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Intracellular Symbionts and Other Bacteria Associated with Deer Ticks (*Ixodes scapularis*) from Nantucket and Wellfleet, Cape Cod, Massachusetts

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The diversity of bacteria associated with the deer tick (*Ixodes scapularis*) was assessed using PCR amplification, cloning, and sequencing of 16S rRNA genes originating from seven ticks collected from Nantucket Island and Wellfleet, Cape Cod, Mass. The majority of sequences obtained originated from gram-negative proteobacteria. Four intracellular bacteria were detected including strains of *Ehrlichia*, *Rickettsia*, and *Wolbachia* and an organism related to intracellular insect symbionts from the *Cytophaga-Flavobacterium-Bacteroides* group. Several strains of members of the *Sphingomonadaceae* were also detected in all but one tick. The results provide a view of the diversity of bacteria associated with *I. scapularis* ticks in the field.

The deer tick, or black-legged tick (*Ixodes scapularis*, also known as *Ixodes dammini*), is a vector for several pathogens of medical and veterinary importance (29). The most prevalent human disease associated with *I. scapularis* is Lyme borreliosis, caused by *Borrelia burgdorferi* sensu stricto. To a lesser extent, deer ticks are also vectors for the agents of human granulocytic ehrlichiosis (HGE; *Anaplasma phagocytophilum*), bartonellosis (*Bartonella* sp.), and babesiosis (*Babesia microti*) (9, 11, 29). More than one of these human pathogens can be carried by a single tick (3). For a variety of reasons including the population dynamics of intermediary hosts and landscape modification, deer ticks are considered to be expanding their range in the eastern half of North America, being reported from Florida to the Canadian maritime provinces, west to North and South Dakota, and south to the state of Coahuila, Mexico (24). The highest density of *I. scapularis* corresponds with the highest incidence of Lyme borreliosis, with 92% of all cases being reported from Connecticut, Delaware, Maryland, Massachusetts, New York, New Jersey, Pennsylvania, Rhode Island, and Wisconsin (http://www.cdc.gov/ncidod/dvbid/lyme/).

In addition to human pathogens, a variety of symbiotic bacteria have been detected in *I. scapularis*. The term symbiotic is used here in the broad sense to indicate two organisms living together without implying an advantage to either. These include members of the *Rickettsiaceae* and *Anaplasmataceae* (7) that are not known to be human pathogens, bacteria belonging to the *Wolbachia persica* group (21, 23, 32, 38), and a variety of other bacteria detected by direct isolation or observation (15, 19). In related ticks, denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA gene fragments has been used to assess bacterial populations (27).

Since blood meals must be obtained from animals for ticks to reach reproductive maturity as adults, bacterial populations associated with ticks might be expected to include organisms derived from contact with plants, animals, and soil during questing behavior, as well as potential or actual human pathogens derived from the blood, skin, or surfaces of animals. Many studies have focused on the diversity and distribution of human pathogens, especially *B. burgdorferi* sensu stricto, associated with *I. scapularis* (35). However, relatively little information is available concerning the broader array of bacteria associated with deer ticks. Here, we report a survey on the populations of bacteria associated with *I. scapularis* nymphs collected from the field using PCR-amplified bacterial 16S rRNA genes. The goal of this study was to provide an overview of the diversity of bacteria associated with ticks in the field. Nymphs were chosen for study since they are the stage primarily responsible for transmitting *B. burgdorferi* and other pathogens to humans (30).

Several hundred deer tick nymphs were collected by flagging vegetation at sites in Wellfleet, Cape Cod, Mass., and Nantucket Island, Mass., during June to July of 2002. Each nymph was placed in a separate sterile 1.5-ml microcentrifuge tube containing 95% ethanol and stored cold until used. Seven ticks were randomly selected for analysis, four from Nantucket (N8 to N11) and three from Wellfleet (W1, W2, and W4). Total DNA was extracted from the nymphs by first rehydrating them in sterile distilled H_2O. The ticks were then placed in 200 μl of Tris-EDTA buffer plus 5% Chelex resin in a 1.5-ml sterile microcentrifuge tube and macerated with a 1,000-μl micropipette tip. Since the purpose of this study was to assess general populations of bacteria associated with the ticks, no procedures to remove surface-associated bacteria were employed. The macerate (about 200 μl) was boiled for 5 min. The preparations were vortexed for 30 s, reboiled for 2 min, quenched on ice, and allowed to cool for 10 min before centrifugation at 12,000 × *g*. The supernatant served as the template for PCR amplification.

For PCR, we used universal 16S ribosomal DNA primers corresponding to positions 8 to 27 (Eubac27F; 5′-AGAGTTT GATCCTGGCTCAG-3′) and 536 to 518 (518R; 5′-GWATT

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ACCGCGGCTGCTGG-3′, with W representing either A or T) of the *Escherichia coli* 16S rRNA gene (6). Reactions were carried out in 25-μl volumes using the Eppendorf MasterMix 2.5× PCR reagents (Brinkmann Instruments, Westbury, N.Y.) plus 0.4 μM (each) primer and 2 μl of template DNA. Amplifications included denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 30 s, and a final extension step at 72°C for 7 min. Negative controls of water and Chelex resin were used to detect if background amplification occurred. The amplification products were cloned using the TOPO TA Cloning kit (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions. Individual clones were collected, and plasmids were prepared using the Qiagen minipreparation kit (Qiagen, Inc., Valencia, Calif.). The inserts in the plasmids were sequenced using T3 and T7 primer sites on the pCR4-TOPO plasmid and the BigDye Terminator Cycle Sequencing kit from Applied Biosystems, Inc. (ABI; Foster City, Calif.). Excess dye was removed with CentriSep spin columns (Princeton Separations, Inc., Adelphia, N.J.) and analyzed on an ABI Prizm 377 XL sequencer. Sequences were edited with SeqEd (ABI) and aligned using ClustalX (34), and taxon assignments were performed using BLASTn 2.2.6 at http://www.ncbi.nlm.nih.gov/BLAST (2).

Seven ticks were prepared individually, and clones were obtained from each tick. A nearest-neighbor comparison of the sequences retrieved in the survey is shown in Table 1. The topographic distribution of sequences among bacterial families is shown in Fig. 1. The sequences obtained were distributed as follows: gram-negative intracellular bacteria, 47%; other gram-negative bacteria, 34%; gram-positive bacteria, 10%; and unclassified organisms, 9%.

A general observation to be made about the population profiles obtained from the ticks is that, among nonintracellular bacteria, relatively few sequences originated from gram-positive strains (19 versus 81% gram negative). The dominance of gram-negative bacteria may be related to the sensitivity of ixodid ticks to desiccation. Few *I. scapularis* ticks survive more than 48 h at humidity levels below 80% (1), and so conditions on and in the tick are likely to permit the persistence of more desiccation-sensitive gram-negative organisms. A previous study of bacterial isolates from *I. scapularis* collected in Maryland (19) yielded mainly gram-positive organisms from the surface of adult ticks but mainly gram-negative isolates from nymphs. Adults appear capable of inhabiting drier environments than nymphs can and are generally found at higher points on vegetation (29). While not directly comparable to the present study, other work on bacterial populations associated with adult *Ixodes ricinus* in Austria using denaturing gradient gel electrophoresis analysis of amplified DNA found several gram-positive potential pathogens as well as strains of *Rickettsia* and *Borrelia* (27). Most of the organisms detected in that study differ from those found here except for strains of *Moraxella*, *Rickettsia*, and *Rhodococcus erythropolis*.

Overall, the most abundant sequence obtained (43 of 107, or 40%) was one related to the spotted fever group of rickettsias. All but one tick yielded the same sequence. The presence of rickettsias in *I. scapularis* ticks is well documented (16–18, 23, 29), and the high incidence of infection supports the idea that they are transmitted transovarially rather than by continual reinfection of larvae (23). Some related tick-borne rickettsias, such as the agents of Rocky Mountain spotted fever (*Rickettsia rickettsii*), cause human disease, although *I. scapularis* has not been implicated in transmitting Rocky Mountain spotted fever. The sequence obtained is nearly identical (99.7%) to ones previously reported from *I. scapularis* in lab colonies (23) and from *I. ricinus* in Europe (28).

A second symbiont detected is closely related to members of the insect-associated alphaproteobacterium *Wolbachia pipientis* group A (99.8% identity to accession no. AY007551, a symbiont of the western corn rootworm, *Diabrotica virgifera virgifera*). Another tick from Nantucket yielded two sequences that are 100% identical to AY007551 in the region amplified (D. R. Benson, unpublished). In other insects, *W. pipientis* strains cause a variety of reproductive alterations including skewed sex ratios through increased production of daughters at the expense of sons and induction of cytoplasmic incompatibility where the reproductive success of uninfected females is impaired, leading to an increase in the frequency of infected cytoplasm in the population (31). Some previous reports of "wolbachiae" in ticks have been based on the detection of *W. persica*, a gammaproteobacterium that had been included in the wolbachiae based on ultrastructural characterization but is unrelated to the common insect symbionts (7, 23). Studies looking specifically for *Wolbachia* (*Anaplasmataceae*) in ticks have generally yielded negative results (12, 22, 23), although a *Wolbachia* sp. sequence in GenBank (AF304445) originated from a semiengorged *Rhipicephalus sanguineus* tick in Okinawa (13). To our knowledge this is the first such report from *I. scapularis* and may provide a rationale for pursuing symbiont-mediated insect control measures that have been suggested for other insects (26).

A third symbiont sequence detected in tick N10 is more than 99% identical to *Anaplasma phagocytophilum* (*Ehrlichia phagocytophila*), the HGE agent (7). Ticks are known to transmit HGE, but the presence of the disease in Nantucket and Cape Cod has only recently been reported (25, 33). The two sequences detected were identical and had three nucleotide differences from the human pathogen. Two of the differences placed the sequences in the variant 1 category previously described for ticks in Wisconsin, Maryland, and the Northeast (4, 20, 21). The third nucleotide difference with the HGE agent was outside the sequence region reported for the variants. The variant 1 strains have not been implicated in human disease (20).

Four clones from one of the Nantucket ticks (N9) originated from an organism closely related (99.7% identical to accession no. AB001518) to a symbiont isolated in cell culture from a Nantucket tick (15). The latter was similar (96% identical) to obligate intracellular symbionts from the *Cytophaga-Flavobacterium-Bacteroides* lineage of Bacteria that recently have been implicated in parthenogenesis of parasitoid wasps and phytophagous mites (Table 1) (36, 37). No information is yet available on the biological role played by this symbiont in ticks.

Aside from the bacteria that are presumed to be intracellular, several others were detected that are related to bacterial pathogens and soil organisms that may dwell on or in ticks. Figure 1 and Table 1 indicate that the distribution of taxa among the ticks examined appears to be relatively nonspecific. Sequences related to bacteria more commonly associated with

TABLE 1. Distribution of sequences among bacterial taxa

Clade and family	Closest BLASTn match (accession no.)[a]	No. identical/total (% Identity)[b,c]	No. by tick:							Accession no.
			W1	W2	W4	N8	N9	N10	N11	
Alphaproteobacteria										
Rickettsiaceae	*Rickettsia* IRS4 (AF141908)	445/447 (99)	5	17	5		9	1	6	AY394611
Anaplasmataceae	*Ehrlichia* sp. (ESP242784)	451/453 (99)						2		AY394612
	Wolbachia sp. (AY007551)	450/451 (99)				1				AY394613
Bradyrhizobiaceae	*Afipia felis* (AF514773)	421/425 (99)					1			AY394614
Caulobacteraceae	Uncultured bacterium (UBA459874)	399/400 (99)		1						AY394615
	Uncultured bacterium (AY221076)	423/431 (98)				1				AY394616
Methylobacteriaceae	*Methylobacterium mesophilicum* (MME400919)	422/426 (99)							1	AY394617
	Methylobacterium sp. strain G296-5 (AF395035)	393/394 (99)							1	AY394618
	Uncultured bacterium (AY268333)	348/362 (96)				1				AY394619
Sphingomonadaceae	*Sphingomonas elodea* (AF503278)	397/397 (99)				2	3			AY394620
	Sphingomonas melonis (AB055863)	433/433 (100)						3		AY394621
	Sphingomonas sp. (AB033945)	425/433 (98)			1					AY394622
	Sphingomonas sp. strain BF2 (SS16SRBF2)	429/433 (99)							2	AY394623
	Sphingomonas sp. strain AV069 (AF385529)	381/382 (99)		1						AY394624
	Sphingopyxis alaskensis (AF378796)	262/267 (98)						1		AY394625
Betaproteobacteria										
Burkholderiaceae	*Burkholderia* (AF247815)	430/437 (98)	1							AY394626
Comamonadaceae	*Delftia acidovorans* (DAC516044)	438/440 (99)	1							AY394627
	Delftia acidovorans WDL34 (AF538930)	456/457 (99)		1						AY394628
	Uncultured bacterium (AY096161)	381/387 (98)						1		AY394629
	Uncultured bacterium (AF469367)	448/452 (99)						2		AY394630
Gammaproteobacteria										
Enterobacteriaceae	*Escherichia coli* (AE016770)	449/450 (99)	1	4						AY394631
Moraxellaceae	*Acinetobacter* sp. strain phenon 4 (ASP293690)	396/400 (99)						2		AY394632
	Acinetobacter sp. (AY177359)	371/380 (97)						1		AY394649
Xanthomonadaceae	*Stenotrophomonas maltophilia* (SMA293468)	438/440 (99)							2	AY394633
CFB[d]										
CFB	*Ixodes scapularis* endosymbiont (AB001518)	482/484 (99)					4			AY394634
Actinobacteria										
Mycobacteriaceae	*Mycobacterium manitobense* (AY082001)	413/425 (97)	2							AY394635
Nocardiaceae	*Rhodococcus erythropolis* strain NVI (AY147846)	430/430 (100)	1	3						AY394636
Propionibacteriaceae	*Propionibacterium acnes* (AB108484)	430/430 (100)		2		1				AY394637
Firmicutes										
Bacillaceae	*Bacillus* sp. (AF497248)	325/327 (99)				1				AY394638
Streptococcaceae	Uncultured *Streptococcus* sp. (AF408263)	360/360 (100)						1		AY394639
Unclassified-uncultured	Uncultured bacterium (AF432816)	369/401 (92)				1			1	AY394640
	Uncultured bacterium (AY145636)	298/335 (88)						1		AY394641
	Uncultured bacterium (AY268338)	459/469 (97)							1	AY394642
	Plastid clone (AY043965)	239/270 (88)				1				AY394643
	Uncultured bacterium (AF145821)	399/410 (97)				1				AY394644
	Uncultured bacterium (AY268299)	365/401 (91)						1		AY394645
	Uncultured bacterium (AY242745)	407/436 (93)						1		AY394646
	Uncultured bacterium (AY274150)	302/328 (92)					1			AY394647
	Uncultured bacterium (AF523889)	315/326 (96)							1	AY394648
Total of 106 sequences			11	29	6	10	18	17	15	

[a] BLAST analysis was done using BLASTn 2.2.6 at http://www.ncbi.nlm.nih.gov/BLAST (2).
[b] Where more than one clone was obtained, the longest sequence run is reported in the percent identity.
[c] The shorter sequences in some clones are due primarily to a low DNA concentration of the prepared clones.
[d] CFB, *Cytophaga-Flavobacterium-Bacteroides* group.

animals included *E. coli* (449 of 450; 99.8%), *Propionibacterium* (430 of 430; 100%), and *Streptococcus* sp. (360 of 360; 100%), as well as some related to occasional human pathogens such as *Acinetobacter*, *Stenotrophomonas*, *Afipia*, and *Burkholderia* species. Sequences belonging to members of the *Sphingomonadaceae* were the only ones, aside from the rickettsias, to be retrieved from all but one of the ticks sampled and accounted for 11% of the total sequences or 23% of the total nonintracellular symbiont population (Fig. 1). Sphingomonads are noted for producing gums in biofilms (14) and also for the biodegradation of a variety of complex xenobiotics (8, 10, 14). They are common soil organisms and perhaps exist as microcolonies adhering to tick surfaces.

The abundance of rickettsial sequences in some ticks tended

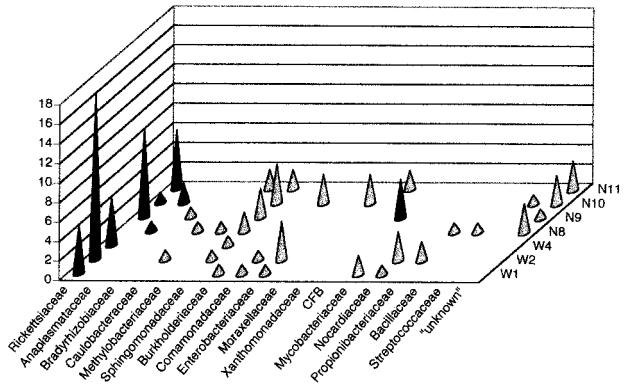

FIG. 1. Landscape summary of the number of representatives of bacterial families in each tick examined. Intracellular symbionts are indicated by filled cones. CFB, *Cytophaga-Flavobacterium-Bacteroides* group.

to overwhelm sequences from other members of the bacterial population; this abundance is noteworthy especially since rickettsial genomes studied have only one copy of 16S rRNA (*rrs*) genes (http://igs-server.cnrs-mrs.fr/mgdb/Rickettsia/). In this regard, *B. burgdorferi* sequences were not obtained even though previous studies using specific primers or other means of detection have shown a rate of infection of about 33% of *I. scapularis* nymphs collected from Nantucket and Cape Cod (M. J. Benson, unpublished data). The absence of *B. burgdorferi* sequences might be related to the forward primer used in this study (Eubac27F); it has one mismatch (C:A) with *Borrelia* 16S ribosomal DNA that occurs three bases from the 3′ end and therefore could be less likely to amplify *Borrelia* 16S sequences from a mixed population. With the Probe Match function at the Ribosomal Database Project website (5) and allowing for one mismatch, 3,631 sequences spanning the domain Bacteria matched Eubac27F, with only the borreliae having a conserved mismatch in the 3′ end of the primer.

In summary, this study has provided an overview of the complex microbiota associated with *I. scapularis* ticks as they exist in the field. Of particular note is the abundance of potential intracellular symbionts, emphasizing the predilection of the tick to host symbionts and occasionally transmit them during feeding. All ticks had at least one intracellular symbiont, and two ticks had more than one. The detection of sequences belonging to the *W. pipientis* lineage and another closely related to the symbiont of wasps and mites that promotes parthenogenesis might indicate that reproductive alterations are taking place within ticks on Nantucket and by extension provides an avenue to explore for controlling tick populations (26).

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