



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

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Rickettsial diseases have long been diagnosed with serum antibodies cross-reactive against *Proteus vulgaris* (Weil–Felix reaction). Although Weil–Felix antibodies are associated with the development of immunity, their rickettsial target and contribution to disease pathogenesis are not established. Here, we developed a transposon for insertional mutagenesis of *Rickettsia conorii*, isolating variants defective for replication in cultured cells and in spotted fever pathogenesis. Mutations in the *polysaccharide synthesis operon* (*psa*) abolish lipopolysaccharide O-antigen synthesis and Weil–Felix serology and alter outer-membrane protein assembly. Unlike wild-type *R. conorii*, *psa* mutants cannot elicit bactericidal antibodies that bind O antigen. The *psa* operon is conserved among rickettsial pathogens, suggesting that bactericidal antibodies targeting O antigen may generate universal immunity that could be exploited to develop vaccines against rickettsial diseases.

transposon mutagenesis | lipopolysaccharide | O antigen | Weil–Felix reaction | *polysaccharide synthesis operon*

Antibodies directed against bacterial surface carbohydrates, i.e., the capsular polysaccharide or the O antigen of lipopolysaccharide (LPS), activate, complement, and promote microbial killing (1). Bactericidal antibodies are acquired during colonization or invasive disease with pathogens such as *Escherichia coli*, *Haemophilus influenzae*, and *Neisseria meningitidis* or following immunization with carbohydrate-conjugate vaccines (2–4). Bactericidal antibodies represent a correlate for immunity; however, disease protection is limited to specific pathogen serotypes and countered by the selection of variants with distinct carbohydrate antigens (5). Rickettsial pathogens rely on hematophagous arthropods for host transmission and disease pathogenesis (6). Due to their obligate requirement for intracellular replication, *Rickettsia* spp. cannot be propagated on laboratory media (7). Rickettsial diseases have therefore been diagnosed with the Weil–Felix serology, the detection of IgG or IgM cross-reactive with *Proteus vulgaris* (8). Increased Weil–Felix serology has been associated with positive clinical outcome and with protective immunity (9). For survivors of epidemic typhus, waning Weil–Felix serology has been observed in patients with recrudescing typhus (Brill–Zinsser disease) (10). Administration of epidemic typhus vaccines, for example extracts from lice infected with *Rickettsia prowazekii*, elicits Weil–Felix antibodies and disease protection (11). Nevertheless, the rickettsial target of Weil–Felix antibodies and its contribution to rickettsial disease pathogenesis are not established.

Here, we developed a transposon mutagenesis technology enabling facile isolation of insertional mutants defective in the obligate intracellular life cycle of *Rickettsia conorii*. We describe that *R. conorii* variants harboring transposon insertions in the conserved *polysaccharide synthesis operon* cannot produce O-antigen polysaccharides and fail to induce Weil–Felix serology with a significant virulence defect in the murine infection model. The results suggest that humans may develop protective immunity against pathogenic rickettsiae by targeting the conserved carbohydrate epitope present in the O antigen of LPS.

## Results

***kkaebi* Transposon Mutagenesis Identifies *R. conorii* Variants Defective in Intracellular Replication.** Earlier work developed transposon mutagenesis for the selection of antibiotic-resistant *Rickettsia* variants. However, the selection for transposon mutants was hindered by rickettsial variants with spontaneous resistance to antibiotic selection (12, 13). To overcome this obstacle, we measured the frequency of spontaneous resistance in *R. conorii* replicating in Vero cells. *R. conorii* rifampin-resistant plaques appeared at a frequency of  $1 \times 10^{-7}$  plaque-forming unit (PFU), whereas chloramphenicol-resistant plaques were not isolated (mutational frequency  $< 1 \times 10^{-8}$  PFU). Using whole-genome sequencing, we identified a missense mutation in the *rpoB* gene of rifampin-resistant *R. conorii* (amino acid substitution D533G [accession no. SRR8404401 [wild-type *R. conorii*] and SRR8404402 [rifampin-resistant *R. conorii*]; refs. 14 and 15). These findings corroborate previous work, demonstrating that *rpoB* missense mutations (R546K) provide for rifampin resistance in *R. prowazekii* (16). However, chloramphenicol does not appear to select for spontaneously resistant *R. conorii* variants and was therefore used for the selection of transposon mutants.

For insertional mutagenesis, we generated pHTRL3, which carries a codon-optimized gene for chloramphenicol acetyltransferase (*cat*) flanked by the inverted repeats (IR) of the Tn5

## Significance

Genetic analysis of *Rickettsia* has been difficult. We developed a transposon and selection scheme to facilitate the isolation of *Rickettsia conorii* mutants with insertional lesions. Here, we demonstrate that the *R. conorii* *polysaccharide synthesis operon* (*psa*) encompasses genetic determinants for biosynthesis of the O antigen, which also affect the composition of outer-membrane proteins, invasion of host cells, and pathogenesis. The O antigen provides essential barrier functions and plays a major role in host–pathogen interactions. Our findings suggest that infected hosts develop protective immunity against *R. conorii* via the production of antibodies targeting the O antigen. Conservation of *psa* among rickettsial species suggests that it may play a universal role in O-antigen synthesis, disease pathogenesis, and the development of immunity.

Author contributions: H.K.K., D.M.M., and O.S. designed research; H.K.K. performed research; R.P. contributed new reagents/analytic tools; H.K.K., D.M.M., and O.S. analyzed data; and H.K.K., D.M.M., and O.S. wrote the paper.

The authors declare no conflict of interest.

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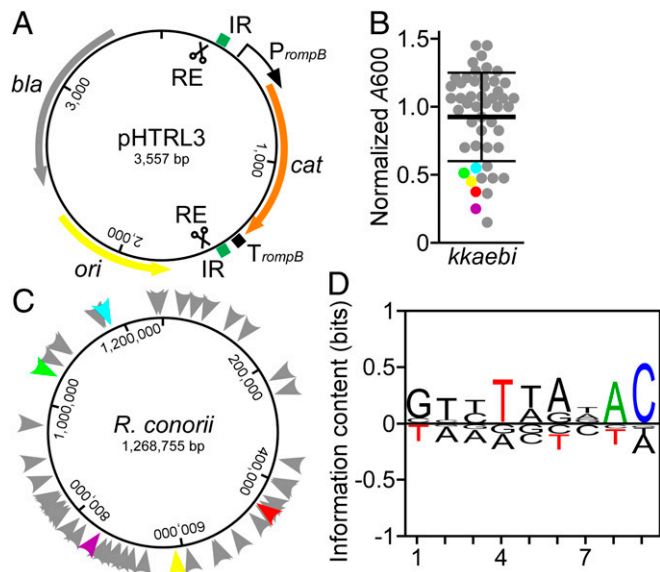
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Data deposition: Whole-genome sequencing data for wild-type *R. conorii* Malish 7 (accession no. SRR8404402) and rifampin-resistant *R. conorii* (accession no. SRR8404401) are deposited in the Sequence Read Archive.

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**Fig. 1.** Transposon mutagenesis in *R. conorii*. (A) Map of pHTRL3 with the *kkaebi* minitransposon. (B)  $A_{600}$  was measured to determine the yield of *R. conorii kkaebi* variants after 4 d growth on Vero cells. Five variants (HK2, HK24, HK27, HK28, and HK50) with defective growth are colored in red, yellow, green, purple, and light blue, respectively. (C) Diagram illustrating the position of *kkaebi* insertion sites on the *R. conorii* chromosome (color code same as in B). (D) Blogo sequence logo plot generated with 53 insertion events of *kkaebi*. Nine bases were used to generate the sequence logo. Nucleotide frequency at each position is indicated by the relative height of each letter; significantly increased or reduced frequency ( $P < 0.05$ ) at a given position is shown in color.

transposon under control of the *R. rickettsii rompB* promoter ( $P_{rompB}$ ) (Fig. 1A). The minitransposon was named *kkaebi*. *kkaebi* DNA was PCR amplified from pHTRL3 template DNA, cleaved with restriction enzyme (RE), and incubated with Tn5 transposase to generate transposome complexes that, when electroporated into *R. conorii* and selected on Vero cell cultures in the presence of chloramphenicol, generated insertional mutations at a frequency of  $5 \times 10^{-8}/\mu\text{g } kkaebi$ . Isolated variants were cataloged with HK numbers in the *R. conorii* mutant library database (SI Appendix, Table S1). After 4 d of plaque expansion in Vero cells, *R. conorii* HK mutants were purified via gradient density centrifugation and absorption at 600 nm ( $A_{600}$ ) was determined. Most of the *kkaebi* mutants replicated to the same level as wild-type *R. conorii* (Fig. 1B). However,  $A_{600}$  and PFU for the HK2, HK24, HK27, HK28, and HK50 variants were lower than those of wild-type *Rickettsia* (Fig. 1B). Sequence analysis of the transposon insertion sites revealed that all *R. conorii* variants harbored single insertions of *kkaebi* distributed randomly across the rickettsial genome (Fig. 1C and SI Appendix, Table S1). Alignment of the 9-nucleotide region for each recorded insertion revealed that transposition of *kkaebi* is not biased by specific nucleotide sequences (Fig. 1D).

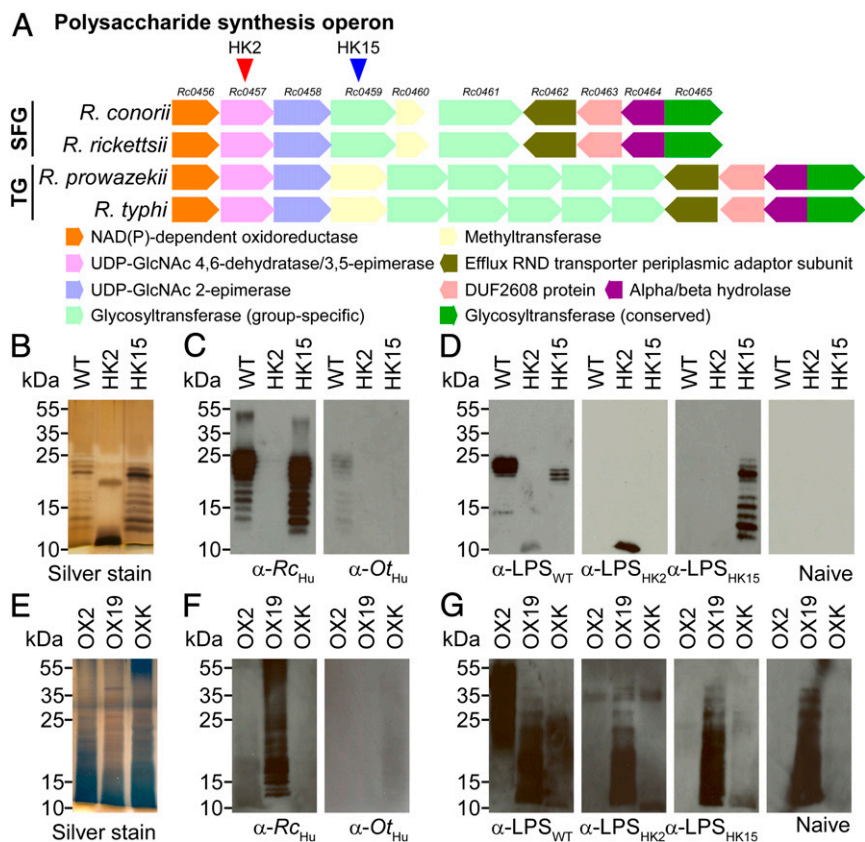
**Genes Involved in the Biosynthesis of the O-Antigen Polysaccharide and Weil-Felix Serology.** Sequence analysis identified 2 variants, HK2 and HK15, with *kkaebi* insertions in *Rc0457* and *Rc0459*. *Rc0457* and *Rc0459* are located in a gene cluster here designated as the rickettsial polysaccharide synthesis operon (*psa*) (Fig. 2A and SI Appendix, Fig. S1). The first 3 genes (*Rc0456* to *Rc0458*) of *psa* are conserved among all pathogenic *Rickettsia* and exhibit sequence homology with 3 gene products from *P. vulgaris* (3074 to 3076) and *Vibrio cholerae* (*wbvB*, *wbvR*, and *wbvD*) (SI Appendix, Fig. S2). When expressed and purified from *E. coli*, WbvB/WbvR/WbvD catalyze synthesis of UDP-L-QuiNAc (*N*-acetyl-L-

quinovosamine), a key constituent of the O antigen of *V. cholerae*, *P. vulgaris*, and *Rickettsia* (SI Appendix, Fig. S2) (17–19). The *Rickettsia typhi* O antigen comprises the polysaccharide repeat  $[\rightarrow 4)\text{-}\alpha\text{-D-Glc-(1}\rightarrow 3)\text{-}\alpha\text{-L-QuiNAc-(1}\rightarrow 4)\text{-}[\alpha\text{-D-Glc-(1}\rightarrow 3)\text{-}\alpha\text{-L-QuiNAc-(1}\rightarrow 4)]_n\text{-}\alpha\text{-D-Glc-(1}\rightarrow 3)\text{-}\alpha\text{-L-QuiNAc-(1}\rightarrow 3)\text{-}\alpha\text{-D-GlcNAc}\rightarrow]$  with a short side chain  $[\text{D-GlcNAc-(1}\rightarrow 3)\text{-}\alpha\text{-L-QuiNAc-(1}\rightarrow 3)\text{-}\alpha\text{-D-GlcNAc}\rightarrow]$  linked to L-QuiNAc (20). Of note, the disaccharide motif,  $[\alpha\text{-L-QuiNAc-(1}\rightarrow 3)\text{-}\alpha\text{-D-GlcNAc}]$ , is also present in the O antigen of *P. vulgaris* OX19 (21). However, in *P. vulgaris* OX2, which does not generate cross-reactive Weil-Felix antibodies with *R. typhi*, the disaccharide unit is linked to the O-antigen repeats via a  $\beta$ -glycosidic bond (21).

To determine whether the *R. conorii* variants HK2 and HK15 exhibit O-antigen synthesis defects, we purified *R. conorii* LPS and analyzed molecules by acrylamide gel electrophoresis and silver staining. As expected, LPS from wild-type *R. conorii* migrated as a spectrum of molecules with tethered O-antigen repeats and ladder-like appearance on polyacrylamide gels (Fig. 2B). LPS isolated from the HK15 mutant exhibited a similar migration pattern (Fig. 2B). In contrast, a single LPS species was purified from the HK2 variant, which migrated faster on the polyacrylamide gel than wild-type and HK15 LPS (Fig. 2B). Immunoblot analysis with *R. conorii*-specific human antisera ( $\alpha\text{-Rc}_{\text{Hu}}$ ) revealed antibodies that bound LPS from wild-type and HK15 *R. conorii*, but not LPS from the HK2 variant (Fig. 2C). Rabbit IgG against *R. conorii* LPS (raised via immunization with purified LPS,  $\alpha\text{-LPS}_{\text{WT}}$ ) bound LPS from wild-type and HK15 *R. conorii*, but displayed only weak binding for HK2 LPS (Fig. 2D). Rabbit IgG raised against purified HK2 and HK15 LPS ( $\alpha\text{-LPS}_{\text{HK2}}$  and  $\alpha\text{-LPS}_{\text{HK15}}$ ) bound to the cognate LPS antigens but did not exhibit cross-reactivity with LPS from wild type or the other mutant strain (Fig. 2D). The LPS O-antigen biosynthesis defect in *R. conorii* HK2 was in part restored by transformation with pHTRL8. This plasmid carries *Rc0457* and upstream promoter sequences as well as *Rc0458* to *Rc0460* (SI Appendix, Fig. S3). *Rc0457* encodes UDP-GlcNAc 4,6-dehydratase/3,5-epimerase, an enzyme that is essential for QuiNAc and O-antigen polysaccharide synthesis in *V. cholerae*. Next, we performed immunoblot analyses with affinity-purified LPS from *P. vulgaris* OX2 and OX19 and *Proteus mirabilis* OXK (Fig. 2E). As expected,  $\alpha\text{-Rc}_{\text{Hu}}$ , but not  $\alpha\text{-Ot}_{\text{Hu}}$  (human immune serum from individuals infected with *Orientia tsutsugamushi*) harbored Weil-Felix antibodies against OX2 and OX19 LPS (Fig. 2F). Finally, rabbit  $\alpha\text{-LPS}_{\text{WT}}$ , but not rabbit  $\alpha\text{-LPS}_{\text{HK2}}$  or  $\alpha\text{-LPS}_{\text{HK15}}$ , recognized *P. vulgaris* OX2 LPS (Fig. 2G). As previously reported, we detected OX19 LPS-specific antibodies in naive rabbit serum, which prohibited further cross-reactivity analysis (22). Together these data indicate that *R. conorii pso* encodes genes for the synthesis of the O antigen of LPS, which represents the rickettsial target of Weil-Felix antibodies.

#### Without O-Antigen Synthesis, *R. conorii* Displays Altered Outer-Membrane Protein Content and Reduced Host Cell Invasion Activity.

We wondered whether the O-antigen synthesis defects of *psa* mutants interfere with the assembly of rickettsial outer-membrane proteins. Outer-membrane extracts of wild-type *R. conorii* and the *psa* mutant strains HK2 and HK15 were analyzed by Coomassie-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which revealed increased abundance of proteins migrating at 190, 120, and 32 kDa from the outer membrane of *R. conorii* HK2 (Fig. 3A). Corresponding gel slices were excised and tryptic peptides of proteins analyzed by mass spectrometry (MS) with in silico comparison of tryptic peptides derived from the *R. conorii* genome (SI Appendix, Table S2). The data identified rOmpA (190 kDa), rOmpB (120 kDa), and rOmpB (32 kDa) as the most abundant species. Of note, the 32-kDa gel slice harbored peptides from 2 additional outer-membrane proteins, rOmpA and Sca1. rOmpA and rOmpB are members of the autotransporter superfamily, forming surface (S) layers that contribute to



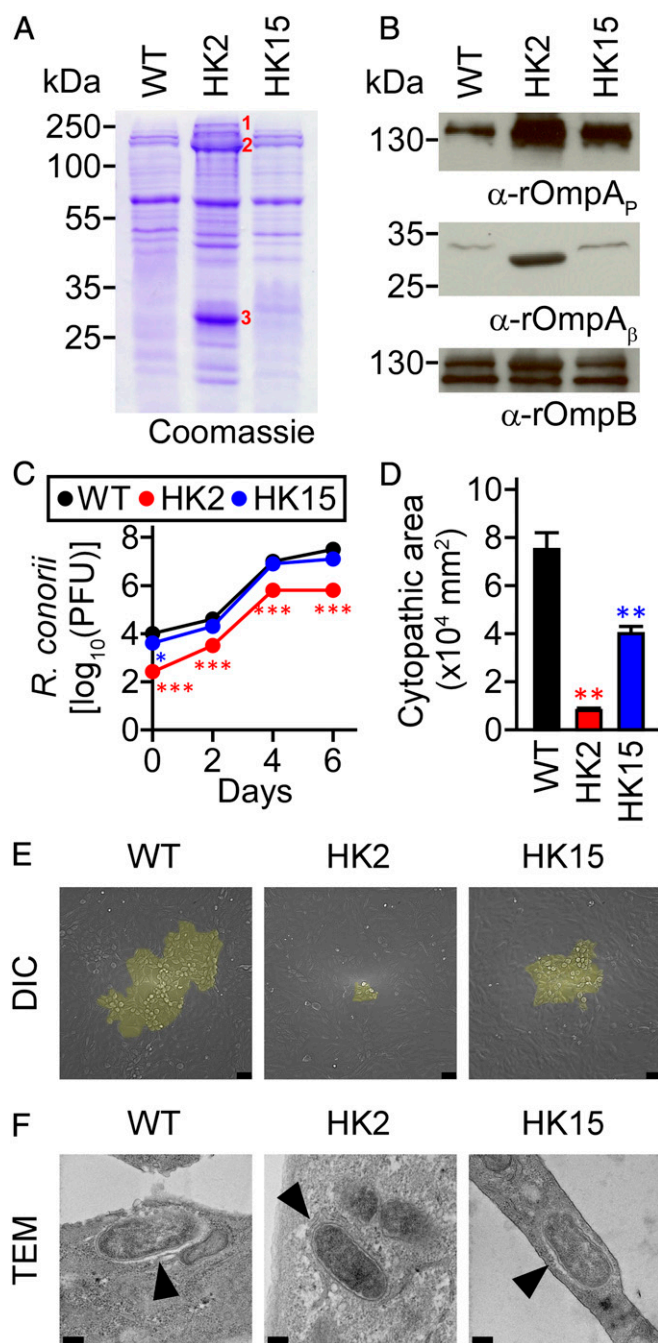
**Fig. 2.** The *Rickettsia pso* is the determinant of Weil-Felix serology. (A) Diagram of the *pso* in pathogenic *Rickettsia*. Arrowheads indicate transposon insertion sites. (B) Silver-stained polyacrylamide gel of affinity-purified LPS from *R. conorii* wild type (WT) or the *pso* variants HK2 and HK15. (C) Immunoblot of *R. conorii* LPS with *R. conorii* ( $\alpha$ -Rc<sub>Hu</sub>) or *O. tsutsugamushi* ( $\alpha$ -Ot<sub>Hu</sub>) convalescent human sera. (D) Rabbit antisera raised against affinity-purified LPS from *R. conorii* wild type ( $\alpha$ -LPS<sub>WT</sub>) or the *pso* variants HK2 ( $\alpha$ -LPS<sub>HK2</sub>) and HK15 ( $\alpha$ -LPS<sub>HK15</sub>) were examined for antibodies against WT, HK2, and HK15 LPS and compared with naive rabbit serum. (E) Silver-stained polyacrylamide gel of affinity-purified LPS from *P. vulgaris* OX2 and OX19 and *P. mirabilis* OXK. (F) Immunoblot of *P. vulgaris* OX2 and OX19 and *P. mirabilis* OXK LPS with  $\alpha$ -Rc<sub>Hu</sub> or  $\alpha$ -Ot<sub>Hu</sub>. (G)  $\alpha$ -LPS<sub>WT</sub>,  $\alpha$ -LPS<sub>HK2</sub>, or  $\alpha$ -LPS<sub>HK15</sub> was examined for antibodies cross-reactive against *P. vulgaris* OX2 and OX19 and *P. mirabilis* OXK LPS.

rickettsial invasion of host cells (23–25). Autotransporters are synthesized as large precursor species with N-terminal signal peptides for secretion via the Sec pathway. The Bam complex subsequently translocates and assembles autotransporters in the outer membrane (26). During assembly, the N-terminal passenger domain of autotransporters is cleaved and displayed on the bacterial surface, while the C-terminal  $\beta$ -barrel domain functions to anchor the passenger domain in the outer membrane. Outer-membrane samples of *R. conorii* strains were subjected to immunoblotting with monoclonal antibodies specific for the 190-kDa passenger domain of rOmpA or polyclonal antipeptide antibodies specific for the 32-kDa  $\beta$ -barrel domain of rOmpA. Compared to wild-type *R. conorii* and the HK15 mutant strain, the abundance of the rOmpA passenger and  $\beta$ -barrel domains was increased in *R. conorii* HK2 (Fig. 3B). Of note, the  $\beta$ -barrel domain exhibited increased mobility in *R. conorii* HK2, suggesting that the O-antigen synthesis defect altered not only the abundance of the autotransporter but also its proteolytic cleavage. Immunoblotting with polyclonal antibodies against rOmpB also revealed an increased abundance of this autotransporter in *R. conorii* HK2 (Fig. 3B). Electroporation of *R. conorii* HK2 with pHTRL8 restored the abundance of rOmpA and rOmpB and of the overall outer-membrane protein content to levels closer to those observed for wild-type *R. conorii* (SI Appendix, Fig. S3).

Defects in O-antigen synthesis and autotransporter display in *R. conorii* HK2 were also associated with reduced attachment to Vero cells (WT, 4.0 log<sub>10</sub> PFU; HK2, 2.4 log<sub>10</sub> PFU,  $P < 0.0001$

at 1-h postinoculation; Fig. 3C). In pairwise comparisons of rickettsial replication at timed intervals, wild-type and HK15 *R. conorii* expanded at similar rates (WT, 7.5 log<sub>10</sub> PFU; HK15, 7.1 log<sub>10</sub> PFU at 6 d postinoculation,  $P > 0.05$ ; Fig. 3C). In contrast, *R. conorii* HK2 replicated at a slower rate (HK2, 5.8 log<sub>10</sub> PFU at 6 d postinoculation; WT vs. HK2,  $P < 0.0001$ ; Fig. 3C). The defects in host cell attachment and intracellular growth were restored when *R. conorii* HK2 was transformed with pHTRL8 [WT, 7.3 log<sub>10</sub> PFU; HK2 (pHTRL7), 5.8 log<sub>10</sub> PFU; HK2 (pHTRL8), 7.0 log<sub>10</sub> PFU at 6 d postinoculation; WT vs. HK2 (pHTRL7),  $P < 0.0001$ ; WT vs. HK2 (pHTRL8),  $P > 0.05$ ; SI Appendix, Fig. S3]. In addition to the attachment defect, the HK2 mutant produced a significantly reduced cytopathic area on Vero cell cultures after 3 d of infection [WT,  $7.5 (\pm 0.6) \times 10^4 \mu\text{m}^2$ ; HK2,  $0.8 (\pm 0.1) \times 10^4 \mu\text{m}^2$ ; HK15,  $4.0 (\pm 0.3) \times 10^4 \mu\text{m}^2$ ; WT vs. HK2,  $P < 0.001$ ; WT vs. HK15,  $P < 0.001$ ; Fig. 3D and E]. Electron microscopy analysis of cytopathic Vero cells identified *R. conorii*, mostly within the cytoplasm of host cells, without significant changes in bacterial size and shape (Fig. 3F and SI Appendix, Fig. S4).

***R. conorii* O-Antigen Synthesis Is Required for Spotted Fever Pathogenesis.** To investigate whether *R. conorii* HK2 or HK15 exhibits virulence defects in the mouse model for acute disease, cohorts of mice were inoculated i.v. with  $1 \times 10^6$  PFU wild-type *R. conorii* or its HK2 or HK15 variants. Animals infected with wild-type *R. conorii* exhibited disseminated vascular disease with



**Fig. 3.** *R. conorii* HK2 is defective for outer-membrane protein display and attachment to host cells. (A and B) Outer-membrane fractions isolated from *R. conorii* wild-type (WT) or the *pso* variants HK2 and HK15 were analyzed by (A) Coomassie-stained PAGE or (B) immunoblotting with monoclonal antibodies against the passenger domain of rOmpA ( $\alpha$ -rOmpA<sub>P</sub>), polyclonal antipeptide antibodies against the  $\beta$ -barrel domain of rOmpA ( $\alpha$ -rOmpA <sub>$\beta$</sub> ), or polyclonal antibodies against rOmpB ( $\alpha$ -rOmpB). (C) *Rickettsia* replication in Vero cells was quantified with the plaque assay ( $n = 3$ ). (D) Quantification of cytopathic areas of Vero cells infected with *R. conorii* variants at day 3 postinfection ( $n = 24$  for WT,  $n = 11$  for HK2, and  $n = 23$  for HK15). Data are the mean ( $\pm$ SEM) of 3 independent determinations. Statistically significant differences were analyzed with (C) 2-way ANOVA with Bonferroni posttests and (D) 1-way ANOVA with Dunnett's posttest. \* $P < 0.05$ ; \*\* $P < 0.001$ ; \*\*\* $P < 0.0001$ . (E) Representative microscopic images of cytopathic areas were identified by differential interference contrast microscopy (DIC) and colored yellow. (Scale bar, 50  $\mu$ m.) (F) Samples were thin-sectioned, stained with uranyl-acetate, and viewed via transmission electron microscopy (TEM). Arrowheads identify intracellular *R. conorii*. (Scale bar, 200 nm.)

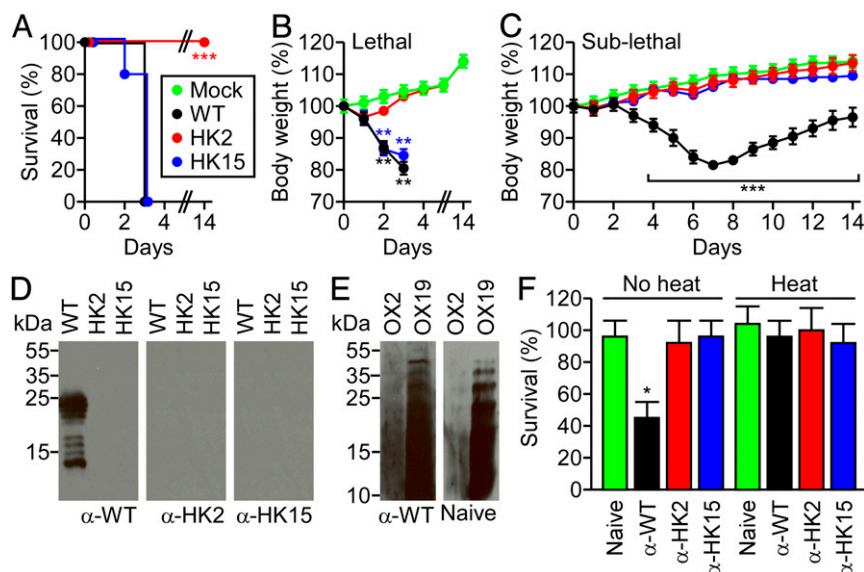
severe body weight loss and lethal outcome within 3 d of inoculation (Fig. 4A and B). The HK15 mutant also caused lethal outcome, whereas inoculation of mice with *R. conorii* HK2 did not produce disease (Fig. 4A and B). Animals infected with  $1 \times 10^3$  PFU of wild-type *R. conorii* exhibited body weight loss (18.5%,  $P < 0.0001$ ) during the first 7 d of infection followed by a slow recovery for the next 7 d (returning to 96.5% of the original body weight). Animals infected with *R. conorii* HK2 or HK15 exhibited only modest weight losses (Fig. 4C). After 14 d, infection with wild-type *R. conorii* ( $\alpha$ -WT) elicited IgG antibody responses against rickettsial LPS that were not observed in animals infected with *R. conorii* HK2 or HK15 (Fig. 4D). However, preexisting antibodies recognizing OX2 and OX19 LPS in naive mice prevented further correlation between *R. conorii* infection and positive Weil-Felix serology (Fig. 4E). Importantly,  $\alpha$ -WT, but neither  $\alpha$ -HK2 nor  $\alpha$ -HK15, promoted complement-mediated killing of *R. conorii* in mouse plasma (Fig. 4F).

## Discussion

Our results demonstrate that the *R. conorii* *pso* locus is responsible for O-antigen biosynthesis, contributes to the pathogenesis, and is essential for the development of bactericidal Weil-Felix antibodies. In light of these findings, bactericidal Weil-Felix antibodies can be assigned a role in protective immunity, supporting earlier clinical observations associating increased Weil-Felix antibodies with survival and convalescence and bactericidal Weil-Felix antibodies with protective immunity in individuals receiving whole-cell Rocky Mountain spotted fever or epidemic typhus vaccines (27, 28). Although the *pso* locus is conserved among spotted fever- or typhus-causing *Rickettsia*, there exist variations in *pso* gene content among the 2 groups. We hypothesize that the genetic differences of the *pso* locus are responsible for differences in Weil-Felix serology as, for example, spotted fever agents, but not typhus agents, elicit antibodies that are cross-reactive with *P. vulgaris* OX2 (29). Nevertheless, similar to carbohydrate-specific bactericidal antibodies in established bacterial vaccines (5), the discovery of the *pso* locus may be exploited to generate O-antigen specific subunit vaccines against spotted fever and typhus agents whose bactericidal antibodies are likely to provide a correlate for protective immunity against the corresponding rickettsial diseases.

We show here that chloramphenicol is a suitable antibiotic to select for *R. conorii* variants with insertional *kkaebi* lesions. Even if the insertional lesion disrupts a gene that contributes to tissue cell invasion or intracellular replication, some of these mutants will exert partial phenotypes of delayed replication, diminished invasion, or reduced cell-to-cell spread that should allow their isolation and phenotypic characterization. Thus, a library of several thousand *R. conorii* mutants with mapped insertional lesions may be tremendously useful for the field of rickettsial biology in assigning function to the more than 800 genes that remain as of yet uncharacterized. Others have initiated similar insertional mutagenesis studies focusing on *Rickettsia parkeri*, *Rickettsia rickettsii*, and *R. prowazekii* (30–33). Together, the results from all of these studies should enable comparative genetic analyses of rickettsial species in the near future, which would be a tremendous boost for a field that for many years was hampered by the lack of tools for genetic studies.

We think it is likely that the insertional disruption of *Rc0457* in *R. conorii* HK2 abolishes the synthesis of the O-antigen repeats within LPS, allowing the mutant to synthesize a rudimentary lipid A core molecule that cannot be further modified. LPS is a major outer-membrane component essential for the growth of many gram-negative bacteria. The biosynthesis and transport of LPS are tightly controlled and coupled to the synthesis and assembly of other cell envelope components, such as peptidoglycan and S-layer proteins, to prevent loss of outer-membrane integrity (34, 35). We envision that the altered composition of the outer



**Fig. 4.** *R. conorii* O-antigen synthesis is required for pathogenesis. (A and B) Kaplan–Meier analysis for survival (A) and body-weight analysis (B) of C3H/HeN mice ( $n = 10$ ) infected with  $1 \times 10^6$  PFU *R. conorii* WT or the *pso* mutants HK2 and HK15 or mock infected (Mock). (C) Body-weight analysis of C3H/HeN mice ( $n = 10$ ) infected with  $1 \times 10^3$  PFU of *R. conorii* WT and *pso* mutant strains. Data are representative of 2 independent experiments. The proportion of survival animals was analyzed using the 2-tailed log-rank test. Two-way ANOVA with Bonferroni posttests were performed to analyze the statistical significance of body-weight change. (D) Immunoblotting of affinity-purified LPS from *R. conorii* WT, HK2, or HK15 with  $\alpha$ -WT,  $\alpha$ -HK2, or  $\alpha$ -HK15 mouse immune serum. (E) Immunoblotting of affinity-purified LPS from *P. vulgaris* OX2 or OX19 with  $\alpha$ -WT or naive mouse sera. (F) Survival of *R. conorii* in mouse plasma mixed with naive,  $\alpha$ -WT,  $\alpha$ -HK2, or  $\alpha$ -HK15 mouse immune serum ( $n = 3$ ). Data are the mean ( $\pm$ SEM) of 3 independent determinations. Statistically significant differences were analyzed with 1-way ANOVA with Dunnett's posttest. \* $P < 0.05$ ; \*\* $P < 0.001$ ; \*\*\* $P < 0.0001$ .

membrane, notably increased surface proteins rOmpA and rOmpB, contributes to the survival of the HK2 variant under high osmolarity pressure. It would be interesting to learn the detailed structure of *R. conorii* O antigen and to compare its structure with relevant O antigens from spotted fever- and typhus-*Rickettsia* as well as *P. vulgaris* and *V. cholerae*. Such analyses, combined with the study of antibodies that cross-react with specific LPS molecules, as is provided here, should resolve remaining questions on how certain bacteria elicit antibodies cross-reactive to rickettsial LPS, where these antibodies bind, and how they may be exploited for the design of immune therapeutics and vaccines against *Rickettsia*.

## Materials and Methods

Detailed information describing materials and methods is provided in *SI Appendix, Materials and Methods*.

***kkaebi* Transposon Mutagenesis.** The *kkaebi* minitransposon DNA was PCR amplified (PCRF, 5'-AAAGACAGCTGTCTTATACACATCTCAACCATCATC-3'; PCRR, 5'-GACAGCTGTCTTATACACATCTCAACCTGAAG-3'), digested with PshAI, purified, and incubated with Tn5 transposase (Lucigen; 10  $\mu$ g *kkaebi* mixed with 2 units Tn5) for 24 h at room temperature to generate transposome complexes. The transposome complexes were dialyzed against 250 mM sucrose prior to electroporation (3 kV $\cdot$ cm $^{-1}$ , 200  $\Omega$ , 25  $\mu$ F, 5.0 ms; Gene Pulser Xcell, Bio-Rad) into electrocompetent *R. conorii* prepared by washing 3 times in cold 250-mM sucrose. *kkaebi* variants were immediately recovered with DMEM supplemented with 5% HI-FBS and inoculated on 6-well tissue culture plates of confluent Vero cells. After 1 h infection at 34  $^{\circ}$ C in 5% CO $_2$  atmosphere, 6-well plates were overlaid with 0.5% agarose in DMEM supplemented with 5% HI-FBS. Infected cells were incubated at 34  $^{\circ}$ C, 5% CO $_2$  for an additional 5 h and treated with chloramphenicol (MP Bio-medicals) at a final concentration of 0.3  $\mu$ g $\cdot$ ml $^{-1}$  to select for *kkaebi* variants. Mutants were isolated via plaque formation after 4 to 15 d of incubation in Vero cell cultures. To expand mutant strains, isolated plaques were resuspended in 2 mL DMEM with 5% HI-FBS and inoculated on each well of 6-well plates of confluent Vero cells. After 1 h infection at 34  $^{\circ}$ C in a 5% CO $_2$  humidified chamber, medium was aspirated and replaced with fresh DMEM with 5% HI-FBS and 0.3  $\mu$ g $\cdot$ ml $^{-1}$  chloramphenicol. At 4 d postinfection, when monolayers of Vero cells were fully infected, Vero cells were mechanically disrupted with 3-mm glass beads, releasing intracellular *R. conorii*. After host

cell debris was removed by centrifugation (1,000  $\times$   $g$ , 4  $^{\circ}$ C, 5 min), the supernatant containing *R. conorii* was transferred to 225-cm $^2$  flasks of confluent Vero cells to expand at 34  $^{\circ}$ C, 5% CO $_2$  for 4 d. *Rickettsiae* were purified from Vero cells by differential centrifugation through 33% MD-76R solution (21,000  $\times$   $g$ , 4  $^{\circ}$ C, 20 min), washed in Sucrose-Phosphate-Glutamate (SPG) buffer (218 mM sucrose, 3.8 mM KH $_2$ PO $_4$ , 7.2 mM K $_2$ HPO $_4$ , 4.9 mM L-glutamate, pH 7.2), and suspended in 1 mL SPG buffer. A $_{600}$  was measured with bacterial samples diluted in SPG buffer.

**Nucleotide Sequence Analysis.** BLogo sequence analysis was conducted with type 2 logos and base representation calculated from the 9-bp nucleotide sequences flanking the *kkaebi* insertion sites (36). The background frequencies of A, C, G, and T used for the BLogo analysis were 0.35, 0.19, 0.17, and 0.29, respectively.

**Outer-Membrane Fractionation.** Outer-membrane fractionation was conducted based on a previously published protocol (37). The centrifugation sediments of *R. conorii* wild-type and *pso* variants or overnight cultures of *P. vulgaris* were suspended in 500  $\mu$ L of buffer A (200 mM Tris-HCl, 1 M sucrose, 1 mM EDTA, pH 8.0) and mixed with 100  $\mu$ L of lysozyme (5 mg $\cdot$ ml $^{-1}$  in dH $_2$ O; Sigma). After 5 min incubation at room temperature, 2 mL of dH $_2$ O was added and incubated for 20 min at room temperature. Then, 3 mL buffer B (50 mM Tris-HCl, 2% Triton X-100, 10 mM MgCl $_2$ , pH 8.0) and 50  $\mu$ L of DNase I (1 mg $\cdot$ ml $^{-1}$  in dH $_2$ O; Sigma) were added and incubated for 20 min at room temperature. The mixture was ultracentrifuged at 160,000  $\times$   $g$  for 60 min at 4  $^{\circ}$ C. The sediment was suspended in 500  $\mu$ L of buffer C (200 mM Tris-HCl, 2% SDS, 10 mM EDTA, pH 8.8) and used for subsequent analyses.

**Affinity Purification of Lipopolysaccharide.** LPS was affinity purified using polymyxin B-agarose (38). Specifically, a fractionated outer-membrane sample was dialyzed (2-kDa molecular weight cutoff; Thermo Scientific) twice against 4 L dH $_2$ O at room temperature. This solution was brought to 50 mM Tris-HCl, pH 7.5, mixed with 20  $\mu$ L of proteinase K (10 mg $\cdot$ ml $^{-1}$  in dH $_2$ O; Sigma), and incubated at 55  $^{\circ}$ C for 5 h. The crude polysaccharide solution was dialyzed (2-kDa molecular weight cutoff; Thermo Scientific) against 4 L dH $_2$ O at 4  $^{\circ}$ C overnight and brought to 100 mM NH $_4$ HCO $_3$ , pH 8.0, 0.9% NaCl. The crude polysaccharide sample was applied to a 2-mL polymyxin B-agarose (Sigma) column and incubated at 4  $^{\circ}$ C for 16 h, followed by washing with 10 mL of wash buffer (100 mM NH $_4$ HCO $_3$ , pH 8.0). LPS was eluted from the column with 10 mL of elution buffer (1% deoxycholic acid in

100 mM  $\text{NH}_4\text{HCO}_3$ , pH 8.0) and extensively dialyzed against 4 L of deoxycholic acid removal buffer (4 mM Tris-HCl, pH 8.8, 0.25% NaCl, 10% EtOH), followed by dialysis against  $\text{dH}_2\text{O}$ . LPS samples were concentrated using a Speed-Vac and stored at 4 °C.

**SDS-PAGE and Immunoblotting.** Samples were mixed with sample buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue, pH 6.8) and boiled at 95 °C for 10 min. Samples were separated on 15% SDS-PAGE gels and stained with Coomassie Brilliant Blue R-250 for detecting outer-membrane proteins (Amsbio). A silver-staining kit was used for detection of LPS (Bio-Rad). For immunoblot analyses, samples were electrophoretically transferred from the gel onto a 0.22- $\mu\text{m}$  PVDF membrane (GE Healthcare). The membrane was immersed in blocking buffer (TBS-T [100 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.1% Tween-20] with 5% milk) for 1 h at room temperature. The membrane was washed and incubated in a solution containing primary antibodies or antisera for 1 h at room temperature. rOmpA-specific mouse monoclonal antibody 13-3 (1:5,000 dilution), rabbit polyclonal antipeptide antibody c240 targeting the transmembrane domain of rOmpA (1:2,000 dilution), or rabbit polyclonal antibody recognizing rOmpB (1:1,000 dilution) was used as previously described (generously provided by Ted Hackstadt, Rocky Mountain Laboratory, Hamilton, MT) (39, 40). Rabbit antisera specific to LPS purified from wild-type *R. conorii*, HK2, and HK15 variants were used at a 1:10,000 (against *R. conorii*) or 1:1,000 (against *P. vulgaris*) dilution in TBS-T. Mouse hyperimmune sera collected from *R. conorii*-infected mice were used at a 1:10,000 (against *R. conorii*) or 1:625 (against *P. vulgaris*) dilution in TBS-T. Human antisera collected from patients with confirmed diagnoses of *R. conorii* or *O. tsutsugamushi* infections were used at a 1:10,000 dilution in TBS-T (kindly provided by Ranjan Premaratna, University of Kelaniya) (41). Of note, consents to store and use human antisera for research and diagnosis purposes

were obtained at the time of sample collection. This study was approved by the ethics review committee, Faculty of Medicine, University of Kelaniya (IRB reference no. P/106/04/2018). The membrane was washed 3 times and incubated with peroxidase-conjugated secondary antibodies (anti-mouse IgG and anti-rabbit IgG [Cell Signaling] and anti-human IgG [Abcam]) at a 1:10,000 dilution in TBS-T for 1 h at room temperature. After a final wash, the membrane was developed using SuperSignal West Pico PLUS (Thermo Scientific) and exposed to Amersham Hyperfilm ECL (GE Healthcare).

***R. conorii* Survival in Mouse Plasma.** Whole blood was collected by cardiac puncture of C3H/HeN mice (Charles River Laboratories) and anticoagulated with 10  $\mu\text{g}\cdot\text{mL}^{-1}$  desirudin (Marathon Pharmaceuticals). Plasma was generated by centrifugation of desirudin-treated blood (1,000  $\times$  g for 5 min at 4 °C, followed by 10,000  $\times$  g for 3 min at 4 °C) for removal of blood cells. The hyperimmune sera samples were heat inactivated at 56 °C for 30 min, followed by incubation on ice for 5 min. Aliquots (50  $\mu\text{L}$ ) of  $5 \times 10^6$  PFU *R. conorii* were opsonized with 50  $\mu\text{L}$  of hyperimmune sera on ice for 10 min and mixed with freshly prepared or heat-inactivated plasma (200  $\mu\text{L}$ ). The infected samples were incubated at 37 °C with rotation for 60 min, at which time all plasma samples were incubated on ice and brought to 1-mL volume with ice-cold DMEM with 5% HI-FBS. Infectious *R. conorii* titers were determined by plaque assay. *R. conorii* survival was calculated as the percentage of the average *R. conorii* initial inoculum at 60 min.

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