTT GTGAATATACAGGATCAGG), and p0491CFw (TATCCCGGGGATACCAT TTTTTCTTGACAGTACAAATCC).

ClustalW alignments and tree formation. Amino acid alignments and phylogenetic trees were constructed based on ClustalW pairwise alignment algorithms on a BLOSUM 30 matrix using MacVector software version 7.2.3. The phylogenetic tree of arginine repressor homologs corresponds to the following GenBank accession numbers (in no particular order): *Lactococcus lactis* subsp. *lactis* Il1403 (NP_267015.1), *Streptococcus pneumoniae* TIGR4 (NP_345670.1), *Bacillus subtilis* (BAA12578.1), *Bacillus stearothermophilus* (1B4AC), *Listeria innocua* Clip11262 (NP_470740.1), *Staphylococcus aureus* subsp. *aureus* N315 (NP_374634.1), *Streptococcus pyogenes* M1 group A streptococcus (GAS) (NP_269620.1), *Haemophilus influenzae* 86-028NP (AAX88207.1), *Pasteurella multocida* subsp. *multocida* strain Pm70 (NP_245486.1), *Escherichia coli* strain K-12 (AAC76269.1), *Vibrio cholerae* O1 biovar eltor strain N16961 (NP_230085.2), *Chlamydophila pneumoniae* J138 (NP_300252.1), *Salmonella enterica* serovar Typhimurium LT2 (P_463323.1), *Thermotoga maritima* (Q9WW19.1), *Mycobacterium leprae* TN (NP_302004.1), *Mycobacterium tuberculosis* CDC1551 (NP_336150.1), and *Pseudomonas aeruginosa* PAO1 (NP_249584.1).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or feature(s)	Source or reference
L. pneumophila strains		
JR32	Am511 salt-sensitive isolate	68
KS79	JR32 Δ*comR*	20
LELA3118	JR32 *dotA*::Tn*903*dII*lacZ*	68
GAH199	KS79 *dotA*::Tn*903*dII*lacZ*	36
GAH280	KS79 Δ*argR*::Km^r	36
GAH318	KS79 Δ*lpg0493*::Km^r	This study
GAH319	KS79 Δ*lpg0495*::Km^r	This study
GAH281	KS79/pXDC31	36
GAH282	GAH199/pXDC31	36
GAH286	GA280/pXDC31	36
GAH339	GAH318/pXDC31	This study
GAH340	GAH319/pXDC31	This study
GAH348	KS79/pXDC95	This study
GAH349	KS79/pGAH140	This study
GAH350	KS79/pGAH141	This study
GAH354	GAH280/pXDC95	This study
GAH355	GAH280/pGAH140	This study
Plasmids		
pMMB207c	pMMB207c *mobA*	70
pXDC31	pMMB207c *Ptac*-GFP	36
pXDC94	pMMB207c *Ptac*-mCherry-2Ω-GFP	This study
pXDC95	pMMB207c *Ptac*-mCherry-GFP	This study
pGAH140	pMMB207c *Ptac*-mCherry-2Ω-*P491*-GFP	This study
pGAH141	pMMB207c *Ptac*-mCherry-2Ω-*P491ΔO*-GFP	This study

Microarray procedure. The *L. pneumophila* microarray has been previously described (GLP7283) (36). Bacterial growth, RNA isolation, and cDNA preparation were performed as described previously (36). Bacterial genomic DNA (gDNA) was used as the reference channel on each slide to allow comparison of each time point and different samples (79). Five micrograms of genomic DNA was labeled with amino-allyl dUTP by using the Klenow fragment and random primers (Invitrogen) at 37°C for 18 h (22). DNA was subsequently coupled to the succinimidyl ester fluorescent dye Alexa Fluor 546 (for cDNA) or Alexa Fluor 647 (for gDNA) (Invitrogen) following the manufacturer's protocols. Hybridization and data acquisition were performed as previously described (36). Local background was removed from spot signal intensity, and normalization was carried out by calculating the fraction over the total signal intensity in both channels as previously described (22). Signal levels that were lower than background were filtered out. Statistical analysis between test and control conditions was performed using unpaired one-tailed Student's *t* test. Genes were considered as differentially expressed if they demonstrated a ratio to the control value of $\log_2 \pm 1.5$-fold with a *P* value of <0.005. Hierarchical clustering diagrams were produced using the TIGR Multiexperiment Viewer (MeV) (69).

Kinetic measurements of growth and transcription. Kinetic measurements were obtained using a TECAN Infinite M200 fluorescence plate reader as described previously (36). Preparation of growth assays in liquid CDM is described above, and the method used is identical to that for assaying transcription during growth in CDM. *A. castellanii* was maintained in PYG (peptone-yeast extract-glucose) medium as described previously (29). The protocol for assaying *L. pneumophila* intracellular multiplication in a microplate spectrofluorometer in *A. castellanii* at a multiplicity of infection (MOI) of 1.0 and the human macrophage cell line THP-1 at an MOI of 0.10 has been described previously, and the experiments were conducted following previously established parameters (36). Measurement of transcription was performed on strains bearing plasmids derived from pXDC94, which contains a copy of the gene encoding the red fluorescence protein mCherry under *Ptac* control and a promoterless copy of a gene encoding green fluorescent protein (GFP). Bacterial growth was measured by the production of mCherry from a *Ptac* promoter induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at an excitation of 580 nm and an emission of 620 nm. Transcription was measured by the production of green fluorescent protein (GFP) at an excitation of 485 and an emission of 520. For each strain background or condition tested, transcription from a control plasmid, pXDC95, from which mCherry and GFP are transcribed from *Ptac* induced with 0.5 mM IPTG, was also analyzed. Transcriptional data analysis during intracellular growth and growth in liquid medium was performed as follows. Relative fluorescence units (RFU) for GFP and mCherry triplicate samples for each time point were averaged, averaged GFP RFU was divided by averaged mCherry RFU, and the value from each time point was normalized (i.e., divided) by the value of *Ptac*-GFP/*Ptac*-mCherry obtained from the pXDC95 control for each strain background or condition. This process of data analysis allows measurement of transcription independent of bacterial growth. Kinetic experiments were performed three times, and the data shown are from one representative experiment. Data points reflect the average value of three biological replicates.

Quantitative real-time PCR during infection. The THP-1 (ATCC TIB-202) cells were maintained in Advanced RPMI (Invitrogen) medium supplemented with 10% (vol/vol) fetal calf serum (Gibco) and 2 mM L-glutamine (Cellgro). A stock culture of these cells was maintained as monocyte-like, nonadherent cells at 37°C in an atmosphere containing 5% (vol/vol) CO_2. For macrophage infection, cells were seeded at 2×10^7 cells per well in petri culture dishes and were differentiated by addition of 10^{-7} M phorbol 12-myristate 13-acetate for 48 h. Before infection, macrophages were treated with antibody raised against the major outer membrane protein (MOMP) of *L. pneumophila* for 30 min. Bacteria were grown overnight shaking in AYE at 37°C and were then added to the cell monolayer at an MOI of 1 and centrifuged for 5 min at $800 \times g$ to synchronize bacterial uptake. After incubation for 2 h at 37°C, the infected cells were washed three times with phosphate-buffered saline (PBS), pH 7.4, and 18 ml of fresh complete RPMI medium containing 100 μg/ml gentamicin was added to each well. After incubation for 1 h at 37°C, cells were washed three times with PBS and the cells were either harvested at 3 h postinfection (T_3) or incubated with 18 ml of fresh complete RPMI medium for a further 6 h (T_9) or 18 h (T_{21}). At each time point, cells were scrapped in 2 ml PBS and 100 μl was removed to determine the CFU. Samples were then centrifuged for 5 min at $1,000 \times g$, and the pellet was lysed in 2 ml TRIzol (Invitrogen) and stored at −80°C. RNA was isolated by using TRIzol reagents as described by manufacturer (Invitrogen). The RNA was subsequently treated with DNase I (Invitrogen) for 1 h at 37°C. The DNase was then inactivated by incubation at 75°C for 5 min and acid phenol-chloroform (Ambion) extracted, and the RNA was precipitated with NaAc-ethanol. The purity and quantity of RNA was assessed by spectrophotometry. cDNA was

synthesized in triplicate using Superscript II (Invitrogen) with random hexamers (Invitrogen), according to the manufacturer's instructions. For each sample, a no-reverse transcriptase reaction served as a no-template control (NTC). qPCRs were performed using the Applied Biosystems StepOne Plus 96-well reverse transcription (RT)-PCR system (qRT-PCR) with Power Syber green PCR master mix following the manufacturer's instructions (Applied Biosystems). For each qPCR run, the calculated threshold cycle (C_T) was normalized to the C_T of the internal control 16S rRNA gene amplified from the corresponding sample and the fold change was calculated as previously described (47). qRT-PCR experiments were performed three times, and the data shown are from one representative experiment. Data points reflect the average value of three biological replicates. The oligonucleotides used in qRT-PCR measurements were lpg0491qF (CAACCAAGCGATAGAAGCTTTAATC) and lpg0491qR (CCTTGTGCCCCATCCATAAG).

RESULTS

Conservation of the *L. pneumophila* ArgR protein. Mutational analysis of putative regulatory genes whose transcription is affected by σ^S resulted in the discovery that the *lpg0490* open reading frame (ORF) is required for efficient intracellular multiplication in the unicellular host *A. castellanii* but not in the macrophage cell line THP-1 (36). The *lpg0490* mutant intracellular growth defect was restored by expressing the gene in *trans* from an IPTG-inducible promoter, thus demonstrating its significance during *A. castellanii* infection (36). Based on conservation of DNA-binding and L-arginine-binding domains in the predicted protein sequence, *lpg0490* was annotated as the *L. pneumophila* arginine repressor (18, 36). Arginine repressor proteins, termed ArgR or AhrC based on their similarity to either the *E. coli* or the *B. subtilis* proteins, respectively, are common in bacteria (19, 46, 55). To determine the relative similarity of the *lpg0490* gene product to the arginine repressors of other bacteria, we used ClustalW to produce a phylogenetic tree of ArgR amino acid sequences from genetically diverse bacterial species (Fig. 1A). The identity of Lpg0490 to the arginine repressors of the bacteria analyzed ranged between 28% and 35%. Overall, the Lpg0490 sequence was slightly more similar to the arginine repressors of both mycobacteria and Gram-positive species such as *B. subtilis*, (30% identity and 54% similarity) than those from Gram-negative bacteria such as *E. coli* (28% identity and 52% similarity).

The structure and function of arginine repressor proteins have been studied extensively in many bacteria, including *E. coli* and *B. subtilis*. Mutational analysis has demonstrated the critical amino acid residues required for ArgR function (41, 52, 82). Alignment of the putative *L. pneumophila* arginine repressor, Lpg0490, shows that the amino acids required for DNA binding, oligomerization, and L-arginine binding of *E. coli* and *B. subtilis* arginine repressors are conserved (Fig. 1B). Based on these observations, *lpg0490* will be called the *argR* gene.

ArgR is not required for growth in media containing L-arginine biosynthetic precursors. Arginine auxotrophy is common among all of the strains of *L. pneumophila* that have been tested (26), including Philadelphia-1, the strain used in this study. Although the chemical composition of the LCV is unknown, *L. pneumophila*'s ability to grow intracellularly, despite its arginine auxotrophy (26), suggests that sufficient L-arginine is available in the LCV to support growth. Alternatively, *L. pneumophila* may be able to utilize the terminal arginine biosynthetic precursors L-ornithine and citrulline during growth (26). It is not known if the ability to utilize L-arginine precur-
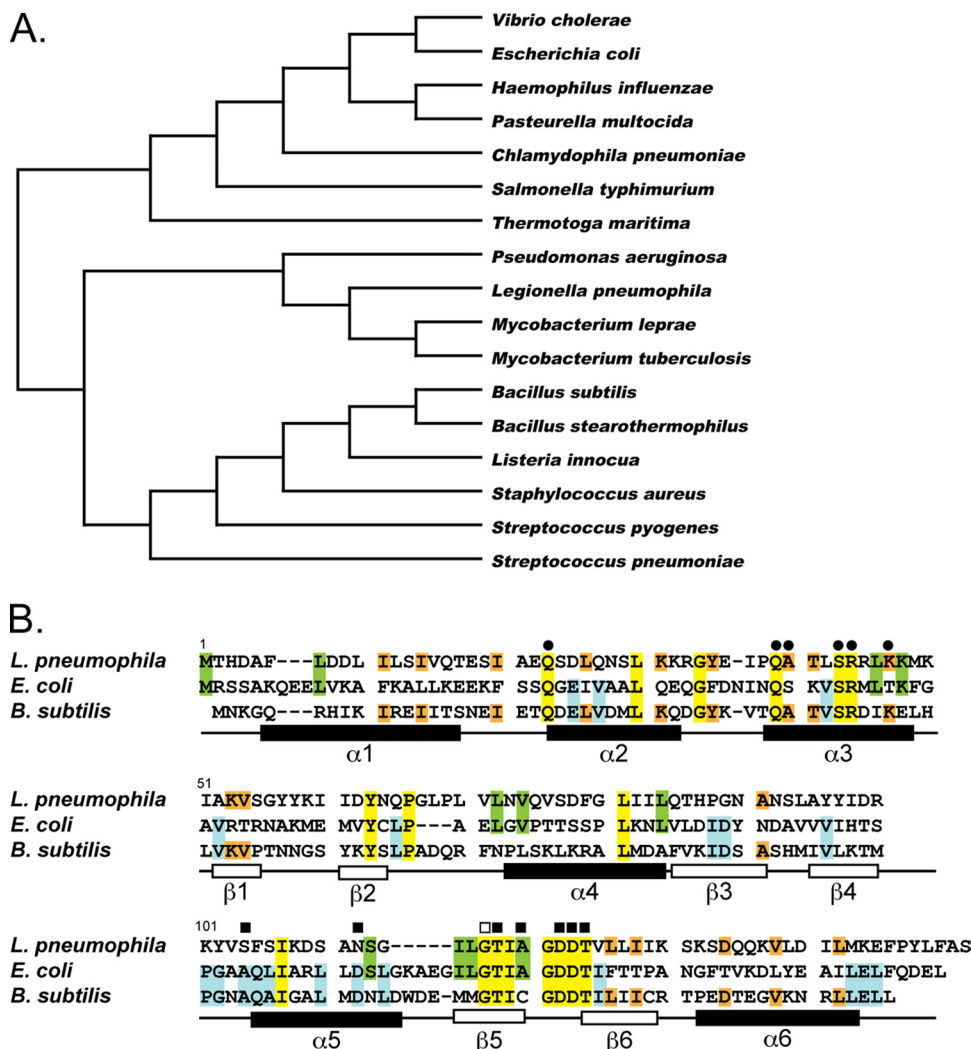
FIG. 1. Conservation of ArgR. A phylogenetic tree of arginine repressor homologs from diverse bacterial species was produced from a ClustalW alignment (A). GenBank accession numbers for all proteins can be found in Materials and Methods. Shown is the ClustalW alignment of amino acid sequences from *E. coli* and *B. subtilis* arginine repressors, ArgR and AhrC, respectively, with the translated sequence from *lpg0490* of *L. pneumophila* (B). Residues conserved among all three bacterial species are highlighted in yellow, those conserved between *E. coli* and *L. pneumophila* are in green, those conserved between *B. subtilis* and *L. pneumophila* are in orange, and those conserved between *E. coli* and *B. subtilis* are in blue (B). Residues required for DNA-binding (●), L-arginine binding (■), or oligomerization (□) demonstrated by mutational analysis in either *E. coli* or *B. subtilis* are indicated above the alignment (B) (8, 41, 76, 82, 83). The general structural features from *B. stearothermophilus* are shown below the amino acid alignments (B) (62).

sors is required for intracellular replication. If mutation of *argR* resulted in an inability to grow in L-arginine or its biosynthetic precursors, this might explain the reported intracellular growth defect (36).

To test the ability of a Δ*argR* mutant to grow in media containing L-arginine or its biosynthetic precursors, chemically defined medium lacking L-arginine (CDM-A) was prepared (64). *L. pneumophila* strains were grown in CDM-A or supplemented with L-arginine (1.0 mg/ml), L-ornithine (1.0 mg/ml), or citrulline (1.0 mg/ml). As expected, wild-type *Legionella* (KS79) is unable to grow in the absence of L-arginine, but the addition of L-arginine or its precursors restored growth (Fig. 2A). The Δ*argR* mutant GAH280 grew similarly to the wild type in CDM-A containing either L-arginine or its biosynthetic precursors (Fig. 2B). Therefore, the intracellular growth defect of the

Δ*argR* mutant does not result from an inability to utilize L-ornithine or L-citrulline as L-arginine precursors.

Global gene expression analysis of an *L. pneumophila* Δ*argR* mutant. Deletion of *argR* results in a significant defect in *L. pneumophila* intracellular multiplication (36). In other bacterial species, ArgR responds rapidly to changes in the intracellular concentration of L-arginine by binding DNA as a transcriptional regulator (78). Using global gene expression analysis, we sought to identify *L. pneumophila* genes regulated by ArgR that contribute to the observed intracellular growth defect as well as those that could be used as reporters of the ArgR response to L-arginine.

RNA was extracted from a Δ*argR* mutant (GAH280) and its isogenic, wild-type (WT) parent strain (KS79) during exponential and postexponential growth in rich media. The transcrip-
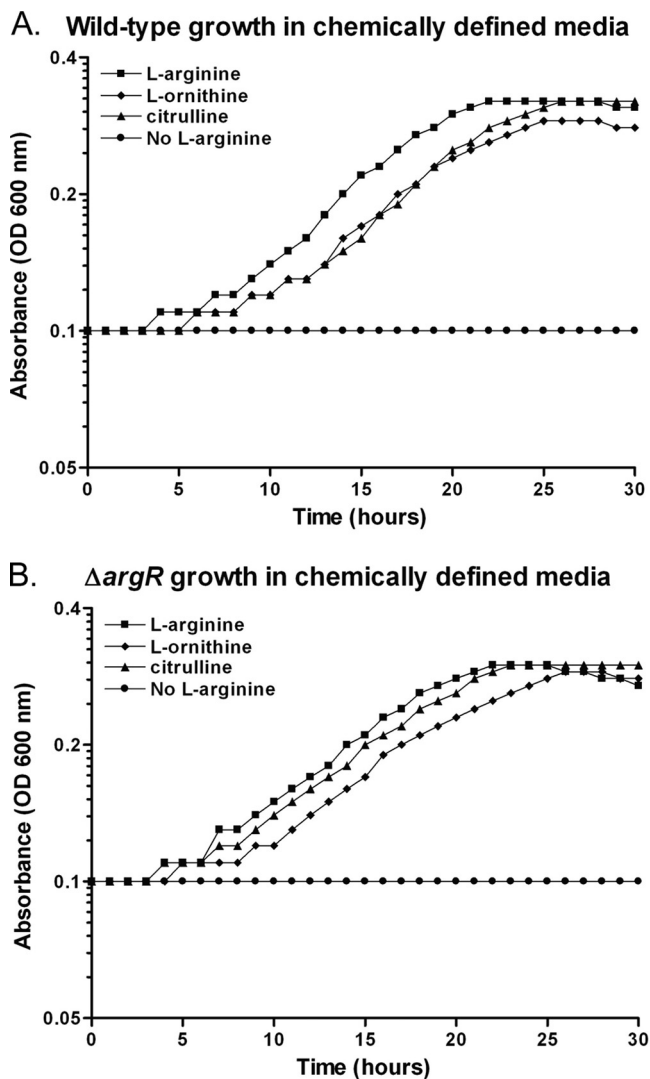
A. Wild-type growth in chemically defined media

B. ΔargR growth in chemically defined media

FIG. 2. Growth of wild-type and ΔargR mutant strains in CDM. Bacterial growth was monitored in a TECAN Infinite M200 plate reader at an absorbance of 600 nm. At time zero, bacteria were washed and diluted back to an OD of 0.10 in chemically defined medium (CDM) prepared either with L-arginine (1.0 mg/ml) (■), without L-arginine (no L-arginine) (●), without L-arginine but including L-ornithine (1.0 mg/ml) (◆), or without L-arginine but including citrulline (1.0 mg/ml) (▲). Absorbance (OD$_{600}$) was monitored for 30 h under the four CDM growth conditions at 37°C for both the ΔargR mutant (GAH280) (B) and its isogenic parent (KS79), the wild type (A).

FIG. 3. The *L. pneumophila* ΔargR microarray. Analysis of the transcriptional profile of a ΔargR mutant (GAH280) in comparison with its isogenic parent, wild-type (WT) strain KS79, during the exponential (E) and postexponential (PE) growth phases in AYE rich medium. ArgR-affected genes, defined by a mutant/wild-type log$_2$ ratio ± 1.5 and $P \leq 0.005$, were clustered using TIGR MeV (B). Depiction of the number of genes positively or negatively affected by ArgR in either phase of growth is shown as a Venn diagram (A). Genes negatively affected during both phases of growth are indicated by a vertical red bar (B).

tional profiles were compared by microarray analysis as previously described (36). For the purpose of this study, significantly affected genes were defined as having a log$_2$ ratio of mutant to wild-type ± 1.5 (3-fold) and with a P value of ≤0.005. The steady-state transcript level of 116 genes was significantly affected by the deletion of the *argR* gene during growth in rich media (Fig. 3A; see Table S1 in the supplemental material). The transcript levels of 12 genes were affected during the exponential phase, 11 of which were increased in the mutant. An increase in transcript level in the mutant background compared to the wild type suggests that the ArgR protein acts negatively on the transcription of these 11 genes. The tran-
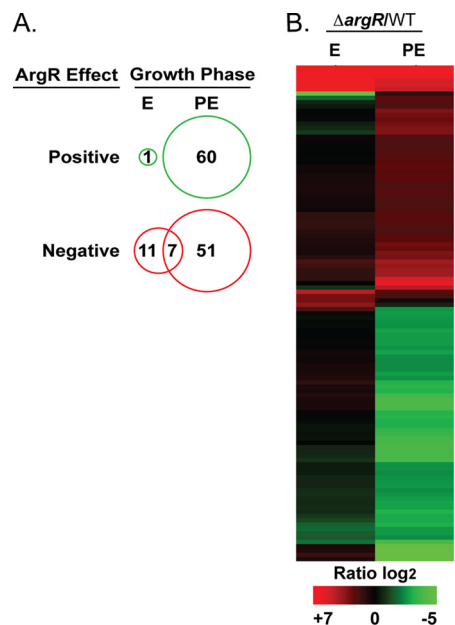
script levels of 111 genes were affected during the postexponential phase, 51 were increased in the mutant compared to the wild type, while the transcripts of another 60 genes were reduced in the mutant background. Reductions in gene transcripts in the mutant compared to the level in the wild type suggest that the ArgR protein acts positively on these genes. Seven of the 11 genes negatively affected by *argR* during the exponential phase are also negatively affected during the postexponential phase, representing the only set of genes that is similarly regulated during both phases of growth (Fig. 3B, red vertical bar). There are no genes that are positively affected by ArgR in one growth phase and negatively in the other. The changes in transcript levels of affected genes could reflect either the direct interaction of ArgR with their promoters or could occur indirectly through ArgR-affected regulators. Although additional experiments are required to determine if ArgR is acting directly on specific gene targets, we were able to identify putative ArgR operator sites in the promoter regions of many of affected genes identified from this microarray (see Table S2 in the supplemental material).

Categories of ArgR-affected genes. The global effects of the *argR* deletion were analyzed by determining the number of genes affected within specific functional categories (18) (Fig. 4A; see Table S1 in the supplemental material). In addition to amino acid metabolism, which was predicted to be affected by ArgR, the categories transport and binding, Icm/Dot-translocated substrates, nucleotide metabolism, and detoxification and stress adaptation also included a notable number of af-

ΔargR/WT		ORF	Protein	PE ΔargR/WT
E	PE			
		lpg2862	LegC8	-3.3
		lpg1948	LegLC4	-2.5
		lpg1702	PpeB	-2.4
		lpg2723	-	-2.3
		lpg1851	Lem14	-2.1
		lpg0483	LegA12	-1.9
		lpg2603	Lem28	-1.8
		lpg2409	Ceg29	-1.7
		lpg2511	SidC	-1.7
		lpg2410	VpdA	-1.7
		lpg2200	CegC4	-1.7
		lpg0634	-	-1.7
		lpg2510	SdcA	-1.7
		lpg0277	Ceg7	-1.6
		lpg2391	SbdC	-1.6
		lpg2137	LegK2	-1.5
		lpg1588	LegC6	-1.5

Pie chart legend (A):
- Transcription & Signal Transduction (2)
- Detoxification & Stress Adaptation (4)
- Nucleotide Metabolism (7)
- Amino Acid Metabolism (10)
- Icm/Dot Translocated Substrates (17)
- Transport & Binding (18)
- Other Functions (20)
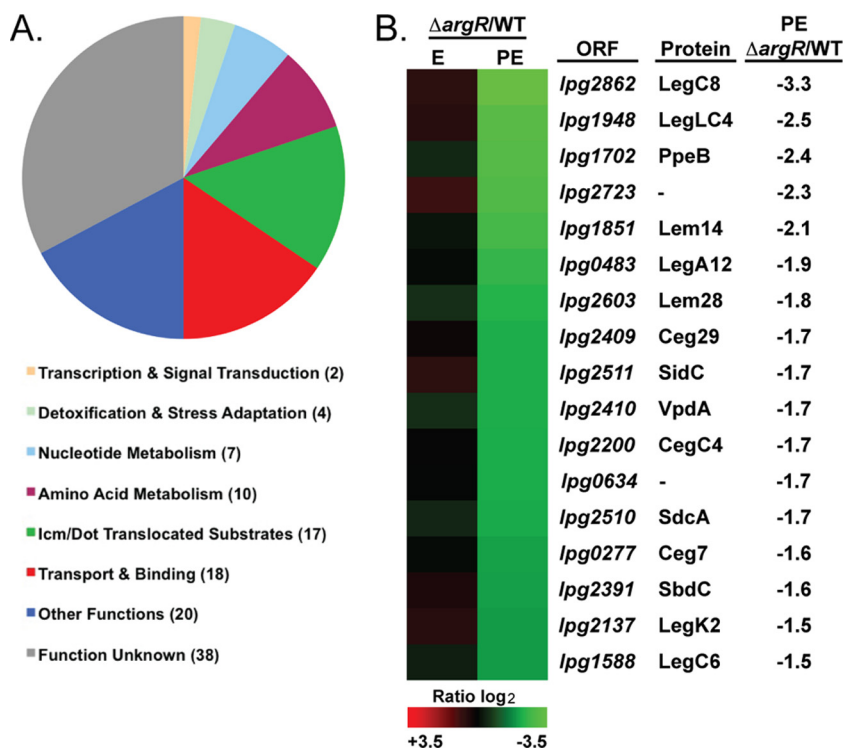- Function Unknown (38)

Ratio log$_2$
+3.5   -3.5

FIG. 4. Categories of ArgR-affected genes. The distribution of ArgR-affected genes in functional categories is illustrated in a pie chart, and the number of affected genes in each category is shown in parentheses (A). Heat mapping depicts ArgR-affected Icm/Dot translocated substrates along with the ORF, protein names, and the log$_2$ ratio (B).

fected genes (Fig. 4A). The deletion of the *argR* gene affected 18 genes categorized with transport and binding (Fig. 4A). The majority of genes categorized as transport and binding genes were negatively affected by ArgR (see Table S1 in the supplemental material) including the most highly ArgR-regulated genes in the genome, *lpg0491* to *lpg0493* (exponential-phase, log$_2$ ratio of +7.6 to 7.3), which encode a putative amino acid ABC transporter (18). In the postexponential phase, ArgR negatively affected components of the type IVA secretion system and TFBSS, which were also categorized with transport and binding (18). Two members of the Lvh Type IVA secretion system, *lvhB8* (*lpg1249*, log$_2$ ratio of 1.7) and *lvHB10* (*lpg1247*, log$_2$ ratio of 1.8), and three members of the Icm/Dot TFBSS, *icmR* (*lpg0443*, log$_2$ ratio of 1.8), *icmQ* (*lpg0444*, log$_2$ ratio of 1.7), and *icmG* (*lpg0452*, log$_2$ ratio of 1.6) were significantly affected in the absence of ArgR. Expression of these components of the Icm/Dot TFBSS of *L. pneumophila* is required for efficient intracellular replication (56, 65, 66, 71). Also included the category of transport and binding were the genes most positively affected by ArgR in the genome, *lpg0174* and *lpg0175* (postexponential phase, log$_2$ ratio of −5.5 and −4.0, respectively), which are predicted to encode pyoverdine biosynthetic proteins, PvcA and PvcB (18). In pseudomonads, pyoverdine is a powerful iron(III) scavenger and transporter (75), and in *L. pneumophila*, the *pvcA/B* genes are regulated by iron concentration (31). As seen previously, deletion of the *pvcA* and *pvcB* genes did not affect *L. pneumophila*'s ability to replicate in *A. castellanii* (3; this study and data not shown).

Deletion of the *argR* gene affected the transcript levels of 17 genes in the category Icm/Dot-translocated substrates (Fig. 4A and B; see Table S1 in the supplemental material). The category Icm/Dot-translocated substrates includes bacterial proteins demonstrated to be translocated into host cells during *Legionella* infection in an Icm/Dot TFBSS-dependent manner (9, 15, 37, 44, 53, 54, 61). To date, the translocation of 140 putative Icm/Dot substrates has been proven; however, the majority of their functions are unknown (9, 15, 37, 44, 53, 54, 61). The transcript level of 17 Icm/Dot-translocated substrates was reduced by the deletion of *argR* (Fig. 4B); thus, the ArgR regulator has a positive affect on the transcription of 12% of the members of this category, either directly or indirectly. Although many of the genes encoding Icm/Dot-translocated substrates are individually dispensable for intracellular multiplication, some have been shown to affect host cell trafficking. Understanding the specific contribution of Icm/Dot substrates and other ArgR-affected genes to *L. pneumophila* intracellular multiplication will require future investigation.

Genomic organization of the *L. pneumophila* ArgR regulon. ArgR has predicted binding sites based on numerous studies in other bacteria. In *E. coli*, the enzymes encoded by the genes *argA* to *argH* synthesize L-arginine from L-glutamate in a process that requires the *carA* and *carB* gene products to form carbamoyl-phosphate (46) (summarized in Fig. 5B). In *E. coli*, the genes required for L-arginine biosynthesis are distributed throughout the genome and transcriptionally repressed by ArgR in the presence of L-arginine, comprising the ArgR regulon (46). *L. pneumophila* lacks homologs of *argA*, *argB*, and *argC*, which explains its L-arginine auxotrophy (18, 26). This study
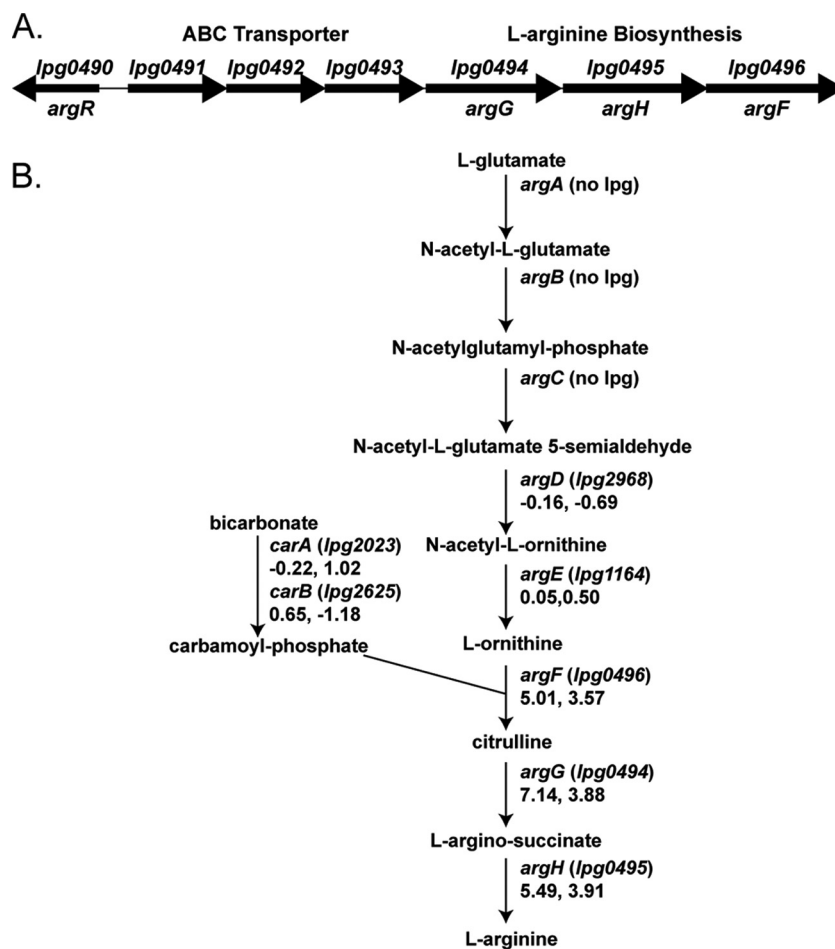
A.

ABC Transporter   **L-arginine Biosynthesis**

lpg0490 *lpg0491* *lpg0492* *lpg0493* *lpg0494* *lpg0495* *lpg0496*

argR   *argG*   *argH*   *argF*

B.

L-glutamate
    argA (no lpg)

N-acetyl-L-glutamate
    argB (no lpg)

N-acetylglutamyl-phosphate
    argC (no lpg)

N-acetyl-L-glutamate 5-semialdehyde
    argD (*lpg2968*)
    -0.16, -0.69

bicarbonate
carA (*lpg2023*)   N-acetyl-L-ornithine
-0.22, 1.02
carB (*lpg2625*)   *argE* (*lpg1164*)
0.65, -1.18   0.05, 0.50

carbamoyl-phosphate   L-ornithine
                                    argF (*lpg0496*)
                                    5.01, 3.57

                                 citrulline
                                    argG (*lpg0494*)
                                    7.14, 3.88

                              L-argino-succinate
                                    argH (*lpg0495*)
                                    5.49, 3.91

                                 L-arginine

FIG. 5. Genomic organization of L-arginine biosynthesis in *L. pneumophila*. The ORF encoding ArgR, *lpg490*, is divergent from a region spanning *lpg0491* to *lpg0496*, in which the genes exhibit strong negative regulation by ArgR (A). *lpg0491* to *-0493* are predicted to encode an amino acid ABC transporter, and *lpg0494* to *-0496* are predicted to encode the final steps of L-arginine biosynthesis (A). The biosynthetic pathway for the conversion of L-glutamate to L-arginine is shown with the *L. pneumophila* ORF associated with each enzymatic step, if present, in parentheses (B). The Δ*argR*/WT log$_2$ ratio is shown at each step below the ORF for the exponential and postexponential phases (B).

reveals that in *L. pneumophila*, the mutation of *argR* does not significantly affect the transcript level of homologs of the predicted ArgR-regulated genes *argD* (*lpg2968*), *argE* (*lpg1164*), *carA* (*lpg2023*), and *carB* (*lpg2625*) (Fig. 5B; log$_2$ ratio microarray data shown under gene names). Deletion of *argR* in *L. pneumophila* does, however, affect the transcript level of genes predicted to encode the terminal steps of L-arginine biosynthesis from L-ornithine; *argF* (*lpg0496*), *argG* (*lpg0494*), and *argH* (*lpg0495*), whose transcript levels are up by as much as 100-fold in the mutant (Fig. 5A; see Table S1 in the supplemental material).

In *L. pneumophila*, the genes predicted to encode the terminal steps of L-arginine biosynthesis are organized in one region of the genome along with a putative amino acid ABC transporter (*lpg0491* to *lpg0496*). The genomic organization of this region is conserved among all sequenced *L. pneumophila* strains (Philadelphia-1, Corby, Lens, and Paris). The transcript levels of *lpg0491* to *lpg0496* are increased by the deletion of *argR* by between 240- and 32-fold (*lpg0492* and *lpg0496*, respectively) in the exponential phase and between 36- and 12-fold (*lpg0492* and *lpg0496*, respectively) in the postexponential

phase (see Table S1 in the supplemental material). Thus, *lpg0491* to *lpg0496* constitute the most highly ArgR-repressed region of the genome and the only set of genes similarly regulated during both phases of growth (Fig. 3B, vertical red bar). In addition, *lpg0491* to *lpg0496* are directly downstream and divergent from the open reading frame (ORF) encoding ArgR, *lpg0490* (Fig. 5A). This organization is in stark contrast to the genomic organization of L-arginine biosynthesis in bacteria such as *E. coli*, in which the genes are in diverse locations throughout the genome and not proximal to the gene encoding the ArgR repressor (11, 17, 39, 45, 50, 62, 82).

To determine which genes in this region may be required for growth of *L. pneumophila* under different conditions, we replaced selected genes with antibiotic selection markers by allelic exchange (36). Deletion of either the putative ATPase of the predicted transporter (*lpg0493*) or the *argH* homolog (*lpg0495*), which is predicted to encode the terminal step of arginine biosynthesis, rendered bacteria unable to grow in CDM in the absence of L-arginine, even if L-ornithine (Fig. 6A) or citrulline (data not shown) were provided in its place. The deletion of the open reading frames *lpg0493* and *lpg0495* did
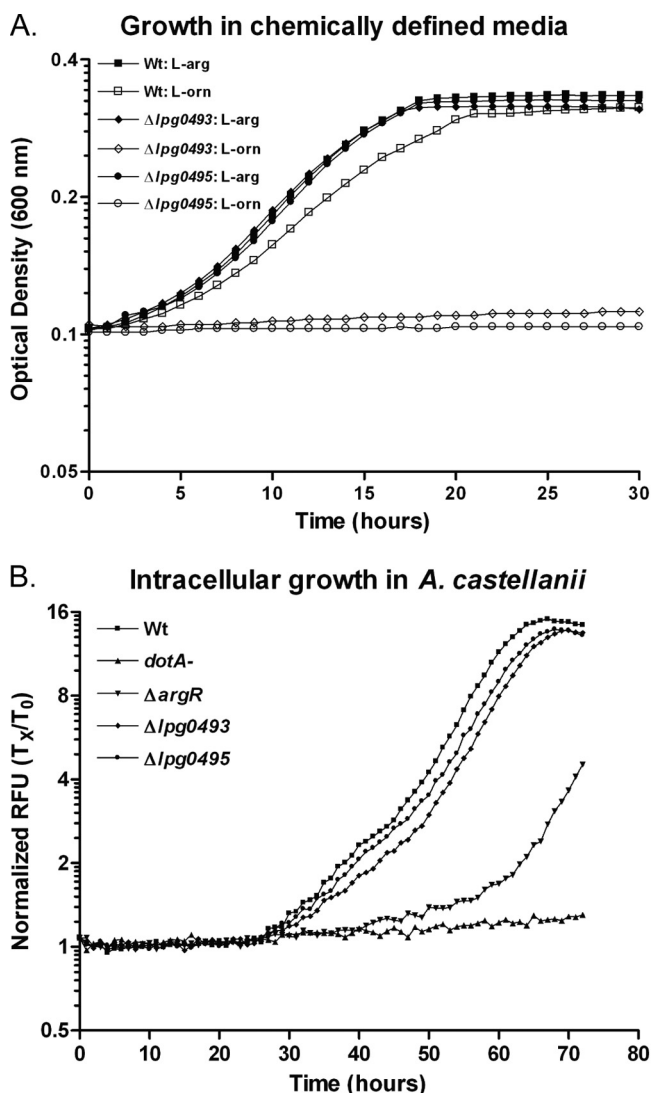
A. Growth in chemically defined media

B. Intracellular growth in *A. castellanii*

FIG. 6. Analysis of growth of the Δ*lpg0493* and Δ*lpg0495* mutants. Growth of the wild-type (Wt) strain KS79 and the Δ*lpg0493* (GAH318) and Δ*lpg0495* (GAH319) mutant strains in CDM containing L-arginine (1.0 mg/ml) (■, ◆, ●, respectively) or in CDM without L-arginine but including L-ornithine (1.0 mg/ml) (□, ◇, ○, respectively) was measured by absorbance at 600 nm in a TECAN Infinite M200 plate reader at 37°C for 30 h (A). The wild-type and mutant strains bearing the GFP⁺ plasmid pXDC31, wild-type strain GAH281 (■), and the *dotA* (GAH282) (▲), Δ*argR* (GAH286) (▼), Δ*lpg0493* (GAH339) (◆), and Δ*lpg0495* (GAH340) (●) mutant strains were used to infect *A. castellanii*, and intracellular growth was monitored by GFP fluorescence in a TECAN Infinite M200 fluorescence plate reader held at 30°C for 72 h following previously described methods (36) (B).
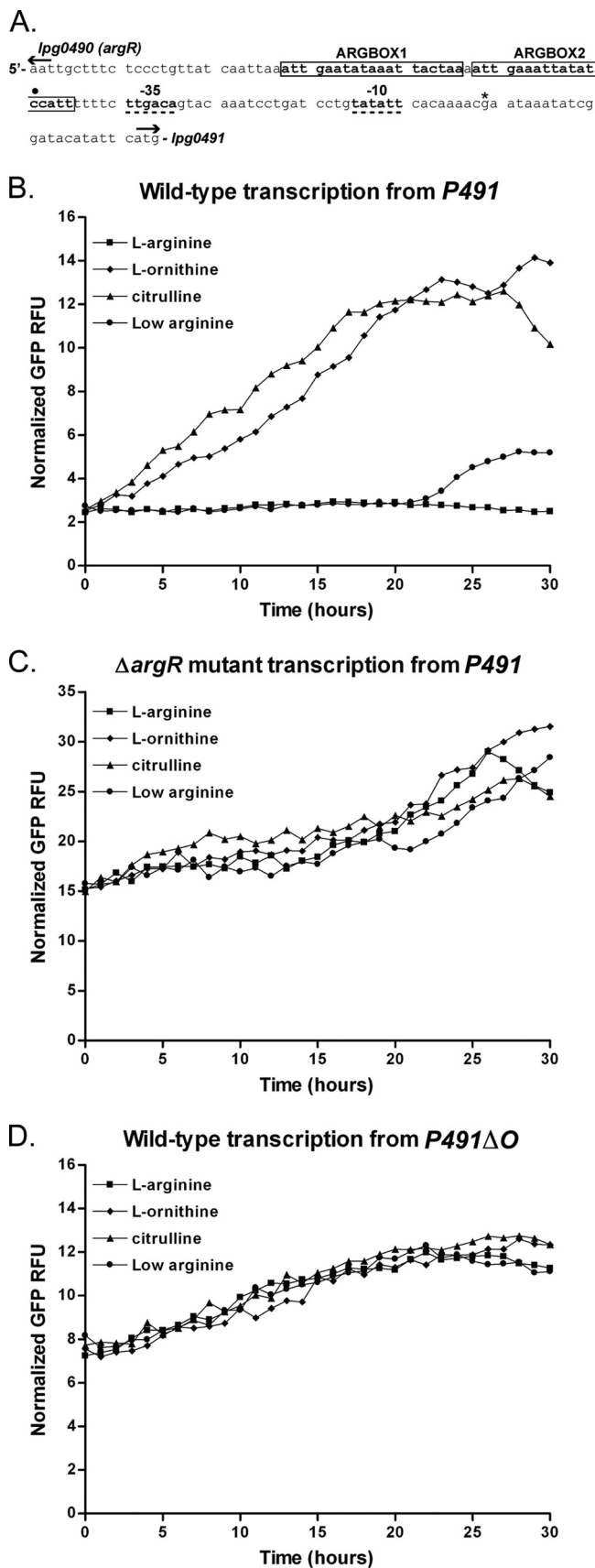
not result in a significant reduction in intracellular growth compared to the wild type (Fig. 6B). Addition of L-arginine to the infection buffer did not alter the wild-type or mutant strains' capacity for intracellular replication (data not shown). Therefore, this region is required for the utilization of L-arginine biosynthetic intermediates, as expected, but is not required for intracellular multiplication, from which we can infer that sufficient L-arginine is available in the LCV to support bacterial growth. Because the promoter of the region from

lpg0491 to *-0496* of the *L. pneumophila* genome is under strong negative control by ArgR and its transcription is predicted to respond to L-arginine, we chose it to use it as a reporter of ArgR function in subsequent studies.

Transcription from the *lpg0491* promoter is repressed by ArgR in the presence of L-arginine. Global gene expression analysis demonstrated that transcripts of genes *lpg0491* to *lpg0496* are highly increased in a Δ*argR* mutant compared to the wild type (see Table S1 in the supplemental material). We wanted to monitor transcription from this region during growth under different environmental conditions, such as low L-arginine concentration in CDM and during intracellular growth. To accomplish this goal, the 129-bp intergenic region between *argR* (*lpg0490*) and *lpg0491* was examined for possible regulatory elements (Fig. 7A). Based on its similarity to the *E. coli* consensus sequence (10), a putative σ⁷⁰ promoter-binding site was identified that ends 34 bp upstream of the *lpg0491* ORF (Fig. 7A, dashed underline). The ArgR repressor is known to bind to a conserved DNA-operator site that is composed of one or more degenerate palindromic sequences (wn GAATwwwwATTCAnw), which are called ARG boxes (13, 84). We identified two putative ARG boxes in the regulatory region preceding *lpg0491* (Fig. 7A), which we predict to be an ArgR operator site.

Transcriptional reporter gene fusions were designed to include the entire *lpg0491* regulatory region (*P491*) or only the putative promoter without the predicted ArgR operator site (*P491ΔO*). Both fragments have the same terminal 3′ base (Fig. 7A, asterisk), 22 bp upstream the *lpg0491* start codon. However, the fragments differ in their 5′ ends, which are 413 bp (outside of the data presented in Fig. 7A) and 71 bp (Fig. 7A, filled circle) upstream from the *lpg0491* start codon for *P491* and *P491ΔO*, respectively. The fragments were cloned into pXDC94, a plasmid containing a promoterless copy of the green fluorescent protein (GFP) gene to report the transcriptional activity of the cloned fragment (shown in Fig. 8A). The same plasmid also includes a copy of the gene encoding the red fluorescence protein mCherry under the control of the *Ptac* promoter and *lacI*�q repressor. When IPTG is added to the growth medium, the levels of mCherry fluorescence are proportional to the number of bacteria.

Reporter plasmids derived from pXDC94, containing *P491* (pGAH140) or *P491ΔO* (pGAH141), were introduced into the wild-type (KS79) and Δ*argR* (GAH280) mutant strains. The strains were grown in either CDM containing L-arginine or CDM in which L-arginine was replaced with L-ornithine, citrulline, or 10-fold less L-arginine (low arginine) in wells of a 96-well plate. The levels of absorbance (600 nm) as well as GFP and mCherry fluorescence were measured every hour for 30 h. In addition, measurements were made with isogenic strains bearing the control plasmid pXDC95, in which *Ptac* promotes the transcription of both GFP and mCherry fluorophores. The transcriptional profiles in Fig. 7 have been normalized to bacterial growth by mCherry (see Fig. S1 in the supplemental material), and the data presented are normalized to the levels of GFP fluorescence from an IPTG-induced *Ptac* promoter, as described in Materials and Methods. Thus, the levels of transcription indicated by the ratio of mCherry to GFP fluorescence shown in Fig. 7B, C, and D are independent of bacterial growth.

A.

lpg0490 (argR)

ARGBOX1   ARGBOX2

5'-aattgctttc tccctgttat caattaa**att gaatataaat tactaa**a**att gaaattatat**

-35 -10
•
ccattctttc **ttgaca**gtac aaatcctgat cctg**tatatt** cacaaaacga ataaatatcg
*

gatacatatt catg- → *lpg0491*

B.

Wild-type transcription from *P491*

C.

Δ*argR* mutant transcription from *P491*

D.

Wild-type transcription from *P491ΔO*

When wild-type *L. pneumophila* is grown in CDM that is replete with L-arginine (1.0 mg/ml), no increase in transcription can be measured from the *P491* fragment (Fig. 7B). However, when L-arginine is absent and L-ornithine or citrulline is provided, an approximate 12-fold increase in transcription is measured from this region that begins following time zero (Fig. 7B). If the CDM contains only one-tenth of the normal concentration of L-arginine (0.1 mg/ml), no increase in transcription is observed until more than 20 h into growth, which is presumably when the CDM begins to be depleted for L-arginine (Fig. 7B). The growth of cultures, measured by mCherry, containing reduced L-arginine ("low arginine") demonstrates a reduction in growth rate at the time corresponding to increased transcription (20 h) (see Fig. S1 in the supplemental material). Alternatively, when the same CDM growth experiment is conducted in the Δ*argR* mutant background, transcription from *P491* is approximately 12-fold higher at time zero than in the wild type. Transcription from *P491* in the Δ*argR* mutant background remains constitutively high regardless of the concentration of L-arginine (Fig. 7C). It is interesting to note that growth of the Δ*argR* mutant was more significantly reduced under the low-arginine condition than that of the wild type (see Fig. S1 in the supplemental material). The reporter fragment that does not include the putative ArgR operator site, *P491ΔO*, was transcriptionally active in the wild type independent of the concentration of L-arginine (Fig. 7D).

Taken together, these data show that transcription from the *lpg0491* promoter region is repressed when the environment is replete with L-arginine and this repression requires a wild-type copy of the *argR* gene. Data from the *P491ΔO* fragment demonstrate that the *lpg0491* promoter resides between 71 and 22 bp upstream from the start codon of the gene. These data also demonstrate that deletion of the region containing the putative ArgR operator site results in transcription that is not L-arginine repressible (Fig. 7D). Because the *P491* region is sensitive

FIG. 7. Transcription from the *lpg0491* promoter region. The intergenic region between *lpg0490* and *lpg0491* contains the putative −10 and −35 sites of the *lpg0491* promoter (bold and dashed underline) and two putative ARG boxes (bold with rectangular outline) (A). The translational start sites for *lpg0490* or *lpg0491* are depicted using divergent arrows (A). The terminal 3' base of transcriptional reporter fragments *P491* and *P491ΔO* is depicted by an asterisk, and the terminal 5' base of the transcriptional reporter fragment *P491ΔO* is depicted by a closed circle (●) (A). The 5' end of the *P491* transcriptional reporter fragment is 413 bp upstream from the translational start of *lpg0491* and is beyond the scope of this figure. The transcriptional reporter fragments *P491* and *P491ΔO* were cloned into pXDC94 (Fig. 8A) to produce pGAH140 and pGAH141, respectively. The growth in CDM (mCherry) (see Fig. S1 in the supplemental material) and GFP-reported transcription from the resulting wild-type and Δ*argR* strains was monitored in the context of a TECAN Infinite M200. CDM was prepared either with L-arginine (1.0 mg/ml) (■), with a reduced concentration of L-arginine ("Low arginine"; 0.1 mg/ml) (●), without L-arginine but including L-ornithine (1.0 mg/ml) (◆), or without L-arginine but including citrulline (1.0 mg/ml) (▲) for 30 h at 37°C (B, C, and D). Transcription data are shown for wild-type (GAH349) (B) and Δ*argR* mutant (GAH355) (C) strains bearing *P491* reporter plasmid (pGAH140) and a wild-type strain (GAH350) bearing the *P491ΔO* reporter plasmid (pGAH141) (D). All conditions have been normalized to growth (mCherry) and *Ptac* transcription as described in Materials and Methods.
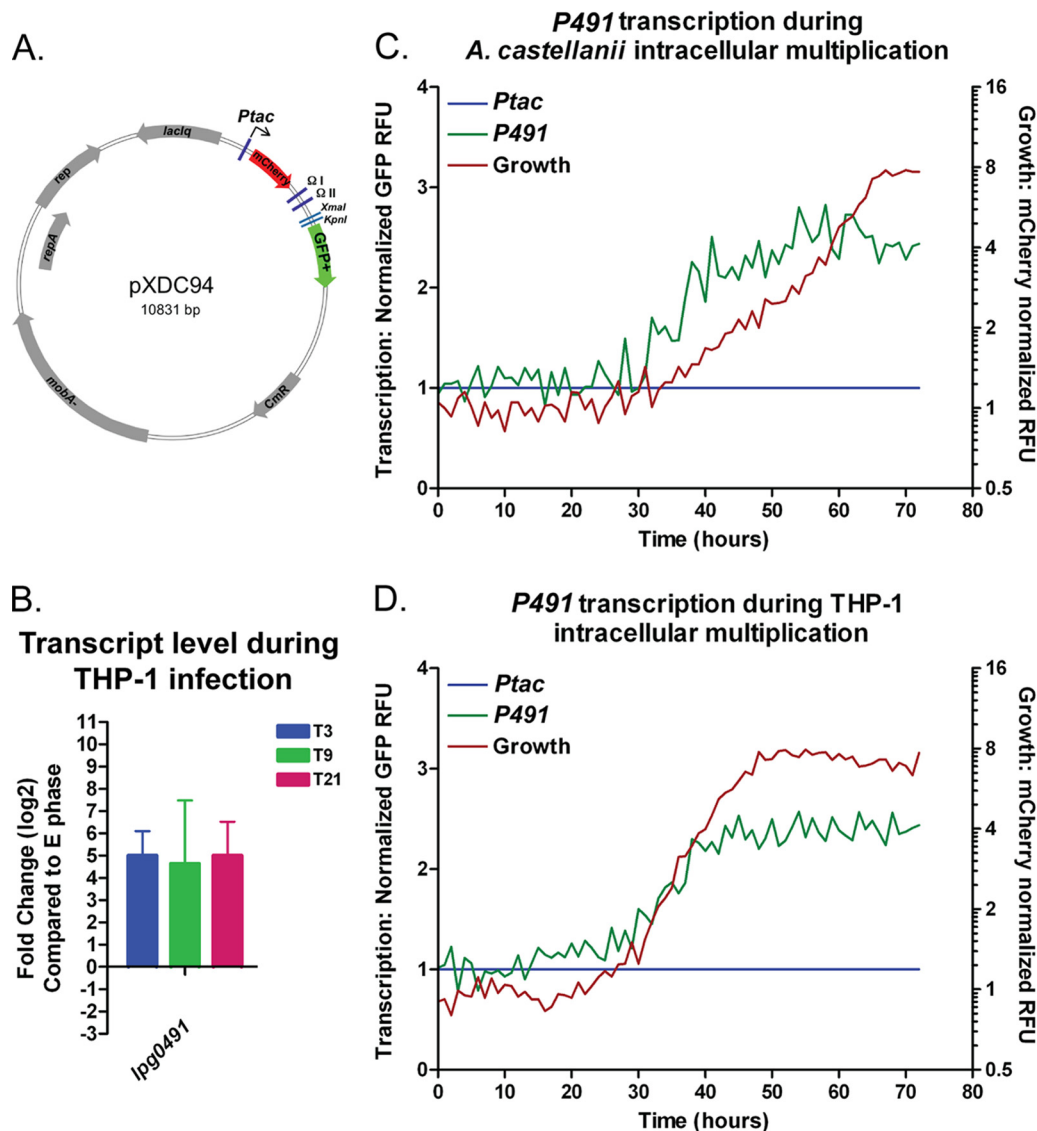
FIG. 8. Transcription during intracellular growth. Map of pXDC94 transcriptional reporter plasmid (A). Reporter fragments were cloned using XmaI and KpnI enzyme sites (A). Following the infection of THP-1 macrophages with wild-type strain KS79, transcripts of *lpg0491* were measure by qRT-PCR at 3, 9, and 21 h postinfection and compared to those of wild-type bacteria during the exponential growth phase (E phase) (B). Infection of *A. castellanii* and THP-1 macrophage cells in the context of a microplate spectrofluorometer was performed as described previously (36). During growth in both *A. castellanii* (C) and THP-1 cells (D) cells of the wild-type strain (GAH349) harboring the *P491* reporter plasmid (pGAH140) were monitored for intracellular replication (mCherry-normalized RFU on the secondary *y* axis; red line) and GFP transcription from *P491* reporter (on primary *y* axis; green line) alongside the wild type (GAH348) harboring pXDC95, from which GFP is *Ptac* transcribed. Transcription data (primary axis; green [*P491*] and blue [*Ptac*] lines) (C and D) was normalized to growth (mCherry; secondary axis red line) and *Ptac* (GFP) transcription, as described in Materials and Methods.

to the L-arginine concentration, in an ArgR-dependent manner, it was subsequently used to determine if L-arginine availability is a transcriptional regulatory signal during intracellular growth.

Transcription of genes coregulated by *argR* and L-arginine during intracellular growth. Deletion of the *argR* gene results in a reduced capacity for *L. pneumophila* intracellular growth in the unicellular protozoan *A. castellanii* (Fig. 6B) (36). Transcriptional repression of the promoter region preceding *lpg0491* (*P491*) is dependent upon ArgR and L-arginine (Fig. 6). These data suggested the possibility that ArgR re-

sponds to changes in the availability of L-arginine during intracellular growth. Having characterized the response of the *lpg0491* promoter region to ArgR and L-arginine (Fig. 6), we sought to determine if transcription from this region was affected during *L. pneumophila* infection. Using reporter plasmids derived from pXDC94 and the normalization method described in the previous section, we measured transcription from the *P491* promoter region, during *L. pneumophila* growth in both *A. castellanii* and THP-1 macrophage host cells (Fig. 8B and D). Due to the low number of bacteria used in this infection method, the transcription of GFP was not detectable prior

to the appearance of measurable levels of bacterial replication, which occurs at approximately 30 h postinfection for *A. castellanii* and 24 h postinfection for THP-1 cells. After this time, the pattern of *P491* transcription can be monitored by GFP production. Following measurable intracellular growth, transcription from *P491* increased over the next 5 h by between 2- and 3-fold in both host cell types (Fig. 8B and D).

To determine if derepression of ArgR-repressed genes occurs at earlier time points, we measured transcripts of *lpg0491* by qRT-PCR at 3, 9, and 21 h following the infection of THP-1 macrophages compared to exponential-phase growth in rich medium. By 3 h postinfection, transcripts of *lpg0491* were increased by a $\log_2$ ratio of approximately 5 (Fig. 8B), which is similar to the level of derepression observed in a Δ*argR* mutant compared to the wild type from the microarray (see Table S1 in the supplemental material). The *lpg0491* transcript levels were maintained through the final measurement at 21 h postinfection (Fig. 8B). Therefore, an increase in transcription during intracellular growth can be observed by either the fluorescence-based (Fig. 8C and D) or qRT-PCR (Fig. 8B) method. These data, in conjunction with the CDM experiments, demonstrate that genes whose transcription is responsive to ArgR and the concentration of L-arginine are derepressed during intracellular multiplication.

DISCUSSION

Intracellular replication of *L. pneumophila* requires the establishment of a specialized compartment within host cells, which occurs through a series of ordered steps that interfere with normal vesicular trafficking (33, 35). *L. pneumophila*'s ability to both survive and control this process is predicted to necessitate the sensing of and response to a myriad of signals (59). Shortly after its entry into host cells, the *L. pneumophila*-containing phagosome evades fusion with the lysosome to establish a prereplicative vacuole called the LCV (40). Determining the chemical and nutrient composition intracellular compartments and therefore identifying molecules that could signal this lifestyle change to a bacterium residing in the LCV is inherently challenging and may have to be achieved by proxy (5). Following our initial report that mutation of the *L. pneumophila argR* homolog resulted in a significant defect in intracellular growth (36), we sought to determine if the L-arginine availability is a previously unrecognized aspect of infection. In this study, we applied microarray analysis to identify the *L. pneumophila* ArgR regulatory targets (Fig. 3), used a promoter tightly controlled by ArgR to report its function (Fig. 7A), demonstrated repression in response to L-arginine (Fig. 7B and C), and showed derepression during intracellular growth from as early as 3 h postinfection (Fig. 8). These data strongly suggest that the concentration of L-arginine in the LCV is a transcriptional regulatory signal sensed by ArgR and thus affects *L. pneumophila* gene expression during intracellular growth.

Utilization of ArgR for the regulation of genes that are not traditionally associated with L-arginine could be a possible explanation for the reduced intracellular growth of an *argR* mutant. The Icm/Dot type IVB section system is required for the evasion of phagolysosome fusion and establishment of the LCV (56, 66). The genes encoding IcmR and IcmQ reside in

their own transcriptional units, and ArgR negatively affects their transcription in the postexponential phase (see Table S1 in the supplemental material). Based on the consensus sequence of *E. coli* ARG boxes (84) and the ARG boxes required for repression of *lpg0491* (Fig. 5 and 6), we identified putative ARG boxes in the promoter regions of both *icmR* and *icmQ* (see Table S2 in the supplemental material). The impact of L-arginine-responsive repression of *icm/dot* genes is unclear, but it could be involved in the proper timing and assembly of the type IVB section system. The Icm/Dot system is responsible for the translocation of protein substrates into the host cell (20). Our investigation found that ArgR positively affected transcripts of 17 of the 140 genes encoding Icm/Dot-translocated proteins during the postexponential phase (Fig. 4; see Table S1 in the supplemental material). This accounts for more than 15% of the total ArgR regulatory affect and 28% of the genes positively affected by ArgR. The genes encoding these Icm/Dot substrates are widely distributed throughout the genome, which makes their coordinate regulation in response to the deletion of the *argR* gene notable. Although the paradigm of ArgR regulation is transcriptional repression through steric inhibition of RNA polymerase promoter binding (19, 84), there is significant precedent for gene activation by ArgR, which also occurs through binding to promoter-proximal ARG boxes (42, 45, 48, 49). Putative ARG boxes for Icm/Dot genes and their substrates as well other ArgR-affected genes were compiled (see Table S2 in the supplemental material) and used to develop a *L. pneumophila* ArgR consensus binding site (see Fig. S2 in the supplemental material). For 6 of the 17 ArgR-affected genes encoding Icm/Dot-translocated substrates, putative ARG boxes were identified. The protein substrates of the Icm/Dot secretion system have been predicted to affect host cell functions, such as endocytic trafficking, in a manner that permits the establishment of the LCV and intracellular replication. Based on the results from this study, it is possible that the L-arginine concentration is a signal for the positive regulation of translocated proteins required for infection.

L-Arginine metabolism is known to be significant for many prokaryotic and eukaryotic intracellular pathogens (14, 27, 38, 57, 67, 80). This is likely due, in part, to the importance of L-arginine in host cell metabolism, especially among pathogens that replicate within macrophages. L-Arginine utilization resides at the crossroads of macrophage lineage determination (7). When activated to become MI macrophages, by lipopolysaccharide (LPS) and the appropriate cytokines, the cationic transporters (CATs) that import L-arginine and the inducible nitric oxide synthase (iNOS) are transcriptionally activated (7). The result is preferential usage of L-arginine by the iNOS pathway and conversion of L-arginine to nitric oxide (NO) and citrulline, the outcome of which can be destruction of intracellular parasites (7). Although CAT induction results in increased intracellular L-arginine, it is not a likely source of L-arginine for pathogens that reside in a vacuole because active transport of L-arginine across the vacuolar membrane is required (5). MII macrophages activate arginase to convert L-arginine into L-ornithine and urea (7). The L-ornithine produced from the arginase pathway is used to make polyamines and proline, which aid the cell in proliferation and collagen production (7). Thus, arginine utilization is a critical determinant in the formation of an MI (killing) versus MII (healing)

macrophage (7). In addition, it has been proposed that macrophages use nutrient deprivation as an intracellular antimicrobial mechanism (5). Precedence for the importance of L-arginine acquisition by an intracellular pathogen was first discovered in *Listeria monocytogenes*. An early study found that the arginine transporter *arpJ* was upregulated intracellularly (43), and more recently microarray analysis showed that transcripts of *L. monocytogenes* arginine biosynthetic genes increase during intracellular growth (14). In addition, ArgR activates the *adi* genes, required for acid tolerance, in *L. monocytogenes* and Δ*argR* mutants have a 10-fold reduction in survival during murine infection (67).

Here we have highlighted the potential significance of L-arginine availability during intracellular growth and presented the first analysis of the *L. pneumophila* ArgR regulon. Although this is a newly discovered aspect of intracellular gene regulation for *L. pneumophila*, it is not without precedent in other intracellular pathogens. Understanding how L-arginine availability functions as a transcriptional signal and determining its intracellular regulatory targets will require further investigation. Additional studies are also needed to fully elucidate the biochemical aspects, associations with other regulators, and involvement in intracellular replication of the *L. pneumophila* ArgR transcriptional regulator.

REFERENCES

1. **Abu Kwaik, Y.** 1996. The phagosome containing *Legionella pneumophila* within the protozoan *Hartmannella vermiformis* is surrounded by the rough endoplasmic reticulum. Appl. Environ. Microbiol. **62:**2022–2028.
2. **Al-Khodor, S., S. Kalachikov, I. Morozova, C. T. Price, and Y. Abu Kwaik.** 2009. The PmrA/PmrB two-component system of *Legionella pneumophila* is a global regulator required for intracellular replication within macrophages and protozoa. Infect. Immun. **77:**374–386.
3. **Allard, K. A., V. K. Viswanathan, and N. P. Cianciotto.** 2006. *lbtA and lbtB* are required for production of the *Legionella pneumophila* siderophore legiobactin. J. Bacteriol. **188:**1351–1363.
4. **Altman, E., and G. Segal.** 2008. The response regulator CpxR directly regulates expression of several *Legionella pneumophila* icm/dot components as well as new translocated substrates. J. Bacteriol. **190:**1985–1996.
5. **Appelberg, R.** 2006. Macrophage nutriprive antimicrobial mechanisms. J. Leukoc. Biol. **79:**1117–1128.
6. **Bachman, M. A., and M. S. Swanson.** 2004. Genetic evidence that *Legionella pneumophila* RpoS modulates expression of the transmission phenotype in both the exponential phase and the stationary phase. Infect. Immun. **72:** 2468–2476.
7. **Bansal, V., and J. B. Ochoa.** 2003. Arginine availability, arginase, and the immune response. Curr. Opin. Clin. Nutr. Metab. Care **6:**223–228.
8. **Burke, M., A. F. Merican, and D. J. Sherratt.** 1994. Mutant *Escherichia coli* arginine repressor proteins that fail to bind L-arginine, yet retain the ability to bind their normal DNA-binding sites. Mol. Microbiol. **13:**609–618.
9. **Burstein, D., T. Zusman, E. Degtyar, R. Viner, G. Segal, and T. Pupko.** 2009. Genome-scale identification of *Legionella pneumophila* effectors using a machine learning approach. PLoS Pathog. **5:**e1000508.
10. **Busby, S., and R. H. Ebright.** 1994. Promoter structure, promoter recognition, and transcription activation in prokaryotes. Cell **79:**743–746.
11. **Caldara, M., D. Charlier, and R. Cunin.** 2006. The arginine regulon of *Escherichia coli*: whole-system transcriptome analysis discovers new genes and provides an integrated view of arginine regulation. Microbiology **152:** 3343–3354.
12. **Cazalet, C., C. Rusniok, H. Bruggemann, N. Zidane, A. Magnier, L. Ma, M. Tichit, S. Jarraud, C. Bouchier, F. Vandenesch, F. Kunst, J. Etienne, P. Glaser, and C. Buchrieser.** 2004. Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. Nat. Genet. **36:**1165–1173.
13. **Charlier, D., M. Roovers, F. Van Vliet, A. Boyen, R. Cunin, Y. Nakamura, N. Glansdorff, and A. Pierard.** 1992. Arginine regulon of *Escherichia coli* K-12. A study of repressor-operator interactions and of in vitro binding affinities versus in vivo repression. J. Mol. Biol. **226:**367–386.
14. **Chatterjee, S. S., H. Hossain, S. Otten, C. Kuenne, K. Kuchmina, S. Machata, E. Domann, T. Chakraborty, and T. Hain.** 2006. Intracellular gene expression profile of *Listeria monocytogenes*. Infect. Immun. **74:**1323–1338.
15. **Chen, J., K. S. de Felipe, M. Clarke, H. Lu, O. R. Anderson, G. Segal, and H. A. Shuman.** 2004. Legionella effectors that promote nonlytic release from protozoa. Science **303:**1358–1361.
16. **Chen, J., M. Reyes, M. Clarke, and H. A. Shuman.** 2007. Host cell-dependent secretion and translocation of the LepA and LepB effectors of *Legionella pneumophila*. Cell. Microbiol. **9:**1660–1671.
17. **Cherney, L. T., M. M. Cherney, C. R. Garen, G. J. Lu, and M. N. James.** 2008. Crystal structure of the arginine repressor protein in complex with the DNA operator from *Mycobacterium tuberculosis*. J. Mol. Biol. **384:**1330–1340.
18. **Chien, M., I. Morozova, S. Shi, H. Sheng, J. Chen, S. M. Gomez, G. Asamani, K. Hill, J. Nuara, M. Feder, J. Rineer, J. J. Greenberg, V. Steshenko, S. H. Park, B. Zhao, E. Teplitskaya, J. R. Edwards, S. Pampou, A. Georghiou, I. C. Chou, W. Iannuccilli, M. E. Ulz, D. H. Kim, A. Geringer-Sameth, C. Goldsberry, P. Morozov, S. G. Fischer, G. Segal, X. Qu, A. Rzhetsky, P. Zhang, E. Cayanis, P. J. De Jong, J. Ju, S. Kalachikov, H. A. Shuman, and J. J. Russo.** 2004. The genomic sequence of the accidental pathogen *Legionella pneumophila*. Science **305:**1966–1968.
19. **Czaplewski, L. G., A. K. North, M. C. Smith, S. Baumberg, and P. G. Stockley.** 1992. Purification and initial characterization of AhrC: the regulator of arginine metabolism genes in *Bacillus subtilis*. Mol. Microbiol. **6:**267–275.
20. **de Felipe, K. S., R. T. Glover, X. Charpentier, O. R. Anderson, M. Reyes, C. D. Pericone, and H. A. Shuman.** 2008. Legionella eukaryotic-like type IV substrates interfere with organelle trafficking. PLoS Pathog. **4:**e1000117.
21. **de Felipe, K. S., S. Pampou, O. S. Jovanovic, C. D. Pericone, S. F. Ye, S. Kalachikov, and H. A. Shuman.** 2005. Evidence for acquisition of Legionella type IV secretion substrates via interdomain horizontal gene transfer. J. Bacteriol. **187:**7716–7726.
22. **Faucher, S. P., S. Porwollik, C. M. Dozois, M. McClelland, and F. Daigle.** 2006. Transcriptome of *Salmonella enterica* serovar Typhi within macrophages revealed through the selective capture of transcribed sequences. Proc. Natl. Acad. Sci. U. S. A. **103:**1906–1911.
23. **Fields, B. S.** 1996. The molecular ecology of legionellae. Trends Microbiol. **4:**286–290.
24. **Fraser, D. W., T. R. Tsai, W. Orenstein, W. E. Parkin, H. J. Beecham, R. G. Sharrar, et al.** 1977. Legionnaires' disease: description of an epidemic of pneumonia. N. Engl. J. Med. **297:**1189–1197.
25. **Gal-Mor, O., and G. Segal.** 2003. Identification of CpxR as a positive regulator of icm and dot virulence genes of *Legionella pneumophila*. J. Bacteriol. **185:**4908–4919.
26. **George, J. R., L. Pine, M. W. Reeves, and W. K. Harrell.** 1980. Amino acid requirements of *Legionella pneumophila*. J. Clin. Microbiol. **11:**286–291.
27. **Gobert, A. P., S. Daulouede, M. Lepoivre, J. L. Boucher, B. Bouteille, A. Buguet, R. Cespuglio, B. Veyret, and P. Vincendeau.** 2000. L-Arginine availability modulates local nitric oxide production and parasite killing in experimental trypanosomiasis. Infect. Immun. **68:**4653–4657.
28. **Grandori, R., T. A. Lavoie, M. Pflumm, G. Tian, H. Niersbach, W. K. Maas, R. Fairman, and J. Carey.** 1995. The DNA-binding domain of the hexameric arginine repressor. J. Mol. Biol. **254:**150–162.
29. **Hales, L. M., and H. A. Shuman.** 1999. The *Legionella pneumophila* rpoS gene is required for growth within *Acanthamoeba castellanii*. J. Bacteriol. **181:**4879–4889.
30. **Hammer, B. K., E. S. Tateda, and M. S. Swanson.** 2002. A two-component regulator induces the transmission phenotype of stationary-phase *Legionella pneumophila*. Mol. Microbiol. **44:**107–118.
31. **Hindre, T., H. Bruggemann, C. Buchrieser, and Y. Hechard.** 2008. Transcriptional profiling of *Legionella pneumophila* biofilm cells and the influence of iron on biofilm formation. Microbiology **154:**30–41.
32. **Horwitz, M. A.** 1983. Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila*) in human monocytes. J. Exp. Med. **158:**1319–1331.
33. **Horwitz, M. A.** 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. **158:**2108–2126.
34. **Horwitz, M. A., and F. R. Maxfield.** 1984. *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. J. Cell Biol. **99:**1936–1943.
35. **Horwitz, M. A., and S. C. Silverstein.** 1980. Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. J. Clin. Invest. **66:**441–450.
36. **Hovel-Miner, G., S. Pampou, S. P. Faucher, M. Clarke, I. Morozova, P. Morozov, J. J. Russo, H. A. Shuman, and S. Kalachikov.** 2009. σˢ controls

multiple pathways associated with intracellular multiplication of *Legionella pneumophila*. J. Bacteriol. **191:**2461–2473.

37. **Ingmundson, A., A. Delprato, D. G. Lambright, and C. R. Roy.** 2007. *Legionella pneumophila* proteins that regulate Rab1 membrane cycling. Nature **450:**365–369.

38. **Iniesta, V., L. C. Gomez-Nieto, and I. Corraliza.** 2001. The inhibition of arginase by N(omega)-hydroxy-l-arginine controls the growth of Leishmania inside macrophages. J. Exp. Med. **193:**777–784.

39. **Jin, L., W. F. Xue, J. W. Fukayama, J. Yetter, M. Pickering, and J. Carey.** 2005. Asymmetric allosteric activation of the symmetric ArgR hexamer. J. Mol. Biol. **346:**43–56.

40. **Kagan, J. C., and C. R. Roy.** 2002. *Legionella* phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. Nat. Cell Biol. **4:**945–954.

41. **Karaivanova, I. M., P. Weigel, M. Takahashi, C. Fort, A. Versavaud, G. Van Duyne, D. Charlier, J. N. Hallet, N. Glansdorff, and V. Sakanyan.** 1999. Mutational analysis of the thermostable arginine repressor from *Bacillus stearothermophilus*: dissecting residues involved in DNA binding properties. J. Mol. Biol. **291:**843–855.

42. **Kiupakis, A. K., and L. Reitzer.** 2002. ArgR-independent induction and ArgR-dependent superinduction of the *astCADBE* operon in *Escherichia coli*. J. Bacteriol. **184:**2940–2950.

43. **Klarsfeld, A. D., P. L. Goossens, and P. Cossart.** 1994. Five *Listeria monocytogenes* genes preferentially expressed in infected mammalian cells: *plcA, purH, purD, pyrE* and an arginine ABC transporter gene, *arpJ*. Mol. Microbiol. **13:**585–597.

44. **Kubori, T., A. Hyakutake, and H. Nagai.** 2008. Legionella translocates an E3 ubiquitin ligase that has multiple U-boxes with distinct functions. Mol. Microbiol. **67:**1307–1319.

45. **Larsen, R., G. Buist, O. P. Kuipers, and J. Kok.** 2004. ArgR and AhrC are both required for regulation of arginine metabolism in *Lactococcus lactis*. J. Bacteriol. **186:**1147–1157.

46. **Lim, D. B., J. D. Oppenheim, T. Eckhardt, and W. K. Maas.** 1987. Nucleotide sequence of the *argR* gene of *Escherichia coli* K-12 and isolation of its product, the arginine repressor. Proc. Natl. Acad. Sci. U. S. A. **84:**6697–6701.

47. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods **25:**402–408.

48. **Lu, C. D., and A. T. Abdelal.** 1999. Role of ArgR in activation of the *ast* operon, encoding enzymes of the arginine succinyltransferase pathway in *Salmonella typhimurium*. J. Bacteriol. **181:**1934–1938.

49. **Lu, C. D., H. Winteler, A. Abdelal, and D. Haas.** 1999. The ArgR regulatory protein, a helper to the anaerobic regulator ANR during transcriptional activation of the *arcD* promoter in *Pseudomonas aeruginosa*. J. Bacteriol. **181:**2459–2464.

50. **Lu, C. D., Z. Yang, and W. Li.** 2004. Transcriptome analysis of the ArgR regulon in *Pseudomonas aeruginosa*. J. Bacteriol. **186:**3855–3861.

51. **Lynch, D., N. Fieser, K. Gloggler, V. Forsbach-Birk, and R. Marre.** 2003. The response regulator LetA regulates the stationary-phase stress response in *Legionella pneumophila* and is required for efficient infection of *Acanthamoeba castellanii*. FEMS Microbiol. Lett. **219:**241–248.

52. **Maas, W. K.** 1994. The arginine repressor of *Escherichia coli*. Microbiol. Rev. **58:**631–640.

53. **Machner, M. P., and R. R. Isberg.** 2007. A bifunctional bacterial protein links GDI displacement to Rab1 activation. Science **318:**974–977.

54. **Machner, M. P., and R. R. Isberg.** 2006. Targeting of host Rab GTPase function by the intravacuolar pathogen *Legionella pneumophila*. Dev. Cell **11:**47–56.

55. **Makarova, K. S., A. A. Mironov, and M. S. Gelfand.** 2001. Conservation of the binding site for the arginine repressor in all bacterial lineages. Genome Biol. **2:**RESEARCH0013.

56. **Marra, A., S. J. Blander, M. A. Horwitz, and H. A. Shuman.** 1992. Identification of a *Legionella pneumophila* locus required for intracellular multiplication in human macrophages. Proc. Natl. Acad. Sci. U. S. A. **89:**9607–9611.

57. **McConville, M. J., D. de Souza, E. Saunders, V. A. Likic, and T. Naderer.** 2007. Living in a phagolysosome; metabolism of *Leishmania* amastigotes. Trends Parasitol. **23:**368–375.

58. **McDade, J. E., C. C. Shepard, D. W. Fraser, T. R. Tsai, M. A. Redus, and W. R. Dowdle.** 1977. Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease. N. Engl. J. Med. **297:**1197–1203.

59. **Molofsky, A. B., and M. S. Swanson.** 2004. Differentiate to thrive: lessons from the *Legionella pneumophila* life cycle. Mol. Microbiol. **53:**29–40.

60. **Muder, R. R., V. L. Yu, and G. D. Fang.** 1989. Community-acquired Legionnaires' disease. Semin. Respir. Infect. **4:**32–39.

61. **Nagai, H., J. C. Kagan, X. Zhu, R. A. Kahn, and C. R. Roy.** 2002. A bacterial guanine nucleotide exchange factor activates ARF on *Legionella phagosomes*. Science **295:**679–682.

62. **Ni, J., V. Sakanyan, D. Charlier, N. Glansdorff, and G. D. Van Duyne.** 1999. Structure of the arginine repressor from *Bacillus stearothermophilus*. Nat. Struct. Biol. **6:**427–432.

63. **Ninio, S., and C. R. Roy.** 2007. Effector proteins translocated by *Legionella pneumophila*: strength in numbers. Trends Microbiol. **15:**372–380.

64. **Reeves, M. W., L. Pine, S. H. Hutner, J. R. George, and W. K. Harrell.** 1981. Metal requirements of *Legionella pneumophila*. J. Clin. Microbiol. **13:**688–695.

65. **Ridenour, D. A., S. L. Cirillo, S. Feng, M. M. Samrakandi, and J. D. Cirillo.** 2003. Identification of a gene that affects the efficiency of host cell infection by *Legionella pneumophila* in a temperature-dependent fashion. Infect. Immun. **71:**6256–6263.

66. **Roy, C. R., K. H. Berger, and R. R. Isberg.** 1998. *Legionella pneumophila* DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. Mol. Microbiol. **28:**663–674.

67. **Ryan, S., M. Begley, C. G. Gahan, and C. Hill.** 2009. Molecular characterization of the arginine deiminase system in *Listeria monocytogenes*: regulation and role in acid tolerance. Environ. Microbiol. **11:**432–445.

68. **Sadosky, A. B., L. A. Wiater, and H. A. Shuman.** 1993. Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. Infect. Immun. **61:**5361–5373.

69. **Saeed, A. I., V. Sharov, J. White, J. Li, W. Liang, N. Bhagabati, J. Braisted, M. Klapa, T. Currier, M. Thiagarajan, A. Sturn, M. Snuffin, A. Rezantsev, D. Popov, A. Ryltsov, E. Kostukovich, I. Borisovsky, Z. Liu, A. Vinsavich, V. Trush, and J. Quackenbush.** 2003. TM4: a free, open-source system for microarray data management and analysis. Biotechniques **34:**374–378.

70. **Segal, G., M. Purcell, and H. A. Shuman.** 1998. Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. Proc. Natl. Acad. Sci. U. S. A. **95:**1669–1674.

71. **Segal, G., J. J. Russo, and H. A. Shuman.** 1999. Relationships between a new type IV secretion system and the *icm/dot* virulence system of *Legionella pneumophila*. Mol. Microbiol. **34:**799–809.

72. **Segal, G., and H. A. Shuman.** 1998. How is the intracellular fate of the *Legionella pneumophila* phagosome determined? Trends Microbiol. **6:**253–255.

73. **Sinai, A. P., and K. A. Joiner.** 1997. Safe haven: the cell biology of nonfusogenic pathogen vacuoles. Annu. Rev. Microbiol. **51:**415–462.

74. **Steinert, M., U. Hentschel, and J. Hacker.** 2002. *Legionella pneumophila*: an aquatic microbe goes astray. FEMS Microbiol. Rev. **26:**149–162.

75. **Stintzi, A., Z. Johnson, M. Stonehouse, U. Ochsner, J. M. Meyer, M. L. Vasil, and K. Poole.** 1999. The *pvc* gene cluster of *Pseudomonas aeruginosa*: role in synthesis of the pyoverdine chromophore and regulation by PtxR and PvdS. J. Bacteriol. **181:**4118–4124.

76. **Sunnerhagen, M., M. Nilges, G. Otting, and J. Carey.** 1997. Solution structure of the DNA-binding domain and model for the complex of multifunctional hexameric arginine repressor with DNA. Nat. Struct. Biol. **4:**819–826.

77. **Swanson, M. S., and B. K. Hammer.** 2000. *Legionella pneumophila* pathogesesis: a fateful journey from amoebae to macrophages. Annu. Rev. Microbiol. **54:**567–613.

78. **Szwajkajzer, D., L. Dai, J. W. Fukayama, B. Abramczyk, R. Fairman, and J. Carey.** 2001. Quantitative analysis of DNA binding by the *Escherichia coli* arginine repressor. J. Mol. Biol. **312:**949–962.

79. **Talaat, A. M., S. T. Howard, W. T. Hale, R. Lyons, H. Garner, and S. A. Johnston.** 2002. Genomic DNA standards for gene expression profiling in *Mycobacterium tuberculosis*. Nucleic Acids Res. **30:**e104.

80. **Talaue, M. T., V. Venketaraman, M. H. Hazbon, M. Peteroy-Kelly, A. Seth, R. Colangeli, D. Alland, and N. D. Connell.** 2006. Arginine homeostasis in J774.1 macrophages in the context of *Mycobacterium bovis* BCG infection. J. Bacteriol. **188:**4830–4840.

81. **Tesh, M. J., S. A. Morse, and R. D. Miller.** 1983. Intermediary metabolism in *Legionella pneumophila*: utilization of amino acids and other compounds as energy sources. J. Bacteriol. **154:**1104–1109.

82. **Tian, G., and W. K. Maas.** 1994. Mutational analysis of the arginine repressor of *Escherichia coli*. Mol. Microbiol. **13:**599–608.

83. **Van Duyne, G. D., G. Ghosh, W. K. Maas, and P. B. Sigler.** 1996. Structure of the oligomerization and L-arginine binding domain of the arginine repressor of *Escherichia coli*. J. Mol. Biol. **256:**377–391.

84. **Wang, H., N. Glansdorff, and D. Charlier.** 1998. The arginine repressor of *Escherichia coli* K-12 makes direct contacts to minor and major groove determinants of the operators. J. Mol. Biol. **277:**805–824.

85. **Warren, W. J., and R. D. Miller.** 1979. Growth of Legionnaires disease bacterium (*Legionella pneumophila*) in chemically defined medium. J. Clin. Microbiol. **10:**50–55.