INFECTION AND IMMUNITY, Jan. 2009, p. 374–386 Vol. 77, No. 1
0019-9567/09/$08.00+0 doi:10.1128/IAI.01081-08
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The PmrA/PmrB Two-Component System of *Legionella pneumophila* Is a Global Regulator Required for Intracellular Replication within Macrophages and Protozoa▽†

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Received 29 August 2008/Returned for modification 5 October 2008/Accepted 10 October 2008

To examine the role of the PmrA/PmrB two-component system (TCS) of *Legionella pneumophila* in global gene regulation and in intracellular infection, we constructed *pmrA* and *pmrB* isogenic mutants by allelic exchange. Genome-wide microarray gene expression analyses of the *pmrA* and *pmrB* mutants at both the exponential and the postexponential phases have shown that the PmrA/PmrB TCS has a global effect on the expression of 279 genes classified into nine groups of genes encoding eukaryotic-like proteins, Dot/Icm apparatus and secreted effectors, type II-secreted proteins, regulators of the postexponential phase, stress response genes, flagellar biosynthesis genes, metabolic genes, and genes of unknown function. Forty-one genes were differentially regulated in the *pmrA* or *pmrB* mutant, suggesting a possible cross talk with other TCSs. The *pmrB* mutant is more sensitive to low pH than the *pmrA* mutant and the wild-type strain, suggesting that acidity may trigger this TCS. The *pmrB* mutant exhibits a significant defect in intracellular proliferation within human macrophages, *Acanthamoeba polyphaga*, and the ciliate *Tetrahymena pyriformis*. In contrast, the *pmrA* mutant is defective only in the ciliate. Despite the intracellular growth defect within human macrophages, phagosomes harboring the *pmrB* mutant exclude late endosomal and lysosomal markers and are remodeled by the rough endoplasmic reticulum. Similar to the *dot*/*icm* mutants, the intracellular growth defect of the *pmrB* mutant is totally rescued *in cis* within communal phagosomes harboring the wild-type strain. We conclude that the PmrA/PmrB TCS has a global effect on gene expression and is required for the intracellular proliferation of *L. pneumophila* within human macrophages and protozoa. Differences in gene regulation and intracellular growth phenotypes between the *pmrA* and *pmrB* mutant suggests a cross talk with other TCSs.

Legionella pneumophila, the causative agent of Legionnaires' disease, is an intracellular bacterium that replicates within protozoa and human macrophages (40, 43, 75). Protozoa are the primary hosts of *L. pneumophila* in the natural aquatic environment (3, 7, 36, 40, 51, 58, 73). Infection of the human host is considered an accidental diversion from the natural life cycle within protozoa (36, 51). When water aerosol containing *L. pneumophila* is inhaled or contaminated water is aspirated, *L. pneumophila* enters the human lung and infects alveolar macrophages and epithelial cells, leading to an atypical pneumonia known as Legionnaires' disease (76, 77). After entry, the *Legionella*-containing phagosomes evade the default endocytic traffic and intercept endoplasmic reticulum (ER) vesicles to establish a replicative niche (16, 42, 45, 59, 70, 74).

Governed by a biphasic life cycle within amoeba host, *L. pneumophila* alternates between a replicative form and a mature intracellular form that is highly infectious to cells and resistant to environmental stress (28, 29, 33, 38). In vitro, this phenotypic modulation triggered upon transition from the exponential (E) to the postexponential (PE) phase requires a delicate regulatory cascade that can be triggered by nutrient limitation (28, 33, 38). At the PE phase, *L. pneumophila* relies on two ppGpp synthases, RelA and SpoT, both of which are essential for differentiation and phenotypic modification at the PE phase. Synthesis of ppGpp in response to amino acid starvation is RelA dependent (33, 34). Whereas *relA* mutant had no defective phenotype in macrophages, the *relA*/*spoT* double mutant is totally defective. The accumulation of the alarmone molecule ppGpp stimulates the LetA/LetS two-component system (TCS), the sigma factors RpoS, RpoN, RpoD, and FliA, and the mRNA-binding repressor protein (CsrA), leading to a phenotypic switch from the intracellular replicative form to the transmissive form (28, 33, 34, 38, 52, 61, 80).

The Dot/Icm type IV secretion system, which is encoded by 26 genes, is required for phagosome biogenesis and intracellular proliferation (27, 63, 64). *L. pneumophila* modulates the trafficking of its phagosome via the action of Dot/Icm-translocated effector proteins (19, 45, 46, 71). The regulation of expression of genes encoding both the Dot/Icm apparatus and some of its substrates has been proposed to be mediated in part by the regulatory cascades triggered at the PE phase (25). Recent work has shown a role for the PmrA/PmrB TCS in the regulation of expression of several genes encoding Dot/Icm-secreted effectors in *L. pneumophila* (79). The PmrA/PmrB TCS is a bacterial signal transduction system that mediates bacterial responses to various stimuli (39), which may be biotic or abiotic and may be triggered via quorum sensing (37). This

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† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 20 October 2008.

TCS consists of a membrane-bound sensor protein (PmrB) that monitors the environment and responds to a specific signal (23) to activate a cognate response regulator protein (PmrA). The response regulator then recognizes and binds to a specific DNA sequence, leading to the modulation of transcription (23). The number of TCSs in *L. pneumophila* is substantially lower than in other bacteria such as *Escherichia coli*, which was estimated to harbor 40 different sensor-regulator pairs (49). The PmrA/PmrB TCS is conserved in all four published genomes of *L. pneumophila*: Lens, Paris, Corby, and Philadelphia-1 (12–15, 65). In *Salmonella enterica* serovar Typhimurium and *Pseudomonas aeruginosa*, the PmrA/PmrB system has been shown to regulate genes that modify lipopolysaccharide and confer bacterial resistance to cationic antimicrobial peptides and polymyxin B and is triggered in response to limiting Mg^{2+} conditions, high levels of Fe^{3+}, and low pH (31, 57). PmrA was identified as a major regulator of the *feoAB* operon in *P. aeruginosa* and *S. enterica* serovar Typhimurium; this locus is known to be involved in iron acquisition and assimilation in *L. pneumophila* as well (22, 48, 56). In *L. pneumophila*, PmrA plays a role in regulating several Dot/Icm-secreted effectors (6, 21, 79), but the environmental signal activating PmrB is unknown.

Zusman et al. previously showed that the PmrA response regulator of *L. pneumophila* promotes the intracellular infection of HL-60 macrophages (79). However, the role of *L. pneumophila* PmrB in the intracellular infection, as well as in the regulation of expression of *L. pneumophila* virulence traits, remains unknown. We characterized here both the *pmrA* and the *pmrB* mutants of *L. pneumophila.* We show that PmrB is involved in the intracellular infection of macrophages and amoebas and that both PmrA and PmrB are necessary for the infection of ciliates. Despite its growth defect, the *pmrB* mutant is not required for evasion of the endocytic pathway, and its defect is totally rescued in the communal phagosome established by the wild-type (WT) strain. The *pmrB* mutant is more sensitive to acidic environments compared to the WT strain, suggesting that low pH may trigger the PmrA/PmrB TCS. Genome-wide microarray analyses suggest a central role for PmrA in the regulation of the *L. pneumophila* life cycle, and a possible cross talk between the PmrA/PmrB TCS and other *L. pneumophila* TCSs is proposed.

MATERIALS AND METHODS

DNA manipulations. DNA manipulations and restriction enzyme digestions were performed by using standard procedures. Restriction enzymes and T4 DNA ligase were purchased from Promega (Madison, WI). *L. pneumophila* chromosomal DNA was prepared by using the Puregene DNA isolation kit from Gentra Systems (Minneapolis, MN). Plasmid preparations were performed with the Bio-Rad Quantum miniprep kit. Purification of DNA fragments from agarose gels for subcloning was carried out with a QIAquick gel purification kit (Qiagen, Inc., Valencia, CA). Fragments containing *L. pneumophila pmrA* and *pmrB* genes were cloned into the plasmid vector pBC-SK^+ (Stratagene, Inc., La Jolla, CA), and the resulting clone was mutagenized by using an EZ-Tn*5*<KAN-2> in vitro transposome insertion kit from Epicentre. Transformation of *E. coli* strain DH5α by electroporation was performed with a BTX ECM 630, as recommended by Invitrogen Corp. (Carlsbad, CA). Mutations of the parental strain AA100 were carried out by allelic exchange with the kanamycin (Kan) insertion mutagenized *pmrA* and *pmrB* clone after natural transformation, as previously described (67, 68). The isogenic *pmrA* and *pmrB* mutants were transcomplemented with the same plasmid vector harboring the corresponding gene that was used as a template for mutagenesis. The primer pairs used to amplify the *L. pneumophila pmrA* and *pmrB* genes by PCR were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and are as follows: *pmrA*, forward (TTACTTGTTG AAGACGATGAAC) and reverse (ACGTATAGTGCGGATAAAGTT) and *pmrB*, forward (CTATTACTACAACATTAACTGCTATT) and reverse (TTTT GGTTTTGTTTTGATGG). Kan insertion was verified by PCR using the manufacturer Kan primers and either the forward or reverse primer for each gene. In both genes, Kan was inserted in the first 300 bp.

Bacterial strains and media. The virulent strain of *L. pneumophila* AA100/130b (ATCC BAA-74) is a clinical isolate that has been described previously (2). The isogenic *dotA* and *htrA* mutants of strain *L. pneumophila* AA100 has been described previously (26, 27, 54). Bacteria were grown from frozen stocks on buffered charcoal-yeast extract (BCYE) agar at 37°C or in buffered yeast extract (BYE) broth at 37°C with shaking (20) for 3 days. The plates and broth used for the cultivation of the mutants were supplemented with 50 μg of Kan/ml. *E. coli* strain DH5α was used as surrogate to clone the *pmrA* and *pmrB* genes. The WT *L. pneumophila* that was used for the confocal rescue studies harbored the plasmid pAM239, which encodes *gfp* (54). The plates for the *gfp*-transformed strains were supplemented with 5 μg/ml of chloramphenicol. *E. coli* strains were cultured with the appropriate antibiotic on Luria-Bertani (LB) agar plates at 37°C with 5% of CO_2 or in LB broth at 37°C with shaking.

Assays for survival under stress conditions. *L. pneumophila* WT strain AA100 and both *pmrA* and *pmrB* mutants were grown for 3 days on BCYE plates at 37°C; 20 ml of BYE medium were then inoculated, and cells were grown for at least 24 h for the stationary-phase stress experiments. The initial CFU count used to inoculate broth for both the E and the PE phases was $\sim 10^8$ per ml. The absorbance at 550 nm was measured by using a thermospectronic spectrophotometer (Thermo Fisher Scientific, Waltham, MA). For E-phase cultures, an optical density (OD) between 0.4 and 0.8 was used; an OD between 3 and 4 was used for the PE-phase cultures. Cells were centrifuged and resuspended in an equal volume of 1× M63 salts to measure the untreated CFU. M63 salts medium contains 22.0 mM KH_2PO_4, 40.2 mM K_2HPO_4, 14.6 mM $(NH_4)_2SO_4$, and 500 nM $FeSO_4$ (pH 6.5). For the different stress conditions, the cell pellet was resuspended in an equal volume solution of 5 M sodium chloride or 0.1 M citric acid at pH 3 for acid stress. Cells were incubated in a 37°C water bath for 30 min. The cells were washed with 1× M63 salts and serially diluted to determine the CFU on BCYE agar plates. To measure the sodium sensitivity of both the WT strain and the mutants, BYE cultures grown to E and PE phases were diluted into H_2O and then plated on BCYE that did or did not contain 100 mM NaCl. The percentage of bacteria that were sodium resistant was calculated as described previously (11).

Microarray analysis. Gene expression analyses of three independent overnight axenic cultures of each of the mutant strains in both E and PE growth phases were performed. The parental strain AA100 and each of the isogenic mutants (*pmrA* and *pmrB*) were inoculated into 50 ml of BYE in 250-ml baffled flasks at an OD at 600 nm (OD_{600}) of 0.05 and grown at 37°C in a rotary shaker at 250 rpm. Each of the cultures was sampled in two 2-ml aliquots at the mid-exponential growth phase ($OD_{600} \sim 0.8$) and then at the PE growth phase (2 to 3 h upon cessation of growth, $OD_{600} = 3$). The bacteria in the collected samples were pelleted by centrifugation and stored frozen at −80°C. The total RNA was isolated from the bacteria by using Qiagen RNeasy RNA isolation procedure. To prepare the samples for microarray hybridization, 20 μg of total RNA from each of the samples was converted to cDNA by reverse transcription in the presence of allylamino-dUTP and fluorescently labeled by coupling the resulting cDNA with the fluorescent dyes Alexa Fluor 546 or Alexa Fluor 647 (Invitrogen) according to the manufacturer's recommendations. The whole-genome gene expression profiling of the mutant strains was done using a custom-made longmer (70-mer) oligonucleotide array designed and manufactured at Columbia Genome Center (Columbia University). A set of 2,977 longmer oligonucleotide probes corresponding to all unique genes identified in the *L. pneumophila* strain Philadelphia-1 was prepared by using the custom oligonucleotide synthesis service from MWG Biotech, Inc. (High Point, NC). The longmer probes were dissolved in 50% dimethyl sulfoxide at 30 μM and spotted in duplicates onto Corning UltraGAPS-coated slides (Corning, Inc., MA) using SpotArray 72 spotting robot (Perkin-Elmer). After the spotting, the microarray slides were stored desiccated at room temperature until further use. The downstream processing and hybridization of the microarrays was performed according to the protocols recommended by Corning, Inc.

After hybridization, the arrays were scanned by using ScanArray Express (Perkin-Elmer, MA) at 5-μm resolution, and the resulting hybridization intensities for all probes from both channels on each array were exported in tab-delimited text file format for further analysis. Raw signal intensities were corrected for dye labeling effects within and between all slides by using the cyclic Lowess procedure implemented in the bioconductor affy microarray analysis package (30). Statistically significant differential expression between the mutants

and the AA100 strain at different growth phases was determined by using the unpaired two-tailed *t* test statistics implemented in the SAM package (72). False discovery rates for the datasets were subsequently estimated as q-values (69). The delta parameters for the q-value cutoffs were set, allowing less than 5% median false positives in a data set. The resulting data were imported for filtering and visualization into the Spotfire DecisionSite for Functional Genomics software suite (TIBCO Spotfire, Inc.). The results were filtered for ScanArray quality scores greater than 2. The resulting sets of differentially expressed genes were further analyzed by using principal component analysis and hierarchical clustering algorithms implemented within Spotfire DecisionSite for Functional Genomics.

Real-time quantitative reverse transcriptase PCR. For analysis of expression of the *flaA* gene in vitro, samples of bacterial cultures from the WT strain AA100 and the *pmrA* and *pmrB* mutants were grown in BYE medium to an OD_{550} of 0.8 to 1 (E phase) or an OD_{550} of 2.0 to 2.2 (PE phase). Total RNA was extracted by using RNeasy minikit (Qiagen) as recommended by the manufacturer. RNA integrity was assessed by visualizing ethidium bromide-stained 0.8% agarose gel. Total RNA was treated with DNase I (Ambion, Austin, TX) at 37°C for 30 min. Equal amounts of RNA were used for cDNA synthesis with Superscript III Plus RNase H^- reverse transcriptase (Invitrogen) and random primers. The generated cDNA was diluted fivefold with RNase-free water. Real-time quantitative PCR was done by using the DNA Engine Opticon System (MJ Research) and carried out in triplicates using a DyNAmo Sybr green quantitative PCR kit in a 20-μl reaction volume, as recommended by the manufacturer (New England Biolabs, Ipswich, MA). The 16S RNA and *flaA* were amplified using primers described previously (32). The 16S RNA was used as an internal normalizing control and to confirm that an equal amount of total RNA was used in each reaction. The PCR conditions were 5 min at 94°C, 15 s at 96°C, and 15 s at 72°C for 30 cycles. The concentration was determined by using the comparative cycle threshold (C_T) method (i.e., the C_T number at the cross-point between the amplification plot and the threshold) and normalized values to the 16S RNA. Relative quantitation by quantitative reverse transcriptase PCR was validated by equivalent and linear amplification of 16S RNA and the *flaA* gene at the assay concentrations. Negative or positive values were considered downregulation or upregulation of *flaA* gene expression, respectively, as represented by a minimum twofold difference.

Cell cultures. Isolation and preparation of the human monocyte-derived macrophages (hMDMs) and macrophage-like U937 cells was carried out as previously described (60). Cells were maintained in RPMI 1640 tissue culture medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum fetal bovine serum (Gibco-BRL). Axenic *Acanthamoeba polyphaga* was cultured as adherent cells in PYG medium as previously described (27). *Tetrahymena pyriformis* was grown in plate count broth (Becton Dickinson Microbiology Systems) (5). With the exception of the growth of *T. pyriformis* at 25°C, all cells were grown at 37°C in the presence of 5% of CO_2.

Intracellular growth kinetics. The bacterial strains were grown in BYE medium to an OD_{550} of 2.0 to 2.2 (PE phase). The mammalian or protozoan cells were infected with the bacteria (27) at a multiplicity of infection (MOI) of 10, but an MOI of 100 was used for *T. pyriformis*. To synchronize the infection, the plates were centrifuged for 5 min at 1,000 rpm using a Centra GP8R Thermo IEC centrifuge. After 1 h of incubation in 5% CO_2 at 37°C, the infected cells were washed three times with the culture medium to remove extracellular bacteria and incubated with 50 μg of gentamicin/ml for 1 h to kill the remaining extracellular bacteria. This step was considered the zero time point (t_0), and the infected cells were subsequently incubated for several time intervals. At the end of each time interval, the culture supernatant was removed, and the macrophages were lysed hypotonically by the addition of 200 μl of sterile water for 10 min or with 0.04% Triton X-100 for the protozoan cells. The supernatant and the lysates were combined, serial dilutions were prepared, and aliquots were plated on BCYE plates for counting. The number of bacteria was expressed as the number of CFU/ml.

CLSM. Confocal laser scanning microscopy (CLSM) and sample analysis were performed with polyclonal rabbit anti-*L. pneumophila* antiserum and Alexa Fluor 488-conjugated donkey anti-rabbit immunoglobulin G (IgG) purchased from Invitrogen. The anti-LAMP-2 (H4B4) monoclonal antibody (developed by J. T. August and J. E. K. Hildreth) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa). To label the lysosomes, monoclonal anti-cathepsin D antibody (BD Transduction, Franklin Lakes, NJ) was used. Mouse anti-KDEL monoclonal antibody purchased from StressGen Biotechnologies (Ann Arbor, MI) was used to label the ER proteins, followed by the addition of Alexa Red 555-conjugated donkey anti-mouse IgG (Molecular Probes). To study the role of PmrA and PmrB in intracellular replication, approximately 5×10^5 hMDMs were grown on circular glass coverslips (Fisher Scientific, Pittsburgh, PA) in 24-well culture plates. An MOI of 10 was used for all experiments for the CLSM except for when formalin-killed bacteria was used as a control, when an MOI of 50 was used. After infections, cells were washed three times with phosphate-buffered saline and processed for confocal microscopy as described previously (60).

For the coinfection experiments, cells were coinfected simultaneously with the WT strain of *L. pneumophila* expressing green fluorescent protein (GFP) and isogenic mutants at an MOI of 10, with the exception of *dotA* mutant, for which an MOI of 20 was used. To synchronize the infection, the plates were centrifuged for 5 min at 1,000 rpm using a Centra GP8R Thermo IEC centrifuge. After 1 h of incubation in CO_2 at 37°C, the infected cells were washed three times with the culture medium to remove extracellular bacteria and then incubated with 50 μg of gentamicin/ml for 1 h to kill the remaining extracellular bacteria. Infected cells were further incubated for 10 h, and the cells were processed for confocal microscopy as described below. All bacteria were labeled with polyclonal rabbit anti-*L. pneumophila* antiserum and Alexa Fluor 555-conjugated donkey anti-rabbit IgG antibody; therefore, the GFP-expressing bacteria become yellow when the two colors are combined, whereas the bacterial strain that did not have GFP is detected by the red fluorescence. The *dotA* and *htrA* mutants were used as positive and negative controls, respectively. The cells were examined by using an Olympus Fv500 laser scanning confocal microscope as described previously (60). On average, 8 to 15 0.2-μm serial Z sections of each image were captured and stored for further analyses using Adobe Photoshop 6.0.

TEM. For transmission electron microscopy (TEM), monolayers in six-well plates were infected with *L. pneumophila* strains at an MOI of 10 for 1 h, followed by 1 h of gentamicin treatment. At 6 h postinfection, the infected monolayers were washed, and the cells were fixed in 3.5% glutaraldehyde, dehydrated in alcohol, processed, and stained for TEM as we described previously (1). Sections were examined with a Hitachi H-7000/STEM electron microscope at 80 kV (26, 50).

Statistical analysis. All experiments were performed at least three times, and the data shown are representative of one experiment. To analyze for statistical significant differences between different sets of data, a two-tailed Student *t* test was used, and the *P* value was obtained.

Microarray data accession number. The processed and raw microarray data for gene expression analysis can be obtained from the NCBI Gene Expression Omnibus database. The accession number is GSE13323. Additional descriptions of the microarray platform and the analysis are posted at http://legionella.cu-genome.org/index.html.

RESULTS

***L. pneumophila* PmrA/PmrB homologs.** We aligned the PmrA response regulator (lpg1292) and PmrB (lpg1291) sensor kinase proteins of *L. pneumophila*, *Coxiella burnetii* (Q83CA1 and Q83CA0), *Salmonella enterica* serovar Typhimurium (AAV92795 and AAA72366), and *Pseudomonas aeruginosa* (Q88F73 and Q88F74) by using CLUSTAL W software. The alignment results showed that *L. pneumophila* PmrA shares 60.8, 45.3, and 55.1% identity and 75.8, 63.6, and 70.7% similarity with *C. burnetii*, *S. enterica* serovar Typhimurium, and *P. aeruginosa*, respectively. PmrA alignments were also performed in other bacteria, including *E. coli*, *Erwinia carotovora* (Q6DB90 and Q6DB91), and *Chromobacterium violaceum* (Q7NZN1 and Q7NZN2). These results showed an average of 85.2% of consensus sequence was shared in all of them (data not shown).

The PmrB sensor shares 47.8, 23.95, and 40.1% identity and 60.7, 38.6, and 60% similarity to *C. burnetii*, *S. enterica* serovar Typhimurium, and *P. aeruginosa*, respectively. Using the SOSUI system (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html), the predicted topology of the PmrB protein in *C. burnettii*, *S. enterica* serovar *Typhimurium*, and *P. aeruginosa* indicated one to two periplasmic domain(s) where the C-terminal region exposed to the outside harbors the sensor domain that becomes activated by a specific environmental signal. Alignment of the PmrB sensor protein sequences of *L. pneumophila*,

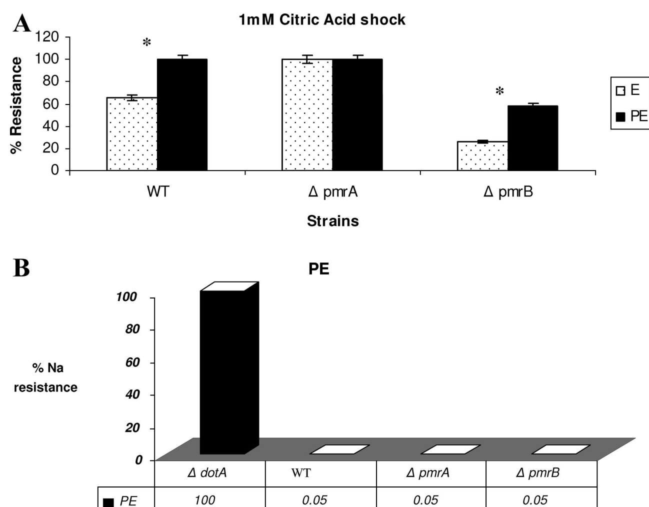

FIG. 1. Phenotypic characteristics of the *pmrA* and *pmrB* mutants under different stress conditions. For the different stress conditions, the cell pellet was resuspended in an equal volume solution of 0.1 M citric acid at pH 3 for acid stress (A) or 5 M sodium chloride (B) and compared to bacteria resuspended in M63 salts. Cells were incubated in a 37°C water bath for 30 min and then washed, serially diluted, and plated on BCYE agar plates to determine the number of resistant bacteria. The experiment was done three times, and the data are representative of one independent experiment. Asterisks represent a significant difference between the WT strain and the *pmrB* mutant.

C. burnetii, and serovar Typhimurium revealed the presence of an EXXE consensus sequence surrounded by a basic amino acid, which is a lysine in *L. pneumophila*. This EXXE motif was shown to be present in the *Saccharomyces* FTR1 iron transporter and in the mammalian ferritin light chain (78), suggesting that iron might be a signal recognized by the PmrB sensor.

Phenotypic characteristics of *pmrA* and *pmrB* mutants under different stress conditions. In *S. enterica* serovar Typhimurium, the sensor kinase PmrB was identified as the primary sensor that activates the PmrA protein when the pathogen experiences strong acid pH, resulting in the transcription of PmrA-activated genes (55). To determine whether low pH may trigger the *pmrAB* TCS in *L. pneumophila*, we exposed both the *pmrA* and the *pmrB* mutants grown to E and PE phases to 1 mM citric acid (pH 3). At 30 min after exposure, the *pmrB* mutant showed a significant growth defect compared to the WT strain and the *pmrA* mutant (Fig. 1A, $P < 0.1$). The complemented *pmrB* mutant was similar to the WT in sensitivity to acidic pH (data not shown).

It was shown that the PE-phase WT strain *L. pneumophila* is sodium sensitive and osmotically resistant (11). However, mutants such as the *dot/icm* mutants of *L. pneumophila* that have lost sensitivity to sodium ion are salt resistant (10, 62). We show here that the *pmrA* and *pmrB* mutants were salt sensitive to the same extent as the WT strain (Fig. 1B). Moreover, when subjected to osmotic stress (5 M NaCl), *L. pneumophila* WT strain and the *pmrA* and *pmrB* mutants showed no significant difference in osmotic resistance at the E or PE phases (data not shown).

Microarray gene analysis of the *pmrA* and *pmrB* mutants. The PmrA response regulator has been recently shown to control the expression of 13 tested substrates exported by the Dot/Icm secretion system (79). The role of this regulator in global regulation of the *L. pneumophila* genome, as well as its transcription profile, is not known. To address this, genome-wide microarray analyses were performed, and gene expression in both the *pmrA* and the *pmrB* mutants was compared to the WT strain AA100 at both the E and the PE phases (see Table S1.1 in the supplemental material). Keeping in mind that the *pmrAB* locus is present in all *L. pneumophila* sequenced genomes and that gene variation among *L. pneumophila* strains usually represents a small proportion of the genome (53), we performed microarray analysis using a Philadelphia-1 strain-specific microarray. A total of 279 genes in both the *pmrA* and the *pmrB* mutants were differentially expressed compared to the WT strain (the findings are summarized in Table 1). The genes were divided into nine groups as follows: (i) *ceg* genes or genes with the consensus sequence for the PmrA binding (32 genes were downregulated in both *pmrA* and *pmrB* mutants, and four genes were upregulated in the *pmrB* mutant at the PE phase); (ii) eukaryotic-like protein encoding genes (10 were downregulated in both mutants during both growth phases, and 3 were upregulated mainly at the PE phase); (iii) type IV secretion apparatus encoding genes and Dot/Icm translocated-effectors (26 genes were downregulated in both mutants at the E and PE phases, and 3 were upregulated at the PE phase); (iv) substrates secreted by the type II secretion system (7 genes show downregulation in both mutants, and only 2 were upregulated in either mutant at the PE phase); (v) stress response genes (13 genes were downregulated mainly at the E phase in the *pmrA* mutant, and 11 were upregulated in both mutants mainly at the PE phase); (vi) PE-phase regulators (2 genes were downregulated at both phases in both the *pmrA* and the *pmrB* mutants); (vii) flagellar genes and other operons (18 genes were downregulated and 15 were upregulated in both mutants at both growth phases; all flagellar synthesis genes [*flgB* to *flgL*] were upregulated in both mutants at

TABLE 1. Summary of genes regulated in a PmrA/PmrB-dependent manner at the E and PE phases

Regulation type	Ceg[b]	No. of genes[a]								Total no. of genes
		Eukaryotic-like	Type IV secretion system	Type II secretion system	Stress response	PE-phase regulators	Flagellar genes, other operons	Metabolic genes	Unknown function	
Positively regulated	32	10	26	7	13	2	18	14	120	242
Negatively regulated	4	3	3	2	11	0	15	4	7	49
Total no.	36	13	29	9	21*	2	24*	18	127	279*

[a] Totals indicated by an asterisk exclude genes that were inversely regulated at the E and PE phases.
[b] Ceg, no. of genes coregulated with the effector encoding gene.

the PE phase); (viii) genes encoding for proteins involved in various metabolic pathways (14 genes were upregulated in the mutants, and 4 were downregulated mainly at the PE, in the *pmrB* mutant only); and (ix) 127 genes of unknown function (see summary in Table 1).

Our data showed that only 36 *ceg* genes were differentially expressed in a PmrA/PmrB-dependent mechanism (see Table S1.2 in the supplemental material). A large number of genes encoding eukaryotic-like proteins, including some ankyrin genes (lpg0038 and lpg2452), a zinc metalloproteinase, and a uracyl DNA glycosylase, were positively regulated by PmrA at both the E and the PE phases (see Table S1.3 in the supplemental material). Upregulation of the expression of ankyrin genes by PmrA and PmrB as derived from the microarrays was confirmed by real-time PCR analyses (data not shown). Our data also showed that the expression of 7 *dot*/*icm* apparatus encoding genes (*dotA*, *icmL*, *icmR*, *icmV*, *icmW*, and *icmX*), as well as 22 Dot/Icm-secreted effector encoding genes, was upregulated in both *pmrA* and *pmrB* mutants at both growth phases. This group includes *sde*-like genes (lpg2153 and lpg2154) that are considered virulence factors of the transmissive phase (44) and most of the *sid*-related genes (*sidC*, *sidD*, *sidE*, and *sidF*). The exceptions were *sidA* and *sidG*, both of which were downregulated in a PmrA-dependent manner. The positive regulation of PmrA over *sidH* (lpg2829) was observed in the PE phase only (see Table S1.4 in the supplemental material). Interestingly, PmrA was found to regulate eight type II-secreted effectors, including *Acph-1*, *icmX*, *lvrE*, *dnaK*, zinc metalloprotease, and chitinase encoding genes and two genes of unknown functions (lpg2526 and lpg1385) (see Table S1.5 in the supplemental material) (18). Therefore, PmrA is a major transcriptional regulator of genes encoding substrates exported by the type IV and type II secretion systems.

Interestingly, the type IV pilin gene, *pilE*, involved in *L. pneumophila* adherence to mammalian and protozoan cells was downregulated in the *pmrB* mutant at the PE phase only (66). Also, three of the *Legionella vir* homologues (*lvh*)—*lvrE*, *virB11*, and *virD4*—were downregulated in the *pmrB* mutant at the PE phase.

Fourteen genes encoding metabolic enzymes (*aroE*, *phbC*, *maeA*, and *bdhA*) were upregulated in a PmrA/PmrB-dependent manner, mainly at the E phase of growth, suggesting that *L. pneumophila* may be using this TCS to couple its differentiation to the metabolic state (see Table S1.9 in the supplemental material). The expression of 20 genes encoding for chaperones and heat and cold shock proteins was downregulated in the *pmrA* mutant in the exponential phase, but this suppression was alleviated at the PE phase. Some of the chaperons genes, including *hslVU* protease subunits (lpg0640 and lpg0641), *hsp10* and *hsp60* (lpg0687 and lpg688), and *dnaK* and *grpE* (lpg2025 and lpg2026), were grouped into operons or located adjacent to each other (see Table S1.6 in the supplemental material).

Two major PE-phase regulators, the CsrA activator of replication and repressor of transmission traits of *L. pneumophila*, as well as the RpoE sigma factor encoding genes (lpg0781 and lpg1577), was regulated in a PmrA/PmrB-dependent manner. The global regulator encoding gene *csrA* was downregulated in both mutants at both growth phases, whereas the *rpoE* was downregulated in both mutants only at the PE phase of growth (see Table S1.7 in the supplemental material).

In addition, several operons controlling the expression of flagellar basal body (*flgB* to *flgL*) and located in the same 10-kb region of the *Legionella* genome spanning loci lpg1216 to lpg1226 are upregulated in the *pmrA* and *pmrB* mutants during the PE phase only (see Table S1.8 in the supplemental material). To confirm the role of the PmrA/PmrB TCS in the expression and regulation of the flagellar genes, we measured the expression levels of the *flaA* gene by real-time PCR. The flagellum subunit protein encoding gene *flaA* was increased by ninefold in the *pmrA* mutant at the PE growth phase compared to the WT strain (see Fig. S1 in the supplemental material). Therefore, regulation of flagellar gene expression, *rpoE*, and the global repressor protein (*csrA*) indicated that PmrA was a major global regulator of *L. pneumophila*, particularly at the PE phase. Interestingly, a group of 41 genes did not show the same pattern of regulation in both the *pmrA* and the *pmrB* mutants, suggesting the presence of cross talk between the PmrA/PmrB and other TCSs (see Table S2 in the supplemental material).

Role of PmrA and PmrB in intracellular growth of *L. pneumophila* within amoebas and ciliates. Previous work has shown that a *pmrA* mutant constructed in the JR32 strain of *L. pneumophila* is defective for intracellular growth in *Acanthamoeba castellanii*, but the defect was not restored by the WT genes *in trans*, suggesting a secondary mutation may have partially resulted in the defect (79). However, the role of PmrB in the intracellular infection remains unknown. We have constructed the *pmrA* and *pmrB* mutants in strain AA100/130b and examined their growth in two different protozoan hosts: *A. polyphaga* and the ciliate *T. pyriformis*. The growth rate of the *pmrA* and *pmrB* mutants in BYE broth showed growth rates similar to that of the WT strain, and there was no difference in the length of the lag phase (data not shown). The *pmrA* mutant grew normally in *A. polyphaga*, whereas the *pmrB* mutant was partially defective, since 10-fold fewer CFU were recovered at both 24 and 48 h (Fig. 2A). In contrast, a more severe growth defect was observed for both mutants in *T. pyriformis*. At 48 h postinfection, there was no detectable growth for the *pmrA* and *pmrB* mutants in the ciliate, and the defect was fully complemented by the WT gene (Fig. 2B). As expected, the *dotA* mutant control was not able to grow in both hosts. We conclude that both proteins played an important role in intracellular growth within the ciliate *T. pyriformis*.

Role of PmrA and PmrB in the intracellular growth of *L. pneumophila* within human macrophages. Zusman et al. (79) have previously reported that a *pmrA* mutant derived from the JR32 *L. pneumophila* strain is partially defective in the HL-60 macrophage cell line. However, whether PmrB plays any role in the intracellular infection of mammalian macrophages is not known. To elucidate the role of both PmrA and PmrB proteins in the intracellular growth of *L. pneumophila* in human macrophages, we examined the intracellular growth kinetics of both mutants. We assessed the role of the two proteins in intracellular replication by examinating the intracellular growth kinetics of the mutants within hMDMs and the U937 macrophage cell line (Fig. 3 and data not shown). Our data showed that the *pmrA* mutant had no detectable intracellular growth defect in both cells. In contrast, the *pmrB* mutant was

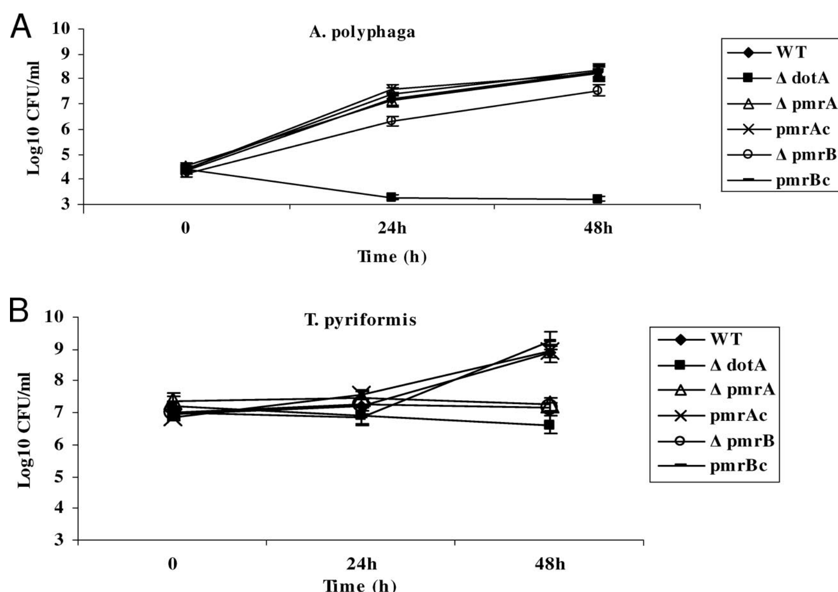

FIG. 2. Intracellular growth kinetics of the *pmrA* and *pmrB* mutants of *L. pneumophila* within protozoa. The intracellular growth kinetics of the *pmrA* and *pmrB* mutant in *A. polyphaga* (A) and *T. pyriformis* (B) were determined. The infection was carried out in triplicates for 1 h, followed by 1 h of gentamicin treatment to kill extracellular bacteria in case of *A. polyphaga*. The infected monolayers were lysed at different time intervals and plated onto agar plates for colony enumeration. The experiment was done three times, and the data are representative of one independent experiment. Error bars represent standard deviations, but some were too small to appear in the figure.

defective in hMDMs, with 200-fold fewer CFU recovered after 48 h compared to the WT strain (Fig. 3). In U937 cells, 2,000-fold fewer CFU were recovered for the *pmrB* mutant compared to the WT strain by 48 h postinfection (data not shown). In both cases, the defect was fully complemented by the WT gene. As expected, the *dotA* mutant control did not grow within any of the macrophages tested (Fig. 3). We conclude that the intracellular growth phenotype of both mutants in human macrophages is different, which further supports our speculation of a possible cross talk between the PmrA/PmrB and other TCSs.

Since the *L. pneumophila pmrB* mutant exhibited a defect in intracellular replication, we examined whether this defect was due to a defect in replication of a subset of bacterial population or to a balanced killing and replication. We performed single-cell analysis to quantitate the number of bacteria per cell at several stages of the intracellular infection of hMDMs. The data showed that at 2 h after infection, ca. 98% of the cells infected with the different strains harbored one organism (Fig. 4A). After 10 h of infection, ca. 70% of the cells harboring the WT strain contained 6 to 15 bacteria. In contrast, the *pmrB* mutant showed less replication, with ca. 68% of the cells harboring three to five bacteria per cell (Fig. 4B). We conclude that the PmrB sensor protein plays an important role in the intracellular growth of *L. pneumophila* within macrophages and that the intracellular growth defect caused by the *pmrB* mutation is homogeneous.

***in cis* rescue of the *pmrB* mutant within communal phagosomes harboring the WT strain.** The *dot*/*icm* mutants are rescued *in cis* for their intracellular defect within communal

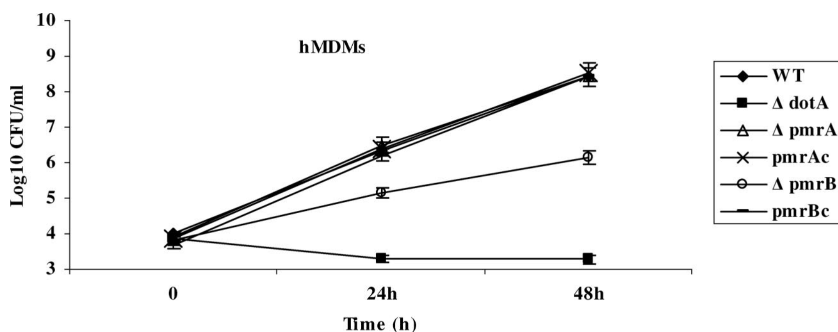

FIG. 3. Intracellular growth kinetics of the *pmrA* and *pmrB* mutants of *L. pneumophila* within macrophages. Intracellular growth kinetics of the WT AA100 strain and the *dotA*, *pmrA*, and *pmrB* mutants in hMDMs. pmrAc and pmrBc represent the *pmrA* and *pmrB* mutant strains complemented with the WT copy of the gene on the pBC plasmid. The infection was carried out in triplicates for 1 h at an MOI of 10, followed by 1 h of gentamicin treatment to kill the extracellular bacteria. The infected monolayers were hypotonically lysed at the indicated time points after infection and plated onto agar plates for colony enumeration. The data are representative of three independent experiments, and error bars represent the standard deviations.

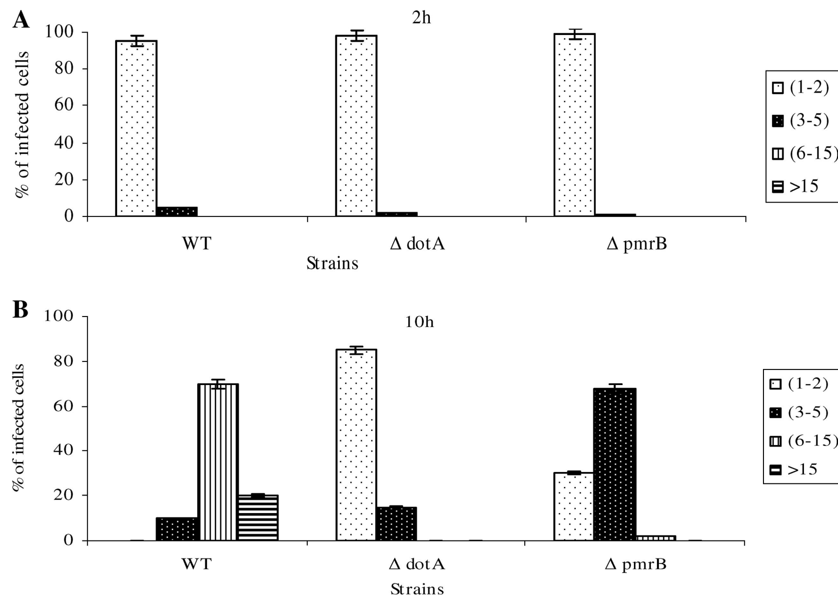

FIG. 4. Single cell analyses of replicative phagosomes. At 2 and 10 h postinfection of hMDMs, 100 infected cells were analyzed by CLSM for the formation of replicative phagosomes. Representative quantitation of the number of bacteria/cell at 2 h (A) and 10 h (B) is shown. The *dotA* mutant was used as a negative control. Infected cells from multiple coverslips were examined in each experiment. The results are representative of three independent experiments performed in triplicates. Error bars represent the standard deviations.

phagosomes harboring the WT strain of *L. pneumophila* that is able to modulate phagosomal biogenesis into a niche suitable for bacterial replication (17). However, *L. pneumophila* mutants defective in intracellular replication due to a defect in stress response genes (such as *htrA* or *rpoS*), which are required for adaptation to the phagosomal microenvironment, are not rescued *in cis* within communal phagosomes harboring the WT strain (4, 17, 54). To examine whether the PmrB protein is required for formation of replicative phagosomes or for adaptation to the phagosomal microenvironment, we coinfected hMDMs with the WT strain and the *pmrB* mutant and determined whether the mutant replicated in communal phagosomes harboring the WT strain. We used coinfection of WT *L. pneumophila* and the two isogenic mutants, the *dotA* or *htrA* mutant, as positive and negative controls, respectively. In all coinfections, only ~10% of the phagosomes were commu-

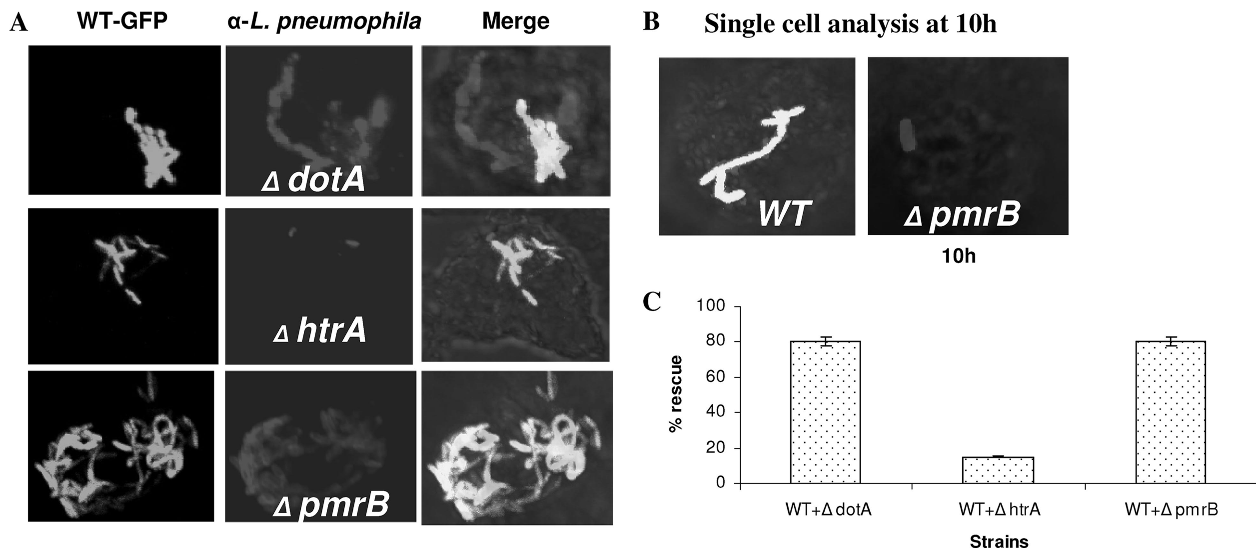

FIG. 5. *in cis* rescue of intracellular growth of the *pmrB* mutant within communal phagosomes harboring the WT strain in hMDMs. The hMDMs were simultaneously infected with the GFP-positive *L. pneumophila* strain AA100 (WT) and one of the mutants—*dotA*, *htrA*, or *pmrB*—followed by fixation at 10 h after infection (see Materials and Methods). Macrophages harboring phagosomes containing both strains (GFP-WT and the mutants) were scored. Representative confocal images and quantitation are shown in panels A and C, respectively. Panel B shows the replication status of single infection by the WT strain and the *pmrB* mutant at 10 h postinfection. The results are representative of three independent experiments performed in triplicates. Error bars represent the standard deviation.

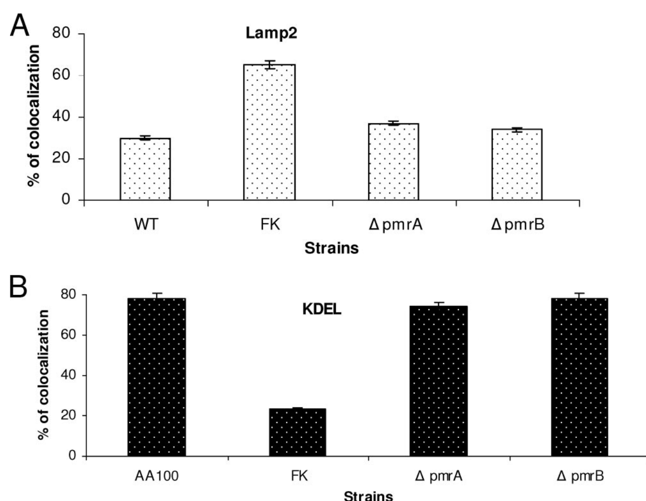

FIG. 6. Quantitative analysis of intracellular trafficking of the *pmrA* and *pmrB* mutants within hMDMs. Quantitation of infected hMDMs for colocalization of the bacterial phagosome for the WT strain AA100 and the *pmrA* and *pmrB* mutants with the late endosomal marker LAMP-2 (A) at 2 h postinfection and the ER marker KDEL at 4 h (B) was performed. Formalin-killed (FK) bacteria were used as a negative control. At least 100 infected cells from multiple coverslips were examined in each experiment by CLSM. The results shown are representative of three independent experiments performed in triplicates. The data represent means ± the standard deviation. There was no significant difference in trafficking of the WT strain and the mutants.

nal phagosomes harboring the two different strains. In the control coinfection of *L. pneumophila* and its *dotA* mutant, replication of the *dotA* mutant was rescued in communal phagosomes containing the WT strain (Fig. 5A and C). Control coinfection of *L. pneumophila* and its *htrA* mutant showed the failure of the WT strain to rescue the *htrA* mutant in communal phagosomes (Fig. 5A and C) (54). Compared to the replication status of a single infection by the WT strain and the *pmrB* mutant at 10 h postinfection (Fig. 5B), our data showed that when the *pmrB* mutant resided in communal phagosomes harboring the WT strain, the mutant replicated robustly, similar to what was observed with the *dotA* mutant (Fig. 5A and C). We conclude that, despite the severe growth defect caused by the *pmrB* mutation, the *pmrB* mutant is able to replicate within a vacuole remodeled by the WT strain similar to the *dot/icm* structural mutants (17).

Intracellular trafficking of the *pmrB* mutant within hMDMs. Since the *pmrB* mutant was defective in intracellular replication, we examined whether its growth defect was caused by a failure to evade the endocytic pathway. To examine the intracellular trafficking of the *pmrB* mutant within hMDMs, we labeled the cells with the late endosomal/lysosomal marker (LAMP-2) and the luminal lysosomal enzyme cathepsin D. CLSM was used to assess the percentage of colocalization of phagosomes harboring the WT strain AA100 or the *pmrB* mutant with the late endosomal and lysosomal compartments (Fig. 6 and 7). Formalin-killed bacteria, which traffic to the phagolysosomes (41, 42), were used as a positive control. The data showed that at 2 h postinfection, phagosomes harboring the WT strain and the *pmrB* mutant colocalized with LAMP-2 at levels of 30 and 34%, respectively, whereas phagosomes containing formalin-killed bacteria showed 65% colocalization (Fig. 6A and 7A). Approximately 62% of the phagosomes harboring formalin killed bacteria colocalized with the lysosomal marker cathepsin D, whereas the WT strain AA100 and the *pmrB* mutant showed 31 to 40% colocalization, respectively (data not shown, Fig. 7B). The slight difference in Lamp2 and cathepsin D colocalization between the WT strain and the *pmrB* mutant was not significant (Student *t* test, $P > 0.1$). The results were similar for the *pmrA* mutant trafficking (Fig. 6). We conclude that, despite its role in intracellular growth, PmrB is not involved in the regulation of genes required for evasion of the endocytic pathway.

Using confocal microscopy, we determined the capacity of the *pmrA* and *pmrB* mutants to decorate their vacuoles with the ER-derived vesicles using an antibody that recognizes the KDEL amino acid sequence, which is the signal for ER retention. The data showed that at 4 h postinfection of hMDMs, 70 to 80% of the phagosomes harboring the WT strain and the *pmrA* and *pmrB* mutants colocalized with the KDEL marker. The formalin-killed bacteria used as a negative control were defective in acquiring the KDEL marker, where only 23% of these phagosomes retained the KDEL maker (Fig. 6B and 7C). These results were consistent with the TEM findings, where we examined the rough endoplasmic reticulum (RER) recruitment to the phagosomes at 6 h postinfection. No significant difference (Student *t* test, $P > 0.1$) was observed between the WT strain (90%) and the *pmrB* mutant (84%) containing vacuoles (Fig. 8). These data indicate that the PmrA/PmrB TCS is not involved in the regulation of genes required for ER recruitment to the phagosome.

DISCUSSION

Expression of *L. pneumophila* virulence factors has been proposed to be mediated by a regulatory cascade that is triggered upon nutrient starvation and driven via two parallel pathways: one involving LetA/LetS and the other involving the RpoS sigma factor (Fig. 9) (8, 9, 47). Recently, the PmrA/PmrB TCS has been shown to control the expression of 13 tested *L. pneumophila* genes encoding Dot/Icm-secreted effectors (79). However, the role of this TCS in the regulation of other virulence factors, as well as its implication in the regulatory cascade of *L. pneumophila* that governs phenotypic transition at the PE phase, is not known.

Based on the rationale that genes present in all four sequenced *L. pneumophila* strains represent the core genome of this species (14, 15), we performed microarray analysis using a Philadelphia-1 strain-specific microarray. The four sequenced genomes of *L. pneumophila* contain each an average of 3,000 genes with 200 to 300 genes specific to each genome, but all contain the *pmrAB* locus. It is important to note that some of the genes present in the clinical strain AA100/130b may be absent in the Philadelphia-1 strain and vice versa and that gene variation among bacterial strains of the same species usually represents a small proportion of the genome (53). Despite potential minor differences between the Philadelphia-1 strain genome and our clinical strain AA100/a30b, ~10% of the core genome appears to be under the regulation of PmrA.

Using a genome-wide microarray, we have shown for the first time that the PmrA response regulator not only regulates

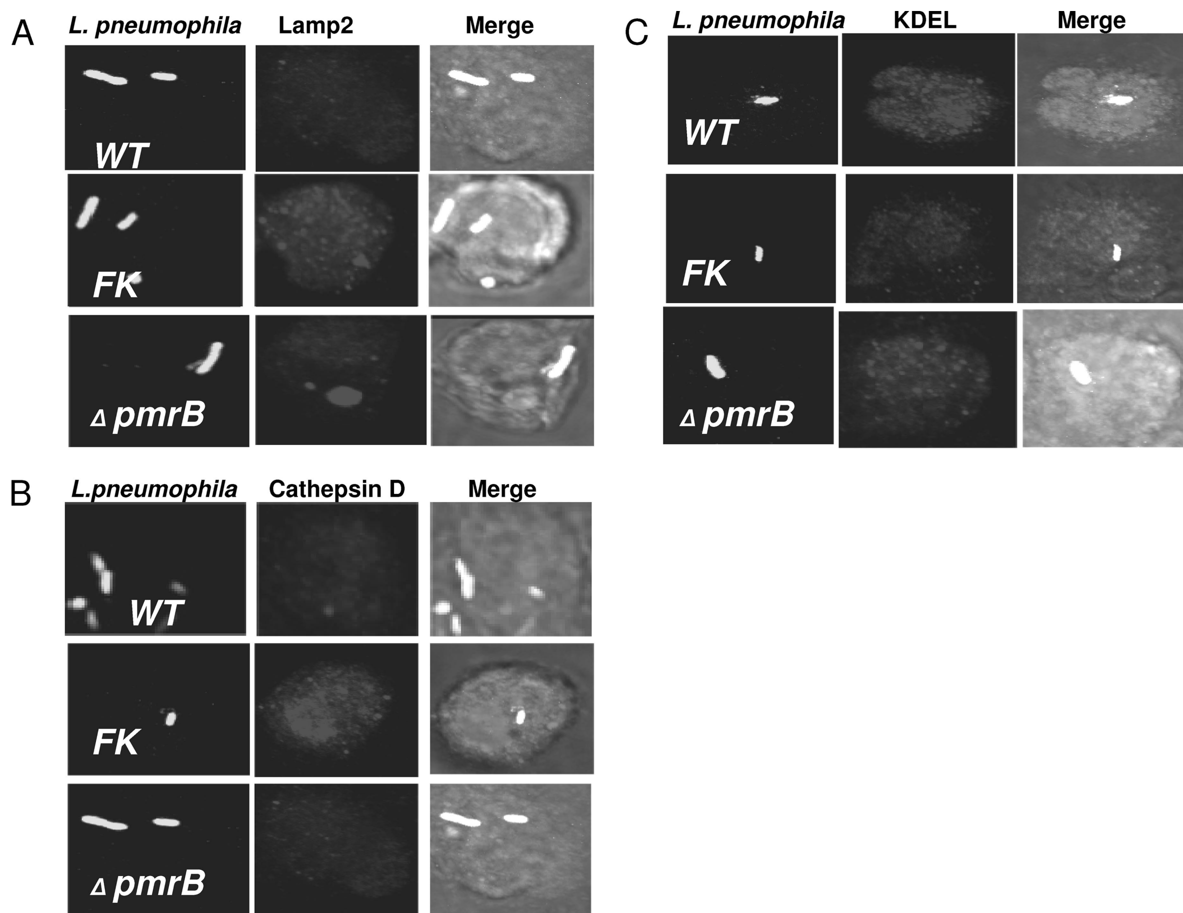

FIG. 7. Intracellular trafficking of the *pmrA* and *pmrB* mutants of *L. pneumophila* within hMDMs. Representative confocal microscopy images of infected hMDMs show colocalization of the bacterial phagosome with the late endosomal marker LAMP-2 (A), the lysosomal enzyme cathepsin D (B), and the ER marker KDEL (C). The bacteria and the LAMP-2, cathepsin D, and KDEL markers were detected by specific antibodies. Formalin-killed (FK) bacteria were used as a negative control. The results shown are representative of three independent experiments performed in triplicates.

the expression of some Dot/Icm secreted effectors but is a global regulator of *L. pneumophila*. The PmrA/PmrB TCS controls the expression of 279 genes of *L. pneumophila*. Although PmrA in *E. coli*, *P. aeruginosa*, and *S. enterica* serovar Typhi has been shown to activate genes involved in the modification of lipopolysaccharide and subsequent resistance to antimicrobial peptides (31), our microarray data do not show any regulation of lipopolysaccharide modification genes by the PmrA/PmrB regulon of *L. pneumophila*.

We propose a working model of the global role of PmrA/PmrB in regulation (Fig. 9). The accumulation of ppGpp stimulates both RpoS and the LetA/LetS cascades of regulation (28, 33, 38). Both cascades result in the expression of many of the virulence traits, causing a major shift in bacterial differentiation from the replicative phase to the transmissive phase (28, 33, 38). This ppGpp alarmone may also induce the PmrA/PmrB cascade, since PmrA expression is induced further at the PE phase of growth (data not shown). Upon expression and/or activation of the PmrA by the PmrB sensor, PmrA modulates the expression of genes that account for ~10% of the core genome. We show that flagellar genes are downregulated in PmrA-dependent manner, which may be acting through that activation of the CsrA repressor. Regulators of the PE phase, LetA/LetS and RpoS, positively regulate flagellar genes upon entry into the PE phase of growth (9, 47). We show here that PmrA is the first negative regulator of expression of flagellar genes at the PE phase. Despite the downregulation of flagellar expression, both the *pmrA* and the *pmrB* mutants were motile (data not shown). Therefore, the role of other regulators, such as RpoS, in inducing flagellar genes at the PE phase of growth tips the balance in favor of flagellar gene expression. Therefore, PmrA/PmrB may act as a negative-feedback loop to fine-tune flagellar gene expression.

We show that metabolic enzymes (PhbC, MaeA, HydG, BdhA, and AroE) are regulated in a PmrA/PmrB-dependent mechanism, suggesting that PmrA/PmrB is involved in certain aspects of the transition of *L. pneumophila* from the E phase to the PE phase upon nutrient starvation (61). Therefore, PmrA-dependent regulation may act as a link between nutrient acquisition and microbial differentiation. Our data clearly show that PmrA acts as a global regulator of genes involved in the transition from the replicative to the transmissive phase of growth and modulates a variety of *L. pneumophila* cellular, metabolic, and physiological processes, particularly

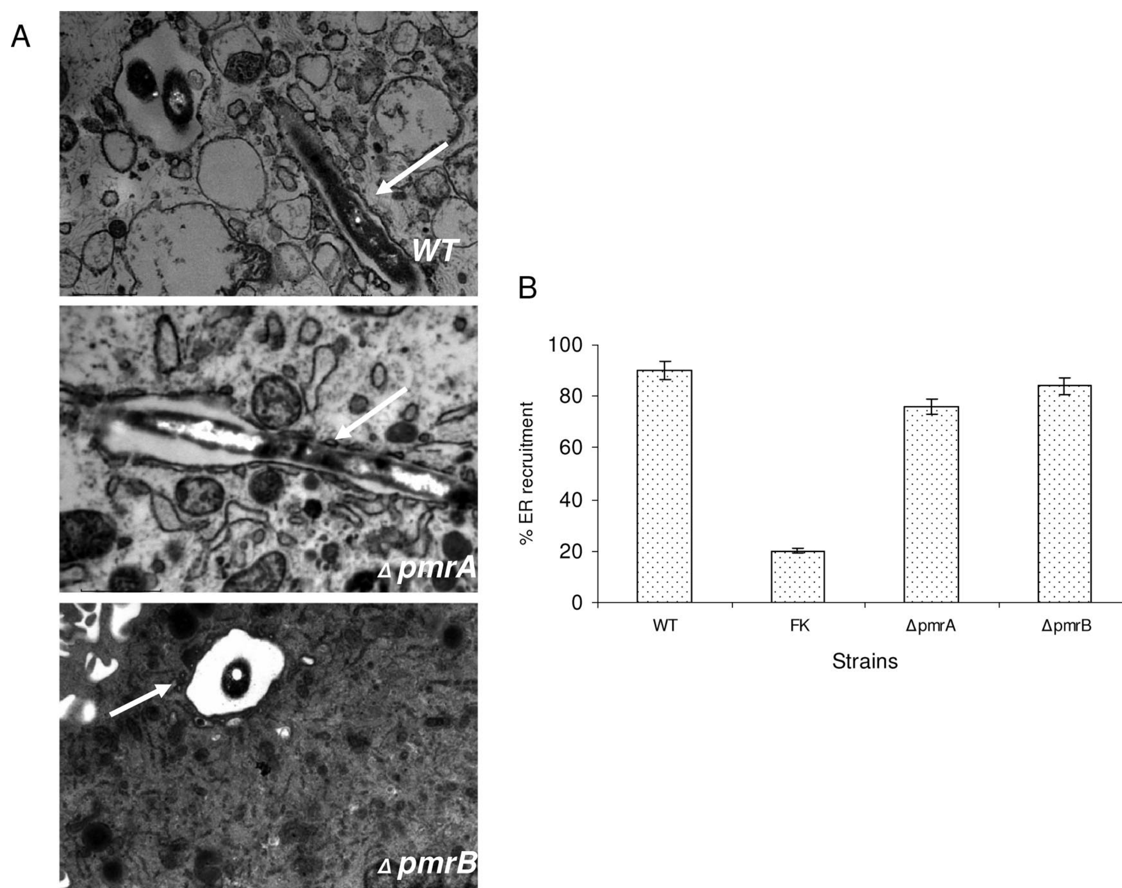

FIG. 8. The *L. pneumophila* PmrA/PmrB mutants are contained within ER-derived phagosomes. (A) Cells were examined by TEM for the presence of the RER studded phagosome at 6 h. The ER is indicated by arrows in the representative electron micrographs shown. (B) Quantitative results for the *pmrA* and *pmrB* mutants compared to the WT strain AA100 and the formalin-killed (FK) WT strain. The results are expressed as percentage of 100 phagosomes surrounded by the RER. The experiment was done three times in triplicate, and error bars represent the standard deviations. There was no significant difference in trafficking of the WT strain and the mutants.

at the PE phase. None of the PE-phase regulators RelA, LetA/S, and RpoS appears to be under the direct regulation of PmrA/PmrB TCS. This suggests that PmrA may be downstream of these regulators or that PmrA is acting through a different regulatory cascade. We speculate that by coupling differentiation to the metabolic state through the PmrA/PmrB TCS, *L. pneumophila* can swiftly acclimate to environmental fluctuations and stress encountered within or outside the host.

A JR32 strain-derived *pmrA* mutant of *L. pneumophila* has been reported to be completely defective for intracellular growth in *A. castellanii* and partially defective for intracellular growth in HL-60-derived human macrophages, but the introduction of a plasmid containing the *pmrA* gene has resulted in only partial complementation of the intracellular growth defect within *A. castellanii* (79). Our data show no detectable defect of the *pmrA* mutant in both *A. polyphaga* and human macrophages, but the *pmrA* mutant is totally defective in the ciliate *T. pyriformis*. The different phenotype of the *pmrA* mutant derived from different parental strain (JR32 and AA100) may be due to the different genetic background of the WT strain and/or a difference in the host cells, but the failure of transcomplementation of the JR32 *pmrA* mutant may indicate a secondary mutation that affected the intracellular growth. The role of PmrB in the intracellular infection is not known. Our data have shown that the *pmrB* mutant exhibits an intracellular growth defect in human macrophages and *A. polyphaga*, but the defect is more pronounced for the *pmrB* mutant within the ciliate *T. pyriformis*. This suggests a possible role for PmrA and PmrB in conferring protozoan host tropism by *L. pneumophila* and enabling the bacteria to adapt to different microenvironmental conditions that may vary with different encountered hosts or ecological niches, such as biofilms.

Despite the severe intracellular growth defect within hMDMs, we show that the *pmrB* mutant evades the endocytic pathway and remodels its phagosome into an ER-derived vacuole. Furthermore, the intracellular growth defect of the *pmrB* mutant is rescued *in cis* within communal phagosomes established by the parental strain similar to the *dot/icm* structural mutants. In contrast, a mutant defective in stress response, such as the *htrA* mutant, is not rescued within the communal vacuoles established by the WT strain. Our data suggest a role for the PmrA/PmrB TCS in controlling genes involved in bacterial adaptation to the phagosomal microenvironment but not genes required for interception of ER-derived vesicles or evasion of the lysosomes (35).

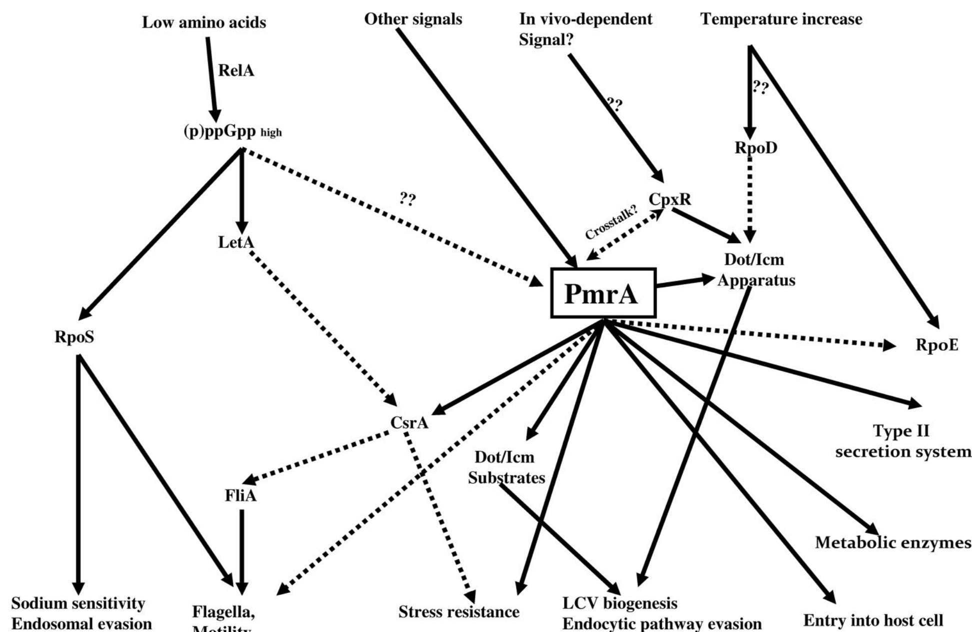

FIG. 9. Working model of the regulatory cascade governing the life cycle phenotypic switch of *L. pneumophila*. Solid lines indicate positive regulation. Dashed lines indicate negative regulation (see Discussion for details).

Our data suggest that PmrA may function through another TCS sensor since the microarray results revealed 41 genes that are differentially regulated in the *pmrA* and *pmrB* mutants, and a dramatic difference in phenotypes of the *pmrA* and *pmrB* mutants is observed in macrophages as well as in protozoa. Interestingly, a cross talk between the PhoPQ and the PmrA/PmrB TCS has been shown in *S. enterica* serovar Typhi, where, in addition to the PmrB sensor, PmrA is activated via PmrD, which is a PhoPQ-regulated protein (55). Even though no homologs of PmrD and PhoPQ TCS are present in *L. pneumophila*, we speculate a possible cross talk with other TCSs such as CpxRA, since some of the PmrA/PmrB-regulated genes have been shown to be regulated by CpxR (*icmR*, *icmV*, and *icmW*) (24).

In summary, we show that the PmrA/PmrB TCS is involved in the intracellular growth of *L. pneumophila*, enabling the bacteria to adapt to environmental fluctuations through triggering of the PmrA global regulator. This regulator triggers the expression of at least nine large families of genes that are involved in various aspects of *L. pneumophila* pathogenesis and modulation of cellular processes in macrophages and protozoa. We propose a cross talk between the PmrA/PmrB TCS and other TCSs. The *pmrB* mutant is defective in mammalian and protozoan hosts, whereas the *pmrA* mutant shows intracellular growth defect only within ciliates, suggesting that PmrA/PmrB may confer a host tropism to *L. pneumophila*. Despite the intracellular growth defect of the *pmrB* mutant, the WT strain rescues its growth defect when both coinhabit the phagosome. Although defective in intracellular proliferation, the *pmrB* mutant evades the endosomal-lysosomal fusion. We show that the PmrA/PmrB TCS is the first global regulator known in *L. pneumophila* to negatively regulate flagellar genes, creating a feedback loop of some positive regulators of flagellar expression such as RpoS and LetA/S, which is likely to fine-tune flagellar expression.

ACKNOWLEDGMENT

We are in debt to Howard Shuman at Columbia University for the microarray analyses that were supported by his NIH grant RO1 (AI064481).

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Editor: A. J. Bäumler