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 retrohoming 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.

*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 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...
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* (Rparr-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 S’ 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 Rparr-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 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 staining of infected Vero cells was performed using MAb 13-2 to rOmpB, MAb 13-3 to rOmpA, and a rabbit polyclonal antibody to

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**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 Rparr-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.
formalin-fixed *R. rickettsii* (αRt). 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 *H9251* 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-
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-
rOmpA Is Nonessential in Mammals

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.
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 α2β1 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 Rpar-2 gene, encoding rifampin resistance, was PCR amplified from the rickettsial transposon vector pMW1650 (54) using primers with 5′ MluI sites. Rpar-2 was digested with MluI and ligated into pACDK4-C to make pACDK4-C.

The chlorohemical acetyltransferase resistance cassette, cat, located on the pACDK4-C rickettsial backbone and originally used for selection in E. coli, was removed. Chlorohemichromesil 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 pACDK4-C plasmid using primers pACD CATmrv F and pACD CATmrv R with 5′ Xmal 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 Xmal and then allowed to self-ligate in a large-volume (100–μl) ligation reaction to promote intragenomic events and create the recircularized plasmid pACDK4-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′ Xmal restriction sites. pADR4-C and the purified bla PCR product were digested with Xmal and ligated to form the penicillin-resistant plasmid pAR4-C.

The T7 promoter was removed through digestion of pAR4-C with Clal and HindIII and replaced with a polynucleotide containing 3 unique restriction sites for the modularity of promoters used to drive the group II intron. The polynucleotide contained (from 5′ to 3′) Clal, DraIII, and HindIII sites and was inserted upstream of the 5′ exon by digestion of pACDK4-C with Clal and HindIII. A 39-bp synthetic oligonucleotide containing an internal DraIII restriction site as well as Clal and HindIII restriction sites at the 5′ and 3′ ends, respectively, was ordered along with the reverse complement. The polynucleotide 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 polynucleotide was digested with Clal and HindIII and ligated to the cut, phosphorylated vector pACR4-C to create pAR4-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 pAR4-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 CBS 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 Mimi-BeadBuster (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 plasmid isolation. Purified R. rickettsii cells were transformed with pARR-C:C::312 or pARR-C:C::528 as previously described (52, 54), with some changes. Specifically, approximately 10⁶ PFU of R. rickettsii were electroprogrammed with 10 μg of either pARR-C:C::312 or pARR-C: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 mg/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 × 10⁴ cells per well. After 18 h, the medium was removed and cells were infected with wild-type or oompA::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 × g for 5 min at 12°C, followed by incubation at 37°C in 5% CO₂. 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 oompA::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 1% agarose gel and stained with ethidium bromide. The ladder standards were marked, and the DNA was transferred to a Hybond-N membrane (GE Healthcare, Pittsburgh, PA) 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 Rparr-2 was generated using primers RfRf and RfRi to amplify the Rparr-2 fragment using PARR-C as a template. The Rparr-2 probe template DNA was combined with dCTP α-32P (3,000 C/mmol, 10 μCi/ml) (PerkinElmer, Shelton, CT) in a synthesis reaction performed using a DECAPrime II labeling kit (Life Technologies) and then purified using Iffilia 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 (Hyperfilm, Amersham) at −80°C in the dark. Membranes were stained with 0.1% amido black to visualize the hybridized DNA. Internal control bands were also included in the membranes.

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.

ACKNOWLEDGMENT

This work was supported by the Intramural Research Program of the NIAID, NIH.

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