Nonselective Persistence of a *Rickettsia conorii* Extrachromosomal Plasmid during Mammalian Infection

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Scientific analysis of the genus *Rickettsia* is undergoing a rapid period of change with the emergence of viable genetic tools. The development of these tools for the mutagenesis of pathogenic bacteria will permit forward genetic analysis of *Rickettsia* pathogenesis. Despite these advances, uncertainty still remains regarding the use of plasmids to study these bacteria in development of these tools for the mutagenesis of pathogenic bacteria will permit forward genetic analysis of *Rickettsia* models of infection, namely, the potential for virulence changes associated with the presence of extrachromosomal DNA and nonselective persistence of plasmids in mammalian models of infection. Here, we describe the transformation of *Rickettsia conorii* Malish 7 with the plasmid pRam18dRGA[AmTrCh]. Transformed *R. conorii* stably maintains this plasmid in infected cell cultures, expresses the encoded fluorescent proteins, and exhibits growth kinetics in cell culture similar to those of nontransformed *R. conorii*. Using a well-established murine model of fatal Mediterranean spotted fever, we demonstrate that *R. conorii*[pRam18dRGA[AmTrCh]] elicits the same fatal outcomes in animals as its untransformed counterpart and, importantly, maintains the plasmid throughout infection in the absence of selective antibiotic pressure. Interestingly, plasmid-transformed *R. conorii* was readily observed both in endothelial cells and within circulating leukocytes. Together, our data demonstrate that the presence of an extrachromosomal DNA element in a pathogenic rickettsial species does not affect either *in vivo* infectivity in models of disease and that plasmids such as pRam18dRGA[AmTrCh] are valuable tools for the further genetic manipulation of *pathogenic rickettsiae*.

*Rickettsia conorii* is a pathogenic member of the spotted fever group (SFG) of the genus *Rickettsia*. Multiple tick-borne SFG *Rickettsia* species can cause a wide range of human and zoonotic diseases, with untreated cases of Rocky Mountain spotted fever (*R. rickettsii*) disease resulting in 20 to 25% mortality rates (1–3). A similar disease in Eurasia, Mediterranean spotted fever (MSF), is caused by *R. conorii* and results in 2 to 32% mortality rates in reported human cases (4). Symptoms of these diseases are manifested 2 to 14 days following arthropod transmission and are the result of widespread bacterial infection of the endothelium with concurrent inflammation (5, 6). While broad-spectrum antibiotic treatment can significantly reduce morbidity and mortality, untreated infections can proceed to more severe disease manifestations, including pulmonary edema, interstitial pneumonia, and multiorgan failure (7).

Historically, analysis of the genetic determinants of SFG *Rickettsia* disease has been hampered by a lack of viable tools for the genetic manipulation of these bacteria. This genetic recalcitrance was largely a consequence of a lack of both an axenic growth medium and selectable markers. However, the development of tools for chromosomal mutagenesis and selectively maintained extrachromosomal DNA in rickettsial species has been demonstrated (8). Plasmids isolated from nonpathogenic *Rickettsia* bacteria have been adapted for replication and selection in both *Rickettsia* and *Escherichia* species (9). These plasmids can potentially be useful for heterologous gene expression, reporter gene expression, and in trans complementation in diverse *Rickettsia* species.

One such plasmid, pRam18dRGA[AmTrCh], is derived from the 18-kbp plasmid from “Candidatus Rickettsia amylovorum” (10, 11). The nonessential portion of the naturally occurring plasmid was removed, and the remaining portion was inserted into an *Escherichia coli* vector. The *gfp*uv and *rpaar2* genes and a multiple cloning site (MCS) were introduced to produce the plasmid pRam18dRGA[MCS] (9, 12, 13). The 948-bp AmTrCh cassette encoding mCherry was subsequently added to create pRam18dRGA[AmTrCh] (9, 14). This plasmid has been successfully transformed into five pathogenic and nonpathogenic *Rickettsia* species (9, 15).

Here, we utilized this plasmid to transform pathogenic *R. conorii* to examine the *in vivo* persistence of an extrachromosomal DNA element in the absence of antibiotic selection and to determine whether the presence of the plasmid affects the virulence of transformed *R. conorii* Malish 7 in a murine model of MSF. These analyses subsequently yielded the identification of new host cell types that are parasitized by *R. conorii in vivo*.

MATERIALS AND METHODS

Bacterial transformation. *R. conorii* strain Malish 7 was electroporated with pRam18dRGA[AmTrCh] in a manner similar to that of Rachek et al. (16). Briefly, approximately 4 × 10⁸ *R. conorii* bacteria were purified by sucrose density centrifugation (17). We made the bacteria electrocompetent by washing them four times with ice-cold 250 mM sucrose. A 10-μl volume of 1 mg/ml pRam18dRGA[AmTrCh] was added to 4 × 10⁷ electro-

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trocoperative R. conorii cells in an electroporation cuvette with a 0.1-cm gap. After 30 min of incubation on ice, the sample was electroporated at 1,700 V, 150 Ω, and 50 μF with a resulting time constant of approximately 6.5 ms. This mixture was immediately transferred to 1 ml of 8 × 10^8 Vero cells in Hanks’ balanced salt solution. After 1 h of incubation at 34°C and 5% CO₂ with rotation, the mixture was transferred to a 75-cm flask containing 15 ml of Dulbecco’s modified Eagle’s medium (DMEM) with 10% bovine serum albumin and 1% nonessential amino acids. After 24 h of incubation at 34°C and 5% CO₂, the medium was changed to complete DMEM with 200 ng/ml rifampin. The medium was replaced every 3 days until bacillary growth was observed by Diff-Quik staining.

**Sorting and recovery of transformants.** Single-bacterial-cell sorting was performed as described in reference 18. Briefly, the peridinin chlorophyll protein (PerCP)/Cy5.5 fluorochrome was conjugated to the anti-R. conorii antibody RC9PA (19) with the Lightning-Link PerCP/Cy5.5 conjugation kit (Innova Biosciences). R. conorii (pRam18dRGA[AmTrCh]) was stained with anti-R. conorii antibody RC9PA-PerCP/Cy5.5 at 10 μg/ml. Labeled R. conorii was applied to a FACSJazz cell sorter with forward scatter (FSC) threshold/trigger, side scatter (SSC) versus FSC graph, and SSC versus log 488-nm excitation 692/30-nm emission graph. Lysed Vero cells and nontransformed R. conorii were used as controls to set negative and positive sort gates. pRam18dRGA[AmTrCh]-transformed R. conorii was applied to the sorter and sorted with 1 sort event into 32 wells, 5 sort events into 32 wells, and 50 sort events into 32 wells of a 96-well plate containing Vero cells in complete DMEM containing 200 ng/ml rifampin. After 5 days of culture, the infected Vero cells were lifted with trypsin-EDTA and expanded into three identical 96-well plates containing Vero cells in complete DMEM with 200 ng/ml rifampin. Flow cytometric analyses and cell sorting of pathogenic R. conorii were performed in a CDC-approved biosafety level 3 (BSL3) laboratory in the Department of Pathobiological Sciences at the Louisiana State University (LSU) School of Veterinary Medicine (SVM) by utilizing protocols approved by the LSU Institutional Biological and Recombinant DNA Safety Committee (IBRDSC).

At 10 total days of growth after sorting, one of the three identical plates was fixed with 4% paraformaldehyde. This plate was stained with the anti-R. conorii antibody RC9PA (19) and an anti-rabbit–Alexa Fluor 546 antibody and observed under ×10 magnification in an Olympus IX71 inverted microscope for the presence of R. conorii (RC9PA) and GFPuv-mediated fluorescence. Subsequently, DNA was extracted as described below from wells containing R. conorii and demonstrating GFPuv-mediated fluorescence. Whole DNA was extracted with the PureLink Genomic DNA kit (Life Technologies) according to the manufacturer’s instructions. The extracted DNA was subjected to PCR with gpFuv_F and gpFuv_R (see Table S1 in the supplemental material), followed by agarose gel electrophoresis to ensure the presence of pRam18dRGA[AmTrCh]. The clone utilized in this study came from a single-event sort into 1 well of a 96-well plate.

**Immunofluorescence microscopy.** R. conorii and R. conorii (pRam18dRGA[AmTrCh]) were cultured in Vero cells. Both types of bacteria were purified by sucrose density centrifugation (17) and mounted onto a single glass slide with a Cytospin Dual sample chamber with a Cytospin centrifuge. The bacteria were fixed with 4% paraformaldehyde and stained with RC9PA with anti-rabbit–Alexa Fluor 350. All microscopy was captured with a Leica DM400B equipped with a 100×/0.4 objective, an EvolutionOE camera, with ImagePro Plus software, and green fluorescent protein (GFP) (excitation wavelength, 488/40 nm; emission wavelength, 527/30 nm), tetramethyl rhodamine isocyanate (TRITC) (excitation wavelength, 515 to 560 nm; emission wavelength, LP590 nm), and 4′,6-diamidino-2-phenylindole (DAPI) (excitation wavelength, 360/40 nm; emission wavelength, 470/40 nm) filters. Analysis of fluorescence intensity was performed with the RGB profile plot plugin within the NIH ImageJ freeware package (http://rsb.info.nih.gov/ij/).

**Flow cytometric analysis.** Sucrose density gradient-purified bacteria were analyzed on a BD FACSJazz equipped with aerosol containment and placed within a Baker class IIA biosafety cabinet within the BSL3 laboratory suite at the LSU SVM. R. conorii (pRam18dRGA[AmTrCh]) was stained with anti-R. conorii antibody RC9PA-PerCP/Cy5.5 at 5 μg/ml. All cytometric events were initially gated for FSC and SSC to eliminate large events. PerCP/Cy5.5-mediated fluorescence was measured at 488-nm excitation and 692/40-nm emission wavelengths. GFPuv-mediated fluorescence was measured at 488-nm excitation and 530/40-nm emission wavelengths. This fluorescence combination did not produce cross-channel background fluorescence, so compensation was not required. Each heat map represents at least 50,000 FSC/SSC gated events.

**Growth in culture.** R. conorii and R. conorii (pRam18dRGA[AmTrCh]) were used to infect Vero cells at a multiplicity of infection (MOI) of 1 bacterium/host cell. Bacteria were allowed to grow at 34°C and 5% CO₂ for a total of 5 days in DMEM–10% FBS supplemented with 200 ng/ml rifampin for the transformed bacteria. At days 1, 3, 5, 7, and 9 postinfection, the infected Vero cells were scraped from the culture plate and DNA was extracted as described above for host, R. conorii, and plasmid quantification.

**R. conorii quantification.** The chromosomal ratio of mouse or Vero (actin) to R. conorii (sc1) DNA was queried by quantitative PCR (qPCR) with TaqMan master mix with a 5-min incubation at 95°C, followed by 55 cycles of 95°C for 15 s, 53°C for 15 s, and 68°C for 30 s. Mouse actin was amplified with actin_Ms_F, actin_Ms_R, and actin_Ms_Max(Vic). Vero actin was amplified with actin_Vero_F, actin_Vero_R, and actin_VHex(Vic). R. conorii sc1 was amplified with sca1_Rc_F, sca1_Rc_R, and sca1_Rc/Rf_Fam (see Table S1 in the supplemental material). All unknowns were quantified by ΔΔCt compared to molar standards.

**Plasmid quantification.** DNA from the above-described growth curve was used to quantify the presence of the pRam18dRGA[AmTrCh] plasmid during growth in culture. The ratio of plasmid (gpFuv) to R. conorii (sc1) was determined by qPCR with SYBR master mix (Roche) by preincubation at 95°C for 5 min, followed by 50 cycles of 95°C for 15 s, 55°C for 15 s, and 68°C for 30 s. Specificity of amplification was validated by melting curve analysis. gpFuv was amplified with gpFuv_F and gpFuv_R. R. conorii sc1 was amplified with sca1_Rc_F and sca1_Rc_R (see Table S1 in the supplemental material).

**Animal infection.** Five- to 7-week-old male C3H/HeN mice were inoculated by retro-orbital injection with 6 × 10^6 (1.5 50% lethal doses [LD₅₀]) of R. conorii (n = 5) or R. conorii (pRam18dRGA[AmTrCh]) (n = 11) or phosphate-buffered saline (PBS) (n = 5) (19). Infected mice were monitored twice daily for signs of disease and daily for weight change. Animals that exhibited symptoms of severe disease that were consistent with not recovering from the infection were removed from the study and scored as succumbing to the infection as previously described (19). These symptoms included ruffled fur, hunched posture, shallow respiration, immobility when touched, and loss of at least 15% of the initial body weight. Animal experiments were conducted in accordance with protocols approved by the IBRDSC and Institutional Animal Care and Use Committee (IACUC) at the LSU SVM. Two predesignated mice were sacrificed at 1, 3, and 5 days postinfection. Spleens, kidneys, livers, hearts, lungs, brains, and blood were aseptically extracted to determine bacterial loads and plasmid copy numbers and assess pathological lesions. Each organ was split into two sections for use in PCR analyses as described above and pathological examination as described below.

**Indirect IHC analysis.** Murine tissues, including those from the lung, liver, and spleen, were collected immediately after euthanasia and fixed in 10% buffered formalin (1:10 tissue-to-formalin ratio). Samples were routinely processed and embedded in paraffin, and 5-μm sections were cut for staining with hematoxylin and eosin (H&E). To localize R. conorii within the infected animals, isolated tissues were additionally subjected to immunohistochemical (IHC) examination with an anti-RC9PA antibody that recognizes several SFG rickettsial species (19) or with an anti-IBa1 macrophage-specific antibody. Primary antibody staining was followed by a biotinylated anti-rabbit IgG secondary antibody (Vector BA 1000 at 1:1,000; Vector Laboratories) and exposure to the detection reagent ( Vect-
A

anti-RcPFA  GFPuv  mCherry  Merge

B

R. conorii

fluorescence intensity

R. conorii

mCherry

FIG 1 In vitro fluorescent properties of R. conorii(pRam18dRGA[AmTrCh]). (A) R. conorii and R. conorii(pRam18dRGA[AmTrCh]) were purified and stained with the anti-Rickettsia antibody RcPFA and a corresponding anti-rabbit–Alexa Fluor 350 antibody (blue). Surface staining of the bacteria is apparent in both anti-RcPFA panels, but only R. conorii(pRam18dRGA[AmTrCh]) demonstrates GFPuv (green)– and mCherry (red)-mediated fluorescence. (B) Unstained R. conorii(pRam18dRGA[AmTrCh]) was analyzed for GFPuv- and mCherry-mediated fluorescence. Analysis of fluorescence intensity along the magenta line indicates that individual bacteria elaborate both GFPuv- and mCherry-mediated fluorescence, as demonstrated by overlapping peaks. However, the mCherry fluorescence is not always above the limit of detection.

Statistics. Bacterial growth in culture was assessed by nonlinear-fit analysis of the slope and intercept. Plasmid contents in culture and in mice were analyzed by one-way analysis of variance. Differences in survival between R. conorii and R. conorii(pRam18dRGA[AmTrCh]) were analyzed by comparison of survival curves via log rank (Mantel-Cox) test in five mice per experimental group. All statistical analyses were performed with the tests indicated within the Prism software package (GraphPad Software Inc.).

RESULTS

Transformation and isolation of pRam18dRGA[AmTrCh]-transformed R. conorii in cell culture. Previous reports had demonstrated the feasibility of using plasmids, including pRam18dRGA[AmTrCh], to transform both recognized human-pathogenic and nonpathogenic members of the genus Rickettsia (9-12, 15). Although successful, these strategies have technical limitations when used to obtain clonal transformed populations. Use of either limiting dilution or plaque assays to isolate Rickettsia clones are time and labor intensive, and repeated passage of these bacteria outside a relevant animal model can potentially contribute to attenuation (20). We sought to determine whether transformation of R. conorii is achievable with this plasmid and whether high-throughput fluorescence-activated cell sorting (FACS) would be useful in isolating clonal transformed R. conorii populations.

The plasmid pRam18dRGA[AmTrCh] (9) was introduced into purified R. conorii Malish 7 by electroporation, and transformed bacteria were subsequently subjected to rifampin selection for 10 days. After outgrowth of Rickettsia bacilli was observed via Diff-Quik staining and microscopic analysis, the infected host cells were lysed by needle passage and unbroken cells were removed with a 2-μm filter. The liberated bacteria were subsequently labeled with a PerCP/Cy5.5-conjugated anti-R. conorii antibody (RcPFA) (19) and subjected to flow cytometric analysis. Rifampin-resistant, fluorescently labeled R. conorii bacilli were individually sorted by extremely stringent single-event sorting with a FACSJazz cell sorter (18). After outgrowth from the single parent bacterium, clonal populations were screened for the presence of GFPuv-positive R. conorii by PCR with primers specific for the gp54 gene on the pRam18dRGA[AmTrCh] plasmid (data not shown). A single R. conorii(pRam18dRGA[AmTrCh]) clone was propagated and purified by sucrose density centrifugation for further analysis.

The pRam18dRGA[AmTrCh] plasmid encodes two fluorescent proteins, GFPuv and mCherry. mCherry expression is driven by the Anaplasma marginale tr promoter, and the encoded protein fluoresces into the TRITC filter set (21, 22). gp54 expression is driven by the R. prowazekii ompA promoter, and GFPuv fluoresces adequately into the FITC filter set (13). Accordingly, we hypothesized that transformed R. conorii(pRam18dRGA[AmTrCh]) should readily fluoresce into the FITC (GFPuv) and TRITC (mCherry) channels as has been previously observed in other Rickettsia species (9, 15). As shown in Fig. 1A, pRam18dRGA[AmTrCh]-transformed R. conorii bacteria labeled with the RcPFA antibody (19) are observable by fluorescence microscopy under TRITC and FITC filter sets, while untransformed bacteria are not visible. Analysis of the fluorescence signal intensity across a portion of an R. conorii(pRam18dRGA[AmTrCh]) plasmid micrograph indicates that individual bacteria elaborate both GFPuv and mCherry signals (Fig. 1B).

Flow cytometric analysis of GFPuv fluorescence. Since GFPuv can be excited under a 488-nm laser and can emit into common...
filter sets (23), we hypothesized that GFPuv-mediated fluorescence should be observable by flow cytometry in R. conorii (pRam18dRGA[AmTrCh]). First, we conjugated the anti-R. conorii antibody RcPFA with the PerCP/Cy5.5 tandem fluorophore. We used RcPFA-PerCP/Cy5.5 to identify R. conorii when excited at 488 nm and detected with a 692/40-nm emission filter. Indeed, purified R. conorii are readily detectible by flow cytometry with RcPFA-PerCP/Cy5.5 (Fig. 2A versus B). Additionally, GFPuv-mediated fluorescence from R. conorii (pRam18dRGA[AmTrCh]) can be detected with FITC filter sets (488-nm excitation and 530/40-nm emission) (Fig. 2A versus C). This is a novel way to identify Rickettsia-specific events and to identify bacteria transformed to GFPuv fluorescence (Fig. 2D).

In vitro growth of transformed R. conorii in cell culture. We next sought to determine if the presence of an extrachromosomal DNA element in R. conorii (pRam18dRGA[AmTrCh]) has a deleterious effect on fitness in cell culture. R. conorii and R. conorii (pRam18dRGA[AmTrCh]) were inoculated into Vero cell cultures at an MOI of 1. Samples were removed from these cultures 1, 3, 5, 7, and 9 days postinoculation, and genomic DNA was extracted from both the host and the bacteria. qPCR analysis of the ratio of R. conorii (sca1) to Vero (actin) DNA contents indicates that transformed and nontransformed bacteria proliferate similarly over 9 days in Vero cell culture (Fig. 3).

We then determined whether the plasmid was stably maintained in vitro without selective antibiotic pressure. The copy number of pRam18dRGA[AmTrCh] was determined by qPCR as the ratio of plasmid (gfpuv) to R. conorii chromosomal (sca1) DNA. The pRam18dRGA[AmTrCh] copy number over the time course of culture indicates that the plasmid is maintained without antibiotic selection (see Table 2). The plasmid copy numbers determined over time are similar to those documented in other transformed Rickettsia species (9, 11, 15).

R. conorii (pRam18dRGA[AmTrCh]) experimental mammalian infection. An important question pertaining to the future of
rickettsial genetics is the persistence of extrachromosomal DNA in animal models of infection when lacking selective pressure. Indeed, pRam18dRGA can be maintained in *R. monacensis* for five passages in tissue culture without antibiotic selection (9), but this phenotype has not been investigated in an *in vivo* model. Additionally, future uses of extrachromosomal DNA will be dependent on establishing that the existence of the plasmid does not, in itself, modulate the infectivity of the bacterium. To address these questions, we utilized a well-established murine model of fatal MSF to examine the pathogenesis of *R. conorii* (pRam18dRGA[AmTrCh]) (19, 24). Susceptible C3H/HeN mice were intravenously infected with 4 × 10^6 infectious units of *R. conorii* or *R. conorii* (pRam18dRGA[AmTrCh]). This dose is 1.5 LD_{50} for nontransformed *R. conorii* (19). Mice were monitored for signs of disease, including: ruffled fur, hunched posture, shallow respiration, immobility, and weight loss. *R. conorii* and *R. conorii* (pRam18dRGA[AmTrCh])-infected mice demonstrated progressive morbidity, as demonstrated by persistent weight loss (Fig. 4A). As demonstrated in Fig. 4B, infected mice succumbed to infection during the normal course of disease with death at days 4 to 5.5 postinfection with a slightly later time of death than the wild type. Nevertheless, this finding establishes that *R. conorii* (pRam18dRGA[AmTrCh]) retains infectivity in a mouse model of infection.

To confirm that *R. conorii* (pRam18dRGA[AmTrCh])-infected mice experienced the normal disease progression, we quantified the bacterial burdens in major organs. Bacterial DNA could be detected and quantified in the mouse spleen and liver after 1 day of infection (Fig. 5A). The total bacterial burden increased after 3 days of infection (Fig. 5B), and this ultimately resulted in rickettsial organ tropism and quantity similar to those previously observed in fatal *R. conorii* infection (Fig. 5C)(19). Most importantly, pRam18dRGA[AmTrCh] was readily detected at all of the time points tested and the plasmid quantity did not decrease over the course of infection (Fig. 5D). Together, these data demonstrate that the pRam18dRGA[AmTrCh] extrachromosomal DNA does not affect *R. conorii* pathogenesis and that the plasmid is stably maintained by *R. conorii* in an animal model of infection.

Pathological examination of animals after 5 days of infection demonstrated *R. conorii* (pRam18dRGA[AmTrCh]) infection of endothelial cells in the heart and lungs (see Fig. S1 in the supplemental material), as has been previously reported (24). The mouse liver additionally demonstrated multifocal to coalescing coagulative hepatic necrosis characterized by the presence of numerous

**FIG 5** *R. conorii* (pRam18dRGA[AmTrCh]) proliferates within infected organs and maintains the extrachromosomal plasmid. *R. conorii* (pRam18dRGA[AmTrCh]) dissemination into mouse organs after 1 (A), 3 (B), or 5 (C) days of infection is shown. Chromosomal equivalents of *R. conorii* *sca1* and mouse actin were determined by qPCR. (D) Nonselective persistence of pRam18dRGA[AmTrCh] throughout the mammalian infection as determined by the qPCR ratio of chromosomal *R. conorii* *sca1* and the plasmid gene *gfpuv*. The change in plasmid copy number is not significant.
We next sought to elucidate the different cell types present within the mammalian bloodstream that are infected by *R. conorii* by determining the locations of GFPuv-positive *R. conorii* within the *ex vivo* white blood cell preparation. GFPuv-positive bacilli were located by fluorescence microscopy without observing the location of the bacteria relative to host cells. After detection of the bacteria, images of the host cells in that microscopic field were acquired. A board-certified veterinary clinical pathologist identified the types of leukocytes in the microscopic field without knowledge of the location of the bacteria. Only after identification of host cells were the locations of the bacteria assigned. Of the 207 GFP-positive *R. conorii* bacteria that could be assigned to a host location, 110 were present in lymphocytes, 67 were in monocytes, 14 were in granulocytic cells, and 16 were not associated with any host cell (see Table 2). Thus, these data suggest that in addition to endothelial cells, cells of nonendothelial origin support *R. conorii* parasitism during fatal mammalian infection.

**DISCUSSION**

Here, we have demonstrated the ability of *R. conorii* to be transformed with the plasmid pRam18dRGA[AmTrCh] (9). *R. conorii* stably maintains this plasmid in culture with no apparent effect on fitness but with the addition of GFPuv- and mCherry-mediated fluorescence phenotypes (Fig. 1 to 3; Table 1). By utilizing a well-characterized mouse model of fatal MSF, we were able to demonstrate that *R. conorii*(pRam18dRGA[AmTrCh]) retains infectivity in a mouse model of fatal infection (Fig. 4). A fatal *R. conorii*(pRam18dRGA[AmTrCh]) challenge results in disseminated infection and, importantly, retention of the extrachromosomal DNA (Fig. 5). Microscopic examination of *R. conorii* (pRam18dRGA[AmTrCh]) within the mouse demonstrated the well-characterized endothelial-cell-targeted infection (see Fig. S1 in the supplemental material) but also within several different cell types in infected target tissues and mammalian host (see Fig. S1 in the supplemental material) but also within several different cell types in infected target tissues and mammalian host.

**TABLE 1** Plasmid copy number during growth in Vero cells

<table>
<thead>
<tr>
<th>Culture time (days)</th>
<th>Avg plasmid copy no. ± SD&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>3</td>
<td>3.09 ± 0.52</td>
</tr>
<tr>
<td>5</td>
<td>2.82 ± 2.03</td>
</tr>
<tr>
<td>7</td>
<td>0.52 ± 0.30</td>
</tr>
<tr>
<td>9</td>
<td>0.55 ± 0.14</td>
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<sup>a</sup>Changes in copy number are not significant.
in the supplemental material), with newly identified infection of mouse macrophages within the liver and infection of nonendothelial cells within the microvascular circulation (Fig. 6). Further microscopic examination showed the presence of GFPuv-positive bacilli within leukocytes (Fig. 7), and pathological examination was used to identify the nature of this host infection (Table 2).

Taken together, these data indicate that the use of pRam18dRGA plasmids will add depth to our analysis of mammalian models of Rickettsia infection. The GFPuv-mediated fluorescence phenotype will permit a more complete analysis of the nature of the pathogenic process and allow us to identify host cells parasitized by pathogenic Rickettsia species.

In vivo maintenance of pRam18dRGA plasmids (Fig. 5 and 7) and the adaptable nature of the pRam18dRGA[MCS] plasmid allow additional ways to examine Rickettsia pathogenesis. The pRam18dRGA[MCS] plasmid contains a multiple cloning site for the insertion of any DNA (9). This design feature makes this plasmid a true shuttle vector, because the additional DNA will be maintained within the bacteria during in vivo Rickettsia infection. First and foremost among potential uses of shuttle vectors is proper and complete examination of gene deletion mutants, because the adaptable nature of the pRam18dRGA [MCS] plasmid will permit a more complete analysis of the nature of the pathogenic process and allow us to identify host cells parasitized by pathogenic Rickettsia species.

In conclusion, we have utilized R. conorii(pRam18dRGA [AmTrCh]) to demonstrate the value of plasmids in the analysis of in vivo Rickettsia pathogenesis. The techniques described here have promoted our identification of novel host cell types parasitized by pathogenic R. conorii in a fatal model of MSF. Therefore, the use of pRam18dRGA[AmTrCh] and related plasmids will strongly influence and enhance the course of future rickettsial research.

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