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Endosymbionts of Ticks and Their Relationship to *Wolbachia* spp. and Tick-Borne Pathogens of Humans and Animals†

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The presence, internal distribution, and phylogenetic position of endosymbiotic bacteria from four species of specific-pathogen-free ticks were studied. These included the hard ticks *Ixodes scapularis* (the black-legged tick), *Rhipicephalus sanguineus* (the brown dog tick), and *Haemaphysalis longicornis* and the African soft tick *Ornithodoros moubata*. PCR assays for bacteria, using two sets of general primers for eubacterial 16S and 23S rRNA genes (rDNAs) and seven sets of specific primers for wolbachial, rickettsial, or *Francisella* genes, indicated that *I. scapularis* possessed symbiotic rickettsiae in the ovaries and that the other species harbored eubacteria in both the ovaries and Malpighian tubules. Phylogenetic analysis based on the sequence of 16S rDNA indicated that the symbiont of *I. scapularis* belonged to the alpha subgroup of proteobacteria and was closely related to the members of the genus *Rickettsia*. The other species had similar microorganisms in the ovaries and Malpighian tubules, which belonged to the gamma subgroup of proteobacteria, and formed a monophyletic group with the Q-fever pathogen, *Coxiella burnetii*. *O. moubata* harbored another symbiont, which formed a monophyletic group with *Francisella tularensis* and *Wolbachia persica*, the latter a symbiont previously isolated from Malpighian tubules of the soft tick *Argas* (*Persicargas*) *arboreus*. Thus, the symbionts of these four tick species were not related to the *Wolbachia* species found in insects. The two symbionts that live in the Malpighian tubules, one closely related to *C. burnetii* and the other closely related to *F. tularensis*, appear to be of ancient origin and be widely distributed in ticks.

Ticks are of considerable medical and veterinary importance, because they harm the host through their feeding action and vector many pathogens. Tick-transmitted bacterial pathogens are quite diverse and include organisms belonging to the genera *Borrelia*, *Rickettsia*, *Francisella*, *Ehrlichia*, *Anaplasma*, *Cowdria*, and *Coxiella* (41). Ticks also harbor unidentified nonpathogenic rickettsia- and wolbachia-like bacteria which are possibly mutualistic endosymbionts (11, 17). Numerous reports have been published on the morphological appearance and distribution of symbionts in various tick species (17). To date, the relationship of the endosymbionts to tick-borne pathogens has been examined mainly by electron microscopy (12, 17–19, 25, 35, 45, 54, 55). The symbionts, usually localized in the Malpighian tubules and/or ovaries, have been identified as being rickettsiae or wolbachiae (17–19, 40, 54).

The relationship of the endosymbionts to the tick-borne pathogenic bacteria of humans and animals and to the wolbachiae of insects remains quite unclear. The genus *Wolbachia* was created by Hertig (20), who studied the rickettsial symbiont found in the gonads of *Culex pipiens* and named it *W. pipientis*. The *Wolbachia* spp. of insects are closely related phylogenetically to the *Rickettsia*, *Ehrlichia*, and *Anaplasma* species transmitted by ticks (32, 36). Endosymbionts of the soft tick *Argus* (*Persicargas*) *arboreus* (previously thought to be *Argas persicus* and hereafter referred to as *A. arboreus*) and the hard tick *Dermacentor andersoni* have been cultured in the yolk sacs of chicken embryos and found to be pathogenic for guinea pigs (7, 43, 44). The *A. arboreus* microbe has been placed in the genus *Wolbachia* and named *W. persica* (43, 44). Since the symbionts in the hard (ixodid) ticks resembled those of *A. arboreus* in distribution and ultrastructure, they have also been considered to be wolbachiae. To further complicate the relationships, there are numerous examples of the long-term maintenance of secondarily acquired pathogenic rickettsiae that behave like endosymbionts (17). It is important to determine the taxonomic status of tick endosymbionts, based on a cladistic analysis, in order to gain insight as to their origin, possible function, and distinction from the tick-transmitted pathogens of humans and animals.

The objectives of this study were to examine the endosymbionts of the hard ticks *Ixodes scapularis*, *Rhipicephalus sanguineus*, and *Haemaphysalis longicornis* and the soft tick *Ornithodoros moubata* and to establish their relationship to pathogenic bacteria transmitted by ticks. We used PCR along with a combination of general and specific PCR primer pairs to detect microorganisms in various organs of these tick species. General primers for bacterial 16S and 23S rRNA genes (rDNAs) were used to detect the presence or absence of eubacteria, and specific primers were used to determine if the symbionts were *Rickettsia*, *Francisella*, or *Wolbachia* spp. We sequenced the small subunit (16S) rDNAs of the symbionts of these four tick species, and the sequences were used to perform a cladistic analysis and determine their phylogenetic positions.

MATERIALS AND METHODS

Arthropods. Three species of hard ticks (suborder Ixodida, family Ixodidae) were used: *I. scapularis*, *R. sanguineus*, and *H. longicornis*. These species were from laboratory colonies maintained on host animals free of tick-borne pathogens. *I. scapularis* was fed on hamsters and adult females on Dutch belted rabbits. *R. sanguineus* was from a colony maintained at Oklahoma State University (provided by S. Ewing). *H. longicornis* was from a colony which was parthenogenetically maintained at Tohoku Agricultural Experiment Station (provided by M. Ohtaishi). The soft tick *O. moubata* (family Argasidae) was provided by Y.

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TABLE 1. Oligonucleotide primers used for PCR

Primer No.[a]	Primer Name	Species	Gene	Nucleotide sequence (5′-3′)	Approx product size (bp)	Reference
1	12Sai	*Drosophila yakuba*	Mitochondrial 12S rRNA	AAACTAGGATTAGATACCCTATTAT	380	39
	12Sbi			AAGAGCGACGGGCGATGTGT		
2	*Ec*16S	*Escherichia coli*	16S rRNA	GCTTAACACATGCAAG	1,198	32
				CCATTGTAGCACGTGT		
3	*Ec*23S	*E. coli*	23S rRNA	CCGAATGGGGAAACCC	1,508	36
				CCACCTGTGTCGGTTT		
4	*Rp*CS.877p	*Rickettsia prowazekii*	Citrate synthase	GGGGGCCTGCTCACGGCGG	381	33
	*Rp*CS.1258n			ATTGCAAAAAGTACAGTGAACA		
5	*Rr*17.61p	*R. rickettsii*	17-kDa genus-common antigen	GCTCTTGCAACTTCTATGTT	434	52
	*Rr*17.492n			CATTGTTCGTCAGGTTGGCG		
6	*Rr*190.70p	*R. rickettsii*	190-kDa antigen (OmpA)	ATGGCGAATATTTCTCCAAAA	532	33
	*Rr*190.602n			AGTGCAGCATTCGCTCCCCCT		
7	*Rr*120	*R. rickettsii*	120-kDa antigen (OmpB)	CTAGTGCAGATGCAAATG	500	16
				GTTTGAAATTGATAATTG		
8	*Wp*16S	*Wolbachia pipientis*	16S rRNA	TTGTAGCCTGCTATGGTATAACT	890	32
				GAATAGGTATGATTTTCATGT		
9	*fts*Z	*Wolbachia* sp.	*ftsZ* homolog	GTATGCCGATTGCAGAGCTTG	769	22
				GCCATGAGTATTCACTTGGCT		
10	F5	*Francisella* species	16S rRNA	CCTTTTTGAGTTTCGCTCC	1,142	14
	F11			TACCAGTTGGAAACGACTGT		

[a] Numbers correspond to those indicated in Fig. 1 and 2.

Chinzei (Mie University). The planthopper *Laodelphax striatellus*, Izumo local strain, which possess a *Wolbachia* sp., was maintained on rice seedlings in the laboratory (31).

Microorganisms. *Escherichia coli* JM109 (competent cells) was purchased from Toyobo Co., Ltd. *Rickettsia rickettsii* HLP2 and *R. montana* M/5-6 were obtained from the NIH Rocky Mountain Laboratories. Both species were propagated in the *I. scapularis* IDE2 or the *Dermacentor albopictus* DALBE3 cell line (29, 30). *W. persica* (ATCC VR-331) was obtained from the American Type Culture Collection. Lyophilized *W. persica* was initially cultured in the *D. albopictus* DALBE3 cell line and after 1 passage grown axenically in tick cell culture medium L15B supplemented with fetal bovine serum (5%), tryptose phosphate broth (10%), cholesterol concentrate (1%), morpholinepropanesulfonic acid (10 mM), and $NaHCO_3$ (1%).

Light microscopy. The dissected organs were smeared on glass slides and air dried. The specimens were fixed twice with methanol and stained with a 5% Giemsa solution buffered with sodium phosphate at pH 6.5 for 30 min at 37°C.

DNA preparation and PCR amplification. Template samples for PCR amplification were prepared as described by O'Neill et al. (32). Briefly, larvae, nymphs, or organs dissected from adult ticks were homogenized in 50 μl of STE buffer (100 mM NaCl, 1 mM EDTA [pH 8.0], 10 mM Tris-HCl [pH 8.0]) and incubated with 3 μl of proteinase K (10 mg/ml) for 40 to 60 min at 37°C. The homogenates were boiled for 3 min to inactivate the proteinase K, centrifuged in a microcentrifuge, and stored at −21°C until needed. Rickettsial and *W. persica* DNAs were prepared as described by Higuchi (21). The DNA of the *Wolbachia* sp. in *L. striatellus* ovaries was prepared as described by O'Neill et al. (32).

Primers used for amplification are shown in Table 1. The primers were synthesized by a DNA synthesizer (model 392 or 394; Applied Biosystems, Inc.). The mitochondrial 12S rDNA primers, 12Sai and 12Sbi (32, 39), were used as a control to check the general integrity of the template DNA preparation and PCR. The *Rp*CS primer set is generic for rickettsiae and amplifies homologous DNA sequences from all species of *Rickettsia* (33). The *Rr*17 primer set is also a generic rickettsial primer set for rickettsiae belonging to the spotted fever group (SFG) or typhus group (52). The *Rr*190 primer set amplifies a region of the gene for the 190-kDa outer membrane protein (OmpA) of *R. rickettsia* and homologous regions of all SFG rickettsiae (33). Similarly, the *Rr*120 primer set is generally reactive with the SFG rickettsiae but not the members of the typhus group (16). Two generic primer sets for the genus *Wolbachia* were used. The *Wp*16S primers are broadly reactive with the *Wolbachia* species found in insects but not with *W. persica* (32). Similarly, the *ftsZ* primer set, based on sequences in a gene coding for a protein that initiates cell division in prokaryotes, is broadly reactive with *Wolbachia* species in insects (22). Finally, we used the F5-F11 set of primers, which is generic for members of the genus *Francisella* and which also amplifies the 16S rDNA of *W. persica* (14).

Amplifications were performed in 50 μl of buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin) with 0.12 mM each deoxynucleoside triphosphate, 20 pmol or 10 μM each primer, 2.5 U of *Taq* polymerase (Perkin-Elmer), and 2 μl of a DNA sample. The PCR cycling conditions were as follows: 1 cycle of 94°C for 30 s; 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 2 min; and a final extension at 72°C for 5 min. PCR products were electrophoresed in a 0.7 to 1.0% agarose gel and stained with ethidium bromide.

rDNA sequencing. To obtain almost-full sequence of small subunit rDNA of symbionts, a primer pair (fD1-rP2 [Table 2]) that amplifies 16S rDNA from most bacteria (48), was used for PCR amplification. The PCR products were treated with a DNA blunting kit (TAKARA) and ligated into pBluescript II (Stratagene).

Plasmid DNA was isolated by the alkaline lysis procedure (38) and used as the sequencing template. The PCR-amplified DNA from the plasmids was also used as a template. The sequences were determined by the *Taq* dye primer and/or the *Taq* dye terminator cycle sequencing method (Applied Biosystems) with a DNA Sequence system (model 373A or 373S; Applied Biosystems). The sequencing primers used in the latter method are shown in Table 2. The sequences of the 16S rDNA were determined for at least two clones having opposite orientations.

Phylogenetic analysis. Homology searches of the 16S rDNA sequences in the GenBank DNA database were performed by using the mpsearch program through e-mail delivery service in the computer center of the Ministry of Agriculture, Fisheries, and Forestry, Tsukuba, Japan. The sequence data of 16S rDNAs for 23 species of bacteria, obtained from GenBank or the DNA Data Base of Japan, were selected for phylogenetic analysis. The representatives of the alpha subclass of the class *Proteobacteria* (42, 53) that we used (and, in parentheses, the DNA database accession numbers) were *R. rickettsii* (L36217), *Rickettsia rhipicephali* (L36216), *Orientia tsutsugamushi* (U17257), *Anaplasma marginale* (M60313), *Ehrlichia equi* (M73223), *W. pipientis* (U23709), *Brucella abortus* (X13695), *Agrobacterium tumefaciens* (M11223), and *Rhodospirillum rubrum* (D30778). Representatives of the beta subclass included *Chromobacterium violaceum* (M22510), *Neisseria polysaccharea* (L06167), and *Pseudomonas testosteroni* (M11224). Representatives of the gamma subclass included *Serratia marcescens* (M59160), *E. coli* (J01859), *Coxiella burnetii* (M21291), *Francisella tularensis* (Z21931), *W. persica* (M21292), *Xenorhabdus nematophilus* (a symbiont of the entomopathogenic nematode *Steinernema carpocapsae*; X82251), *Arsenophonus nasoniae* (an endosymbiont of the wasp *Nasonia vitripennis*; M90801), *Buchnera aphidicola* (the primary endosymbiont of the aphid *Acyrthosiphon pisum*; M27039), the primary endosymbiont of a whitefly, *Bemisia tabaci* (Z11925), and an endosymbiont of the clam *Codakia orbicularis* (X84979). The gram-positive eubacterium *Bacillus thuringiensis* (D16281) was used as an outgroup species.

Stretches of about 1,450 bp of the 16S rDNA sequences were aligned by using CLUSTAL W software (46) for multiple sequence alignment. A large gap and two small gaps in the alignment sequences were deleted manually. Tree topology was built by the neighbor-joining method of PHYLIP (Phylogeny Inference Package), version 3.5C (13). Confidence values for individual branches of the resulting tree were determined by a bootstrap analysis in which 500 bootstrap trees were generated from resampled data.

Nucleotide sequence accession numbers. The GSDB, DDBJ, EMBL, and NCBI accession numbers for the nucleotide sequences of the 16S rDNAs of symbionts are D84558 (*I. scapularis*), D84559 (*R. sanguineus*), AB001519 and AB001520 (*H. longicornis*), and AB001521 and AB001522 (*O. moubata*).

TABLE 2. Oligonucleotide primers used for sequencing 16S rDNA

Primer	Nucleotide sequence (5′-3′)	Position in *E. coli*	Reference
Forward			
fD1	AGA GTT TGA TCC TGG CTC AG	8–27	48
f16S3	AGT GGG GAA TAT TGG ACA ATG	356–376	37
f16S5	GTG CCA GCA GCC GCG G	515–530	36
f16S7	GTA GAG GTG AAA TTC GTA AA	685–704	
f16S9	ATT AGA TAC CCT (AG)GT AGT CC	787–806	15
f16S11	AAA CT(CT) AAA (GT)GA ATT GAC GG	907–926	24
f16S13	TTT AAT TCG ATG CAA CGC G	955–973	
f16S15	GAG GAA GGT GGG GAT GAC GTC AA	1175–1197	
Reverse			
r16S2	CAT TGT CCA ATA TTC CCC ACT	376–356	
r16S4	CCG CGG CTG CTG GCA C	530–515	
r16S6	TTT ACG AAT TTC ACC TCT AC	704–685	36
r16S8	GGA CTA C(CT)A GGG TAT CTA AT	806–787	
r16S10	CCG TCA ATT C(AC)T TT(AG) AGT TT	926–907	
r16S12	CGC CTT GCA TCG AAT TAA A	973–955	37
r16S14	TTG ACG TCA TCC CCA CCT TCC TC	1197–1175	15
r16S16	CCT TGT TAC GAC TTC ACC C	1505–1487	15
rP2	ACG GCT ACC TTG TTA CGA CTT	1511–1491	48

RESULTS

PCR detection of symbionts in ticks. The fidelity of the primer pairs was first confirmed by using positive control DNA prepared from the bacterium *E. coli*, two species of rickettsiae (*R. montana* and *R. rickettsii*), and a *Wolbachia* sp. (from the planthopper *L. striatellus*). The general primers for eubacterial 16S and 23S rDNAs amplified DNA fragments of the predicted sizes from all four microbes. The primer sets *Rp*CS, *Rr*17, *Rr*190, and *Rr*120 generated fragments of the appropriate sizes with *R. montana* and *R. rickettsii* but not with the planthopper *Wolbachia* sp. and *E. coli*. The generic *Wolbachia* primers *Wp*16S and *ftsZ* amplified the predicted DNA products from only the *Wolbachia* sp. Nonspecific DNA bands or minor fragments were observed in some reactions, but the positive amplifications, which gave major fragments of the appropriate sizes, were easily recognized.

Ten unfed *I. scapularis* larvae were examined for the presence of symbiotic microorganisms by using PCR amplification with the nine primer sets (Table 1, primers 1 to 9). All larvae amplified fragments of the appropriate sizes for the eubacterial 23S rDNA, the rickettsial *Rp*CS and *Rr*190, and seven individuals amplified fragments consistent with rickettsial *Rr*17 (data not shown). None of the larvae amplified DNA with the *Rr*120, *Wp*16S, or *ftsZ* primer pair. These results indicated that all larvae were infected with an organism belonging to the genus *Rickettsia*. We then examined 10 individual nymphs by using the 23S rDNA-*Rp*CS-*Rr*17-*Rr*190 combination. Six of the 10 nymphs gave positive reactions (data not shown), indicating that some of the nymphs, possibly the males, did not possess endosymbionts. It has been previously reported that the rickettsial symbionts of *I. ricinus* are restricted to the ovarian primordia of females and absent in testicular primordia of males (55). This possibility was considered further by examining selected organs of adult *I. scapularis* for rickettsiae by using PCR amplification. The salivary glands, midgut, Malpighian tubules, ovaries, testes, and forelegs were each tested separately, using the nine primer pairs. The mitochondrial 12S rDNA product from tick mitochondria was detected with all tissues. Amplification of fragments with the primers designed to detect eubacterial genes occurred only with ovarian tissues. The other organs and tissues, including the testes and Malpighian tubules, did not yield amplified products (Fig. 1). With ovaries, PCR products of appropriate sizes were obtained with the 16S and 23S rDNA primers (Fig. 1, lanes 2 and 3) and three of the rickettsial primers (*Rp*CS, *Rr*17, and *Rr*190) (Fig. 1, lane 4 to 6). As with larvae, the ovarian samples did not amplify fragments with the *Rr*120 primer pair. These results strongly suggested that *I. scapularis* females harbored a *Rickettsia* species, possibly a member of the SFG, in the ovarian tissues.

R. sanguineus nymphs yielded prominent amplification products with the primers for eubacterial 16S and 23S rDNAs. Strong positive PCR amplifications with the eubacterial 23S rDNA primers were obtained in all eight individual nymphs tested. The adult organs were also examined by using the nine pairs of primers. Positive fragments were amplified in the ovary and Malpighian tubules with the eubacterial 16S and 23S rDNA primers (Fig. 2, lanes 2 and 3), indicating the presence of bacteria in these two organs. The salivary glands, midgut, testes, and forelegs gave no specific PCR-amplified products. As with the nymphs, none of the adult tissues amplified prod-

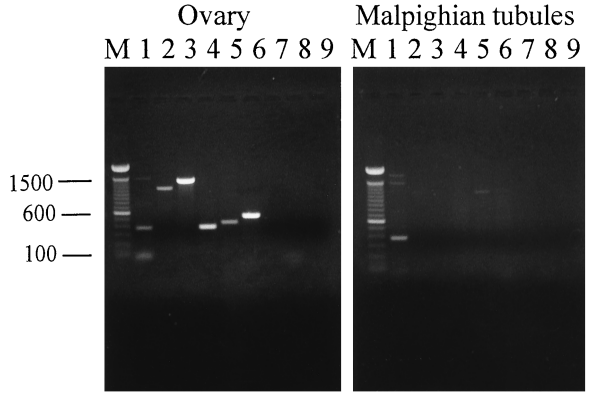

FIG. 1. PCR-amplified products from the ovary and Malpighian tubules of *I. scapularis*. M, 100-bp DNA ladder. Numbers 1 through 9 refer to the primer pairs shown in Table 1. Numbers on the left indicate sizes in base pairs.

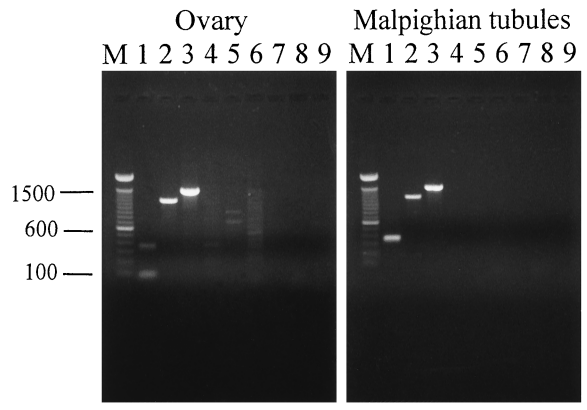

FIG. 2. PCR-amplified products from the ovary and Malpighian tubules of *R. sanguineus*. M, 100-bp DNA ladder. Numbers 1 through 9 refer to the primer pairs shown in Table 1. Numbers on the left indicate sizes in base pairs.

ucts in assays using any of the primer sets designed to detect rickettsiae or *Wolbachia* species.

The *H. longicornis* ovarian and Malpighian tubule homogenates yielded PCR amplifications with the eubacterial 16S and 23S rDNA primers but not with the rickettsial and wolbachial primers (data not shown). This finding indicated that *H. longicornis* harbored eubacteria in these organs.

Results with *O. moubata* were similar to those obtained with *R. sanguineus* and *H. longicornis* in that amplified products of the appropriate sizes were obtained from the ovaries and Malpighian tubules with the 16S and 23S rDNA primers. The bacterial 16S and 23S primer pairs, however, amplified two sets of products closely related in molecular size (data not shown). The amplification of two distinct products indicated that these tissues were infected with two (or more) different kinds of microorganisms. *W. persica*, which was isolated from the Malpighian tubules of another soft tick, *A. arboreus* (44), has recently been found to be a *Francisella* species (14). Thus, we tested a generic *Francisella* primer set (Table 1). The ovaries and Malpighian tubules of *O. moubata* amplified a 1,150-bp product with the *Francisella*-specific oligonucleotides (data not shown). In addition, the axenically cultured *W. persica* was also confirmed to yield a fragment of the expected size for members of the *Francisella* genus.

Observation of symbionts. We examined Giemsa-stained smear preparations of various organs for microorganisms by light microscopy. The ovarian tissues of *I. scapularis* were heavily infected with coccoid or short rod-shaped rickettsia-like microorganisms as described for *I. ricinus* (28). In *R. sanguineus*, similar microorganisms were observed in both the ovaries and Malpighian tubules. The anterior two thirds of the Malpighian tubules were heavily infected, but the posterior parts connected to the rectal bladder were free of microorganisms. The *R. sanguineus* symbionts varied shape and size and were pleomorphic as previously described (10). Some were coccoid, some were rod shaped, and others were quite elongated. These results confirmed that those tissues that gave positive PCR results were indeed colonized by microbes.

Nucleotide sequences of 16S rDNA of the symbionts. We obtained an almost full-sized 16S rDNA fragment of the predicted size (approximately 1.5 kbp) by using primer pair fD1-rP2 (48); 1,420 and 1,460 nucleotides excepting primer regions were sequenced for the 16S rDNAs in the symbionts of *I. scapularis* and *R. sanguineus* (DNA database accession no. D84558 and D84559), respectively. The restriction endonuclease (*Eco*RV, *Hin*cII, *Sac*II, *Sma*I, and *Xba*I) digestions of the PCR-amplified products supported the evidence that the products from either *I. scapularis* or *R. sanguineus* were composed of one species of DNA. The PCR fragments amplified from the ovaries and Malpighian tubules of *R. sanguineus* also gave the same restriction endonuclease digestion patterns (data not shown). In addition, the sequences of the 16S rDNA cloned from the ovarian and Malpighian tubule fragments were the same.

In contrast, PCR amplification using the fD1 and rP2 primers amplified two distinct products in *O. moubata*, similar to the results with the primers for 16S and 23S rDNAs. We cloned the PCR products and partially sequenced nine clones from the ovaries and seven clones from the Malpighian tubules. They fell into two species; the ovaries yielded five clones of one species and four clones of the other, while the Malpighian tubules yielded two and five clones, respectively. Sequences of 1,560 and 1,448 nucleotides were determined for the two 16S rDNA PCR products of what we considered to be two different symbionts, A and B (DNA database accession no. AB1521 and AB1522).

We partially sequenced seven clones derived from the ovarian PCR products of one female *H. longicornis* and found four clones had the same sequence whereas three clones differed from these four clones and from each other. The complete sequences of the 16S rDNA inserts of the similar four clones were determined (1,436 bp, excepting the primer regions; DNA database accession no. AB001520). A homology search showed that the sequence was that for an organism belonging to the beta subclass of proteobacteria but that it had no close relatives in the databases. We then cloned and sequenced the PCR products from the ovary of another female. This time we obtained five clones, all having the same sequence. This sequence was identical to that of one of the three clones isolated in the previous sample but different from that of the microorganism belonging to the beta subclass. The PCR products from the *H. longicornis* Malpighian tubules were also cloned, and the sequences of eight clones were identical to those of the five clones isolated from the ovary of the second female and to the clone from the ovary of the first female. The 1,465-nucleotide sequence of the 16S rDNA of this latter *H. longicornis* microbe (DNA database accession no. AB001519) was similar to those of the *R. sanguineus* symbiont and the symbiont A of *O. moubata.*

Phylogeny of the symbionts. The homology search for 16S rDNA of the *I. scapularis* symbionts revealed that these microbes belonged to the alpha subdivision of proteobacteria and were most closely related to members of the genus *Rickettsia* belonging to the SFG, for example, *Rickettsia* sp. (accession no. L36102), *R. massiliae* (L36106 and L36214), and *R. rhipicephali* (L36216). The symbionts of *R. sanguineus*, *H. longicornis*, and *O. moubata* (symbiont A), in contrast, were related to bacteria belonging to a distinctly different lineage of the proteobacteria, especially *C. burnetii* (M21291) and the clam symbiont *Codakia orbicularis* (X84979) and *Lucina nassula* (X95229). Symbiont B of *O. moubata* was closely related phylogenetically to *W. persica* (M21292) and *F. tularensis* (L26086, L26084, and Z21931).

The phylogenetic interrelationships of these symbionts with other bacteria were compared by the neighbor-joining method of PHYLIP, using the 23 bacterial species selected for cladistic analysis. The phylogenetic tree (Fig. 3) showed a well-authorized figure in which the proteobacteria formed three major lineages, (the alpha, beta, and gamma subclasses), using *B. thuringiensis* as the outgroup species. The symbiont of *I. scapularis*, which belonged to the alpha subclass of proteobacteria,

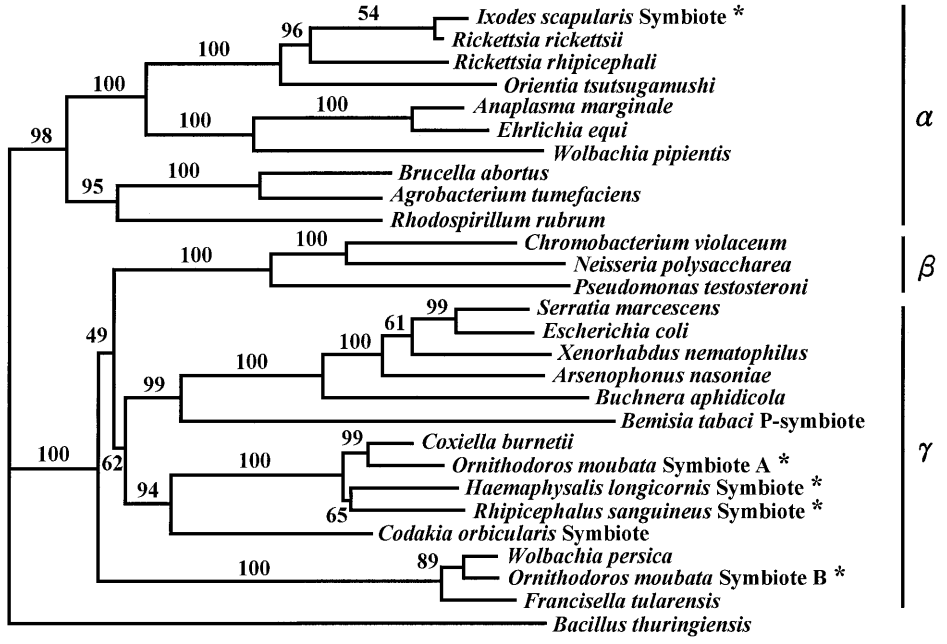

FIG. 3. Phylogenetic relationships of the symbionts in *I. scapularis*, *R. sanguineus*, *H. longicornis*, and *O. moubata* to 23 selected species of eubacteria. The gram-positive bacterium *B. thuringiensis* was selected as an outgroup species, and other proteobacteria were divided into three subdivisions: alpha, beta, and gamma. Asterisks show the tick symbionts analyzed in this study. The tree was constructed by a neighbor-joining procedure with the software package PHYLIP, version 3.5C, by using nucleotide sequences of 16S rDNA. Numbers indicate bootstrap percent confidence values.

was genetically distant from other symbionts of ticks, which belonged to the gamma subclass.

The *I. scapularis* symbiont formed a monophyletic group very closely linked with two members of the genus *Rickettsia*, *R. rickettsii* and *R. rhipicephali*. This result indicates that the symbiont of *I. scapularis* is a member of the genus *Rickettsia* and belongs to the SFG. Among the four species of symbionts which belonged to the gamma subclass, three species, the symbionts of *R. sanguineus* and *H. longicornis* and symbiont A of *O. moubata*, formed a monophyletic group with *C. burnetii*. Symbiont A of *O. moubata* and *C. burnetii* were more closely related than the symbionts from the two ixodid ticks, *R. sanguineus* and *H. longicornis*. Symbiont B of *O. moubata* showed a close relationship with the *A. arboreus* symbiont, *W. persica*, and the tularemia organism *F. tularensis*. The bacterial symbionts of the whitefly (*Bemisia tabaci* P-symbiont), aphid (*Buchnera aphidicola*), wasp (*Arsenophonus nasoniae*), and nematode (*X. nematophilus*) constitute a different lineage from the symbionts of ticks. *W. pipientis* was genetically distant and belonged to the alpha subclass.

DISCUSSION

Bacterial symbionts appear to occur in most species of argasid and ixodid ticks regardless of geographical origin. These microbes are transovarially transmitted and do not appear to be pathogenic for ticks. Cowdry (10) and Mudrow (28) noted that these relatively large gram-negative and pleomorphic bacteria were present during all life stages of ticks. Symbionts have been identified as wolbachiae or rickettsiae, on the basis of their ultrastructure (17).

The symbiont of *I. scapularis* was found to belong to the alpha subclass of proteobacteria and be a member of the SFG rickettsiae. Reports on the presence and location of symbionts in *Ixodes* spp. vary. Balashov (4) detected symbionts in both the ovaries and Malpighian tubules of *I. ricinus* and *I. persulcatus*. However, Mudrow (28) found symbionts in the ovaries and Malpighian tubules of *I. hexagonus* but not in *I. ricinus*. Rickettsiae have been observed only in the ovaries of *I. scapularis* and *I. ricinus* (26, 55). Zhu et al. (55) failed to detect any rickettsiae in the primordial testicular tissues of fed and molting male larvae or nymphs in *I. ricinus*. The *Rickettsia*-specific primers demonstrated the symbionts of *I. scapularis* were present in all flat unfed larvae and only half of the nymphs. Taken together, these results indicate that male *I. scapularis* ticks lose their symbionts during the feeding or molting process. In the adults, we detected rickettsiae only in the females, in their ovaries but not Malpighian tubules. On the basis of its 16S rDNA sequence, the *I. scapularis* symbiont is closely related to *R. rickettsii*, the agent of Rocky Mountain spotted fever, and *R. rhipicephali*, a nonpathogenic rickettsia originally isolated from *R. sanguineus*. In another study, we sequenced the rickettsial *ompA* gene (domain 1) of the *I. scapularis* symbiont. A phylogenetic analysis classified the symbiont as closely related to *R. montana* (51).

The 16S rDNA sequences of symbiont A of *O. moubata* and the symbionts of *R. sanguineus* and *H. longicornis* were closely related to that for the Q-fever organism, *C. burnetii*. On the basis of its ultrastructure, the *R. sanguineus* symbiont has previously been identified as a rickettsia (40) and a wolbachia (17, 18). However, the symbionts of *R. sanguineus* failed to amplify primer pairs diagnostic for these bacteria. *C. burnetii*, a mammalian pathogen (3) that naturally infects over 40 species of ticks (2), is also an obligate intracellular bacterium that develops in the phagolysosomes of host cells (1, 9). *C. burnetii* multiplies extensively within the body of the tick. In addition to invading the Malpighian tubules and the ovaries, *C. burnetii* invades the gut, salivary glands, and hemolymph. In chicken and mammalian cells, *C. burnetii* is highly pleomorphic and displays vegetative development (27). The vegetative forms of *C. burnetii* (27) are ultrastructurally similar to the symbionts of

R. sanguineus (18, 40). It is difficult to differentiate between the symbionts and *C. burnetii* on the basis of morphology (4).

We found that symbiont B of *O. moubata* belongs to the genus *Francisella* and confirmed that *W. persica* is also a *Francisella* species (14). *F. tularensis*, the tularemia agent, is transmitted by both hard and soft ticks, multiplies in the gut cells during feeding, invades the Malpighian tubules, and is transmitted transovarially (4, 5, 8). Reinhardt et al. (34) reported that the ovaries and Malpighian tubules of *O. moubata* harbor two kinds of symbionts: one a rickettsia-like microbe and the other a coccoid bacterium. PCR amplification and sequence analyses indicated that both the ovaries and the Malpighian tubules were infected with two different microorganisms, symbiont A and symbiont B. Symbiont A may be the rickettsia-like microbe, phylogenetically close to *C. burnetii*, and symbiont B may be the coccoid microbe, phylogenetically related to *F. tularensis*, identified in the ovaries by Reinhardt et al. (34).

The 16S rDNA sequence of a bacterium in the ovaries of one of the *H. longicornis* was quite distinct. It falls into the beta subclass but is unlike any of the 16S rDNA sequences that have been deposited in the databases to date. Since the sequence was not obtained with the PCR products from the second female, this sequence appears to be not that for an obligate symbiont but rather that for a tick associated microbe or fortuitous contaminant.

The primers for wolbachial 16S rDNA and *ftsZ* did not amplify DNA from any of the tick symbionts or *W. persica*. These primer sets specifically amplify *Wolbachia* spp. that have been found in a variety of arthropods (22, 32), including acarines (mites) (6, 23, 47). The genus *Wolbachia* currently consists of three species, *W. pipientis*, *W. melophagi*, and *W. persica*, but the taxonomic position of *W. persica* has not been resolved (50). *W. pipientis* is the type species for the genus, and recent phylogenetic studies based on 16S rDNA has revealed that *W. persica* is genetically distant from *W. pipiens* and other insect associated wolbachia. *W. persica* belongs to the gamma subclass of proteobacteria, and *W. pipiens* belongs to the alpha subclass (32, 37, 49). In addition to the inconsistency in the phylogenetic relationship between *Wolbachia* spp. of insects and ticks, there is also discrepancy between *W. persica* and the *R. sanguineus* symbiont, which has been called a *Wolbachia* spp. (17). The *R. sanguineus* endosymbiont, together with the endosymbiont of *H. longicornis* and the symbiont A of *O. moubata*, has a lineage different from that of *W. persica*. Consequently, the genus name *Wolbachia* is situated in three different lineages in the tree. We propose that the genus name *Wolbachia* be restricted to the group of bacteria which have been demonstrated to belong to the alpha subclass of proteobacteria and which constitute a distinct lineage in the monophyletic group containing members of the genus *Ehrlichia* and *Anaplasma* as shown in Fig. 3.

In summary, we have found that the endosymbiotic bacteria of four tick species are closely related to bacterial pathogens transmitted by ticks. The infection of Malpighian tubules with symbionts is observed in many soft (argasid) and hard (ixodid) ticks. The microorganisms in the Malpighian tubules also infect the ovaries and appear to be of more ancient origin, while the rickettsial symbiont in the ovaries of *I. scapularis* appears to be a more recent association. The ancestral origin of the endosymbionts remains to be defined, but we speculate that they were originally animal pathogens acquired by ticks during their feeding on a bacteremic host. The ancestral species of these tick-associated microorganisms may have divided along two lines: the endosymbionts which became completely adapted and confined to the internal milieu of the ticks, and the pathogenic bacteria which retained or acquired the ability to infect and propagate in both the tick and vertebrate host. It seems that the endosymbionts have become specialists and the pathogens have become generalists in their evolution for host selection. In this regard, it is of interest that the endosymbionts isolated from the Malpighian tubules of *A. arboreus* (*W. persica*) and *D. andersoni* (unidentified) were found to be pathogenic for chicken embryos and guinea pigs (7, 43, 44). The potential for the endosymbionts in ticks to emerge (or reemerge) under natural conditions as human or animal pathogens remains to be determined.

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