

# Characterization of Gut-Associated Bacteria in Larvae and Adults of the Southern Pine Beetle, *Dendroctonus frontalis* Zimmermann

ARCHANA VASANTHAKUMAR,<sup>1,2</sup> ITALO DELALIBERA, JR.,<sup>1,3</sup> JO HANDELSMAN,<sup>4</sup>  
KIER D. KLEPZIG,<sup>5</sup> PATRICK D. SCHLOSS,<sup>4,6</sup> AND KENNETH F. RAFFA<sup>1</sup>

Environ. Entomol. 35(6): 1710–1717 (2006)

**ABSTRACT** We report the first study of gut-associated bacteria of bark beetles using both culture-dependent and culture-independent methods. These insects are major pests of pine trees but also contribute to important ecological functions such as nutrient cycling. We found members of the  $\alpha$ - and  $\gamma$ -Proteobacteria and Firmicutes in larvae of the southern pine beetle, *Dendroctonus frontalis* Zimmermann. Sequences from three larval guts were grouped into one to three operational taxonomic units (OTUs) at 3% difference among sequences. Communities in adult southern pine beetle guts consisted solely of members of the  $\gamma$ -Proteobacteria. These could be grouped into three to five OTUs at 3% difference between sequences. These gut communities have relatively low species richness, which may reflect the specialization needed to exploit a nutrient-poor food source, colonize a chemically complex habitat, and maintain consistent associations with mutualistic fungi. However, there is considerable variation in gut microbiota composition among individual insects, suggesting the need for additional studies on sources of variation and potential substitutability among species performing similar functions.

**KEY WORDS** bark beetle, gut microbiota, 16S ribosomal RNA, rarefaction analysis, distance-based operational taxonomic unit and richness determination

Insects can harbor gut microbial communities that range from simple to complex (Cruden and Markovetz 1987, Leadbetter et al. 1999, Lilburn et al. 2001, Handelsman et al. 2005). Most studies to date have focused on defining community membership (Cazemier et al. 1997, Domingo et al. 1998, Broderick et al. 2004) as a first step toward understanding the roles of community members in the insect's biology and in the functioning of the community itself. In a few systems that have been studied in more detail, important contributions to the host insect's physiology and life history have been attributed to gut-associated microbes (Leadbetter et al. 1999, Dillon et al. 2000, Lilburn et al. 2001, Moran et al. 2005).

Little is known about the diversity, physiology, and ecology of microorganisms associated with the guts of the diverse groups of beetles that develop within the wood and bark of trees (Moore 1972a, b, Brand et al. 1975, Bridges 1981, Bridges et al. 1984, Delalibera et al.

2005). Bark beetles (Coleoptera: Scolytidae; alt. Curculionidae: Scolytinae) comprise an economically and ecologically important group that inhabit the subcortical tissues of trees, especially the phloem and outer bark (Wood 1982). Some species feed on decaying or dead trees and play beneficial ecological roles, such as assisting in decomposition and nutrient cycling, providing a major food base for birds and other wildlife, and fostering gap formation in forests (Schowalter 1981, Raffa et al. 1993). Others attack living trees and play important roles in ecological processes such as fire and succession (Romme et al. 1986, Raffa and Berryman 1987, Rykiel et al. 1988). These latter species are also significant forest pests, causing large scale losses that extend over millions of hectares of contiguous forests (Coulson 1979, Wallin and Raffa 2004). Their impacts are expected to increase markedly and expand into new latitudinal, altitudinal, and host species ranges as atmospheric temperatures rise (Logan and Powell 2001, Carroll et al. 2004). In addition to such native species, bark beetles are the most commonly intercepted exotic insects, a number of which are established invasive species causing severe environmental and economic costs (Haack 2002, Chornesky et al. 2005).

One such economically important native bark beetle is the southern pine beetle, *Dendroctonus frontalis* Zimmermann. This insect can attack and kill a variety of *Pinus* species, including both weakened and healthy trees (Coulson 1979, Paine et al. 1984). Adults tunnel

<sup>1</sup> Department of Entomology University of Wisconsin, Madison, WI 53706.

<sup>2</sup> Corresponding author: Department of Entomology, 1630 Linden Dr., 345 Russell Labs, University of Wisconsin, Madison, WI 53706 (e-mail: ava@entomology.wisc.edu).

<sup>3</sup> Current address: University of São Paulo, Piracicaba, SP, Brazil.

<sup>4</sup> Department of Plant Pathology, University of Wisconsin, Madison, WI 53706.

<sup>5</sup> USDA Forest Service, Southern Research Station, Pineville, LA 71360.

<sup>6</sup> Current address: Department of Microbiology, University of Massachusetts, Amherst, MA 01003.

through the phloem and cambium, disrupting the tree's vascular system. After mating, they oviposit along the galleries. Larvae feed in the phloem initially and later extend their feeding galleries into the outer bark. Mature larvae pupate in chambers at the ends of these galleries. Young adults emerge from the dead tree and repeat the cycle by searching for new hosts.

Most knowledge of microorganisms associated with bark beetles involves fungi, particularly those transported externally on the exoskeleton or in specialized structures known as mycangia (Paine et al. 1997, Krokene and Solheim 1998, Hsiau and Harrington 2003, Lim et al. 2004, 2005). Such fungi may provide a food source for larvae (Six and Klepzig 2004), assist in digestion of host materials (Ayes et al. 2000), or assist beetles in overcoming host defenses (Krokene and Solheim 1998), whereas others can be antagonistic to developing larvae (Barras 1970, Klepzig and Wilkens 1997). In contrast, little is known about gut symbionts of bark beetles. Previous studies have suggested a role in pheromone synthesis (Brand et al. 1975), although the significance of this relationship is unclear (Conn et al. 1984), and in protection from gallery-invading fungi (Cardoza et al. 2006). The importance of symbiotic fungi in the life cycles and population dynamics of bark beetles (Hofstetter et al. 2006), the nutrient-poor substrate on which they feed, and previous studies documenting cellulolytic and nitrogen-fixing bacteria in wood-boring insects (Bridges 1981, Delalibera et al. 2005) suggest that gut symbionts could play important roles in the biology of bark beetles.

The goal of this study was to define and characterize the microorganisms associated with guts of larvae and adults of the southern pine beetle, using classical microbiological methods and culture-independent molecular techniques. This information will provide the basis for subsequent studies on the roles of these microorganisms in bark beetle development, ecology, and management.

### Materials and Methods

**Insect Collection and Gut Dissection.** *D. frontalis* adults and larvae were collected from loblolly pine trees in the Bienville National Forest near Forest, MS, in October 2002, July 2003, and September 2003. Insects were transported overnight in a cooler with an ice pack and dissected the following day. Adult beetles had emerged 24–72 h before shipping from field-collected logs held in an environmental chamber. Larvae used were late-instar larvae. The insects were surface sterilized in 70% ethanol for 1 min and rinsed in sterile water before dissection. Insects were dissected in 10 mM sterile phosphate-buffered saline (PBS) inside a sterile laminar flow hood using dissection scissors and fine-tipped forceps. The head and last abdominal segment of each larva were severed, and pressure was applied anterior to the crop to release the gut. The thorax of adult beetles was held with forceps, and the head was pulled away from the thorax until the entire gut was stretched out of the insect body but still attached. The gut was separated from the body by

cutting its extremities in a drop of sterilized PBS. Both larval and adult guts were milky white in color, indicating a lack of cellulose. Guts were washed in PBS to avoid possible contaminants derived from other tissues and either pooled or transferred individually to 1.5-ml microfuge tubes containing 50, 100, or 500  $\mu$ l of PBS. The guts were sonicated (50/60 Hz, 117 V, 1.0 Amps; Branson Ultrasonics, Danbury, CT) for 30 s, macerated with a plastic pestle, and vortexed at medium speed for 10 s to separate bacterial cells from the gut wall.

**Isolation of Bacteria and DNA Extraction.** Each larval gut was placed in 100  $\mu$ l of 10 mM PBS. Adult guts were placed in 500  $\mu$ l of 10 mM PBS. Serial 10-fold dilutions were spread on duplicate plates of one-tenth strength TSA (3 g/liter tryptic soy broth; Difco Laboratories; 15 g/liter agar, pH 7.0). Plates were incubated in a growth chamber at 28°C for 3–5 d; bacterial colonies were categorized based on morphology and counted across all plates on the three lowest countable dilutions. Pure cultures of bacterial isolates were obtained and used in further analyses.

Bacterial cultures were grown in 5 ml of Luria-Bertani broth (10 g/liter, Bacto Tryptone; 5 g/liter Bacto-yeast extract; 5 g/liter NaCl, pH 7.0) at 28°C for 2 d. A previously described protocol was used to extract DNA (Broderick et al. 2004). Cell suspensions were lysed using chemical detergents and Proteinase K (Promega, Madison, WI). DNA was isolated using phenol chloroform extractions and isopropanol precipitation.

Individual guts from larvae and pooled guts from 30 or 10 adult beetles were placed in 1.5-ml tubes containing 50  $\mu$ l PBS and maintained at 4°C until DNA extraction. The tubes were centrifuged at low speed to pellet the insect tissues, and DNA was extracted from the bacteria in the supernatant. DNA from the gut bacterial community was extracted using 200  $\mu$ l of InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's directions. Briefly, 200  $\mu$ l of matrix was combined with gut extracts, incubated at 56°C for 1 h and mixed on a vortex mixer at high speed for 10 s. The mixture was heated in a boiling water bath for 10 min to lyse cells and centrifuged at high speed to separate the matrix, which adsorbs cell lysis products, from the DNA. Twenty microliters of the resulting DNA was used in a 50- $\mu$ l polymerase chain reaction (PCR) reaction.

**PCR Amplification and 16S rRNA Gene Libraries.** DNA extracted from individual cultured bacterial colonies was diluted 1/20, and the 16S rRNA genes were amplified by PCR using primers 27f and 1492r (Broderick et al. 2004). Final concentrations for 50- $\mu$ l PCR reactions were as follows: 2  $\mu$ l diluted DNA (10–100 ng), 0.2  $\mu$ M of each primer, 0.2 mM dNTPs, 5 U of *Taq* polymerase, and 1 $\times$  *Taq* polymerase buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100 and 1.5 mM MgCl<sub>2</sub>; Promega). The reaction conditions were 94°C for 3 min, 35 cycles at 94°C for 30 s, 55°C for 1.5 min, 72°C for 2.5 min, and a final extension at 72°C for 5 min. PCR products were purified using AMPure

magnetic beads (Agencourt Bioscience, Beverly, MA).

16S rRNA genes were amplified by PCR from total DNA isolated from gut extracts as described above. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), and cloned into a pGEM-T vector (Promega) according to the manufacturer's directions. One hundred clones from each sample were transferred to plates of Luria-Bertani agar amended with 50 mg/liter ampicillin and incubated at 37°C for 48 h. Crude lysates of clones were prepared by suspending each colony in 50  $\mu$ l of lysis buffer (50 mM NaOH, 0.25% sodium dodecyl sulfate) in a 96-well microplate and heating at 95°C for 15 min. Lysates were diluted 1/10 in sterile water and used as DNA template for PCR amplification of the insert using M13 vector primers (Broderick et al. 2004). PCR reaction conditions consisted of an initial denaturation step at 94°C for 3 min, five cycles at 94°C for 30 s, 57°C for 1.5 min, 72°C for 2.5 min, five cycles at 94°C for 30 s, 56°C for 1.5 min, 72°C for 2.5 min, 25 cycles at 94°C for 30 s, 55°C for 1.5 min, 72°C for 2.5 min, and a final extension cycle at 72°C for 7 min. PCR products were purified using AMPure magnetic beads (Agencourt BioScience).

#### Sequencing Cultured Bacterial Isolates and Clones.

At least two bacterial isolates representative of each morphology were chosen for analysis by amplified ribosomal DNA restriction analysis (ARDRA) using separate *MspI* and *TaqI* digests. PCR products (9  $\mu$ l) were digested independently with restriction enzymes *MspI* and *TaqI* (Promega) for 2 h according to the manufacturer's specifications. The restriction fragments were separated by gel electrophoresis. The ARDRA restriction patterns of isolates were compared visually and grouped. At least one bacterial isolate from each distinct ARDRA pattern was selected for sequencing. Clones to be sequenced were chosen randomly.

Bacterial isolates were sequenced using 16S rRNA gene primers 27 F, 704 F, 787R, or 1492R (Broderick et al. 2004). Clones with inserts of the right size ( $\approx$ 1,500 bp) were sequenced using vector primers SP6 and T7 and 16S rRNA gene primers 704 F and 787R (Broderick et al. 2004). Sequencing reactions were performed in a total volume of 15  $\mu$ l consisting of 0.5  $\mu$ l DNA, 0.37  $\mu$ l (10  $\mu$ M) of primer, 1.0 or 1.1  $\mu$ l BigDye (Perkin-Elmer, Wellesley, MA) 3  $\mu$ l 5 $\times$  buffer (Perkin-Elmer), 0.75  $\mu$ l DMSO, and 9.28 or 9.38  $\mu$ l nuclease-free water. The reaction conditions were 95°C for 3 min, 50 cycles at 96°C for 20 s, 46°C for 30 s, and 60°C for 2 min, and a final extension at 72°C for 7 min. Sequenced products were purified either with Sephadex G-50 columns (Pharmacia Biotech, Piscataway, NJ) or CleanSEQ magnetic beads (Agencourt Bioscience). Sequences were determined on an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA) at the University of Wisconsin-Madison Biotechnology Center.

**Sequence Analyses.** Sequences were compiled using SeqMan (DNASTAR, Madison, WI) and compared with the nonredundant GenBank library using BLAST

(Altschul et al. 1997). Voucher specimens of bacterial colonies were preserved in the forest entomology laboratory in the Department of Entomology of the University of Wisconsin. Clone sequences were tested for chimeric structures by using RDP Check\_Chimera (Cole et al. 2005) and Bellepheron (Huber et al. 2004), and chimeras were excluded from further analyses.

Sequences from each library were aligned in ARB (Ludwig et al. 2004) using the RDP 7.1 phylogenetic tree (Cole et al. 2005) for comparison. The sequences were automatically aligned in the ARB sequence editor, and alignments were manually corrected if necessary. Aligned sequences were added to the phylogenetic tree using a maximum parsimony method integrated in ARB. Taxonomic descriptions were determined based on the position of each aligned sequence in the phylogenetic tree. A distance matrix was computed in ARB using the Jukes-Cantor correction and used as the input file in the software package distance-based operational taxonomic unit and richness determination (DOTUR) to calculate operational taxonomic units (OTUs) and construct rarefaction curves at distance levels of 20, 10, 5, 3, and 1% (Schloss and Handelsman 2005). These are considered to correspond to phylum, class, genus, species, and strain levels (Schloss and Handelsman 2004). Rarefaction analyses are useful in gauging adequacy of sampling. Rarefaction curves are built by plotting the total number of OTUs found versus the total number of sequences sampled. Generally, the slope of a rarefaction curve is high in the beginning and gradually decreases, causing the curve to level off, at which point, sampling is considered adequate. We present rarefaction curves for OTUs at a difference of no more than 3% between sequences, which is widely used to approximate species-level similarity. Although rarefaction analyses are useful in estimating the adequacy of sampling, they are not considered to provide true estimates of the richness in a community. Therefore, the Chao1 richness estimator was also calculated in DOTUR (Schloss and Handelsman 2005). This nonparametric richness estimator estimates diversity of a community based on the number of singletons (OTUs represented by only one sequence) and doubletons (OTUs represented by two sequences) found in a sample (Bohannon and Hughes 2003). Terminal Chao1 richness estimates are reported for 3% difference between sequences.

Nucleotide sequences analyzed in this study were deposited in GenBank under the accession numbers DQ314782–DQ314800, DQ316909–DQ316958, and DQ321537–DQ321658.

## Results

### Bacterial Species in Southern Pine Beetle Larvae.

Five bacterial genera were associated with larval guts in culture-independent analyses (Table 1). Sequence alignments in ARB revealed that all three larval guts contained sequences that clustered with the closely related genera, *Rahnella*, *Serratia*, and *Yersinia*. These genera accounted for 100% (total number of se-

**Table 1.** Bacterial taxa associated with guts of larvae and adults of the southern pine beetle in culture-dependent and culture-independent analyses

Bacterial taxa	Larvae			Adults		
	Culture-independent		Culture-dependent	Culture-independent		Culture-dependent
	Insects	Clones	Insects	Insects	Clones	Insects
<i>n</i>	3	69	5	4	95	3
$\alpha$ -Proteobacteria						
<i>Mycoplana</i> sp.	0	0	1	0	0	0
<i>Rhodobacter</i> sp.	0	0	1	0	0	0
$\gamma$ -Proteobacteria						
<i>Acinetobacter</i> sp.	0	0	0	1	1	0
<i>Cedecea neteri</i>	0	0	1	0	0	0
<i>Enterobacter</i> spp. <sup>a</sup>	0	0	3	1	5	0
<i>Erwinia</i> sp.	0	0	0	1	1	0
<i>Hafnia alvei</i>	0	0	0	1	1	0
<i>Klebsiella</i> spp.	0	0	2	1	1	2
<i>Pantoea</i> spp. <sup>b</sup>	1	2	0	0	0	0
<i>Pseudomonas</i> spp. <sup>c</sup>	0	0	0	2	35	3
<i>Rahnella</i> spp. <sup>d</sup>	2	14	4	4	41	0
<i>Salmonella</i> sp.	0	0	0	2	6	0
<i>Serratia</i> spp. <sup>e</sup>	1	21	2	1	4	1
<i>Strenotrophomonas maltophilia</i>	0	0	0	0	0	1
<i>Yersinia</i> sp.	2	27	0	0	0	0
Firmicutes						
<i>Bacillus cereus</i>	0	0	4	0	0	0
<i>Enterococcus faecalis</i>	1	5	1	0	0	0
<i>Lactobacillus</i> sp.	0	0	1	0	0	0
<i>Leuconostoc mesenteroides</i>	0	0	2	0	0	0

Individual guts from five larvae and three adults were analyzed by culturing. The 16S rRNA genes of 33 isolates from larval guts and 15 isolates from adult guts were sequenced. In culture-independent analyses, three individual larval guts or four pooled adult gut samples were analyzed. All sequences analyzed were at least 95% similar to previously published sequences in GenBank. Sample sizes of insects refer to the total no. of larval or adult samples analyzed. Sample sizes of clones refer to the total no. of clones analyzed. The no. of insects from which the clones were derived is also noted under culture-independent analyses. The no. of samples or clones in which each genus was observed is noted.

<sup>a</sup> *Enterobacter aerogenes* and unidentified *Enterobacter*.

<sup>b</sup> *Pantoea agglomerans* and unidentified *Pantoea*.

<sup>c</sup> *Pseudomonas libaniensis* and unidentified *Pseudomonas*.

<sup>d</sup> *Rahnella aquatilis* and unidentified *Rahnella*.

<sup>e</sup> *Serratia marcescens* and unidentified *Serratia*.

quences in library,  $n = 19$ ;  $n = 22$ ) of all clones in two 16S rRNA gene libraries and 75% ( $n = 28$ ) of all clones in the third larval library. Eighteen percent of the clones from the third clone library clustered with *Enterococcus faecalis* and 7% with *Pantoea* spp. The *Rahnella* sequences included *R. aquatilis* and members that could not be characterized at the species level (Table 1).

Rarefaction analyses indicated that there is a relatively simple community associated with southern pine beetle larval guts (Fig. 1a). The rarefaction curves for two libraries, spbL10 and spbL15, leveled off at 3% difference between sequences, whereas the curve for spbL12 rose but with a relatively low slope. When sequences were analyzed at no more than 5% difference among them, the rarefaction curves for two libraries were flat lines, and the third one was flattening at three OTUs. To further assess whether these three larval guts have been sufficiently sampled, the nonparametric richness estimator, Chao1, was calculated. The collector's curves for the Chao1 estimator at 3% difference between sequences had leveled off, indicating that most of the species had been sampled already. The terminal Chao1 richness estimates for the three larval libraries were one, two, and three OTUs, where an OTU was defined as a group of sequences differing by no more than 3%.

Eleven bacterial genera were found by culturing larval gut extracts (Table 1). Culturing revealed genera from the  $\gamma$ -Proteobacteria and  $\alpha$ -Proteobacteria that were not found in culture-independent analyses. *Rahnella* spp. and *Serratia* spp. were found in larval guts in both culture-based and culture-independent analyses. In contrast, all of the gram-positive genera isolated by culturing, except *E. faecalis*, were absent from the 16S rRNA gene libraries.

**Bacterial Species in Southern Pine Beetle Adults.** Both culture-independent and culture-dependent analyses of adult guts revealed organisms that affiliate with the  $\gamma$ -Proteobacteria (Table 1). Sequences that affiliated with nine bacterial genera were found in 16S rRNA gene libraries constructed from adult guts. In sequence alignments, all four clone libraries contained sequences that clustered with the genus *Rahnella*, accounting for 88, 40, 50, and 11% of the clones in each of the libraries (total number of sequences in each library,  $n = 17, 25, 26,$  and  $27,$  respectively). Again, these included *R. aquatilis* and members that could not be identified to the species level. The genus *Pseudomonas* accounted for 46 and 85% ( $n = 26$  and  $27,$  respectively) of the clones in two libraries. No gram-positive bacteria were found in 16S rRNA gene libraries derived from adult guts. Three of four bacterial genera found by culturing adult gut extracts were

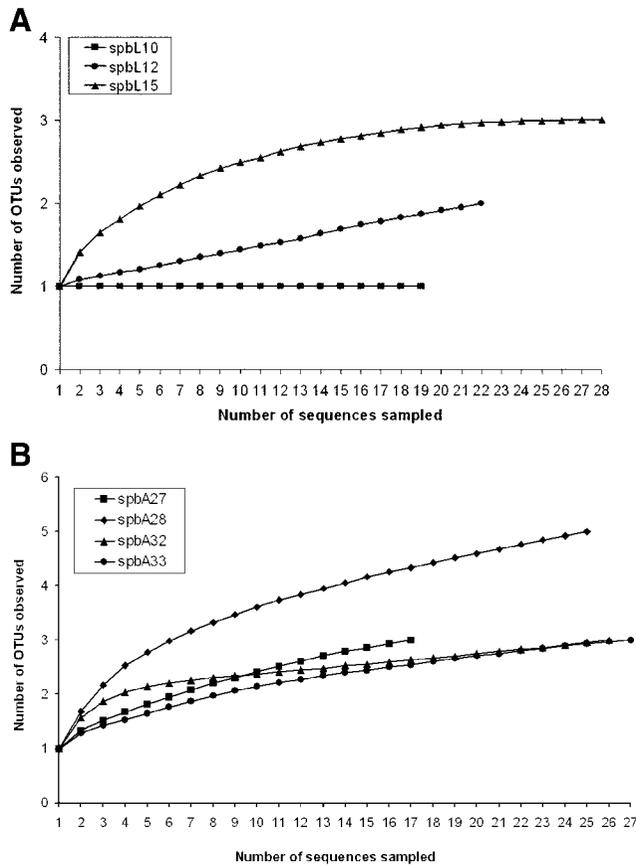


Fig. 1. Rarefaction analyses of 16S rRNA gene libraries constructed from (a) individual larval or (b) pooled adult guts. Rarefaction curves were constructed based on analyses performed in DOTUR using the furthest neighbor assignment algorithm. Rarefaction curves represent 97% sequence similarity among sequences within larval (spbL10, spbL12, and spbL15) and adult (spbA27, spbA28, spbA32, and spbA33) libraries.

represented in the culture-independent studies as well.

The rarefaction curves for all four libraries from adult guts have low slopes at 3% difference between sequences (Fig. 1b). Rarefaction curves at 5% difference between sequences leveled off for three libraries and had a low slope for the fourth library.

At 10 and 20% difference among sequences, the curves were largely flattened, suggesting that we sampled almost all the classes and phyla associated with adult southern pine beetle guts. The collector's curves for the Chao1 richness estimator had leveled off at 3% sequence difference among sequences. The terminal Chao1 richness estimates for the adult gut samples were three, three, three, and six OTUs when an OTU was defined as a group of sequences differing by no more than 3%.

### Discussion

Based on culture-dependent and culture-independent analyses, the gut microbiota of the southern pine beetle adults and larvae differ substantially in composition. In both life stages, however, the overall spe-

cies richness was relatively low. In culture-dependent and independent assessments, larval guts contained organisms that affiliate with the  $\alpha$ -Proteobacteria,  $\gamma$ -Proteobacteria, and Firmicutes, whereas adult guts contained organisms that affiliate with the  $\gamma$ -Proteobacteria (Table 1). By comparison, the midgut microbiota of the gypsy moth, a tree-feeding folivore, consists of 15 phylotypes that affiliate with  $\alpha$ -Proteobacteria,  $\gamma$ -Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria (Broderick et al. 2004). Interestingly, neither life stage of the southern pine beetle contained obligate anaerobic microorganisms such as members of Bacteroidetes, which have been commonly found in other insect guts (Moran et al. 2005).

Moore (1972) isolated *Enterobacter aerogenes*, *Pseudomonas fluorescens*, *Serratia marcescens*, and *Bacillus* spp. from the alimentary canal of the southern pine beetle, which is consistent with our findings. However, he did not observe the most common species in our study, *Rahnella aquatilis*, or any member of that genus. Some of this variation could be caused by location, time of sampling, and different culture conditions. *Bacillus* spp., *Serratia*, and *Pseudomonas* were

pathogenic to the beetles (Moore 1972a) and are common inhabitants of many insect guts (Brand et al. 1975, Broderick et al. 2004). *S. marcescens* is a facultative enteropathogen of many insects (Steinhaus 1959).

Many of the bacterial genera that we found in the southern pine beetle have been isolated from other invertebrate guts. Bacteria similar to *Rahnella*, the most common genus represented in these southern pine beetle larvae, occur in the intestines of snails, slugs, and earthworms (Muller et al. 1995a, b). This bacterium was also isolated from the larvae of a xylophagous beetle, *Saperda vestita* Say, the linden borer (Delalibera et al. 2005). *E. faecalis* was found to be consistently associated with other insect guts (Egert et al. 2003, Broderick et al. 2004) and is usually a harmless member of the human gut (Hooper et al. 2001). Other members of the Firmicutes such as *Lactobacillus* and *Leuconostoc* spp. have been found in other wood-boring species, such as the linden borer and Asian Longhorned beetle, *Anoplophora glabripennis* (Motschulsky) (Schloss et al. 2006).

Culture-independent methods have commonly revealed a greater diversity of bacterial phylotypes than culture-dependent methods (Paster et al. 2001, Broderick et al. 2004, Eckburg et al. 2005). In our study, some bacterial genera from the Firmicutes isolated on media were not found in DNA-based analyses (Table 1). This result is not unusual and has been observed in other environments (Dunbar et al. 1999). A possible explanation for this difference could be bias introduced by DNA extraction, PCR amplification, cloning, or culturing methods. Alternatively, it might reflect the low abundance of these genera in the community.

The rarefaction analyses based on the available data suggest that there is relatively low richness in the larval and adult gut communities of the southern pine beetle, but also that additional diversity remains to be characterized (Fig. 1). Because other studies have suggested that rarefaction analyses should be combined with richness estimators to make reliable conclusions about the total richness in the community (Hughes et al. 2001), we also calculated the nonparametric Chao1 richness estimates, which corresponded with the results of rarefaction analyses. Although the differences in community membership between larval and adult guts were consistent at the phylum level, they were more variable at the genus and species levels. Further studies are required to quantify this variability.

The presence of gram-positive bacteria such as *Bacillus* and *Leuconostoc* sp. in larval but not adult guts suggests that these bacteria might play an important role in growth and development. Feeding is the most important activity of growing larvae, whereas adults feed only minimally. Previous studies found no evidence for cellulolytic activity among bacteria associated with larval and adult guts of southern pine beetle (Delalibera et al. 2005), indicating that this important nutritional role is likely performed by the beetles themselves or other microbial associates. Nitrogen-fixing *Enterobacter* spp. have been isolated from the southern pine beetle, which, together with some fun-

gal associates, may concentrate nitrogen for developing larvae (Bridges 1981, Ayres et al. 2000, Klepzig and Six 2004). *Rahnella aquatilis*, *Klebsiella* spp., and *Pantoea* spp., which were commonly found in *D. frontalis* larvae, are known to fix nitrogen in other environments (Behar et al. 2005) and hence might be involved in nitrogen fixation in the southern pine beetle. Another important role might be detoxification of conifer defensive compounds, which consist primarily of monoterpenes, diterpene acids, and phenolics (Lewinsohn et al. 1991, Raffa et al. 2005), groups known to be metabolized by bacteria (Martin et al. 1999, Yu and Mohn 1999).

Our initial characterization of the gut microbial community of the southern pine beetle, albeit based on a limited sample size, provides a basis for addressing further questions about functions and interactions. Particular issues needing to be resolved include (1) variation of this community among beetles within and among regions, seasons, tree species, and population phases, (2) functions of various bacteria in insect development, host exploitation, reproduction, and interactions with other organisms, (3) interactions among various members of the gut community, (4) resilience of these communities, and (5) applying an understanding of gut symbioses to improve pest management.

#### Acknowledgments

We thank B. Burwitz and E. Cepaitis for technical support and the researchers at the USDA Forest Service, Southern Research Station in Pineville, LA, who collected and shipped the southern pine beetles. Funding for this research was provided by the USDA Forest Service Southern Research Station, the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service (CSREES) Grant 2003-35302-13528, UW-Madison College of Agricultural and Life Sciences, and McIntire-Stennis WIS 04529. The critical reviews of two anonymous reviewers are greatly appreciated.

#### References Cited

- Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389–3402.
- Ayres, M. P., R. T. Wilkens, J. J. Ruel, M. J. Lombardero, and E. Vallery. 2000. Nitrogen budgets of phloem-feeding bark beetles with and without symbiotic fungi. *Ecology* 81: 2198–2210.
- Barras, S. J. 1970. Antagonism between *Dendroctonus frontalis* and the fungus *Ceratocystis minor*. *Ann. Entomol. Soc. Am.* 63: 1187–1190.
- Behar, A., B. Yuval, and E. Jurkevitch. 2005. Enterobacteria-mediated nitrogen fixation in natural populations of the fruit fly *Ceratitidis capitata*. *Mol. Ecol.* 14: 2637–2643.
- Bohannan, B.J.M., and J. Hughes. 2003. New approaches to analyzing microbial biodiversity data. *Curr. Opin. Microbiol.* 6: 282–287.
- Brand, J. M., J. W. Bracke, A. J. Markovetz, D. L. Wood, and L. E. Browne. 1975. Production of verbenol pheromone

- by a bacterium isolated from bark beetles. *Nature* (Lond.) 254: 136–137.
- Bridges, J. R. 1981. Nitrogen-fixing bacteria associated with bark beetles. *Microb. Ecol.* 7: 131–137.
- Bridges, J. R., J. E. Marler, and B. H. McSparrin. 1984. A quantitative study of the yeasts and bacteria associated with laboratory-reared *Dendroctonus frontalis* Zimm (Coleoptera, Scolytidae). *J. Appl. Entomol.* 97: 261–267.
- Broderick, N. A., K. F. Raffa, R. M. Goodman, and J. Handelsman. 2004. Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Appl. Environ. Microbiol.* 70: 293–300.
- Cardoza, Y. J., K. D. Klepzig, and K. F. Raffa. 2006. Bacteria in oral secretions of an endophytic insect inhibit antagonistic fungi. *Ecol. Entomol.* (in press).
- Carroll, A. L., S. W. Taylor, J. Regniere, and L. Safranyik. 2004. Effects of climate change on range expansion by the mountain pine beetle in British Columbia: challenges and solutions. Proceedings of the Mountain Pine Beetle Symposium, 30–31 Oct., 2004 Kelowna, British Columbia, Canada.
- Cazemier, A. E., J.H.P. Hackstein, H.J.M. Op den Camp, J. Rosenberg, and C. van der Drift. 1997. Bacteria in the intestinal tract of different species of arthropods. *Microb. Ecol.* 33: 189–197.
- Chornesky, E.A., A. M. Bartuska, G. H. Aplet, K. O. Britton, J. Cummings-Carlson, F. W. Davis, J. Eskow, D. R. Gordon, K. W. Gottschalk, R. A. Haack, A. J. Hansen, R. N. Mack, F. J. Rahel, M. A. Shannon, L. A. Wainger, and T. B. Wigley. 2005. Science priorities for reducing the threat of invasive species. *BioScience* 55: 335–348.
- Cole, J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and J. M. Tiedje. 2005. The ribosomal database project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res.* 33: 294–296.
- Conn, J. E., J. H. Borden, D.W.A. Hunt, J. Holman, H. S. Whitney, O. J. Spanier, H. D. Pierce, and A. C. Oehlschlager. 1984. Pheromone production by axenically reared *Dendroctonus ponderosae* and *Ips paraconfusus* (Coleoptera, Scolytidae). *J. Chem. Ecol.* 10: 281–290.
- Coulson, R. N. 1979. Population dynamics of bark beetles. *Annu. Rev. Entomol.* 24: 417–477.
- Cruden, D. L., and A. J. Markovetz. 1987. Microbial ecology of the cockroach gut. *Annu. Rev. Microbiol.* 41: 617–643.
- Delalibera, I., J. Handelsman, and K. F. Raffa. 2005. Contrasts in cellulolytic activities of gut microorganisms between the wood borer, *Saperda vestita* (Coleoptera: Cerambycidae), and the bark beetles, *Ips pini* and *Dendroctonus frontalis* (Coleoptera: Curculionidae). *Environ. Entomol.* 34: 541–547.
- Dillon, R. J., C. T. Vennard, and A. K. Charnley. 2000. Pheromones—exploitation of gut bacteria in the locust. *Nature* (Lond.) 403: 851–851.
- Domingo, J.W.S., M. G. Kaufman, M. J. Klug, and J. M. Tiedje. 1998. Characterization of the cricket hindgut microbiota with fluorescently labeled rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* 64: 752–755.
- Dunbar, J., S. Takala, S. M. Barns, J. A. Davis, and C. R. Kuske. 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. *Appl. Environ. Microbiol.* 65: 1662–1669.
- Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K. E. Nelson, and D. A. Relman. 2005. Diversity of the human intestinal microbial flora. *Science* 308: 1635–1638.
- Egert, M., B. Wagner, T. Lemke, A. Brune, and M. W. Friedrich. 2003. Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). *Appl. Environ. Microbiol.* 69: 6659–6668.
- Haack, R. A. 2002. Intercepted bark- and wood-boring insects in the United States: 1985–2000. *Newslett. Mich. Entomol. Soc.* 47: 14–15.
- Handelsman, J., C. J. Robinson, and K. F. Raffa. 2005. Microbial communities in lepidopteran guts: from models to metagenomics, pp. 143–168. *In* M. J. McFall, B. Henderson, and E. G. Ruby (eds.), *The influence of cooperative bacteria on animal host biology*. Cambridge University Press, New York.
- Hemingway, R. W., G. W. McGraw, and S. J. Barras. 1977. Polyphenols in *Ceratocystis minor* infected *Pinus taeda*: fungal metabolites, phloem and xylem phenols. *J. Agric. Food Chem.* 25: 717–722.
- Hofstetter, R. W., J. T. Cronin, K. D. Klepzig, J. C. Moser, and M. P. Ayres. 2006. Antagonisms, mutualisms, and commensalisms, affect outbreak dynamics of the southern pine beetle. *Oecologia* (Berl.) 145: 679–691.
- Hooper, L. V., and J. I. Gordon. 2001. Commensal host-bacterial relationships in the gut. *Science* 292: 1115–1118.
- Hsiau, P.T-W., and T. C. Harrington. 2003. Phylogenetics and adaptations of basidiomycetous fungi fed upon by bark beetles (Coleoptera:Scolytidae). *Symbiosis* 34: 111–131.
- Huber, T., G. Faulkner, and P. Hugenoltz. 2004. Bellero-phon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* 20: 2317–2319.
- Hughes, J. B., J. J. Hellmann, T. H. Ricketts, and B.J.M. Bohannan. 2001. Counting the uncountable: statistical approaches to estimating microbial diversity. *Appl. Environ. Microbiol.* 67: 4399–4406.
- Klepzig, K. D., and R. T. Wilkens. 1997. Competitive interactions among symbiotic fungi of the southern pine beetle. *Appl. Environ. Microbiol.* 63: 621–627.
- Klepzig, K. D., and D. L. Six. 2004. Bark beetle fungal symbiosis: context dependency in complex interactions. *Symbiosis* 37: 189–206.
- Krokene, P., and H. Solheim. 1998. Pathogenicity of four blue stain fungi associated with aggressive and nonaggressive bark beetles. *Phytopathology* 88: 39–44.
- Leadbetter, J. R., T. M. Schmidt, J. R. Graber, and J. A. Breznak. 1999. Acetogenesis from H<sub>2</sub> plus CO<sub>2</sub> by spirochetes from termite guts. *Science* 283: 686–689.
- Lewinsohn, E., M. Gijzen, T. J. Savage, and R. Croteau. 1991. Defense mechanisms of conifers: relationship of monoterpene cyclase activity to anatomical specialization and oleoresin monoterpene content. *Plant Physiol.* 96: 38–43.
- Lilburn, T. C., K. S. Kim, N. E. Ostrom, K. R. Byzek, J. R. Leadbetter, and J. A. Breznak. 2001. Nitrogen fixation by symbiotic and free-living spirochetes. *Science* 292: 2495–2498.
- Lim, Y. W., S. M. Alamounti, J. J. Kim, and C. Breuil. 2004. Multigene phylogenies of *Ophiostoma clavigerum* and closely related species from bark beetle-attacked *Pinus* in North America. *FEMS Microbiol. Lett.* 237: 89–96.
- Lim, Y. W., J. J. Kim, M. Lu, and C. Breuil. 2005. Determining fungal diversity on *Dendroctonus ponderosae* and *Ips pini* affecting lodgepole pine using cultural and molecular methods. *Fungal Divers.* 19: 79–94.
- Logan, J. A., and J. A. Powell. 2001. Ghost forests, global warming, and the mountain pine beetle (Coleoptera: Scolytidae). *Am. Entomol.* 47: 160–173.
- Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W.

- Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer. 2004. ARB: a software environment for sequence data. *Nucleic Acids Res.* 32: 1363–1371.
- Martin, V.J.J., Z. T. Yu, and W. W. Mohn. 1999. Recent advances in understanding resin acid biodegradation: microbial diversity and metabolism. *Arch. Microbiol.* 172: 131–138.
- Moore, G. E. 1972a. Microflora from the alimentary tract of healthy southern pine beetles, *Dendroctonus frontalis* (Scolytidae), and their possible relationship to pathogenicity. *J. Invertebr. Pathol.* 19: 72–75.
- Moore, G. E. 1972b. Pathogenicity of ten strains of bacteria to larvae of the southern pine beetle [*Dendroctonus frontalis*]. *J. Invertebr. Pathol.* 20: 41–45.
- Moran, N. A., P. Tran, and N. M. Gerardo. 2005. Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterial phylum Bacteroidetes. *Appl. Environ. Microbiol.* 71: 8802–8810.
- Muller, H. E., G. R. Fanning, and D. J. Brenner. 1995a. Isolation of *Ewingella americana* from mollusks. *Curr. Microbiol.* 31: 287–290.
- Muller, H. E., G. R. Fanning, and D. J. Brenner. 1995b. Isolation of *Serratia fonticola* from mollusks. *Syst. Appl. Microbiol.* 18: 279–284.
- Paine, T. D., F. M. Stephen, and H. A. Taha. 1984. Conceptual model of infestation probability based on bark beetle abundance and host tree susceptibility. *Environ. Entomol.* 13: 619–624.
- Paine, T. D., K. F. Raffa, and T. C. Harrington. 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annu. Rev. Entomol.* 42: 179–206.
- Paster, B. J., S. K. Boches, J. L. Galvin, R. E. Ericson, C. N. Lau, V. A. Levanos, A. Sahasrabudhe, and F. E. Dewhirst. 2001. Bacterial diversity in human subgingival plaque. *J. Bact.* 183: 3370–3383.
- Raffa, K. F., and A. A. Berryman. 1987. Interacting selective pressures in conifer-bark beetle systems: a basis for reciprocal adaptations? *Am. Nat.* 129: 234–262.
- Raffa, K. F., T. W. Phillips, and S. M. Salom. 1993. Strategies and mechanisms of host colonization by bark beetles, pp. 102–128. In T. Schowalter and G. Filip (eds.), *Beetle-pathogen interactions in conifer forests*. Academic, San Diego, CA.
- Raffa, K. F., B. H. Aukema, N. Erbilgin, K. D. Klepzig, and K. F. Wallin. 2005. Interactions among conifer terpenoids and bark beetles across multiple levels of scale: an attempt to understand links between population patterns and physiological processes. *Rec. Adv. Phytochem.* 39: 80–118.
- Romme, W. H., D. H. Knight, and J. B. Yavitt. 1986. Mountain pine beetle in the Rocky Mountains: regulators of primary productivity? *Am. Nat.* 127: 484–494.
- Rykiel, E. J., R. N. Coulson, R. N., P.J.H. Sharpe, T.F.H. Allen, and R. O. Flamm. 1988. Disturbance propagation by bark beetles as an episodic landscape phenomenon. *Landscape Ecol.* 1: 129–139.
- Schloss, P. D., and J. Handelsman. 2004. Status of the microbial census *Microbiol. Mol. Biol. Rev.* 68: 686–691.
- Schloss, P. D., and J. Handelsman. 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl. Environ. Microbiol.* 71: 1501–1506.
- Schloss, P. D., I. Delalibera, Jr., J. Handelsman, and K. F. Raffa. 2006. Bacteria associated with the guts of two wood boring beetles: *Anoplophora glabripennis* and *Saperda vestita* (Cerambycidae). *Environ. Entomol.* 35: 625–629.
- Schowalter, T. D. 1981. Insect herbivore relationship to the state of the host plant: biotic regulation of ecosystem nutrient cycling through ecological succession. *Oikos* 37: 126–130.
- Six, D. L., and K. D. Klepzig. 2004. *Dendroctonus* bark beetles as model systems for studies on symbiosis. *Symbiosis* 37: 207–232.
- Steinhaus, E. A. 1959. *Serratia marcescens* Bizio as an insect pathogen. *Hilgardia* 28: 351–380.
- Wallin, K. F., and K. F. Raffa. 2004. Feedback between individual host selection behavior and population dynamics in an eruptive insect herbivore. *Ecol. Monogr.* 74: 101–116.
- Wood, D. L. 1982. The role of pheromones, kairomones, and allomones in the host selection and colonization behavior of bark beetles. *Annu. Rev. Entomol.* 27: 411–446.
- Yu, Z. T., and W. W. Mohn. 1999. Isolation and characterization of thermophilic bacteria capable of degrading dehydroabietic acid. *Can. J. Microbiol.* 45: 513–519.

Received for publication 22 March 2006; accepted 3 October 2006.