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Highly Prevalent *Coxiella* sp. Bacterium in the Tick Vector *Amblyomma americanum*▽

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Laboratory-reared and field-collected *Amblyomma americanum* ticks were hosts of a *Coxiella* sp. and a *Rickettsia* sp. While the *Coxiella* sp. was detected in 50 of 50 field-collected ticks, the *Rickettsia* sp. was absent from 32% of ticks. The *Coxiella* sp. showed evidence of a reduced genome and may be an obligate endosymbiont.

The lone star tick *Amblyomma americanum* is a common pest of humans and domestic animals in the southern United States, and its range now extends into the Northeast. In parts of New Jersey *A. americanum* ticks are more commonly encountered than the deer tick *Ixodes scapularis* (13). While originally considered a nuisance species, *A. americanum* is now recognized as a vector of *Ehrlichia chaffeensis*, the agent of human monocytic ehrlichiosis, and *E. ewingii*, a cause of granulocytic ehrlichiosis in humans and dogs (16). *A. americanum* is also the vector of *Borrelia lonestari* (1), an organism that has been implicated but not proven (5, 17) as a cause of a tick-associated rash illness in the southern United States (2, 9). According to Childs and Paddock, the "public health relevance of lone star ticks is no longer in question" (3). Yet comparatively little is known about the biology of this or other *Amblyomma* spp.

As part of a genetics study of *A. americanum*, we produced a cDNA library from RNA extracted from the midguts of laboratory-reared female *A. americanum* ticks. Upon sequencing cDNA clones, we found that 4% of ~500 nonredundant cDNA sequences had closest matches to sequences of the Q-fever agent *Coxiella burnetii* (unpublished findings). These included coding sequences for DnaK (DQ912980); FusA, elongation factor G (DQ908900); RpsF, ribosomal protein S6 (DQ908901); RpsG, ribosomal protein S7 (DQ908902), and 16S rRNA gene (AY939824). *Coxiella*-like bacteria have been reported to have been present in the hard ticks *Haemaphysalis longicornis* and *Rhipicephalus sanguineus* and the soft tick *Ornithodoros moubata* (12), but these had not been further characterized. Figure 1 is a phylogram of 16S rRNA sequences of the *Amblyomma* bacterium, *C. burnetii* and related bacteria (12), other members of *Legionellales*, and two other γ-proteobacteria, *Escherichia coli* and the methanotroph *Methylococcus capsulatus*. The analysis indicates that *C. burnetii*, the bacteria of *Haemaphysalis*, *Rhipicephalus*, and *Ornithodoros* ticks, and the *Amblyomma* bacterium are monophyletic and that they, along with a symbiont of the marine dinoflagellate *Heterocapsa circularisquama*, constitute a clade separate from *Legionella* spp. and from a clade that comprises entomopathogenic *Rickettsiella* spp. and the protozoan-associated bacterium *Aquicella siphonis*. Similar phylogenetic analyses of the alignments of the housekeeping genes *fusA*, *rpsF*, and *rpsG* of the

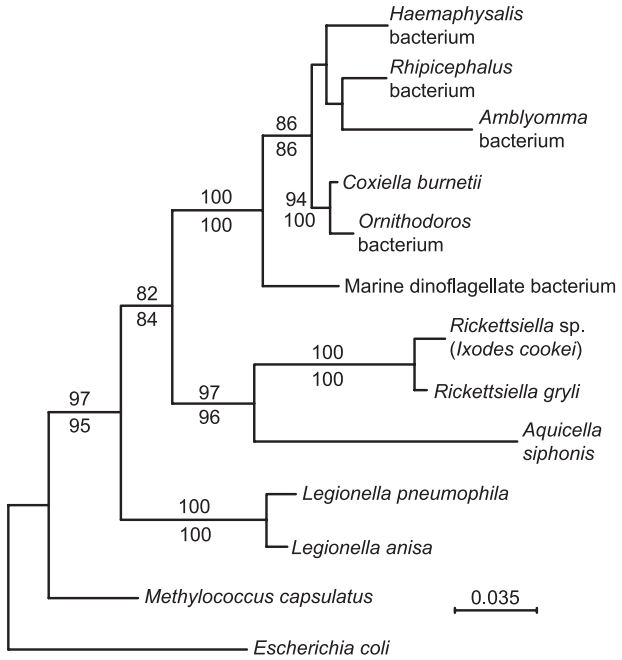

FIG. 1. Phylogram of partial 16S rRNA genes of an *Amblyomma americanum* bacterium (accession number AY939824) and selected other γ-proteobacteria: *Coxiella burnetii* bacterium (NC_002971), *Haemaphysalis longicornis* bacterium (AY342035), *Rhipicephalus sanguineus* bacterium (D84559), *Ornithodoros moubata* bacterium (AB001521), a marine dinoflagellate bacterium (AB058918), *Rickettsiella grylli* (U97547), a *Rickettsiella* sp. of *Ixodes woodi* (AF383621), the protozoan-associated bacterium *Aquicella siphonis* (AY359283), *Legionella pneumophila* (NC_006369), *Legionella anisa* (AY744776), *Methylococcus capsulatus* (NC_002977), and *Escherichia coli* K-12 (NC_000913). Nucleotide positions with gaps in the aligned sequences were excluded. Bootstrap values of nodes with >80% values by maximum likelihood (500 replicates; shown above the line) or neighbor-joining (1,000 replicates; shown below the line) distance criteria are indicated and were estimated using PHYLO_WIN phylogenetic analysis software (http://pbil.univ-lyon1.fr/software/phylowin.html). Bar, nucleotide distance.

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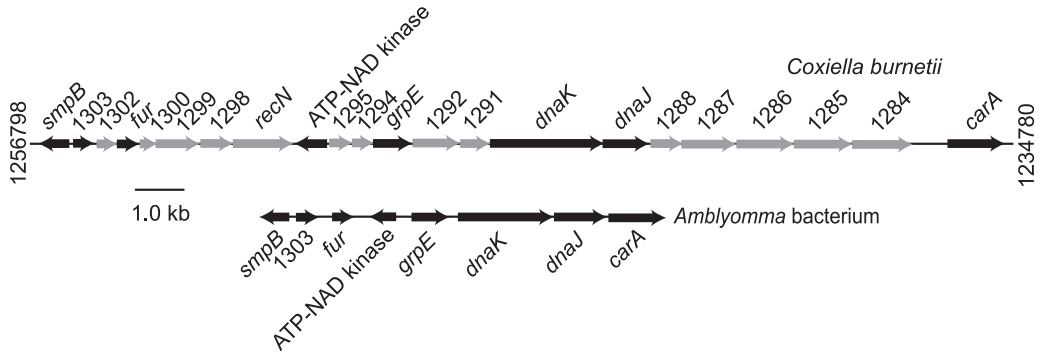

FIG. 2. Physical maps of 22 kb of the chromosome of *Coxiella burnetii* (accession number NC_002971) and a corresponding 9-kb region of an *Amblyomma americanum* bacterium (DQ912980). The arrows correspond to the lengths of the open reading frames (ORFs) and the presumed direction of transcription. Black arrows indicate ORFs found in both sequences. The numbers at the ends of the *C. burnetii* sequence are the genome nucleotide positions. ORFs with matches to known genes, e.g., *dnaK*, or with a protein family of presumed function are indicated. ORFs designated by numbers alone indicate hypothetical proteins of *C. burnetii* (14). Bar, sequence length.

Amblyomma bacterium and corresponding genes of *E. coli* K-12 (NC_000913), *M. capsulatus* (NC_002977), *L. pneumophila* (NC_002942), and *C. burnetii* (NC_002971) confirmed that the *A. americanum* bacterium is a sister taxon to *C. burnetii* (data not shown). Nucleotide sequence identities between the *Amblyomma* bacterium and *C. burnetii* for *fusA*, *rpsF*, and *rpsG* were 72%, 64%, and 69%, respectively.

We next screened a lambda bacteriophage library of *A. americanum* genomic DNA, which we had previously produced from eggs (7), with the cloned *dnaK* cDNA of the symbiont as a probe, as described previously (7). A hybridizing clone was isolated, and the insert was sequenced over both strands on a CEQ 8000 automated sequencer (Beckman Coulter, Fullerton, CA) with custom primers. Figure 2 shows physical maps of 8,332 nucleotides of the *Amblyomma* bacterium (DQ912980) and 22,018 nucleotides of a homologous region of *C. burnetii* (NC_002971) (14). These two genome fragments had the same gene order for genes in common: *smpB*, *fur*, *grpE*, *dnaK*, *dnaJ*, and *carA*. But the *Amblyomma* bacterium's fragment lacked nearly all the hypothetical proteins, which are designated by number alone in the figure, of *C. burnetii* in this region. The *Amblyomma* bacterium may also lack the DNA repair function provided by *recN*. These findings provided further evidence that the tick bacterium is in the same lineage as *C. burnetii* but also that it has a reduced genome, a common feature of obligate endosymbionts of invertebrates (10).

Using quantitative PCR with specific primers and dye-labeled probes, we then characterized the distribution, prevalence, and copy numbers of the *Coxiella* sp. in *A. americanum*. We compared these results with those obtained for a *Rickettsia bellii*-like bacterium that had been identified in *A. americanum* (U11012) and *Amblyomma* spp. in Brazil (8). The targets for the *Coxiella* sp. and the *Rickettsia* sp. were the *fusA* gene and the citrate synthase gene (*gltA*; accession no. AY388956), respectively. The internal control for both PCR assays was an exon of the *A. americanum* gene for macrophage inhibitory factor (MIF), which we had previously demonstrated to be present in this species (7). The ratios of copies of *fusA* or *gltA* to the MIF gene provided estimates of the burden of the *Coxiella* sp. and *Rickettsia* sp. in the ticks. Total DNA was obtained by freezing and pulverizing adult ticks in liquid nitrogen and then extracting DNA as described previously (15). The 6-carboxyfluorescein-labeled probe, forward primer, and reverse primer specific for *fusA* were 5′ATTTACCTGCACC TACTGATATACCTGAT3′, 5′AGCCTTATTAGATGCTG TGGTTGA3′, and 5′CGTCTGCTTCTTCACCTCGAA3′, respectively. The probe, forward primer, and reverse primer for the MIF gene were 5′CACTGATGACCCATGCGCTATTG

TABLE 1. Prevalences and relative numbers of *Coxiella* sp. and *Rickettsia* sp. bacteria in female *A. americanum* ticks collected in five state parks in the United States[a]

State	State park	No. of ticks with *Coxiella* sp. detected by PCR/ total no. of ticks	Mean *fusA*/MIF ratio (95% CI)[b]	No. of ticks with *Rickettsia* sp. detected by PCR/ total no. of ticks	Mean *gltA*/MIF ratio (95% CI)[b]
Maryland	Elk Neck	10/10[c]	52 (7–97)	5/10[c]	2.8 (0.8–4.9)
Georgia	Fort McAllister	10/10	106 (8–205)	7/10	4.1 (0.7–7.4)
Georgia	Hard Labor Creek	10/10	79 (9–150)	6/10	2.0 (0.1–4.0)
Kentucky	Green River Lake	10/10	47 (4–91)	9/10	2.2 (0.4–4.0)
Oklahoma	Osage Hill	10/10	75 (15–135)	7/10	2.3 (0.7–3.9)
Total		50/50	72 (43–101)	34/50	2.7 (1.8–3.6)

[a] *fusA*, *Coxiella* sp. elongation factor G gene; MIF, *A. americanum* macrophage inhibitory factor gene; *gltA*, *Rickettsia* sp. citrate synthase gene.
[b] Values represent mean ratios of *fusA* or *gltA* to MIF gene copies per positive tick result (95% confidence interval).
[c] The value for the Cochran-Mantel-Haenszel statistic of prevalence of *Coxiella* sp. versus *Rickettsia* sp. among sampled ticks, stratified by state park, was 18.7 with 1 degree of freedom (two-tailed test; $P < 10^{-6}$) (StatXact v. 6, Cytel Software Corp.).

CAAAT3′, 5′CCACATCAACGCCGATCAG3′, and 5′TTTG TTCTCCTTTGGACTCAGACA3′, and the probe, forward primer, and reverse primer for *gltA* were 5′ATGCTTCTACT TCAACAGTCCGAATTGCCG3′, 5′TCCTACATGCCGAC CATGAG3′, and 5′AAAGGGTTAGCTCCGGATGAG3′, respectively. PCR was carried out using a Rotor-Gene RG-3000 apparatus (Corbett Research, San Francisco, CA). The reactions were carried out with 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphates, 3 U *Taq* polymerase (Roche Diagnostics, Mannheim, Germany), and 0.1 μM of primers, and conditions were 1 cycle at 95°C for 4 min, 40 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min, and finally 72°C for 7 min. For standard curves, the PCR targets were cloned into the plasmid pCR2.1-TOPO (Invitrogen, San Diego, CA).

We found no evidence of this *Coxiella* sp. in five each of field-collected adult *Ixodes scapularis* and *Dermacentor variabilis* ticks by the *fusA* assay, and the MIF gene assay was specific for *A. americanum* (data not shown). Of 10 laboratory-reared *A. americanum* females at 3 months postmolt, which were obtained from Oklahoma State University, all were positive for the *Coxiella* sp. by PCR; the mean (95% confidence interval) *fusA*/MIF gene copy number ratio was 85 (range, 75 to 95). We then dissected four adult females as described previously (6) and extracted DNA. We found that the mean *fusA*/MIF ratios were 381 (range, 252 to 512) in midguts, 79 (range, 39 to 298) in ovaries, and 12 (range, 11 to 14) in salivary glands.

We examined the distribution of the *Coxiella* sp. and *Rickettsia* sp. in natural populations of *A. americanum* by randomly sampling 10 female ticks from collections of ticks from five different state parks in the United States in Maryland, Georgia, Kentucky, and Oklahoma. The ticks were collected in 2004 by drag sampling (4) and stored in 70% ethanol until DNA extraction and quantitative PCR were performed as described above. The results are shown in Table 1. The *Coxiella* sp. was found in all ticks at each location. In contrast, 16 (32%) of the 50 ticks did not have a detectable *Rickettsia* sp. at a ≥0.01 *gltA*/MIF copy ratio (2-tailed chi-square test; $P < 0.0001$). A mean *fusA*/MIF ratio of 129 (range, 81 to 177) for the *Coxiella* sp. approximated what was observed in laboratory ticks. Among field-collected ticks with the *Rickettsia* sp., the mean *gltA*/MIF copy ratio was 4.3 (range, 2.7 to 5.9), 30-fold lower than observed for the relative copy number of the *Coxiella* sp.

In summary, we identified a hitherto unknown *Coxiella* sp. in all laboratory-reared and field-collected *A. americanum* females. The bacterium was more prevalent and in higher densities than an *R. bellii*-like species in lone star ticks. The ubiquity of the *Coxiella* sp. and its presence in a genomic library derived from eggs indicate that it is an endosymbiont (11). Evidence of a reduced genome for the bacterium supports this proposal. The consequences of this bacterium for the fitness of its tick host, including nutrient provisioning, remain to be determined.

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