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Characterization of an Endosymbiont Infecting Wood Ticks, *Dermacentor andersoni*, as a Member of the Genus *Francisella*

MARK L. NIEBYLSKI,[1]* MORT G. PEACOCK,[1] ELIZABETH R. FISCHER,[2] STEPHEN F. PORCELLA,[1] AND TOM G. SCHWAN[1]

Laboratory of Microbial Structure and Function[1] and Rocky Mountain Microscopy Branch,[2] Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana 59840

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A microorganism (*Dermacantor andersoni* symbiont [DAS]) infecting Rocky Mountain wood ticks (*D. andersoni*) collected in the Bitterroot Mountains of western Montana was characterized as an endosymbiont belonging to the genus *Francisella*. Previously described as *Wolbachia* like, the organism's DNA was amplified from both naturally infected tick ovarial tissues and Vero cell cultures by PCR assay with primer sets derived from eubacterial 16S ribosomal DNA (rDNA) and *Francisella* membrane protein genes. The 16S rDNA gene sequence of the DAS was most similar (95.4%) to that of *Francisella tularensis* subsp. *tularensis*. Through a combination of Giménez staining, PCR assay, and restriction fragment length polymorphism analysis, 102 of 108 female ticks collected from 1992 to 1996 were infected. Transovarial transmission to female progeny was 95.6%, but we found no evidence of horizontal transmission.

Hereditarily transmitted symbionts commonly infect numerous species of ticks and have been described as *Wolbachia* or *Wolbachia* like (5, 8, 17, 41). First recognized by R. Koch in the late 1800s, these bacteria replicate intracellularly within putative endosomes or phagosomes (3, 5, 17, 18, 35, 39, 44). Microscopic examinations determined that they are highly pleomorphic and range in size from 0.6 to 3.4 μm (5, 17, 34). The first isolation of a tick symbiont was made in 1961 from argasid ticks, later identified as *Argas arboreus*, collected from heron rookeries near Cairo, Egypt (40). This symbiont was classified as "*Wolbachia persica*" (alpha subdivision of *Proteobacteria*, family *Rickettsiaceae*, tribe *Wolbachieae*) based on its lack of reaction in serological tests with known tick-borne agents, predilection for reproductive tissues, inability to grow on cell-free media, and morphological similarities observed by light microscopy to a variety of fastidious insect endosymbionts known as the *Wolbachia pipientis* group or cytoplasmically inherited microorganisms (39, 40, 43). Subsequently, analysis of 16S ribosomal DNA (rDNA) gene sequences determined that "*W. persica*" was phylogenetically distant from the *W. pipientis* group and, in fact, related to members of the genus *Francisella* (gamma subdivision of *Proteobacteria*, *Francisella* group) (11, 28).

In 1973, a microorganism (*Dermacentor andersoni* symbiont [DAS]) isolated in chicken embryos was found to infect nearly all Rocky Mountain wood ticks (*D. andersoni*) in the Bitterroot Valley of Montana (5). These intracellular bacteria were abundant in ovarial tissues of naturally infected ticks (5, 17, 18). Although described as a *Wolbachia*-like symbiont, this bacterium was pathogenic for vertebrates, unlike most members of the *W. pipientis* group (5, 43). Guinea pigs and golden hamsters inoculated intraperitoneally with 0.1 ml of a 50% suspension of infected yolk sac exhibited elevated body temperatures (>40°C), while higher doses (0.5 ml) were lethal (5). Inoculation of wood ticks intracoelomically with infectious yolk sac suspensions resulted in a generalized and massive infection throughout the tick which invariably proved fatal (5). Complement fixation and fluorescent-antibody tests suggested that the organism was distinct from but related to "*W. persica*" (5).

In this report, we characterize this DAS as a new species of the genus *Francisella*. A PCR assay employing primer sets specific for *F. tularensis* subsp. *tularensis* membrane protein (TUL4) and eubacterial 16S rDNA genes amplified products from infected tick tissues and cell cultures. PCR assay, DNA sequencing, and restriction fragment length polymorphism (RFLP) analysis, techniques which have been successful in detecting, identifying, and differentiating bacteria including *Francisella* strains associated with hematophagous arthropods (1, 10, 11, 13, 14, 28), were used to characterize the DAS. Despite its capacity to infect guinea pigs following inoculation and its high infection rate in wood ticks, we found no evidence that the DAS was transmissible via tick bite. Members of the *W. pipientis* group were not detected in any wood tick tissues examined and, to our knowledge, no tick-associated microorganism has been definitively identified as a true *Wolbachia* sp.

MATERIALS AND METHODS

Bacterial strains and nucleotide sequence accession numbers. The DAS was characterized directly from naturally infected *D. andersoni* tick ovarial tissues and infected laboratory tissue cell cultures. For comparison, several other bacterial strains were employed in this study, as follows: DNAs from *F. tularensis* subsp. *tularensis* (LVS) and *F. tularensis* subsp. *novicida* (KM9) and Vero cell cultures of "*W. persica*" (YM) and *Rickettsia rickettsii* (R), the causative agent of Rocky Mountain spotted fever. The GenBank accession numbers for the 16S rDNA sequences used to distinguish the bacterium from reference *Proteobacteria* associated with biting arthropods are as follows: the DAS, AF001077; *F. tularensis* subsp. *tularensis* (LVS), L26086; *F. tularensis* subsp. *novicida* (ATCC 15482), L26084; *F. philomiragia* (ATCC 25017), L26085; "*W. persica*" (ATCC VR 331), M21292; *Coxiella burnetii*, M21291; *Bartonella quintana* (Fuller), M11927; *Anaplasma marginale*, M60313; *Cowdria ruminantium* (CS isolate), X61659; *R. rickettsii* (R), L36217; *Ehrlichia chaffeensis* (91HE17), U23503; *W. pipientis*, U23709; *Escherichia coli*, V00348.

Screening of tick tissues. Adult *D. andersoni* ticks were collected from Blodgett Canyon in the Bitterroot Mountain Range, Ravalli County, Mont., during 1992 and 1994 to 1996 by dragging a white flannel cloth (1 m^2) across vegetation where ticks quested for hosts. Additional specimens were collected

* Corresponding author. Mailing address: Rocky Mountain Laboratories, NIAID-NIH, 903 S. Fourth St., Hamilton, MT 59840. Phone: (406) 363-9376. Fax: (406) 363-9204. E-mail: mark_niebylski@nih.gov.

during 1995 from Poudre Canyon, Larimer County, Colo. A laboratory colony was established from ticks collected in Montana during 1995. Ticks were blood fed, and replete females were allowed to oviposit (22). Approximately 250 larvae, 100 nymphs, or 17 adults (12 females, five males) were fed per guinea pig.

Both field-collected and laboratory colony ticks were surface sterilized by three sequential washes in 30% hydrogen peroxide, 95% ethanol, and sterile distilled water (dH_2O) prior to dissections. Next, tick salivary glands and reproductive tissues were removed with sterile forceps and rinsed three times in dH_2O. Portions of tissue samples were smeared on and heat fixed to slides, stained by the Giménez method (15), and microscopically examined under oil immersion (magnification, ×1,000) for evidence of intracellular bacteria. The remaining portion of each sample was stored in 200 μl of brain heart infusion (BHI) at −5°C for PCR assay.

Electron microscopic studies. Ovarial tissues from a laboratory colony female tick (RML-296) infected with the DAS and from a tick (RML-795) coinfected with both the DAS and a *Rickettsia* sp. were examined with a transmission electron microscope (Philips CM-10). 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 1% aqueous (aq) tannic acid and stained overnight en bloc in 1% uranyl acetate (aq). Samples were dehydrated through a graded ethanol series and embedded in Spurr's resin. Thin sections were cut with an ultramicrotome (RMC MT-7000), stained with 1% uranyl acetate (aq) and Reynold's lead citrate, and observed at 80 kV. In addition, a whole female tick (RML-096) from the laboratory colony infected with the DAS was fixed, stained, and sectioned as described previously (16). Ovarial tissues, salivary glands, hemocytes, midgut, Malpighian tubules, and hypodermal and endodermal tissues from this tick were then examined with an electron microscope (Hitachi HU-11E) at 75 kV.

Isolation and cultivation. Ovarial tissues from six laboratory colony ticks infected with the DAS were triturated with glass grinders in 5 ml of BHI. Approximately 0.2 ml of suspension and two 10-fold serial dilutions were inoculated directly onto *D. albipictus* tick (DALBE3) cells (provided by U. G. Munderloh, University of Minnesota, Minneapolis) by standard methods (30). Inoculations were performed in triplicate. DALBE3 cells were cultivated in closed 75-cm^2 flasks (Corning Inc., Corning, N.Y.) with a special medium (25) and incubated at 27°C. DALBE3 cells were harvested on day 10 postinoculation and passaged into primary chicken embryo (PCE) cell cultures (30). PCR cells were harvested and passaged into Vero cells. Both of these cell cultures were incubated for 9 days at 34°C with 40 ml of M199 medium (Gibco, Grand Island, N.Y.), 2% fetal bovine serum, and 10 mM L-glutamine. All cell culture harvests were screened for the DAS by the Giménez method and a PCR assay. Cultivation of the DAS on cell-free medium was attempted by streaking triturates of infected tick ovarial tissues across blood agar plates which were then incubated in a candle jar at 21, 28, and 37°C.

PCR methods. Reproductive tissues and salivary glands from field-collected and laboratory colony ticks along with Vero cell cultures were analyzed by PCR assay. In these tests, we assayed 15 male and 165 female ticks, which included those used to generate electron micrographs, RFLP profiles, and sequence data for the DAS. Initially, individual tick tissue samples were prepared for PCR assay as described previously (14). Next, tissues were screened by PCR assay with five separate primer sets. Two of these were based on a previously published sequence of the gene for a 17-kDa *F. tularensis* subsp. *tularensis* membrane protein (TUL4) (37). The first primer set (TUL4-A) amplified an approximately 250-bp fragment corresponding to positions 393 through 642 on the forward strand of the TUL4 gene (24, 37). A TUL4-B primer set, 5′-GAATATGTCAAAGG TAGG (*F. tularensis* subsp. *tularensis* TUL4 positions 147 to 165) and 5′-TCAG AAGCGATTACTTCT (*F. tularensis* subsp. *tularensis* TUL4 positions 984 to 966R), amplified an approximately 838-bp fragment. A portion of the PCR products amplified by this primer set were further analyzed by RFLP analysis as described below. Lastly, a primer set (99F and 994R) specific for detecting members of the genus *Wolbachia* (alpha subdivision of *Proteobacteria*) (28, 29), a primer set (RpCS.877F and 1258R) specific for detecting members of the genus *Rickettsia* (33), and a primer set (Rr190.70F and 602R) specific for detecting spotted fever group rickettsiae (33) were tested on tick tissues and cell cultures.

To further characterize the DAS, a PCR assay employing a eubacterial 16S rDNA gene primer set, *E. coli* positions 45 to 62 and 1242 to 1227R (28), was selectively tested on tick ovarial tissues and Vero cell cultures. This primer set amplified an approximately 1,187-bp fragment corresponding to positions 29 through 1186 on the forward strand of the *F. tularensis* subsp. *tularensis* 16S rDNA gene. PCR products amplified by this primer set were sequenced as described below.

All PCR assays were performed with a GeneAmp PCR reagent kit in 0.5-ml GeneAmp reaction tubes in accordance with the manufacturer's recommendations (Perkin-Elmer/Cetus, Norwalk, Conn.). Each PCR sample consisted of a DNA template (5 μl), two primers suspended at a concentration of 30 pmol/μl (0.5 μl of each), dH_2O (34.75 μl), PCR kit 10× reaction buffer (5 μl), all four deoxynucleoside triphosphates at 200 μM (1 μl of each), 1.25 U of *Taq* polymerase (0.25 μl), and an overlay of mineral oil (25 μl). In addition to tick tissue suspensions, *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *novicida*, "*W. persica*," *R. rickettsii*, cell cultures infected with the DAS, and salivary gland suspensions inoculated with infected ovarial tissue triturates were tested. Uninfected tick tissues or cell cultures were included in all PCR assays as negative controls. Each PCR sample was amplified in a thermocycler (Perkin-Elmer/Cetus) for 35 repeated cycles with a temperature profile of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min per cycle.

To confirm *Francisella* infections, PCR products generated by the TUL4-A primer set from *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *novicida*, "*W. persica*," and 11 ovarial tissues infected with the DAS were electrophoresed in 0.7% agarose gels and then subjected to Southern blot analyses employing a γ[r-^{32}P]dATP 5′-end-labeled FT443 oligonucleotide probe corresponding to positions 443 through 468 on the forward strand of the *F. tularensis* subsp. *tularensis* TUL4 gene (24, 37) through standard methods (36, 38). ϕX174 replicative-form DNA cleaved with *Hae*III was used as a molecular size marker. Ticks tested by this method included those (BL-294, RML-395, and RML-495) used to generate RFLP profiles and sequence data.

Restriction enzyme analysis of PCR amplification products and staining of gels were carried out as described previously (14), with minor modifications. The DNA fragments used in RFLP analyses were PCR products amplified by the TUL4-B primer set from *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *novicida*, and "*W. persica*" and 15 infected ovarial tissues, including those from field-collected (BL-192, BL-294, BL-595, and BL-196) and laboratory colony (RML-395, RML-495, and RML-296) ticks, and infected cell cultures. First, 10 μl of each PCR mixture were electrophoresed in 0.7% agarose gels. Samples with fragments of the appropriate size (838 bp) were further analyzed. Ten microliters of each PCR mixture was digested overnight at 37°C with restriction enzyme *Cla*I, *Dra*I, or *Hin*dIII and the appropriate enzyme buffer (New England Biolabs, Beverly, Mass.). Digested PCR products were mixed with Ficoll loading buffer (0.25% Orange G, 60 mM EDTA, 10 mM Tris, and 15% Ficoll 400 in dH_2O) and loaded onto 8% polyacrylamide gels (1.5 mm thick) for electrophoretic analysis by standard methods (36).

Sequencing of PCR products. Both strands for each of three PCR amplification products generated from the DAS by the 16S rDNA primer set were sequenced (Sequenase Version 2.0; United States Biochemical, Cleveland, Ohio). Following purification by MicroSpin S-400 HR columns (Pharmacia Biotech Inc., Piscataway, N.J.), two PCR products were sequenced directly, one amplified from the ovarial tissues of a field-collected tick (BL-294) and the other amplified from Vero cells infected with the DAS cultivated from a laboratory colony tick (RML-395). A third partial 16S rDNA PCR product was amplified from an infected laboratory colony tick (RML-495), cloned into a pCRII plasmid DNA vector (TA cloning kit version 2.0; Invitrogen, San Diego, Calif.), and sequenced. Cultured clones were harvested, and their DNAs were purified by cesium chloride equilibrium density gradient centrifugation (36). Appropriate oligonucleotide primers were designed based on prior sequence readings and purchased commercially (Genemed Bio, San Francisco, Calif.). Nucleotide sequences were analyzed and manipulated by using the MacVector (version 4.1.1, International Biotechnologies Inc.-Kodak, New Haven, Conn.) software packages, Lasergene (DNASTAR, Madison, Wis.), and Genebee (Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia). The algorithms used to construct phylogenetic relationships were Cluster (7, 20, 45), Topological (7, 45), and Jotun Hein (19). Sequence alignments were performed and similarities were calculated by using Clustal V (20) and BestFit (University of Wisconsin Genetics Computer Group-LITE, Madison) (9). The partial 16S rDNA gene sequence obtained for the DAS was submitted as a query in the GenBank sequence database to assist in identifying the most similar sequences.

Vertical and horizontal transmission studies. Progeny from an infected adult wood tick (BL-295) were reared to adulthood and tested to determine the DAS's rate of transstadial and transovarial transmission. Adult female ticks were screened for the DAS by Giménez stain, PCR assay, and RFLP analysis.

An indirect fluorescent-antibody (IFA) test was employed to screen sera from guinea pigs used in tick blood feedings for seroconversion to the DAS and thereby provide evidence of transmission via tick bite (31, 32). In these tests, Vero cell cultures infected with the DAS, along with ovarial tissues from infected laboratory ticks (RML-296), were smeared on and acetone fixed to slides. Uninfected Vero cells, *R. rickettsii*, and uninfected tick ovarial tissues were included as negative controls. Each sample was incubated separately with serum from each of 38 guinea pigs which include: (i) sera from 35 separate guinea pigs fed upon by either larval, nymphal, or adult wood ticks infected with the DAS, (ii) serum from a guinea pig inoculated on days 1 and 10 with 1 ml of Vero cells infected with the DAS, (iii) serum from a guinea pig inoculated on days 1 and 10 with a 1.0-ml suspension of chicken embryo infected with the DAS (5), and (iv) serum from a guinea pig inoculated with five infected tick ovarial tissues triturated in 1.0 ml of BHI. Sera were harvested 4 weeks postinoculation or post-tick feeding. Prior to use, 1.0 ml of uninfected Vero cell lysate was added to 3.0 ml of sera from guinea pigs inoculated with infected Vero cells to adsorb out nonspecific antibodies reactive with the Vero cells. Following incubation with the preadsorbed guinea pig sera, samples were stained with fluorescein isothiocyanate-labeled rabbit anti-guinea pig serum (31). Each conjugate was diluted 1:50 in sterile dH_2O, incubated for 30 min at 27°C, and then washed three times in phosphate-buffered saline. IFA slide preparations were examined with a fluorescence microscope under oil immersion (×1,000) for evidence of the DAS.

TABLE 1. Summary of intracellular bacterial infections in female *D. andersoni* ticks from the Bitterroot Mountain Range of Montana as determined by Giménez staining and PCR assay[a]

Assay	No. of tissue samples screened/no. positive[b]			
	Field collected		Laboratory colony	
	Salivary glands	Ovarial tissue[c]	Salivary glands	Ovarial tissue[c]
Giménez stain	3/108	105/108	0/45	43/45
PCR primer sets				
Francisella (TUL4-A)	0/108	102/108	0/45	43/45
Rickettsia (RpCS.877/1258R)	3/108	3/108	0/45	0/45
SFG rickettsiae (Rr190.70/602R)	3/108	3/108	0/45	0/45
Wolbachia (99F/994R)	0/29	0/29	0/20	0/20

[a] Salivary glands and reproductive tissues from 12 female ticks collected in Colorado and 15 male ticks from the Bitterroot Mountain Range of Montana also were screened, but no PCR products were amplified and no organisms were observed following Giménez staining.

[b] The values represent the numbers of samples positive for intracellular bacteria as determined by Giménez staining and PCR assay, respectively.

[c] RFLP analysis of PCR products amplified with a *Francisella* membrane protein primer set (TUL4-B) from 15 separate tick ovarial tissues gave identical profiles that were different from those of other *Francisella* spp.

RESULTS

Massive infections with the DAS were observed in *D. andersoni* tick ovarial tissues and infected cell cultures following Giménez staining. Staining and PCR assay determined that a minimum of 102 of 108 field-collected ticks from the Bitterroot Mountains were infected (minimal field infection rate, 94.4%) (Table 1). Identical tests on the laboratory colony of ticks determined that 43 of 45 female progeny (minimal transovarial transmission rate, 95.6%) were infected through the adult stage (Table 1). The DAS was detected neither in ovarial tissues and salivary glands from 12 female wood ticks collected in Colorado nor in gonadal tissues and salivary glands from 15 male ticks collected in the Bitterroot Mountains. The DAS was successfully cultivated in DALBE3 tick cells and passaged into PCE and Vero cells and previously in chicken yolk sacs (5). It failed to grow on blood agar plates.

Groups of the putative DAS were found to be restricted to ovarial tissue within apparent phagosomes by electron microscopy (Fig. 1) (5). These pleomorphic bacteria measured on average, approximately 1.2 μm in diameter, while the vesicles harboring them were as large as 10 μm in diameter. Their morphology was consistent with that of *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *novicida*, and "*W. persica*," which also are found in phagocytic vesicles (2, 5, 34). Both the outer membrane of the DAS surrounding a cortical zone and inner medullary zone and a vesicular limiting membrane were clearly visible (Fig. 1A and B) (5). Associated with the DAS and encompassing vesicles were small inclusions noted previously with *F. tularensis* subsp. *tularensis* (Fig. 1B) (2). In ovarial tissues coinfected with the DAS and *Rickettsia* sp., both microorganisms were observed within the same cell (Fig. 1C and D) (5). Other than the DAS and *Rickettsia* sp., we observed no morphologically distinct intracellular organisms in any of the tissues we examined by electron microscopy.

When the partial 16S rDNA gene sequence of the DAS was submitted as a query in the GenBank sequence database, the most closely related sequence was that of *F. tularensis* subsp. *tularensis*. Sequence alignment comparisons performed by Clustal (20) also determined that the DAS was closely related to *F. tularensis* subsp. *tularensis* with 95.4% similarity (Table 2). Further, the DAS exhibited 92.5% similarity with both "*W. persica*" and *F. tularensis* subsp. *novicida* (Table 2). For comparison, *F. tularensis* subsp. *tularensis* exhibited 92.0 and 97.0% similarity to "*W. persica*" and *F. tularensis* subsp. *novicida*, respectively (Table 2). Only 64.2% similarity was observed between the DAS and *W. pipientis* (alpha subdivision of *Proteobacteria*) (Table 2). Similarity values calculated by BestFit were consistent with the results generated by Clustal (data not shown). The partial 16S rDNA gene sequences generated for the DAS infecting a field-collected tick, a laboratory colony tick, and Vero cell cultures were 1,195 bp long and identical.

Phylogenetic analysis of partial 16S rDNA gene sequences determined that the DAS was tightly clustered among the members of the genus *Francisella* and evolutionarily closest to "*W. persica*" (Fig. 2). All of the algorithms used in phylogenetic analyses produced the same general results with regard to the distance between groups and the order of node connections (data not shown). These results support our findings obtained from similarity studies in that the DAS exhibited the shortest evolutionary distances as calculated from nucleotide substitution values and the highest levels of sequence similarity with members of the *Francisella* group, to including "*W. persica*" (Table 2 and Fig. 2). *Coxiella burnetii* was phylogenetically the next closest relative (Fig. 2).

PCR products were amplified with the eubacterial 16S rDNA and *F. tularensis* subsp. *tularensis* membrane protein gene primer sets (TUL4-A and TUL4-B) from tick ovarial tissues but not from salivary glands. Salivary gland suspensions inoculated with infected ovarial tissue triturates tested positive for the DAS, indicating that salivary glands, by themselves, were uninfected rather than inhibiting the PCR assay, resulting in false negatives. Further, no products were amplified from any tick tissues or cell culture using a *Wolbachia* genus-specific primer set (Table 1). Rickettsia-specific primer sets determined that 2.8% of the ticks collected in the Bitterroot Mountains had both salivary glands and ovarial tissues infected with *Rickettsia* sp. (Table 1). These bacteria, which are phylogenetically distant from the *Francisella* group (11, 28), were subsequently identified as *R. rickettsii* and *R. bellii* by standard methods (14) (data not shown). This rickettsial infection rate in wood ticks from this locale was comparable to that reported previously where 4.0% were infected with either *R. rickettsii*, *R. bellii*, or *R. rhipicephali* (14). No rickettsiae were detected by PCR assay in cell cultures infected with the DAS. In turn, the TUL4 primer sets failed to amplify any products from cell cultures infected with *R. rickettsii* (data not shown).

PCR products were amplified by both the TUL4-A and TUL4-B primer sets from cell cultures and 15 separate tick ovarial tissues infected with the DAS. Products amplified by the TUL4-A primer set were approximately 250 bp long and indistinguishable from the product amplified from *F. tularensis* subsp. *tularensis* (Fig. 3). Southern blot analysis with an internal oligonucleotide probe hybridized to DNA amplified from the DAS infecting 11 ovarial tissues, which confirmed that the amplified fragments originated from a TUL4 gene (Fig. 4).

RFLP analyses of products amplified by the TUL4-B primer set from the DAS gave identical restriction enzyme digestion profiles (Fig. 5). The PCR product amplified from both the DAS and "*W. persica*" were not cut by *Cla*I and were similar when digested with *Dra*I (Fig. 5). Yet, *Hin*dIII digests of these same products gave distinct patterns for the two bacteria (Fig. 5). These profiles of the DAS and "*W. persica*" were different in all cases from the nearly identical patterns observed for *F.*

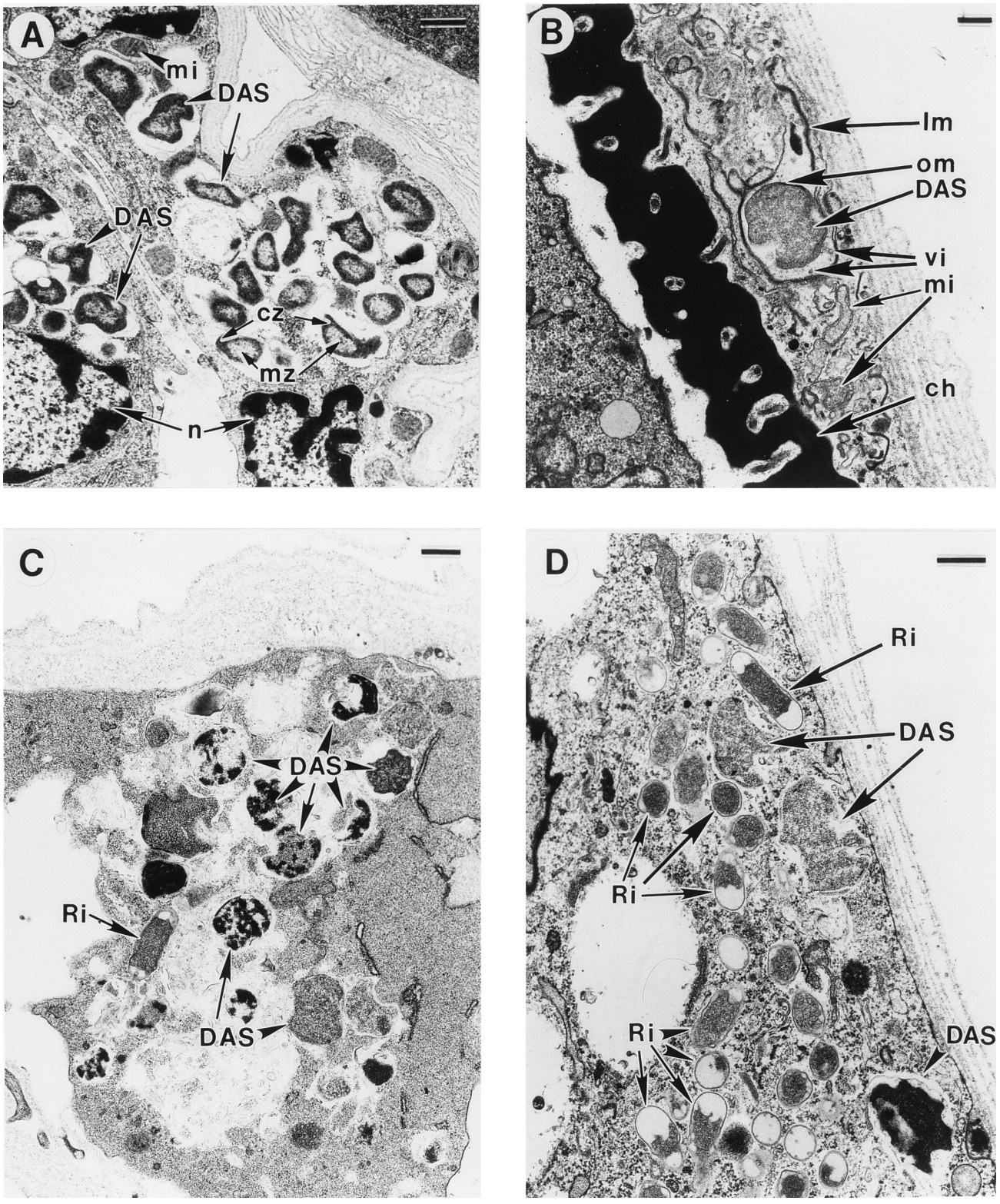

FIG. 1. Transmission electron micrographs of *D. andersoni* ovarial tissue cells infected with the DAS. (A) Aggregation of the DAS beside the nucleus. (B) Phagocytic vesicle containing the DAS near the ovarial cell surface beside a developing tick oocyte. (C) Aggregation of the DAS in phagocytic vesicles. Note the single intracellular *Rickettsia* sp. nearby. (D) Cell coinfected with the DAS and *Rickettsia* sp. Abbreviations indicate the ovarial cell nucleus (n), cortical zone (cz), medullar zone (mz), chorion of a developing oocyte (ch), mitochondria (mi), outer membrane (om), vesicle limiting membrane (lm), vesicular inclusions (vi), and *Rickettsia* sp. (Ri). Bars, 0.5 μm.

TABLE 2. Levels of sequence similarity based on alignment of 16S rRNA gene sequences from the DAS and some reference *Proteobacteria* associated with biting arthropods, including *Francisella* spp. and "*W. persica*"

Organism (strain)	% Sequence similarity[a]											
	DAS	*F. tularensis* subsp. *tularensis* (LVS)	*F. philomiragia* (ATCC 25015)	"*W. persica*" (ATCC VR 331)	*F. tularensis* subsp. *novicida* (ATCC 15482)	*C. burnetii*	*B. quintana* (Fuller)	*A. marginale*	*R. rickettsii* (R strain)	*C. ruminantium* (CS isolate)	*E. chaffeensis* (91HE17)	*W. pipientis*
DAS	100	95.4	95.0	92.5	92.5	77.0	75.1	68.4	68.2	67.8	67.3	64.2
F. tularensis subsp. *tularensis* (LVS)		100	97.2	92.0	97.0	77.3	71.5	69.9	67.9	67.9	67.4	65.2
F. philomiragia (ATCC 25015)			100	90.3	94.6	76.5	70.3	69.4	69.1	67.3	65.8	65.6
"*W. persica*" (ATCC VR 331)				100	89.0	75.2	67.3	65.8	64.7	65.4	64.1	61.6
F. tularensis subsp. *novicida* (ATCC 15482)					100	75.0	67.5	66.9	64.6	64.9	64.3	61.9
C. burnetii						100	69.2	68.1	67.6	66.7	65.7	65.0
B. quintana (Fuller)							100	75.6	77.7	75.1	75.1	72.1
A. marginale								100	79.6	89.4	88.5	82.0
R. rickettsii (R)									100	80.6	79.7	78.0
C. ruminantium (CS isolate)										100	96.7	83.9
E. chaffeensis (91HE17)											100	81.7
W. pipientis												100

[a] The sequences compared correspond to positions 29 to 1186 on the forward sense strand of the *F. tularensis* subsp. *tularensis* (LVS) 16S rDNA gene sequence. The percent similarities calculated by Clustal V are lower than those determined by other methods, as this alignment program penalizes for base deletions.

tularensis subsp. *tularensis* and *F. tularensis* subsp. *novicida* (Fig. 5). The observed RFLP profile for *F. tularensis* subsp. *tularensis* was consistent with that predicted based upon its TUL4 gene sequence, in which *Cla*I would cut at a single site to produce two fragments of 360 and 481 bp, *Dra*I would cut at two sites to produce three fragments of 200, 258, and 383 bp, and *Hin*dIII would cut at three sites to produce four fragments of 79, 138, 205, and 419 bp (37).

The exact sizes of the uncut and digested fragments amplified from the various samples were difficult to determine due to a previously noted gel artifact which resulted in overestimation of fragment sizes (33). For example, the uncut PCR products amplified from *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *novicida*, "*W. persica*," and the DAS were of various sizes and all were apparently larger than the predicted 838 bp when electrophoresed in polyacrylamide gels (Fig. 5). Yet, the same uncut fragments electrophoresed in 0.7% agarose gels ran true to size and all measured approximately 840 bp (data not shown). Despite its inaccuracy in predicting DNA fragment sizes, the RFLP typing procedure is advantageous for determining pattern similarity through side-by-side visual comparisons (33) and can reliably discriminate between *Francisella* spp. (Fig. 5).

Guinea pigs exhibited mild fever (>40.0°C) with onset on day 3 and defervescence on day 6 after intraperitoneal inoculation with suspensions of tick ovarial tissue, Vero cell cultures, and chicken yolk sac suspensions (5) infected with the DAS. In contrast, guinea pigs fed upon by infected ticks and those inoculated with either uninfected chicken yolk sac suspensions (5) or uninfected Vero cell suspensions failed to develop fever or become symptomatic. IFAs failed to detect antibodies to the DAS in serum samples from 35 guinea pigs fed upon by either infected larval, nymphal, or adult wood ticks. Yet, sera from guinea pigs inoculated with suspensions of infected cell cultures, chicken embryos, and tick ovarial tissues reacted strongly to the DAS, indicating seroconversion to the organism.

DISCUSSION

The DAS was found to be a highly pleomorphic microorganism which infects and replicates with *D. andersoni* ovarial tissues, specifically in phagocytic vesicles. Based on 16S rDNA gene sequence data, the DAS appears to be related to but different from *F. tularensis* subsp. *tularensis*, an important vector-borne pathogen causing tularemia worldwide (3, 23), and other members of the *Francisella* group (Table 2 and Fig. 2). This conclusion also is supported by the presence of an *F. tularensis* subsp. *tularensis* membrane protein (TUL4) gene in the organism. Restriction enzyme digests of the PCR product amplified with the TUL4-A primer set from the DAS gave an RFLP profile distinct from those of other members of the genus *Francisella*. These findings, along with comparable morphological features observed by electron microscopy, suggest that the DAS should be classified in the *Francisella* group (gamma subdivision of *Proteobacteria*), which is comprised principally of *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *novicida*, and *F. philomiragia*. Similarly, our results also indicate that "*W. persica*" is related to the *Francisella* group (Table

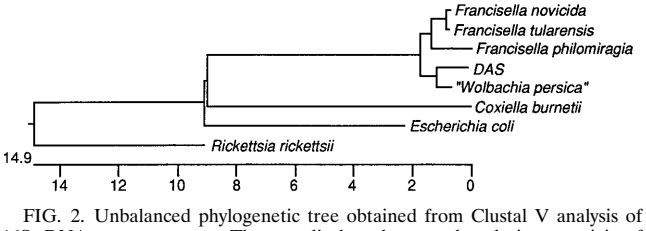

FIG. 2. Unbalanced phylogenetic tree obtained from Clustal V analysis of 16S rDNA gene sequences. The tree displays the rooted evolutionary origin of phylogenies assuming a biological clock. The evolutionary relationship of the DAS to "*W. persica*" and other members of the *Francisella* group (gamma subdivision of *Proteobacteria*) is shown. *R. rickettsii* (alpha subdivision of *Proteobacteria*) was used as an outgroup, while *C. burnetii* and *E. coli* were included for further outlier comparisons. The scale represents the number of nucleotide substitutions calculated against the number of compared nucleotide sites.

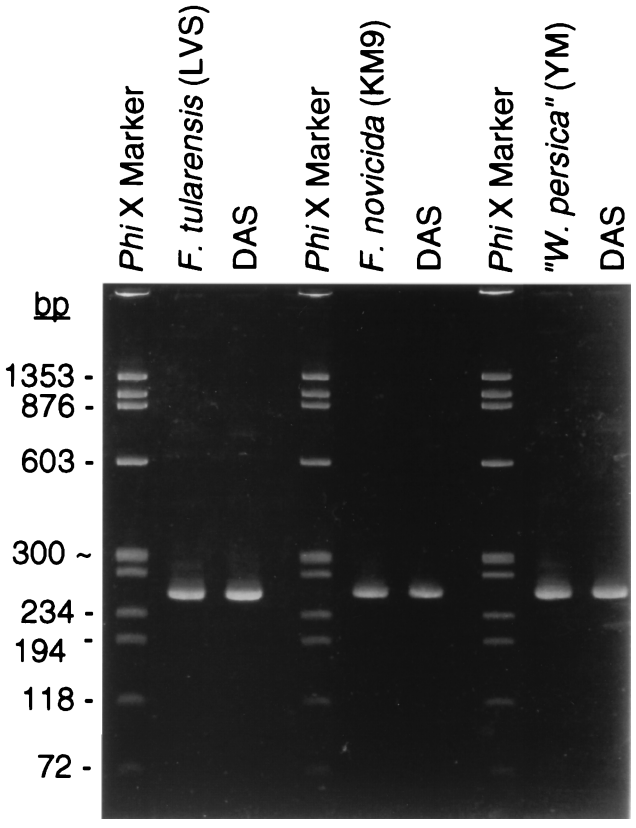

FIG. 3. PCR products amplified by the *F. tularensis* subsp. *tularensis* membrane protein gene primer set (TUL4-A) from the DAS compared with those amplified from *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *novicida*, and "*W. persica*." Molecular weight standards are indicated on the left.

2 and Fig. 2) and should be reclassified (11, 28). Concurrently with our studies, a symbiont of *Ornithodoros moubata* was characterized and its partial 16S rDNA gene sequence was determined to be 97.9 and 95.4% similar to those of the DAS and *F. tularensis* subsp. *tularensis*, respectively (26).

PCR amplification with the TUL4-A primer set, followed by Southern blot hybridization, confirmed that the DAS possesses the *Francisella* TUL4 gene (Fig. 3 and 4). However, this procedure was unable to discriminate among infections with the DAS, *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *novicida*, and "*W. persica*." RFLP typing of PCR products amplified with the TUL4-A primer set successfully differentiated among the DAS, *F. tularensis* subsp. *tularensis*, and "*W. persica*" (Fig. 5). Since the primer set is specific for a *Francisella* membrane protein (37), this technique may be more applicable than RFLP analyses of 16S rDNA sequences (10, 11) in identifying *Francisella* spp. associated with ticks since eubacterial primers amplify products from a broad spectrum of tick-borne bacteria (28).

The inability of the *Wolbachia*-specific primer set to amplify any products from wood tick tissues or inoculated cell cultures indicates that members of the *W. pipientis* group were not present. To date, no tick-associated microorganisms have been definitively identified as true members of the genus *Wolbachia*. Hence, we caution against the tendency to label apparent arthropod symbionts as members of the genus *Wolbachia* until definitive typing is performed.

The DAS was found to be transovarially maintained in nearly all of the wood ticks examined from the Bitterroot Mountains but restricted principally to the ovarial tissue (Table 1) (5, 17). Beyond lack of salivary gland infection, wood ticks failed to transmit the DAS horizontally while feeding on guinea pigs in the laboratory. For this reason, it has been deemed an endosymbiont. Lack of detectable infection in male ticks has been demonstrated previously with symbionts of other tick species (3).

Conversely, *F. tularensis* subsp. *tularensis* can infect nearly all tick tissues of both sexes but field infection rates in *Dermacentor* ticks are generally below 2.5%, with high mortality in those infected (3, 12, 21, 23). Tularemia is commonly transmitted to vertebrates via the bite or excretions of infected hard ticks and tabanids, drinking of contaminated water, and contact with infected vertebrate tissues or excretions (12, 23). Although transstadial transmission of *F. tularensis* subsp. *tularensis* in ticks is common, evidence for transovarial transmission remains dubious (3, 21). The disease has been well documented in the Bitterroot Valley (23), but the annual number of human cases and tick infection rates in this locale are unknown. The factors required by members of the *Francisella* group to be transmitted to vertebrates also remains an issue. Regardless, our findings emphasize the need for caution in removing attached ticks, specifically to avoid rupturing the chitinous exoskeleton and exposing the potentially pathogenic microorganisms that ticks may harbor.

The interaction of the DAS with other tick-borne bacteria in the arthropod host and its ultimate impact on the transmission and maintenance of other tick-borne pathogens, such as those causing tularemia and Rocky Mountain spotted fever, warrant investigation. Given the high infection rate in ovarial tissues, it

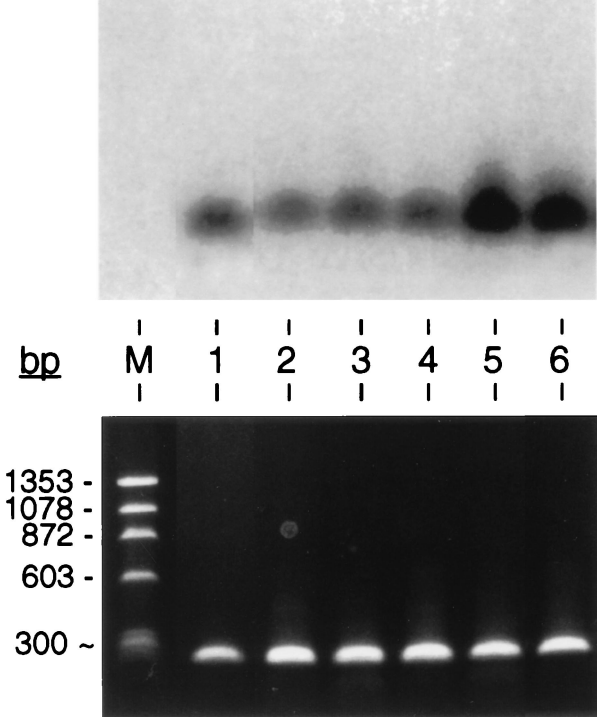

FIG. 4. Southern blot analysis with an oligonucleotide probe specific for an internal site in the *F. tularensis* subsp. *tularensis* membrane protein (TUL4) gene and 0.7% agarose gel electrophoresis of PCR products amplified by the TUL4-A primer set. Lanes: 1, *F. tularensis* subsp. *tularensis*; 2, *F. tularensis* subsp. *novicida*; 3, "*W. persica*"; 4, the DAS in a tick collected 1992; 5, the DAS in a tick collected 1994; 6, the DAS in a laboratory colony tick. Molecular size standards for a ϕX marker (lane M) are indicated on the left.

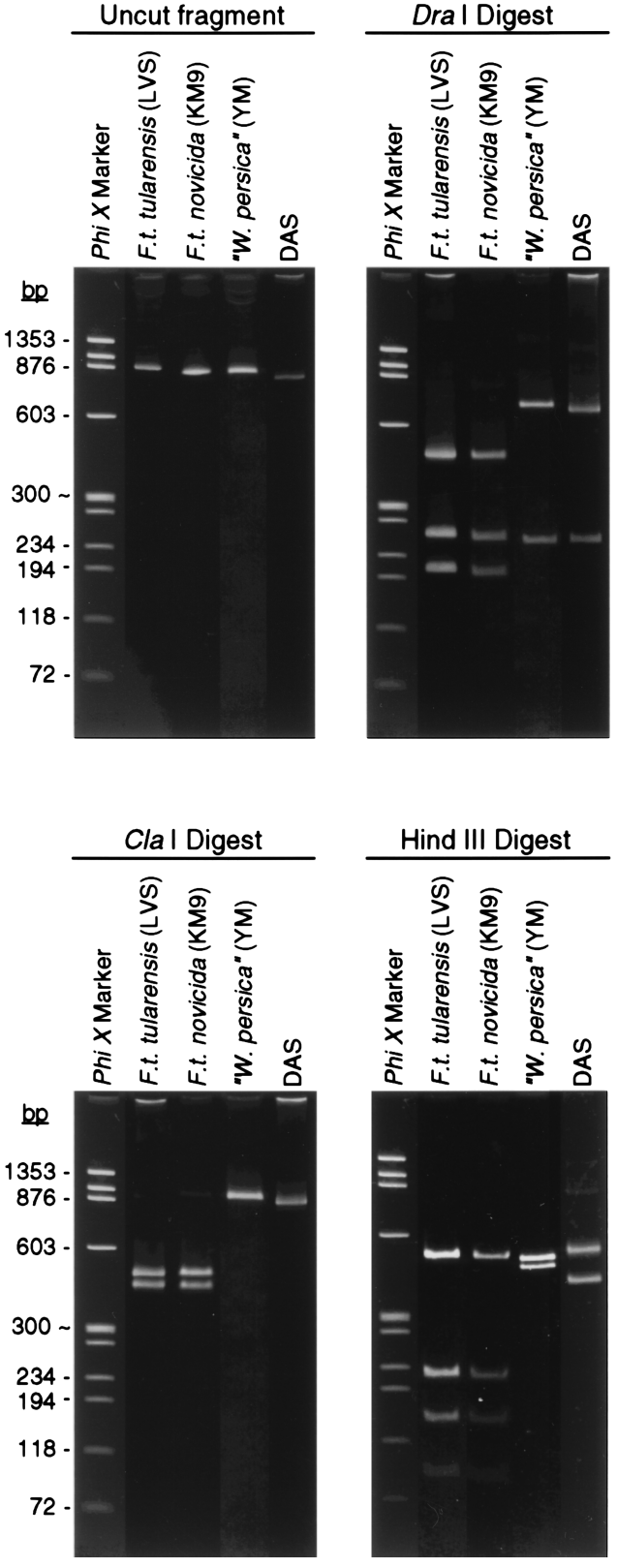

FIG. 5. RFLP analysis of PCR products amplified by the *F. tularensis* subsp. *tularensis* membrane protein gene primer set (TUL4-B) for the DAS, *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *novicida*, and "*W. persica*." Molecular size standards are indicated on the left.

is plausible that the DAS and symbionts of other tick species may limit the capacity for extrinsic tick-borne bacteria to invade and multiply in tick ovaries. The ultimate result may be a reduction in vertical transmission and maintenance of infectious bacteria by the arthropod host, a phenomenon theorized to occur between species of rickettsiae (6).

Although microorganisms commonly described as *Wolbachia*-like symbionts have been recognized in multiple tick species, including members of the *Dermacentor*, *Ixodes*, *Amblyomma*, *Rhipicephalus*, *Haemaphysalis*, *Hyalomma*, *Argas*, *Ornithodoros*, and *Boophilus* genera (3, 17, 44), most still remain uncharacterized and others may await discovery. The role that these microorganisms play in providing nutrition to ticks, as well as the impact they may have on host population sex ratios and infection rates, as described for other arthropod symbionts (4, 27, 42), is unexplored.

Description of the DAS. We propose that the DAS be classified as a unique *Francisella* species. Gene sequence data, the presence of a *Francisella* membrane protein gene, and electron micrographs indicate the DAS is a member of the *Francisella* group which can be discriminated from its close relatives by RFLP typing. Its localization in tick ovarial tissues, inability to be transmitted by tick bite, and stable maintenance via transstadial and transovarial transmission in wood ticks suggest that the DAS is an endosymbiont whose biology contrasts with that of other previously identified *Francisella* spp. Continued studies on the DAS and other tick symbionts may reveal if heretofore uncharacterized *Francisella* spp. reside elsewhere and the potential public health impact they may have.

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