Examination of Rickettsial Host Range for Shuttle Vectors Based on dnaA and parA Genes from the pRM Plasmid of Rickettsia monacensis

Nicole Y. Burkhardt,* Lisa D. Price,* Xin-Ru Wang,* Chan C. Heu,* Gerald D. Baldridge,* Ulrike G. Munderloh,* Timothy J. Kurtti*

ABSTRACT The genus Rickettsia encompasses a diverse group of obligate intracellular bacteria that are highly virulent disease agents of mankind as well as symbionts of arthropods. Native plasmids of Rickettsia amblyommatis (AaR/SC) have been used as models to construct shuttle vectors for genetic manipulation of several Rickettsia species. Here, we report on the isolation of the complete plasmid (pRM658B) from Rickettsia monacensis IrR/Munich mutant Rmona658B and the construction of shuttle vectors based on parA and dnaA. To identify regions essential for replication, we made vectors containing the dnaA and parA genes of pRM with various portions of the region surrounding these genes and a selection reporter cassette conferring resistance to spectinomycin and expression of green fluorescent protein. Rickettsia amblyommatis (AaR/SC), R. monacensis (IrR/Munich), Rickettsia bellii (RML 369-C), Rickettsia parkeri (Tate’s Hell), and Rickettsia montanensis (MS/6) were successfully transformed with shuttle vectors containing pRM parA and dnaA. PCR assays targeting pRM regions not included in the vectors revealed that native pRM was retained in R. monacensis transformants. Determination of native pRM copy number using a plasmid-carried gene (RM_p5) in comparison to chromosomally carried gltA indicated reduced copy numbers in R. monacensis transformants. In transformed R. monacensis strains, native pRM and shuttle vectors with homologous parA and dnaA formed native plasmid-shuttle vector complexes. These studies provide insight on the maintenance of plasmids and shuttle vectors in rickettsiae.

IMPORTANCE Rickettsia spp. are found in a diverse array of organisms, from ticks, mites, and fleas to leeches and insects. Many are not pathogenic, but others, such as Rickettsia rickettsii and Rickettsia prowazekii, can cause severe illness or death. Plasmids are found in a large percentage of nonpathogenic rickettsiae, but not in species that cause severe disease. Studying these plasmids can reveal their role in the biology of these bacteria, as well as the molecular mechanism whereby they are maintained and replicate in rickettsiae. Here, we describe a new series of shuttle plasmids for the transformation of rickettsiae based on parA and dnaA sequences of plasmid pRM from Rickettsia monacensis. These shuttle vectors support transformation of diverse rickettsiae, including the native host of pRM, and are useful for investigating genetic determinants that govern rickettsial virulence or their ability to function as symbionts.

KEYWORDS Rickettsia plasmids, Rickettsia monacensis, Rickettsia amblyommatis, shuttle plasmids, transformation, host range, Rickettsia, plasmids, shuttle vector
Some *Rickettsia* spp. are unique among the *Rickettsiales* in harboring plasmids (1). More than 30 plasmids have been identified in a wide range of *Rickettsia* species and strains (2–6). The diversity of *parA* on rickettsial plasmids suggests that foreign plasmids have invaded rickettsiae (7) via a conjugation system (8, 9). As many as 4 distinct plasmids, each with a distinct partitioning system, can coexist in a given rickettsial strain (2, 10). Plasmids are more commonly associated with avirulent and less virulent rickettsiae and are absent in highly virulent strains (see Table S1 in the supplemental material). Rickettsial plasmids have undergone reductive evolution, and to date, virulence has not been linked to the presence of plasmids (5, 11). These features indicate that shuttle vectors modeled after rickettsial plasmids can be developed as tools to introduce foreign genes into rickettsiae, as we have demonstrated previously (12).

The discovery of low-copy-number plasmids in many *Rickettsia* spp. (1, 2, 13) spurred development of the first shuttle vectors, a fundamental advance in rickettsial transformation technologies (12, 14). Their potential has been realized by generation of transformant rickettsiae expressing selectable markers and fluorescent proteins that have enabled study of rickettsial interactions with host cells in unprecedented detail. Examples with arthropod endosymbionts include *R. tamurae* subsp. *buchneri* and *Rickettsia peacockii* in tick host cells (15), as well as *Rickettsia bellii* expressing a plasmid-encoded heterologous *rickA* gene (16). Although the typhus group rickettsiae are among those *Rickettsia* spp. that do not harbor a native plasmid, shuttle vectors have also been used to transform and study typhus group rickettsiae, including the epidemic typhus agent, *Rickettsia prowazekii*, in L929 murine fibroblast cells (17) and the endemic typhus agent, *Rickettsia typhi*, in CB17 SCID mice and in association with CD8 cells (18). The first-generation rickettsial shuttle vectors are now an important component of the still limited toolbox for genetic manipulation of rickettsiae (19, 20), underscoring the need for further expansion and optimization of the vectors and for the study of plasmid biology in the genus *Rickettsia*.

The design of plasmid-based bacterial transformation vectors was influenced by discovery of incompatibility, which resulted in classification of plasmids into incompatibility groups determined by their ability to be simultaneously maintained within a single host cell. Incompatibility arises when both foreign and native plasmids carry the same or a similar *parA* gene, causing both plasmids to become unstable (21, 22). *R. tamurae* subsp. *buchneri* and *Rickettsia amblyommatis* have multiple independent plasmids, each with a distinct *parA* gene (2, 3, 10). The role of *parA* in plasmid partitioning and incompatibility in rickettsiae remains largely unexplored and likely has practical consequences for use of rickettsial shuttle vectors. In contrast, *R. monacensis* contains only one native plasmid, pRM, and phylogenetic analysis shows it contains a *parA* gene with no significant similarity to those from 19 other rickettsial plasmids (3), although the plasmid pRAS01 from *Rickettsia asembonensis* strain NMRCii has a *parA* gene with 66% nucleotide identity to *parA* from pRM (23). The predicted amino acid sequence of *ParA* from pRM is most similar to *ParA*-like predicted proteins from *Mycobacterium* and *Bartonella* spp. (25 to 53% BLASTP identities). Thus, shuttle vectors from pRM should theoretically be compatible for transformation of an expanded range of rickettsiae.

Our aim is to both expand the repertoire of shuttle vectors for use in the study of *Rickettsia* spp. and improve our understanding of the mechanisms of plasmid maintenance in diverse rickettsial species. Here, we report isolation of pRM from *Rickettsia monacensis* in *Escherichia coli* and its development as a family of shuttle vectors, which were used to transform several rickettsiae, including *R. monacensis* and *R. amblyommatis* AaR/SC, whose pRAM plasmids have also been developed as rickettsial shuttle vectors (12). Quantitative PCR (qPCR) results indicated that introduction of the pRM shuttle vector reduced copy numbers but did not eliminate endogenous pRM in transformed *R. monacensis*. The first-generation pRAM-based shuttle vector family and second-generation pRM-based shuttle vector family carry unrelated *parA* genes and thus diversify the range of vector choices available for transformation of rickettsiae.
RESULTS

Identification of minimal coding sequences required for pRM shuttle vector replication and partitioning. Because coding sequences that support plasmid replication and partitioning are often clustered together, we identified the minimal region required for rickettsial shuttle vector replication and partitioning by constructing three shuttle vectors with various portions of RM_p16-21 coding sequences: pRMdSGK clone 1 (pRMΔ1), pRMdSGK clone 2 (pRMΔ2), and pRMdSGK clone 3 (pRMΔ3) (Fig. 1). All three clones contained coding sequence for the pRM DnaA-like protein (RM_p16) and ParA (RM_p18), which function in DNA replication and chromosomal stability, as well as RM_p17 and RM_p19, hypothetical proteins (HPs) of unknown function, although RM_p19 contains domains with similarity to those of the HTH_XRE family transcriptional regulators. The smallest test construct, pRMΔ1, included the gene cluster RM_p16 through RM_p19 and partial coding sequence (359 bp of 507 bp) for RM_p20 (green bar in Fig. 1C), a second likely HTH_XRE family protein with 74% similarity to RM_p19. The pRMΔ2 construct extended the same dnaA/para region through an intact RM_p20 coding sequence and an amino-terminal fragment of RM_p21 (orange bar in Fig. 1C). The pRMΔ3 construct further encoded an intact RM_p21, a likely Sca12 cell surface antigen, and approximately 1 kbp of downstream noncoding sequence (llac bar in Fig. 1C).

Transformation trials of five SFG Rickettsia spp. The smallest test construct, pRMΔ1, transformed only R. monacensis (Table 1), which carries pRM as its native plasmid. In contrast, in trials with pRMΔ2 (encoding the same proteins from RM_p16 through RM_p19 as well as the intact RM_p20 XRE family protein with its upstream region) and pRMΔ3 (extended to encode the RM_p21 Sca12 cell surface antigen), both constructs transformed all five Rickettsia species tested (Table 1). Species successfully transformed included four spotted fever group (SFG) rickettsiae, notably R. amblyomatis AaR/SC, which contains 3 native plasmids, as well as R. bellii, which occupies a more basal phylogenetic position (24). The cluster from RM_p16 through RM_p19 thus supported transformation of the parental R. monacensis, but inclusion of the RM_p20 locus and/or a short upstream sequence extended the range of a pRM-based shuttle vector to other SFG and ancestral group rickettsiae. In contrast to RM_p20, RM_p21 and the associated noncoding 1-kbp sequence conferred no apparent advantage.

These results indicated that the loci from RM_p16 through RM_p20 of pRM contained the minimum necessary DNA sequence and protein coding capacities for a shuttle vector capable of transforming a wide range of SFG and ancestral group rickettsiae. The promoter prediction program BPROM (25) indicated a single promoter upstream of the RM_p20 locus, but none for RM_p17, -18, or -19, consistent with functional dependence of RM_p18 expression on sequences upstream of RM_p20 and a likely operon extending through the RM_p16 locus (Fig. 1D). The predicted transcription start site was at bp 19395 of pRM, with a −10 box from 19402 to 19410 and a −35 box from 19425 to 19430. A comparison of growth rates of wild-type (WT) R. monacensis and pRMΔ2-transformed R. monacensis using three separate growth curve analyses indicated no significant differences between the growth rates of WT and transformants (doubling times of 17.5 and 18 h, respectively).

Evaluation of GFP expression in wild-type and transformed R. monacensis using confocal microscopy. The cell-free R. monacensis WT strain and transformants were stained with NucBlue Live Cell Stain ReadyProbes (Thermo Fisher Scientific, Waltham, MA) to visualize all rickettsiae, whether transformed or not, then gently mounted onto slides by using a Cytospin centrifuge (Thermo Fisher) and observed by confocal microscopy. The left column of Fig. 2A with a DAPI (46-diamidino-2-phenylindole) filter shows all the rickettsiae present, while the middle column shows only gfpuv-expressing transformed rickettsiae (fluorescein isothiocyanate [FITC] filter). The third column (Fig. 2B) illustrates the overlap of fluorescence in the DAPI and FITC fields. Visually, the degree of colocalization indicated that nearly all the rickettsiae present were transformed. To confirm these observations, we used Pearson’s coefficient (PCC) and Manders’ colocalization coefficient (MCC) (26, 27) to evaluate the colocalization of the DAPI and FITC fluorescence. For the PCC analysis, the correlation coefficient was measured on all pixels in an individual
FIG 1 Construction of *R. monacensis* pRM shuttle vectors. (A) Schematic diagram depicting construct pJAZZ[pRM658B], the *R. monacensis* plasmid pRM with pMOD658 transposon cloned into the linear plasmid pJAZZ. The two arms formed by digested pJAZZ are indicated by brown double-ended arrows, and pRM is represented by a red double-ended arrow; genes present on pRM are denoted with black arrows. The pMOD658 transposon is shown in green; resistance to chloramphenicol (CAT) and the ability of the clone to express green fluorescent protein (GFPuv) are conferred by the transposon inserted in pRM. The unique SmaI site with which pRM was linearized for cloning is located in the transposon. Pink balloons indicate the restriction enzyme sites used for subcloning the *dnaA/parA* region of pRM. (B) The 5.6-kbp selection reporter cassette SGK was ligated with three pRM fragments of various sizes containing *dnaA* and *parA*, yielding the *R. monacensis* pRM shuttle vectors pRM1, -2, and -3 (C). The numbered black line in panel C represents bp 14400 to 21600 of the 23,486-bp *R. monacensis* plasmid pRM, and the thick black horizontal arrows beneath it indicate predicted genes and their orientations. Colored vertical arrows indicate the restriction enzyme sites used during pRM subcloning, and colored bars above the numbered black line represent the three fragments of pRM contained in the finished shuttle vectors shown in panel D.
The MCC assay measures the proportion of rickettsial DNA that localizes with transformed plasmid fluorescence (M1) and vice versa (M2). Almost 100% colocalization is indicated by the very similar M1 and M2 values calculated for each of the three transformed R. monacensis strains (Fig. 2C).

**Presence of shuttle vector in pRM-transformed R. amblyommatis and R. parkeri and conservation of native plasmids in transformed R. amblyommatis.** Undigested DNA from R. amblyommatis and Rickettsia parkeri transformed with pRMΔ2 and pRMΔ3 was separated by pulsed-field gel electrophoresis (PFGE) (Fig. 3A) and transferred onto Zeta-Probe membranes. Because R. parkeri does not contain native plasmids, hybridization of R. parkeri pRMΔ2 and pRMΔ3 transformants with digoxigenin-labeled gfpuv probe (28) identified the unaltered shuttle vectors, with asterisks indicating the nicked linear forms of pRMΔ2 and pRMΔ3 at 10.81 and 12.814 kbp, respectively (Fig. 3B). The R. amblyommatis transformants show a band pattern similar to that of R. parkeri; however, extra bands were present in both R. amblyommatis pRMΔ2 and pRMΔ3 hybridized with the gfpuv probe (Fig. 3B). Stripped Southern blots were hybridized with recombinase, hsp2, and helicase digoxigenin-labeled probes specific for R. amblyommatis strain AaR/SC plasmids pRAM18, pRAM23, and pRAM32, respectively (12). Bands of the appropriate size were observed for all 3 native pRAM plasmids (pRAM18, 18.344 kbp; pRAM23, 22.852 kbp; and pRAM32, 31.972 kbp) in the transformed R. amblyommatis (marked with asterisks in Fig. 3A, C, D, and E), but not in R. parkeri. Thus, pRM shuttle vector and all three native R. amblyommatis plasmids are conserved in the pRM-transformed R. amblyommatis.

**Testing for the presence of native pRM and shuttle vectors in pRMΔ-transformed R. monacensis.** To assess native pRM in shuttle vector transformants, genomic DNA from WT and transformant R. monacensis strains was PCR amplified with native pRM-specific primer sets (Table 2). All three R. monacensis transformants contained native pRM, as indicated by the presence of amplicons from genes present in native pRM but not in the shuttle vectors (Fig. 4A, B, and C). The dGFPuvF2/R2 primer amplicons confirmed the presence of the shuttle vectors (Fig. 4D). R. amblyommatis AaR/SC pRMΔ2 DNA was used as a control to confirm primer specificity; as expected, it yielded no amplicon with native pRM primer sets but was positive with dGFPuvF2/R2 primers.

**Copy number ratios of native pRM and pRM shuttle vectors in transformed R. monacensis.** Quantitative PCR (qPCR) estimation of the relative ratio of the single-copy pRM-encoded resolvase RM_p5 (qRM_p5F/qRM_p5R primer set in Table 2) and chromosome-carried gltA genes (29) indicated 1.5 copies of pRM for each chromosome copy in WT R. monacensis, which decreased to a ratio of 0.5 to 0.8 in pRMΔ1-, -2-, and -3-transformed R. monacensis (Fig. 5). This ratio was mirrored by the 0.5-to-0.9 ratio of

---

**TABLE 1 Transformation of Rickettsia with plasmid constructs containing pRM dnaA and parA**

| Strain typea | pRAM18_dRGA, 10.3 kbp | pRMΔ1 (RM_p16-20), 10.4 kbp | pRMΔ2 (RM_p16-21), 10.9 kbp | pRMΔ3 (RM_p16-21), 12.8 kbp |
|--------------|------------------------|-------------------------------|-------------------------------|-------------------------------|
| Strains with endogenous plasmids | | | | |
| R. monacensis Ir/R/Munich (pRM, 23 kb) | + | - | + | + |
| R. amblyommatis AaR/SC (pRAM; 18, 23, and 32 kb) | + | - | + | + |
| Plasmid-free strains | | | | |
| R. parkeri Oktibbeha | + | - | + | + |
| R. montanensis M5/6 | + | - | + | + |
| R. belli 369C | + | - | + | + |

---

aThe native plasmid name is given in parentheses, along with the approximate native plasmid size(s).
bThe construct designation and size are shown, with the pRM genes included in parentheses. An italicized gene number indicates the sequence does not represent the complete gene.
shuttle vector (qGFPuvF/qGFPuvR primers from shuttle vector-carried gfpuv in Table 2) to chromosome in the transformant rickettsiae (Fig. 5). There were no further significant changes in copy number ratios during serial passage of the transformant rickettsia (up to 20 passages in the case of pRMΔ2) (data not shown).

Presence of shuttle vector and native plasmid complexes in pRM-transformed *R. monacensis*. To confirm the PCR-indicated presence of native pRM in pRMΔ shuttle vector-transformed *R. monacensis*, undigested genomic DNA from WT and pRMΔ1, -2,
and -3 transformants was separated by pulsed-field gel electrophoresis (Fig. 6) and transferred onto Zeta-Probe membranes. Nicked linear, circular, and multimeric forms of pRM were present in the WT *R. monacensis* lanes (Fig. 6A and C, bands with white asterisks). The presence of the shuttle vector in *R. monacensis* transformed with pRM was demonstrated by hybridization with digoxigenin-labeled *gfpuv* probe (absent in the WT) (Fig. 6B). The *gfpuv* probe localized to bands in the ~40-kbp region (arrowhead) rather than at 10 to 12 kbp (the size of the shuttle vectors), indicating that there are no detectable monomeric pRM1, pRM2, or pRM3 in transformant populations.

![FIG 3 Pulsed-field gel electrophoresis and Southern blot analysis of *R. amblyommatis* and *R. parkeri* transformed with pRM shuttle vectors pRMΔ2 and pRMΔ3, *gfpuv*, and pRAM digoxigenin-labeled probes were used to detect the presence of shuttle vector and/or plasmid pRAM18, pRAM23, or pRAM32 in transformants. (A) PFGE gel. White asterisks mark the nicked linear forms of pRAM18, pRAM23, and pRAM32, respectively. (B) Southern blot analysis of pulsed-field gel hybridized with digoxigenin-labeled *gfpuv* probe. The nicked linear forms of pRMΔ2 and pRMΔ3 are denoted by black and white asterisks, respectively. Higher-molecular-weight bands are supercoiled and multimer forms of the shuttle vectors pRMΔ2 and pRMΔ3. Note the absence of labeling in the WT *R. amblyommatis* and *R. parkeri* lanes. (C) Southern blot analysis of panel A hybridized with digoxigenin-labeled pRAM18 probe (invertase). (D) Southern blot analysis of panel A hybridized with digoxigenin-labeled pRAM23 probe (Hsp2). (E) Southern blot analysis of panel A hybridized with digoxigenin-labeled pRAM32 probe (RecD). Black asterisks in panels C, D, and E denote nicked linear forms of pRAM18, -23, and -32, respectively.

Table 2

| Primer | Primer ID | Sequence | Amplicon size (bp) |
|--------|-----------|----------|--------------------|
| RM_p22 specific | RM_p22 F | TCTACACGGCCTTCTTTTC | 335 |
| | RM_p22 R | CTCAATCTGGTCTTGCGAGGC | |
| RM_p1 specific | RM_p prez1F | TCTGATTTTGGTTTGCTGGGC | 677 |
| | RM_p prez3R | CATTCTGTCCTTGGCTCCTTTG | |
| Shuttle vector specific | dGFpuxF | TTTGTCCTGGAGAGGTTGGAAGGT | 399 |
| | dGFpuxR | CCTTTTTTCTTTTGCTTGC | |
| Citrate synthase qPCR a | qCS-F | TCGCAAATGTTCACGGTACTTT | 74 |
| | qCS-R | TCGTGCATTTCTTTCCATTGTG | |
| GFPuv qPCR b | qGFpuxF | CAGTGGAGAGGTTGGAAGGTGAC | 114 |
| | qGFpuxR | ACCATAAGAGAAAGTAGTGACAAGTGTTGGC | |
| RM_p5 qPCR | qRM_p5F | CACACATCGGAGAGGTGATT | 106 |
| | qRM_p5R | CGAATAAGTCGGCAGCTAAGA | |

aSee reference 28 for details.
bSee reference 12 for details.
The PFGE gel shown in Fig. 6C was transferred to a membrane and hybridized with a probe containing digoxigenin-labeled RM_p23 (13), a gene present on native pRM but absent on the shuttle vectors (Fig. 6D). In the WT lane, the probe hybridized to the 20- to 30-kbp linear nicked and multimeric forms (black asterisks) of pRM. In the transformant lanes, the RM_p23 probe hybridized in the ~40-kbp region (Fig. 6D, arrowhead) at the same relative positions as the gfpuv probe (Fig. 6B). The strong hybridization of the RM_p23 probe at the 40-kbp position in transformant rickettsia lanes versus weak hybridization at the lowest position (lowest black asterisk in panel D) indicates the likely presence of native pRM that is predominantly complexed with shuttle vector, as transformants lacking pRM would not hybridize with RM_p23.

![PFGE gel](image_url)

**FIG 4** PCR results demonstrating the presence of native pRM in pRMΔ-transformed *R. monacensis*. PCR amplicons were electrophoresed on a 1% agarose gel stained with GelGreen (Biotium, Hayward CA). (A, B, and C) PCR results using primers specific for native pRM genes (RM_p22, RM_p1, and RM_p3) not present in pRM shuttle vectors. (D) PCR products using gfpuv-specific primers (dGFPuvF2/dGFPuvR2) to detect shuttle vector. Lane designations: 1, no-template control; 2, pRMΔ2 passage 1 transformant; 3, pRMΔ2 passage 3 transformant; 4, pRMΔ1 passage 3 transformant; 5, pRMΔ3 passage 3 transformant; 6, WT *R. monacensis*; 7, *R. amblyommatis* pRMΔ2 transformant.

![Plasmid-to-chromosome copy number ratios](image_url)

**FIG 5** Plasmid-to-chromosome copy number ratios in transformed *R. monacensis*. Native plasmid (pRM), shuttle vector, and chromosomal copy numbers were quantified by qPCR of RM_p5, gfpuv, and gltA, respectively. Passage numbers (e.g., p17) used for this assay were as follows: WT, p17 (all 3 replications); pRMΔ2, p10 (all 3 replications); pRMΔ1, one replication with p3 and two replications with p9; pRMΔ3, one replication with p3 and two replications with p7.
DISCUSSION

Although plasmids have been extensively studied in other bacteria, the discovery of plasmids in rickettsiae is fairly recent (1, 2, 13), and their biological function is largely unexplored. Genomic analysis has shown that rickettsiae have undergone reductive evolution (30–33), resulting in such characteristics as AT enrichment, high conservation of genome sequences among species, higher levels of virulence, and variable presence and numbers of plasmids (3). Rickettsial plasmids have likewise undergone reductive evolution and mirror rickettsial genomes in relative size and GC content (34). It has been proposed that a rickettsial ancestor supported a plasmid system that was lost in some species due to their unique obligate intracellular life cycle (3, 34), but other species presumably retained plasmids due to an advantage conferred by their presence. Plasmids are known agents of horizontal gene transfer, facilitating host adaptation/virulence, antibiotic and stress resistance, and genetic plasticity (1, 34–36). Although the origins and functions of many rickettsial plasmid gene sequences have been inferred from similarities to those of other bacteria, the role of plasmids and their interactions in rickettsiae remain to be elucidated. Real-time PCR and whole-genome sequencing showed that these are low-copy-number plasmids (2), while creation of shuttle vectors from pRAM18 and pRAM32 and their subsequent transformation into rickettsiae confirmed that parA and dnaA were essential for plasmid replication and maintenance (12). However, the mechanism by which these genes function is yet to be identified.

We have developed two new Rickettsia plasmid shuttle vectors, pRMΔ2 and pRMΔ3, that can be used to transform plasmid-free Rickettsia spp. as well as those carrying native plasmids. In conjunction with pRMΔ1, they collectively contained various regions of pRM surrounding the dnaA and parA genes. Analysis of their relative efficacies in transformed rickettsiae allowed us to identify plasmid sequences important for the replication and
maintenance of the shuttle vectors in rickettsiae. Our data indicated that RM_p20 or its immediate upstream sequence was required for shuttle vector replication in rickettsiae. Specifically, R. parkeri, R. bellii, Rickettsia montanensis (plasmid free), and R. amblyommatis strain AaR/SC (carrying 3 plasmids) were not transformed with shuttle vector pRMΔ1 (Fig. 1C, containing RM_p16 through RM_p19 and the 3’ end of RM_p20) but were transformed with shuttle vectors pRMΔ2 and Δ3 (containing RM_p16 through RM_p20 and pRM21, respectively). These data support the prediction (37) that the region of pRM containing RM_p17 to RM_p20 forms an operon. It is possible that RM_p19 and -20 are not themselves required because absence of a single promoter upstream of RM_p20, as predicted by BPROM (25), would prevent expression of RM_p18 (parA) in pRMΔ1. Without expression of the ParA chromosomal stability protein, the shuttle vector would not be maintained, consistent with the observed phenotype. In contrast, R. monacensis was transformed with pRMΔ1 when the other species were not, likely due to the presence of native pRM providing the necessary ParA for maintenance of the shuttle vector.

Partition systems usually include three features: a site that acts like a centromere, a centromere-binding protein (CBP) (usually encoded by parB), and an NTPase (parA) (38). As none of the genes in the pRM operon have similarity to known parB genes, the pRM partitioning system could work in one of several ways. The partitioning system could rely on the chromosomal parB to work or represent a new type of system, as in R388, which functions with a single protein and a centromere site (38). Alternatively, the pRM hypothetical genes could act in the capacity of parB as CPBs are not necessarily significantly similar in sequence but are typically dimers of helix-turn-helix (HTH) or ribbon helix-helix DNA-binding proteins (38). Both RM_p19 and -20 have HTH_XRE domains and might function as CPBs.

The pRM operon containing parA appears to be unique among known rickettsial plasmids. A BLASTN search of the genes RM_p17 and -20 showed no rickettsial homology, while RM_p19 only had homology with the rickettsial endosymbiont of ixodes pacificus plasmid (pREIP). Interestingly, translation of the nucleotide sequence for RM_p19 and -20 and subsequent BLASTP of the amino acid sequence shows a low level of similarity to pREIP (36% identity and 59% positive for RM_p19; 40% identity and 60% positive for RM_p20) and to rickettsial endosymbionts of a variety of arthropods, including beetles such as Platysa sonomae and Bembidion nr Transversale, bedbugs (Cimex lectularius), and midges (Culicoides impunctatus). These Torix clade Rickettsia spp. (39) have 2 or 3 different regions of similarity for both RM_p19 and -20 and have identities ranging from 30 to 40%, with 53% to 62% positive.

The ParA protein encoded on R. monacensis pRM is sufficiently distinct from those of pRAM18, -23, and -32 (the native plasmids of R. amblyommatis) that it should support uptake and maintenance of the pRM shuttle vector in R. amblyommatis. In their comparison of rickettsial plasmid genes, El Karkouri et al. (3) showed that pRM parA was distinct from the parA of all other sequenced rickettsial plasmids. A BLASTP search of the ParA protein from pRM identified only one rickettsial homolog (R. asembonensis, with 46% identity), while its closest match was from a Mycoplasmataceae bacterium, with 53% identity. Lack of interactions between nonhomologous ParA proteins may explain why R. amblyommatis strain AaR/SC was transformed with pRM shuttle vectors, despite its three native plasmids and their potential for causing plasmid incompatibility. On the other hand, the successful transformation of R. monacensis with its own pRM shuttle vectors was unexpected. It is possible that the presence of identical ParAs from native plasmids and shuttle vectors supports mutual plasmid maintenance in rickettsiae rather than promoting incompatibility. These results also highlight the fact that plasmid transformation of different Rickettsia species is by no means a routine activity with universally predictable results. For example, the construct pRAM18/Rif/GFPuv, containing full-length pRAM18, successfully transformed R. bellii and R. parkeri (12), but for unknown reasons, shuttle vectors that incorporated full-length pRM instead of pRAM18 (data not shown) were unable to transform R. montanensis, R. monacensis, R. peacockii, and R. parkeri or 3 strains of R. amblyommatis. Thus, there is still much to
learn about the role of these rickettsial plasmids in the functioning of rickettsiae and the mechanisms by which they operate.

The studies reported here give us a better understanding of the mechanisms of rickettsial plasmid maintenance in diverse rickettsial species. We explored the basis for the ability of pRM-based shuttle vectors to transform R. monacensis. Our PCR results detected the presence of shuttle vector in transformants, which continued to persist through serial transferring. They also indicated a decrease in the ratio of pRM to the chromosome, suggesting that transformants harbored an average of two or more chromosome copies per cell or that plasmids were cured from some of the rickettsiae. Nevertheless, data from Fig. 2 suggest that almost 100% of the rickettsiae contained the shuttle vector. Furthermore, the results showed that the use of homologous rickettsial parA regions leads to the formation and maintenance of complexes between shuttle vectors and native plasmids, suggesting possible defects in partitioning of plasmids carrying the same parA genes. The surprising ability of R. monacensis to be transformed by shuttle vectors containing the parA gene from its native plasmid emphasizes the need for further study of plasmid maintenance and incompatibility in rickettsiae, and further studies are needed to elucidate the molecular basis for the apparent linkage of shuttle vectors with pRM present in R. monacensis.

MATERIALS AND METHODS

**Bacterial strains and plasmids.** The *Rickettsia monacensis* strain IrR/Munich′ (40) WT was used at passages 10 to 70 after initial isolation from a tick. It was propagated in *Ixodes scapularis* cells, line i56E, as described previously (16). *R. amblyommatis* (strain AaR/SC), *R. bellii* (strain RML 369-C), *R. parkeri* (strain Tate′s Hell), and *R. montanensis* (strain M5/6) were propagated in the same manner as *R. monacensis*.

The *R. monacensis* plasmid pRM was cloned in *E. coli* as previously described (37). Briefly, electroporation of *R. monacensis* with the pMOD658 transposon yielded the Rmona658B transformant, in which the pMOD658 transposon encoding chloramphenicol acetyltransferase (CAT) and carrying a gfp<sub>ω</sub> fluorescent marker was inserted into pRM (37). The mutated pRM was cloned in its entirety by chloramphenicol resistance conferred by the aminoglycoside adenyltransferase gene, driven by a rickettsial spectinomycin/streptomycin resistance conferred by the aminoglycoside adenyltransferase gene, (ii) (strain RML 369-C), (iii) (strain AaR/SC), and (iv) (strain RML 369-C), were propagated in the same manner as in *E. coli* as well as rickettsial ompA gene promoter, and (iii) "K" indicates kanamycin resistance from the pET-28a<sup>+</sup> vector (Novagen, EMD Millipore, Bedford, MA), adapted as described below. Specific nucleotide spans from pRM are from accession no. EF564599 (37). Multiple cloning sites (MCSs) are designated "MCS." Promoters are designated "p." The following abbreviations are used to designate plasmids and their derivatives: (i) "S" indicates spectinomycin/streptomycin resistance conferred by the aminoglycoside adenyltransferase gene, aadA, driven by a rickettsial ompA promoter, (ii) "G" indicates green fluorescent protein (GFP) encoded by gfp<sub>ω</sub> under regulation of the rickettsial ompA gene promoter, and (iii) "K" indicates kanamycin resistance from the pET-28a<sup>+</sup> vector (Novagen, EMD Millipore, Bedford, MA), adapted as described below. Specific nucleotide spans from pRM are from accession no. EF564599 (37). Multiple cloning sites (MCSs) are designated "MCS." Promoters are designated "p.

**Construction of pRM-based shuttle vectors.** (i) Preparation of selection reporter cassette. We constructed a cassette into which pRM fragments could be cloned. The SGK selection reporter cassette (see Fig. S1C in the supplemental material) was derived from the previously constructed shuttle vector pRAM18-RGA(MCS) (12) (Fig. S1A) and contained genes needed for replication and antibiotic selection in *E. coli* as well as reporter and antibiotic selection genes for use in rickettsiae (Fig. S1C). Replacement of pGEM with a 3,159-kbp DraIII/PshA fragment of the pET-28a<sup>+</sup> vector was prompted by previous experiments indicating that some genes cloned into the pRAM18 shuttle vector MCS were less stable in pGEM than pET. Because spectinomycin and streptomycin are water soluble and not treated rickettsioses, but rifampin may be, we replaced the spectinomycin/streptomycin resistance marker cassette with the pET-28a<sup>+</sup> vector (Novagen, EMD Millipore, Bedford, MA), adapted as described below. Specific nucleotide spans from pRM are from accession no. EF564599 (37). Multiple cloning sites (MCSs) are designated "MCS." Promoters are designated "p.

(ii) Subcloning pRM. To identify which region(s) of pRM yielded the most effective shuttle vector for rickettsial transformation, we cloned specific fragments of pRM to create a family of deletion constructs: pRMΔ1, pRMΔ2, and pRMΔ3. To construct pRMΔ1 and pRMΔ3, pJAZZ(pRM658B) was digested with BamHI/Paci or BamHI/PFLM (Fig. 1A and B), and the desired 4,671-bp and 7,177-bp fragments containing either RM_p16 to -20 (pRM bp 14561 to 19231) or RM_p16 through -21 (pRM bp 14561 to 21646) were gel purified (Zymoclean Gel DNA Recovery kit; Zymo Research, Irvine, CA). The fragment ends were blunted (DNATerminator) and ligated into the blunt and dephosphorylated selection reporter cassette (SGK), creating the 10,368-bp pRMΔ1 and 12,797-bp pRMΔ3, respectively (Fig. 1D). To construct pRMΔ2, QS DNA polymerase (New England Biolabs) was used to PCR amplify RM_p16 through RM_p20 and into the 5′ end of RM_p21 (pRM bp 14512 through 19585) with primers RM_p16 FOR and NheI/digested SGK, to yield the 10,881-bp shuttle vector pRMΔ2 (Fig. 1D).

To predict the location of rickettsial promoters in the pRM fragments cloned into the shuttle vectors, we used BPROM (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gbfinddb) (25).
Preparation of shuttle vector plasmid DNA. Endotoxin-free maxipreps (Qiagen, Valencia, CA) were prepared for all pRM shuttle vectors as per the manufacturer’s recommendations, for use in transforming rickettsiae. The integrity and orientation of inserted genes were reconfirmed by sequencing fragment junctions and by restriction digest analysis. (See Table 3 for the sequencing primers.)

Transformation of rickettsiae. Rickettsiae were purified and electroporated as described previously (16). Rickettsial transformants were selected using growth medium containing spectinomycin and streptomycin, each at a final concentration of 100 μg/mL. Cultures were monitored for expression of the GFPuv reporter on a weekly basis by using an inverted microscope (Nikon Diaphot TMD with Y-FL-epi-fluorescence attachment and a sapphire GFP 31043 filter) or by examining wet mounts on an upright Nikon Eclipse E400 microscope with a B-2E/C FITC filter (Nikon, Melville, NY).

Growth rate analysis of R. monacensis with pRMΔ2. Multinuclear plates of ISE6 cells were inoculated with R. monacensis WT at a 1:25 dilution and sampled at selected times (2.8, 48.5, 72.8, 96.4, 119.7, 143.7, 168.9, and 191.4 h postinoculation [hpi]). Two plates of the R. monacensis WT diluted 1:50 were sampled at 0, 22.8, 47.2, 70.3, 94.5, 119.8, 137.7, and 167 hpi. One plate of ISE6 cells inoculated with the R. monacensis pRMΔ2 transformant diluted 1:25 was sampled at 4, 49.5, 97.2, 145.3, 215.5, 264.2, 311.5, and 359.5 hpi. Two additional plates seeded with the same transformant were diluted 1:10 and 1:25 and sampled at 3.3, 72.1, 98.5, 121.2, 145, 167.2, 217.8, and 265.4 hpi. Growth rates were based on qPCR-determined levels of chromosomal single-copy gene gltA (29) from the average of 3 growth curve analyses.

Evaluation of GFP expression in WT and transformed R. monacensis using confocal microscopy. Cell-free WT and transformed R. monacensis cells were resuspended in complete medium and incubated with NucBlue Live Cell Stain ReadyProbes reagent (1: 50 dilution) (Thermo Fisher Scientific) in the dark for 30 min at room temperature. Fifty-microliter aliquots of cell-free R. monacensis were deposited onto microscope slides (Cytospin centrifuge; Thermo Fisher) at 200 rpm for 3 min. The slides were mounted for 30 min at room temperature. Fifty-microliter aliquots of cell-free WT and transformed R. monacensis WT diluted 1:25 were sampled at 4, 49.5, 97.2, 145, 167.2, 217.8, and 265.4 hpi. Growth rates were based on qPCR-determined levels of chromosomal single-copy gene gltA (29) from the average of 3 growth curve analyses.

TABLE 3 Sequencing primers for shuttle vector pRMΔ2

| Primer ID | Sequence | pRM coordinates |
|-----------|----------|-----------------|
| RM_p[16-20] seq F1 | 5'-TCTTATTGTCGTGCTG-3' | bp 15048–15065 |
| RM_p[16-20] seq F2 | 5'-TCTTATTGTCGTGCTG-3' | bp 15655–15672 |
| RM_p[16-20] seq F3 | 5'-TTACCCTATCCATACCG-3' | bp 16484–16501 |
| RM_p[16-20] seq F4 | 5'-CGTGGTAGAATGAGGAGTC-3' | bp 16787–16806 |
| RM_p[16-20] seq F5 | 5'-TACGCCACTATTCCGCCTG-3' | bp 17415–17432 |
| RM_p[16-20] seq F6 | 5'-AGAATAATCCGACCAGCAG-3' | bp 18018–18035 |
| RM_p[16-20] seq F7 | 5'-GATTTTCCTACACACAACCC-3' | bp 18209–18226 |
| RM_p[16-20] seq F8 | 5'-CAGGAAGTGTAAGAGCTCTT-3' | bp 18958–18977 |
Real-time quantitative PCR (qPCR) was used to estimate relative copy number ratios (43, 44) of single-copy genes specific to the R. monacensis chromosome (gtf4 encoding citrate synthase) to analyze the native pRM plasmid (RM_pS locus for transposon resolve), and the pRM-derived shuttle vectors (GFP uv) by using three primer pairs (Table 2). With the exception of a 56°C annealing temperature, reactions and copy number estimates were executed as previously described (2).

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS
This research was supported by grants (R01 AI049424 and R01 A1081690) to U.G.M. from the U.S. National Institutes of Health.

We thank Roderick Felsheim for technical advice and assistance.

REFERENCES
1. Ogata H, Renesto P, Audic S, Robert C, Blanc G, Fournier P-E, Parinello H, Claverie J-M, Raoult D. 2005. The genome sequence of Rickettsia felis identifies the first putative conjugative plasmid in an obligate intracellular parasite. PLoS Biol 3:e248. https://doi.org/10.1371/journal.pbio.0030248.

2. Baldridge GD, Burkhart NY, Labruna MB, Paccheco RC, Paddock CD, Williams PC, Billingsley PM, Felsheim RF, Kurtti TJ, Munderloh UG. 2010. Widespread dispersal and possible multiple origins of low-copy-number plasmids in Rickettsia species associated with blood-feeding arthropods. Appl Environ Microbiol 76:1718–1731. https://doi.org/10.1128/AEM.02988-09.

3. El Karkouri K, Pontarotti P, Raoult D, Fournier P-E. 2016. Origin and evolution of rickettsial plasmids. PLoS One 11:e0147492. https://doi.org/10.1371/journal.pone.0147492.

4. El Karkouri K, Kowalczewska M, Armstrong N, Azza S, Fournier P-E, Raoult D. 2017. Multi-omics analysis sheds light on the evolution and the intracellular lifestyle strategies of spotted fever group Rickettsia spp. Front Microbiol 8:3163. https://doi.org/10.3389/fmicb.2017.01363.

5. Diop A, Raoult D, Fournier P-E. 2018. Rickettsial genomics and the paradigm of genome reduction associated with increased virulence. Microbes Infect 20:401–409. https://doi.org/10.1016/j.micinf.2017.11.009.

6. Diop A, Raoult D, Fournier P-E. 2019. Paradigmatic evolution of rickettsial genomes.Ticks Tick Borne Dis 10:462–469. https://doi.org/10.1007/jttbdiss2018.11.007.

7. Felsheim RF, Kurtti TJ, Munderloh UG. 2009. Genome sequence of the endosymbiont Rickettsia peacockii and comparison with virulent Rickettsia rickettsii: identification of virulence factors. PLoS One 4:e8361. https://doi.org/10.1371/journal.pone.0008361.

8. Ogata H, La Scola B, Audic S, Renesto P, Blanc G, Robert C, Fournier P-E, Claverie J-M, Raoult D. 2006. Genome sequence of Rickettsia bellii illuminates the role of amoebae in gene exchanges between intracellular pathogens. PLoS Genet 2:e67. https://doi.org/10.1371/journal.pgen.0020076.

9. Heu CC, Kurtti TJ, Nelson CM, Munderloh UG. 2015. Transcriptional analysis of the conjugal transfer genes of Rickettsia bellii RML369-C. PLoS One 10:e0137214. https://doi.org/10.1371/journal.pone.0137214.

10. Gillespie JJ, Joardar V, Williams KP, Driscoll T, Hostetler JB, Nordberg E, Paddock CD, Williamson PC, Billingsley PM, Heu CC, Felsheim RF, Kurtti TJ, Munderloh UG. 2011. Development of shuttle vectors for transformation of diverse Rickettsia species. PLoS One 6:e29511. https://doi.org/10.1371/journal.pone.0029511.

11. Baldridge GD, Burkhart NY, Felsheim RF, Kurtti TJ, Munderloh UG. 2008. Plasmids of the pRM/pRF family occur in diverse Rickettsia species. Appl Environ Microbiol 74:645–652. https://doi.org/10.1128/AEM.02262-07.

12. Wood DO, Hines A, Tucker AM, Woodard A, Driskill LO, Burkhart NY, Kurtti TJ, Baldridge GD, Munderloh UG. 2012. Establishment of a replicating plasmid in Rickettsia prowazekii. PLoS One 7:e34715. https://doi.org/10.1371/journal.pone.0034715.

13. Kurtti TJ, Burkhart NY, Heu CC, Munderloh UG. 2016. Fluorescent protein expressing Rickettsia buchneri and Rickettsia peacockii for tracking symbiont-tick cell interactions. Vet Sci 3:34. https://doi.org/10.3390/vetsci3030034.

14. Oliver JD, Burkhart NY, Felsheim RF, Kurtti TJ, Munderloh UG. 2014. Morbidity characteristics are altered for Rickettsia bellii transformed to overexpress a heterologous rickA gene. Appl Environ Microbiol 80:1170–1176. https://doi.org/10.1128/AEM.03352-13.

15. Kurtti TJ, Burkhart NY, Heu CC, Munderloh UG. 2016. Fluorescent protein expressing Rickettsia buchneri and Rickettsia peacockii for tracking symbiont-tick cell interactions. Vet Sci 3:34. https://doi.org/10.3390/vetsci3030034.

16. Hauptmann M, Burkhart N, Munderloh U, Kuehl S, Richardt U, Krasemann S, Hartmann K, Krech T, Fleischer B, Keller C, Osterloh A. 2017. GFPuv-expressing recombinant Rickettsia typhi: a useful tool for the study of pathogenesis and CDB T cell immunology in R. typhi infection. Infect Immun 85:e00156-17. https://doi.org/10.1128/IAI.00156-17.

17. Riley SP, Macaluso KR, Martinez JJ. 2015. Electrotropism and clonal isolation of Rickettsia species. Curr Protoc Microbiol 39:3A.6.1–3A.6.20. https://doi.org/10.1002/9780471729259.mc03a06s39.

18. Riley SP, Fish AL, Garza DA, Banajee KH, Harris E, del Piero C, Martinez JJ. 2016. Nonselective persistence of a Rickettsia conori plasmid during mammalian infection. Infect Immun 84:790–797. https://doi.org/10.1128/IAI.00125-15.

19. Bignell C, Thomas CM. 2001. The bacterial ParA-ParB partitioning proteins. J Bacteriol 193:1–34. https://doi.org/10.1128/JB.193.1.00129-0.

20. Bouet JY, Nordström K, Lane D. 2007. Plasmid partition and incompatibility—the focus shifts. Mol Microbiol 65:1405–1414. https://doi.org/10.1111/j.1365-2958.2007.05882.x.

21. Maina AN, Luce-Fedrow A, Omulo S, Hang J, Chan TC, Ade F, Jima DO, Ogola E, Ge H, Reinman RF, Njenga MK, Richards AL. 2016. isolation and characterization of a novel Rickettsia species (Rickettsia asembonensis sp. nov.) obtained from cat fleas (Ctenocephalides felis). Int J Evol Microbiol 66:451–457. https://doi.org/10.1099/ijem.0.003182.

22. Gillespie JJ, Williams K, Shukla M, Snyder EE, Nordberg EK, Ceraf ML, Dharmanoll C, Rainey D, Sonjia J, Shollam JM, Shikanahut WD, Wattam R, Purkayastha A, Czar M, Czsta O, Setubal JC, Azaf WD, Sobral BS. 2008. Rickettsia phylogenomics: unwinding the intricacies of obligate intracellular life. PLoS One 3:e2018. https://doi.org/10.1371/journal.pone.0002018.

23. Solovyev V, Salamov A. 2011. Automatic annotation of microbial genomes and metagenomic sequences, p 61–78. In Li RW (ed), Metagenomics and its applications in agriculture, biomedicine and environmental studies. Nova Science Publishers, Hauppauge, NY.

24. Manders EMM, Verbeek FJ, Aten JA. 1993. Measurement of co-localization of objects in dual-colour confocal images. J Microsc 169:375–386. https://doi.org/10.1111/j.1365-2818.1993.tb03313.x.

25. McDonald JH, Dunn KW. 2013. Statistical tests for measures of colocalization in biological microscopy. J Microsc 252:295–302. https://doi.org/10.1111/jmi.12093.

26. Baldrige GD, Burkhart N, Herron MJ, Kurtti TJ, Munderloh UG. 2005. Analysis of fluorescent protein expression in transformants of Rickettsia monacensis, an obligate intracellular tick symbiont. Appl Environ Microbiol 71:2095–2105. https://doi.org/10.1128/AEM.71.4.2095-2105.2005.
29. Stenos J, Graves SR, Unsworth NB. 2005. A highly sensitive and specific real-time PCR assay for the detection of spotted fever and typhus group rickettsiae. Am J Trop Med Hyg 73:1083–1085. https://doi.org/10.4269/ajtmh.2005.73.1083.

30. Andersson S, Zomorodipour A, Andersson J, Sicheritz-Pontén T, Alsmark CM, Podowski RM, Näslund AK, Eriksson A, Winkler HH, Kurland CG. 1998. The genome sequence of Rickettsia prowazekii and the origin of mitochondria. Nature 396:133–140. https://doi.org/10.1038/24094.

31. Andersson JO, Andersson SGE. 1999. Genome degradation is an ongoing process in Rickettsia. Mol Biol Evol 16:1178–1191. https://doi.org/10.1093/oxfordjournals.molbev.a026208.

32. Blanc G, Ogata H, Robert C, Audic S, Suhre K, Vestris G, Claverie JM, Raoult D. 2007. Reductive genome evolution from the mother of Rickettsia. PLoS Genet 3:e14. https://doi.org/10.1371/journal.pgen.0030014.

33. Merhej V, Raoult D. 2011. Rickettsial evolution in the light of comparative genomics. Biol Rev Camb Philos Soc 86:379–405. https://doi.org/10.1111/j.1469-185X.2010.00151.x.

34. Gillespie JJ, Beier MS, Rahman MS, Ammerman NC, Shallom JM, Purkayastha A, Sobral BS, Azad AF. 2007. Plasmids and rickettsial evolution: insight from Rickettsia felis. PLoS Genet 3:e14. https://doi.org/10.1371/journal.pgen.0030014.

35. Regnery RL, Spruill CL, Plikaytis BD. 1991. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. J Bacteriol 173:1576–1589. https://doi.org/10.1128/jb.173.5.1576-1589.1991.

36. Baldridge GD, Burkhardt NY, Selheim RF, Kurtti TJ, Munderloh UG. 2007. Transposon insertion reveals pRM, a plasmid of Rickettsia monacensis. Appl Environ Microbiol 73:4984–4995. https://doi.org/10.1128/AEM.00988-07.

37. Bouet JY, Funnell BE. 12 June 2019, posting date. Plasmid localization and partition in Enterobacteriaceae. EcoSal Plus 2019. https://doi.org/10.1128/ecosalplus.ESP-0003-2019.

38. Simser JA, Palmer AT, Fingerle V, Wilksie B, Kurtti TJ, Munderloh UG. 2002. Rickettsia monacensis sp. nov., a spotted fever group rickettsia, from ticks (Ixodes ricinus) collected in a European city park. Appl Environ Microbiol 68:4559–4566. https://doi.org/10.1128/AEM.68.9.4559-4566.2002.

39. Regnery RL, Spruill CL, Plikaytis BD. 1991. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. J Bacteriol 173:1576–1589. https://doi.org/10.1128/jb.173.5.1576-1589.1991.

40. Baldridge GD, Burkhardt NY, Simser JA, Kurtti TJ, Munderloh UG. 2004. Sequence and expression analysis of the ompA gene of Rickettsia peacockii, an endosymbiont of the Rocky Mountain wood tick, Dermacentor andersoni. Appl Environ Microbiol 70:6628–6636. https://doi.org/10.1128/AEM.70.11.6628-6636.2004.

41. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(DDelta CT) method. Methods 25: 402–408. https://doi.org/10.1006/meth.2001.1262.

42. Lee C, Kim J, Shin SG, Hwang S. 2006. Absolute and relative QPCR quantification of plasmid copy number in Escherichia coli. J Biotechnol 123: 273–280. https://doi.org/10.1016/j.jbiotec.2005.11.014.