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Essential Role for the Response Regulator PmrA in *Coxiella burnetii* Type 4B Secretion and Colonization of Mammalian Host Cells

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Successful host cell colonization by the Q fever pathogen, *Coxiella burnetii*, requires translocation of effector proteins into the host cytosol by a Dot/Icm type 4B secretion system (T4BSS). In *Legionella pneumophila*, the two-component system (TCS) PmrAB regulates the Dot/Icm T4BSS and several additional physiological processes associated with pathogenesis. Because PmrA consensus regulatory elements are associated with some *dot/icm* and substrate genes, a similar role for PmrA in regulation of the *C. burnetii* T4BSS has been proposed. Here, we constructed a *C. burnetii pmrA* deletion mutant to directly probe PmrA-mediated gene regulation. Compared to wild-type bacteria, *C. burnetii* $\Delta pmrA$ exhibited severe intracellular growth defects that coincided with failed secretion of effector proteins. Luciferase gene reporter assays demonstrated PmrA-dependent expression of 5 of 7 *dot/icm* operons and 9 of 11 effector-encoding genes with a predicted upstream PmrA regulatory element. Mutational analysis verified consensus sequence nucleotides required for PmrA-directed transcription. RNA sequencing and whole bacterial cell mass spectrometry of wild-type *C. burnetii* and the $\Delta pmrA$ mutant uncovered new components of the PmrA regulon, including several genes lacking PmrA motifs that encoded Dot/Icm substrates. Collectively, our results indicate that the PmrAB TCS is a critical virulence factor that regulates *C. burnetii* Dot/Icm secretion. The presence of PmrA-responsive genes lacking PmrA regulatory elements also suggests that the PmrAB TCS controls expression of regulatory systems associated with the production of additional *C. burnetii* proteins involved in host cell parasitism.

The zoonotic disease agent *Coxiella burnetii* causes human Q fever by invading and replicating within mononuclear phagocytes, such as alveolar macrophages (1, 2). Once internalized by a host cell, this highly infectious intracellular bacterium replicates exclusively within a membrane-bound compartment, or parasitophorous vacuole (PV). Cumulative evidence from studies of infected primary and continuous macrophage cell cultures indicates that the mature PV has phagolysosomal characteristics, including the presence of several late-endosomal/lysosomal membrane markers, an acidic pH, and active cathepsins that correlate with degradative activity capable of destroying *Escherichia coli* trafficked to the vacuole (2, 3). The PV is a specialized growth-permissive compartment that requires *C. burnetii* protein synthesis for biogenesis (4–6). *C. burnetii* actively manipulates vesicular trafficking pathways to sequester structural components for PV expansion and, presumably, nutrients for pathogen growth (5–8). Pronounced PV fusogenicity is associated with recruitment of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) syntaxin-8 and VAMP7 (5, 8). Small interfering RNA (siRNA) studies also suggest important roles for syntaxin-17 and retromer function (9).

New genetic tools have verified that the *C. burnetii* Dot/Icm type 4B secretion system (T4BSS), analogous to the well-defined Dot/Icm system of *Legionella pneumophila*, is essential for PV formation. *C. burnetii dotA*, *dotB*, *icmD*, *icmL*, and *icmX* mutants all fail to replicate in mammalian host cells (10–13). Intracellular growth of the *icmD* mutant is rescued if it cooccupies the PV with wild-type bacteria, indicating that *dot/icm* mutants are capable of intracellular growth if functions of T4BSS effector proteins are provided in *trans* (11).

Approximately 130 *C. burnetii* T4BSS substrates have been identified using *L. pneumophila* as the surrogate host and adenylate cyclase (CyaA)- or β -lactamase-based secretion assays (10,

13–19). Lists of candidate effector genes for screening were assembled based primarily on bioinformatic criteria (13–19). Bioinformatic predictors of effector genes/proteins include the presence of eukaryote-like motifs/domains (14, 16–18), a glutamate-rich C-terminal secretion signal (E block) (13, 15, 20, 21), and/or a regulatory element recognized by the response regulator PmrA (13, 14, 21, 22).

Advances in *C. burnetii* genetics now allow direct screening of potential Dot/Icm substrates in *C. burnetii* (12, 23, 24). Cytosolic translocation by *C. burnetii* has been verified for 27 of the *C. burnetii* Dot/Icm substrates originally identified using *L. pneumophila*, and dependency on at least one *dot/icm* gene has been demonstrated for secretion of 17 substrates (10–14, 17, 25). Furthermore, screening of a *C. burnetii* *Himar1* transposon mutant library recently revealed mutations in five *cir* (*Coxiella* effector for intracellular replication) genes among the *C. burnetii* effector gene pool that have severe replication defects in J774A.1 macrophages (13).

Although progress has been made in identifying substrates translocated by the *C. burnetii* T4BSS, little information is available on the effector functions of these proteins. The biological roles of Cir proteins remain unresolved (13); however, three *C. burnetii* T4BSS substrates (AnkG, CeaA, and CeaB) have known

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antiapoptotic activities (26, 27). Ectopic expression in mammalian cells and gain of function by *L. pneumophila* demonstrate that the ankyrin repeat-containing protein AnkG inhibits apoptosis through binding of the proapoptotic mitochondrial protein p32 (gClqR) (26). Ectopic expression experiments also show that CaeB blocks apoptotic signals originating from the mitochondria and that CeaA inhibits apoptosis by an unknown mechanism (27). Recently, the effector protein CvpA was demonstrated to subvert clathrin-coated vesicle trafficking by binding to the clathrin adaptor protein AP2 (28). Cooption of clathrin transport processes by CvpA is predicted to promote acquisition of endolysosomal membrane for PV formation (28).

There are also considerable gaps in our knowledge with regard to regulation of *C. burnetii* type 4B secretion. In *L. pneumophila*, a complex interplay between the two-component systems (TCSs) PmrAB, CpxRA, LqsRS, and LetAS regulate expression of Dot/Icm effector-encoding genes (29). A prototypical TCS consists of a membrane-localized sensory histidine kinase and a cognate cytosolic response regulator. Environmental signals trigger autophosphorylation of the histidine kinase at a conserved histidine residue. Phosphotransfer to a conserved aspartate on the response regulator then initiates an output response that commonly involves DNA binding and transcriptional regulation (30), although output domains can also have enzymatic activity that directly transduces signals (e.g., diguanylate cyclases and methyltransferases) (31). Most genes encoding TCS kinase-regulator pairs occur in operons. Currently, *L. pneumophila* PmrAB and CpxRA are known to directly control expression of 43 (22, 29, 32) and 11 (29, 33) effector-encoding genes, respectively, with consensus nucleotide binding sequences identified for the response regulators PmrA (22) and CpxR (33). LqsRS is a quorum-sensing TCS that indirectly regulates expression of 12 effector-encoding genes (29, 34). The sensor kinase LqsS is stimulated by the autoinducer molecule LAI-1, resulting in activation of the response regulator LqsR (35). However, LqsR lacks a DNA-binding motif; consequently, the precise mechanism of gene regulation is unresolved (34). LetAS controls expression of 26 effector-encoding genes by inducing expression of two small RNAs (sRNAs) (RsmY and RsmZ) that inhibit the RNA-binding protein CsrA, a posttranscriptional inhibitor of effector gene expression (29, 36–38). LetS is a hybrid histidine kinase that utilizes a four-step phosphorelay to activate the response regulator LetA (30, 39–41). LetA belongs to the NarL family of response regulators that contain a helix-turn-helix DNA-binding motif (31, 42). *letA* and *letS* are unlinked and consequently considered orphan TCS genes (30).

C. burnetii lacks homologs of CpxRA and LqsRS. Clear homologs of LetAS are also not evident; however, *C. burnetii* encodes four orphan hybrid histidine kinases (39) and four orphan NarL family response regulators (31, 42). Thus, it is conceivable that a TCS derived from this group of proteins functions similarly to LetAS (40). Interestingly, *C. burnetii* encodes two CsrA proteins but lacks predicted CsrA-regulating sRNAs (43). *C. burnetii* encodes homologs of *L. pneumophila* PmrAB (QseBC), with both response regulator PmrAs containing a typical CheY-like receiver domain and an OmpR family winged-helix DNA-binding output domain (42). Based on the presence of the upstream PmrA regulatory element, cTTAATatT-N₂-cTTAATatT (where lowercase letters indicate less conserved nucleotides), Zusman et al. (22) predicted 68 PmrA-regulated genes in the *C. burnetii* genome, including *icmD*, the *CoxigA* gene, *dotD*, *icmV*, and *icmW*, encod-

ing components of the secretion apparatus. Furthermore, they showed that the *CoxigA* gene and *icmW* are expressed in a PmrA-dependent fashion by wild-type *L. pneumophila* and by an *L. pneumophila* *pmrA* deletion mutant expressing the *C. burnetii* PmrA homolog (22). A second permutation of the PmrA regulatory element proposed by Chen et al. (14) expanded the number of potential *C. burnetii* PmrA-regulated genes to 126. Subsequent testing of these candidate genes in *L. pneumophila* revealed that approximately 27% encode bona fide Dot/Icm substrates (13, 14).

In the current study, we employed a *C. burnetii* *pmrA* deletion mutant to evaluate the role of the PmrAB operon in the intracellular replication of *C. burnetii* and regulation of the T4BSS. *C. burnetii* Δ *pmrA* exhibits severe intracellular growth defects compared to wild-type bacteria. Luciferase gene reporter assays, effector translocation assays, RNA sequencing (RNA-seq), and mass spectrometry (MS) confirm that the defective intracellular growth of the Δ *pmrA* mutant is associated with dysregulation of the Dot/Icm T4BSS. However, transcriptome and proteome data also reveal new Dot/Icm substrates and several PmrA-regulated genes potentially involved in virulence that lack a PmrA regulatory element. Thus, the PmrAB TCS may cross talk with other TCSs and/or indirectly control expression of additional regulatory systems. This study begins to unravel the transcriptional regulatory networks associated with *C. burnetii* virulence.

MATERIALS AND METHODS

Bacterial strains and mammalian cell lines. The bacterial strains used in this study are listed in Table 1. The *C. burnetii* Nine Mile phase II (clone 4, RSA439) strain was utilized throughout this work. Wild-type *C. burnetii* and genetic transformants were grown microaerobically in ACCM-2 as previously described (24). For storage, bacteria were pelleted following 6 days of growth, washed 3 times in phosphate-buffered saline (PBS; 1 mM KH₂PO₄, 155 mM NaCl, 3 mM Na₂HPO₄, pH 7.4), and then suspended in cell-freezing medium (RPMI 1640 medium containing 10% dimethyl sulfoxide and 10% fetal bovine serum [FBS]; Invitrogen, Carlsbad, CA) and frozen at -80°C . *E. coli* Stellar (BD Clontech, Mountain View, CA) and PIR1 (Invitrogen) cells were used for recombinant DNA procedures and cultivated in Luria-Bertani (LB) broth. *E. coli* transformants were selected on LB agar plates containing 50 μg of kanamycin/ml or 10 μg of chloramphenicol/ml. African green monkey kidney (Vero) cells (CCL-81; ATCC) and THP-1 cells (TIB-202; ATCC), a human acute monocytic leukemia cell line, were maintained in RPMI 1640 medium containing 10% FBS at 37°C and 5% CO₂. *C. burnetii* replication in host cells or in ACCM-2 was measured by quantitative PCR of genome equivalents (GE) as previously described (3, 44) using a probe specific to *dotA*.

Generation and complementation of a *pmrA* mutant. The plasmids and oligonucleotide primers used in this study are listed in Tables 1 and 2, respectively. Restriction enzymes were obtained from New England Biolabs (Ipswich, MA). PCR was performed using Accuprime *Pfx* or *Taq* polymerase (Invitrogen). PCR primers were obtained from Integrated DNA Technologies (San Diego, CA). All cloning procedures were conducted using an In-Fusion PCR cloning system (BD Clontech), and DNA was transformed into either *E. coli* Stellar or PIR1 competent cells. For targeted inactivation of *C. burnetii* *pmrA* (*cbu1227*), the 5'- and 3'-end-flanking regions of the gene were first amplified from genomic DNA by PCR using the upstream and downstream oligonucleotide pairs CBU1227-5'-F/CBU1227-5'-R and CBU1227-3'-F/CBU1227-3'-R, respectively. The 5' and 3' fragments were cloned into BamHI/SalI-digested pJC-CAT (12) by In-Fusion PCR, resulting in the formation of an internal AgeI site between the 5' and 3' regions and the creation of pJC-CAT::CBU1227-5'3'. The P1169-Kan cassette was amplified from pJB-Kan (24) by PCR with P1169-Kan-AgeI-KO-rev-F and P1169-Kan-AgeI-KO-rev-R

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or phenotype	Source or reference
Strains		
<i>E. coli</i> Stellar	F ⁻ <i>endA1 supE44 thi-1 recA1 relA1 gyrA96 phoA</i> ϕ 80 Δ <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 Δ (<i>mrr-hsdRMS-mcrBC</i>) Δ <i>mcrA</i> λ ⁻	Clontech
<i>E. coli</i> PIR1	F ⁻ Δ <i>lac</i> -169 <i>rpoS</i> (Am) <i>robA1 creC510 hsdR514 endA recA1 uidA</i> (Δ MluI):: <i>pir</i> -116	Invitrogen
<i>C. burnetii</i> Nine Mile (RSA439)	Phase II, clone 4	Beare et al. (12)
<i>C. burnetii</i> Δ <i>pmrA</i>	Nine Mile, phase II containing a <i>pmrA</i> (<i>cbu1227</i>) deletion; Kan ^r	This study
<i>C. burnetii</i> Δ <i>dotA</i>	Nine Mile, phase II containing a <i>dotA</i> (<i>cbu1648</i>) deletion; Kan ^r	Beare et al. (12)
Plasmids		
pJB-Kan	pJB2581 containing <i>kan</i> driven by <i>P1169</i> ; Kan ^r	Omsland et al. (24)
pJC-CAT	pJC84 containing <i>cat</i> driven by <i>P1169</i> ; Cm ^r	Beare et al. (12)
pJC-CAT:: <i>cbu1227</i> -5'3'	5'- and 3'-end-flanking DNA from <i>cbu1227</i> cloned into pJC-CAT; Cm ^r	This study
pJC-CAT:: <i>cbu1227</i> -5'3'-Kan	<i>P1169</i> -Kan cassette cloned into pJC-CAT:: <i>cbu1227</i> -5'3'; Cm ^r Kan ^r	This study
pTnS2:: <i>P1169</i> - <i>tmsABCD</i>	<i>cbu1169</i> promoter cloned into pTnS2; Amp ^r R6K <i>ori</i>	Beare et al. (12)
pMiniTn7T-CAT	<i>P1169</i> -CAT cloned into pUC18R6K-mini-Tn7T-Gm; Cm ^r Amp ^r R6K <i>ori</i>	Beare et al. (12)
pMiniTn7T-CAT:: <i>cbu1227</i> /1228comp	<i>cbu1227</i> /1228comp fragment cloned into pMiniTn7T-CAT; Cm ^r Amp ^r	This study
pGSV4	Vector containing the <i>luxCDABE</i> operon	Warawa et al. (45)
pMiniTn7T-CAT:: <i>luxCDABE</i>	Promoterless <i>luxCDABE</i> fragment cloned into pMiniTn7T-CAT; Cm ^r Amp ^r	This study
pMiniTn7T-CAT::P0021- <i>luxCDABE</i>	<i>cbu0021</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P0794- <i>luxCDABE</i>	<i>cbu0794</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P0814- <i>luxCDABE</i>	<i>cbu0814</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::PenhC- <i>luxCDABE</i>	<i>enhC</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P1213- <i>luxCDABE</i>	<i>cbu1213</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P1226- <i>luxCDABE</i>	<i>cbu1226</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P <i>pmrA</i> - <i>luxCDABE</i>	<i>pmrA</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P1314- <i>luxCDABE</i>	<i>cbu1314</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P <i>gltA</i> - <i>luxCDABE</i>	<i>gltA</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P1556- <i>luxCDABE</i>	<i>cbu1556</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P1636- <i>luxCDABE</i>	<i>cbu1636</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P <i>rpoS</i> - <i>luxCDABE</i>	<i>rpoS</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P1751- <i>luxCDABE</i>	<i>cbu1751</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P1758- <i>luxCDABE</i>	<i>cbu1758</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P1823- <i>luxCDABE</i>	<i>cbu1823</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P2052- <i>luxCDABE</i>	<i>cbu2052</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P <i>icmH</i> - <i>luxCDABE</i>	<i>icmH</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P <i>icmW</i> - <i>luxCDABE</i>	<i>icmW</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P <i>icmV</i> - <i>luxCDABE</i>	<i>icmV</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P <i>dotD</i> - <i>luxCDABE</i>	<i>dotD</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P <i>icmT</i> - <i>luxCDABE</i>	<i>icmT</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P <i>coxigA</i> - <i>luxCDABE</i>	<i>CoxigA</i> gene promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pMiniTn7T-CAT::P <i>icmD</i> - <i>luxCDABE</i>	<i>icmD</i> promoter cloned into pMiniTn7T-CAT- <i>luxCDABE</i>	This study
pJB-CAT-CyaA	<i>C. burnetii</i> CyaA fusion vector; Cm ^r Amp ^r	Beare et al. (12)
pJB-CAT-CyaA- <i>cbu0122</i>	<i>cbu0122</i> cloned into pJB-CAT-CyaA	This study
pJB-CAT-CyaA- <i>cbu0409</i>	<i>cbu0409</i> cloned into pJB-CAT-CyaA	This study
pJB-CAT-CyaA- <i>cbu0508</i>	<i>cbu0508</i> cloned into pJB-CAT-CyaA	This study
pJB-CAT-CyaA- <i>cbu0705</i>	<i>cbu0705</i> cloned into pJB-CAT-CyaA	This study
pJB-CAT-CyaA- <i>cbu1231</i>	<i>cbu1231</i> cloned into pJB-CAT-CyaA	This study
pJB-CAT-CyaA- <i>cbu1530</i>	<i>cbu1530</i> cloned into pJB-CAT-CyaA	This study
pJB-CAT-CyaA- <i>cbu1540</i>	<i>cbu1540</i> cloned into pJB-CAT-CyaA	This study
pJB-CAT-CyaA- <i>cbu1614</i>	<i>cbu1614</i> cloned into pJB-CAT-CyaA	This study
pJB-CAT-CyaA- <i>cbu1651</i>	<i>cbu1651</i> cloned into pJB-CAT-CyaA	This study
pJB-CAT-CyaA- <i>cbu1685</i>	<i>cbu1685</i> cloned into pJB-CAT-CyaA	This study
pJB-CAT-CyaA- <i>cbu1686</i>	<i>cbu1686</i> cloned into pJB-CAT-CyaA	This study
pJB-CAT-CyaA- <i>cbu1752</i>	<i>cbu1752</i> cloned into pJB-CAT-CyaA	This study
pJB-CAT-CyaA- <i>cpeD</i>	<i>cbuA0015</i> cloned into pJB-CAT-CyaA	Beare et al. (12)
pJB-CAT-CyaA- <i>cpeE</i>	<i>cbuA0016</i> cloned into pJB-CAT-CyaA	Beare et al. (12)

oligonucleotides and cloned into AgeI-digested pJC-CAT::CBU1227-5'3' to create pJC-CAT::CBU1227-5'3'-Kan. For complementation of *C. burnetii* Δ *pmrA*, the *cbu1227* promoter region, along with *cbu1227* and *cbu1228*, was amplified from genomic DNA by PCR using the oligonucleotides CBU1227comp-F and CBU1227comp-R. The resulting fragment was cloned into EcoRI-digested pMini-Tn7T-CAT (12) by In-Fusion PCR to create pMini-Tn7T-CAT::*cbu1227*/1228comp.

Electroporation of *C. burnetii* was conducted as previously described (24). Selection of *C. burnetii* "loop-in" pJC-CAT::CBU1227-5'3'-Kan transformants with chromosomal integration of the suicide plasmid was

conducted by culture of bacteria in ACCM-2 containing kanamycin (final concentration, 350 μ g/ml) and chloramphenicol (final concentration, 3 μ g/ml). Resolution of the *sacB*-bearing plasmid cointegrant was accomplished by subculture of transformants for 4 days in ACCM-2 supplemented with 1% sucrose and kanamycin. *C. burnetii* Δ *pmrA* was subsequently expanded by culture in ACCM-2 containing kanamycin. Clonal isolation was achieved by limiting dilution in ACCM-2, and deletion of *pmrA* was verified by PCR. PCR validation was accomplished by amplifying an internal *pmrA* gene fragment with specific primer pairs, with wild-type and mutant genomic DNAs as the templates. Generation and verifi-

TABLE 2 Oligonucleotide primers used in this study

Category and primer	Sequence (5' to 3')
Primers for gene deletion, analysis, and complementation	
CBU1227-5'-F	CGGTACCCGGGGATCCGCTGCTCACCTACTACGCGCAAC
CBU1227-5'-R	CACCACCGGTGACGCGAGCGTACGCCCTGACGGTCTTGGGCATC
CBU1227-3'-F	CGTCGACCGGTGGTGGCGATGTACGTCGGTAGAAAAAAGTCTGAATGACG
CBU1227-3'-R	GAACCTGTTTGTGCGACTGAACTCGCTATTGGCTTCAAG
P1169-Kan-Agel-KO-rev-F	CATGCGCACCACCGGTATGGCTTCGTTTCGCAGCG
P1169-Kan-Agel-KO-rev-R	GCTCGCGTCGACCGGTTTATCAGAAGAACTCGTCAAGAAGG
CBU1227-F	CTTGTTGAAGATGATGAATTTCTCG
CBU1227-R	TTCAGACTTTTTTTCTACCATGTAAC
CBU1227-comp-F	TACTCAATGGAATTCGGTATAGCATCAACTCCCAGTG
CBU1227-comp-R	GCTTCTCGAGGAATTCCTATCCACAGAAATTGTGAATAAGGG
Primers for luciferase vector construction	
miniTn7-Lux-LR-noP-F	GTATCGATAAGCTAGCGGATCCCAGTCTGCAGATGGCAAATATGACTAAAAAATTCAT TCATTATTAACGG
miniTn7-Lux-LR-noP-R	TGCCAACAGATGTACAGATTTACCTTTTCGGAAAAAGCC
Primers for luciferase promoter fusions	
PCBU0021-Lux-F	ATAAGCTAGCGGATCCGCATGAACGACCTCCTCATTAC
PCBU0021-Lux-R	GTCATATTTGCCATCGTTTATCTCCAGCGCTTACG
PCBU0794-Lux-F	ATAAGCTAGCGGATCCCATAAAAATCAAGATACAAGTTGGG
PCBU0794-Lux-R	GTCATATTTGCCATCTTTTTTATCCACCTTACAAAAATTTAGAG
PCBU0814-Lux-F	ATAAGCTAGCGGATCCGCGGAACACTTATTTTATGGATGACC
PCBU0814-Lux-R	GTCATATTTGCCATCAAAACCATATTTCTCATTTTCAATTGCTTTAATG
PenhC-Lux-F	ATAAGCTAGCGGATCCCACGGTTAGATGTAGAAAGCC
PenhC-Lux-R	GTCATATTTGCCATCCCATTAAATTTTCCCTAGTTAAAAATATAGC
PCBU1213-Lux-F	ATAAGCTAGCGGATCCTGGTGCCCCAAGGCGATTCTAC
PCBU1213-Lux-R	GTCATATTTGCCATCTATAAGCTGCTCACTGGGAAAC
PCBU1226-Lux-F	ATAAGCTAGCGGATCCCGAGGTGCGAGGATAATAATATC
PCBU1226-Lux-R	GTCATATTTGCCATCAATGGATACCCCTGTATC
PpmrA-Lux-F	ATAAGCTAGCGGATCCCGACTTTTTCAACGATTCTATC
PpmrA-Lux-R	GTCATATTTGCCATCCACTCTCACCTTACTTACTGCG
PCBU1314-Lux-F	ATAAGCTAGCGGATCCGTTATGAAAAATGATTTATTCATC
PCBU1314-Lux-R	GTCATATTTGCCATCGAGGTAACCTCCATTTTATAC
PglA-Lux-F	ATAAGCTAGCGGATCCCCTTATCTTCTCAAACGCC
PglA-Lux-R	GTCATATTTGCCATCTCGTGCTCCTGGCGGGTG
PCBU1556-Lux-F	ATAAGCTAGCGGATCCGAAGACGCTCTCTCATTAC
PCBU1556-Lux-R	GTCATATTTGCCATCAAAATACCCTTTTTATTTTCGTTTTTCGG
PCBU1636-Lux-F	ATAAGCTAGCGGATCCTGAATTATATTTTCATCCAAGCTTAC
PCBU1636-Lux-R	GTCATATTTGCCATCCCTGTTTTTCCCTCCGAAGTTAGTC
PrpoS-Lux-F	ATAAGCTAGCGGATCCCTTCCGATGGAGTTGTTGTATATAG
PrpoS-Lux-R	GTCATATTTGCCATCAGTCTGCTGCTCCTTAACCCG
PCBU1751-Lux-F	ATAAGCTAGCGGATCCGACAACCCACATAGCGGGAG
PCBU1751-Lux-R	GTCATATTTGCCATCATTCCAACCTCTCACCGAAC
PCBU1758-Lux-F	ATAAGCTAGCGGATCCGTCCTGCTGCTGCTTACCTTACCC
PCBU1758-Lux-R	GTCATATTTGCCATCAAAAACCTCCCTTTTATAGTAAAGAG
PCBU1823-Lux-F	ATAAGCTAGCGGATCCGTTCCGCGGATCTAAATGACCGCC
PCBU1823-Lux-R	GTCATATTTGCCATCATTTCCTCCGCAATAGCTTAGG
PCBU2052-Lux-F	ATAAGCTAGCGGATCCCAGGAATGGCGAGATTCTTTC
PCBU2052-Lux-R	GTCATATTTGCCATCTAAGGCCCTATCAAAGAAATG
PicmH-Lux-F	ATAAGCTAGCGGATCCGCCCCGATCCCCGTAACCTTACG
PicmH-Lux-R	GTCATATTTGCCATCTACGGCGTATTATACCTGCAATAAATTTG
PicmW-Lux-F	ATAAGCTAGCGGATCCCAACGATATTTCTGATGGTTTTG
PicmW-Lux-R	GTCATATTTGCCATCGACTTCTCCGCTATTTAGGGTC
PicmV-Lux-F	ATAAGCTAGCGGATCCGTTTCGATCTCGGGATTGCCATC
PicmV-Lux-R	GTCATATTTGCCATCTCATGCTAGCACCCTTCTTAAAG
PdotD-Lux-F	ATAAGCTAGCGGATCCTGGGGCAACGTACGAAGATAC
PdotD-Lux-R	GTCATATTTGCCATCTTTCAGTCTGCTTAAATCAAGTAAGG
PicmT-Lux-F	ATAAGCTAGCGGATCCCTACGGTGGATACATACTGAG
PicmT-Lux-R	GTCATATTTGCCATCAAAAGACCCTTTATACTGGTTTTAAATTTAAC

(Continued on following page)

TABLE 2 (Continued)

Category and primer	Sequence (5' to 3')
PcoxigA-Lux-F	ATAAGCTAGCGGATCCGCCCTCTGAGGAGGAAAGCTCAAC
PcoxigA-Lux-R	GTCATATTTGCCATCGTCAATACCCTTATTCGTAGATTAATCC
PicmD-Lux-F	ATAAGCTAGCGGATCCGCAGCTCATTCCGCCAATTATTG
PicmD-Lux-R	GTCATATTTGCCATCGATGACTAATCTCCAGAAATAATAATTTTTCTAC
Primers for CyaA fusions	
CBU0122-CyaA-F	TTCCGGCTATGTCGACATGAGTACGGCTAATAATTGTG
CBU0122-CyaA-R	GCATGCCTCAGTCGACCTATTTTTGGGGGTAGAAAAAAGCTCG
CBU0409-CyaA-F	TTCCGGCTATGTCGACATGGCCACAGTCCCAGTCATAGG
CBU0409-CyaA-R	GCATGCCTCAGTCGACTTACTGCCCCCTGCATGTTTATTTTTAGG
CBU0508-CyaA-F	TTCCGGCTATGTCGACATGCCAACAAATTTAGAAACCG
CBU0508-CyaA-R	GCATGCCTCAGTCGACTTAATGAGTAATCAGAAACCGCCGC
CBU0705-CyaA-F	TTCCGGCTATGTCGACATGAATAGTCCAACAGGAAGTAGC
CBU0705-CyaA-R	GCATGCCTCAGTCGACCTAAATCATTTCACGGCTCACC
CBU1231-CyaA-F	TTCCGGCTATGTCGACATGTCAAGGCAATCATTTCATCACC
CBU1231-CyaA-R	GCATGCCTCAGTCGACCTAAAAGAAAAACCCCAATAGTCCACC
CBU1530-CyaA-F	TTCCGGCTATGTCGACATGCCTTTATCTAAAGAAGAAATTC
CBU1530-CyaA-R	GCATGCCTCAGTCGACTTAAATAGTCTAGCTCGGAGAAAATCTAC
CBU1540-CyaA-F	TTCCGGCTATGTCGACATGCTTTTCGTTATTTTGCTTCAAAGG
CBU1540-CyaA-R	GCATGCCTCAGTCGACCTATGGATAGTAAATTTTATTATAAGTG
CBU1614-CyaA-F	TTCCGGCTATGTCGACATGCTCAATCTATATTTCCAGAAGC
CBU1614-CyaA-R	CATGCCTCAGTCGACTCAATTAATGTTTTATTGAACAAGAACAGC
CBU1651-CyaA-F	TTCCGGCTATGTCGACATGAATAAATATCTTTTAAATAGGAACG
CBU1651-CyaA-R	GCATGCCTCAGTCGACTTAAAACAGTGAATATTAGATGAATC
CBU1685-CyaA-F	TTCCGGCTATGTCGACATGAGAACACCATCTCAACCGTG
CBU1685-CyaA-R	GCATGCCTCAGTCGACTTATTTAATTTAATGCTAGAAATGG
CBU1686-CyaA-F	TTCCGGCTATGTCGACATGCCCTCTAGTTCTAAAGATTTAAGAAAAAGTTAAAAAAC
CBU1686-CyaA-R	GCATGCCTCAGTCGACTTACTAGGGTTGATATCCGTTTG
CBU1752-CyaA-F	TTCCGGCTATGTCGACATGAGAGATCCAGATCAAGAAATGC
CBU1752-CyaA-R	GCATGCCTCAGTCGACTTATGAAGGGCCGAATGCCGGGAC

cation of a Tn7-based genetic complement of *C. burnetii* Δ pmrA were performed as previously described (12).

Genes conferring resistance to chloramphenicol, kanamycin, or ampicillin were approved for *C. burnetii* genetic transformation studies by the Rocky Mountain Laboratories Institutional Biosafety Committee and the Centers for Disease Control and Prevention, Division of Select Agents and Toxins Program.

Construction of luciferase reporter strains. The operon encoding luciferase (*luxCDABE*) was amplified by PCR from pGSV4 (45) using the primers miniTn7-Lux-LR-noP-F and miniTn7-Lux-LR-noP-R. The promoterless *luxCDABE* PCR fragment was cloned into NheI- and EcoRI-digested pMiniTn7T-CAT by using In-Fusion PCR to create pMiniTn7T-CAT::*luxCDABE*. Promoters for testing in the luciferase assay were amplified by PCR using the primers listed in Table 2. The corresponding fragments were cloned by In-Fusion PCR into BamHI/PstI-digested pMiniTn7T-CAT::*luxCDABE* to create the plasmids listed in Table 1. These plasmids were used to transform *C. burnetii* and generate strains expressing single-copy *lux* promoter fusions.

Luciferase assays. Individual wells of a 12-well tissue culture plate containing 2 ml of ACCM-2 were inoculated with *C. burnetii* harboring pMiniTn7T-CAT-*luxCDABE* promoter constructs at a cell density of 1×10^6 GE/ml. After 4 days of incubation, the growth medium was thoroughly mixed by pipetting and 200 μ l of each culture was added to a Microtiter 2, white, 96-well plate (Thermo Scientific). Luminescence was measured over 5 s using a Safire2 microplate reader (Tecan).

Indirect immunofluorescence. Infected Vero cells were fixed for 20 min in 4% paraformaldehyde plus PBS, followed by permeabilization for 5 min in 0.1% saponin in PBS. Cells were stained for indirect immunofluorescence as previously described (4, 46). Rabbit anti-*C. burnetii* serum and a mouse monoclonal antibody directed against LAMP3 (CD63) (clone H5C6; BD Biosciences) were used as primary antibodies. Alexa

Fluor 488 and 594 IgG (Invitrogen) were used as secondary antibodies. Coverslips were mounted using ProLong Gold containing 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) to visualize nuclei. Microscopy was conducted using a modified Perkin-Elmer UltraView spinning-disk confocal system connected to a Nikon Eclipse Ti-E inverted microscope. Images were obtained using MetaMorph software (Molecular Devices, Inc., Downingtown, PA) and processed with ImageJ software (written by W. S. Rasband at the U.S. National Institutes of Health, Bethesda, MD, and available from <http://rsb.info.nih.gov/ij/>).

Construction of cyaA plasmids and translocation assays. Genes encoding potential Dot/Icm substrates were amplified by PCR from *C. burnetii* genomic DNA using the oligonucleotides listed in Table 2. The resulting PCR products were then cloned by In-Fusion PCR into the unique Sall site of pJB-CAT-CyaA to create the plasmids listed in Table 1. THP-1 cells (1×10^5 per well) in 24-well plates were differentiated into macrophage-like cells by incubation overnight in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum (FBS) and 200 nM phorbol myristate acetate (PMA) (Sigma-Aldrich). Cells were washed once with RPMI 1640 plus 10% FBS, infected with 1×10^6 *C. burnetii* transformants expressing CyaA fusion proteins, and incubated in RPMI 1640 plus 10% FBS for 48 h. The concentration of cyclic AMP (cAMP) in lysates from infected cells was determined using the cAMP enzyme immunoassay (GE Healthcare) as previously described (11). Positive secretion of CyaA fusion proteins was scored as a cytosolic cAMP level ≥ 2.5 -fold higher than that for cells infected with *C. burnetii* expressing CyaA alone (17).

Immunoblotting. *C. burnetii* strains were cultivated for 4 days in ACCM-2 and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting to assess expression of IcmD, IcmK, and DotA. As a loading control, blots were also probed with monoclonal antibody against elongation factor Ts (EF-Ts; generously provided by James Samuel, Texas A&M University). Rabbit

anti-IcmK and -DotA antibodies were a generous gift of Edward Shaw, Oklahoma State University, and rabbit anti-IcmD antibody has been previously described (11). Following incubation of membranes with primary antibody, reacting proteins were detected using anti-rabbit or anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase (Pierce, Rockford, IL) and chemiluminescence using ECL Pico reagent (Pierce).

RNA-seq. Wild-type *C. burnetii* and the $\Delta pmrA$ knockout strain were inoculated in triplicate into 20 ml of ACCM-2 at a concentration of 1×10^6 GE/ml. Bacteria were grown in ACCM-2 for 4 days and then harvested by centrifugation ($10,000 \times g$) for 10 min at 4°C. Pellets were suspended in 1 ml of TRIzol and frozen at -80°C . RNA was extracted as previously described (47) prior to rRNA depletion using the MICROExpress kit (Ambion, Life Technologies). Purified and ribosome-depleted RNAs were sequenced and analyzed by the Oregon State University Center for Genome Research and Biocomputing (OSU CGRB). Samples were processed and multiplexed using an Illumina TruSeq RNA kit, followed by paired-end, 100-bp sequencing using an Illumina HiSeq 2000 system. Three samples each of purified and ribosome-depleted RNAs were sequenced to produce an average of 28.3 million paired-end traces each. Sequences were aligned to the *C. burnetii* Nine Mile (RSA493) genome (GenBank accession number [GI:71066702](https://www.ncbi.nlm.nih.gov/nuccore/GI:71066702)) using TopHat2 (48). Gene annotations for alignment were created from the GenBank file using custom Perl (practical extraction and reporting language) scripts. Read counts per gene (fragments per kilobase of transcript per million reads mapped) and differential expression were calculated with the Cuffdiff 2 algorithm (49). The R bioconductor package cummeRbund (50) was used to identify differentially expressed genes.

Microcapillary reverse-phase high-performance LC-MS/MS. Wild-type *C. burnetii* and the $\Delta pmrA$ mutant were grown for 4 days in ACCM-2, and then equal numbers of bacteria (based on GE) were pelleted, washed 3 times in PBS, and suspended in $2 \times$ Laemmli sample buffer. Samples were boiled prior to one-dimensional SDS-PAGE, conducted by the Research Technologies Branch of the National Institute of Allergy and Infectious Diseases. Each sample lane was cut into 24 pieces for identification of separated proteins. Protein identification was performed on reduced and alkylated, trypsin-digested samples prepared by standard mass spectrometry (MS) protocols. The supernatant and two washes (5% formic acid in 50% acetonitrile) of the gel digests were pooled and dried in 200 μl polypropylene auto-sampler vials (SUN-SRI, Rockwood, TN) using a SpeedVac (Labconco, Kansas City, MO). Recovered peptides were resuspended in 5 μl of solvent A (0.1% formic acid, 2% acetonitrile, and 97.9% water). Prior to mass spectrometry, the resuspended peptides were chromatographed directly on a column without trap cleanup. The bound peptides were separated at 500 nl/min, generating 8 to 12 million Pa of pressure, using a C_{18} reverse-phase medium packed in a pulled-tip, nano-chromatography column (0.100 mm [internal diameter] by 150 mm [length]) from Precision Capillary Columns (San Clemente, CA). Chromatography was performed in line and at room temperature with an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, West Palm Beach, FL) using a mobile phase consisting of a linear gradient prepared from solvent A and solvent B (0.1% formic acid, 2% water, and 97.9% acetonitrile). Nano-electrospray liquid chromatography–tandem MS (LC-MS/MS) was performed with a Proxeon Easy-nLC II multidimensional liquid chromatograph and a temperature-controlled Ion Max Nanospray source (Thermo Fisher Scientific) in line with the LTQ Orbitrap Velos mass spectrometer.

Computer-controlled, data-dependent, automated switching to MS/MS by Xcalibur 2.1 software was used for data acquisition and provided the peptide sequence information. UniProt KB/Swiss-Prot (August 2013) and the UniProtKB/TrEMBL (August 2013) databases containing *C. burnetii* genomic sequences were searched using Mascot software (Matrix Science, Beachwood, OH), with one allowed missed cleavage and mass tolerances of 10 ppm and 0.8 Da for the precursor and fragment ions, respectively. Carbamidomethylation of cysteine was set as a fixed modification, while oxidation of methionine, deamidation of asparagine and

glutamine, and N-terminal acetylation of protein were searched as dynamic modifications. The resulting search files were reclustered against the same sequence database for further analysis using ProteoIQ software (PREMIER Biosoft, Palo Alto, CA). Assignments were filtered using the Protein Prophet algorithm as implemented within ProteoIQ, with cutoff filters set to 95% for proteins. Normalization was carried out by both total sampling and normalized spectral abundance factors (NSAF) (51). Two spectra per peptide and two peptides per protein minimum were used to refine the results, as were protein and peptide probability filters of 0.95 and 0.5, respectively. Protein NSAF are calculated as the number of spectral counts for protein X (SpC) divided by the number of amino acids (*L*) in protein X divided by the sum of SpC/*L* for all proteins in the experimental data set.

Statistical analysis. Statistical analyses were performed using a one-way analysis of variance (ANOVA) and Prism software (GraphPad Software, Inc., La Jolla, CA).

RESULTS

***C. burnetii* requires PmrA for intracellular replication.** Previous studies using *L. pneumophila* as a surrogate host indicated that the response regulator PmrA has an important role in the transcription of *C. burnetii* Dot/Icm genes. Furthermore, Dot/Icm function is necessary for the intracellular growth of *C. burnetii* (10, 11, 13). To directly examine the importance of PmrA in the replication of *C. burnetii* in mammalian host cells, we generated a *pmrA* mutant by targeted gene deletion (12). Using Tn7, the mutant was genetically complemented in *cis* with a single copy of *pmrAB* under the control of its native promoter. Levels of growth of wild-type *C. burnetii*, the $\Delta pmrA$ mutant, and the complemented mutant were indistinguishable in the axenic medium, ACCM-2 (Fig. 1A). However, *C. burnetii* $\Delta pmrA$ replication was significantly reduced in Vero cells at 6 days postinfection, as shown by a 3-fold increase in GE, relative to a 300-fold increase for wild-type *C. burnetii* (Fig. 1B). As in *C. burnetii* Dot/Icm mutants (12), defective intracellular replication of *C. burnetii* $\Delta pmrA$ correlated with the formation of small, tight-fitting LAMP3-positive vacuoles in Vero cells (Fig. 1C). Complementation by Tn7::*pmrAB* rescued both intracellular replication and PV biogenesis, which confirmed that the altered phenotypes were due to the absence of *pmrA* (Fig. 1B and C). These data indicate that PmrA is essential for the intracellular growth of *C. burnetii*.

Transcription of *pmrA* declines following entry into stationary phase. We next employed the luciferase bioluminescent reporter system to explore PmrA function in *C. burnetii*. The plasmid pMiniTn7T-CAT::*luxCDABE* was constructed and used to generate strains expressing a single copy of *luxCDABE* (*lux*) transcriptionally fused to selected promoter regions. To gain insight into the temporal regulation of *pmrA*, luciferase assays were first conducted on wild-type *C. burnetii* carrying *lux* fused to the *pmrA* promoter region. Luciferase activity was measured after 2, 3, 4, 6, 10, and 14 days of growth in axenic medium. Luciferase activity was maximal and roughly the same through 6 days of growth (Fig. 2). Significant reductions in activity occurred during stationary-phase growth at 10 and 14 days (24). Accordingly, a 4-day time point, representing the late exponential phase of *C. burnetii*'s growth cycle, was used throughout this study to characterize PmrA-mediated gene regulation.

Deletion of PmrA reduces expression of the Dot/Icm apparatus and substrate-encoding genes. Based on the presence of consensus regulatory elements, Zusman et al. (22) predicted a *C. burnetii* PmrA regulon and demonstrated in *L. pneumophila* that two

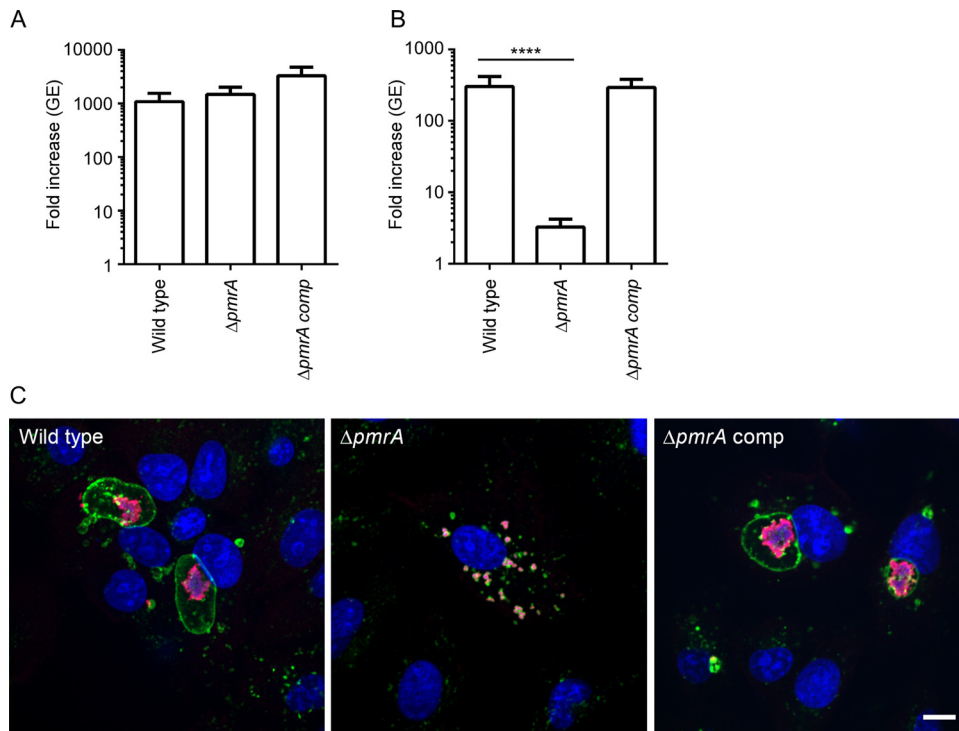


FIG 1 *C. burnetii* $\Delta pmrA$ has severe intracellular growth defects. Replication of wild-type *C. burnetii*, the $\Delta pmrA$ mutant, and the complemented mutant (comp) in ACCM-2 (A) and Vero cells (B). Fold increases in *C. burnetii* genome equivalents (GE) after 6 days of growth are depicted. Results are expressed as the means of results from two biological replicates from four independent experiments. Error bars indicate the standard deviations from the means, and asterisks indicate a statistically significant difference ($P < 0.0001$) compared to values for wild-type *C. burnetii*. (C) Confocal fluorescence micrographs of Vero cells infected for 4 days with wild-type *C. burnetii*, the $\Delta pmrA$ mutant, or the complemented mutant. LAMP3 (green) and *C. burnetii* (red) are stained by indirect immunofluorescence, and DNA (blue) is stained with DAPI. Bar, 5 μ m.

C. burnetii *dot/icm* genes are regulated by PmrA. To explore PmrA's regulation of the Dot/Icm system directly in *C. burnetii*, we constructed *lux* fused to the promoter regions of the Dot/Icm apparatus and substrate-encoding genes. *C. burnetii* and the $\Delta pmrA$ mutant were transformed with Tn7 constructs bearing *lux* fusions, and then luciferase assays were conducted following 4

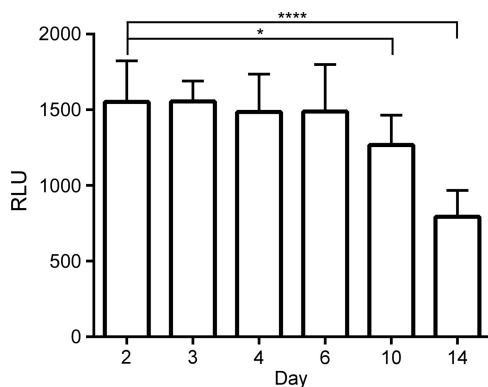


FIG 2 Transcription of *pmrA* declines following entry of *C. burnetii* into stationary phase. Luciferase assays were conducted after 2, 3, 4, 6, 10, and 14 days of growth in axenic medium of wild-type *C. burnetii* carrying *lux* fused to the *pmrA* promoter region. Bioluminescent readings are expressed as relative light units (RLU). Results are expressed as the means of results from two biological replicates from three independent experiments. Error bars indicate the standard deviations from the means, and asterisks indicate a statistically significant difference (*, $P < 0.05$; ****, $P < 0.0001$).

days of growth in axenic medium. Tested genes fell into three groups. Group 1 is comprised of five control genes that lack a PmrA regulatory element: *cbu1136* (*enhC*), *cbu1226*, *cbu1227* (*pmrA*), *cbu1410* (*gltA*), and *cbu1669* (*rpoS*). *cbu1226*, encoding an NAD-specific glutamate dehydrogenase, was included in this group to determine if deletion of closely linked *pmrA* affected its transcription. Group 2 consists of *icmH* (*cbu0321*), *icmD* (*cbu1624*), the CoxigA gene (*cbu1634a*), *icmT* (*cbu1641*), *dotD* (*cbu1643*), *icmV* (*cbu1649*), and *icmW* (*cbu1650*), which are the first structural genes of the seven predicted *dot/icm* operons (Fig. 3A) (52). With the exception of *icmT* and *icmH*, these genes contain an upstream PmrA regulatory element (22). Group 3 contains 11 genes with an upstream PmrA regulatory element that encodes proteins that are positive for Dot/Icm secretion by *L. pneumophila*: *cbu0021* (*cig2*), *cbu0794* (*cig20*), *cbu0814* (*cig22*), *cbu1213* (*cig33*, *ankI*), *cbu1314* (*cig37*), *cbu1556* (*cig50*), *cbu1636* (*cig55*), *cbu1751* (*cig57*), *cbu1758* (*cig58*, *ankM*), *cbu1823* (*cig61*), and *cbu2052* (*cirD*) (13, 15, 16, 18, 22). Furthermore, *cbu1636* and *cbu1823* have been demonstrated to be regulated by PmrA in *L. pneumophila* (22).

All group 1 control genes showed the same level of expression in wild-type *C. burnetii* and the $\Delta pmrA$ mutant. Of note, equal levels of transcription of the $P_{pmrA}::luxCDABE$ reporter fusion indicated that PmrA does not regulate itself. Within group 2, *icmD*, the CoxigA gene, *dotD*, *icmV*, and *icmW* were all expressed in a PmrA-dependent fashion (Fig. 3A and B). The *icmTS* and *icmHF* operons were transcribed independently of PmrA (Fig. 3A

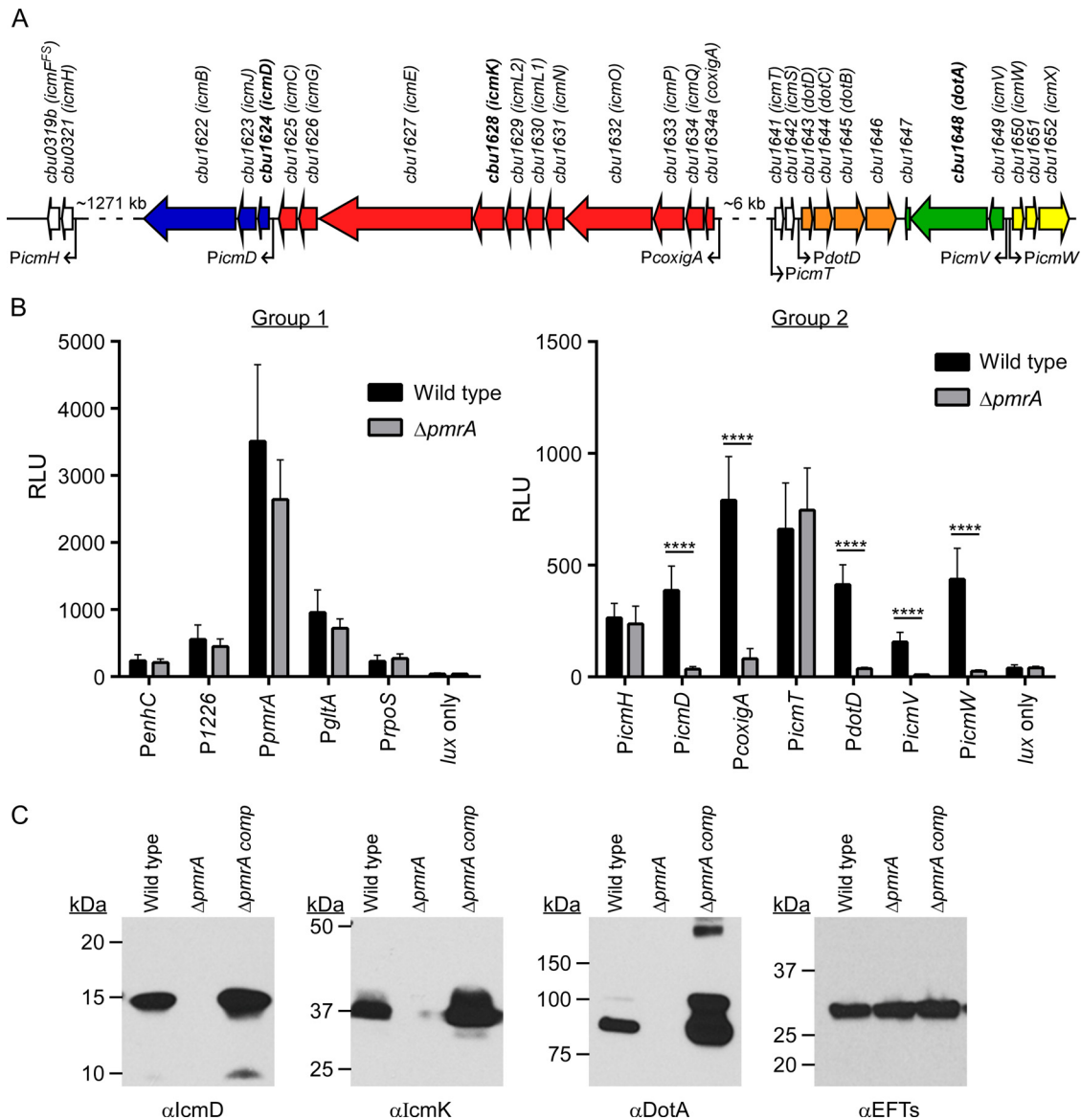


FIG 3 The *C. burnetii* dot/icm locus is regulated by PmrA. (A) Linkage and predicted operon structures of *C. burnetii* dot/icm genes. Operons with upstream PmrA regulatory elements are colored. Operon-specific promoters are indicated with an arrow below the first gene of each predicted operon. *icmF* is truncated due to a frameshift (*icmF^{FS}*). Antibodies specific for proteins encoded by boldface genes were used in immunoblotting, described below. (B) Luciferase activities of the *lux* operon transcriptionally fused to the promoter regions of control genes (left) and dot/icm genes (right). Assays were conducted after 4 days of growth in axenic medium of wild-type *C. burnetii* and the $\Delta pmrA$ mutant expressing *lux* fusions. Bioluminescent readings are expressed as relative light units (RLU). Results are expressed as the means of results from two biological replicates from three independent experiments. Error bars indicate the standard deviations from the means, and asterisks indicate a statistically significant difference ($P < 0.0001$) between wild-type *C. burnetii* and the $\Delta pmrA$ mutant. (C) Immunoblots of lysates of wild-type *C. burnetii* (lane 1), the $\Delta pmrA$ mutant (lane 2), and the complemented mutant (lane 3) probed with anti-IcmD (α IcmD), -IcmK, and -DotA antibodies. Probing for EF-Ts was conducted as a loading control.

and B). Immunoblotting confirmed that production of IcmD, IcmK, and DotA was PmrA dependent (Fig. 3C). Nine group 3 Dot/Icm substrate-encoding genes were regulated by PmrA, while two genes (*cbu0814* and *cbu1758*) were not (Fig. 4). Thus, *lux* assays collectively show that 14 of 16 genes with predicted PmrA regulatory elements are regulated in *C. burnetii* in a PmrA-dependent fashion.

PmrA is required for secretion by the T4BSS. To confirm that PmrA is required for assembly of a functional Dot/Icm secretion apparatus, CyaA translocation assays were performed on wild-type *C. burnetii* and the $\Delta pmrA$ mutant expressing adenylate cy-

clase fused to the defined *C. burnetii* effector proteins CpeD and CpeE (17). Expression of CyaA alone was used as a negative control. With these constructs, fusion protein expression is driven by the *C. burnetii* *hsp20* promoter (17). CpeD and CpeE fusion proteins were secreted in THP-1 macrophages by wild-type *C. burnetii*, as indicated by the ≥ 2.5 -fold increase in cAMP levels relative to those in organisms expressing CyaA alone (Fig. 5). Conversely, cAMP levels generated by the $\Delta pmrA$ mutants did not exceed the CyaA-alone negative-control level (Fig. 5). Negative secretion was not due to a lack of CyaA fusion protein, as immunoblotting revealed equal amounts of fusion protein produced by wild-type *C.*

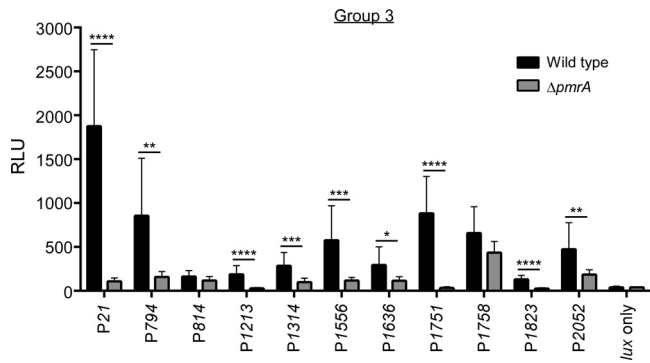


FIG 4 Expression of *C. burnetii* Dot/Icm substrates is regulated by PmrA. Luciferase activities of the *lux* operon transcriptionally fused to the promoter regions of Dot/Icm substrate-coding genes with predicted *pmrA* regulatory elements. Assays were conducted after 4 days of growth in axenic medium of wild-type *C. burnetii* and the $\Delta pmrA$ mutant expressing *lux* fusions. Bioluminescent readings are expressed as relative light units (RLU). Results are expressed as the means of results from two biological replicates from three independent experiments. Error bars indicate the standard deviations from the means, and asterisks indicate a statistically significant difference between wild-type *C. burnetii* and the $\Delta pmrA$ mutant (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.0001$).

burnetii and the $\Delta pmrA$ mutant (data not shown). Collectively, these data agree with *lux* assays and suggest that PmrA-dependent transcriptional activation is essential for secretion by the Dot/Icm T4BSS.

Mutational analysis defines nucleotides required for PmrA-regulated expression. Consensus PmrA regulatory element sequences have been proposed for *C. burnetii* based on experiments conducted with *L. pneumophila* (22). To define nucleotides recognized by PmrA in *C. burnetii*, promoter regions of *pmrA*-regulated *cbu0021* and the *CoxigA* gene were mutated. Two conserved TTAA nucleotide regions in the predicted PmrA-binding regions (22) were changed individually to TACA. In addition, the predicted -10 promoter regions TAGAAT (*cbu0021*) and TATAAT (*CoxigA* gene) were changed to CTGAAT and CTTTCT, respectively. Luciferase assays were conducted after 4 days of growth in axenic medium of wild-type *C. burnetii* expressing *lux* fused to mutated or nonmutated promoter regions. As depicted in Fig. 6, all regulatory element/promoter mutations resulted in dramatically reduced levels of expression compared to that of *lux* fused to the nonmutated promoter. Diminished expression levels were comparable to those associated with the $\Delta pmrA$ mutant expressing *lux* fused to nonmutated *cbu0021* or the *CoxigA* gene promoter regions.

RNA-seq and whole bacterial cell mass spectrometry reveal constituents of the *C. burnetii* PmrA regulon and new Dot/Icm substrates. To obtain an extended view of the PmrA regulon, transcriptional profiling of wild-type *C. burnetii* and the $\Delta pmrA$ mutant cultivated for 4 days in medium was performed using RNA-seq. This procedure identified 49 genes that were downregulated greater than 2-fold in *C. burnetii* $\Delta pmrA$ relative to levels in wild-type bacteria (Table 3 and see Data Set S1 in the supplemental material). No genes were upregulated greater than 2-fold. Twenty-one downregulated genes contained previously identified upstream PmrA regulatory elements (14, 22), including *icmD*, *icmQ*, *dotD*, *icmV*, and *icmW*. Accordingly, 17 additional genes were downregulated due to the polycistronic nature of *dot/icm*

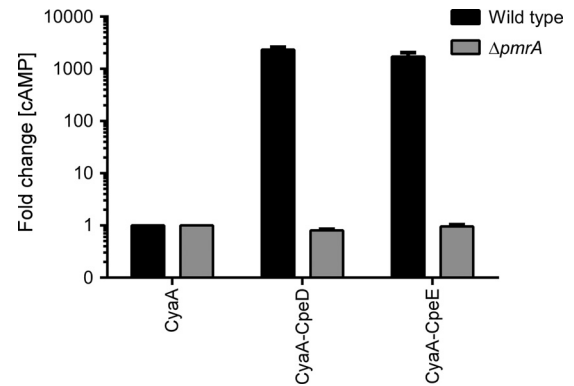


FIG 5 PmrA is required for secretion by the Dot/Icm T4BSS. Cytosolic levels of cAMP were measured following infection of THP-1 macrophages for 2 days with wild-type *C. burnetii* or the $\Delta pmrA$ mutant expressing CyaA alone or CyaA fused to the previously defined Dot/Icm substrates CpeD and CpeE. Elevated levels of cAMP indicating secretion were observed only with wild-type *C. burnetii* expressing CyaA-CpeD or -CpeE fusion proteins. Results shown are from one experiment conducted in duplicate and are representative of three independent experiments. Error bars indicate the standard deviations from the means.

transcription. Eight downregulated genes with PmrA motifs were previously identified as T4BSS substrates (10, 13, 15, 28). The remaining eight downregulated genes with PmrA elements were tested elsewhere for Dot/Icm secretion in *L. pneumophila*, with negative results (13, 15). Twelve downregulated genes (not including *dot/icm* genes) did not contain discernible PmrA motifs. Four of these genes encoded hypothetical proteins with a predicted C-terminal E block translocation signal, one of which (*cbu1686*) encodes a substrate secreted by the *L. pneumophila* Dot/Icm system (13, 15). Replacement of *pmrA* with a kanamycin cassette in the $\Delta pmrA$ mutant correlated with decreased expression of downstream *pmrB*, a result consistent with the predicted operon structure of *pmrAB* (52).

To determine the degree to which transcription correlated with protein synthesis, we performed LC-MS/MS on whole cells of wild-type *C. burnetii* and the $\Delta pmrA$ mutant cultivated for 4 days in axenic medium. Compared to proteins in wild-type bacteria, 39 proteins were ≥ 2 -fold less abundant and 23 were undetected in *C. burnetii* $\Delta pmrA$. Twenty-nine of these proteins (46.8%) are encoded by genes transcriptionally downregulated ≥ 2 -fold in the $\Delta pmrA$ mutant according to RNA-seq results, including 17 and 5 Dot/Icm apparatus proteins and substrates, respectively (Table 3 and see Data Set S2 in the supplemental material). Transcript levels of genes encoding the 33 remaining proteins did not vary more than 2-fold, although 11 of these genes contained PmrA motifs (13, 22). Moreover, 6 proteins within this group are previously described Dot/Icm substrates (10, 13, 15). Proteins encoded by 19 PmrA-regulated genes were not detected in wild-type *C. burnetii* or the $\Delta pmrA$ mutant. Possible explanations for this result include posttranscriptional inhibition of translation and/or protein production below the level of detection by mass spectrometry. Overall, transcriptome and proteome data correspond with the bioinformatically based PmrA regulon (13, 22) but also reveal new regulon genes without PmrA regulatory elements.

RNA-seq revealed six genes (*cbu0409*, *cbu1231*, *cbu1540*, *cbu1614*, *cbu1651*, and *cbu1752*) downregulated in the $\Delta pmrA$ mutant that encode hypothetical proteins, lack PmrA regulatory

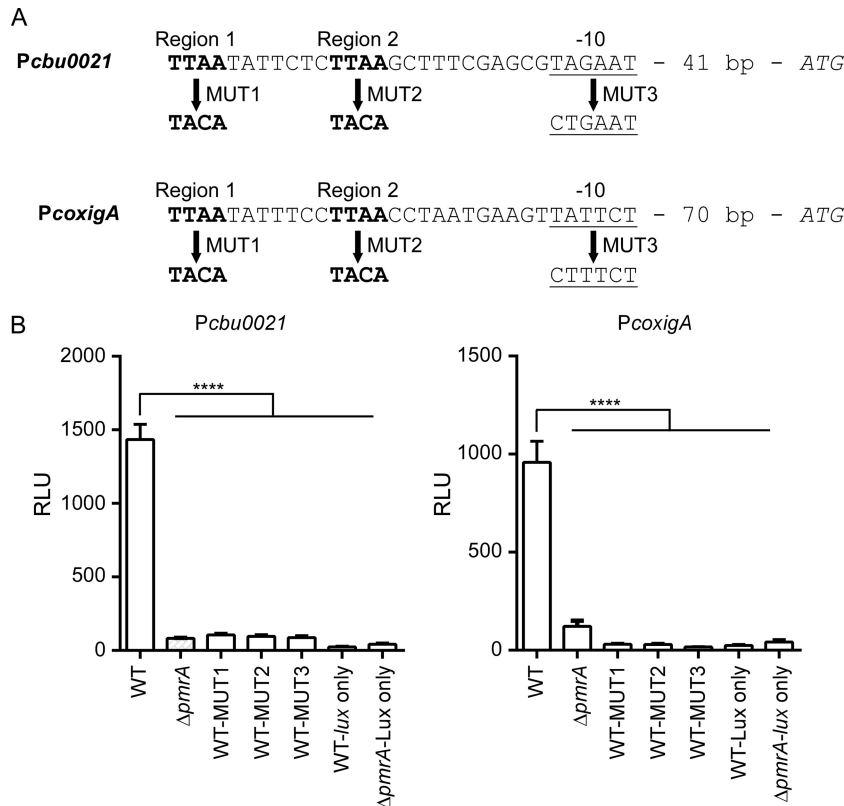


FIG 6 Mutational analysis defines regulatory element nucleotides required for PmrA-driven expression. (A) Regions upstream of *pmrA*-regulated *cbu0021* and the *CoxigA* gene. Boldface nucleotides show mutational changes of the two TTAA regions within the predicted PmrA regulatory element (MUT1 and MUT2), while underlined nucleotides show mutational changes within the -10 promoter region (MUT3). (B) Luciferase activity of wild-type (WT) *C. burnetii* expressing transcriptional proteins of the *lux* operon fused to nonmutated promoter regions or regions containing the MUT1, MUT2, or MUT3 mutation. Luciferase activity was also assessed for the $\Delta pmrA$ mutant expressing *lux* fused to nonmutated promoters and wild-type *C. burnetii* and the $\Delta pmrA$ mutant expressing *lux* alone. Assays were conducted after 4 days of growth in axenic medium. Bioluminescent readings are expressed as relative light units (RLU). Results are expressed as the means of results from two biological replicates from three independent experiments. Error bars indicate the standard deviations from the means, and asterisks indicate a statistically significant difference ($P < 0.0001$) with respect to wild-type *C. burnetii* expressing a nonmutated promoter *lux* fusion.

elements, and have not been previously tested for Dot/Icm-mediated secretion (Table 3). We were curious whether any of these proteins are secreted by the *C. burnetii* Dot/Icm T4BSS. CyaA translocation assays were performed on wild-type *C. burnetii* and a $\Delta dotA$ mutant (11) expressing adenylate cyclase fused to the encoded proteins. Expression of CyaA alone was used as a negative control. As depicted in Fig. 7, CBU1614 and CBU1752 fusion proteins were secreted in THP-1 macrophages by wild-type *C. burnetii*, as indicated by the ≥ 2.5 -fold increase in cAMP levels relative to levels in organisms expressing CyaA alone. Levels of cAMP generated by the $\Delta dotA$ mutant expressing Cya fusion proteins did not exceed the CyaA-alone negative-control level, confirming Dot/Icm-dependent translocation. We extended this analysis to proteins within our PmrA-regulated list with an associated PmrA (CBU0508 and CBU1530) or E block (CBU0122 and CBU0705) motif that were reported as negative for Dot/Icm secretion by *L. pneumophila* (13). Secretion was also assessed for CBU1685 (PmrA motif), and CBU1686 (E block motif), for which there are conflicting reports on secretion by *L. pneumophila* (13, 15). As shown Fig. 7, CBU0122, CBU1530, CBU1685, and CBU1686 fusion proteins were secreted in a Dot/Icm-dependent fashion by *C. burnetii*. Thus, comparative transcriptomics and proteomics of wild-type *C. burnetii* and the $\Delta pmrA$ mutant can be used to identify new candidate substrates of the *C. burnetii* Dot/

Icm system and to clarify disparate secretion results obtained with the surrogate *L. pneumophila* system.

DISCUSSION

Due to a historic lack of molecular methods, genetic analysis of *C. burnetii* regulatory systems required for host cell parasitism and disease pathogenesis has not been possible. For this study, we developed a luciferase-based gene reporter system for use in *C. burnetii* and showed, using a *pmrA* deletion mutant, that the PmrA response regulator of the PmrAB TCS is a critical positive regulator of the *C. burnetii* Dot/Icm T4BSS. Consequently, the severe growth defect of *C. burnetii* $\Delta pmrA$ in mammalian cells is similar to that of *dot/icm* mutants, with little to no replication occurring in LAMP3-positive PVs. Mutation of predicted regulatory elements confirmed nucleotides required for promoter recognition by PmrA, and differential gene expression between wild-type *C. burnetii* and the isogenic $\Delta pmrA$ mutant revealed new Dot/Icm substrates.

There are notable differences between PmrA regulation of *L. pneumophila* and *C. burnetii* Dot/Icm systems. In *L. pneumophila*, *dot/icm* genes lack PmrA motifs and are not directly transcriptionally regulated by PmrA (22). However, *dot/icm* genes are down-regulated in an *L. pneumophila* $\Delta pmrA$ mutant (22, 32), suggesting indirect regulation, possibly through PmrAB cross talk with

TABLE 3 Genes and proteins downregulated ≥ 2 -fold by *C. burnetii* $\Delta pmrA$ compared to their levels of expression in wild-type bacteria

Category and identifier	Product	Gene(s)	Size (aa) ^a	Motif (reference[s])	Dot/Icm substrate (reference[s]) ^b	Fold change	
						RNA-seq	MS ^c
Downregulated by RNA-seq and MS							
CBU0021	Hypothetical protein	<i>cig2</i>	809	PmrA (13, 22)	Yes (15)	-5.1	-14.0
CBU0077	Hypothetical membrane-spanning protein		263	PmrA (13)	Yes (10, 25), ^d no (13)	-2.4	-2.5
CBU0505	Ribosomal protein-alanine acetyltransferase	<i>cig14</i>	205	PmrA (13, 22)	No (13)	-2.9	-8.2
CBU0560	Hypothetical cytosolic protein		410		No (15)	-2.8	-3.5
CBU1103	Membrane-bound lytic murein transglycosylase D precursor	<i>cig29</i>	426	PmrA (13, 22)	No (13)	-4.7	NP
CBU1228	Sensor protein PmrB	<i>pmrB</i>	478			-3.4	NP
CBU1366	Hypothetical exported protein ^e	<i>cig40</i>	110	PmrA (14, 22)	No (14)	-3.1	NP
CBU1622	IcmB protein	<i>icmB</i>	1,003			-3.2	-8.0
CBU1623	IcmJ protein	<i>icmJ</i>	212			-3.2	NP
CBU1624	IcmD protein	<i>icmD</i>	138	PmrA (13, 22)	No (13)	-3.1	-5.7
CBU1626	IcmG protein	<i>icmG</i>	244			-2.6	-5.6
CBU1627	IcmE protein	<i>icmE</i>	1,039			-2.7	-5.3
CBU1628	IcmK protein	<i>icmK</i>	351			-2.8	-6.3
CBU1629	IcmL protein	<i>icmL</i>	218			-3.1	-10.5
CBU1630	IcmL protein	<i>icmL</i>	207			-3.0	-4.4
CBU1631	IcmN protein	<i>icmN</i>	212			-3.5	-16.8
CBU1632	IcmO protein	<i>icmO</i>	792			-3.7	NP
CBU1633	IcmP protein	<i>icmP</i>	387			-4.1	NP
CBU1634	IcmQ protein	<i>icmQ</i>	238	PmrA (13, 22)		-5.0	-18.1
CBU1643	DotD protein	<i>dotD</i>	169	PmrA (13, 22)	No (13)	-2.3	-6.3
CBU1644	DotC protein	<i>dotC</i>	274			-2.5	-6.6
CBU1648	DotA protein	<i>dotA</i>	814			-4.0	-594.3
CBU1649	IcmV protein	<i>icmV</i>	164	PmrA (13, 22)	No (13)	-5.5	NP
CBU1650	IcmW protein	<i>icmW</i>	149	PmrA (13, 22)	No (13)	-6.1	NP
CBU1651	Hypothetical membrane-associated protein		152			-4.7	NP
CBU1685	Hypothetical protein		473	PmrA (13)	Yes (13), no (15)	-4.3	-9.6
CBU1686	Hypothetical protein		773	E block (13)	Yes (15), no (13)	-4.0	-8.1
CBU1751	Hypothetical protein	<i>cig57</i>	420	PmrA (13, 22)	Yes (13)	-3.0	-7.2
CBU1752	Hypothetical protein		412			-3.0	-7.8
Downregulated by RNA-seq							
CBU0122	Hypothetical membrane-associated protein		92	E block (13)	No (13)	-5.2	—
CBU0273	Hypothetical protein	<i>cig9</i>	248	PmrA (13, 22)	No (13, 15)	-2.2	—
CBU0409	Hypothetical protein		51			-3.5	—
CBU0410	Hypothetical membrane-spanning protein	<i>cig12</i>	578	PmrA (13, 22)	Yes (13)	-2.5	—
CBU0436	Hypothetical membrane-spanning protein	<i>cig13</i>	258	PmrA (13, 22)	No (13)	-2.2	—
CBU0508	Hypothetical membrane-spanning protein		237	PmrA (14)	No (13)	-3.8	—
CBU0665	Hypothetical protein	<i>cig18, cvpA</i>	328	PmrA (13, 22)	Yes (28)	-2.6	—
CBU0705	Hypothetical protein		109	E block (13)	No (13)	-2.2	—
CBU0706	Hypothetical protein		236	E block (13)	No (13)	-2.5	—
CBU0860	Hypothetical protein		153	PmrA (13)	No (13)	-2.8	—
CBU1231	Hypothetical membrane-associated protein		232			-2.6	—
CBU1530	Hypothetical membrane-spanning protein	<i>cig47</i>	645	PmrA (13, 22)	No (10, 13)	-2.1	—
CBU1540	Hypothetical protein		109			-2.6	—
CBU1543	Hypothetical protein	<i>cig49</i>	188	PmrA (13, 22)	Yes (13)	-2.3	—
CBU1614	Hypothetical protein		139			-2.2	—
CBU1625	IcmC protein	<i>icmC</i>	169			-2.0	—
CBU1634a	CoxigA protein	<i>coxigA</i>	49			-6.8	—
CBU1645	ATP-binding protein DotB	<i>dotB</i>	372			-2.1	-1.9
CBU1652	IcmX protein	<i>icmX</i>	376			-4.8	—
CBU2052	Hypothetical protein	<i>cirD</i>	300	PmrA (13)	Yes (10, 13)	-2.3	—
Downregulated by MS							
CBU0027	Acyltransferase family protein		296			0.3	-2.3
CBU0044	Hypothetical exported protein		251			-0.3	-4.7
CBU0049	Hypothetical protein		436			-1.7	-9.7
CBU0084	Phosphoglycerol transferase MdoB	<i>mdoB, cig3</i>	638	PmrA (13, 22)	No (13)	-1.4	NP
CBU0138	Cell division protein FtsQ	<i>ftsQ</i>	243			-0.4	-2.7
CBU0156	Type 4 major prepilin protein PilA	<i>pilA</i>	140			0.3	NP
CBU0280	DNA polymerase IV	<i>dinP</i>	372			-0.1	-2.3
CBU0378	Hypothetical membrane-associated protein		140			-0.9	-4.5
CBU0388	Hypothetical protein		1,392	PmrA (13)	Yes (13, 15)	-1.4	NP
CBU0432	Transporter, MFS superfamily		428			0.5	-2.7

(Continued on following page)

TABLE 3 (Continued)

Category and identifier	Product	Gene(s)	Size (aa) ^a	Motif (reference[s])	Dot/Icm substrate (reference[s]) ^b	Fold change	
						RNA-seq	MS ^c
CBU0447	Ankyrin repeat protein	<i>ankF</i>	184		Yes (10, 18)	0.3	-2.7
CBU0459	Potassium/proton antiporter RosB	<i>rosB</i>	406			0.4	-2.0
CBU0644	Plasmid stabilization system toxin protein		112			-0.4	-2.3
CBU0787	Peptide synthetase		514			-1.5	-4.5
CBU0794	Hypothetical protein	<i>cig20</i>	464	PmrA (13, 22)	Yes (13)	-0.8	NP
CBU0881	Hypothetical cytosolic protein	<i>cig23</i>	221	PmrA (13, 22)	Yes (13)	-0.8	NP
CBU0970	Hypothetical exported membrane-spanning protein		351	E block (13)	No (13)	-1.1	-2.6
CBU1098	Hypothetical cytosolic protein	<i>cig28</i>	274	PmrA (13, 22)	No (13)	-1.6	-5.3
CBU1179	Multidrug resistance transporter, Bcr family		398			0.1	-2.5
CBU1370	Hypothetical membrane-associated protein		328	PmrA (13)	No (13)	-1.6	-5.9
CBU1439	NADH-quinone oxidoreductase chain J	<i>nuoJ</i>	201			0.1	-3.2
CBU1493	Hypothetical protein	<i>cig46</i>	557	PmrA (22)		-0.4	NP
CBU1636	Hypothetical protein	<i>cig55</i>	391	PmrA (13)	Yes (13)	-1.0	NP
CBU1641	IcmT protein	<i>icmT</i>	85			-0.3	NP
CBU1719	10-kDa chaperonin GroES	<i>groES</i>	116			1.2	-2.8
CBU1768	Hypothetical exported protein		153		No (10)	-0.2	NP
CBU1794	Hypothetical protein	<i>cig59</i>	272	PmrA (13, 22)	No (13)	-1.6	NP
CBU1823	Hypothetical protein	<i>cig61</i>	349	PmrA (13, 22)	Yes (10, 13)	-0.9	NP
CBU1843	Hypothetical exported protein		134			0.5	NP
CBU1850	Hypothetical protein		161			0.2	-2.7
CBU1863	Hypothetical membrane-spanning protein	<i>cig62</i>	603	PmrA (13, 22)	No (13)	-1.4	-2.9
CBU1896	Macrolide efflux protein		412			0.5	NP
CBU2032	GGDEF family protein		254			0.1	NP

^a aa, amino acids.

^b CBU0122, CBU1530, CBU1614, CBU1685, CBU1686, and CBU1752 were subsequently shown to be positive for Dot/Icm-mediated secretion by *C. burnetii* (Fig. 7). CBU0409, CBU0508, CBU0705, CBU1213, CBU1540, and CBU1651 were subsequently shown to be negative for Dot/Icm-mediated secretion by *C. burnetii* (Fig. 7).

^c NP, no peptide fragments were detected in the $\Delta pmrA$ mutant; —, no peptide fragments were detected in wild-type *C. burnetii* or the $\Delta pmrA$ mutant.

^d Carey et al. (10) and Newton et al. (25) demonstrated that CBU0077 is a Dot/Icm substrate of *L. pneumophila* and *C. burnetii*, respectively.

^e Zusman et al. (22) demonstrated that *cbu1366* is regulated by PmrA in *L. pneumophila*.

CpxR (22). In *C. burnetii*, operons initiating with *icmD*, the *CoxigA* gene, *dotD*, *icmV*, and *icmW*, all of which contain a PmrA motif, are expressed in a PmrA-dependent fashion. *icmTS* and *icmHF* do not require PmrA for expression, which is consistent with the lack of PmrA motifs. The reason for this regulatory disconnect is unclear. *icmHF* is located over 1 megabase from the

linked *dot/icm* locus. Moreover, *icmF* is annotated as a pseudo-gene because of an internal stop codon and is unlikely to produce a functional protein. Unlike most Dot/Icm proteins that are evolutionarily related to proteins involved in IncI plasmid conjugation, homologs of IcmH and IcmF are common among pathogenic bacteria, and they have an origin different from that of Dot/

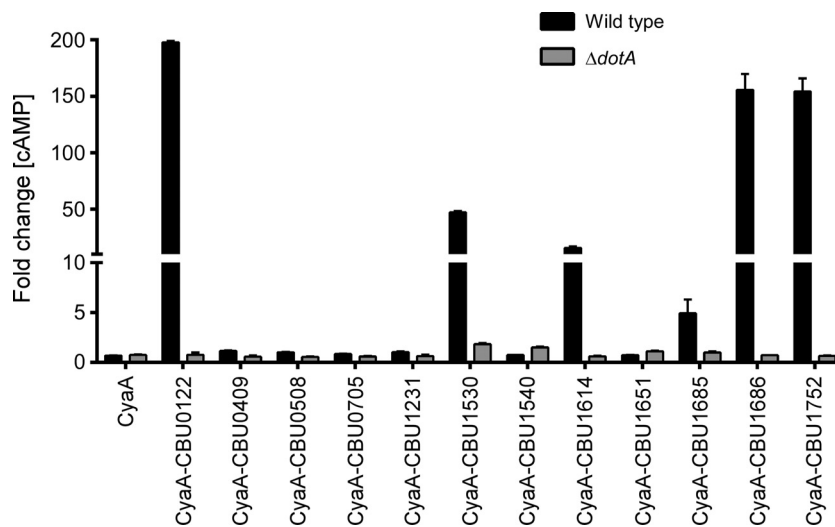


FIG 7 RNA-seq reveals new Dot/Icm substrates. Cytosolic levels of cAMP were measured following infection of THP-1 macrophages for 2 days with wild-type *C. burnetii* or the $\Delta dotA$ mutant expressing CyaA alone or CyaA fused to possible Dot/Icm substrates. Elevated levels of cAMP indicating secretion were observed for CBU0122, CBU1530, CBU1614, CBU1685, CBU1686, and CBU1752. Results shown are from one experiment conducted in duplicate and are representative of three independent experiments. Error bars indicate the standard deviations from the means.

Icm proteins (53). In *L. pneumophila*, IcmH and IcmF apparently function together and mutation of either coding region results in severe growth defects in amoebae but only moderate growth defects in human macrophages (53). *C. burnetii* does not use amoebae as an environmental replication niche (54), and the truncation of IcmF presumably results in a nonfunctional IcmHF complex. Thus, *icmHF* may not be required for *C. burnetii* Dot/Icm function. IcmT is necessary for the intracellular growth of *L. pneumophila* and mediates pore formation, which enables pathogen egress from macrophages (55, 56). A concerted lytic event is not associated with *C. burnetii* release from host cells (57); however, whether IcmT serves an essential role in *C. burnetii* Dot/Icm secretion is unknown. *L. pneumophila* IcmS and IcmW form a heterodimer that serves as a chaperone for translocation of a subset of substrates (15, 58). *C. burnetii* IcmS also interacts with IcmW, and the complex is predicted to have chaperone-like activity (59). The lack of PmrA regulation of *C. burnetii icmS* may indicate that the protein is constitutively expressed. Consequently, PmrA interactions with temporally regulated *icmW* and other undefined chaperone-like proteins might promote secretion of distinct subsets of effectors at different time points during the infectious cycle (60). *L. pneumophila icmS* is also not directly or indirectly regulated by PmrA (22).

The *L. pneumophila* PmrA regulon appears to be more complex than the *C. burnetii* counterpart. Microarray transcriptome analysis of wild-type *L. pneumophila* and an isogenic $\Delta pmrA$ mutant in the exponential and postexponential growth phases revealed PmrA regulation of 279 genes, including genes encoding the Dot/Icm apparatus and substrates, type II secretion substrates, flagellar components, and multiple metabolic enzymes (32). We found 82 *C. burnetii* genes/proteins regulated by PmrA during late-exponential-phase growth that are associated primarily with type IV secretion. Interestingly, one of these genes, *cbu1103*, encodes a predicted membrane-bound lytic murine transglycosylase. Specialized lytic transglycosylases generate gaps in the peptidoglycan that allow assembly of both type III and type IV secretion systems (61). Thus, it is intriguing to speculate that *cbu1103* is PmrA regulated to ensure coexpression with the *dot/icm* locus. The *C. burnetii* genome is roughly 60% of the size of *L. pneumophila*'s, with a less complex repertoire of Dot/Icm effectors; thus, a reduced PmrA regulon is not unexpected. Moreover, we recognize the caveat that the single late-exponential-phase time point used in our study provides only a snapshot of the PmrA regulon. Several time points over the complete *C. burnetii* growth cycle are required to gain a comprehensive view of the PmrA regulon. Nonetheless, these data suggest that the *C. burnetii* PmrAB TCS primarily regulates the T4BSS.

As predicted, a high percentage of *C. burnetii* PmrA-regulated genes contain PmrA regulatory elements, and several code for previously defined Dot/Icm substrates. However, several regulated genes do not contain a PmrA regulatory element, implicating indirect regulation via PmrAB cross talk with other TCSs and/or control of expression of other regulatory factors (22, 29, 62). We demonstrated that, among the hypothetical proteins encoded by genes lacking PmrA motifs, CBU0122, CBU1686, CBU1614, and CBU1752 are secreted by *C. burnetii* in a Dot/Icm-dependent fashion. Secretion of CBU1686 and CBU1752 was not altogether unexpected, as their encoding genes are downstream (in predicted two-gene operons [52]) from PmrA motif-containing and Dot/Icm substrate-coding *cbu1685* and *cbu1751*, respectively (13).

Moreover, CBU1686, along with secreted CBU0122, contain an E block motif; however, both proteins were previously reported by Weber et al. (13) as negative for secretion by *L. pneumophila* by the β -lactamase translocation assay. We also demonstrate that CBU1530 (PmrA motif), which is negative for secretion by *L. pneumophila* (10, 13), is secreted by the *C. burnetii* Dot/Icm system. Our data show that, as previously reported for *L. pneumophila* (63), transcriptome data can be used to identify new Dot/Icm substrates. Furthermore, our secretion results that conflict with published data using the surrogate *L. pneumophila* system reinforce the idea that secretion assays should be conducted directly in *C. burnetii*, which is now amenable to genetic manipulation.

Relative to free-living bacteria, *C. burnetii* encodes few TCSs. This likely reflects the stable environment encountered by *C. burnetii* as a natural obligate intracellular bacterium requiring few adaptive responses (30). *C. burnetii* encodes, in addition to PmrAB, PhoBR and an EnvZ-like histidine kinase paired with an OmpR family response regulator (42). In *E. coli*, PhoBR senses environmental phosphate concentrations (64), while EnvZ/OmpR responds to environmental osmolarity (65). As mentioned above, *C. burnetii* also encodes four orphan hybrid histidine kinases (CBU0634, CBU0789, CBU1084, and CBU1761) (31, 39, 42) and four orphan response regulators with output domains containing a DNA-binding motif (CBU0712, CBU0780, CBU0955, and CBU1043) (31, 39, 42). Only CBU1761 contains a histidine phosphotransferase domain required for phosphoryl group transfer to a cognate response regulator (30, 39). However, CBU0351 contains this domain (39) and consequently may catalyze phosphotransfer from CBU0634, CBU0789, and/or CBU1084 (39). A fifth unclassified orphan response regulator, CBU0760, contains a phosphoryl group receiver domain but lacks an output domain (39). Following activation, this type of response regulator engages downstream effectors via direct protein-protein interactions (66).

The presence of *csrA-1* and *csrA-2* in the *C. burnetii* genome suggests that some combination of *C. burnetii* orphan histidine kinases and response regulators functions analogously to *L. pneumophila* LetAS. The LetAS-RsmYZ-CsrA regulatory cascade is intricately woven into *L. pneumophila* stationary-phase physiology, controlling expression of transmissive-phase traits in addition to Dot/Icm effector proteins (67). Both LetAS and RpoS (σ^s) positively respond to the alarmone ppGpp generated during periods of low amino acid abundance (67). RpoS positively regulates expression of RsmYZ, thereby potentiating LetAS-mediated relief of posttranscriptional repression by *csrA* (36). Interestingly, the majority of LetAS-RsmYZ-CsrA-regulated Dot/Icm substrates are known, or predicted, effectors of eukaryotic vesicular trafficking (29). For productive infection, *L. pneumophila* must escape the default endolysosomal pathway and generate a specialized endoplasmic reticulum-derived replication vacuole within minutes of internalization (68). Thus, preloading the stationary-phase, transmissive form of *L. pneumophila* with these effectors is an elegant pathogenic strategy to ensure proper trafficking immediately upon host cell internalization. Unlike *L. pneumophila*, *C. burnetii* requires transit through the canonical endolysosomal pathway for productive infection. Nonetheless, *C. burnetii* undergoes a biphasic developmental cycle superficially similar to that of *L. pneumophila* by generating cell types specifically adapted for intracellular replication and extracellular survival (67, 69). Indeed, the nonrep-

licating and environmentally stable *C. burnetii* small-cell variant that develops during stationary phase is biologically analogous to the *L. pneumophila* transmissible form (70). A stationary-phase regulatory network with an integrated TCS comprised of an orphan sensor/regulator pair may therefore control *C. burnetii* developmental transitions.

This work further expands the *C. burnetii* genetics toolbox to include a reporter system based on *Photobacterium luminescens* luciferase. This luciferase system has advantages over other reporter systems in being highly sensitive and responsive, detectable in living cells without addition of exogenous substrate, and capable of generating a signal that does not stably accumulate (71, 72). Luciferase is comprised of LuxAB, which oxidizes cellular substrates to generate visible light of approximately 490 nm. LuxCDE comprises a reductase complex that generates a reduced substrate for LuxAB (71, 72). Expression of the five Lux subunits required for luciferase activity does not negatively affect *C. burnetii* growth (P. A. Beare and R. A. Heinzen, unpublished data). Thus, the *lux* system should have broad utility in the study of *C. burnetii*-host interactions, including assessment of gene expression during various stages of macrophage invasion, intracellular survival of mutant strains, and bioluminescent imaging of *C. burnetii* during animal infection (73).

This research sets the stage for identification of the environmental stimuli that activate the *C. burnetii* PmrAB TCS and production of the T4BSS. In *Salmonella enterica*, PmrAB is activated by submillimolar Fe^{3+} and low pH (~5.8) (74). Low pH has also been implicated as an activator of *L. pneumophila* PmrAB in *L. pneumophila* (32). It is logical to suspect that acidification of the *C. burnetii* PV as it transits through the endolysosomal cascade also stimulates the PmrAB TCS, although this effect would have to be distinguished from the global activation of *C. burnetii* metabolism that occurs under acidic conditions (75, 76). Nonetheless, the increasing genetic tractability of *C. burnetii* now allows elucidation of the roles that TCSs and other regulatory factors play in Q fever pathogenesis.

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