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Phylogenetic Analysis of the Spirochetes *Borrelia parkeri* and *Borrelia turicatae* and the Potential for Tick-Borne Relapsing Fever in Florida

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Isolates of *Borrelia turicatae*, *Borrelia parkeri*, and the Florida canine borrelia (FCB) were examined to further phylogenetically characterize the identities of these spirochetes in the United States. DNA sequences of four chromosomal loci (the 16S rRNA gene, *flaB*, *gyrB*, and *glpQ*) were determined for eight isolates of *B. turicatae* and six isolates of *B. parkeri*, which grouped the spirochetes into two distinct but closely related taxa (>98% sequence identity) separate from *Borrelia hermsii*. The FCB was clearly separated with the group identified as *B. turicatae*, confirming this bacterium as a relapsing fever spirochete. Therefore, the potential for tick-borne relapsing fever in humans and other animals exists in Florida and future efforts are needed to determine the enzootic hosts and distribution of this spirochete in the southeastern United States. Analysis of plasmids demonstrated both linear and circular forms in *B. turicatae* but only linear plasmids in *B. parkeri*, which should be of interest to investigators concerned with plasmid diversity and evolution within this group of spirochetes.

Tick-borne relapsing fever of humans in the United States was first recognized nearly 90 years ago (47). However, the diagnosis is still often missed, illness is underreported (30), and regions of endemicity not previously known to exist continue to be identified (58). Areas of risk occur in scattered foci of endemicity throughout the western United States and southern British Columbia, Canada (29, 30), where three species of spirochetes, *Borrelia hermsii*, *Borrelia turicatae*, and *Borrelia parkeri*, are maintained in enzootic cycles with their specific and respective tick vectors, *Ornithodoros hermsi*, *Ornithodoros turicata*, and *Ornithodoros parkeri* (26, 31).

In the past, patients were assumed to be infected with the species of spirochete associated with the geographical area and ecological setting where a specific tick was found. Hence, if people developed relapsing fever after frequenting a cave in Texas where spirochete-infected *O. turicata* ticks were found, they were assumed to be infected with *B. turicatae* (28, 51). Relapsing fever patients with exposure to ticks while sleeping in a cabin at a higher elevation in a coniferous forest were assumed to be infected with *B. hermsii* (30). Today, with the ability to culture these spirochetes (4, 5, 44) and a variety of molecular analysis-based identification techniques (5, 15, 16, 36, 73), the laborious method of xenodiagnosis using live ticks is no longer needed. Historically, spirochetes identified as *B. hermsii* have been isolated from many humans (3, 11, 35, 54, 58), yet we are unaware of any isolates of *B. turicatae* or *B. parkeri* from human patients and only a few tick isolates of these two species have been described (38). The epidemiological evidence for *B. turicatae* causing human infections is strong (30, 51), while *B. parkeri* has only rarely been implicated (19, 27).

The impact that these three species of spirochetes have on the health of wild and domestic animals is less understood than for humans. Rodents are the natural vertebrate hosts for *B. hermsii*, and this spirochete was isolated from two chipmunks, *Tamias umbrinus*, during an investigation of an interstate outbreak of relapsing fever that originated in Colorado (71). One report without isolation implicated a relapsing fever spirochete, which was closely related to *B. hermsii* based on the DNA sequence of the 16S rRNA gene, with the death of a northern spotted owl in Washington (69). *B. turicatae* is pathogenic in dogs (14, 51), and *B. parkeri* may be pathogenic in horses (72). In 1979, Schalm presented a case report of a spirochetemic dog that resided in the foothills of Kern County, California (52). No isolation was attempted, spirochetes were not characterized, and this dog could have been exposed to either *O. hermsi* or *O. parkeri* as both of these species of ticks have been collected in the general area (25, 37). Additionally, spirochetes not consistent with *Borrelia burgdorferi*, an agent of Lyme disease, were observed in blood smears of two sick dogs in Florida (13). Spirochetes were cultured from the blood of one dog and designated the Florida canine borrelia (FCB) (13). Interestingly, a disjunct population of *O. turicata*, the vector of *B. turicatae* in the western United States, exists in Florida (10, 25). The occurrence of a non-Lyme disease borrelia that produced acute-onset illness and detectable spirochetemia in dogs and the presence of *O. turicata* ticks in the same region of Florida suggested that the FCB might be *B. turicatae*. If this were true, then the potential for relapsing

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TABLE 1. Designations of the *B. turicatae* and *B. parkeri* isolates used in this study and their biological sources, years of isolation, and geographic origins

Species and isolate	Host source (no. and stage)[a]	Yr	Locality[b]
B. turicatae			
RML	*O. turicata* (?)	?	Kansas
91E135	*O. turicata* (3N, 3A)	1991	Crockett Co., Tex.
95PE-570	*O. turicata* (1N, 1A)	1995	Atascosa Co., Tex.
99PE-1807	*O. turicata* (2A)	1999	Real Co., Tex.
PE1-926	*O. turicata* (1A)	1999	Real Co., Tex.
TCB-1	Domestic dog	1999	Clay Co., Tex.
TCB-2	Domestic dog	2001	Lubbock Co., Tex.
FCB	Domestic dog	1992	Sumter Co., Fla.
B. parkeri			
RML	*O. parkeri* (?)	?	California
CA216	*O. parkeri* (1M)	1991	Monterey Co., Calif.
CA218	*O. parkeri* (1N)	1991	Monterey Co., Calif.
CA219	*O. parkeri* (1F)	1991	Monterey Co., Calif.
CA220	*O. parkeri* (1M)	1991	Monterey Co., Calif.
CA221	*O. parkeri* (1N)	1991	Monterey Co., Calif.

[a] A, adult, sex unknown; M, male; F, female; N, nymph.
[b] Co., County.

fever to occur in humans would also exist in Florida. Recent surveys of ixodid ticks and small mammals in Georgia, South Carolina, and northern Florida found several species of *Borrelia* but no relapsing fever spirochetes (24, 46). Thus, we undertook a study to compare the FCB with other recent isolates of *B. turicatae* from dogs and ticks from Texas and with *B. parkeri* isolates from ticks from California.

DNA-DNA hybridization studies with single isolates of each species led Hyde and Johnson to suggest that *B. hermsii*, *B. turicatae*, and *B. parkeri* may be conspecific (43). However, these investigators concluded also that additional work was required before any taxonomic changes were made, a conclusion supported by others (50). Here we identify the FCB as *B. turicatae* and demonstrate its close relationship to other isolates of this species obtained from dogs and ticks. We also show that while *B. turicatae* and *B. parkeri* are closely related based on the DNA sequences of four chromosomal loci, *B. parkeri* differs from both *B. turicatae* and *B. hermsii* by the lack of circular plasmids in its genome, and we support the current nomenclature of these spirochetes as three distinct species.

MATERIALS AND METHODS

***Borrelia* strains and cultivation.** Fifteen isolates of borreliae were studied, including eight isolates of *B. turicatae* and six isolates of *B. parkeri* that originated from ticks or domestic dogs in Texas, Kansas, California, and Florida (Table 1). One isolate of *B. hermsii* (DAH) was included for comparison, which originated from a human relapsing fever patient in eastern Washington (3).

B. parkeri CA216 to CA221 were isolated from a single nymph or adult *O. parkeri* tick collected from the burrows of the California ground squirrel, *Spermophilus beecheyi*, at the University of California Hastings Natural History Reservation in Carmel Valley, California (Monterey County), in early September 1991. Ticks were collected by placing dry ice at the entrances of burrows, allowing the dry ice to sublimate for up to an hour, then examining soil from the burrow entrances in a white enamel pan for the presence of ticks. In total, 117 nymphs and 25 adults (10 males, 15 females) of *O. parkeri* were collected. Ticks were identified using the key to *Ornithodoros* adults and late-stage nymphs in reference 37.

Ticks were prepared and dissected as discussed previously (40). Briefly, ticks were surface sterilized in 3% hydrogen peroxide for 30 s, then in 70% ethanol for 30 s, rinsed in sterile phosphate-buffered saline, and embedded in paraffin. Ticks were dissected in sterile phosphate-buffered saline, and smears prepared from the midgut diverticulum, central ganglion, and salivary glands of each specimen were examined by a direct immunofluorescent-antibody (DFA) assay with a fluorescein isothiocyanate-labeled anti-*B. burgdorferi* conjugate prepared in a domestic rabbit.

The remaining tissues of each tick were placed in 1 ml of BSK II medium (4) containing 12.5 μg of rifampin per ml. The cultures were kept at 34.5°C and examined weekly for 1 month for the presence of spirochetes by dark-field microscopy at ×400. Positive cultures were incubated until the density of spirochetes reached 5 to 10 organisms per ×400 field, and 1 ml of the culture was passed into 9 ml of BSK II medium. After the spirochetal density of the first passage reached ~50 organisms per field, 1 ml containing 20% glycerol–80% BSK II medium was prepared for storage at −75°C. The DFA assays detected spirochetes in the salivary glands or central ganglion in only 2 of 20 adults and in none of 49 nymphs. One of the DFA-positive ticks and four other specimens produced the five isolates from 1991 described here.

The isolates of *B. turicatae* from Texas ticks originated from ticks collected from soil in caves or under houses in three counties that people had frequented prior to contracting relapsing fever. Ticks were attracted with dry ice and prepared as described above. Tissues from one to six DFA-positive ticks were inoculated intraperitoneally into a laboratory mouse. The mouse was bled daily, and blood from the tail vein was examined microscopically for the presence of spirochetes, and when found infected, the blood was inoculated into BSK-H medium (Sigma-Aldrich Co., St. Louis, Mo.). The isolations of spirochetes from the two dogs from Texas were done also by first injecting mice intraperitoneally with the infected dog blood and then inoculating the infected mouse blood into BSK-H medium. The isolation of the FCB was described previously (13).

SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Whole-cell lysates of spirochetes were prepared as described previously (57). Proteins were separated by one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with Laemmli buffer (45) and a vertical gel apparatus (Bethesda Research Laboratories-GIBCO, Gaithersburg, Md.). Proteins were blotted onto nitrocellulose membranes with Towbin buffer (70) and a Trans-Blot Cell (Bio-Rad Laboratories). The membranes were blocked overnight at room temperature with TSE-Tween (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% Tween 20) and incubated with monoclonal antibodies H9724 (8) and H9826 (55). Bound antibodies were detected by ^{125}I-labeled protein A autoradiography.

PCR amplification and DNA sequencing. Total genomic DNA was purified from 500-ml cultures of all the isolates as previously described (62), quantified by UV spectrophotometry, and diluted to 0.05 μg for each 50-μl reaction mixture. *Taq* polymerase, deoxynucleoside triphosphates, and buffer were used as recommended by the manufacturer (Promega Corp., Madison, Wis.). PCRs began with heating at 94°C for 3 min, followed with 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 3 min. After the 35th cycle, an additional 7-min extension was done at 72°C. PCR amplification products were visualized in agarose gels stained with ethidium bromide. Reaction products that contained a single amplicon of the predicted size were processed with a QIAGEN PCR Purification kit (QIAGEN Inc., Valencia, Calif.). DNA sequencing reactions were performed with ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction mix (Applied Biosystems, Inc., Foster City, Calif.) with slight modifications of the manufacturer's protocol to reduce the final volume to 15 μl. The mixtures were run for 45 cycles with a PTC-225 DNA Engine Tetrad Thermal Cycler (MJ Research, Inc., Waltham, Mass.). DNA sequences were determined for the 16S rRNA gene (1,272 bp), *flaB* (1,002 bp), *gyrB* (1,902 bp), and *glpQ* (1,014bp) loci in each isolate with a model 3700 automated DNA sequencer (Applied Biosystems, Inc.) and multiple primers (Invitrogen Corp., Carlsbad, Calif.) (Table 2). *flaB* encodes a protein subunit of the periplasmic flagella, *gyrB* encodes a subunit of the bacterial enzyme DNA gyrase, and *glpQ* encodes the enzyme glycerophosphodiester phosphodiesterase. Nucleotide and deduced amino acid sequences were analyzed with the Sequencher 4.1 (Gene Codes Corp., Ann Arbor, Mich.) and MacVector 6.0 (Oxford Molecular, Beaverton, Oregon) software packages. Concatenated DNA alignments were constructed with the Megalign and ClustalV programs in the Lasergene software package (DNASTAR, Madison, Wis.) and transferred into the MacClade program (46a) for manual correction. MacClade output files were opened in PAUP (67a), where maximum-likelihood neighbor-joining trees were created. Trees were rooted by making the outgroup paraphyletic with respect to the ingroup. To test the robustness of clade designations, a full heuristic search with 1,000 bootstrapping replicates was performed.

Agarose gel electrophoresis. Total genomic DNA samples were electrophoresed in 1% agarose gels with 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 10 mM EDTA) without ethidium bromide. Plasmids were resolved in a reverse-pulse electrical field provided with a PPI-200 programmable power inverter (MJ Research, Inc.). DNA was electrophoresed at 100 V for 15 min and run on

TABLE 2. Oligonucleotide primers used for gene amplification and DNA sequencing

Gene and primer designation	Sequence (5′ to 3′)	Base positions[a]
16S rRNA[c]		
FD3[b]	AGAGTTTGATCCTGGCTTAG	−89 to −70
T50[b]	GTTACGACTTCACCCTCCT	+127 to +109
Rec4[b]	ATGCTAGAAACTGCATGA	533–550
Rec9[b]	TCGTCTGAGTCCCCATCT	1052–1035
16s-1	TAGAAGTTCGCCTTCGCCTCTG	641–620
16s-2	TACAGGTGCTGCATGGTTGTCG	939–960
flaB[d]		
Fla-ans5′	TGTGATATCCTTTTAAAGAGACAAATGG	−81 to −54
Btur-fla3′	GGTTCCTGACTTTAACACTAGCC	+160 to +138
Fla+1A	AGAGCTTGGAATGCAACCTG	447–466
Fla−1B	TGCTTCATCCTGATTTGC	552–535
Fla-alt3′	TCTAAGCAATGACAATACATATTGAGG	1002–976
gyrB[e]		
gyrB 5′ A-1	TTTATTGGTTTTAAGTCAAGTTGAATATGTC	−120 to −90
gyrB 3′	GGCTCTTGAAACAATAACAGACATCGC	+116 to +90
gyrB 5′	GGTTTATGAGTTATGTTGCTAGTAATATTCAAGTGC	−5 to 31
gyrB 5′+1	TTATCAAAGAGACTTAGGGAACTTGC	547–572
gyrB 5′+2	GAAAGATGTTCCAAGTCTTACATTAGATG	906–934
gyrB 5′+3	GCTGATGCTGATGTTGATGG	1480–1499
gyrB 3′+1	TGCCCATTCTCAATTAACTCCC	1568–1547
gyrB 3′+2−2nd	GCACTTTTTTTTCTCTCAGACTCTC	1184–1160
gyrB 3′+3	TTCTCTTTTCCCGATCTCCTATC	629–607
glpQ[f]		
glpQ F+1	GGGGTTCTGTTACTGCTAGTGCCATTAC	−234 to −207
Rev-2	CAATACTAAGACCAGTTGCTCCTCCGCC	+135 to +108
Rev-1	GCACAGGTAGGAATGTTGGAATTTATCCTG	485–514
glpQ F−1	CAATTTTAGATATGTCTTTACCTTGTTGTTTATGCC	568–533

[a] For *flaB, gyrB*, and *glpQ*, the minus numbers represent positions upstream of the A in the ATG start codon; plus numbers represent positions downstream of the last base in the stop codon; numbers with no plus or minus sign are within the open reading frame, beginning with the A in the start codon.
[b] Primer from reference 50. Minus and plus positions flank the sequence used in the analysis.
[c] PCR, 1,489 bp; trimmed to 1,272 bp.
[d] PCR, 1,246 bp; open reading frame, 1,005 bp.
[e] PCR, 2,141 bp; open reading frame, 1,905 bp.
[f] PCR, 1,386 bp; open reading frame, 1,017 bp.

program 3 for 18 h with recirculation of the buffer in ice. The gels were stained with ethidium bromide and visualized with UV transillumination. Two-dimensional agarose gel analysis for identification of circular plasmids was performed as previously described (60).

Southern blot analysis. Undigested genomic DNA samples from the borreliae were examined for *blyA* and *bdrC1* by Southern's method (63) and the DIG Luminescent Detection Kit (Roche Applied Science, Indianapolis, Ind.). *blyA* is on a cp32 circular plasmid of *B. hermsii* (64) and encodes a pore-forming hemolysin. *bdrC1* is a member of a large paralogous family containing internal direct repeats, with paralogues on both linear and circular plasmids (21, 74). The gel-bound DNA was depurinated in 0.25 N HCl for 10 min, denatured in 1.5 M NaCl–0.5 M NaOH for 40 min, and neutralized in 0.5 M Tris (pH 7.5)–1.5 M NaCl for 40 min. DNA was transferred overnight by capillary action onto MagnaGraph nylon membranes (Micron Separations Inc., Westborough, Mass.) with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7) and cross-linked to the membrane with a UV Stratalinker 1800 (Stratagene, La Jolla, Calif.). The membranes were prehybridized at 65°C for 6 h in 60 ml of 5× SSC–0.1% (wt/vol) *N*-lauroylsarcosine sodium salt–0.02% SDS–6% blocking reagent (Roche Applied Science).

Hybridization probes were produced with the PCR DIG Probe Synthesis Kit (Roche Applied Science) following the instructions of the manufacturer. Primers for *blyA* were BlyA5′ (GTATTAATAACATATTAGATTTTTTACTTCAAC) and BlyA3′ (CATAAATCCTCCTTTTCTTTGTCTC) (64) and were predicted to amplify a 184-bp fragment. Primers for *bdrC1* were BdrC5′ (CCAATAGTT ACTCAACAAATGG) and BdrC3′ (GGTATCTATTTTGTTATCG) (21) and were predicted to amplify a 321-bp fragment. Genomic DNA of *B. hermsii* DAH was used as a template to produce the *blyA* probe, and DNA of *B. parkeri* RML was used to produce the *bdrC1* probe. PCR amplification included initial denaturation at 96°C for 3 min; 35 cycles of 94°C for 30 s, 55 to 56°C for 30 s, and 72°C for 2.5 min; and a final extension at 72°C for 7 min. The digoxigenin-labeled probes were denatured at 98°C for 10 min, added to 6 ml of fresh hybridization buffer with the membrane, and incubated at 55°C for 18 h. The membranes were washed with 2× SSC–0.1% SDS for 10 min at room temperature and with 0.3× SSC–0.1% SDS for 30 min at 65°C. The blots were incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase and developed with the CDP-*Star* chemiluminescent substrate (both from Roche Applied Science). HyperfilmECL high-performance chemiluminescence film (Amersham Biosciences Corp., Piscataway, N.J.) was exposed to membranes and developed to display the pattern of hybridization.

Electron microscopy. Borreliae were examined for the presence of circular DNA molecules in total genomic extractions. DNA samples were prepared for electron microscopy as previously described (39). Grids were examined with a JEOL 100B electron microscope at 40 kV at a magnification of ×7,000. Plasmid pBR322 was used as a calibration standard, and contour lengths were measured with a Numonics Graphic calculator and a Tektronico 4052A computer.

Nucleotide sequence accession numbers. The 49 new nucleotide sequences of the four loci from the eight isolates of *B. turicatae* and six isolates of *B. parkeri* have been deposited in the GenBank database under accession numbers AY934594 to AY934642. Accession numbers for additional sequences used and deposited previously for a few of these isolates and *B. hermsii* DAH include L37837, U40762, AY597657, AY597716, AY597777, AY604974, AY604975, AY604979, AY604980, AF247156, and AF247157.

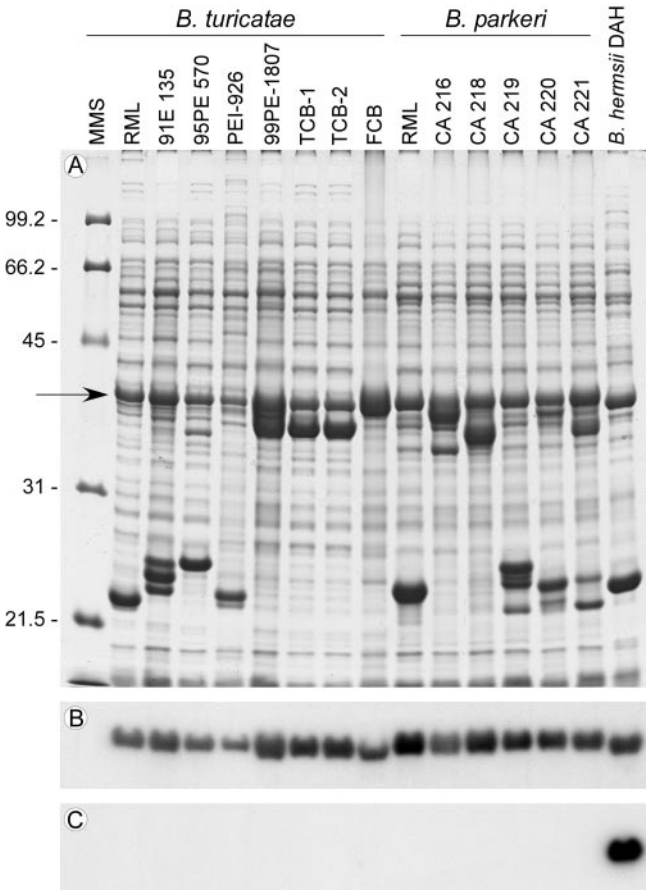

FIG. 1. Protein profiles of whole-cell lysates of *B. turicatae* and *B. parkeri* (A), immunoblot reactivity with *Borrelia* genus-specific monoclonal antibody H9724 (B), and lack of reactivity with *B. hermsii*-specific monoclonal antibody H9826 (C). The arrow indicates the position in the stained gel of flagellin, to which the monoclonal antibodies bind. *B. hermsii* DAH is the only lysate recognized by H9826. Molecular mass standards (MMS) are shown at the left (sizes are in kilodaltons).

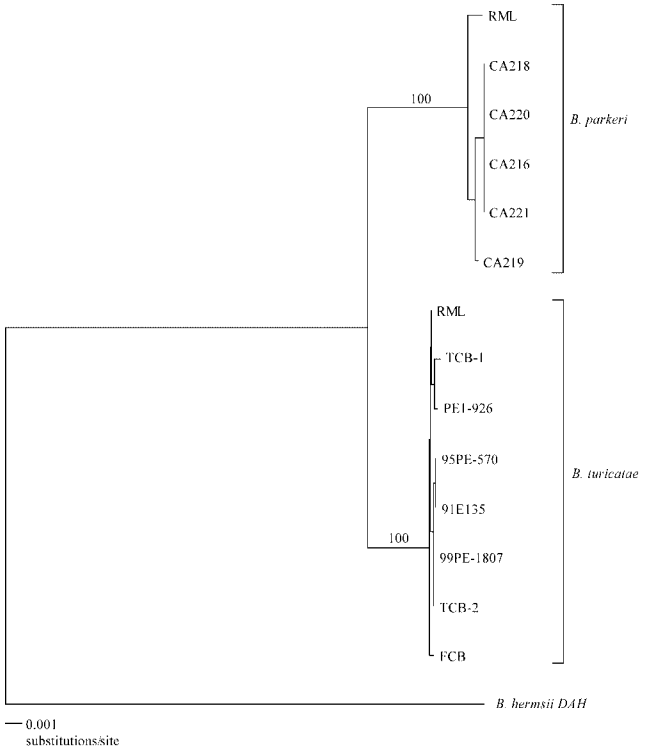

FIG. 2. Phylogenetic tree of the concatenated DNA sequences for the four loci in *B. turicatae* and *B. parkeri* with *B. hermsii* DAH used for the outgroup. The tree was constructed with ClustalV and the neighbor-joining method to distinguish the two species. The reliability of the tree topology was gauged by performing bootstrap analysis using 1,000 replicates. Bootstrap values of 100% at nodes, where the configuration to the right was found, are shown. The bar shown in the graph provides scale to the branch lengths and represents the number of substitutions per site (0.001). The FCB clearly falls within the *B. turicatae* cluster.

RESULTS

Protein profiles and reactivity with monoclonal antibodies. Proteins and immunoblot reactivities were analyzed to characterize and identify the isolates of spirochetes. Whole-cell lysates of the 11 isolates from soft ticks and 3 isolates from dogs had similar protein profiles, except for variation in the number of proteins in the low 20-kDa and high 30-kDa range (Fig. 1). This heterogeneity most likely represented differences in the variable small proteins and variable large proteins produced by the in vitro population of spirochetes at the time the lysates were prepared. The higher-passage RML isolates of *B. turicatae* and *B. parkeri* had only a single dominant protein in the low 20-kDa range. This phenotype may be due to a switch and stabilization to a single variable small protein in culture, as has been demonstrated for *B. hermsii* (9, 67) and which is associated with persistent infection in ticks (56). All of these isolates reacted with monoclonal antibody H9724 (Fig. 1B) but not with H9826 (Fig. 1C), which identified these spirochetes in the genus *Borrelia* but not *B. hermsii*.

DNA sequence analysis. Multilocus sequence typing was performed to further characterize the isolates at the molecular level. Four loci totaling 5,190 bases were determined for the 14 isolates in the two species. The 16S rRNA gene was the least powerful at separating the two species, as there was only one base difference between them in the 1,272 bp determined (99.92% identity). The complete sequences for *flaB*, *gyrB*, and *glpQ* all separated the two species with the same branch pattern; therefore, we show only the tree based on the concatenated sequence for all four loci compared to *B. hermsii* (Fig. 2). The 1,000 bootstrap replicates of the concatenated sequences demonstrated that 100% of the trees produced identical nodes for the *B. turicatae* and *B. parkeri* clusters (Fig. 2). The FCB clearly grouped with the other isolates of *B. turicatae*, which identified this spirochete to the species level. There were 52 base positions in the three protein-coding loci examined that were unique to all isolates in either species; however, these spirochetes are closely related, with high DNA identity values also for these three loci (*flaB* = 98.8%, *gyrB* = 98.84%, and *glpQ* = 98.22%). Within each species, there were only a few base positions that varied, and when single base substitutions were present, only a few of these resulted in amino acid substitutions (Table 3).

TABLE 3. Number of variable DNA sequence positions and amino acid changes in four loci among 14 isolates of *B. turicatae* and *B. parkeri*

Locus (no. of bases)	No. of base positions that vary[a]	
	B. turicatae	*B. parkeri*
16S rRNA (1,272)	0	0
flaB (1,002)	1 (N in FCB)	0
gyrB (1,902)	5 (4S, 1N in FCB)	6 (5S, 1N in RML and CA 219)
glpQ (1,014)	2 (1N in 4 isolates, 1N in TCB-1)	5 (4S, 1N in RML)

[a] S = synonymous substitution with no amino acid change; N = nonsynonymous substitution (amino acid change[s] followed by the isolates with amino acid substitutions).

Analysis of plasmids. The genomes of borreliae are extremely complex, with numerous linear and circular plasmids that vary among species and may be diagnostic. A diversity of linear plasmids was observed among the isolates by reverse-field agarose gel electrophoresis of uncut genomic DNA (Fig. 3A). All the spirochetes had a small linear plasmid of approximately 13 to15 kbp and numerous larger linear plasmids varying in size from approximately 20 to 55 kb. A single large linear plasmid ranging from 180,000 to 220,000 bp has been reported for some tick-borne relapsing fever spirochetes (22, 41), but this was not distinguishable from the sheared chromosomal DNA. *B. turicatae* FCB and five of the *B. parkeri* isolates had an additional plasmid approaching 100 kb. *B. turicatae* 91E135 had 10 resolved linear plasmids, but the number of plasmids in most of the other isolates was difficult to determine precisely as some of the more intensely stained bands certainly represented doublets or triplets of nearly comigrating molecules. Also, there may be families of multiple plasmids of similar sizes, as has been described for *B. burgdorferi* (23, 34), that were not detectable by electrophoresis.

While the linear plasmids in *B. parkeri* appeared to be slightly larger than in *B. turicatae*, the variability in size and uncertainty in the number of these plasmids made identifying a unique pattern for either species difficult. However, all the *B. turicatae* isolates had diffuse bands of slower-migrating DNA that were suggestive of circular molecules that ran aberrantly as much larger molecules than their true size (32, 61). None of these bands was observed in the *B. parkeri* isolates (Fig. 3A), which suggested that *B. parkeri* lacked circular plasmids. To pursue this further, we performed a Southern blot assay with the DNA transferred from the gel shown in Fig. 3A and a probe to *blyA*, a gene which encodes a proposed holin and is located on the cp32 circular plasmids of *B. burgdorferi* and *B. hermsii* (23, 64). This probe hybridized strongly to the diffuse, slower-migrating DNA in *B. hermsii* (Fig. 3B), which confirmed the aberrant gel location of the 30-kb circular plasmids in this species. A similar pattern was observed for all the *B. turicatae* isolates but for none of the *B. parkeri* isolates, in which the probe hybridized only to a single linear plasmid varying in size from approximately 40 to 55 kb (Fig. 3B). The probe also hybridized to linear plasmids in *B. turicatae* and *B. hermsii*,

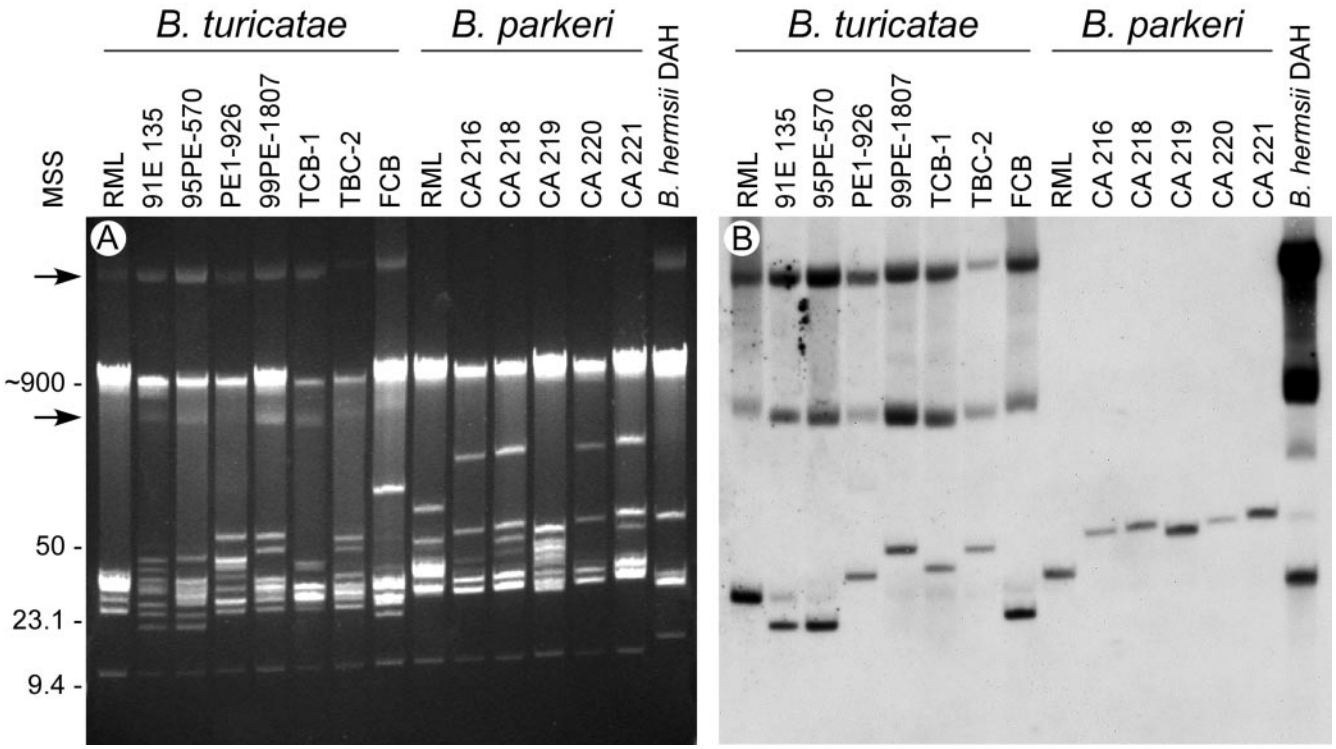

FIG. 3. Reverse-field agarose gel of total genomic DNA for plasmids in *B. turicatae* and *B. parkeri* (A) and Southern blot assay with the *blyA* probe to identify the approximately 30-kb circular plasmids (B). Arrows on the left indicate the positions of the aberrantly migrating 30-kb circular plasmids in *B. turicatae* and *B. hermsii* that hybridized with the probe but were not present in *B. parkeri*. Positions of molecular size standards (MSS) are shown on the left (sizes are in kilobases).

which, given the high stringency of the blot, suggested that there may be a second copy of *blyA* or other paralogous sequences on the linear plasmids. A second Southern blot was performed with the *bdrC* probe, which hybridized to multiple linear plasmids in all the samples and to the aberrant, slower-migrating circular DNAs in *B. turicatae* and *B. hermsii* but not with any *B. parkeri* DNA in this region of the gel (data not shown). This result also suggested that *B. parkeri* lacked circular plasmids.

Next we performed two-dimensional agarose gel analysis to confirm the presence of circular plasmids by their retarded migration in the second dimension after being nicked to open circles with UV irradiation. We examined total genomic DNAs from 14 species of borreliae and identified circular plasmids in *B. burgdorferi* B31, *B. garinii* G2, *B. afzelii* VS461, *B. bissettii* DN127, *B. valaisiana* VS116, *B. japonica* HO14, *B. hermsii* DAH, *B. turicatae* 91E135, *B. coriaceae* Co53, and *B. crocidurae* CR2A (data not shown). We were unable to examine *B. duttonii*; however, a circular plasmid has been found in this African borrelia (68). However, no circular plasmids were detected in the six isolates of *B. parkeri*, *B. anserina* BA2, or *B. recurrentis* 132. Therefore, we performed Southern blot assays with a selected set of DNA samples after electrophoresis in two dimensions (Fig. 4). The stained gel clearly demonstrated circular plasmids in *B. hermsii* and both low- and high-passage *B. turicatae* but not in *B. parkeri*, *B. anserina*, or *B. recurrentis* (Fig. 4A). Southern blot assays with the *blyA* probe (Fig. 4B) and the *bdrC* probe (Fig. 4C) demonstrated strong hybridization with the retarded circular DNAs in *B. hermsii* and *B. turicatae* but not with the other species, including *B. parkeri* CA216 or the five other isolates of this species. The presence of circular plasmids in the *B. turicatae* isolate after 1 year of continuous cultivation and 100 passages suggested that the apparent absence of circular plasmids in the low-passage *B. parkeri* isolate was not a result of artificial growth.

Finally, DNA samples from a few species were examined by electron microscopy for the presence of circular molecules. Circular DNAs with contour lengths corresponding to approximately 30 kb were found in *B. turicatae* 91E135, *B. coriaceae* Co53, and *B. crocidurae* CR2A but not in *B. parkeri* RML and CA218 or *B. anserina* BA2. These results supported the electrophoretic and Southern blot analysis finding that circular plasmids were absent in at least three species of *Borrelia*, including *B. parkeri*.

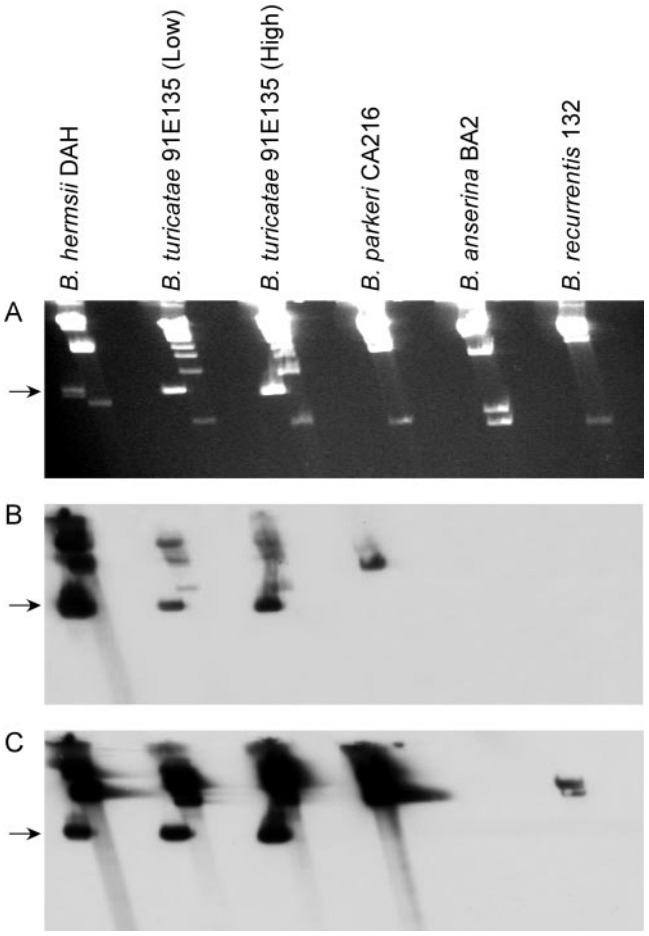

FIG. 4. Two-dimensional agarose gel electrophoresis to determine the presence of circular plasmids in several species of *Borrelia* (A) and Southern blot assays utilizing the same gel samples transferred and hybridized with the *blyA* probe (B) and the *bdrC* probe (C). Arrows on the left identify the circular plasmids with retarded migration in the second dimension that hybridized with both probes. Two samples of the same isolate of *B. turicatae* were included before (Low) and after (High) 1 year of continuous in vitro cultivation.

DISCUSSION

The *B. turicatae* and *B. parkeri* isolates partially characterized prior to this study are rare, numbering only four for both species. Our literature review, as well as GenBank and PubMed searches, found only *B. turicatae* RML, 91E135 and its Ozona clone (Oz1) (18, 20), and M2007 and *B. parkeri* RML, M3001, 6230, and 6232 (38), all of which originated from ticks. A recent study to type relapsing fever spirochetes based on a 663- to 685-bp intergenic spacer sequence included only two isolates of *B. turicatae* (RML/Kansas and Oz1) and none of *B. parkeri* (16). The new isolates from ticks and dogs, including the FCB (13), and results described here help strengthen the foundation for these two distinct taxa.

All analyses identified the FCB as *B. turicatae*, thus confirming the potential for tick-borne relapsing fever in humans and other animals in Florida, where the infected dogs were first identified (13). These spirochetemic dogs were infected 31 months apart at two locations 72 km from each other, which suggested that a focus of endemicity of this spirochete was present (13). The animals involved in the maintenance of this spirochete in nature are not known, although laboratory mice and guinea pigs are susceptible to infection and produce microscopically detectable spirochetemias and pathological abnormalities including myocarditis, dermatitis, and lymphoid hyperplasia (12). We suspect the tick vector in Florida is *O. turicata*, which has been collected from locations near the infected dogs and several other counties in this state (1, 25). Additional field and laboratory work is needed to determine the distribution of these ticks, the presence and prevalence of spirochetes in them, and other animals involved in the enzootic cycle.

Serological surveys may also be fruitful to assess prior exposure to *B. turicatae* in this region. The initial description of the

FCB also included a serological survey that demonstrated that 17 of 99 (17%) dog serum samples were reactive with the spirochete (13). A serological test for anti-GlpQ antibodies, which discriminates relapsing fever from Lyme disease, might also help delineate the distribution of past infections (49, 59). And as with the first identified dogs, microscopic examination of blood smears for the presence of spirochetes is the most rapid method to confirm relapsing fever in humans and other animals (17).

An unexpected finding was the absence of circular plasmids in *B. parkeri*, as well as *B. anserina* and *B. recurrentis*. Hyde and Johnson were the first to identify plasmids in borreliae in 1984 (43), although the complexity of 21 plasmids (12 linear and 9 circular) as exemplified by *B. burgdorferi* was not fully appreciated until the structure of its genome was determined (23, 34). Linear plasmids were first identified in *B. hermsii* (48) and *B. burgdorferi* (7). The *B. turicatae* and *B. parkeri* isolates we examined contained numerous linear plasmids, which are abundant and ubiquitous among all members of the genus (66). A comprehensive survey for both linear and circular plasmids in all species of *Borrelia* has not been done, and why some bacteria have extrachromosomal DNAs in both circular and linear forms is not clear (65). *B. burgdorferi* loses some linear and circular plasmids quickly when grown continuously in vitro (6, 53, 62), although the cp32 circular plasmid family is relatively stable (66). Plasmid instability during in vitro growth has not been reported for relapsing fever spirochetes. Here, we examined one *B. turicatae* isolate before and after 100 passages during continuous growth for 1 year. We saw no apparent reduction in hybridization of the *blyA* and *bdrC* probes with the cp32 circular plasmids in the high-passage preparation. Additionally, five of the six isolates of *B. parkeri* we examined had been passaged only four to seven times after isolation from ticks; therefore, the absence of the cp32 circular plasmids from these spirochetes was unlikely an in vitro artifact.

Our results conflict with those of a previous study investigating the large family of *bdr* (borrelia direct repeat) genes, in which an oligonucleotide probe was stated to hybridize with a 32-kb circular plasmid in *B. parkeri* (21). The one *B. parkeri* isolate used for the *bdr* analysis (21), while not identified, originated from our laboratory and is the same isolate we designated RML in the present work. Because of this discrepancy, we performed multiple Southern blot assays with our six isolates of *B. parkeri*, *blyA* and *bdrC* probes, and DNA samples resolved in both one- and two-dimensional gels. We failed to find evidence for the presence of circular plasmids in *B. parkeri*, and we believe the lack of such extrachromosomal elements uniquely distinguishes this species from the other primary tick-borne relapsing fever spirochetes in North America. The demonstration here that three species of borrelia lack circular plasmids may also be of value to investigators interested in plasmid diversity, structure, and evolution within the genus.

Finally, the four loci we sequenced in all isolates each separated the spirochetes into two groups, although only one base difference in the 16S rRNA gene was found between them. As discussed by others (33), this highly conserved, non-protein-coding locus may not be sufficient to discriminate between closely related species. Therefore, we also examined *flaB*, *gyrB*, and *glpQ*, which are loci with utility in defining borreliae or other prokaryote taxa (2, 36, 42, 49). Each of these loci separated the spirochetes into the same two closely related groups (>98% sequence identity), with little variation in either cluster. Yet both species of spirochetes are transmitted by different tick species (26), and as described herein there is a fundamental difference in the structures of the genomes, with circular plasmids present in *B. turicatae* but not in *B. parkeri*. Therefore, in spite of the high DNA sequence identity shared by these two groups of spirochetes, we believe these two species are likely of recent evolutionary separation, just as their two tick vectors probably are (25), and the specific status of these spirochetes should be retained. Future efforts should be directed toward elucidating to what extent these spirochetes cause relapsing fever infections among humans and other animals.

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