Supplementary Information

***Rickettsia conorii* O-antigen is the target of bactericidal Weil-Felix antibodies**

Hwan Keun Kim[a,b,1], Ranjan Premaratna[c], Dominique M. Missiakas[a,b], Olaf Schneewind[a,b]

[a]Howard Taylor Ricketts Laboratory, Argonne National Laboratory, 9700 South Cass Avenue, Lemont, IL 60649, USA.

[b]Department of Microbiology, University of Chicago, 920 East 58th Street, Chicago, IL 60637, USA.

[c]Department of Medicine, Faculty of Medicine, University of Kelaniya, Ragama, Sri Lanka.

[1]**Correspondence author**. Hwan Keun Kim, 9700 South Cass Avenue, HTRL Rm 1109, Lemont, IL 60649, Phone (630) 252-2387; E-mail: hkim4@bsd.uchicago.edu

www.pnas.org/cgi/doi/10.1073/pnas.1911922116

SI Materials and Methods

Cell lines and bacterial strains. Vero cells (African green monkey kidney cells, ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) at 37°C in a 5% CO_2 atmosphere. *E. coli* DH5α and XL-1 were grown in Luria-Bertani Miller broth (Difco) at 37°C, supplemented with ampicillin (100 $\mu g \cdot ml^{-1}$), rifampin (20 $\mu g \cdot ml^{-1}$), or chloramphenicol (20 $\mu g \cdot ml^{-1}$) where appropriate. *P. vulgaris* OX2 (ATCC 7829), *P. vulgaris* OX19 (ATCC 6898), and *P. mirabilis* OXK (ATCC 15146) strains were grown in Luria-Bertani Miller broth (Difco) at 37°C. *Rickettsia rickettsii* Sheila Smith (Rocky Mountain Laboratory, Dr. Ted Hackstadt), *R. conorii* Malish 7 (ATCC VR-613) and *kkaebi* variants were grown in Vero cells in the presence of rifampin (0.2 $\mu g \cdot ml^{-1}$) and/or chloramphenicol (0.3 $\mu g \cdot ml^{-1}$) where appropriate. *Rickettsiae* were purified from Vero cells by differential centrifugation through 33% MD-76R solution (816 mM meglumine diatrizoate, 157 mM sodium diatrizoate hydrate, 1 mM NaH_2PO_4, pH 7.0; 21,000 × g, 4°C, 20 min) and stored at -80°C in SPG buffer (218 mM sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2HPO_4, 4.9 mM L-glutamate, pH 7.2).

Genomic and plasmid DNA purification. Rickettsial genomic DNA (gDNA) and plasmid vectors were extracted using commercially available kits (PureLink genomic DNA extraction kit and QIAGEN plasmid kits) following the manufacturers' instructions.

Plasmid construction. Plasmids were constructed using the primers and restriction enzymes indicated in *SI appendix*, Table S3. pHTRL1 and pHTRL2 were sequentially constructed by directional cloning of polymerase chain reaction (PCR) amplified transcription promoter (P_{rompB}) and terminator (T_{rompB}) of *R. rickettsii rompB* into the multiple cloning sites (MCS) of the pMOD-2<MCS> transposon construction vector (Epicentre). The sequence of the *cat* gene was

codon-optimized for use in *R. conorii*, synthesized by Integrated DNA Technologies, Inc and PCR cloned into pHTRL2, creating the *kkaebi* mini-transposon flanked by 19-bp Tn*5* inverse repeat (IR) sequences in pHTRL3. The *R. prowazekii*-optimized *arr2* rifampin resistance cassette, derived from pRAM18dRGA (kindly provided by Dr. Ulrike Munderloh, University of Minnesota) (1), was inserted into pHTRL2, generating pHTRL5. The 1.6 kbp fragment, which was created by overlap extension PCR of *colE1* origin of replication and P_{rompB}-*cat*-T_{rompB}, was used for generation of a *R. conorii*-*E. coli* shuttle vector (pHTRL4) by ligating with a 5.6 kbp fragment containing a multiple cloning site, *dnaA* and *parA* derived from pRAM18dRGA. Another shuttle vector (pHTRL6) was constructed by replacing the *cat* gene with another antibiotic cassette, *arr2*, from pHTRL5. For pHTRL7, a gene coding for green fluorescent protein (GFP_{UV}) driven by the *rompA* promoter (P_{rompA}) was cloned into pHTRL6. A gene fragment containing *R. conorii Rc0457–Rc0460* was PCR amplified from *R. conorii* template DNA and cloned into MCS of pHTRL7.

Inverse PCR. Transposon insertion sites were identified by inverse PCR (iPCR). Chromosomal DNA samples, isolated from *kkaebi* variants, were digested with Nde*I* at 37°C for one hour, followed by heat-inactivation at 65°C for 20 min and then self-ligated with T4 DNA ligase (NEB) at 16°C for 16 hours. Following ligation, the plasmid DNA was isolated with a PCR purification kit (Qiagen) and used as a template in an iPCR (iPCRF, 5’-GCTGTAATATCAAGCTGAACAGTCTGG-3’; iPCRR, 5’-CAAGGTGATAAGGTATTAATGCCACTTG-3’). DNA sequencing of the obtained PCR

fragments revealed the *kkaebi* insertion sites within the chromosome of *R. conorii* (GenBank Accession no. NC_003103.1).

RNA extraction and cDNA synthesis. Three independent cultures of the *R. conorii* wild-type, HK2 pHTRL7, and HK2 pHTRL8 variants were grown on Vero cells at 34°C with 5% CO_2 for 2 days. Vero cells were mechanically disrupted with 3 mm glass beads. After host cell debris was removed by centrifugation (1,000 ×*g*, 4°C, 5 min), the supernatant containing *R. conorii* was treated with RNAprotect Bacteria Reagent (Qiagen). Total RNA was extracted using RNeasy Mini Kit (Qiagen) with on-column DNase I digestion, according to the manufacturer's instructions. 100 ng of total RNA was used for cDNA synthesis with random hexamers (iScript cDNA synthesis kit, Bio-Rad). Primers used for PCR amplification of *Rc0459* is listed in *SI appendix*, Table S3.

Growth and plaque assay. Growth curves were generated by infecting wild-type *R. conorii* and *kkaebi* variants into monolayers of Vero cells in 6-well plates at a multiplicity of infection of 0.01. At 2-day intervals, Vero cells in each well were dislodged with 3 mm glass beads and lysed by vortexing with 3 mm glass beads. *R. conorii* infectious titer was determined by infecting monolayers of Vero cells with 10-fold serial dilutions of lysates containing *Rickettsia* in DMEM supplemented with 5% HI-FBS. Upon infection, Vero cells were incubated at 34°C with 5% CO_2 for 60 min to allow attachment and overlaid with DMEM containing 5% HI-FBS and 0.5% agarose. Antibiotics were added six hours post-infection and incubation continued at 34°C for four-five days prior to isolation or direct enumeration of plaques with MTT staining (Acros).

Microscopy analyses. Cytopathology in Vero cell cultures was analyzed by microscopy. On day 3 post-infection, differential interference contrast (DIC) images of Vero cells infected with *R. conorii* variants in multiple fields of 6-well plates were captured with a charge-coupled device

camera, contrast adjusted, and pseudo-colored using Affinity Photo. Areas of cytopathic Vero cells were assessed by NIH ImageJ software. For electron microscopic studies, Vero cells infected with *R. conorii* variants for three days were fixed with 2% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, embedded in epoxy, and thin sectioned (Advanced Electron Microscopy Facility, University of Chicago). Sections were stained with uranyl acetate and viewed using a Tecnai F30 (Philips/FEI) transmission electron microscope (field emission gun, 300 kV accelerating voltage, and magnification of × 25,000) and a high-performance charge-coupled device camera with 4,000-by-4,000 resolution. Images were acquired using Gatan Digital Micrograph software, contrast adjusted, and processed using Affinity Photo. Rickettsial cell width and length were measured using NIH ImageJ software.

Transformation. Approximately 5×10^8 PFU of purified *R. conorii* were electroporated with 5 μg of plasmids, immediately plated onto Vero cell monolayers, and then allowed to infect Vero cells for 60 min at 34°C, after which the medium was removed and replaced with fresh DMEM containing 5% HI-FBS and 0.5% agarose. Six hours post-infection, medium containing antibiotics was added for the selection of transformants. Individual plaques isolated and expanded in Vero cell monolayers in the presence of antibiotics. Typical yields for plasmid transformation are shown in *SI Appendix*, Table S4**.**

Rabbit immunization. Affinity purified LPS samples (10 U per injection) were emulsified with complete Freund's adjuvant (Difco) and injected subcutaneously into New Zealand white rabbits (Charles River Laboratories). At 21-day intervals, rabbits received booster immunizations of LPS emulsified with incomplete Freund's adjuvant (Difco).

Mouse model of *R. conorii* systemic infection. C3H/HeN mice (male, 6-week, N=10 per group, Charles River Laboratories) were infected by intravenous retro-orbital injection with lethal (1 ×

10^6 PFU) or sub-lethal (1×10^3 PFU) doses of *R. conorii* in 0.1 ml SPG buffer (2). Infected mice were monitored twice daily for signs of disease and daily for weight loss. Blood samples were collected on day 14 post-infection to determine serum antibody titers by western blot analyses against rickettsial antigens.

Biosafety and biosecurity. Animal research was performed in accordance with institutional guidelines following experimental protocol review, approval, and supervision by the University of Chicago's Institutional Biosafety Committee (IBC) and the Institutional Animal Care and Use Committee (IACUC). Animals were managed by The University of Chicago Animal Resource Center, which is accredited by the American Association for Accreditation of Laboratory Animal Care and the Department of Health and Human Services (DHHS number A3523-01). Animals were maintained in accordance with the applicable portions of the Animal Welfare Act and the DHHS "Guide for the Care and Use of Laboratory Animals". Veterinary care was under the direction of full-time resident veterinarians boarded by the American College of Laboratory Animal Medicine. Experiments with *Rickettsia* were performed in biosafety level 3 (BSL3) or animal BSL3 (ABSL3) containment. The University of Chicago Institutional Biosafety Committee and National Institute of Health Recombinant DNA Advisory Committee reviewed and approved the use of a gene encoding chloramphenicol resistance for the selection of mutants with insertional lesions in *R. conorii*.

Statistical analyses. All statistical analyses were performed using Prism software (GraphPad). The proportion of survival animals was analyzed using the two-tailed Log-rank test. Two-way ANOVA with Bonferroni post-tests was performed to analyze the statistical significance of body-weight change and *R. conorii* growth data. Measurements of *R. conorii* survival in plasma,

cytopathogenic sizes, and relative transcription levels were analyzed for statistical significance using one-way ANOVA with Dunnett's multiple comparison test.

Materials and data availability. The data and unique materials that support the findings of this study are available from the corresponding author upon request. Whole-genome-sequencing data (wild-type *R. conorii* Malish 7 and rifampin-resistant *R. conorii*) are deposited to the Sequence Read Archive database (Accession PRJNA513326).

SI **Tables.**

Table S1. ***kkaebi*** **insertion sites in** ***R. conorii*****.**

Name	Inactivated gene	Gene annotation
HK1	Intergenic	n/a
HK2	*Rc0457*	UDP-GlcNAc 4,6-dehydratase/3,5-epimerase
HK3	*Rc0521*	Hypothetical protein
HK4	Intergenic	n/a
HK5	*Rc0610*	Hypothetical protein
HK6	*Rc0820*	Pseudo gene
HK7	*Rc1371*	Hypothetical protein
HK8	*Rc0690*	DNA adenine methylase
HK9	Intergenic	n/a
HK10	*Rc0690*	DNA adenine methylase
HK11	*Rc0441*	Hypothetical protein
HK12	Intergenic	n/a
HK13	*Rc0059*	ATP-dependent chaperone, ClpB
HK14	Intergenic	n/a
HK15	*Rc0459*	Glycosyltransferase
HK16	Intergenic	n/a
HK17	*Rc0553*	Cytochrome c oxidase subunit I
HK18	*Rc1263*	Pseudo gene
HK19	*Rc0452*	Paraslipin
HK20	*Rc0401*	DNA mismatch repair protein, MutS
HK21	Intergenic	n/a
HK22	*Rc0779*	Bifunctional folypolyglutamate synthase
HK23	*Rc0401*	DNA mismatch repair protein MutS
HK24	*Rc0629*	Lon protease
HK25	Intergenic	n/a
HK26	*Rc0722*	Nucleotide exchange transporter, Tlc3
HK27	*Rc1141*	HlyC/CorC family transporter
HK28	*Rc0784*	BrnT/AT system
HK29	*Rc_RS07925*	Recombinase, RecF
HK30	*Rc0744*	Type 2 isopentenyl-diphosphate Delta-isomerase
HK31	*Rc0247*	Pseudo gene
HK32	*Rc_RS07845*	Pseudo gene
HK33	*Rc0858*	Transcriptional regulator, MraZ
HK34	Intergenic	n/a
HK35	*Rc0835*	Alpha-2-macroglobulin family protein
HK36	*Rc0901*	ATP-dependent DNA helicase RecG
HK37	*Rc0901*	ATP-dependent DNA helicase RecG
HK38	*Rc0110*	Autotransporter domain-containing protein, Sca2
HK39	Intergenic	n/a
HK40	*Rc0400*	3’(2’),5’-bisphosphate nucleotidase, CysQ
HK41	*Rc0744*	Type 2 isopentenyl-diphosphate Delta-isomerase
HK42	Intergenic	n/a

HK43	*Rc0078*	Tellurite resistant protein
HK44	*Rc0472*	AsmA family protein
HK45	*Rc0363*	Hsp20/alpha crystalline family protein
HK46	Intergenic	n/a
HK47	*Rc1201*	Palindromic element RPE1 domain-containing protein
HK48	Intergenic	n/a
HK49	Intergenic	n/a
HK50	*Rc1273*	Autotransporter outer membrane protein
HK51	*Rc0143*	Hypothetical protein
HK52	*Rc0759*	Transcription termination factor Rho
HK53	*Rc0622*	Pseudo gene

Table S2. Protein identification by mass spectrometry.

Gel slice[a]	Gene[b]	Protein (Accession)[c]	PSM[d]
1	*Rc1273*	rOmpA (14916620)	250
	Rc0182	RpoC (14916697)	133
	Rc0181	RpoB (499283791)	98
	Rc1085	rOmpB (499284603)	69
	Rc0144	VirB6c (81528530)	45
2	*Rc1085*	rOmpB (499284603)	513
	Rc0181	RpoB (499283791)	420
	Rc0182	RpoC (14916697)	341
	Rc1218	Hypothetical protein (499284723)	51
	Rc0667	Sca4 (15619756)	42
3	*Rc1085*	rOmpB (499284603)	980
	Rc1281	Putative surface protein (499284783)	343
	Rc1273	rOmpA (14916620)	132
	Rc0185	ATPase (15619234)	85
	Rc0019	Sca1 (499283647)	81

[a]Coomassie-stained SDS-gel slices (Fig. 3*A*) were excised and proteins identified via LC-MS/MS analyses of tryptic peptides.

[b]Genes and [c]proteins identified by data comparison with *in silico* translational products derived from the genome sequence of *R. conorii* Malish 7 (NC_003103.1).

[d]Peptide spectrum match (PSM) indicates the total number of identified peptide sequences for the protein, including those redundantly identified.

Table S3. List of oligonucleotides used in this study.

Name	Target	Sequence
pHTRL1-1	*rompB* terminator	AAATCTAGATCGCTCCCGATTAGCAAG
pHTRL1-2	*rompB* terminator	AAACTGCAGGAGCCATGCAACAAGGC
pHTRL2-1	*rompB* promoter	AAAGAATTCATCCCGCGGTCAAGCCACGG
pHTRL2-2	*rompB* promoter	AAAGGTACCTGTCCTATATTTAAGTTAAAATTTAGTACC
pHTRL3-1	*cat*	AAAGGTACCATGGAGAAAAAGATAACTGGTTATAC
pHTRL3-2	*cat*	AAAGGATCCTTATGCACCACCTTGCCAT
pHTRL4-1	*colE1*	AAAGCTAGCCGCGTTGCTGGCGTTTTTCC
pHTRL4-2	*colE1*	CCGTGGCTTGACCGCGGGATGCATGCCCCCGTAGAAAAAGATCAAAGGATCTTCTTG
pHTRL4-3	P_{rompB}-*cat*-T_{rompB}	CAAGAAGATCCTTTGATCTTTTCTACGGGGGCATGCATCCCGCGGTCAAGCCACGG
pHTRL4-4	P_{rompB}-*cat*-T_{rompB}	AAAAACGTTGAGCCATGCAACAAGGCATGTAGGCG
pHTRL5-1	*arr2*	AAAGGTACCATGGTAAAAGATTGGATTCCTATTTCTC
pHTRL5-2	*arr2*	AAAGGATCCTTAATCTTCAATAACATGTAAACCACGA
pHTRL6-1	P_{rompB}-*arr2*-T_{rompB}	AAAGCATGCATCCCGCGGTCAAGCCAC
pHTRL6-2	P_{rompB}-*arr2*-T_{rompB}	AAAGCATGCATCCCGCGGTCAAGCCAC
pHTRL7-1	P_{rompA}-GFP_{UV}-T_{rompA}	AAAAACGTTTCCTCGCAATGACGCTTCTCAAAACTAC
pHTRL7-2	P_{rompA}-GFP_{UV}-T_{rompA}	AAAAACGTTGCAACAAGGCCAACACCGCTAC
pHTRL8-1	*Rc0457-Rc0460*	AAACCTAGGGTGAGTTTAAGTAAATTGTCATTGCGAATATAGGC
pHTRL8-2	*Rc0457-Rc0460*	AAACCTAGGGTAAGTTTGTGCTAAAGCACTATTTATAGCTTCTTTC
Rc0459F	*Rc0459*	CGTTACTGGATGATATAATGGTGTCAG
Rc0459R	*Rc0459*	CGATAAATGCAGCAGGGTTTTC

Table S4. Selecting for *R. conorii* plasmid transformants

Plasmid[a]	Antibiotic[b]	PFU[c]
Pre-electroporation	–	5.7×10^{8} ($\pm5.8\times10^{7}$)
Post-electroporation	–	4.7×10^{8} ($\pm2.9\times10^{7}$)
pHTRL6	Rifampin	53 (±20)
pHTRL4	Chloramphenicol	287 (±21)

[a]*R. conorii* Malish 7 was electroporated with 5 µg of pHTRL4 or pHTRL6 in 100 µl SPG buffer in 0.1 cm-gap sterile cuvettes at 3.0 kV, 200 ohms, 25 µF, 5 ms using a Gene Pulser (Bio-Rad).

[b]Vero cells infected with *R. conorii* transformants in the presence of rifampin (0.2 $\mu g \cdot ml^{-1}$) or chloramphenicol (0.3 $\mu g \cdot ml^{-1}$).

[c]Data are the mean plaque forming units (PFU, ±SEM) of *R. conorii* propagated on Vero cells (N=3).

SI **Figures.**

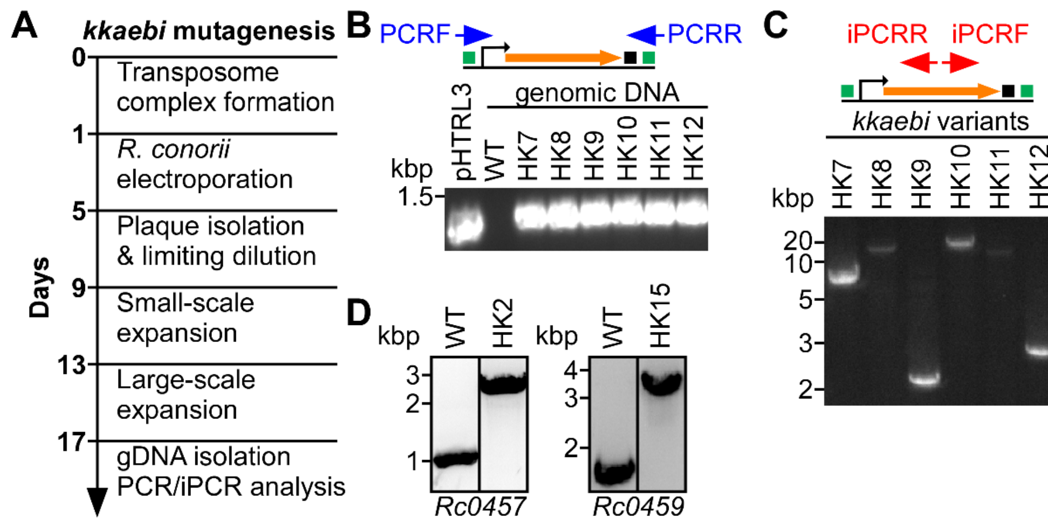
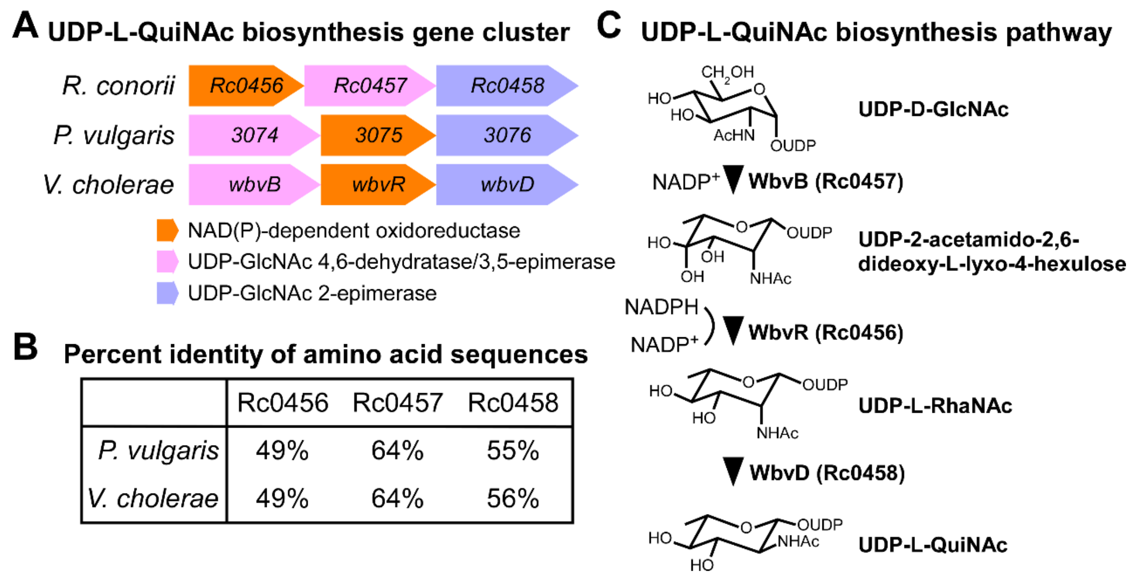


Fig. S1. Generation of *kkaebi* variants and identification of HK2 and HK15. (*A*) Schematic overview of *kkaebi*-mutagenesis. (*B*) PCR amplification of the *kkaebi*-transposon from the genomic DNA of *R. conorii* variants. (*C*) Inverse PCR analysis to identify *kkaebi* insertion sites in the chromosome of *R. conorii* variants. (*D*) PCR amplification of *Rc0457* and *Rc0459* from genomic DNA of *R. conorii* wild-type (WT), HK2, and HK15.



	Rc0456	Rc0457	Rc0458
P. vulgaris	49%	64%	55%
V. cholerae	49%	64%	56%

Fig. S2. The *N*-acetyl-L-quinovosamine (L-QuiNAc) biosynthesis gene cluster in *R. conorii*. (*A*) Diagram of three conserved genes in *R. conorii* (NC_003103.1), *P. vulgaris* (JPIX01000008.1) and *V. cholerae* (AF390573.1). (*B*) Percent identity of amino acid sequences. (*C*) Proposed pathway for UDP-L-QuiNAc biosynthesis.

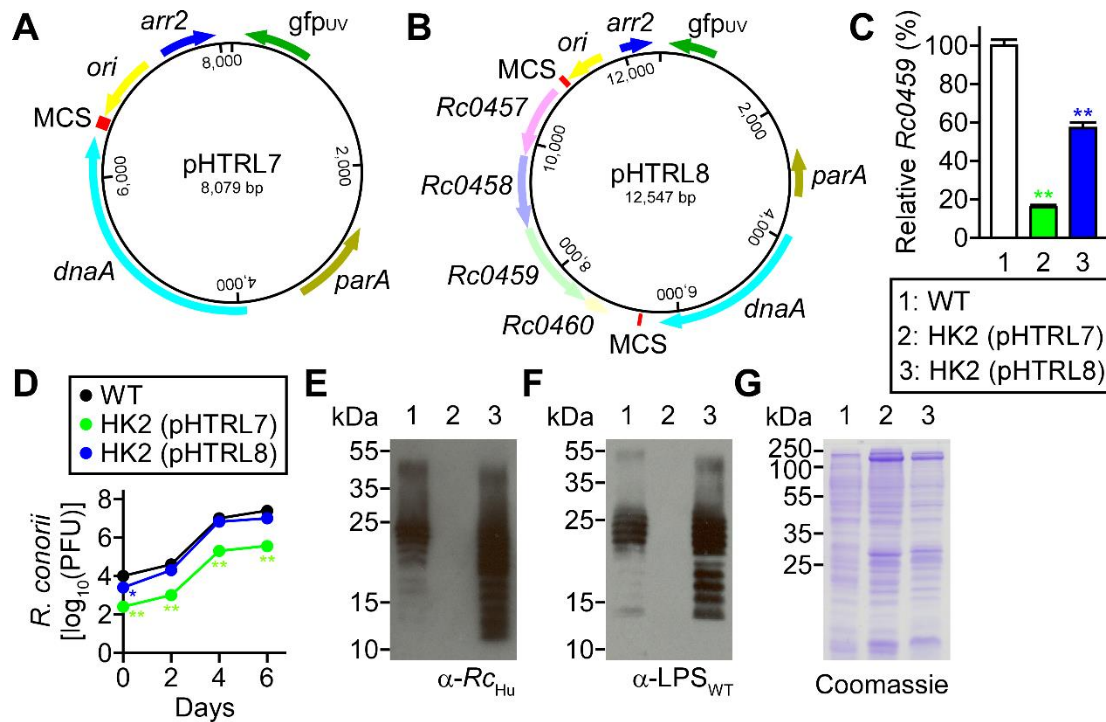


Fig. S3. Plasmid complementation analysis with *R. conorii* HK2. (*A*) Maps of pHTRL7 encoding codon-optimized *arr2* and (*B*) pHTRL8 carrying *Rc0457–Rc0460*. (*C*) Relative transcription level of *Rc0459* in *R. conorii* variants isolated from Vero cells at 2 days post inoculation (N=3). (*D*) Quantification of plaque forming units (PFU) in Vero cell cultures inoculated for variable incubation times with *R. conorii* WT, HK2 (pHTRL7) or HK2 (pHTRL8) (N=3). Immunoblot analyses of affinity purified LPS from *R. conorii* wild-type (WT) or the *pso* mutants HK2 and HK15 with (*E*) human immune serum from individuals convalescent from *R. conorii* infection (α-Rc_{Hu}) or (*F*) rabbit serum raised against purified wild-type *R. conorii* LPS (α-$\mathrm{LPS}_{\mathrm{WT}}$). (*G*) Coomassie-stained SDS-PAGE analysis of outer membrane fractions from *R. conorii* WT, HK2 (pHTRL7) or HK2 (pHTRL8). Data are the mean (±SEM) of three independent determinations. Statistical significances for *Rc0459* transcription levels and rickettsial growth were analyzed with one-way ANOVA with Dunnett's multiple comparison test and two-way ANOVA with Bonferroni post-tests, respectively. *, $P<0.05$; **, $P<0.0001$.

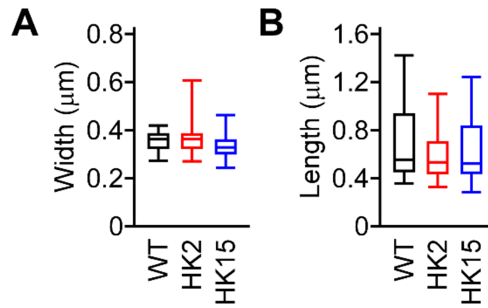


Fig. S4. Size measurements of *R. conorii* variants. (*A*) Bacterial widths and (*B*) lengths were determined using transmission electron microscopic images from Vero cells infected for three days with *R. conorii* wild-type (WT), HK2, or HK15. One-way ANOVA with Dunnett's post-test was used to identify statistically significant differences (N=34 for WT, N=31 for HK2, and N=32 for HK15; $P>0.05$; center line, median; box limits, upper and lower quartiles; whiskers, minimum and maximum values).

***SI* references.**

1. Burkhardt NY*, et al.* (2011) Development of shuttle vectors for transformation of diverse *Rickettsia* species. (Translated from eng) *PLoS One* 6:e29511 (in eng).
2. Walker DH, Popov VL, Wen J, & Feng HM (1994) *Rickettsia conorii* infection of C3H/HeN mice. A model of endothelial-target rickettsiosis. (Translated from eng) *Lab. Invest.* 70:358-368 (in eng).