

Bacterial Diversity in *Amblyomma americanum* (Acari: Ixodidae) With a Focus on Members of the Genus *Rickettsia*

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ABSTRACT The lone star tick, *Amblyomma americanum* (Acari: Ixodidae), is commonly reported from people and animals throughout the eastern U.S. and is associated with transmission of a number of emerging diseases. To better define the microbial communities within lone star ticks, 16S rRNA gene based analysis using bacteria-wide primers, followed by sequencing of individual clones ($n = 449$) was used to identify the most common bacterial operational taxonomic units (OTUs) present within colony-reared and wild *A. americanum*. The colony-reared ticks contained primarily sequence affiliated with members of the genus *Coxiella* (89%; 81/91), common endosymbionts of ticks, and *Brevibacterium* (11%; 10/91). Similarly, analysis of clones from unfed wild lone star ticks revealed that 96.7% (89/92) of all the OTUs identified were affiliated with *Coxiella*-like endosymbionts, as compared with only 5.1–11.7% (5/98–9/77) of those identified from wild lone star ticks after feeding. In contrast, the proportion of OTUs identified as *Rickettsia* sp. in wild-caught ticks increased from 2.2% (2/92) before feeding to as high as 46.8% (36/77) after feeding, and all *Rickettsia* spp. sequences recovered were most similar to those described from the spotted fever group *Rickettsia*, specifically *R. amblyommii* and *R. massiliae*. Additional characterization of the Rickettsiales tick community by polymerase chain reaction, cloning, and sequencing of 17 kDa and *gltA* genes confirmed these initial findings and suggested that novel *Rickettsia* spp. are likely present in these ticks. These data provide insight into the overall, as well as the rickettsial community of wild lone star ticks and may ultimately aid in identification of novel pathogens transmitted by *A. americanum*.

KEY WORDS *Amblyomma americanum*, bacterial diversity, 16S gene, *Rickettsia*

Amblyomma americanum (Acari: Ixodidae), the lone star tick, is an important arthropod pest and disease vector that has dramatically increased in both number and geographic distribution in recent years largely because of expansion of white-tailed deer populations (Childs and Paddock 2003, Paddock and Yabsley 2007). Although deer serve as the keystone host for both immature and adult stages of this aggressive tick, lone star ticks also readily bite humans and a variety of other animals; indeed, *A. americanum* is the tick most commonly reported from people in the southern United States, accounting for up to 83% of human tick bites in some surveys (Felz et al. 1996). Historically restricted to relatively isolated pockets primarily in the southeastern and south-central United States, lone star ticks have expanded regionally and nationally in recent years, with populations now established as far north as Maine (Keirans and Lacombe 1998, Ginsberg

et al. 2002, Childs and Paddock 2003, Paddock and Yabsley 2007). At the same time, a number of novel *A. americanum*-associated pathogens and diseases have been described in the last two decades (Ewing et al. 1971, Goddard and Norment 1986, Anderson et al. 1991, Kirkland et al. 1997, Reeves et al. 2008). This expanding geographic range together with our increasing awareness of the pathogens and diseases associated with *A. americanum* lends a sense of urgency to efforts to understand the bacterial diversity present in this arthropod in natural systems.

A. americanum has been associated with several human diseases, including ehrlichioses, spotted fever caused by rickettsial agents, and Southern Tick Associated Rash Illness (STARI). Lone star ticks are well established as the primary vector of at least three distinct ehrlichial pathogens: *Ehrlichia chaffeensis*, the causative agent of human monocytotropic ehrlichiosis (HME) (Anderson et al. 1991), *E. ewingii*, the cause of human granulocytic ehrlichiosis (Buller et al. 1999), and an *E. ruminantium*-like organism referred to as the Panola Mountain *Ehrlichia* (PME) (Reeves et al. 2008). Human ehrlichiosis, particularly when caused by *E. chaffeensis*, is potentially life-threatening and is

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considered the most common tick-borne disease of people in the southern U.S. (McQuiston et al. 2003, Olano et al. 2003, Manangan et al. 2007, Miura and Rikihisa 2007, Wimberly et al. 2008). The historical literature also links Rocky Mountain spotted fever caused by *R. rickettsii* with *A. americanum* (McDade and Newhouse 1986), but more recent data suggest another rickettsial agent, such as *R. amblyommii*, may be associated with a spotted fever like illness in people (Apperson et al. 2008).

Perhaps the most perplexing of the lone star tick-associated diseases is STARI, a Lyme borreliosis-like condition in which people develop an erythema migrans rash after the bite of a lone star tick which cannot be attributed to infection with *Borrelia burgdorferi*, the only known cause of Lyme borreliosis in North America (Wormser et al. 2005a,b, Philipp et al. 2006). In endemic areas, erythema migrans after a tick bite is considered pathognomonic for Lyme borreliosis and a physician-diagnosed erythema migrans is considered sufficient evidence to warrant report as a confirmed case for surveillance purposes (Centers for Disease Control [CDC] 1997). However, in areas where Lyme borreliosis is not known to be endemic, such as the southern U.S., the etiology of *A. americanum*-associated erythema migrans remains elusive (Blanton et al. 2008, Masters et al. 2008). Attempts to attribute STARI to infection with *B. lonestari*, a relapsing fever-like spirochete present in wild lone star ticks and associated with a single case of STARI, or with *R. amblyommii*, have not been confirmed with clinical microbiological studies to date (James et al. 2001, Wormser et al. 2005a,b, Billeter et al. 2007, Nicholson et al. 2009).

A full characterization of the spectrum of the bacterial community in *A. americanum* is an important first step to establish a basis for further investigations of this tick as a vector of known and yet to be discovered pathogens. Recently, multiple studies have convincingly demonstrated the ubiquitous presence of a *Coxiella*-endosymbiont in wild and colony-reared lone star ticks (Jasinskas et al. 2007, Klyachko et al. 2007, Zhong et al. 2007, Clay et al. 2008). However, attempts to characterize complete microbial communities in wild lone star ticks have not included colony-reared *A. americanum* as controls or evaluated changes in diversity of microbes induced by tick feeding (Clay et al. 2008). For the study reported here, our goal was to document differences in the degree of microbial diversity present in colony-reared ticks and wild caught ticks before and after feeding. The population of vector-borne pathogens increases within arthropod vectors in response to blood feeding (de Silva and Fikrig 1995, Azad and Beard 1998), and so we used blood feeding as a means to increase the proportion of bacteria that were perhaps more likely to be transmitted to hosts. Our results show that microbial diversity is severely limited in colony-reared ticks, and somewhat restricted in unfed, wild caught ticks, but that feeding greatly increases the microbial diversity detected, particularly for the members of the Rickettsiales, in wild caught ticks that presumably fed on infected vertebrates in their immature stages.

Materials and Methods

Samples/Source of Ticks. Colony-reared adult *A. americanum* were obtained from the Oklahoma State University Tick Laboratory (Stillwater, OK) that has maintained the same line of lone star ticks since 1976 with the last introduction of new colony material 2 yr before this study. (L. Coburn, personal communication). Wild adult lone star ticks were collected from Lake Carl Blackwell Recreational Area, OK (site 1) and Panola Mountain State Park, GA (site 2) in May of 2008 using dry ice traps and drags as previously described (Lockhart et al. 1997). A subset of 100 adult lone star ticks from site 1 were fed in two groups of 50 (25 males and 25 females) on each of two pathogen-free class A beagle dogs that had been shown to be free of infection with all pathogens of interest before tick feeding as determined by specific polymerase chain reaction (PCR) for *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., and *Borrelia* spp. on whole blood as described below. In addition, dogs were evaluated for evidence of antibodies to *Ehrlichia*, *Anaplasma*, and *B. burgdorferi* via a commercial ELISA (SNAP 4Dx, IDEXX Laboratories Inc., Westbrook, ME) according to manufacturer's instructions, and by indirect fluorescent antibody assay for *R. rickettsii* using a commercial laboratory service (Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, OK). A subset of 100 colony-reared lone star ticks were fed in two groups of 50 (25 males, 25 females) of each of two specific-pathogen free New Zealand white rabbits. Ticks were allowed to feed until females were replete and then collected for PCR.

Nucleic Acid Extraction. From each of the pools of unfed adults, 100 adults (50 males and 50 females) were dissected and all internal tissues transferred into a sterile 1.5 ml tube containing 0.8 ml $1\times$ phosphate-buffered saline (PBS) and total DNA was extracted using a commercially available kit (FastDNA SPIN Kit for Soil, MP Biomedicals, Solon, OH) according to the manufacturer's instructions. From the two pools of fed ticks (site 1, colony-reared), ticks were dissected and all internal tissues and contents from feeding transferred into a sterile vial containing $1\times$ PBS. Because of large differences in body mass post feeding, fed males and fed females were pooled and evaluated separately. The volume of PBS used was adjusted according to body weight of the ticks dissected to reach the same ratio (body weight:PBS). Total DNA was extracted from fed ticks via a modified lysis bead-beating protocol as previously described (Dojka et al. 1998). Total nucleic acid was extracted from 100 μ l of whole blood using the GFX genomic blood DNA purification kit (Amersham Biosciences, Buckinghamshire, United Kingdom) according to manufacturer's directions.

PCR Amplification, Cloning, and Sequencing. Multiple DNA targets were used to identify organisms in these samples, including bacteria-wide 16S rRNA gene, *Rickettsia*-specific 17 kDa fragment, and rickettsial citrate synthase gene (*gltA*). All pools were also tested for individual organisms using PCR protocols

Table 1. Gene targets, primer sequences, and references for PCR protocols used in this study

| Targeted gene | Primer | Sequence | Reference |
|--|--|---|--|
| Bacteria-wide 16S rDNA | 8F 805R | 5'-AGAGTTTGATCTGGCTCAG-3' 5'-GACTACCAGGCTATCTAATCC-3' | Elshahed et al. 2003 |
| <i>Borrelia</i> flagellin (flaB) | FLALL FLARL FLALS FLARS | 1° 5'-ACATATTCAGATGCAGACAGAGCT-3' 5'-GCAATCATAGCCATTGCAGATTGT-3' 2° 5'-AACAGCTGAAGAGCTTGAATG-3' 5'-CTTTGATCACTTATCATTCTAATAGC-3' | Barbour et al. 1996 |
| <i>Ehrlichia</i> and <i>Anaplasma</i> 16S rDNA | ECB ECC | 1° 5'-CGTATTACCGGCTGCTGGCA-3' 5'-AGAACGAACGCTGGCGCAAGCC-3' | Dawson et al. 1996, Anderson et al. 1992a, Anderson et al. 1992b |
| <i>E. chaffeensis</i> 16S rDNA | HE3 HE1 | 2° 5'-TATAGGTACCGTCATTATCTTCCCTAT-3' 5'-CAATTGCTTATAACCTTTTGGTTATAAAAT-3' | |
| <i>E. ewingii</i> 16S rDNA | HE3 EE72 | 2° 5'-TATAGGTACCGTCATTATCTTCCCTAT-3' 5'-CAATTGCTTATAAGTCTCTGACTATT-3' | |
| <i>A. phagocytophilum</i> 16S rDNA | GE9f GAIUR | 2° 5'-AACGGATTATCTTTATAGCTTGCT-3' 5'-GAGTTTGCCGGGACTTCTTCT-3' | |
| <i>A. platys</i> 16S rDNA | DGA GAIUR | 2° 5'-TTATCTCTGTAGCTTGCTACG-3' 5'-GAGTTTGCCGGGACTTCTTCT-3' | |
| <i>E. ruminantium</i> citrate synthase | Ehr3CS-185F Ehr3CS-777R Ehr3CS-214F Ehr3CS-619R | 1° 5'-GCCACCCGAGATAGTTAGGGA-3' 5'-TTCGTGCTCGTGGATCATAGTTTT-3' 2° 5'-TGTCATTTCCACAGCATTCTCATC-3' 5'-TGAGCTGGTCCCACAAAGTT-3' | Yabsley et al. 2008 |
| <i>Rickettsia</i> 17 kDa | 17k-5 17K-3 17KD1 17KD2 | 1° 5'-GCTTTACAAAATTCTAAAAACCATATA-3' 5'-TGTCTATCAATTCCAACTTGCC-3' 2° 5'-GCTCTTGCAACTTCTATGTT-3' 5'-CATTGTTCCTCAGGTTGGCC-3' | Stothard 1995 |
| <i>Rickettsia</i> citrate synthase (gltA) | RpCS.877 RpCS.1258 | 5'-GGGGCCCTGCTCACGGCCG-3' 5'-ATTGCAAAAAGTACAGTGAACA-3' | Regnery et al. 1991 |

established for *Borrelia* spp., *E. chaffeensis*, *E. ewingii*, *E. ruminantium*, *A. platys*, and *A. phagocytophilum*. All primers, amplification protocols, and references are listed in Table 1.

A 16S rRNA gene fragment (≈ 800 bp) was amplified as previously described (Elshahed et al. 2003) from the bulk community DNA of each tick pool using primers 8 F and 805R (Table 1) in 50 μ l reactions containing the following (final concentrations): 26.7 μ l pure water, 10 μ l of 5 \times buffer (Promega, Madison, WI), 5 μ l MgCl₂, 2 μ l deoxynucleoside triphosphate mixture (10 μ M), 2 μ l of the 8 F and 805R primers (Invitrogen Corp., Carlsbad, CA), and 1 μ l (1.5 U) of GoTaq Flexi DNA polymerase (Promega), and 2 μ l DNA. PCR amplification was carried out on a Gene Amp PCR system 9700 thermocycler. The 16S rRNA gene amplification used a protocol involving initial denaturation for 5 min at 94°C and 39 cycles of 92°C for 0.5 min, 50°C for 1 min, and 72°C for 2 min, followed by a final extension at 72°C for 20 min. PCR products obtained were visualized on a 2% agarose electrophoresis gel.

Amplification of the 17 kDa fragment (434 bp) of *Rickettsia* sp. was achieved using a nested PCR protocol using the external primers 17K-5 and 17k-3 (Table 1) in a 25 μ l reaction that contained 13.5 μ l molecular biology-grade water, 2.5 μ l 25 μ M MgCl₂ (Roche Applied Biosystems, Indianapolis, IN), 2.5 μ l 10X PCR Gold Buffer (Roche), 0.25 μ l 10 μ M dNTPs (Promega), 0.5 μ l of each primer (10 μ M), 0.25 μ l AmpliTaq Gold (Roche), and 5 μ l of sample DNA. Cycling conditions in the primary reaction were 95°C for 3 min followed by 40 cycles of 95°C for 15 s, 48°C for 30 s, 70°C for 30 s, followed by 72°C for 7 min. For the secondary reaction, 5 μ l of primary product was used as template in a 25 μ l reaction containing the

same PCR components with the exception of the primers 17 kD1 and 17 kD2 (Table 1) and cycling conditions in the secondary reaction were 95°C for 3 min followed by 40 cycles of 95°C for 15 s, 50°C for 30 s, 70°C for 30 s, followed by 72°C for 7 min.

To detect the citrate synthase (gltA) fragment (380 bp) of *Rickettsia* sp., PCR primers RpCS.877 and RpCS.1258 (Table 1) were used in a 25 μ l reaction that contained 17.5 μ l molecular biology-grade water, 2.5 μ l 25 μ M MgCl₂ (Roche), 2.5 μ l 10X PCR Gold Buffer (Roche), 0.25 μ l 10 μ M dNTPs (Promega), 0.5 μ l of each primer (10 μ l), 0.25 μ l AmpliTaq Gold (Roche), and 1 μ l of sample DNA. Cycling conditions were 95°C for 5 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 45 s, followed by 72°C for 5 min.

The amplicons produced in all reactions were directly cloned using a TOPO-TA cloning kit according to the manufacturer's instructions (Invitrogen). Plasmids from a total of 96 clones from each amplification protocol and each pool were submitted for sequencing (1,152 total sequence requests); sequencing and assembly procedures were performed at the Advanced Center for Genome Technology at the University of Oklahoma as previously described in detail (Elshahed et al. 2003).

Phylogenetic Analysis. For each of the three gene targets (16S, gltA, 17 kDa), sequences were initially compared with entries in GenBank database using BLASTnr (Altschul et al. 1997) for rough determination of phylogenetic affiliation. Sequences were also aligned in Greengenes NAST aligner to a 7,862-character global alignment (DeSantis et al. 2006b), and ran through Greengenes classifier (DeSantis et al. 2006a). In addition to Greengenes classifier output, the NAST-aligned sequences were imported to Greengenes May 2007 ARB database and added to the ARB universal

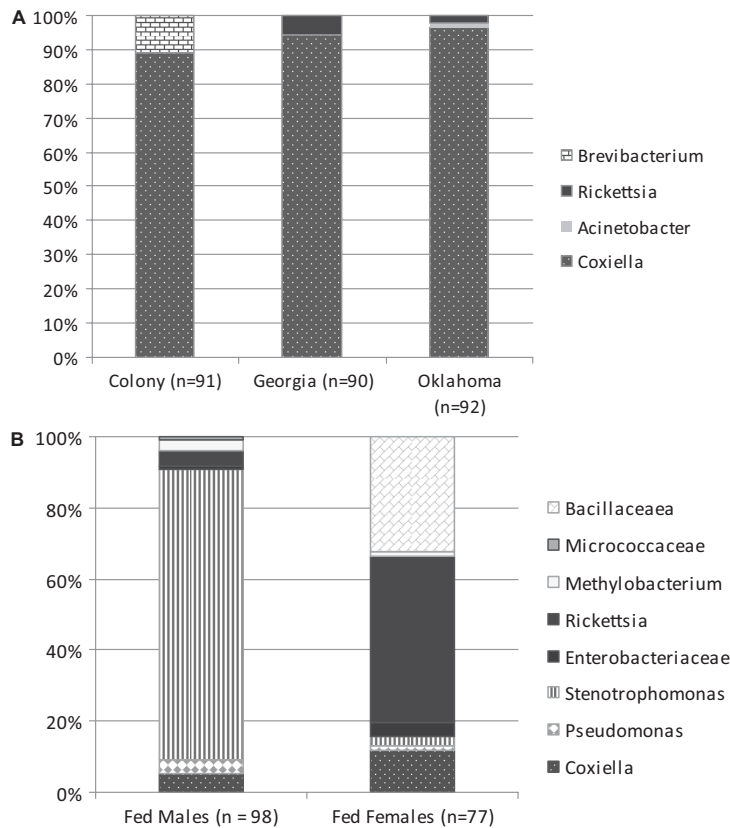


Fig. 1. (A) Bacterial community structure in colony reared and wild *A. americanum* from two different locations. (B) Effect of feeding on bacterial community structure within male and female wild *A. americanum* collected from Lake Carl Blackwell, OK (site 1).

dendrogram using ARB parsimony function to determine their position in the global phylogenetic tree (Ludwig et al. 2004).

For OTU assignment and phylogenetic tree construction, sequences were aligned using ClustalX (Thompson et al. 1997), and the alignments were exported to PAUP (Version 4.01b10; Sinauer associates, Sunderland, MA). A pair-wise distance matrix generated in PAUP was exported to DOTUR (Schloss and Handelsman 2005), and used for assignment of operational taxonomic units (OTUs) at the standard 97% sequence similarity cutoff for analysis of the 16S sequences, 98% for *Rickettsia*-specific 17 kDa gene, and 99% for the *gltA* *Rickettsia* gene. The cutoff for 16S rRNA gene sequences was based on widely accepted species cutoff values for such gene (DeSantis et al. 2006b). The cutoffs for 17 kDa and *gltA* rickettsial genes was based on typical average sequence divergence between *gltA* and 17 kDa genes identified in various rickettsial genomes and PCR based analysis of pure cultures of rickettsial species. Basic diversity measurements, for example, Shannon index, average nucleotide diversity, and Good's coverage were calculated as previously described (Good 1953, Martin 2002, Magurran 2004). Phylogenetic trees were constructed using OTUs from this study as well as repre-

sentatives of closely related reference sequences. Distance neighbor joining trees with no corrections, F-84 corrections, and Jukes-Cantor corrections were constructed using PAUP, and all gave similar tree topologies.

Nucleotide Sequence Accession Number. Sequences generate in this study were deposited in GenBank database under the accession numbers GQ302888-GQ302959.

Results

Bacterial Diversity in Unfed Colony-Reared and Wild-Caught *A. americanum*. In the clone library from colony-reared lone stars, the majority of 16S rRNA gene sequences obtained (80/91 clones, 20 OTUs, 87.9%) were members of the genus *Coxiella*, class γ -*Proteobacteria* (Fig. 1). These clones were most closely related (94–98% sequence similarity) to *Coxiella* endosymbionts of *A. americanum* (Fig. 2) reported in previous studies examining bacterial diversity in lone star ticks (Clay et al. 2008, Jasinskas et al. 2007, Klyachko et al. 2007, Zhong et al. 2007). In addition to *Coxiella*-affiliated clones, a few 16S rRNA gene sequences (10/91 clones, 3 OTUs) belonged to the family *Brevibacteriaceae*, within the

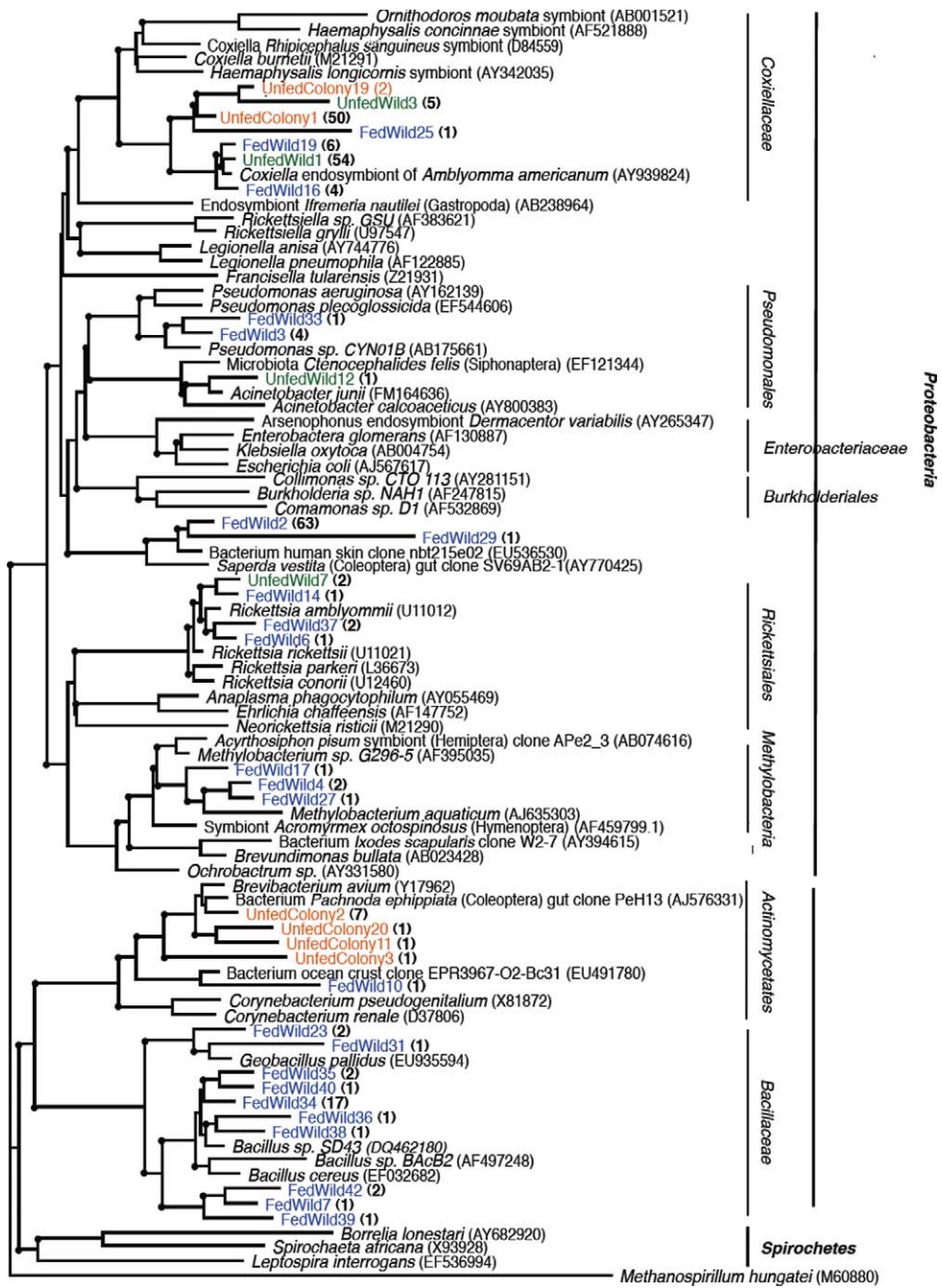


Fig. 2. Distance neighbor-joining tree based on the 16S rRNA gene sequences of bacterial OTUs in colony reared and wild-caught lone star ticks (site 1) before and after feeding encountered in clone libraries constructed in this study (excluding members of the order Rickettsiales). Bold circles indicate a bootstrap value of >50%. Bootstrap values were based on 1,000 replicates. The frequency of occurrence of each OTU is reported in parentheses. (Online figure in color.)

phylum *Actinomycetes*, with their closest relatives being members of the aerobic, heterotrophic genus *Brevibacterium* (97% sequence similarity to *Brevibacterium avium* strain 3055, isolated from diseased poultry (Y17962) (Pascual and Collins 1999) (Figs. 1 and 2).

Both populations of wild lone star ticks evaluated from two distant geographical regions within the U.S. (Oklahoma, GA) had similar bacterial communities, with 16S rRNA gene sequences of *Coxiella* endosymbionts again being the most abundant (94.4% Georgia and 96.7% Oklahoma) (Fig. 1). While the most abun-

Table 2. Diversity estimates obtained from bacterial 16S rRNA clone libraries generated in this study

| | Clones (n) | Shannon | ACE avg | Goods coverage |
|----------------|------------|---------|---------|----------------|
| Colony-reared | 91 | 2.01 | 48.2 | 87.9 |
| Georgia | 90 | 1.73 | 19.7 | 92.2 |
| Oklahoma | 92 | 1.44 | 24.8 | 93.5 |
| Fed ticks (OK) | 175 | 2.59 | 68.9 | 64.3 |

dant bacterial group within both colony-reared and wild ticks from two distinct locations were similar, the less abundant members were completely different. No *Brevibacteriaceae*-affiliated sequences were identified in the wild caught ticks. Instead, sequences affiliated with the genus *Acinetobacter* (Order *Pseudomonales*, γ -*Proteobacteria*), as well as spotted fever group *Rickettsia* sp. (closely affiliated to *R. amblyommii*, and *R. massiliae*) were identified as the minor components of the bacterial communities (Figs. 1 and 2). No significant differences in diversity estimates were observed between wild and colony reared ticks (Table 2).

Change in Microbial Population Distribution Upon Feeding Adult Ticks. Analysis of sequence of 175 clones (98 clones from pooled male ticks and 77 clones from pooled female ticks) from fed, wild-caught ticks (site 1) showed that blood feeding led to significantly higher diversity, when compared with unfed controls. In addition to observing previously undetected phylogenetic groups upon feeding (Fig. 1b), diversity estimates clearly indicate that feeding results in an increase in the community diversity within lone star ticks, as evident by increase in diversity indices (Shannon, ACE), as well as the decrease in the library coverage (Table 2).

In both male and female wild ticks, feeding resulted in a significantly lower proportion of *Coxiella* (Fig. 1), and hence a more even bacterial distribution resulting in a higher ACE estimate. In addition, feeding resulted in the identification of various OTUs not detected in clone libraries from unfed ticks, including members of the genus *Pseudomonas* (Order *Pseudomonales*, γ -*Proteobacteria*), members of the genus *Methylobacterium* (class α -*Proteobacteria*), as well as sequences affiliated with the genus *Stenotrophomonas*, family *Enterobacteriaceae*, and families *Micrococcaceae*, and *Bacillaceae* within the Phylum *Firmicutes*. Most importantly, the proportion of clones affiliated with the genus *Rickettsia*, order *Rickettsiales*, within the α -*Proteobacteria* significantly increased, particularly in the fed females (2.2 to 46.8%). *Rickettsial* clones belonged to the spotted fever group, with *R. amblyommii*, *R. massiliae*, and *R. rickettsii* str. Iowa the closest relatives to our sequences (Figs. 1 and 3). Although *Coxiella* (20%) and *Bacillaceae* (23.6%), and a diverse array of other bacteria were present in the colony raised ticks after feeding, no sequences associated with members of the genus *Rickettsia*, *Pseudomonas*, *Methylobacterium*, *Stenotrophomonas*, *Enterobacteriaceae*, or *Micrococcaceae* were found in the colony-raised fed ticks (data not shown).

Characterization of Rickettsial Populations in Wild *A. americanum* Before and After Feeding Using 17 kDa and *gltA* as Group Specific Targets. Because members of the *Rickettsia* sp., a lineage with multiple pathogenic members, was stimulated by feeding, we decided to further characterize the rickettsial population in ticks using two *Rickettsiales*-specific phylogenetic markers. To this end, we cloned and sequenced PCR products of genes encoded outer membrane protein (17 kDa) and citrate synthase (*gltA*) in DNA pools from wild ticks (site 1) before and after feeding.

In general, both phylogenetic trees from both gene sequences gave similar topologies, with rickettsial populations identified that belong to two main lineages. The first lineage is comprised of close relatives of the *R. amblyommii* and *R. japonica* group, and the other is a relatively minor component that may represent a novel rickettsial species. Analysis of results using both targets revealed a higher proportion of clones within the *R. amblyommii* group. In unfed ticks, 56.3% (17 kDa) to 80% (*gltA*) were *R. amblyommii* affiliated; upon feeding, this proportion increased to 95.2% (17 kDa) to 95.6% (*gltA*).

Other Pathogenic Microbes Described From Lone Star Ticks. All tick pools (before and after feeding) were tested for specific pathogens using established PCR protocols and were found to be PCR negative for *E. chaffeensis*, *E. ewingii*, *E. ruminantium*, *Anaplasma* sp., *B. burgdorferi*, and *B. lonestari*. In addition, both dogs failed to seroconvert to *Ehrlichia*, *Anaplasma*, *B. burgdorferi*, or *Rickettsia*.

Discussion

In this study, we characterized the spectrum of bacteria present in *A. americanum* from an established laboratory colony and compared it to that found in wild lone star ticks before and after feeding to identify both common and less frequent components of the natural tick microfauna. As expected from previous reports (Jasinskas et al. 2007, Klyachko et al. 2007), *Coxiella* was very commonly identified in both colony-reared and wild caught ticks although the proportion decreased upon tick feeding because of increase in other organisms, most notably *Rickettsia* sp. (Fig. 1). The near-ubiquitous presence of *Coxiella* sp. in multiple-organ systems suggests that these might be obligate endosymbionts for lone star ticks. A reduced genome compared with the pathogen *C. burnetii*, as well as reduced reproductive success of lone star ticks without *Coxiella*, support this hypothesis (Jasinskas et al. 2007, Klyachko et al. 2007, Zhong et al. 2007). Colony ticks, but not wild ticks, also harbored *Brevibacterium*, a gram-positive soil organism not previously described from lone star ticks (Clay et al. 2008). Because it was absent from wild ticks, we suspect this organism may be present or maintained because of the colony rearing process.

Wild lone star ticks, which presumably feed on a variety of wild vertebrates as larvae and nymphs and thus acquire a complex microfauna, harbored bacteria

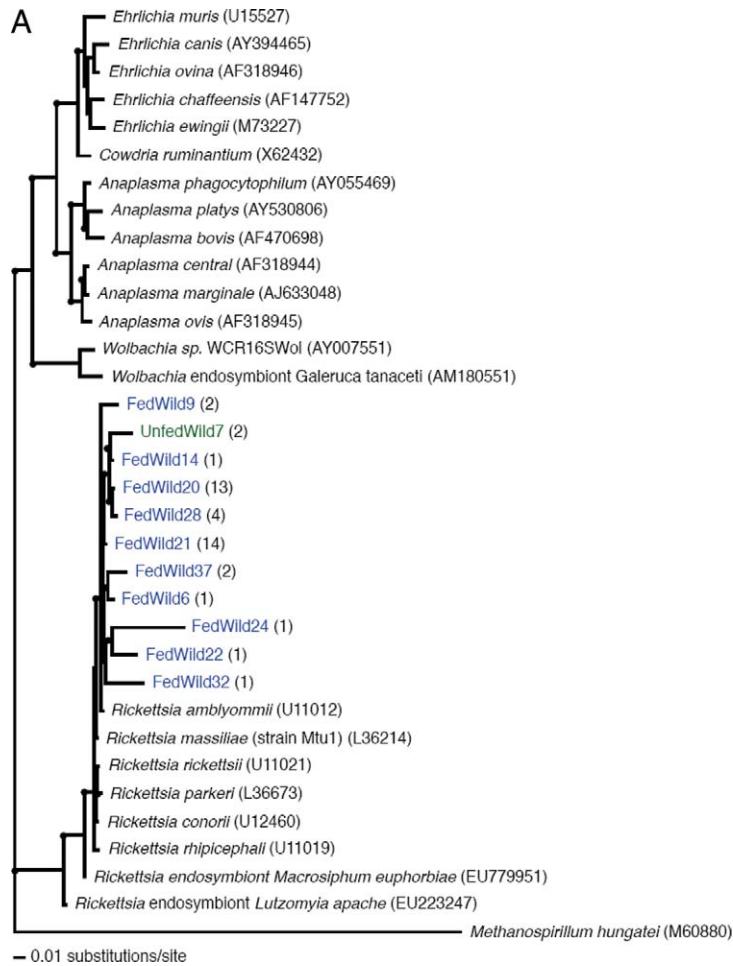


Fig. 3. (A) Distance neighbor-joining tree based on the 16S rRNA gene sequences of OTUs affiliated with members of the order Rickettsiales encountered in clone libraries constructed in this study. Bold circles indicate a bootstrap value of >50%. Bootstrap values were based on 1,000 replicates. The frequency of occurrence of each OTU is reported in parentheses. (Online figure in color.)

not found in the colony ticks, namely spotted fever group *Rickettsia* spp. and *Acinetobacter* spp. Another recent study investigated the bacterial community of unfed adult lone star tick and identified four bacterial components: the *Coxiella* endosymbiont, *R. amblyommii*, *Pantoea agglomerans*, and an *Arsenophonus* endosymbiont (Clay et al. 2008). However, numbers of clones evaluated, relative proportion of the population comprised by each bacteria, and overall diversity estimates were not reported (Clay et al. 2008), precluding direct comparison to our results. *Acinetobacter*, a gram-negative soil organism occasionally associated with nosocomial infections, was previously reported from larval but not adult lone star ticks (Clay et al. 2008).

Upon blood feeding, the microbial diversity in the wild *A. americanum* increased, allowing detection of organisms not identified in the ticks before feeding, including those in the genera *Pseudomonas*, *Stenotrophomonas*, and *Methylobacterium*. Members of the

closely related genera *Pseudomonas* and *Stenotrophomonas* are widespread gram-negative bacteria that can induce clinically relevant opportunistic infections (Toleman et al. 2007, Crossman et al. 2008, Looney et al. 2009); both genera have been previously reported from Ixodid ticks (Benson et al. 2004, Moreno et al. 2006). To our knowledge, this is the second report of *Pseudomonas* spp. (Clay et al. 2008) and the first of *Stenotrophomonas* from lone star ticks. *Methylobacterium* spp. are commonly found in soil and on the surface of plants; infection of immunocompromised individuals has been reported but this genus has not been previously identified in ticks although another Rhizobiales (*Ochrobactrum* spp.) was reported from larval lone star ticks (Clay et al. 2008). Fed adult *A. americanum* in the current study also harbored bacteria in the families *Bacillaceae*, *Enterobacteraceae*, and *Micrococcaceae*. Two other members of the *Enterobacteraceae*, *Enterobacter agglomerans*, and an *Arsenophonus* endosymbiont were reported from unfed

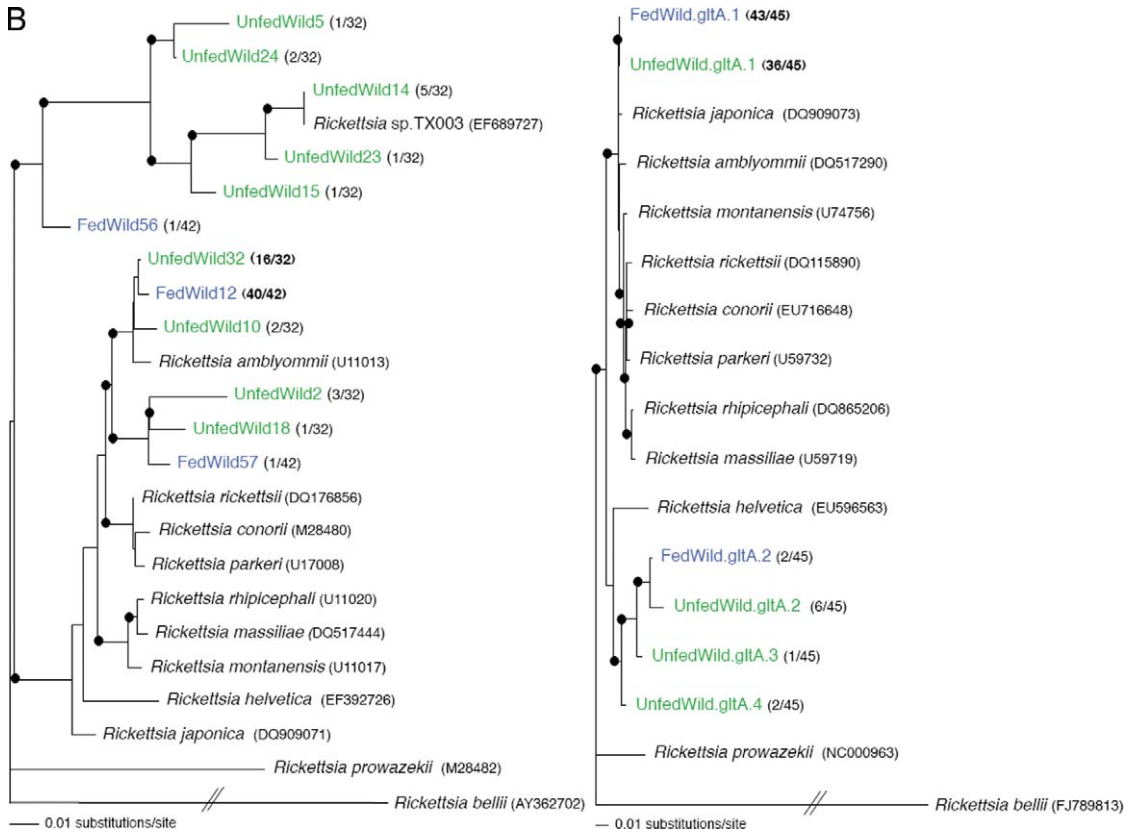


Fig. 3. Continued. (B) Distance neighbor-joining tree based on the 17 kDa gene sequences obtained in this study. Bold circles indicate a bootstrap value of >50%. Bootstrap values were based on 1,000 replicates. The frequency of occurrence of each OTU is reported in parentheses. (C) Distance neighbor-joining tree based on the gltA gene sequences obtained in this study. Bold circles indicate a bootstrap value of >50%. Bootstrap values were based on 1,000 replicates. The frequency of occurrence of each OTU is reported in parentheses. (Online figure in color.)

lone star ticks previously (Clay et al. 2008) but the sequences from our *A. americanum* were distinct.

Male and female ticks differed, in some instances dramatically, in both type and the relative proportion of bacteria present. Overall, fed male lone star ticks had a comparatively smaller proportion of *Rickettsia* spp. and more bacteria that are thought to be environmental or commensals. For example, *Stenotrophomonas* was much more common in male (81.6% of sequences) than female (2.6% of sequences) lone star ticks, suggesting surface contamination may have been responsible for its presence. In contrast, *Rickettsia* spp. and the *Coxiella* endosymbiont comprised a larger share of the sequences found in fed female ticks, an observation consistent with the fact that these organisms are transovarially transmitted and increase in number with tick feeding (de Silva and Fikrig 1995, Azad and Beard 1998, Macaluso et al. 2001, Stromdahl et al. 2008, Zanetti et al. 2008).

Analysis of *Rickettsia* spp. by three different genes (16S, 17 kDa, gltA) showed at least three distinct spotted fever group *Rickettsia* were present in these ticks. Known pathogens in this group share a common clinical presentation, including fever, headache, and

dermal rash, which can be combined with an eschar at the site of the tick bite (Parola et al. 2009). Sequences from our ticks were most closely related to *R. amblyommii*, *R. massiliae*, and *R. rickettsii*. Additionally, our analysis revealed a higher proportion of clones within the *R. amblyommii* group in the fed ticks, suggesting that this group is selectively stimulated by feeding or that it has a higher relative growth rate than other *Rickettsia* species. Further, it appears that feeding selects for a single or very few OTUs within this group, as evident by the prevalence of a single OTU in fed libraries of both genes, as opposed to a relatively more diverse, even distribution within unfed libraries of both genes. Thus feeding appears to stimulate only specific strains with the rickettsial community of *A. americanum*.

R. amblyommii is widely distributed in *A. americanum* populations, and is suspected to account for a high rate of seropositivity to the spotted fever group because of cross-reactivity (Apperson et al. 2008, Stromdahl et al. 2008). This agent has been associated with a rash in a human patient after a tick bite (Billeter et al. 2007) although its role as a human pathogen has not yet been fully established (Parola et al. 2009). To

date, *R. massiliae* has only been associated with *Rhipicephalus sanguineus*, *R. turanicus*, and *Ixodes ricinus* (Eremeeva et al. 2006, Fernandez-Soto et al. 2006, Ogawa et al. 2006, Blanc et al. 2007). To our knowledge, this is the first report of *R. massiliae* in lone star ticks.

In North America, *R. rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF), is vectored by *Dermacentor* spp. and, occasionally, *R. sanguineus* (Demma et al. 2006). However, *Amblyomma* spp. ticks infected with *R. rickettsii* have been reported from South America (Guedes et al. 2005). Historic reports of lone star ticks as a vector of RMSF in North America are thought largely because of the presence of related spotted fever group *Rickettsia* spp. in *A. americanum*, such as *R. amblyommii* (Burgdorfer 1969). Our finding in this study of sequences that most closely resemble, yet are distinct from, *R. rickettsii* (96%) suggests novel spotted fever group *Rickettsia* species may be present in lone star ticks although additional specific data are required to confirm that assertion. Novel spotted fever group *Rickettsia* continue to be reported (Fournier et al. 2006, Fujita et al. 2006, Mediannikov et al. 2008), underscoring the importance of continuing to pursue the role of these organisms as human and veterinary pathogens.

Specific PCRs optimized for detection of *Ehrlichia* spp., *Anaplasma* spp., and *Borrelia* spp. did not identify these individual pathogens in these lone star ticks. These results were somewhat surprising as *E. chaffeensis*, *E. ewingii*, and *B. lonestari* are known to be present in white-tailed deer and lone star ticks from this area (Mixon et al. 2006). Pooling of the ticks before DNA extraction may have suppressed detection of infrequent organisms. As a basic rule, the greater the diversity within sequences isolated from a distinct microbial population, the more likely minor populations are masked (Schloss and Handelsman 2006). This concept is supported in the current study by the statistics shown in Table 2. Pooling ticks before analysis has been previously demonstrated to decrease prevalence estimates of *E. chaffeensis* infection in wild lone star ticks (Lockhart et al. 1997). Moreover, tick populations with the same geographical background show shifts in their pathogen load over time (Varela et al. 2004, Bacon et al. 2005), and thus at the time of collection these ticks may not have contained the pathogens.

Direct comparison of colony-raised, unfed, and fed wild-caught adult *A. americanum* reflect the dynamics within the bacterial community associated with this tick. Environmental influences appear to have a less significant influence on the proportion inhibited by the different groups of bacteria compared with the feeding process. While some bacteria are acquired from the skin of the host, which is incorporated in the feeding lesion, feeding causes pathogens, especially *Rickettsia* spp. to multiply. The findings of this study establish a basis for further investigations on the background of various diseases in human and veterinary medicine associated with *A. americanum*.

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