Rickettsia peacockii sp. nov., a New Species Infecting Wood Ticks, Dermacentor andersoni, in Western Montana†

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Rickettsia peacockii, a new species of spotted fever group rickettsiae, was identified from Rocky Mountain wood ticks (Dermacentor andersoni) collected in the Sapphire Mountain Range on the eastern side of Bitterroot Valley, Montana. DNA from R. peacockii Skalkaho† (T = type strain) in naturally infected tick tissue was amplified by a PCR assay with primer sets derived from subcapsular 16S ribosomal DNA (rDNA), rickettsial citrate synthase, and 190-kDa surface antigen (rOmpA) genes. Partial 16S rDNA and rOmpA gene sequences exhibited levels of similarity of 99.7 and 93.2%, respectively, with the sequences of the spotted fever agent Rickettsia rickettsii. By using Giménez staining, fluorescent antibody tests, a PCR assay, and a restriction fragment length polymorphism analysis, 76 of 115 female ticks (minimal field infection rate, 66.1%) collected between 1992 and 1995 were found to be infected. The organism is passed transstadially and transovarially (minimal vertical transmission rate, 73.3%), and infections are localized in ovarian tissues. Attempts to cultivate R. peacockii were unsuccessful.

Rickettsiae are gram-negative bacteria that are maintained in nature by replication in cells of small mammals and arthropods (5, 7, 23, 32, 47). At least 23 Rickettsia species (alpha subdivision of the Proteobacteria, family Rickettsiaceae) have been or are being described (40, 43), while undoubtedly more remain to be discovered. Of these species, Rickettsia rickettsii, the etiological agent of Rocky Mountain spotted fever (RMSF), is the most significant public health threat in the western hemisphere. Human RMSF cases were first recognized in 1873 in Bitterroot Valley in western Montana (34). Through a series of classic experiments begun in 1906, H. T. Ricketts and coworkers clearly established that the etiology of RMSF involves R. rickettsii in Rocky Mountain wood ticks (Dermacentor andersoni) (39). Transmission to humans occurs principally through bites of infected ticks. Between 1981 and 1992, 4,217 confirmed human RMSF cases (minimum case/fatality ratio, 4.0%) were reported in the United States (9).

Since the pioneering work of Ricketts, several other species of rickettsiae have been recognized in Bitterroot Valley. A survey of 3,705 adult D. andersoni ticks collected in the valley during 1977 revealed four distinct serotypes of hemocyte-associated rickettsiae. The results of DNA sequencing and a restriction fragment length polymorphism (RFLP) analysis, techniques which have been successful in helping to characterize rickettsiae and other bacteria associated with hematophagous arthropods (1, 3, 10, 14, 20, 38, 41, 46), indicated that the organism is a unique SFG rickettsial species. The sequence results suggest that strain Skalkaho† (T = type strain) either lacks or has a truncated form of the major rickettsial 190-kDa surface antigen (rOmpA). Unsuccessful attempts to cultivate the bacterium are also described below.

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† This paper is dedicated to Mort G. Peacock in recognition of his lifelong contributions to rickettsiology, inimitable cell culture talents, and help provided to us and many others in studying rickettsiae.
‡ Present address: Division of Zoonotic Diseases, Centers for Disease Control and Prevention, Ft. Collins, CO 80522.
Individual heat-fixed smears of tissue samples were stained by the Gimbénez method (15) and were microscopically examined under oil immersion (magnification, ×1,000) for rickettsiae. In the IFA test performed to confirm rickettsial infections, smears of each aseptically collected tissue sample were stained with anti-rickettsia guinea pig serum and then with fluorescein isothiocyanate-labeled mouse anti-guinea pig serum (31). A second IFA test was selectively performed on fixed ovular tissues from field-collected ticks (Skalkaho and lab colony ticks) (RML-295 and RML-795) which were used to obtain sequence data for the bacterium. In this IFA test, acetone-fixed tissue smears were stained separately with monoclonal antibody (Mab) 13-2 to rOmpA and Mabs 14-13 and 13-3 to rOmpA as described previously (2). Samples were then stained with fluorescein isothiocyanate-labeled rabbit anti-mouse serum. Each conjugate was diluted 1:50 in sterile D2O, incubated for 30 min at 27°C, and then washed three times in phosphate-buffered saline. Cell-cultured R. rickettsii and rickettsia-free tick ovular tissues were included in IFA tests as positive and negative controls, respectively. For comparison, R. rickettsii and five other rickettsial strains were stained with Mabs. IFA slide preparations were examined with a fluorescence microscope under oil immersion (magnification, ×1,000) for evidence of rickettsiae.

Morphological studies. Tick ovarian tissues from infected (RML-295 and RML-795) and uninfected (RML-195) females were examined for rickettsiae with a transmission electron microscope (model CM-10; Phillips). The tissues were fixed overnight in 4% paraformaldehyde–2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4). Cells were postfixed in 0.5% osmium tetroxide–0.8% potassium ferricyanide and then in 1% aqueous tannic acid and stained overnight in bloc 1% aqueous uranyl acetate. Samples were dehydrated through a graded ethanol series and embedded. Spurr’s sections were cut with an ultramicrotome (model RMC MT-7000), stained with 1% aqueous uranyl acetate and Reynolds’s lead citrate, and observed at 80 kV.

Isolation and cultivation attempts. Tick ovarian tissues (ten samples) collected with Skalkaho were triturated with glass grinders in 5 ml of 4% sucrose–phosphate–glutamate buffer. Next, 0.25 ml of the resulting suspension was inoculated into a tripodically six-into-one flask (M. pennsylvanicus) and 1 ml into five-into-one flasks containing four male and six male mice (Mus musculus). Blood, liver, spleen, kidney, and tunica vaginalis tissue samples were removed on day 5 postinoculation. Blood clots and tissues were first screened for rickettsial infection by performing an IFA test. Samples were then trypsinized separately in 5 ml of sucrose-phosphate-glutamate buffer, and 0.25 ml of each suspension was inoculated into Vero, M. pennsylvanicus tunica vaginalis (MTV), and Porousus tridens marquesi kidney epithelial (PTK2) cell monolayers. In parallel, infected tick ovarian tissues (nine samples) were triturated in 4 ml of BHI and 0.2 ml of each suspension and two 10-fold serial dilutions were inoculated directly onto cell monolayers (28). The cell lines inoculated were Vero, MTV, PTK2, D. albipunctatus tick embryo, primary chicken embryo (PCE), mouse connective fibroblast, and mouse histiocytoma cell lines. Cultivation in chicken embryos was attempted previously without success (8). We attempted to establish a primary cell line from tick ovarian tissues by using standard methods (50).

Cells were cultivated in closed 75-cm² flasks (Corning, Inc., Corning, N.Y.) that were incubated at 34°C and contained 40 ml of M199 medium (Gibco, Grand Island, N.Y.) supplemented with 2% fetal bovine serum and 10 mM l-glutamine. Tick cell cultures were grown in a special medium (25). Inoculated MTV and tick cells were also grown at 27°C. In addition, separate flask containing inoculated Vero, MTV, and PTK2 cell cultures were supplemented with 0.005% dextran sulfate, 0.01% hemin, and 0.025% ferric pyrophosphate (Sigma Chemical Co., St. Louis, Mo.). All cell cultures were inoculated with mammalian or ovarian tissue samples in duplicate. One of each of these cell cultures was randomly screened for the presence of ricketsiae by Gimbénez staining and IFA test on days 4 through 20 postinoculation. Cells in the second flask were harvested on day 10 postinoculation and passed into Vero and MTV cells (28). A maximum of four sequential passages were performed.

A modification of the centrifugation-shell vial technique was also employed in attempts to isolate and cultivate the organism (19). Briefly, infected ovarian tissue triturates were inoculated in triplicate into shell vials containing Vero, MTV, and PCE cell monolayers on M199 medium containing 2% fetal bovine serum and 10 mM l-glutamine. The shell vials were screened for the presence of ricketsiae on days 4 and 7 postinoculation. If one shell vial was found to be infected with rickettsia-like organisms, cells from a duplicate vial were passage in new shell vials.

Attempts to plaque purify the organism were performed by using standard methods (49). Vero cells grown in six-well trays (Corning) with agar overlays were inoculated with serial dilutions of infected ovarian tissue (two samples) suspended in 1 ml of BHI. One shell vial was observed daily for 2 weeks for evidence of plaque formation.

PCR assay. Tick reproductive tissues, salivary glands, amputated legs, midguts, Malpighian tubules, midgut, and hypodermal tissues of adult male ticks were also tested. Portions of individual tick tissues were either smeared on slides and fixed for staining and the IFA test or stored in 200 μl of BHI at –20°C for the PCR assay.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nucleotide sequence accession no.</th>
<th>R OmpA sequence</th>
<th>16s rDNA sequence</th>
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</tr>
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<td></td>
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<td>U21460</td>
<td></td>
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<td>U43795</td>
<td>L36213</td>
<td></td>
</tr>
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<td></td>
</tr>
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<td>U43807</td>
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<td>IXA</td>
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* The sequences compared correspond to positions 91 through 602 and positions 29 through 1185 on the forward strand of the R. rickettsii R OmpA and 16s rDNA genes, respectively.

** SFG rickettsial strain used in RFLP analyses.

† The partial rOmpA gene sequences of R. peacocki generated from four infected ticks (laboratory colony tick RML-295 and field-collected ticks KG-392, SNK-10, and SNK-295) were identical and were represented by a single sequence submitted for Skalkaho.

‡ Sequence generated in this study (all other sequences were obtained from GenBank).

§ The two 16s rDNA sequences of R. peacocki generated from two separate ticks (for both the rOmpA and 16s rDNA genes) submitted for Skalkaho.

†† Not applicable.

### MATERIALS AND METHODS

**Rickettsial strains.** The rickettsial species described here was characterized directly from infected D. andersoni tick ovaries, and Skalkaho was designated the type strain (the strain designation refers to the local from which infected ticks were collected). For comparison, a number of SFG rickettsial strains were also employed in this study (Table 1); these strains included several organisms isolated from ticks in Montana (4, 30, 33).

**Ticks and screening of tick tissues for rickettsiae.** Adult D. andersoni ticks were collected during May 1992, 1994, and 1995 by dragging a white flannel cloth (area, 1 m²) across vegetation where ticks were questing for hosts. All ticks were reared to adulthood (21).

Following oviposition, female ticks were surface sterilized by sequentially washing them for 10 min in hydrogen peroxide, 70% ethyl alcohol, and sterile distilled water (D2O). Both field-collected and laboratory colony ticks were screened for rickettsiac infections. First, ticks were tested for the presence of hemocytic-associated rickettsiae by hemolymph test (16). These amputated legs were stored individually in 100 μl of brain heart infusion (BHI) at –20°C for a subsequent PCR assay. Next, tick salivary glands and ovarian tissues were removed with sterile forceps, washed three times in sterile D2O, and tested for the presence of rickettsiae. Malpighian tubules, midguts, and hypodermal tissues of a small proportion of the dissected ticks were also removed and screened for the presence of rickettsiae. Similarly, hemolymph, salivary glands, testicular tissues, Malpighian tubules, midguts, and hypodermal tissues from adult male ticks were also tested. Portions of individual tick tissues were either smeared on slides and fixed for staining and the IFA test or stored in 200 μl of BHI at –20°C for the PCR assay.
TABLE 2. Summary of R. peacockii infections in tissues from field-collected and laboratory colony D. andersoni ticks as determined by Giménez staining, IFA tests, and PCR assays

<table>
<thead>
<tr>
<th>Ticks</th>
<th>Sex</th>
<th>Hemolymph</th>
<th>Salivary glands</th>
<th>Reproductive tissue</th>
<th>Malpighian tubules</th>
<th>Midguts</th>
<th>Hypodermal tissues</th>
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<td>Field collected</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Female</td>
<td>0/NT/0 (115)</td>
<td>0/0/0 (115)</td>
<td>76/76/81 (115)</td>
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<td>0/0/0 (36)</td>
<td>0/0/0 (36)</td>
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<tr>
<td>Laboratory colony</td>
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<td></td>
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<tr>
<td>Female</td>
<td>0/NT/0 (45)</td>
<td>0/0/0 (45)</td>
<td>33/33/34 (45)</td>
<td>1/1/0 (10)</td>
<td>1/1/0 (10)</td>
<td>0/0/0 (10)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0/NT/NT (65)</td>
<td>0/0/0 (65)</td>
<td>0/0/NT (65)</td>
<td>0/NT/NT (65)</td>
<td>0/NT (65)</td>
<td></td>
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</tr>
</tbody>
</table>

a In the IFA tests a polyclonal antibody to R. rickettsii R was used, and in the PCR assays both rickettsial CS and rOmpA gene primer sets were used.

b Five ovarial tissue samples that were negative for rickettsiae as determined by Giménez staining and IFA tests were positive as determined by PCR assays. The organisms in these samples and 35 other samples were identified as R. peacockii by an RFLP analysis. All tissues that were positive for rickettsiae gave amplification products with both primer sets.

c Number of positive samples as determined by Giménez staining/number of positive samples as determined by IFA tests/number of positive samples as determined by PCR assays (number of samples tested). NT, not tested.

RESULTS

Massive infections of Skalkaho were observed only in D. andersoni ovarial tissues. Of 115 field-collected ticks, 76 were infected (minimal field infection rate, 66.1%), with ovarial tissues testing positive for rickettsiae by Giménez staining, an IFA test in which a polyclonal antibody to R. rickettsii was used, and PCR assays in which two separate rickettsial gene primer sets were used (Table 2). Identical tests performed with laboratory colony ticks revealed that 33 of 45 female offspring (minimal transovarial transmission rate, 73.3%) from the same parent were infected through the adult stage (Table 2). Transstadial transmission was also observed as Skalkaho has been maintained in laboratory ticks for two generations.

No rickettsiae were detected by the PCR assays in adult female tick hemocytes, salivary glands, midguts, Malpighian tubules, or hypodermal tissues (Table 2). However, individual, nonintracellular, and very rare rickettsia-like organisms were observed associated with a few female tick midgut tissues and Malpighian tubules following nonspecific Giménez and IFA staining (Table 2). A PCR assay performed with the rickettsial CS gene and rOmpA gene primer sets failed to amplify any products from these samples, suggesting that they were not infected with SFG rickettsiae. The identity of the organisms found remains uncertain. The hemolymph, salivary glands, testicular tissues, Malpighian tubules, midguts, and hypodermal tissues of field-collected and laboratory colony adult male ticks were also apparently free of rickettsial infection (Table 2). In contrast, in both males and females the same tick tissues are susceptible to infection with R. rickettsii and other SFG rickettsiae (14, 16, 29).

The infection rates in tissues stained by the Giménez method were identical to those determined by IFA tests in which a polyclonal antibody to R. rickettsii was used. In PCR assays performed with the rickettsial CS gene and rOmpA gene primer sets and these samples we detected rickettsiae in all of the tissues that were positive as determined by staining and IFA tests, and amplification products of the appropriate sizes were generated by both primer sets. An additional five samples that were negative as determined by staining and IFA
tests gave PCR amplification products whose RFLP profiles were consistent with those of SkalkahoT.

The reactivities of SkalkahoT and several other SFG rickettsial strains to MAbs are summarized in Table 3. Both heat-sensitive MAb 14-13 and heat-resistant MAb 13-5 to rOmpA failed to bind to SkalkahoT, while at least one of the two MAbs bound strongly to all other rickettsial strains tested (Table 3). Conversely, MAb 13-2 to rOmpB bound strongly to SkalkahoT and related rickettsiae with MAbs to rOmpA and rOmpB

The levels of sequence similarity between the new organism and other rickettsiae were comparable to the levels of similarity observed among the SFG relatives of SkalkahoT (Table 4). The 16S rDNA sequence of SkalkahoT was most similar (level of similarity, 99.7%) to the sequences of R. peacockii, R. japonica, and R. rickettsii, followed by R. slovaca (99.3%) and R. conorii (99.4%). The sequence of SkalkahoT was 99.7% similar to the sequence of R. rickettsii and 99.6% similar to the sequence of R. peacockii.

### Table 3. Reactivity of R. peacockii SkalkahoT and related rickettsiae with MAbs to rOmpA and rOmpB

<table>
<thead>
<tr>
<th>Strain</th>
<th>rOmpA MAb 13-2</th>
<th>rOmpA MAb 14-13</th>
<th>rOmpA MAb 13-5</th>
</tr>
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<tbody>
<tr>
<td>R. peacockii SkalkahoT</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R. rickettsii</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R. rickettsii HLP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R. conorii Malish 7</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>R. montana M5-6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R. rhipicephali 85TW</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>WB8-2</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

- *Infected ovarian tissues from laboratory colony ticks (RML-295 and RML-795) and field-collected ticks (SK-594 and SK-295) were tested and elicited identical reactivities.

### Table 4. Levels of 16S rDNA gene sequence similarity for R. peacockii SkalkahoT and several SFG rickettsiae

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</tr>
<tr>
<td>R. slovaca 13-B</td>
<td>100</td>
<td>99.7</td>
<td>99.7</td>
<td>99.4</td>
<td>99.2</td>
<td>99.3</td>
<td></td>
</tr>
<tr>
<td>R. conorii Malish 7</td>
<td>100</td>
<td>99.6</td>
<td>99.3</td>
<td>99.3</td>
<td>99.3</td>
<td>99.0</td>
<td></td>
</tr>
<tr>
<td>R. japonica YM</td>
<td>100</td>
<td>99.2</td>
<td>99.0</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>R. montana M5-6</td>
<td>100</td>
<td>99.0</td>
<td>99.0</td>
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</table>

* The two partial 16S rDNA sequences determined for R. peacockii, a sequence amplified from a tick (SK-594) collected in 1994 and a cloned sequence from a tick collected in 1995 (SK-295), were identical.

Attempts to cultivate organisms in mammals and cell cultures failed to establish rickettsial colonies. The organism was rare but detectable by PCR and IFA tests through three passages in MTV cells. No rickettsia-induced plaques were detected in Vero cells with agar overlays. Attempts to establish a primary D. andersoni tick ovarian tissue cell line were also unsuccessful.

Transmission electron microscopy of D. andersoni ovarian tissue revealed packets of putative SkalkahoT cells replicating intracellularly (Fig. 1) and within developing eggs. The organism was also abundant in the interstitial spaces of the ovaries and was observed only in those ovarian tissues that were positive for rickettsiae as determined by staining and PCR. These rod-shaped rickettsiae were approximately 1.2 μm long and 0.4 μm wide.

The partial 16S rDNA sequence of SkalkahoT generated from a PCR fragment amplified from a tick collected in 1994 was identical to the sequence of a cloned PCR product amplified from a tick collected in 1995. For the aligned sequences, the base signatures for SkalkahoT were T and C at positions 474 and 532, respectively, on the forward strand of the 16S rDNA gene sequence. Furthermore, the four partial rOmpA gene sequences generated for the bacterium from three separate ticks collected in 1992, 1992, and 1995, as well as a laboratory colony tick, were identical. The rOmpA gene sequence of SkalkahoT was most similar (93.0%) to the sequence of R. slovaca 13-B (Table 5). A lower level of similarity (91.6%) was observed with R. rickettsii HLP and R strains (Table 5). For comparison, the rOmpA gene sequence of R. slovaca 13-B exhibits 93.0% similarity to the Rickettsia japonica YM sequence, while the R. rickettsii R sequence exhibits 93.2

**FIG. 1.** Transmission electron micrograph of R. peacockii SkalkahoT (arrows 1) in a D. andersoni ovarian cell beside a developing tick egg (arrowhead 2). Magnification, ×39,000. Bar = 1 μm.
and 92.6\% similarity to the R. conorii Malish 7 and R. japonica YM sequences, respectively (Table 5).

The partial sequence of the rOmpA gene of Skalkaho\(^7\) indicated that there was a deletion of a single base at either position 403 or position 404 on the forward sense strand. This deletion prematurely ends the open reading frame which begins at position 70 in the gene by converting what would be a translational stop codon in Skalkaho\(^7\) to Met. This data for Skalkaho\(^7\) which predicted that RsaI would not cut the fragment due to lack of an appropriate digestion site while PstI would cut at positions 119 and 278 on the forward strand to produce three fragments that are 118, 159, and 255 bp long. AluI enzyme digests (data not shown) of PCR products amplified by the rickettsial CS gene primer set confirmed the presence of rickettsiae in 35 tick ovaries, and the digestion profiles were indistinguishable from those of other SFG rickettsiae (14).

### DISCUSSION

The ability to detect and differentiate Skalkaho\(^7\) by molecular techniques provides a promising approach for investigating the cycling, behavior, and interaction of rickettsiae in nature. In particular, the low incidence of RMSF on the eastern side of Bitterroot Valley has never been adequately explained; however, given its abundance there, it seems plausible that Skalkaho\(^7\) may be responsible for this phenomenon (8). In ongoing studies addressing this hypothesis, workers are trying to determine the incidence of Skalkaho\(^7\) on the western side of the valley and whether massive infections of this rickettsia in tick ovarian tissue inhibit transovarial transmission of virulent R. rickettsii. Another potential limiting factor of R. rickettsii is that the mammalian reservoir of suitable RMSF-hosting hosts on the eastern side may be resistant to rickettsial infection due to prior exposure and seroconversion to Skalkaho\(^7\). This interference via cross-protection has been demonstrated for low- and high-virulence strains of R. rickettsii (35, 36) and for distant related SFG rickettsiae (11, 13), but no interference was observed for Skalkaho\(^7\) since guinea pigs inoculated with triturates of wood ticks harboring the ES agent remained susceptible to infection with R. rickettsii (8). Studies to determine the capacity of the organism to be transmitted to mammals during tick feeding were inconclusive (8).

Considering the high level and stable maintenance of Skalkaho\(^7\) in ticks, this bacterium may be beneficial to ovarial cells or may bias ticks toward infection, as has been demonstrated with some arthropod endosymbionts, such as the AB agent, a
rickettsial species associated with male killing in the ladybird beetle (48), and other members of the Rickettsiaceae (26, 45). If this is true, the result would be selection for a greater percentage of Rickettsia peacockii tick ovarian cells infected with Skalkaho3. The biological properties which the organism may share with other rickettsia-like endosymbionts (44, 48) remain to be determined.

Description of Rickettsia peacockii sp. nov. We propose the name Rickettsia peacockii (pea.cock’ii. M.L. gen. n. peacockii of Peacock, named after rickettsiologist M. G. Peacock) for a new SFG rickettsial species which maintains natural infections in wood ticks via transstadial and transovarial transmission. Although Rickettsia peacockii could not be maintained in laboratory tissue culture, there is precedent for naming and describing uncultivable prokaryotes (1, 22). A type strain (SkalkahoT) was characterized directly from an infected D. andersoni tick (SK-594) which was used to generate 16S rDNA and rOmpA gene sequences (Tables 4 and 5). RFLP profiles (Fig. 2), and IFA reactivity data (Table 3) and to establish an infected laboratory tick colony (RML-795).

The localization of R. peacockii Skalkaho3 in tick ovarian tissue, our inability to maintain infections in mammalian systems, the apparent lack or truncation of the rOmpA surface antigen, and the gene sequence data indicate that this fastidious microorganism is a unique rickettsial species. With its stable maintenance in ticks, R. peacockii Skalkaho3 appears to be an endosymbiont whose biology contrasts with the biology of other rickettsiae. Continued studies on the organism should contribute to our understanding of rickettsia-vector-mammalian host dynamics and the mechanisms which virulent rickettsiae employ to infect and ultimately damage host cells.

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