SUPPLEMENTAL MATERIAL

Table S1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or phenotype	Source
Strains		
E. coli Stellar cells	F-, *endA1*, *supE44*, thi-1, *recA1*, *relA1*, *gyrA96*, *phoA*, Φ80D *lacZ*Δ *M15*, Δ (*lacZYA - argF*) U169, Δ(*mrr - hsdRMS - mcrBC*), Δ*mcrA*, λ–	Clontech
E. coli TOP10	F-, *mcrA* Δ*(mrr-hsdRMS-mcrBC),* φ*80lacZΔM15,* Δ*lacX74, nupG, recA1, araD139,* Δ*(ara-leu)7697, galE15, galK16, rpsL(StrR,) endA1,* λ-	Invitrogen
C. burnetii RSA439	phase II, clone 4	(1)
Plasmids for ectopic expression of *C. burnetii* genes in eukaryotic cells		
pENTR/D-TOPO	Gateway entry vector for directional cloning of a blunt-end PCR products	Invitrogen
pENTR/N-CBU0021	CBU0021 inserted into pENTR/D-TOPO	This study
pENTR/N-CBU1556	CBU1556 inserted into pENTR/D-TOPO	This study
pENTR/N-CBU1818	CBU1818 inserted into pENTR/D-TOPO	This study
pENTR/N-CBU1863	CBU1863 inserted into pENTR/D-TOPO	This study
pT-Rex-DEST30/N-mCherry	Anhydrotetracycline-inducible expression of N-terminal mCherry fusions in mammalian cells; Amp^r	(2)
pT-Rex-DEST30/N-GFP	Anhydrotetracycline-inducible expression of N-terminal GFP fusions in mammalian cells; Amp^r	This study
pT-Rex-DEST30/N-GFP-CBU0021	CBU0021 inserted into pT-Rex-DEST30/N-GFP	This study
pT-Rex-DEST30/N-mCherry-CBU0021	CBU0021 inserted into pT-Rex-DEST30/N-mCherry	This study
pT-Rex-DEST30/N-mCherry-CBUD0487	CBUD0487 inserted into pT-Rex-DEST30/N-mCherry	This study
pT-Rex-DEST30/N-GFP-CBU0534	CBU0534 inserted into pT-Rex-DEST30/N-GFP	This study
pT-Rex-DEST30/N-mCherry-CBUK0790	CBUK0790 inserted into pT-Rex-DEST30/N-mCherry	This study
pT-Rex-DEST30/N-mCherry-CBU0885	CBU0885 inserted into pT-Rex-DEST30/N-mCherry	This study
pT-Rex-DEST30/N-GFP-CBUD0886	CBUD0886 inserted into pT-Rex-DEST30/N-GFP	This study
pT-Rex-DEST30/N-mCherry-CBU1493	CBU1493 inserted into pT-Rex-DEST30/N-mCherry	This study
pT-Rex-DEST30/N-GFP-CBU1543	CBU1543 inserted into pT-Rex-DEST30/N-GFP	This study
pT-Rex-DEST30/N-GFP-CBU1556	CBU1556 inserted into pT-Rex-DEST30/N-GFP	This study
pT-Rex-DEST30/N-mCherry-CBU1556	CBU1556 inserted into pT-Rex-DEST30/N-mCherry	This study
pT-Rex-DEST30/N-mCherry-CBU1676	CBU1676 inserted into pT-Rex-DEST30/N-mCherry	This study
pT-Rex-DEST30/N-GFP-CBU1818	CBU1818 inserted into pT-Rex-DEST30/N-GFP	This study
pT-Rex-DEST30/N-mCherry-CBU1818	CBU1818 inserted into pT-Rex-DEST30/N-mCherry	This study
pT-Rex-DEST30/N-GFP-CBU1863	CBU1863 inserted into pT-Rex-DEST30/N-GFP	This study
pT-Rex-DEST30/N-mCherry-CBU1863	CBU1863 inserted into pT-Rex-DEST30/N-mCherry	This study
pcDNA 6/TR	Constitutively expresses the anhydrotetracycline repressor protein	Invitrogen
Plasmids for targeted gene deletion		
pJC-CAT	pJC84 containing *cat* driven by P1169 ;Cm^r	
pJC-CAT::CBU0021-5′3′	5' and 3' flanking DNA from CBU0021 cloned into pJC-CAT; Cm^r	This study
pJC-CAT::CBU1556-5′3′	5' and 3' flanking DNA from CBU1556 cloned into pJC-CAT; Cm^r	This study
pJC-CAT::CBU1818-5′3′	5' and 3' flanking DNA from CBU1818 cloned into pJC-CAT; Cm^r	This study
pJC-CAT::CBU1863-5′3′	5' and 3' flanking DNA from CBU1863 cloned into pJC-CAT; Cm^r	This study
pJB-Kan	pJB2581 containing *kan* driven by P1169; Kan^r	(3)
pJC-CAT::CBU0021-5′3′-Kan	P1169-Kan cassette cloned into pJC-CAT::CBU0021-5'3'; Cm^r, Kan^r	This study
pJC-CAT::CBU1556-5′3′-Kan	P1169-Kan cassette cloned into pJC-CAT::CBU1556-5'3'; Cm^r, Kan^r	This study

pJC-CAT::CBU1818-5′3′-Kan	P1169-Kan cassette cloned into pJC-CAT::CBU1818-5'3'; Cm^r, Kan^r	This study
pJC-CAT::CBU1863-5′3′-Kan	P1169-Kan cassette cloned into pJC-CAT::CBU1863-5'3'; Cm^r, Kan^r	This study
pTnS2::P1169-*TnsABCD*	CBU1169 promoter cloned into pTnS2; Amp^r, R6K ori	(3)
pMini-Tn7T-CAT	P1169-CAT cloned into pUC18R6K-mini-Tn7T-Gm; Cm^r, Amp^r, R6K ori	(3)
pMini-Tn7T-CAT::CBU1818comp	CBU1818comp fragment cloned into pMiniTn7T-CAT; Cm^r, Amp^r	This study
pMini-Tn7T-CAT::CBU1863comp	CBU1863comp fragment cloned into pMiniTn7T-CAT; Cm^r, Amp^r	This study
Plasmids for adenylate cyclase translocation assays		
pJB-CAT-CyaA	Plasmid for expression of N-terminal CyaA fusions in *C. burnetii* driven by P1169; Cm^r, Amp^r	(1)
pJB-CAT-CyaA-CBU0021	CBU0021 cloned into pJC-CAT-CyaA	This study
pJB-CAT-CyaA-CBUD0487	CBUD0487 cloned into pJC-CAT-CyaA	This study
pJB-CAT-CyaA-CBU0534	CBU0534 cloned into pJC-CAT-CyaA	This study
pJB-CAT-CyaA-CBUK0790	CBUK0790 cloned into pJC-CAT-CyaA	This study
pJB-CAT-CyaA-CBU0885	CBU0885 cloned into pJC-CAT-CyaA	This study
pJB-CAT-CyaA-CBUD0886	CBUD0886 cloned into pJC-CAT-CyaA	This study
pJB-CAT-CyaA-CBU1457	CBU1457 cloned into pJC-CAT-CyaA	This study
pJB-CAT-CyaA-CBU1493	CBU1493 cloned into pJC-CAT-CyaA	This study
pJB-CAT-CyaA-CBU1543	CBU1543 cloned into pJC-CAT-CyaA	This study
pJB-CAT-CyaA- CBU1556	CBU1556 cloned into pJC-CAT-CyaA	This study
pJB-CAT-CyaA- CBU1676	CBU1676 cloned into pJC-CAT-CyaA	This study
pJB-CAT-CyaA- CBU1818	CBU1818 cloned into pJC-CAT-CyaA	This study
pJB-CAT-CyaA- CBU1819	CBU1819 cloned into pJC-CAT-CyaA	This study
pJB-CAT-CyaA- CBU1863	CBU1863 cloned into pJC-CAT-CyaA	This study

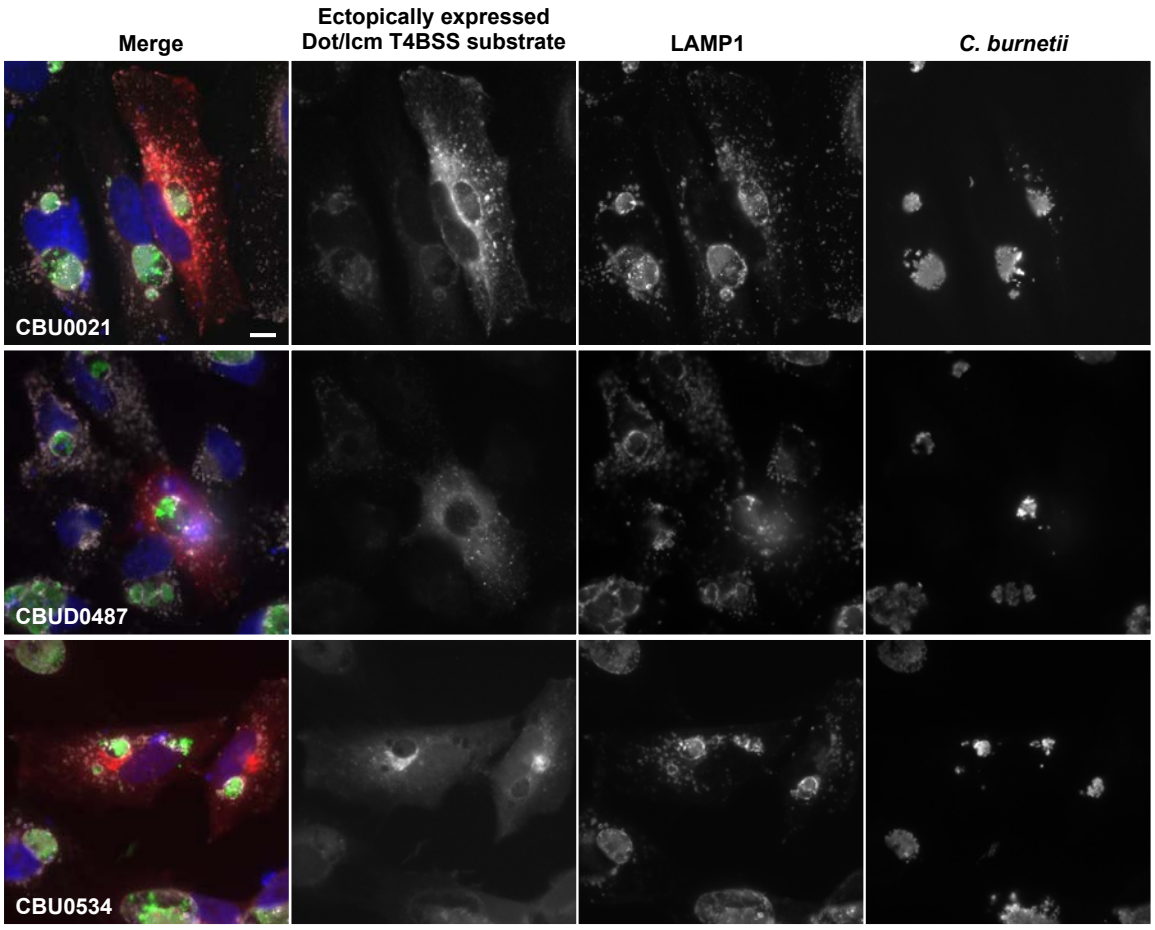
Table S2. Oligonucleotide primers used in this study

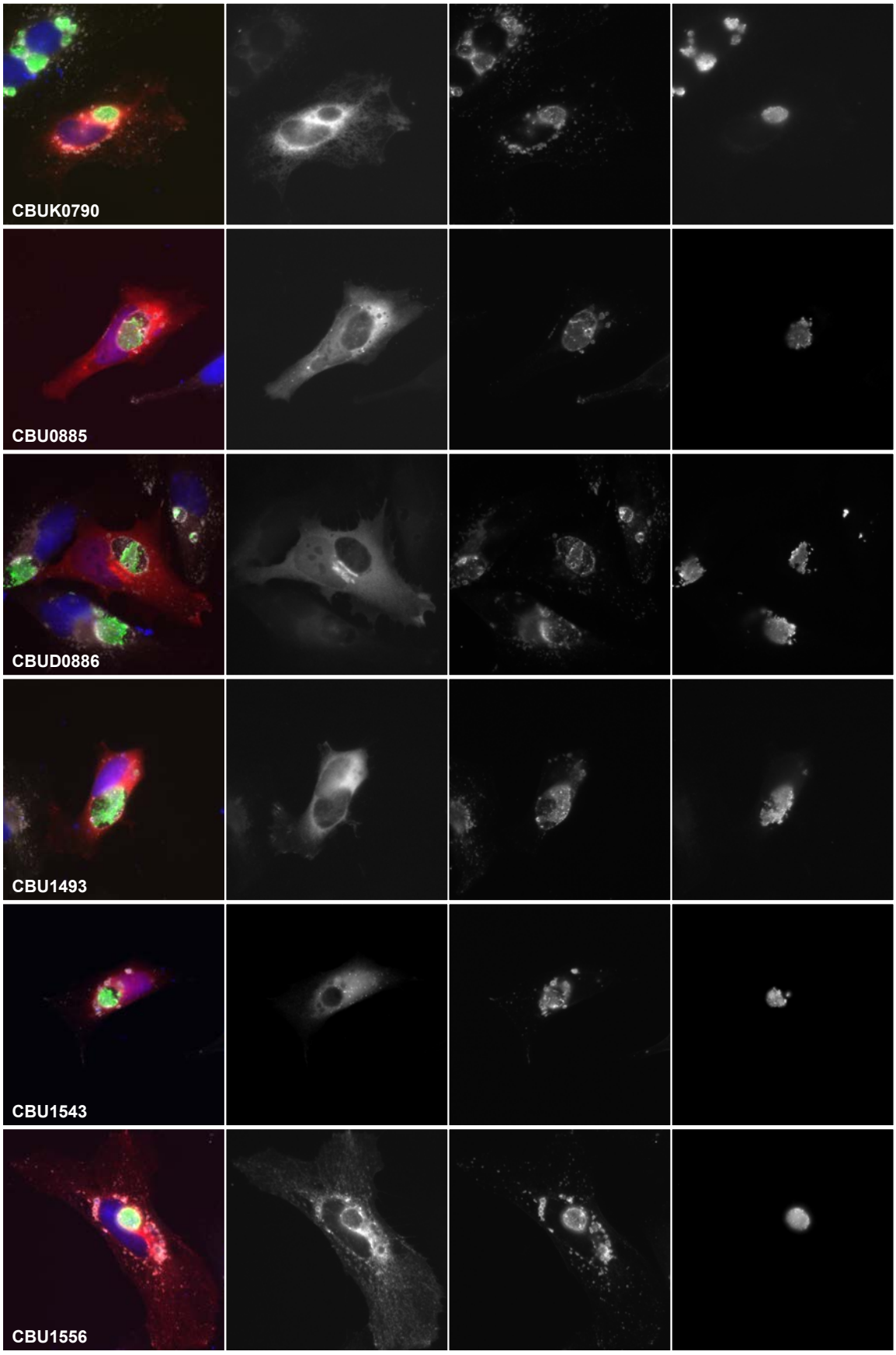
Primer	Sequence (5′ to 3′)
Primers for gene deletion	
CBU0021-5′-F	CGGTACCCGGGGATCCCGATTGCATTCAGTCGATTAAAC
CBU0021-5′-R	CACCCATATGCGACGCGAGCGTCGAGGTTTATCTCCAGCGCTTTACGCG
CBU0021-3′-F	CGTCGCATATGGGTGCGCATGTACGTCTAATTTACTTTAAAAATTTTTTATTGACCG
CBU0021-3-′R	GAACCTGTTTGTCGACTCGCGTGAGTCCATCGAAAG
CBU1556-5′-F	CGGTACCCGGGGATCCCGCCGTGGTAAAATGACAGG
CBU1556-5′-R	CACCCATATGCGACGCGAGCGTCGAGAAAATACCCTTTTTATTTTCGTTTTCGGCG
CBU1556-3′-F	CGTCGCATATGGGTGCGCATGTACGTCATTTATCCAATCTATTGCTCGCG
CBU1556-3-′R	GAACCTGTTTGTCGACGGAGCAATCACAATGCTGTTAATC
CBU1818-5′-F	CGGTACCCGGGGATCCCATCATCCAGGCTTGAGCGCG
CBU1818-5′-R	GCACCACCGGTCGACGTCGCCGCAATCTAGACATTTTTAACCTCG
CBU1818-3′-F	CGTCGACCGGTGGTGCCGAGGGGAAACTAGTCATTTCAGGTATTGCAG
CBU1818-3-′R	GAACCTGTTTGTCGACGAACAGCGACCCTGCC
CBU1863-5′-F	CGGTACCCGGGGATCCCTTGGAGCGAAGCTATAGAGTG
CBU1863-5′-R	CACCCATATGCGACGCGAGCGACGATTGGGAAGAACTTCCC
CBU1863-3′-F	CGTCGCATATGGGTGCGCATGCTCTGATCCCGCTGCGCAAGCG
CBU1863-3-′R	GAACCTGTTTGTCGACCGAAACTTTCGGGACTAATTCCCG
P1169-Kan-NdeI-KO-F	CGCTCGCGTCGCATATGATGGCTTCGTTTCGCAGCG
P1169-Kan-NdeI-KO-R	GCATGCGCACCCATATGTTATCAGAAGAACTCGTCAAGAAGG
P1169-Kan-AgeI-KO-F	GGCGACGTCGACCGGTATGGCTTCGTTTCGCAGCGAACTTGG
P1169-Kan-AgeI-KO-R	CCTCGGCACCACCGGTTTATCAGAAGAACTCGTCAAGAAGGC
Primers for gene complementation	
CBU1818comp-F	GCTTCTCGAGGAATTCGGCTTAATCAAAATTCGATTTG
CBU1818comp-R	TTACTCAATGGAATTCTTAGAAAGCCTGGCGGCC
CBU1863comp-F	GCTTCTCGAGGAATTCAAGCTCAATAACTCTAATTTTGAG
CBU1863comp-R	TTACTCAATGGAATTCTTACGCAGTAAGTGCAGAAGGTACAG
Primers for Cloning into pENTR	
pENTR/N- CBU0021-F	CACCATGAGCAGACAGCCATCATTGACG
pENTR/N- CBU0021-R	TCACTTAGTGAAAGAAGCAATGGGC
pENTR/N- CBUD0487-F	CACCATGTTGAGCTCGCCGCCATCTGTACTC
pENTR/N- CBUD0487-R	TCATAAACGAGGCTTAGGCGCAG
pENTR/N- CBU0534-F	CACCATGATGAGAATCAACGTAGATTCC
pENTR/N- CBU0534-R	TCAACGTGGCTGCGGGTTTTG
pENTR/N- CBUK0790-F	CACCATGGCGAGACAACCCAGTGACAGTGATC
pENTR/N- CBUK0790-R	TCACGATGCACCAAGACTCTGAAAGCG
pENTR/N- CBU0885-F	CACCATGGTTGATTTAAATGATCTTCTCAGAATT
pENTR/N- CBU0885-R	TCAACGACTCATTGAACACACGTGC
pENTR/N- CBUD0886-F	CACCATGCCTAAATCTATCAATGAAGC
pENTR/N- CBUD0886-R	TCATTGTAACAAACAACAGGTACG
pENTR/N- CBU1493-F	CACCATGGTCAAGCCGCAGGATGAC
pENTR/N- CBU1493-R	TCAGGAGCAAGTACAGGTTGTTTG
pENTR/N- CBU1543-F	CACCATGCAACCCACCGCAGAGACTC
pENTR/N- CBU1543-R	TCATTTTCCAAGGGACTCGCTTTG

pENTR/N- CBU1556-F	CACCATGCCTTCTGATAGAAACGGCG
pENTR/N- CBU1556-R	TCAAACGGGAGCATAATTAACCGG
pENTR/N- CBU1676-F	CACCATGCCCCAAAATTTACTCATCATTGAC
pENTR/N- CBU1676-R	TCACAACCCTCGAGAAAAACGGC
pENTR/N- CBU1818-F	CACCATGTCTAGATTGCCATCCAAAAC
pENTR/N- CBU1818-R	TCAGTTTCCCAGACGCAACG
pENTR/N- CBU1819-F	CACCATGCGTCCAATTCATATGGCTGG
pENTR/N- CBU1819-R	TCAGAAAGCCTGGCGGCC
pENTR/N- CBU1863-F	CACCATGCGAAATGATGATGATACTCATTC
pENTR/N- CBU1863-R	TCACGCAGTAAGTGCAGAAGG
Primers for cloning into pJB-CAT-CyaA	
CyaA- CBU0021-F	TTCCGGCTATGTCGACATGAGCAGACAGCCATCATTGACG
CyaA- CBU0021-R	GCATGCCTCAGTCGACTCACTTAGTGAAAGAAGCAATGGGC
CyaA- CBUD0487-F	TTCCGGCTATGTCGACATGTTGAGCTCGCCGCCATCTGTACTC
CyaA- CBUD0487-R	GCATGCCTCAGTCGACTCATAAACGAGGCTTAGGCGCAG
CyaA- CBU0534-F	TTCCGGCTATGTCGACATGATGAGAATCAACGTAGATTCC
CyaA- CBU0534-R	GCATGCCTCAGTCGACTCAACGTGGCTGCGGGTTTTG
CyaA- CBUK0790-F	TTCCGGCTATGTCGACATGGCGAGACAACCCAGTGACAGTGATC
CyaA- CBUK0790-R	GCATGCCTCAGTCGACTCACGATGCACCAAGACTCTGAAAGCG
CyaA- CBU0885-F	TTCCGGCTATGTCGACATGGTTGATTTAAATGATCTTCTCAGAATTC
CyaA- CBU0885-R	GCATGCCTCAGTCGACTCAACGACTCATTGAACACACGTGC
CyaA- CBUD0886-F	TTCCGGCTATGTCGACATGCCTAAATCTATCAATGAAGC
CyaA- CBUD0886-F	GCATGCCTCAGTCGACTCATTGTAACAAACAACAGGTACG
CyaA- CBU1457-F	TTCCGGCTATGTCGACATGCCTTACCCTTACGAAGCTAAC
CyaA- CBU1457-R	GCATGCCTCAGTCGACTCATGTTTTTACCCTATCATTACCCAC
CyaA- CBU1493-F	TTCCGGCTATGTCGACATGGTCAAGCCGCAGGATGAC
CyaA- CBU1493-R	GCATGCCTCAGTCGACTCAGGAGCAAGTACAGGTTGTTTG
CyaA- CBU1543-F	TTCCGGCTATGTCGACATGCAACCCACCGCAGAGACTC
CyaA- CBU1543-R	GCATGCCTCAGTCGACTCATTTTCCAAGGGACTCGCTTTG
CyaA- CBU1556-F	TTCCGGCTATGTCGACATGCCTTCTGATAGAAACGGCG
CyaA- CBU1556-R	GCATGCCTCAGTCGACTCAAACGGGAGCATAATTAACCGG
CyaA- CBU1676-F	TTCCGGCTATGTCGACATGCCCCAAAATTTACTCATCATTGAC
CyaA- CBU1676-R	GCATGCCTCAGTCGACTCACAACCCTCGAGAAAAACGGC
CyaA- CBU1818-F	TTCCGGCTATGTCGACATGTCTAGATTGCCATCCAAAAC
CyaA- CBU1818-R	GCATGCCTCAGTCGACTCAGTTTCCCAGACGCAACG
CyaA- CBU1819-F	TTCCGGCTATGTCGACATGCGTCCAATTCATATGGCTGG
CyaA- CBU1819-R	GCATGCCTCAGTCGACTCAGAAAGCCTGGCGGCC
CyaA- CBU1863-F	TTCCGGCTATGTCGACATGCGAAATGATGATGATACTCATTC
CyaA- CBU1863-R	GCATGCCTCAGTCGACTCACGCAGTAAGTGCAGAAGG

[a] The locations of restriction enzyme cut sites are underlined.

Fig. S1. Subcellular localization of Dot/Icm T4BSS substrates in *C. burnetii*-infected HeLa cells. Epifluorescence micrographs of *C. burnetii*-infected HeLa cells ectopically expressing Dot/Icm T4BSS substrates N-terminally fused to GFP or mCherry fluorescent proteins (red). At 72 h post infection, cells were immunostained for LAMP1 (gray) and *C. burnetii* (green). Nucleic acid was stained with DAPI (blue). Scale bar, 10 μm.




CBUK0790
CBU0885
CBUD0886
CBU1493
CBU1543
CBU1556

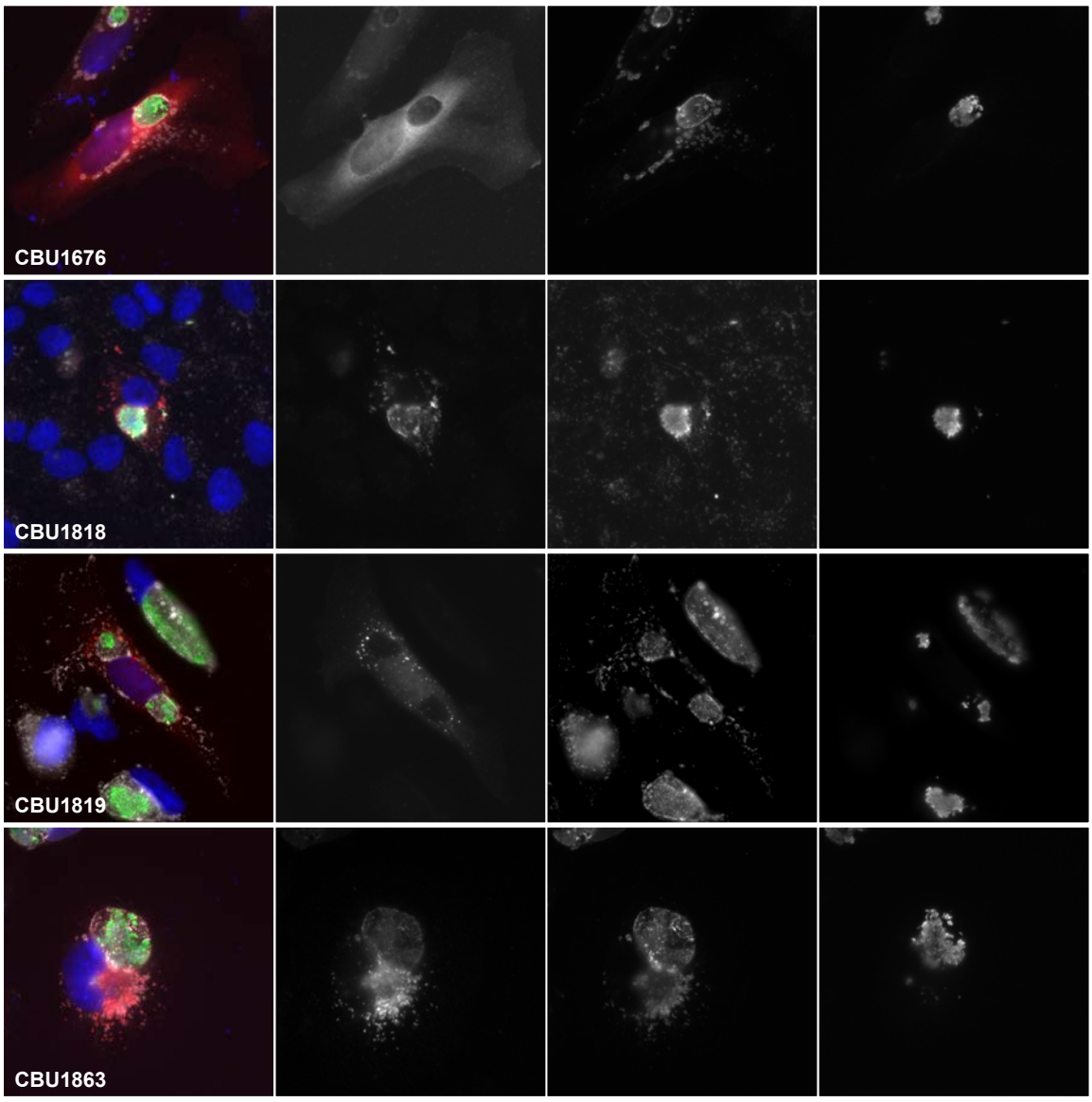

CBU1676
CBU1818
CBU1819
CBU1863

Fig. S2. *C. burnetii* localizes with ectopically expressed Cvp effectors early after infection. Confocal fluorescence micrographs of HeLa cells ectopically expressing CvpB, CvpC, CvpD, or CvpE fused to mCherry fluorescent protein (red). Cells were infected with *C. burnetii* at an MOI of 50 for 6 h, then immunostained with antibodies against LAMP (gray) or *C. burnetii* (green). Nucleic acid was stained with DAPI (Blue). Scale bar, 10 μm.

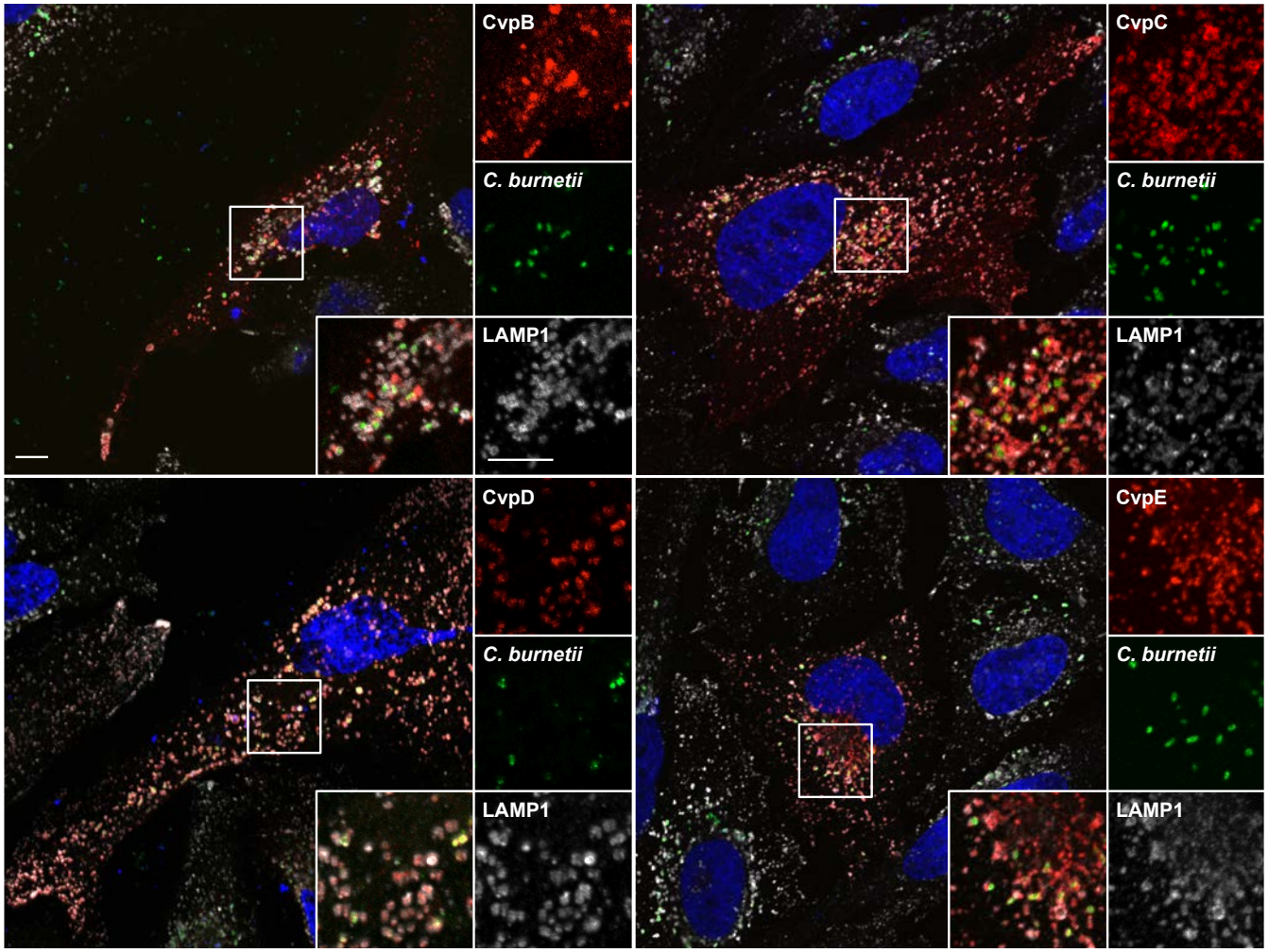
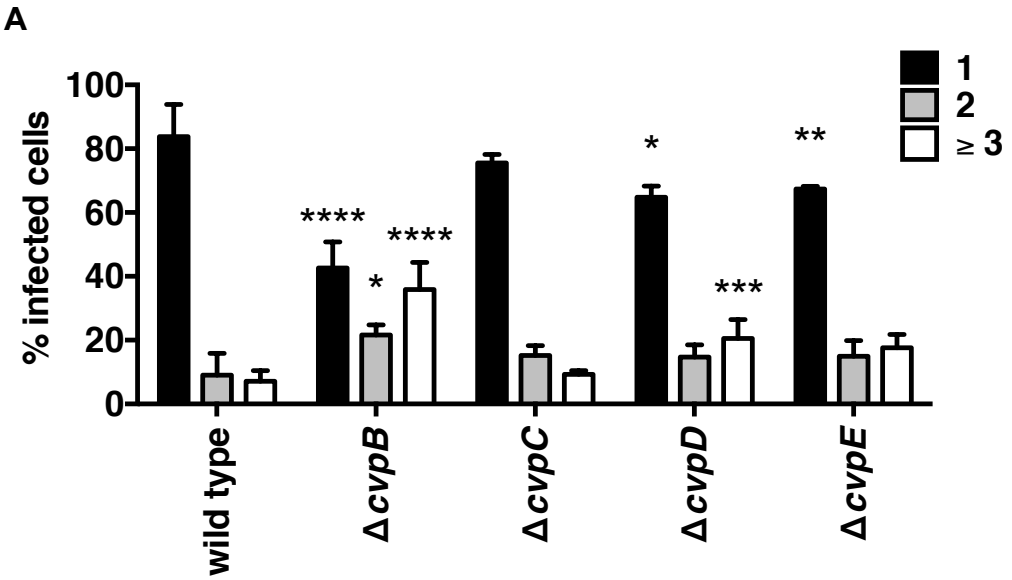


Fig. S3. PV generated by the *C. burnetii* Δ*cvp* mutants acidify, but are deficient in homotypic fusion. Vero cells were infected at an MOI of 100 with wild type *C. burnetii* or the Δ*cvp* mutants, then incubated for six days. (A) *C. burnetii* Δ*cvpB, -D,* and *-E* exhibit defects in homotypic fusion. The number of PVs in each cell (N = 100) were counted and the mean percentage of cells containing 1, 2, or ≥ 3 PV were plotted from three independent experiments. Error bars represent the standard deviation from the means and asterisks indicate a statistically significant difference (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001) when compared to values for cells infected with wild type *C. burnetii* as judged by two-way ANOVA. (B) *C. burnetii* Δ*cvp* mutants occupy acidified PV. Cells were incubated with LysoTracker DND-26 (green) prior to immunostaining for LAMP1 (gray) and *C. burnetii* (red). Nucleic acid was stained with DAPI (blue). Shown are maximum intensity projections of z-stacks collected by confocal fluorescence microscopy of each channel and the merged image (Scale bar, 20 μm).



B

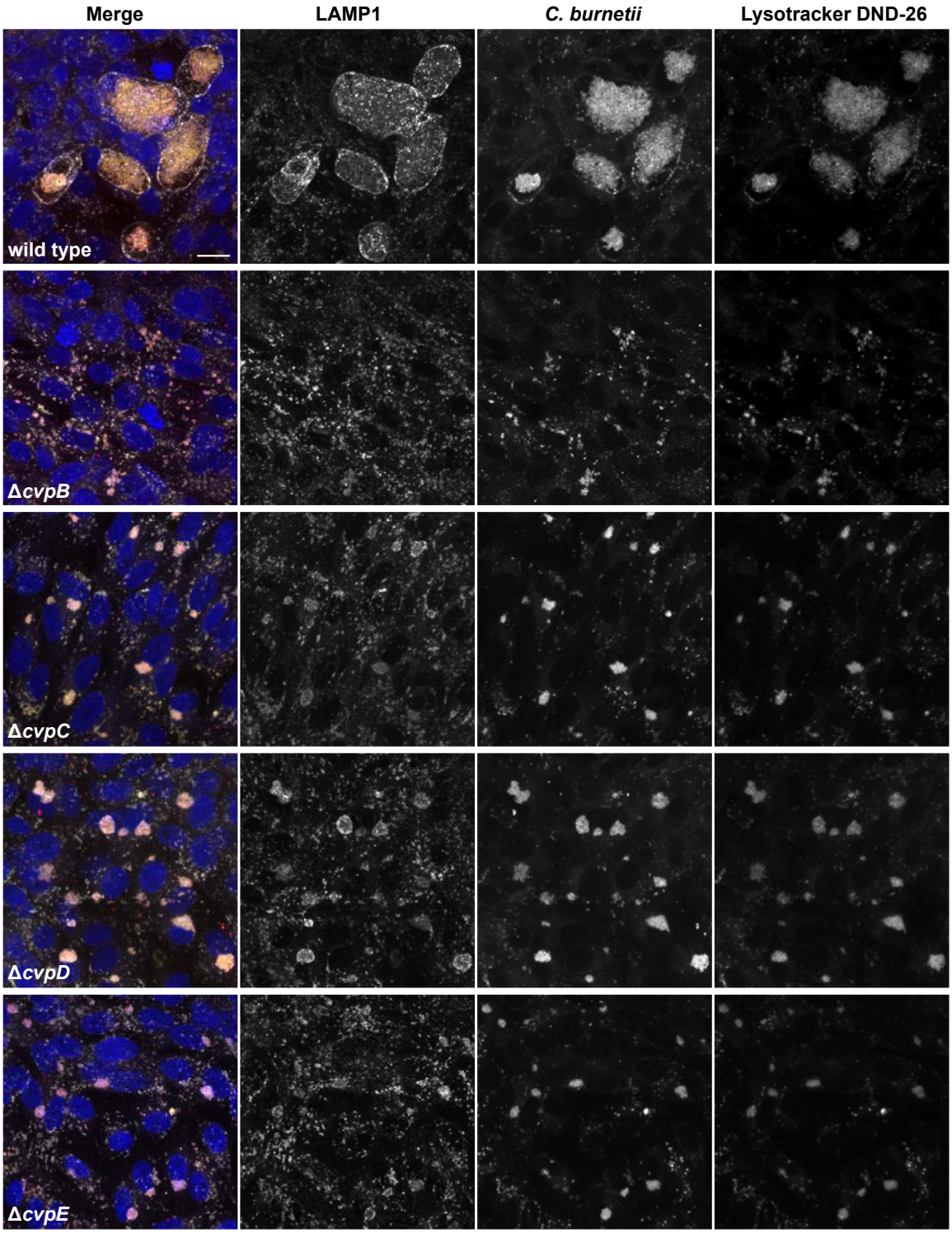
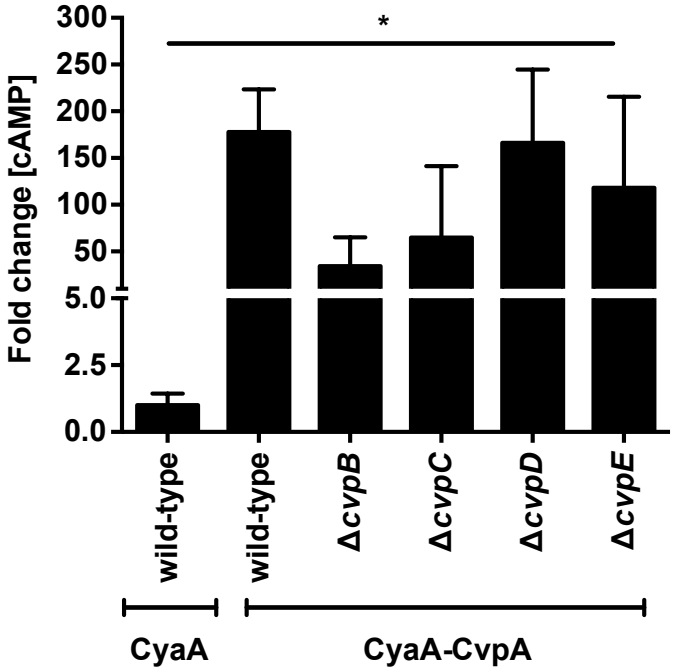

Merge
LAMP1
C. burnetii
Lysotracker DND-26
wild type
ΔcvpB
ΔcvpC
ΔcvpD
ΔcvpE

Fig. S4. Cyosolic translocation of a Dot/Icm effector by *C. burnetii* Δ*cvp* mutants. Cellular concentrations of cAMP were determined after infection of THP-1 macrophages for 48 h with wild type *C. burnetii* or the Δ*cvp* mutants expressing a CyaA fusion with the Dot/Icm effector CvpA. Results are expressed as fold change relative to wild type *C. burnetii* expressing CyaA alone from one experiment conducted in duplicate and representative of three independent experiments. Error bars indicate the standard deviation from the means. Asterisks indicates a statistically significant difference ($P < 0.05$) when compared to values for the CyaA alone negative control as judged by one-way ANOVA.



References

1. **Beare PA, Gilk SD, Larson CL, Hill J, Stead CM, Omsland A, Cockrell DC, Howe D, Voth DE, Heinzen RA.** 2011. Dot/Icm type IVB secretion system requirements for *Coxiella burnetii* growth in human macrophages. mBio **2:**e00175-00111.
2. **Larson CL, Beare PA, Howe D, Heinzen RA.** 2013. *Coxiella burnetii* effector protein subverts clathrin-mediated vesicular trafficking for pathogen vacuole biogenesis. Proc. Natl. Acad. Sci. USA **110:**E4770-4779.
3. **Beare PA, Larson CL, Gilk SD, Heinzen RA.** 2012. Two systems for targeted gene deletion in *Coxiella burnetii*. Appl. Environ. Microbiol. **78:**4580-4589.