RESEARCH ARTICLE

Targeted Knockout of the *Rickettsia rickettsii* OmpA Surface Antigen Does Not Diminish Virulence in a Mammalian Model System

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ABSTRACT **Strains of *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF), differ dramatically in virulence despite >99% genetic homology. Spotted fever group (SFG) rickettsiae produce two immunodominant outer membrane proteins, rickettsial OmpA (rOmpA) and rOmpB, which are conserved throughout the SFG and thought to be fundamental to pathogenesis. rOmpA is present in all virulent strains of *R. rickettsii* but is not produced in the only documented avirulent strain, Iowa, due to a premature stop codon. Here we report the creation of an isogenic *ompA* mutant in the highly virulent strain Sheila Smith by insertion of intronic RNA to create a premature stop codon 312 bp downstream of the 6,747-bp open reading frame initiation site (int312). Targeted insertion was accomplished using an LtrA group II intron retrohoming system. Growth and entry rates of Sheila Smith *ompA*::int312 in Vero cells remained comparable to those of the wild type. Virulence was assessed in a guinea pig model by challenge with 100 PFU of either *ompA*::int312 Sheila Smith or the wild type, but no significant difference in either fever peak (40.5°C) or duration (8 days) were shown between the wild type and the knockout. The ability to disrupt genes in a site-specific manner using an LtrA group II intron system provides an important new tool for evaluation of potential virulence determinants in rickettsial disease research.**

IMPORTANCE ***R. rickettsii* rOmpA is an immunodominant outer membrane autotransporter conserved in the spotted fever group. Previous studies and genomic comparisons suggest that rOmpA is involved in adhesion and may be critical for virulence. Little information is available for rickettsial virulence factors in an isogenic background, as limited systems for targeted gene disruption are currently available. Here we describe the creation of an rOmpA knockout by insertion of a premature stop codon into the 5′ end of the open reading frame using a group II intron system. An isogenic rOmpA knockout mutation in the highly virulent Sheila Smith strain did not cause attenuation in a guinea pig model of infection, and no altered phenotype was observed in cell culture. We conclude that rOmpA is not critical for virulence in a guinea pig model but may play a role in survival or transmission from the tick vector.**

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Rickettsia rickettsii is a Gram-negative obligate intracellular pathogen transmitted to humans through an arthropod vector. *R. rickettsii* is the causative agent of Rocky Mountain spotted fever (RMSF), the most severe of the spotted fevers (1–3) and a reemerging disease with increasing prevalence in the United States (4). Since its earliest recognition, cases of RMSF have been known to differ dramatically in severity (5). Even today, cases of RMSF which do not receive immediate and suitable treatment can approach mortality rates as high as 20% (3, 6). Although the pathology of RMSF is well documented, the molecular basis of *R. rickettsii* virulence is not well understood.

In 1984, Anacker et al. (7) described two surface antigens of 190 and 120 kDa in *R. rickettsii*; monoclonal antibodies (MAbs) specific to these antigens protected mice against a lethal challenge of *R. rickettsii*, while MAbs to lipopolysaccharide (LPS) offered no protective effect (8, 9). Furthermore, vaccination of guinea pigs with recombinant rickettsial OmpA (rOmpA) protected against challenge with virulent *R. rickettsii* (10). Similar results were reported for *Rickettsia conorii*, although there were differences in the apparent molecular masses of the antigens (11). Discrepancies in the described masses of both surface antigens eventually led to the nomenclature of "rOmpA" for the 190-kDa antigen and "rOmpB" for the 120-kDa antigen (10–14). The open reading frames of both *ompA* and *ompB* predict protein sizes considerably higher than what is observed. Both are autotransporters (15); proteolytic processing of rOmpB into a smaller mature form and associated β-fragment has been described (16). Processing of rOmpA has not yet been confirmed.

rOmpA is conserved throughout the spotted fever group (SFG), while rOmpB is conserved in both the SFG and typhus group (TG). Studies have suggested that rOmpA plays a role in adhesion, and rOmpB has been implicated in both adhesion and

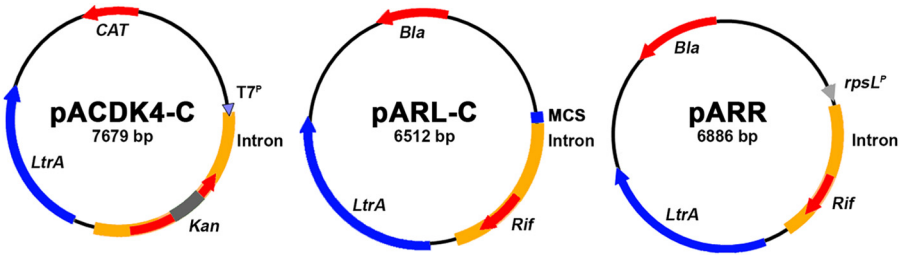

FIG 1 Construction of the pARR plasmid. Sigma's TargeTron vector pACDK4-C was altered for experimentation in *Rickettsia*. The selectable marker *cat* and the intron kanamycin RAM marker were removed and replaced with *bla* and Rp*arr-2*, respectively. The T7 promoter was removed and replaced with a multiple cloning site (MCS) to create pARL-C. The strong rickettsial *rpsL* promoter was cloned into the MCS to create pARR.

invasion (17–19). rOmpA and rOmpB are members of a family of surface cell antigens (Sca), autotransporter proteins found throughout the rickettsiae (15). *R. rickettsii* contains a significant number of Sca proteins in both the S-layer and outer membrane (15, 20, 21), with the S-layer made up largely of rOmpB (22–24). rOmpA and rOmpB are prominent outer membrane proteins in the SFG (7), with rOmpB found in greater abundance than rOmpA, calculated at an approximate 9:1 molar ratio, respectively (25).

Highly similar strains (>99%) of *R. rickettsii* can vary significantly in virulence (7, 26). A comparison of the virulent *R. rickettsii* Sheila Smith and avirulent Iowa strains revealed two notable differences, the absence of rOmpA in Iowa due to a premature stop codon and a partial defect in the processing of rOmpB (27), suggesting that these disruptions contribute to avirulence in Iowa. Furthermore, *R. peacockii*, an avirulent tick-borne rickettsia, also contains multiple premature stop codons in the *ompA* open reading frame (28). *R. rickettsii* Sheila Smith is the virulent rickettsia most closely related to *Rickettsia peacockii*. These findings suggest that rOmpA and rOmpB may be critical for virulence in SFG rickettsiae. Here we describe the use of a group II intron system in *R. rickettsii* to disrupt genes in a targeted manner, with the creation of an isogenic rOmpA knockout mutation in the highly virulent *R. rickettsii* Sheila Smith.

RESULTS

***ompA*::int312 knockout.** Group II intron retrohoming was employed to create a directed knockout in *R. rickettsii* Sheila Smith. The pARR suicide vector used here is a derivative of the TargeTron (Sigma Aldrich) vector pACDK4-C. pACDK4-C uses LtrA, a multifunctional reverse transcriptase, to insert intronic RNA at a specific DNA target site. pACDK4-C was modified to conform to restrictions for genetic modification in *R. rickettsii* (Fig. 1). For selection in rickettsiae, the kanamycin retrotransposition-activated marker (RAM) cassette was removed from pACDK4-C and replaced with the *arr-2* gene from pMW1650 codon-optimized for use in *Rickettsia prowazekii* (Rp*arr-2*) (29), encoding rifampin resistance. Chloramphenicol is an effective treatment for RMSF and is currently used as a secondary management option (30, 31), and rickettsiae can exhibit increasing resistance to chloramphenicol (32). Therefore, the chloramphenicol resistance gene *cat* was removed from the plasmid backbone and replaced with an XmaI restriction endonuclease site. A β-lactamase gene conferring ampicillin resistance was inserted into the XmaI site for selection in *Escherichia coli*. A promoter system like the T7/DE3 system used for inducible retrohoming in *E. coli* is not feasible for use in rickettsiae. The T7 promoter was therefore removed and replaced with a polylinker directly upstream of the 5′ exon site. The constitutive rickettsial promoter *rpsL* (29) was inserted into the polylinker. Other promoters, including the *ompB* promoter (25, 33) and citrate synthase promoter (34), were also tested and found to induce expression of the group II intron and give positive transformants. The final plasmid, pARR, contains the *rpsL* promoter, as it gave the greatest number of Rp*arr-2*$^+$ transformants per transformation (data not shown). pARR was retargeted to *ompA* by either of the two predicted group II insertion points in *ompA* (at nucleotide 312 [int312] or 528) using primers designed through the Sigma TargeTron insertion prediction algorithm software.

R. rickettsii Sheila Smith was transformed via electroporation with pARR targeted to either site 312 or site 528, followed immediately by plaque cloning in Vero cells under rifampin selection. After 10 to 16 days, plaques were picked and placed into individual T25 flasks of Vero cells. Each plaque was tested using PCR primers specific to the genomic region flanking the *ompA* insertion point (Fig. 2). After growth, Vero cells were lysed and a portion of the lysate was frozen for storage, with the remainder being used for additional rounds of plaque cloning. The process of plaque cloning was repeated a total of 4 times to ensure a clonal strain of *R. rickettsii* Sheila Smith *ompA*::int312 for further characterization.

Characterization of the *ompA*::int312 mutant. The disruption of *ompA* in Sheila Smith was initially confirmed using PCR to amplify the genetic region surrounding the predicted group II insertion point and then sequenced to verify correct insertion (Fig. 2). The group II intron inserted either 312 bp or 528 bp downstream of the start codon according to the original targeting sequence. Each insert created a 1,374-bp insertion with in-frame stop codons in all 3 reading frames. Insertion site 312, which gave the most truncated rOmpA protein (truncated 116 amino acids downstream of the start codon), was chosen for this study. We observed no phenotypic or genotypic difference, aside from insertion location, between *ompA* knockout clones containing insertions at nucleotide 312 or 528 (data not shown).

Coomassie brilliant blue staining of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel with equally loaded protein lysates of wild-type Sheila Smith, Sheila Smith *ompA*::int312, and Iowa demonstrated the absence of the rOmpA band from the Sheila Smith *ompA*::int312 clone and Iowa but its abundance in the wild type (Fig. 3A). Immunofluorescence of infected Vero cells was performed using MAb 13-2 to rOmpB, MAb 13-3 to rOmpA, and a rabbit polyclonal antibody to

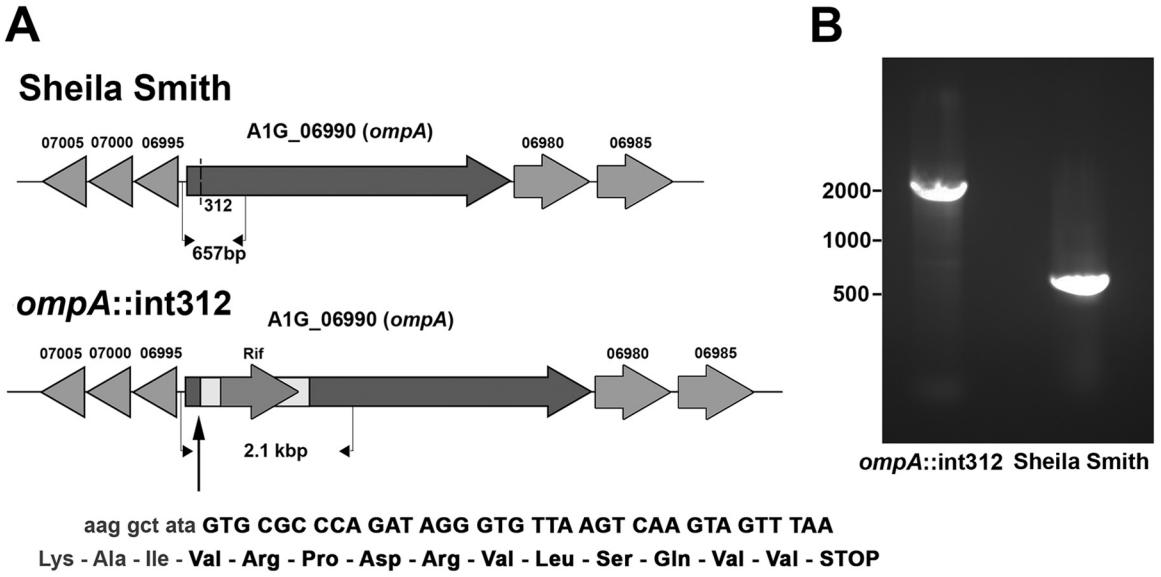

FIG 2 Intron insertion into *ompA*. (A) The insertion target for the group II intron was 312 bp downstream of the *ompA* start site. DNA primers flanking $ompA_{312}$ were used for identification and sequencing of *R. rickettsii* transformant plaques. Sequence data of the intronic disruption of *ompA* revealed a stop codon in the open reading frame 354 bp downstream of the *ompA* start site, predicting a truncated 116-amino-acid rOmpA protein lacking the passenger and autotransporter domains. (B) PCR amplification was performed to identify initial *ompA* mutants. With the correctly targeted insertion present, *ompA* produces a band 1,400 bp larger than the wild-type band.

formalin-fixed *R. rickettsii* (αRr). The staining patterns were consistent for Sheila Smith, Sheila Smith *ompA*::int312, and Iowa; only wild-type Sheila Smith exhibited fluorescence when probed with 13-3 antibody (Fig. 3B). All three populations exhibited a strong signal with 13-2 and αRr antibodies. Western blots using monoclonal antibodies specific to both rOmpA (13-3) and rOmpB (13-2) (8) were performed on protein lysates of density-gradient-purified clonal rickettsial populations using wild-type Sheila Smith as a positive control and Iowa as a negative control. No rOmpA protein was detected via Western blotting in the *ompA*::int312 mutant (Fig. 3C). Immunoblots repeated using additional monoclonal antibodies specific to rOmpA with differing specificities (8) gave results similar to those obtained with 13-3 (data not shown).

Southern blotting was performed to confirm insertion of the group II intron at a single point in the genome (Fig. 3D). Autoradiography revealed a single insertion point in the *ompA*::int312 genome and none in the wild type.

Growth and entry of Sheila Smith *ompA*::int312 in Vero cells. The growth kinetics and invasion of the *ompA*::int312 Sheila Smith mutant were characterized in cell culture prior to commencement of animal trials. Vero cell monolayers were infected with either wild-type Sheila Smith or Sheila Smith *ompA*::int312 and the cultures lysed and replated on fresh monolayers to determine numbers of PFU. There was no significant difference in growth rates between the wild-type and *ompA*::int312 strains (Fig. 4A).

Entry rates were also compared between the mutant and wild type using in/out assays in Vero cells to detect the percentages of intracellular bacteria between 0 and 60 min postinfection (Fig. 4B). No significant difference was detected between the wild-type and mutant strains at any time point, with approximately 55% of rickettsiae from both groups internalized at 30 min. This corresponds with previous measurements of induced phagocytosis in *R. conorii* (35).

Virulence of the *ompA*::int312 Sheila Smith mutant in a guinea pig model. Two independent guinea pig challenge experiments were conducted to assess the fever response of a Sheila Smith *ompA*::int312 mutant (Fig. 4C). The initial challenge involved 5 guinea pigs per group. The lack of attenuation observed in the knockout strain was unexpected; thus, 1 random animal from each of the wild-type and knockout groups was sacrificed at peak fever (day 8) to obtain spleens for culture of rickettsiae to confirm that reversion or selection of wild-type rOmpA had not occurred (Fig. 5). No rOmpA or intact *ompA* was detected in these reisolated rickettsiae by immunofluorescence or PCR, respectively. A second experiment was performed using 6 guinea pigs per group. Both trials were conducted under identical conditions of guinea pig strain/sex/age, rickettsial dose, and fever measurement. Specifically, the wild-type Sheila Smith and Sheila Smith *ompA*::int312 strains were injected intradermally at 100 PFU into female 6- to 8-week-old Hartley guinea pigs and their fever responses monitored. Inoculum was quantitated prior to infection and after infection using both direct and plaque counting. The two trials gave very similar results; thus, the results of both trials were combined to form a single fever response curve. Both wild-type Sheila Smith and the *ompA*::int312 strain induced a fever response, with no significant difference between the wild type and knockout. Recovery of viable rickettsiae from the spleen near the peak of the fever (day 6) showed 1,932 ± 121 and 1,561 ± 260 PFU/g for wild-type Sheila Smith and the *ompA*::int312 strain, respectively.

DISCUSSION

Strains of *R. rickettsii* have a reduced genome (~1.3 Mbp), with high interstrain homology but very different degrees of virulence (7, 26, 36). *R. rickettsii* produces two immunodominant high-

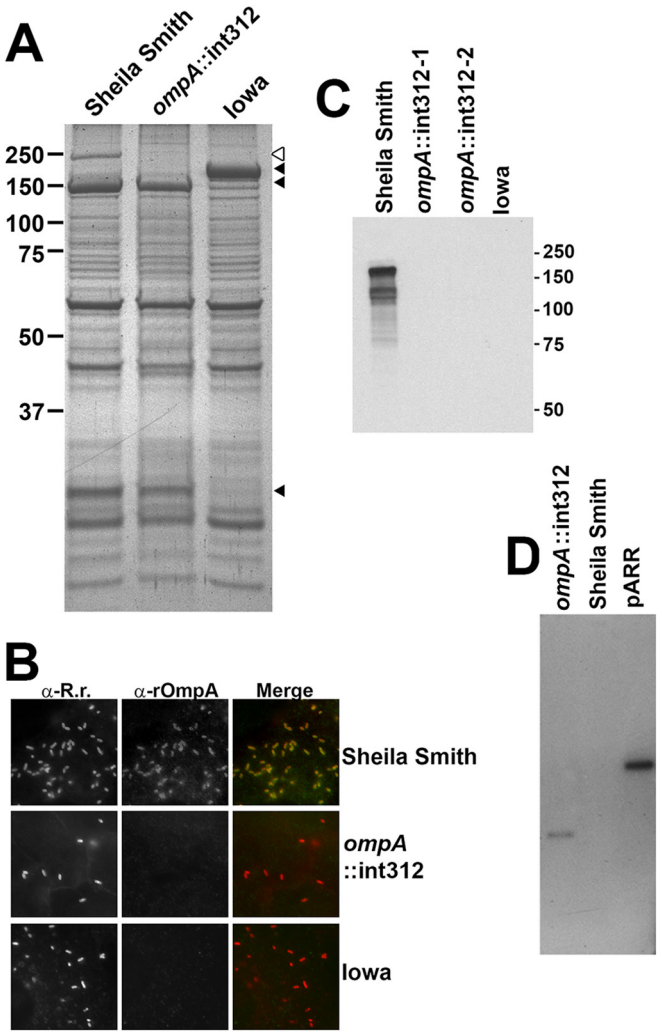

FIG 3 Characterization of *R. rickettsii* Sheila Smith and Sheila Smith *ompA*::int312. (A) SDS-PAGE gel stained with Coomassie brilliant blue. Protein lysates were equalized to 1×10^7 rickettsiae per lane. No rOmpA (210 kDa) was detected in Sheila Smith *ompA*::int312 or Iowa (open arrow) but was detected in Sheila Smith. rOmpB was identified in all three samples. Sheila Smith and Sheila Smith *ompA*::int312 displayed the postprocessed 120-kDa and 32-kDa rOmpB subunits (black arrows), while Iowa displayed the characteristic defective processing of rOmpB, with the predominant form as the unprocessed 168-kDa precursor. Molecular masses (in kilodaltons) are noted at the left. (B) Immunofluorescence of *R. rickettsii* using a species-specific polyclonal antibody and a monoclonal antibody specific to rOmpA. Vero cell monolayers on coverslips were infected with *R. rickettsii* Iowa, Sheila Smith, or Sheila Smith *ompA*::int312 and probed with MAb 13-3 to rOmpA. No rOmpA was detected in any sample of Iowa or Sheila Smith *ompA*::int312. (C) Protein lysates normalized to 1×10^7 rickettsiae per lane were resolved on a 10% polyacrylamide gel, transferred to nylon membranes, and blotted with MAb 13-3 specific to rOmpA. No rOmpA was detected in any sample except Sheila Smith. Molecular masses (in kilodaltons) are noted at the right. (D) Southern hybridization of 5 μg of purified genomic DNA from the *ompA*::int312 mutant and Sheila Smith and linearized pARR plasmid DNA using α-P^{32}-labeled probe specific to the intron marker R*parr-2*. No bands were detectable in the Sheila Smith lane, while a single band was detected in Sheila Smith *ompA*::int312 and linearized-pARR lanes.

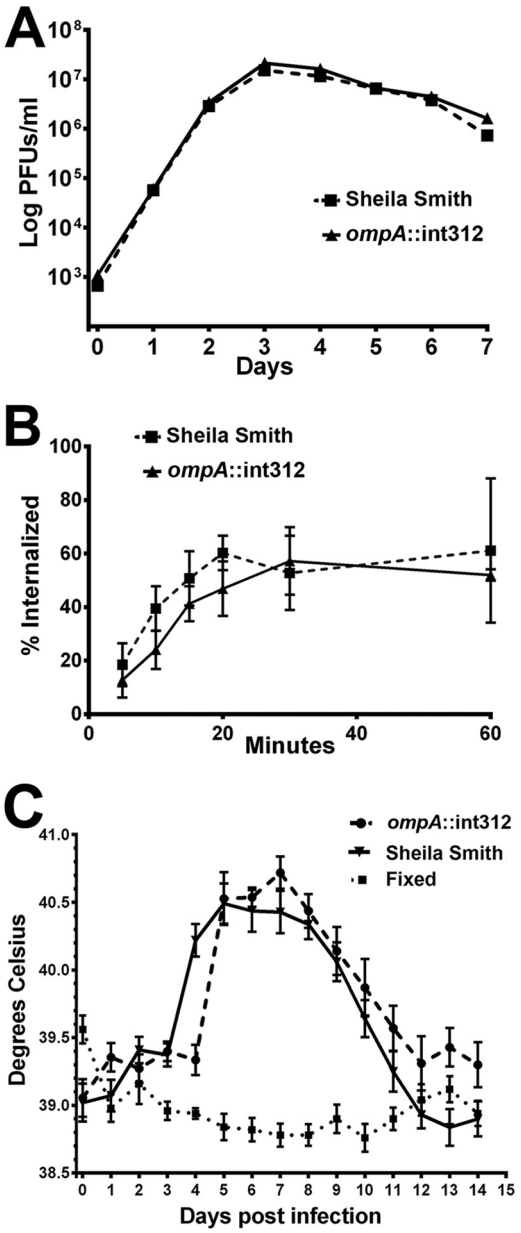

FIG 4 Growth rate and infectivity of the *ompA*::int312 mutant. (A) *R. rickettsii* Sheila Smith and its *ompA*::int312 mutant were assessed for growth in Vero cell culture using a plaque assay. Vero monolayers were infected at an MOI of 0.25 in serial dilutions. No difference in growth was observed between the rOmpA knockout and the wild type, with both strains reaching peak densities at 3 to 4 days. (B) An in/out assay was used to assess internalization. Approximately 50% of the rickettsiae were internalized by 30 min. Each time point was tested in triplicate; no significant difference was detected between Sheila Smith and its *ompA*::int312 mutant. (C) Guinea pig fever responses to challenges with 100 PFU of Sheila Smith, Sheila Smith *ompA*::int312, and fixed Sheila Smith. No significant difference was detected between Sheila Smith and Sheila Smith *ompA*::int312, while fixed Sheila Smith did not produce any observable fever response. Peak fever was observed between days 5 and 7 for both strains, and all guinea pigs were afebrile by day 12.

molecular-mass outer membrane proteins that have been correlated with virulence, rOmpA (190 kDa) and rOmpB (132 kDa) (14, 15, 18, 21, 27, 37–39). To date, however, no molecular basis for the reported differences in virulence has been determined. *R. rickettsii* strain Iowa is the only avirulent strain of *R. rickettsii* with a published genome. A previous genomic comparison of Iowa and virulent Sheila Smith revealed 188 coding nonsynonymous single nucleotide polymorphisms (SNPs) and 47 coding in-

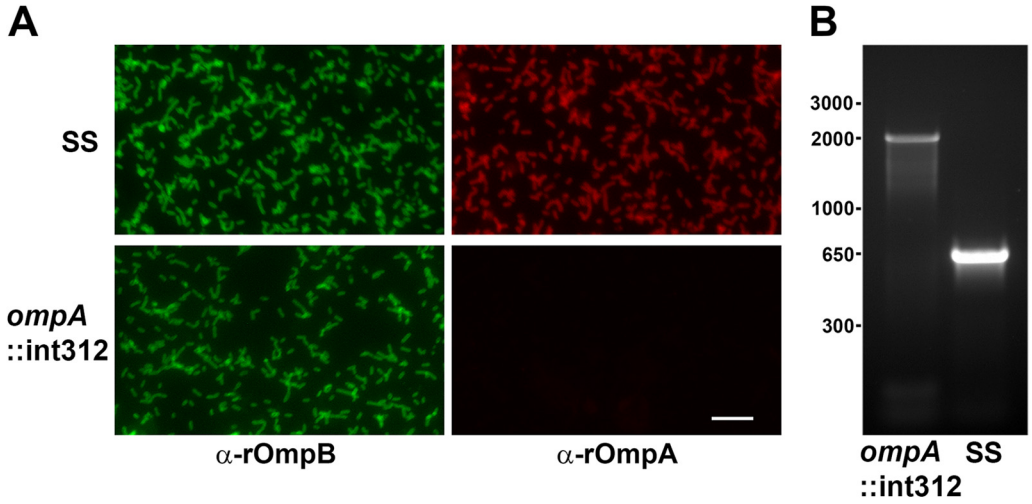

FIG 5 Immunofluorescence microscopy and PCR of guinea pig spleens recovered 8 days after infection with *R. rickettsii* Sheila Smith (SS) and Sheila Smith *ompA*::int312. (A) Immunofluorescence microscopy of *R. rickettsii* isolated and purified from guinea pig spleen removed at peak fever. Slides were incubated with MAb 13-3, specific to rOmpA, as well as polyclonal antibody αRr. No rOmpA fluorescence was observed from spleens isolated from guinea pigs infected with Sheila Smith *ompA*::int312, confirming that no wild-type reversion or mixed-population infections had occurred. Bar = 5 μm. (B) PCR amplification of the *ompA* region of *R. rickettsii* from spleen lysates (taken from guinea pigs during peak fever). No wild-type amplicon was detected in Sheila Smith *ompA*::int312-challenged guinea pigs. Sizes (in bp) are noted at the left.

sertions/deletions (27). One of the notable differences was a deletion in *ompA* in Iowa, leading to an early stop codon and the absence of the protein in the outer membrane. This deletion was the only insertion/deletion unique to Iowa when the comparative analysis was extended to include several other strains (Morgan, R, Hino) of varying virulence and geographic distribution (26). Interestingly, a deletion in *ompA* is also present in avirulent *R. peacockii* compared to its most closely related pathogenic spotted fever group rickettsia, *R. rickettsii* Sheila Smith (37). Despite suggestive evidence that rOmpA may be a virulence determinant in rickettsiae, an isogenic rOmpA knockout of Sheila Smith does not reduce virulence in a mammalian model of Rocky Mountain spotted fever.

In addition to the absence of rOmpA, a feature unique to Iowa is the partially defective proteolytic processing of rOmpB into a mature form (16). Genomic comparisons of Iowa to strains showing normal processing of rOmpB determined that SNPs at and around the cleavage site were not responsible for the loss of processing (16). It was later hypothesized that rOmpA might contribute to the processing of rOmpB into a mature form (27). Here we observed no processing defect of rOmpB in the *ompA*::int312 mutant. Furthermore, rOmpB from the Morgan strain was identical to that in the Iowa strain (26); thus, there are likely other factors involved in the proteolytic processing of rOmpB. There are a number of hypothetical genes in Iowa which have nonsynonymous SNPs compared to the virulent strains Sheila Smith, Morgan, and R (26) which may contribute to the failure of Iowa to effectively process rOmpB. The lack of attenuation of an rOmpA knockout was surprising and counterintuitive in light of results from previous studies of rOmpA in rickettsiae. The *ompA* gene is conserved throughout the SFG rickettsiae despite reduction of the rickettsial genome (27). *ompA* is not found in the typhus group; however, a 369-bp fragment of *ompA* remains in *R. prowazekii* (40). Molecular phylogenetic analysis has indicated that the passenger domain of *ompA* has undergone positive selective pressure (15), suggesting that the protein is active and necessary for some aspect of the natural history of spotted fever group rickettsiae. Although no growth defect was observed in cell culture and no decrease in virulence was observed in an animal model system, it is possible that rOmpA is necessary for maintenance in and dissemination by tick vectors. Arthropod vectors constitute the main environment of *Rickettsia* for the majority of their life cycle, while humans represent a dead-end host. rOmpA does not appear to be critical for the survival and reproduction of spotted fever group rickettsiae in ticks, as *R. peacockii* lacks rOmpA yet was isolated from *Dermacentor andersoni* (37, 41). Although *R. peacockii* is transmitted transovarially in ticks, there is no evidence that it is transmitted from ticks to mammals, and it has not been propagated in mammalian cell culture (41, 42). There are certain considerations that must be addressed when assessing the function of rOmpA in an arthropod host using comparative genomics. There is a significant lack of synteny in *R. peacockii* compared to *R. rickettsii*, and several genes are absent, including *sca1*, the protease II gene, and *rickA* (37). It is likely that rickettsial pathogenesis is a multifactorial process. The presence or absence of rOmpA in the original Iowa isolate is difficult to predict but may have been an artifact of attenuation of Iowa through more than 200 serial egg passages. Iowa was originally isolated from guinea pig spleen following injection with *Dermacentor variabilis* suspensions (43). The initial *D. variabilis* Iowa suspension showed only mild infectivity in Guinea pigs but exhibited greatly increased virulence following 11 to 15 yolk sac (YS) passages. Enhanced virulence was maintained for ~50 YS passages before attenuation was observed. By 1941, Iowa had undergone 240 YS passages and no longer induced fever in guinea pig models of infection (43).

Approximately 40% of the passenger domain of *ompA* is made up of 13 tandem repeats (13, 44). Regularly repeating structural units have been shown to have possible relationships with attachment and invasion (45–48). Several studies have reported the adhesin-like capabilities of rOmpA. Monoclonal antibodies to

rOmpA inhibited rickettsial attachment up to 90% in L-929 cells, and purified rOmpA competitively inhibited attachment (18). A fragment of the rOmpA passenger domain cloned into an *E. coli* heterologous expression system and expressed as a truncated membrane protein was found to interact with the $\alpha 2\beta 1$ integrin, and expression of the truncated rOmpA protein was sufficient to mediate adherence to endothelial cells (49). Although we did not examine the attachment *per se* of the *ompA*::int312 mutant, no invasion or growth defect was observed in Vero cells. This is not surprising, as Iowa also displays no altered growth phenotype in cell culture and replicates at rates equivalent to those of virulent strains (27). The significance of rOmpA's role as an adhesin should be reassessed, as several other adhesins have been identified in *Rickettsia*, suggesting that rickettsial attachment is most likely mediated through multiple proteins (50, 51).

Recent advances in the genetic manipulation of *Rickettsia* have provided unprecedented opportunities to define virulence determinants in rickettsiae. A lack of a media suitable for cell-free growth limits disruption of essential genes for intracellular survival and replication, but certain factors required for virulence, such as Sca2 (52) and phospholipase D (53), can be disrupted without deleterious effects on *in vitro* replication. The ability of avirulent strains to infect without causing apparent disease but conferring protective immunity (27) offers the potential of live vaccine strains. The recognition of a relatively limited number of genetic differences between some virulent and avirulent strains of *R. rickettsii* (26) offers new opportunities to definitively identify additional rickettsial virulence determinants. The possibility of site-directed mutagenesis using a group II intron system allows for a more direct approach to studying rickettsial pathogenesis.

MATERIALS AND METHODS

Plasmid construction. All restriction enzymes and ligases, phosphatases, and DNA polymerases were purchased from New England BioLabs (Beverly, MA) unless otherwise specified. Oligonucleotides and primers used in this study were purchased from Integrated DNA Technologies (Skokie, IL) unless otherwise specified. Primer and probe sequences are shown in Table S1 in the supplemental material. All cloning was performed in *E. coli* DH5α MAX Efficiency competent cells (Life Technologies, Carlsbad, CA).

The TargeTron pACDK4-C plasmid was purchased from Sigma-Aldrich (Atlanta, GA) and modified for intron integration in rickettsiae (Fig. 1). The kanamycin RAM cassette for postintegration selection was removed by digestion with MluI. The Rp*arr-2* gene, encoding rifampin resistance, was PCR amplified from the rickettsial transposon vector pMW1650 (54) using primers with 5′ MluI sites. Rp*arr-2* was digested with MluI and ligated into pACDK4-C to make pACDR4-C.

The chloramphenicol acetyltransferase resistance cassette, *cat*, located on the pACDK4-C plasmid backbone and originally used for selection in *E. coli*, was removed. Chloramphenicol is used as a secondary treatment option for RMSF, and genes conferring resistance are not suitable as recombinant material for rickettsial transformation. The *cat* gene was removed by whole-plasmid PCR amplification of the pACDR4-C plasmid using primers pACD CATrmv F and pACD CATrmv R with 5′ XmaI sites and sequences specific to the up- and downstream regions immediately flanking *cat.* Direction of amplification proceeded away from the *cat* gene so that a linear DNA product of 6,013 bp was formed after PCR amplification. The DNA product was purified and digested with XmaI and then allowed to self-ligate in a large-volume (100-μl) ligation reaction to promote intraligation events and create the recircularized plasmid pADR4-C. Resistance to penicillin provides a suitable alternative for plasmid propagation in *E. coli*, as β-lactams are ineffective against rickettsiae (55; unpublished data) and not used for treatment of RMSF. The penicillin resistance gene *bla* was amplified from plasmid pBOMB4 (56) using primers pACD Bla Xma F and pACD Bla Xma R, containing 5′ XmaI restriction sites. pADR4-C and the purified *bla* PCR product were digested with XmaI and ligated to form the penicillin-resistant plasmid pAR4-C.

The T7 promoter was removed through digestion of pAR4-C with ClaI and HindIII and replaced with a polylinker containing 3 unique restriction sites for the modularity of promoters used to drive the group II intron. The polylinker contained (from 5′ to 3′) ClaI, DraIII, and HindIII sites and was inserted upstream of the 5′ exon by digestion of pACDR4-C with ClaI and HindIII. A 39-bp synthetic oligonucleotide containing an internal DraIII restriction site as well as ClaI and HindIII restriction sites at the 5′ and 3′ ends, respectively, was ordered along with the reverse complement. The polylinker was created by hybridizing the two individual oligonucleotides together as follows. Two micrograms of each oligonucleotide was added to 1× annealing buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 5 mM EDTA), heated at 95°C for 5 min, and then annealed by slowly cooling the reaction mixtures to room temperature (approximately 45 min). The polylinker was digested with ClaI and HindIII and ligated to the cut, phosphorylated vector pAR4-C to create pARL-C. The strong rickettsial *rpsL* promoter (29) was amplified from pMW1650 using primers pACD rpsL F/R, containing DraIII and HindIII sites incorporated into the forward and reverse primers, respectively. The PCR product was digested with DraIII and HindIII, purified, and then ligated to a DraIII/HindIII-digested pARL-C vector to create pARR-C.

The TargeTron software algorithm (http://www.sigma-genosys.com/targetron/) (Sigma Aldrich), which predicts high-specificity group II intron insertion points in DNA sequences, was used to determine appropriate sites of intron insertion into *ompA*. Only two sites in the 6,747-bp open reading frame were identified, with E values under 0.5 at 312 and 528 bp downstream of the start codon. These sites had E values of 0.419 and 0.469, respectively. The EBS2, EBS1d, and IBS primers that were needed to retarget the pARR-C vector to these two target sites were ordered, and pARR-C was targeted to each site according to TargeTron's suggested protocol.

Bacterial strains, growth, and purification. *R. rickettsii* strains Sheila Smith and Iowa (43) were grown and propagated in Vero cells in M199 medium and purified by Renografin density gradient centrifugation (57). Growth curves were performed by infecting wild-type and *ompA*::int312 Sheila Smith into Vero cell monolayers in T-25 flasks, with one flask infected for each day of the growth curve. Flasks were scraped on the appropriate day, and cells were lysed by disruption using a MiniBeadBeater (Biospec, Inc., Bartlesville, OK) for 10 s using 1-mm glass beads. Lysates containing either Sheila Smith or Sheila Smith *ompA*::int312 were then serially diluted for a plaque assay (58) to determine PFU and acridine orange stained for direct rickettsial counts.

Transformation and clonal isolation. Purified *R. rickettsii* cells were transformed with pARR-C::312 or pARR-C::528 as previously described (52, 54), with some changes. Specifically, approximately 10^9 PFU of *R. rickettsii* were electroporated with 10 μg of either pARR-C::312 or pARR-C::528, immediately plated onto Vero cell monolayers, and then allowed to infect for 30 min at 37°C, after which M199 medium was added. After 9 h, medium was removed and replaced with M199 medium containing 5% fetal bovine serum (FBS), 0.05% agarose, and 200 ng/ml rifampin. The infection was allowed to progress until plaque formation (approximately 10 days). Clonal transformants were obtained by 4 repetitions of picking individual plaques, expanding the plaques in Vero cell monolayers with M199 and 200 ng/ml rifampin for PCR verification, and then recloning as previously described (52, 58).

Genotypic analysis. Genomic DNA was purified from clonal transformants as previously described (52). PCR was run using primers ompAChk-F/R, which flank and amplify the first 657 bp of the *ompA* gene to detect insertions in either the bp 312 or bp 528 site. Genomic DNA was sequenced using the same primers and performed by the Rocky Mountain Laboratories Genomics Unit (Hamilton, MT).

In/out assay. Rates of entry were determined essentially as described previously (17). Specifically, Vero cells were seeded onto coverslips at 1×10^5 cells per well. After 18 h, the medium was removed and cells were infected with wild-type or *ompA*::int312 Sheila Smith at a multiplicity of infection (MOI) of 10 in 100 μl of Dulbecco's modified Eagle's medium (DMEM) in triplicate for each time point. Synchronization of bacterial attachment was accomplished by centrifugation at 400 $\times$ *g* for 5 min at 12°C, followed by incubation at 37°C in 5% CO_2. Cells were fixed in 3.7% paraformaldehyde (PFA). A 60-min time point was also included. Following fixation, coverslips were probed with monoclonal antibody (MAb) 13-2, washed, permeabilized, and reprobed with a rabbit polyclonal antibody to formalin-fixed *R. rickettsii* (αRr). After being washed, coverslips were incubated with secondary anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594. Coverslips were observed under a Nikon Eclipse 80i microscope, and rickettsiae staining positive for αRr and negative for 13-2 were considered internalized while bacteria positively stationed for both antibodies were considered external. A total of 200 rickettsiae were counted for each time point, and results are expressed as percentages of internalized rickettsiae.

Southern blotting. Five-microgram samples of genomic DNA of wild-type and *ompA*::int312 Sheila Smith were digested to completion with HindIII, which cuts the 1.23-Mbp genome into an average size of 1,250 bp. Five hundred nanograms of the purified pARR vector was also linearized by digestion with HindIII and used as a positive control. Digested DNA was resolved in a 5-mm-thick 1% agarose gel and stained with ethidium bromide. The ladder standards were marked, and the DNA was transferred to a Hybond-N (GE Healthcare, Pittsburgh, PA) nylon membrane via capillary transfer with 20$\times$ SSC (3M NaCl, 0.3 M sodium citrate, pH 7.0) overnight. UV cross-linking was accomplished using a Stratalinker 1800 (Stratagene, Los Angeles, CA) set to auto-cross-link for 40 s. The Hybond-N membrane was dried after irradiation and immediately probed.

The 458-bp probe specific to Rp*arr-2* was generated using primers RifF and RifR to amplify the Rp*arr-2* fragment using pARR-C as a template. The Rp*arr-2* probe template DNA was combined with dCTP α-^{32}P (3,000 Ci/mmol, 10 mCi/ml) (PerkinElmer, Shelton, CT) in a synthesis reaction performed using a DECAPrime II labeling kit (Life Technologies) and then purified using Illustra microspin G-25 Sephadex columns (GE Healthcare, Pittsburgh, PA). Half the column eluent (approximately 5 μl) was used to probe the nylon membrane. After hybridization, the probed nylon membrane was dried and exposed to autoradiography film CL-Xposure film (Thermo Scientific, Atlanta, GA) for 12 h.

Antibodies, immunoblotting, and immunofluorescence. Western blots were performed using MAbs 13-2 (to rOmpB) and 13-3 (to rOmpA) as previously described (8). Specifically, purified rickettsiae were prepared by heat inactivation and resuspended in Laemmli buffer to a concentration of 10^9 rickettsiae/ml. Ten microliters of each sample was loaded onto 1-mm 10% polyacrylamide gels, run at 120 V until completion, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were probed with a 1:1,000 MAb dilution and washed, and peroxidase-conjugated anti-mouse secondary antibody was applied. After a final wash, blots were developed using an ECL Prime detection kit (GE Healthcare) and exposed to CL-Xposure film (Thermo Scientific).

Immunofluorescence was observed using MAbs 13-2, 13-3 (8), and a rabbit polyclonal antibody, αRr. Vero cells were grown on coverslips and infected with rickettsiae at an MOI of 5 overnight at 37°C in M199 medium. Monolayers were fixed in 3.7% PFA and permeabilized in phosphate-buffered saline (PBS) with 0.01% Triton X-100 and 0.05% SDS. Fixed coverslips were stained with αRr and either 13-2 or 13-3, washed, and reprobed with anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594. Images were acquired on a Nikon Eclipse 80i microscope with a 60$\times$ 1.4-numerical-aperture oil immersion objective and a Nikon DS-Qi1Mc camera.

Guinea pig infection. All animal work protocols used in this study were approved by the Rocky Mountain Laboratories Animal Care and Use Committee prior to the initiation of experimentation. Six-week-old Hartley strain guinea pigs were purchased from Charles River Laboratories (Wilmington, MA) and housed in the animal biosafety level 3 laboratory. Guinea pigs were inoculated intradermally with 100 PFU of either wild-type *R. rickettsii* Sheila Smith, Sheila Smith *ompA*::int312, K36 (control), or formalin-fixed *R. rickettsii* Sheila Smith (control). The remainder of the inoculum was immediately replated onto Vero cell monolayers and then assessed for PFU numbers by plaque assay. Temperatures were monitored rectally for 14 days beginning the day of infection.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00323-15/-/DCSupplemental.

Table S1, DOCX file, 0.02 MB.

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