Composition of the bacterial community in the gut of the pine engraver, Ips pini (Say) (Coleoptera) colonizing red pine. Symbiosis

ARTICLE *in* SYMBIOSIS · JANUARY 2007

Impact Factor: 1.44

CITATIONS
31

READS
83

7 AUTHORS, INCLUDING:

Italo Delalibera
University of São Paulo
81 PUBLICATIONS **909** CITATIONS
SEE PROFILE

Archana Vasanthakumar
Harvard University
14 PUBLICATIONS **183** CITATIONS
SEE PROFILE

Kier D Klepzig
US Forest Service
81 PUBLICATIONS **2,372** CITATIONS
SEE PROFILE

Kenneth F. Raffa
University of Wisconsin–Madison
332 PUBLICATIONS **9,509** CITATIONS
SEE PROFILE

SYMBIOSIS (2007) 43, 97–104 ©2007 Balaban, Philadelphia/Rehovot ISSN 0334-5114

Composition of the bacterial community in the gut of the pine engraver, *Ips pini* (Say) (Coleoptera) colonizing red pine

Italo Delalibera Jr.[1,2*], Archana Vasanthakumar[1], Benjamin J. Burwitz[1], Patrick D. Schloss[3,5], Kier D. Klepzig[6], Jo Handelsman[3,4], and Kenneth F. Raffa[1]

[1]Department of Entomology, [3]Department of Plant Pathology, and [4]Department of Bacteriology, University of Wisconsin-Madison, Madison, WI 53706, USA;
[2]Department of Entomology, Plant Pathology and Agricultural Zoology, ESALQ-University of São Paulo, Av. Pádua Dias 11, CP 9 Piracicaba-SP 13418-900, Brazil, Tel. +55-19-3429-4260 (ext. 316), Fax. +55-19-3429-4338, Email. italo@esalq.usp.br;
[5]Current address: Department of Microbiology, University of Massachusetts, Amherst, MA 01003, USA;
[6]USDA Forest Service, Southern Research Station, Pineville, LA 71360, USA

(Received February 10, 2007; Accepted April 9, 2007)

Abstract
The gut bacterial community of a bark beetle, the pine engraver *Ips pini* (Say), was characterized using culture-dependent and culture-independent methods. Bacteria from individual guts of larvae, pupae and adults were cultured and DNA was extracted from samples of pooled larval guts. Analysis of 16S rRNA gene sequences amplified directly from the gut community suggests that the gut bacterial communities associated with *I. pini* are relatively simple, compared to many other systems. Six bacterial genera from four classes were detected by culturing gut bacteria from larvae, pupae and adults. Two genera, *Pantoea* and *Stenotrophomonas* (γ-Proteobacteria) were found in all life stages of *I. pini*, consistently in larvae and adults, and less commonly in pupae. Sequences that affiliate with the Enterobactereaceae of the γ-Proteobacteria were found in 95% of the clones sampled. The Enterobactereaceae genera, *Pantoea* and *Erwinia,* accounted for 88% of all clone sequences. These results are consistent with previous work indicating that another bark beetle, the southern pine beetle, *Dendroctonus frontalis* Zimmerman, also has a relatively simple gut flora, compared with wood colonizing insects such as wood borers and termites. The composition and abundance of bacteria associated with different life stages of *I. pini* are possibly associated with specific functions of the gut bacterial communities of larvae, pupae, and adults.

Keywords: Bark beetle, gut microbiota, 16S ribosomal RNA, rarefaction analysis

1. Introduction

Bark beetles (Coleoptera: Curculionidae: Scolytinae; altern. Scolytidae) have a variety of relationships with symbiotic microorganisms (Paine et al., 1997). Some microorganisms serve as food for larvae (Six and Klepzig, 2004), some assist in digestion, and some increase nitrogen concentration in phloem surrounding the larvae (Ayres et al., 2000). Symbionts also detoxify plant compounds (Dowd, 1992) and assist in overcoming host defenses (Krokene and Solheim, 1998). Some symbionts produce compounds that are used as aggregation pheromones by bark beetles (Brand et al., 1975), whereas others produce volatile metabolites that can be exploited by natural enemies of bark beetles (Sullivan and Berisford, 2004).

Despite the large body of knowledge on microbial symbionts of bark beetles, most of this information concerns external and mycangial fungi. In contrast, relatively little is known about the composition and function of the gut microbiota, especially bacteria. Moreover, most previous studies on the diversity of gut microbiota of bark beetles relied on culture dependent techniques and microscopic observations. Those studies revealed nitrogen-fixing bacteria (Bridges, 1981), fungi such as the blue-stain fungus, *Ophiostoma ips* (Rumbold) Nannfeldt (Furniss et al., 1995) and yeasts (Moore, 1972; Brand et al., 1977). More recently, gut bacteria of the southern pine beetle, *Dendroctonus frontalis* Zimmermann (Vasanthakumar et al., 2006), and bacteria in oral egestions

*The author to whom correspondence should be sent.

of the spruce beetle, *Dendroctonus rufipennis* (Kirby) were characterized using both culture dependent and culture independent methods (Cardoza et al., 2006).

Knowledge of bacterial species composition facilitates studies of the functions of the gut microbiota. In addition, defining interactions among various members of the gut community can lead to the development of novel pest management strategies (Broderick et al., 2000). The aim of this study was to characterize the gut bacterial communities throughout the life cycle of the pine engraver, *Ips pini* (Say), using classical microbiological methods and culture-independent molecular techniques.

2. Materials and Methods

Source of insects

All *I. pini* were reared from a laboratory colony at the University of Wisconsin at Madison, WI, USA that was maintained on red pine, *Pinus resinosa* Ait. harvested from the field, and continuously replenished with wild insects, using the method of Raffa and Dahlsten (1995). Logs were stripped of their bark, and beetles were extracted directly from the galleries. Late instar larvae, pupae at late stage (close to eclosion, so that the gut could be easily removed) and teneral adults were analyzed.

Dissection of insect guts

Immediately following extraction from the logs, insects were surface sterilized in 70% ethanol for 1 min. After sterilization the insects were rinsed in 10 mM sterilized phosphate buffered saline (PBS) prior to dissection. To check the quality control of surface sterilization some specimens were placed in an Eppendorf tube with the sterile PBS buffer and then removed and rolled on plates with GYP medium (20 g/liter glucose, 10 g/liter peptone, 5 g/liter yeast extract, 1 g/liter $MgSO_4 \times 7H_2O$, 15 g agar, pH 6.9). The PBS used to wash the insects was also plated on GYP medium. Insects were dissected inside a sterile laminar flow hood using dissection scissors and fine-tipped forceps according to the method described by Vasanthakumar et al. (2006). The head and last abdominal segment of each larva and pupa were severed, and pressure was applied anterior to the crop to release the gut. The thorax of each adult beetle was held with forceps, and the head was pulled away until the entire gut was stretched out of the insect body but still attached. The gut was then separated from the body by cutting its extremities in a drop of sterilized PBS. Guts were washed in PBS and either pooled or transferred individually to 1.5-ml microfuge tubes containing 50 µl, 100 µl, or 500 µl 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. The larval gut contents used for DNA extraction were filtered using 12-µm polycarbonate syringe filters (GE Osmonics Inc., Trevose, PA) to eliminate the insect tissues and undigested food.

Culturing bacteria

Bacteria were cultured from individual guts of five larvae, five pupae and seven adults. Each larval gut was placed in a microfuge tube containing 100 µl 10 mM PBS. Pupal and adult guts were placed in 500 µl PBS.

Eight serial ten-fold dilutions were spread on duplicate plates of one-tenth strength tryptic soy agar (TSA, 3 g/liter tryptic soy broth, Difco Laboratories, 15 g/liter agar, pH 7.0) and CMC agar (5 g/liter carboxymethyl-cellulose (CMC), 12 g/liter agar and 0.2 g /liter yeast extract). Plates were incubated in a growth chamber at 28ºC. Bacterial colonies were categorized based on morphology (shape, elevation, surface, size, opacity, pigmentation, etc.) and microscopic observations and then counted across all plates on the three lowest countable dilutions. Based on the number of colonies per dilution plate, the volume of PBS used to mix the gut and the volume plated on the serial dilution plates, the mean number of colonies was converted to cfu/gut. Pure cultures of bacterial isolates were obtained by single colony isolation and used in further analyses. DNA was extracted from samples of each bacterial type and then sequenced to confirm the identity of the bacteria.

Isolated microorganisms were tested for the ability to degrade CMC. Plates were treated with Congo red to confirm presence or absence of endoglucanase activity accordingly to Teather and Wood (1982). Voucher specimens of bacterial colonies were preserved in the forest entomology laboratory in the Department of Entomology of the University of Wisconsin-Madison. Bacterial densities between larvae and adult stages were compared by Student's *t*-test.

DNA extraction from pooled gut extracts and cultured bacteria

Three pooled gut samples from *I. pini* larvae, named IPSL8, IPSL15 and IPSL16, were used in the culture-independent analysis. IPSL8 consisted of pooled guts from 10 larvae dissected and extracted in October 2002, and IPSL15 and IPSL16 contained 20 and 30 pooled guts, respectively, extracted in September 2003. The tubes were centrifuged at low speed to remove the insect tissue, and DNA was extracted from the bacteria and other microorganisms in the supernatant. Microbial DNA from IPSL8 was extracted using 200 µl of InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's directions. Briefly, 200 µl of matrix was combined with gut contents, incubated at 56°C for 1 h, and

vortexed at high speed for 10 s. The mixture was heated in a boiling water bath for 10 min and then centrifuged at high speed to separate the matrix from the gut contents. Twenty microliters of the resulting DNA was used in a 50-µl PCR reaction to constructed a 16S rRNA gene library. DNA from cells isolated from samples IPSL15 and IPSL16 was extracted using the FastDNA SPIN kit for soil (Qbiogene, Inc., Carlsbad, CA) according to the manufacturer's directions, which involves mechanical lysis by bead beating followed by purification of DNA on a silica matrix.

For DNA extraction from cultured bacteria, 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 28ºC for two days. DNA was extracted using a modified CTAB protocol described previously (Broderick et al., 2004). Cell suspensions were lysed using chemical detergents and Proteinase K (Promega Corp. Inc, Madison, WI). DNA was then isolated using phenol-chloroform extractions and isopropanol precipitation.

PCR amplification and construction of 16S rRNA gene libraries

16S rRNA gene libraries were constructed from total DNA isolated from pooled larval gut extracts. General primers for bacteria 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') (Lane, 1991) were used to amplify 16S rRNA genes from DNA extracted from individual cultured bacterial colonies, and from total DNA isolated from gut extracts, as in Broderick et al. (2004). Final concentrations for 50-µl PCR reactions were as follows: 2 µl diluted DNA (10–100 ng) (or 20 µl for DNA extracted using InstaGene™ Matrix), 0.2 µM of each primer, 0.2 mM dNTPs, 5 units of *Taq* polymerase and 1X *Taq* polymerase buffer (50 mM KCl, 10 mM Tris-HCl, [pH 9.0], 0.1% Triton X-100 and 1.5 mM $MgCl_2$, Promega Corp., Madison, WI, USA). The reaction conditions were: 94°C for 3 min, 35 cycles at 94°C for 30 s, 55°C for 1.5 min, and 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 Corp., Beverly, MA) or QIAquick® PCR purification Kit (Qiagen Inc., Valencia, CA), and cloned into a pGEM-T vector (Promega Corp., Madison, WI) according to the manufacturer's directions. 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 µl of lysis buffer (50 mM NaOH, 0.25% sodium dodecyl sulfate) in a 96-well plate and heating at 95°C for 15 min. Lysates were diluted 10-fold in sterile water and used as DNA template for PCR amplification of the insert using M13 vector primers. PCR products were purified using AMPure magnetic beads (Agencourt BioScience Corp., Beverly, MA).

Sequencing 16S rRNA genes from cultured bacterial isolates and clone libraries of pooled gut extracts

Cultured bacterial isolates were selected for sequencing of their 16S rRNA genes based on distinct ARDRA (amplified ribosomal DNA restriction analysis) restriction patterns. At least two bacterial isolates representative of each colony morphology were chosen for these analyses. PCR products (9 µl) were digested independently with the restriction enzymes *Msp*I and *Taq*I (Promega) for 2 h according to the manufacturer`s specifications. The restriction PCR fragments were separated by electrophoresis in agarose (a mixture of 1.75% low melting agarose and 0.5% PCR grade agarose) in 1X Tris-borate-EDTA buffer at 4 V/cm. The gels were stained with ethidium bromide and visualized under UV light. The ARDRA restriction patterns of isolates were compared visually and grouped. At least one cultured bacterial isolate from each distinct ARDRA pattern was selected for sequencing.

16S rRNA genes from cultured bacterial isolates were sequenced using 16S rRNA gene primers 27F, 704F (5'-GTAGCGGTGAAATGCGTAGA-3'), 787R (5'-CTACCAGGGTATCTAAT-3'), or 1492R (Lane, 1991). Sequencing reactions were performed in a total volume of 15 µl consisting of 0.5 µl DNA, 0.37 µl (10 µM) of primer, 1.0 or 1.1 µl BigDye (Perkin-Elmer Corp., Wellesley, MA), 3 µL 5x buffer (Perkin-Elmer Corp., Wellesley, MA), 0.75 µL DMSO and 9.28 or 9.38 µl nuclease-free water. The reaction conditions were: 95°C for 3 min, 50 cycles at 96°C for 20s, 46°C for 30s, and 60°C for 2 min, and a final extension at 72°C for 7 min. Clones containing full-length inserts (ca. 1,500 bp) were sequenced using vector primers SP6 and T7 and 16S rRNA gene primers 704F and 787R as in Broderick et al. (2004). Sequencing reactions were performed using BigDye (Perkin-Elmer Corp., Wellesley, MA). Sequenced products were purified either with Sephadex G-50 columns (Pharmacia Biotech, Piscataway, NJ) or CleanSEQ™ magnetic beads (Agencourt Bioscience Corp., Beverly, MA). Sequences were determined on an ABI 377 DNA sequencer (Applied Biosystems., Foster City, CA) at the University of Wisconsin-Madison Biotechnology Center.

Sequence analyses of 16S rRNA genes

Compiled 16S rRNA gene sequences (SeqMan, DNASTAR Inc., Madison, WI) were tested for chimeric structures by using RDP Check_Chimera (Cole et al., 2005) and Bellepheron (Huber et al., 2004). Nonchimeric sequences were aligned and analyzed with the ARB software package (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.

Table 1. Taxonomic affiliation and population density of culturable bacteria from guts of larvae, pupae and adults of *Ips pini*.

Bacterial taxon[a]		Larvae (N[b]=5) (cfu/gut[d])		Pupae (N=5)		Adults (N=7) (cfu/gut[d])	
	%[c] Guts	Mean	Range	% Guts	% Guts	Mean	Range
γ-Proteobacteria							
Pantoea sp.	100	3.6×10^5	$5.3\times10^3 - 1.7\times10^6$	40	71.4	3.9×10^4	$<50 - 2.7\times10^5$
Stenotrophomonas sp.	100	9.0×10^5	$3.9\times10^3 - 4.4\times10^6$	20	100	2.2×10^4	$50 - 8.5\times10^4$
Pseudomonas sp.	0	<100		20	0	<50	
Actinobacteria							
Frigoribacterium sp.	0	<100		20	0	<50	
Bacteroidetes							
Chryseobacterium sp.	40	2.9×10^4	$<100 - 1.2\times10^5$	0	0	<50	
Firmicutes							
Bacillus sp.	0	<100		0	57.1	105	(<50–575)

[a]Bacteria were cultured on tryptic soy agar (TSA) and identified based on their 16S rRNA gene sequences. [b]N = number of single gut samples analyzed. [c]Percentage of insects containing bacterial taxa. [d]Mean colony forming units (cfu) per larvae or adult gut samples. Very low densities of bacteria were detected from guts of pupae, so densities of bacteria were not estimated for this life stage. Detection limits for larvae, adults and pupae were 100, 50 and 5 cfu/gut, respectively.

Table 2. Composition of gut bacterial communities of pooled gut samples from *Ips pini* larvae based on culture-independent 16S rRNA gene sequence analysis.

Bacterial taxon[a]	# of representatives in libraries:		
	IPSL8	IPSL15	IPSL16
γ-Proteobacteria			
Pantoea sp.	9	4	14
Stenotrophomonas sp.	2	0	3
Erwinia sp.	10	27	0
α-Proteobacteria			
Ochrobacter sp.	1	0	0
Firmicutes			
Streptococcus mitis	0	0	3
Total # of clones sequenced in each library	22	31	20

[a]Sequences were affiliated with taxa using the ARB database phylogenetic tree.

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 DOTUR to calculate Operational Taxonomic Units (OTUs) and to construct rarefaction curves (= the total number of OTUs versus total number of sequences sampled) at distance levels of 20, 10, 5, 3 and 1% (Schloss and Handelsman, 2005). These arbitrary cut-offs are used to represent phylum, class, genus, species and strain levels, respectively (Schloss and Handelsman, 2004). Rarefaction analyses are useful in gauging adequacy of sampling. The Chao1 richness estimator was also calculated in DOTUR (Schloss and Handelsman, 2005). This nonparametric index estimates diversity of a community based on the number of singletons (OTUs represented by only one sequence) and doubletons (OTUs represented by two sequences) in a sample (Bohannan and Hughes, 2003). Terminal Chao1 richness estimates are reported for 5% difference between sequences.

Nucleotide sequence accession numbers

Nucleotide sequences were deposited in GenBank under the accession numbers DQ836634 to DQ836713.

3. Results

Analysis by culture-dependent methods

I. pini larval gut communities were dominated by four phyla: Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes. Six bacterial genera from these phyla were detected by culturing gut bacteria from larvae (3 taxa), pupae (4 taxa) and adults (3 taxa) of *I. pini* (Table 1). Four of these genera (*Bacillus* sp., *Chryseobacterium* sp., *Frigoribacterium* sp. and *Pseudomonas* sp.) were detected in only one life stage, whereas *Pantoea* sp. and *Stenotrophomonas* sp. (γ-Proteobacteria) were found across all life stages of *I. pini*. They were consistently found in larvae and adults, and commonly found in pupae.

I. pini larval guts were reddish-brown and microscopic observations of the gut content indicate the presence of

cellulose fibers. Both pupal and adult guts were milky white in color, indicating a lack of cellulose. Except for *Stenotrophomonas* sp., there was poor growth on CMC (carboxymethyl-cellulose) medium. Therefore, estimates of bacterial population density are presented for TSA medium only. The densities of bacteria were highly variable among insect samples of the same life stage. Very few bacteria were observed in only a few dilution plates from guts of pupae, so densities of bacteria were not estimated in this stage. Detection limits for bacteria in the guts of larvae, adults and pupae were 100, 50 and 5 bacterial colony-forming units (cfu) per gut, respectively. The total bacterial population size per adult gut ($6.2\times10^4 \pm 5.1\times10^4$, mean cfu ± SE) was not significantly different from that of the larval gut ($1.3\times10^6 \pm 1.2\times10^6$) (T=1.0, P=0.37). The densities of *Pantoea* sp. and *Stenotrophomonas* sp. were $3.6\times10^5 \pm 3.3\times10^5$ and $9\times10^5 \pm 8.7\times10^5$ per gut, respectively, in larvae, and $3.9\times10^4 \pm 3.9\times10^4$ and $2.2\times10^4 \pm 1.4\times10^4$ in adults. There were no consistent differences in bacterial densities between these life stages (T=0.95, P=0.4 for *Pantoea* sp. and T=1.01, P=0.37, for *Stenotrophomonas* sp.). The population densities of *Chryseobacterium* sp. and *Bacillus* sp. were lower than those of other bacteria found in the larval and adult stages, respectively.

Culture-independent 16S rRNA gene sequence analysis

Proteobacteria and Firmicutes were the only phyla found among 73 16S rRNA gene sequences in clone libraries constructed from three different gut samples of *I. pini* larvae. Sequences that affiliate with the Enterobactereaceae of the γ-Proteobacteria were found in 94% of the clones sampled. Sequence alignments in ARB revealed that five bacterial taxa, *Pantoea* sp., *Stenotrophomonas* sp., *Erwinia* sp., *Ochrobacter* sp. and *Streptococcus mitis*, were associated with larval guts (Table 2). All three libraries contained sequences that clustered with *Pantoea*. Two out of the three libraries contained clones that clustered with *Erwinia*. These two closely related genera accounted for 87% of all clones sequenced. Nine percent and 15% of the sequences from two clone libraries clustered with *Stenotrophomonas* sp. Two other taxa, *Ochrobacter* sp. and *Streptococcus mitis*, were only detected in a single clone library each, comprising only 5% and 15% of the library in which it appeared.

Rarefaction analyses demonstrated that the gut bacterial communities associated with *I. pini* are relatively simple. The rarefaction curves for two libraries, IPSL15 and IPSL16, leveled off at 5% difference between sequences, while the curve for IPSL8 continued to rise but with a relatively low slope (Fig. 1). To determine whether these three libraries had been sufficiently sampled, the nonparametric richness estimator, Chao 1, was calculated. The terminal Chao1 richness estimates for the three larval libraries were 5, 4 and 3 OTUs (IPSL8, IPSL15 and IPSL16, respectively), where an OTU was defined as a group of sequences differing by no more than 5%, which is roughly equivalent to a genus cut-off. The collector's curves for the Chao1 estimator at 5% difference between sequences had leveled off, indicating that further sampling would be unlikely to reveal more genera (data not shown).

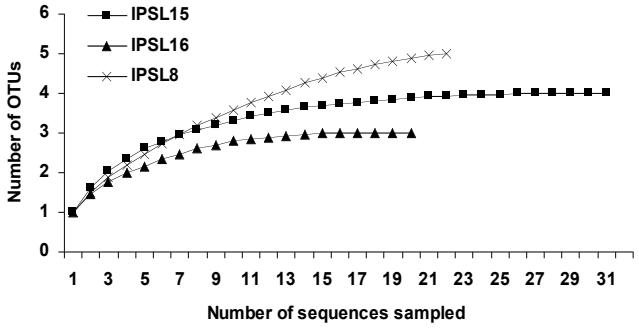

Figure 1. Rarefaction analyses of 16S rRNA gene libraries constructed from larval guts of *Ips pini*. IPSL8, IPSL15 and IPSL16 are pooled larval gut samples containing 10, 20 and 30 larval guts, respectively. Rarefaction curves were constructed based on analyses performed in DOTUR using the furthest neighbor assignment algorithm. Rarefaction curves represent 95% sequence similarity among sequences.

4. Discussion

The overall species richness of bacteria in the guts of all life stages of *I. pini* was relatively low and the species all affiliate with known genera. Similarly, Moore (1972) cultured only eight bacteria, seven fungi and one yeast from another beetle species, *D. frontalis*, and never more than five species from a single insect. Rarefaction analyses based on culture-independent methods likewise suggest that the species richness in *D. frontalis* is relatively low (Vasanthakumar, 2006). By comparison, the midgut microbiota of larvae of the wood-boring beetle, *Anoplophora glabripennis* (Motschulsky), consists of 23 phylotypes that affiliate with α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Firmicutes, Actino-bacteria, and Bacteroidetes (Schloss et al., 2006). The gut microbial community of termites, another wood colonizing group of insects, is also more complex than the community reported here, represented by 19 bacterial phyla in which most of the sequences had less than 90% 16S rRNA gene sequence identity with known species (Ohkuma and Kudo, 1996; Hongoh et al., 2005).

Certain bacterial taxa appear to be widespread in the guts of bark and wood-boring beetles. *Stenotrophomonas* sp., *Pantoea* sp. and *Bacillus* sp., the most common bacteria found in *I. pini,* were also found in *D. frontalis*. However, *Pantoea* sp. and *Bacillus* sp. were only found in *D. frontalis*

larvae and *Stenotrophomonas* sp. only in *D. frontalis* adults. *Pantoea agglomerans* has been found in larvae of the wood-boring beetle, *Saperda vestita* (Delalibera et al., 2005). *Stenotrophomonas maltophilia*, *Chryseobacterium* and *Pseudomonas putida* were detected in *S. vestita* adults.

Changes in diversity and abundance of gut bacteria across life stages have been studied in very few holometabolous insects. Here, we demonstrated that *Pantoea* and *Stenotrophomonas* were commonly found across all life stages of *I. pini*, suggesting that these bacteria can be maintained during metamorphosis. However, there are pronounced declines during the pupal stage. Transtadial transmission was also demonstrated for *Phlebotomus duboscqi* Neveu-Lemaire (Diptera: Psychodidae) (Volf et al., 2002) and three *Anopheles* species (Pumpuni et al., 1996). Nardi et al. (2006) observed that many of the cuticular features of the hindgut epithelium morphology of the wood feeding beetle, *Odontotaenius disjunctus* (Illiger) are conserved during the transition from larva to adult. These conserved anatomical regions could likely retain bacteria during metamorphosis. The housefly, *Musca domestica* L., and the sugar beet root maggot, *Tetanops myopaeformis* (von Roder) also shed the cuticular lining of their guts during pupation, displacing the majority of the bacteria within the pupa to the inner surface of the pupal case (Greenberg, 1959).

Stenotrophomonas sp. was the only bacterium able to grow vigorously in CMC medium (carboxymethyl-cellulose agar). However, we found no evidence that this bacterium was able to degrade CMC. The absence of cellulolytic activity across all types of bacteria isolated from *I. pini* (Delalibera et al., 2005) may reflect the feeding substrate of this insect. In contrast to beetles that feed mostly on sapwood in addition to bark and phloem and harbor cellulolytic bacteria (e.g. *Saperda vestita*), *I. pini* feeds mostly on phloem contents and fungi, and also bark found near the surface of the tree using different strategies to increase nutrient acquisition (Delalibera et al., 2005).

Species characterization, even though based on a limited sample size, is a first step in understanding the functions and interactions of the gut microbial community of *I. pini*. Several associations with particular *I. pini* life stages are consistent with known functions of related bacteria. For example, *Bacillus cereus,* present in *Ips paraconfusus* Lanier guts, can convert host α-pinene into *cis*-verbenol, an adult aggregation pheromone (Brand et al., 1975). Interestingly, we found *Bacillus* only in *I. pini* adults, the stage that synthesizes pheromone. Conversely, *Pantoea* spp. are known to fix nitrogen in *D. frontalis* (Behar et al., 2005) and *Pantoea* spp. were present in all stages of *I. pini*. Additionally, oral secretions of adult *D. rufipennis* contain Actinobacteria, Firmicutes, Gammaproteobacteria and Betaproteobacteria, including *Pseudomonas* and *Bacillus,* that inhibit antagonistic fungi invading beetle galleries (Cardoza et al., 2006), and these were distributed across adult and larval *I. pini,* but more prevalent in adults.

16S rRNA gene clone libraries have often revealed a much greater diversity of microbes than cultivation-based techniques in environments such as seawater, freshwater, and soil (Ward et al., 1990; Rappé and Giovannoni, 2003; Handelsman, 2004). Identification of the dominant members of the bacterial communities of *I. pini* larvae revealed similar numbers of bacterial taxa by culture-dependent and culture-independent methods. This is similar to results from *D. frontalis* (Vasanthakumar et al., 2006), and to a lesser extent with a lepidopteran defoliator, the gypsy moth, *Lymantria dispar* (L.), in which 77% of morphotypes were culturable (Broderick et al., 2004). Differences between results from culturing and culture-independent techniques can be caused by DNA extraction, PCR amplification, cloning or by culturing methods (e.g. bacteria were only cultivated under aerobic conditions).

The broad geographic distribution and host range of *I. pini* have made this insect a model for biogeographic approaches to chemical ecology (Cognato et al., 1999), and could likewise serve a parallel purpose for analyses of gut microbiota. In particular, the differences we observed among life stages can provide a framework for studies of microbiota functionality across the entire life history of an insect host. Further research is needed to quantify sources of variation in the gut microbiota of *I. pini*, evaluate the stability of the microbial community, and determine the various functions bacteria may play in the host's biology.

Acknowledgements

We appreciate technical support by Egle Cepaitis, UW-Madison Dept. Entomol. 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, and by the UW-Madison College of Agricultural and Life Sciences and McIntire-Stennis WIS 04529.

REFERENCES

Ayres, M.P., Wilkens, R.T., Ruel, J.J., Lombardero, M.J., and Vallery, E. 2000. Nitrogen budgets of phloem-feeding bark beetles with and without symbiotic fungi. *Ecology* **81**: 2198–2210.

Behar, A., Yuval, B., and Jurkevitch, E. 2005. Enterobacteria-mediated nitrogen fixation in natural populations of the fruit fly *Ceratitis capitata*. *Molecular Ecology* **14**: 2637–2643.

Bohannan, B.J.M. and Hughes, J. 2003. New approaches to analyzing microbial biodiversity data. *Current Opinion in Microbiology* **6**: 282–287.

Brand, J.M., Bracke, J.W., Markovetz, A.J., Wood, D.L., and Browne, L.E. 1975. Production of verbenol pheromone by a bacterium isolated from bark beetles. *Nature* **254**: 136–137.

Brand, J.M., Schultz, J., Barras, S.J., Edson, L.J., Payne, T.L., and Hedden, R.L. 1977. Bark-beetle pheromones: Enhancement of *Dendroctonus frontalis* (Coleoptera: Scolytidae) aggregation pheromone by yeast metabolites in laboratory bioassays. *Journal of Chemical Ecology* **3**: 657–666.

Bridges, J.R. 1981. Nitrogen-fixing bacteria associated with bark beetles. *Microbial Ecology* **7**: 131–137.

Broderick, N.A., Goodman, R.M., Raffa, K.F., and Handelsman, J. 2000. Synergy between Zwittermicin A and *Bacillus thuringiensis* subsp. *kurstaki* against gypsy moth (Lepidoptera: Lymantriidae). *Environmental Entomology* **29**: 101–107.

Broderick, N.A., Raffa, K.F., Goodman, R.M., and Handelsman, J. 2004. Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Applied and Environment Microbiology* **70**: 293–300.

Cardoza, Y.J., Klepzig, K.D., and Raffa, K.F. 2006. Bacteria in oral secretions of an endophytic insect inhibit antagonistic fungi. *Ecological Entomology* **31**: 636–645.

Cognato, A.I., Seybold, S.J., and Sperling, F.A.H. 1999. Incomplete barriers to mitochondrial gene flow between pheromone races of the North American pine engraver, *Ips pini* (Say) (Coleoptera : Scolytidae). *Proceedings Royal Society London Series Biological Sciences* **266**: 1843–1850.

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McGarrell, D.M., Garrity, G.M., and Tiedje, J.M. 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Research* **33**: 294–296.

Delalibera Jr., I., Handelsman, J., and Raffa, K.F. 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). *Environmental Entomology* **34**: 541–547.

Dowd, P.F. 1992. Insect fungal symbionts: A promising source of detoxifying enzymes. *Journal of Industrial Microbiology* **9**: 149–161.

Furniss, M.M., Harvey, A.E., and Solheim, H. 1995. Transmission of *Ophiostoma ips* (Ophiostomatales: Ophiostomataceae) by *Ips pini* (Coleoptera: Scolytidae) to ponderosa pine in Idaho. *Annals of the Entomological Society of America* **88**: 653–660.

Greenberg, B. 1959. Persistence of bacteria in the developmental stages of the housefly. III. Quantitative distribution in prepupae and pupae. *American Journal of Tropical Medicine and Hygiene* **8**: 613–617.

Handelsman, J. 2004. Metagenomics: Application of genomics to uncultured microorganisms. *Microbiology and Molecular Biology Reviews* **68**: 669–684.

Hongoh, Y, Deevong, P., and Inoue, T. 2005. Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. *Applied and Environment Microbiology* **71**: 6590–6599.

Huber, T., Faulkner, G., and Hugenholtz, P. 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* **20**: 2317–2319.

Krokene, P. and Solheim, H.1998. Pathogenicity of four blue-stain fungi associated with aggressive and nonaggressive bark beetles. *Phytopathology* **88**: 39–44.

Lane, D.J. 1991. 16S/23S rRNA sequencing. In: *Nucleic Acid Techniques in Bacterial Systematics*. Stackebrandt, E. and Goodfellow, M., ed. John Wiley & Sons, Inc., New York, NY, pp. 115–175.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N. , Vilbig, A., Lenke, M., Ludwig, T., Bode, A., and Schleifer, K.H. 2004. ARB: a software environment for sequence data. *Nucleic Acids Research* **32**: 1363–1371.

Moore, G.E. 1972. Microflora from the alimentary tract of healthy southern pine beetles, *Dendroctonus frontalis* (Scolytidae), and their possible relationship to pathogenicity. *Journal of Invertebrate Pathology* **19**: 72–75.

Nardi, J.B., Bee, C.M., Miller, L.A., Nguyen, N.H., Suh, S., and Blackwell, M. 2006. Communities of microbes that inhabit the changing hindgut landscape of a subsocial beetle. *Arthropod Structure and Development* **35**: 57–68.

Ohkuma, M. and Kudo, T. 1996. Phylogenetic diversity of the intestinal bacterial community in the termite *Reticulitermes speratus*. *Applied and Environment Microbiology* **62**: 461–468.

Paine, T.D., Raffa, K.F., and Harrington, T.C. 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* **42**: 179–206.

Pumpuni, C.B., DeMaio, J., Kent, M.J., Davis, R., and Beier, J.C. 1996. Bacterial population dynamics in three anopheline species: impact on *Plasmodium* sporogonic development. *American Journal of Tropical Medicine and Hygiene* **54**: 214–218.

Raffa, K.F. and Dahlsten, D.L. 1995. Differential responses among natural enemies and prey to bark beetle pheromones. *Oecologia* **102**: 17–23.

Rappé, M.S. and Giovannoni, S.J. 2003. The uncultured microbial majority. *Annual Review of Microbiology* **57**: 369–94.

Schloss, P.D. and Handelsman, J. 2004. Status of the microbial census. *Microbiology and Molecular Biology Reviews* **68**: 686–691.

Schloss, P.D. and Handelsman, J. 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Applied and Environment Microbiology* **71**: 1501–1506.

Schloss, P.D., Delalibera Jr., I., Handelsman, J., and Raffa, K.F. 2006. Bacteria associated with the guts of two wood boring beetles: *Anoplophora glabripennis* and *Saperda vestita* (Cerambycidae). *Environmental Entomology* **35**: 625–629.

Six, D.L. and Klepzig, K.D. 2004. *Dendroctonus* bark beetles as model systems for studies on symbiosis. *Symbiosis* **37**: 207–232.

Sullivan, B.T. and Berisford, C.W. 2004. Semiochemicals from fungal associates of bark beetles may mediate host location behavior of parasitoids. *Journal of Chemical Ecology* **30**: 703–717.

Teather, R.M. and Wood, P.J. 1982. Use of Congo redpolysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Applied and Environment Microbiology* **43**: 777–780.

Vasanthakumar, A., Delalibera Jr., I., Handelsman, J., Klepzig, K.D., Schloss, P.D., and Raffa, K.F. 2006. Characterization of gut-associated bacteria in larvae and adults of the southern pine beetle, *Dendroctonus frontalis* Zimmermann. *Environmental Entomology* **35**: 1710–1717.

Volf, P., Kiewegova, A., and Nemec, A. 2002. Bacterial colonization in the gut of *Phlebotomus duboscqi* (Diptera:

Psychodidae): transtadial passage and the role of female diet. *Folia Parasitologica* **49**: 73–77.

Ward, D.M., Weller, R., and Bateson, M.M. 1990. 16S rRNA reveal numerous uncultured microorganisms in a natural community. *Nature* **345**: 63–65.

Wright, S.A.I., Zumoff, C.H., Schneider, L., and Beer, S.V. 2001. *Pantoea agglomerans* strain EH318 produces two antibiotics that inhibit *Erwinia amylovora in vitro*. *Applied and Environment Microbiology* **67**: 284–292.

Zhang, L. and Birch, R.G. 1997. The gene for albicidin detoxification from *Pantoea dispersa* encodes an esterase and attenuates pathogenicity of *Xanthomonas albilineans* to sugarcane. *Plant Biology* **94**: 9984–9989.