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)

ITALO DELALIBERA, JR.,[1, 2] JO HANDELSMAN,[3] AND KENNETH F. RAFFA[1]

Environ. Entomol. 34(3): 541–547 (2005)

ABSTRACT The goal of this study was to characterize the cellulose-degrading microorganisms in the guts of wood-inhabiting beetles. We enriched for cellulose-degrading microorganisms by inoculating filter paper in liquid growth medium with macerated guts from larvae and adults of the wood-boring longhorned beetle, *Saperda vestita*, and the phloeophagous bark beetles, *Ips pini* and *Dendroctonus frontalis*. After incubation, microorganisms were isolated in pure culture and tested for their ability to digest carboxymethylcellulose (CMC). Isolates were identified based on their ribosomal RNA gene or intergenic region sequences. Bacteria and fungi from the gut of *S. vestita* degraded filter paper, whereas the microorganisms from the phloeophagous insects did not. The only bacterium detected that was able to degrade CMC is closely related to *Sphingobium yanoikuyae*, a member of the α-Proteobacteria class. We found this species in all field-collected *S. vestita* larvae in 2002 and 2003. Population densities of cellulolytic bacteria in *S. vestita* ranged from 2.4×10^5 to 3.6×10^6 CFU/gut. Bacteria isolated from the phloeophagous beetles *I. pini* and *D. frontalis* did not degrade CMC. Two fungi isolated from the gut of *S. vestita* adults had strong degradative activity. Sequences of the ITS1, 5.8S, and ITS2 rRNA regions indicated that these fungi are highly similar to *Fusarium culmorum* and *Penicillium crustosum*, respectively. This study provides the first description of the gut microbial community of *S. vestita* and the first documentation of association between cellulolytic microorganisms and a wood-boring beetle.

KEY WORDS cellulose degradation, longhorned beetles, bark beetles, microbial diversity

CELLULOSE IS A COMPLEX CARBOHYDRATE polymer, and its complete degradation requires a set of enzymes that act synergistically. In insects, cellulose digestion is accomplished by a complex of three types of enzymes: C_1-cellulases, C_x-cellulases, and cellobiases (Martin 1983). Many insects synthesize their own C_x-cellulases and cellobiases, but few can synthesize C_1-cellulases. Several insect cellulases have been purified and characterized (Genta et al. 2003). A diverse assemblage of protozoan, fungal, and bacterial species living in the guts of insects produces cellulases. These microbes play important roles in cellulose degradation and also enable some insects to exploit cellulose-rich substrates that might otherwise be unsuitable because of low levels of nitrogen and other important nutrients. Many aerobes and facultative anaerobes that degrade cellulose have been isolated from termites (Breznak and Brune 1994, Varma et al. 1994) and cockroaches (Cruden and Markovetz 1979).

Two diverse groups of endophytic insects are the longhorned beetles, which bore in the sapwood and phloem, and the bark beetles, which bore in the phloem, of woody plants. Many play important roles in ecosystem processes, ranging from nutrient cycling to fire cycles and succession (Schowalter and Filip 1993, Hanks 1999). Some longhorned beetles cause substantial economic losses to commercial forests, wood products, and landscape ornamentals, and bark beetles are generally recognized as the most damaging insects affecting North American forests (Schowalter and Filip 1993). Because of their cryptic habits, and in some cases, slow development time, this group is especially prone to accidental introduction into new regions, where they can be very damaging (Hoebeke 1994, Haack and Poland 2001). Some recent examples include the Asian longhorned beetle, pine shoot beetle, and banded elm beetle in the United States, the brown spruce longhorned beetle in Canada, the eastern five-spined ips in Australia, and the red turpentine beetle, *Dendroctonous valens* (LeConte), in China. Moreover, the environmental and economic injuries incurred by indigenous species are likely to intensify

[1] Department of Entomology, University of Wisconsin, Madison, WI 53706.

[2] Corresponding author: Departamento de Entomologia Fitopatologia e Zoologia Agrícola, ESALQ-USP, CP 9, Piracicaba-SP 13418-900, Brazil (e-mail: id24@cornell.edu).

[3] Department of Plant Pathology, University of Wisconsin, Madison, WI 53706.

0046-225X/05/0541–0547$04.00/0

with global atmospheric warming (Logan and Powell 2001).

Despite the economic and ecological importance of beetles in general, and wood-boring beetles in particular, little is known about the diversity of their gut microbiota. In addition, nothing is known about the potential role of gut microorganisms in cellulose digestion by wood-boring beetles. Therefore, the guts of wood-boring beetles represent an unexplored habitat for the discovery of new microbes and new insect-microorganism symbioses. Given the nutrient-poor composition of the sapwood and phloem substrates, it seems likely that microbes provide nutritional supplements that benefit their hosts.

The purpose of this study was to isolate, identify, and quantify cellulolytic microbes from the guts of the endophytic beetles, the pine engraver, *Ips pini* (Say), the southern pine beetle, *Dendroctonus frontalis* Zimmermann, and the linden borer, *Saperda vestita* Say. Although both groups live underneath the bark of trees, the major components of their diets differ. Bark beetles feed mostly on phloem and fungi throughout their development. Longhorned beetle larvae feed initially in phloem, but later deep in the sapwood. The higher cellulose content of sapwood led us to hypothesize that *S. vestita* is more likely to contain cellulose-degrading microbes than *I. pini* or *D. frontalis.*

Materials and Methods

Insects. *Sapera vestita* were collected from colonized linden trees (*Tilia cordata* cultivar "Greenspier") in Oak Creek and Waukesha, WI, on 26 September 2002 and 1 July 2003, respectively. Six larvae from Oak Creek and three adults and five larvae from Waukesha were used. *D. frontalis* were collected in July and August 2003 from Loblolly pine trees (*Pinus taeda*) in Forest, MS. Insects were transported overnight and dissected on the following day. Guts from three larvae and five adults were individually used for inoculation. *I. pini* (five larvae and seven adults) were obtained in October 2003 from a laboratory colony maintained on red pine (*Pinus resinosa*) that is replenished with wild stock from Madison, WI, several times per year (Raffa and Dahlsten 1995). All gut extractions were performed in a sterile ventilation hood within 2 d after collection from the logs.

The insects were surface sterilized by submersion in 70% ethanol for 1 min and rinsed in sterile water before dissection. Insects were dissected in 0.01 M sterile phosphate buffered saline (PBS; 0.138 M NaCl and 0.0027 M KCl, pH 7.4) using dissection scissors and fine-tipped forceps. Gut extractions of *I. pini* and *D. frontalis* larvae were performed by severing the head and the last abdominal segment and applying pressure anterior to the crop up to the end of the body. After the body contents were released from the cuticle, the gut was washed with PBS. The guts of bark beetle adults were extracted by holding the thorax with forceps and pulling the head from the thorax until the entire gut was stretched out of the body but still attached to the head and the rest of the body. The gut was separated from the body by cutting its ends in a sterile drop of PBS. Because of the linden borer's large size, the guts of adults and larvae were easily removed after dissection by cutting the body laterally. The guts were washed in PBS and transferred individually to 1.5-ml microfuge tubes with 0.1 or 0.5 ml of PBS. The tubes were sonicated (50/60 Hz, 117 V, 1.0 A; Branson Ultrasonics, Danbury, CT) for 30 s, macerated with a plastic pestle, and vortexed at medium speed for 10 s to separate microbial cells from the gut wall.

Isolation of Cellulolytic Microorganisms. Immediately after dissection, 100 μl of the microbial suspension with the gut debris was transferred to 15-ml test tubes with 3 ml of medium 1a—5 g/liter filter paper strips (Whatman), 40 mg/liter Bacto yeast extract (Becton Dickinson, Sparks, MD), 100 mg/liter malt extract (Becton Dickinson), and 2 g/liter $CaCO_3$, pH 7.0—and also medium 1b—5 g/liter carboxymethylcellulose sodium salt (CMC, low viscosity; Sigma, St. Louis, MO), 30 mg/liter yeast extract, 100 mg/liter malt extract, and 2 g/liter $CaCO_3$, pH 7.0. Methods and media were adapted from Wenzel et al. (2002). Each insect sample and medium was replicated three times.

The tubes were incubated aerobically and shaken at 100 rpm at 28°C in the dark. After the filter paper was visibly degraded, indicating the presence of cellulases, or after 6 wk (whichever came first), serial dilutions of the cultures were transferred to two solid media: medium 2 (5 g/liter CMC, 0.2 g/liter yeast extract, and 12 g/liter agar, pH 7.0) and medium 3 (5 g/liter CMC, 30 mg/liter Bacto Tryptic soy broth [Becton Dickinson, Sparks, MD], 10 g/liter malt extract, and 12 g/liter agar, pH 7.0). Pure cultures of representatives of morphologically distinct bacterial colonies were obtained by multiple subsequent subculturing on solid media. The cellular morphology of the microorganisms in each colony was examined at 1000× magnification. At least two representatives of each colony type were grown on media 2 and 3 at 28°C.

CMC Assay. Isolated microorganisms were tested for the ability to degrade CMC. Plates with single colonies and plates used for serial dilution were tested with the CMC assay on solid media 2 or 3 by covering the petri dishes with Congo red dye (ICN Biomedicals, Aurora, OH) (Teather and Wood 1982). Carboxymethylcellulose degradation was indicated by a clear zone around the colonies. Enzyme activity was indexed as the diameter of the colony plus the clear zone around it divided by the diameter of the colony. Two measurements were taken from each colony, with at least two colonies from the same bacterial isolate. Microbes with carboxymethylcellulase activity were inoculated in medium 1a to confirm their ability to degrade filter paper.

Identification of Microorganisms by rRNA Gene Sequencing. Isolated bacterial colonies were transferred to test tubes containing 5 ml of Luria-Bertani media (10 g/liter Bacto Tryptone, 5 g/liter Bacto-yeast extract, and 5 g/liter NaCl, pH = 7.0), incubated at 28°C for 2 d, and used for DNA extraction using the CTAB protocol adapted from Ausubel et al. (1994), described elsewhere (Broderick et al. 2004). The DNA

was diluted 20-fold and amplified by polymerase chain reaction (PCR) in a thermocycler (PTC-100; MJ Research, Waltham, MA) using primers 27f and 1492r (Lane 1991). Final concentrations for 50-μl PCR reactions were as follows: 2 μl diluted DNA (10–100 ng), 0.2 μM of each primer, 0.2 mM dNTPs, 5 U of *Taq* polymerase, and 1× *Taq* polymerase buffer (Promega, Madison, WI). 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 Promega's AMPure magnetic beads (AMPure; Promega).

Candidates for DNA sequencing were selected based on ARDRA (amplified rRNA restriction analysis). 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 1× Tris-borate-EDTA buffer at 4 V/cm. The gels were stained with ethidium bromide and visualized under UV light. The ARDRA restriction pattern of each clone was compared by visually grouping the similar patterns and sequencing selected bacterial isolates that had different patterns.

Sequencing reactions were conducted using the BigDye reaction mix (Perkin-Elmer, Boston, MA). Conditions for a 15-μl reaction were 0.5 μl DNA, 0.37 μl (10 μM) of primer 27 F, 704 F, 787R, or 1492R (Lane 1991), 1.0 μl BigDye, 3 μl 5× buffer, 0.75 μl DMSO, and 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. Similar procedures were conducted with fungal isolates, except that primers ITS1 and ITS4 (White et al. 1990) and a 50°C annealing temperature were used in PCR reactions. The fragment amplified by the ITS primers comprised a partial sequence of the 18S ribosomal RNA gene; complete sequences of the internal transcribed spacer 1, 5.8S ribosomal RNA gene, and the internal transcribed spacer 2; and a partial sequence of the 28S ribosomal RNA gene. Samples were cleaned with CleanSEQ reaction clean-up (Agencourt Bioscience, Beverly, MA). Sequence determination was performed on an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA) at the UW Biotechnology Center. Sequences were compiled using DNAStar (DNASTAR, Madison, WI) and deposited in GenBank under accession nos. AY770408–AY770432. Each sequence was compared with the nonredundant GenBank library using a BLAST search. The closest relative of each sequence was identified based on sequence comparison with the rDNA sequences in the database.

Quantification of CMC-Degrading Bacteria. The total number of cultivable bacteria and the number of CMC-degrading bacteria in the guts of linden borer larvae were estimated on TSA medium (3 g/liter tryptic soy broth and 15 g/liter agar, pH 7) and medium 2, respectively. The guts of five larvae were placed individually in PBS, and 100 μl of each dilution was plated on each medium (three replicates, eight-fold serial dilutions). Bacterial colonies were categorized based on morphology. The total number of colonies and the number of colonies of each bacterial type were counted across all plates for the three highest countable dilutions for that type. Plates with medium 2 were subjected to a CMC test to determine the number of CMC-degrading bacteria, but enzyme activity was not easily observed on the plates of less diluted bacteria. As an alternative approach to estimating the number of cellulolytic bacteria, ≈200 bacterial colonies from dilution plates (10^{-1} to 10^{-3}) were transferred to new plates with medium 2 (25 colonies per plate). Before plates were used in the Congo red test, colonies were characterized based on morphology, marked at the bottom of the petri dishes, and inoculated in a new plate. The rRNA genes of some of these bacteria also were sequenced.

Results

Filter paper in medium 1a was visibly degraded 19 d after inoculation with the gut microbiota of *S. vestita* larvae. Filter paper degradation and CMC-degrading bacteria were detected in all 11 *S. vestita* larvae examined. Among the several isolates of cellulolytic bacteria obtained in 2002 and 2003, only one ARDRA pattern was detected, and the 16S rRNA gene from one representative isolate from each year was analyzed. The 16S rRNA gene sequences of these two isolates had 99% identity to the α-Proteobacterium, *Sphingobium yanoikuyae* (Yabuuchi et al.) (Table 1). The enzyme activity index of 10 colonies of this bacterium (AY770418 and AY770419) was 2.3 ± 0.3 in medium 2 and 1.2 ± 0.2 in medium 3.

The density of cellulolytic bacteria ranged from 0.24 to 3.57 × 10^6 CFU/gut. Populations of cellulolytic bacteria were much lower than the total number of cultured bacteria (0.86–1.98 × 10^9 CFU/gut). The 16S rRNA genes of the most common types of non-CMC degrading bacteria were sequenced, and eight different sequences were identified. Three different sequences were detected on the dilution plates directly from gut content, and they were 99% identical to *Pantoea agglomerans* (Ewing and Fife), *Raoutella terrigena* (Izard et al.), and *Rahnella aquatilis* (Izard et al.). All other bacteria listed in Table 1 were from cellulose-enriched media. Some of these bacteria partially cleared the substrate under the colony. Limited fungal growth was observed in media 1a, 1b, and 2 inoculated with the larval gut of *S. vestita.* Two fungi and a yeast were isolated from *S. vestita* larvae, and two of them had limited CMC degradation activity (Table 2).

Filter paper degradation was observed in media where the gut contents of two different adult *S. vestita* were inoculated. A fungal mycelium mass was observed covering the pieces of paper after 3 wk. Two species of fungi were isolated, and CMC digestion was confirmed for both species. A fungus closely related to *Fusarium culmorum* (Smith) Saccardo was isolated from two adult linden borers and *Penicillium crusto-*

Table 1. Bacteria isolated from the gut of the linden borer, *S. vestita*, identified by 16S rRNA sequence analysis

Insect GenBank accession no.	Bacterial phylum	Closest known taxa—GenBank accession no.	Identity (%)
Linden borer larvae			
AY770418[a]	α-Proteobacteria	*Sphingobium yanoikuyae*—U37524.1	99
AY770419[a]	α-Proteobacteria	*Sphingobium yanoikuyae*—U37525.1	99
AY770420	β-Proteobacteria	*Acidovorax* sp.—AY093698.1	97
AY770421	γ-Proteobacteria	*Rahnella* sp.—U88435.1	99
AY770417	γ-Proteobacteria	*Raoultella planticola*—AF181574.1	99
AY770416	γ-Proteobacteria	*Klebsiella oxytoca*—U78183.1	100
AY770422	γ-Proteobacteria	*Enterobacter intermedius*—AF310217.1	98
AY770431	γ-Proteobacteria	*Pantoea agglomerans*—AF373197.1	99
AY770415	γ-Proteobacteria	*Raoultella terrigena*—Y17670.1	99
AY770432	γ-Proteobacteria	*Rahnella aquatilis*—U90757.1	99
Linden borer adult			
AY770423	α-Proteobacteria	*Brucella* sp.—AY331581.1	96
AY770424	α-Proteobacteria	*Brucella* sp.—AY331581.1	97
AY770430	β-Proteobacteria	*Delftia acidovorans*—AB020186.1	99
AY770429	β-Proteobacteria	*Pseudomonas putida*—AB180734.1	99
AY770425	γ-Proteobacteria	*Stenotrophomonas maltophilia*—AY040357.1	100
AY770426	γ-Proteobacteria	*Acinetobacter* sp.—AB020207.1	99
AY770427	Bacteroidetes	Uncultured *Sphingobacterium* sp.—AB076874.1	98
AY770428	Bacteroidetes	*Chryseobacterium joosteii*—AJ271010.1	97

[a] CMC-degrading bacteria; enzyme activity, indexed as the diameter of the colony plus the clear zone around it divided by the diameter of the colony, was 2.3 ± 0.3 in medium 2 and 1.2 ± 0.2 in medium 3.

sum Thom from a single adult linden borer. The clearing halos of *F. culmorum* and *P. crustosum* isolated from the guts of adult of *S. vestita* were the largest among all fungi tested, indicating release of enzyme into the medium (enzyme activity index, 2.15 and 1.76 in medium 2, respectively). The region between the 18S rRNA and 28S rRNA genes (ITS1, 5.8S, ITS2) of a noncellulolytic ascomycetous yeast (AY770414) diverged deeply (92% identity) from all known sequences, indicating that it may belong to a genus whose rRNA genes have not been sequenced previously. No cellulose-degrading bacteria were isolated from adults. Several noncellulolytic bacteria were isolated, and eight different DNA sequences were identified.

In both *S. vestita* larvae and adults, almost all bacteria isolated belong to the α-, β-, and γ-Proteobacteria. The only exceptions were AY770427 and AY770428, both of which are affiliated with the Bacteroidetes and were only found in adult guts. All 16S rRNA sequences had >96% identity to known sequences. The larval and adult gut communities of *S. vestita* had no bacteria or fungi in common.

Filter paper degradation was not observed in media in which the gut contents of *I. pini* or *D. frontalis* larvae or adults were inoculated. CMC digestion was not detected in tests with any bacteria isolated from these insect species. The noncellulolytic bacteria isolated from the bark beetles were not identified. Two fungal isolates, each originating from a single *I. pini* and *D. frontalis* larva, had CMC-degrading activity. The rDNA sequences (ITS1, 5.8S, ITS2) of these two phylotypes are 100% identical to *P. crustosum*, *P. chrysogenum* Thom, and *P. commune* Thom. In fact, the fungi AY770411, AY770408, and AY770409 isolated from *D. frontalis* larva, *I. pini* larva, and *S. vestita* adult were <1% divergent. We also isolated a few noncellulolytic

Table 2. Fungi (phylum Ascomycota) isolated from the guts of *S. vestita*, *I. pini*, and *D. frontalis* identified by ITS1, 5.8S, and ITS2 rRNA sequence analysis

Insect Sequence ID/GenBank accession no.	E.A.[a]	Fungal subphylum	Closest known taxa—GenBank accession no.	Identity (%)
S. vestita larvae				
AY770412	1.20	Pezizomycotina	*Nectria haematococca*—AF130142	98
AY770413	1.14	Pezizomycotina	*Neosartorya stramenia*—AF459730	98
AY770414	ND	Saccharomycotina	*Candida atlantica*—CAT539368	92
S. vestita adult				
AY770410	2.15	Pezizomycotina	*Fusarium culmorum*—AY147334.2	99
AY770409	1.76	Pezizomycotina	*Penicillium crustosum*—AY373907.1, *P. griseoroseum*, *P. expansum*, *P. commune*	100
I. pini larva				
AY770408	NM[c]	Pezizomycotina	*Penicillium crustosum*—AY373907.1, *P. commune*	100
D. frontalis larva				
AY770411	1.31	Pezizomycotina	*Penicillium chrysogenum*—AY373903.1, *P. commune*	100

[a] Enzyme activity was indexed as the diameter of the colony plus the clear zone around it divided by the diameter of the colony on cellulose containing medium 2.

ND, not detected; NM, not measured.

yeasts from the three insect species, but these did not digest CMC and were not identified.

Discussion

Filter paper degradation by intestinal microbes was detected in media inoculated with guts of larvae and adults of the longhorned beetle *S. vestita* but not the bark beetles *D. frontalis* and *I. pini*. *S. yanoikuyae* was the only bacterium that digested CMC. This bacterium can be considered a member of the indigenous biota of *S. vestita* larvae because it was present in all field-collected individuals during 2 consecutive yr. Interestingly, this bacterium was not detected in the guts of adults. *S. yanoikuyae* has been extensively studied because of its extreme versatility in catabolism (Kim et al. 1997). *S. yanoikuyae* B1 can metabolize a wide spectrum of aromatic hydrocarbons including *m*-xylene, biphenyl, naphthalene, anthracene, and phenanthrene as the sole carbon and energy source and is also capable of co-oxidizing a wide variety of polycyclic aromatic hydrocarbons after induction by either biphenyl or *m*-xylene (Gibson et al. 1973, Kim and Zylstra 1995, Borde et al. 2003). The ability of *S. yanoikuyae* to digest cellulose has not been documented previously, although an uncultured cellulolytic strain closely related to *Sphingomonas* sp. (*Sphingobium* was recently proposed to accommodate some *Sphingomonas* species, including *S. yanoikuyae*) was found in the gut of the termite *Zootermopsis angusticollis* (Hagen) (Wenzel et al. 2002).

Five fungi and one yeast isolated in this study digested CMC. The ones isolated from bark beetles and *S. vestita* larvae may be transient microbes that were passing through on ingested food material, given the low frequency of the fungi detected in a single insect. *S. vestita* adults harbor two fungal species that produced a large mycelium mass in medium 1a and had high CMC-degrading activity in medium 2. More individuals need to be studied to determine whether the fungi in *S. vestita* are indigenous or transient species derived from ingested food. Two of the fungi are closely related to *Nectria hematococca* Berk. and Br. and to *F. culmorum*, which are phytopathogenic filamentous fungi with cellulolytic activity (Manka 1988, Moubasher and Mazen 1991, Kang and Buchenauer 2000, 2002).

Wood-feeding termites and wood-feeding cockroaches are the only insects in which the cellulose-degrading gut microbiota has been extensively studied. The cellulose- and hemicellulose-degrading bacterial species isolated from termite guts (Saxena et al. 1993, Varma et al. 1994, Schäfer et al. 1996, Wenzel et al. 2002) and cockroaches (Cruden and Markovetz 1979) were not found in the guts of the beetle species described here. Cellulose-degrading fungi that are members of the *Fusarium* and *Penicillium* genera were isolated from the gut of *S. vestita* in this study and have also been isolated from termite hills and nests (Zoberi and Grace 1990), but not from the termite gut.

CMC is a cellulose derivative and a standard substrate in assays of endoglucanase activity (Karlsson et al. 2002). Most of the work to identify cellulolytic microbes has employed CMC sodium salt because of its high solubility in water. However, there are some indications that the ability of microorganisms to use native cellulose is more restricted than their ability to use its derivatives (Reese et al. 1950). Cultures of microorganisms presenting CMC-degrading activity in our study weakly digested filter paper strips. This was also observed in strains of *Clostridium*, *Eubacterium*, *Serratia*, *Citrobacter*, and *Klebsiella* isolated from the cockroach, *Eublaberus posticus* (Erichson) (Cruden and Markovetz 1979).

Degradation of filter paper in medium inoculated with the macerated gut, which contained the complete microbiota, indicates that a set of microbes producing a mixture of enzymes is probably necessary for complete digestion of cellulose that could not be accomplished by single isolates. The fermentation products of some species may serve as substrates for other bacterial reactions, and the bacteria that did not degrade CMC may contribute these substrates. An analogous relationship has been documented in termites, which harbor many bacteria and yeasts with hemicellulose-degrading activities, although none of the community members produces all of the hydrolytic enzymes required to digest hemicellulose completely (Schäfer et al. 1996).

The density of cellulolytic bacteria in insect guts has been determined only in a few species. Cruden and Markovetz (1979) estimated that CMC-producing organisms constitute up to 1% of the cultured bacteria in the gut of *E. posticus* (up to 1.0×10^7 aerobic and 2.0×10^8 anaerobic cellulolytic bacteria/ml). Wenzel et al. (2002) estimated that the gut of *Z. angusticollis* contains up to 10^7 cellulolytic bacteria per milliliter, indicating that these bacteria represent a substantial proportion of the gut community and therefore may contribute to cellulose degradation, augmenting the activity of cellulolytic flagellates and the termite's own cellulases. The populations of aerobic and facultative aerobic bacteria that we observed in *S. vestita* were lower than in *E. posticus*. The density and diversity of strictly anaerobic cellulolytic bacteria and those that are firmly attached to the epithelium, and therefore removed from the sample that was analyzed, remain to be studied.

The apparent absence of cellulolytic microbes in *I. pini* and *D. frontalis* is consistent with the microhabitat and ecology of the insect hosts. Cellulose fibers and undigested bark were not present in the guts of the adults and larvae of *D. frontalis* and adult *I. pini* examined, although some were present in *I. pini* larvae. Although these beetles live beneath bark in pine trees, they feed mostly on phloem contents and fungi, using different strategies to increase nutrient acquisition. For example, symbiotic mycangial fungi concentrate dietary nutrients for larval *D. frontalis* and *Dendroctonus ponderosae* (Hopkins), and nonmycangial fungi facilitate nitrogen use by *I. pini* (Barras 1973, Six and Paine 1998, Ayres et al. 2000). Compared with the bark beetles, the wood-feeding longhorned beetle, *S. vestita*, harbors cellulolytic bacteria, has a longer life span,

and lives inside the cambium feeding on a suboptimal diet high in cellulose, with slow food throughput during much of the larval stage.

The function of the gut microbiota has been explored in only a small number of insect species, especially with regard to cellulose digestion (Dillon and Dillon 2004). The presence of the cellulolytic *S. yanoikuyae* in all *S. vestita* individuals tested from two different sites and during different seasons suggests that this bacterium might contribute to cellulose digestion. However, insects that harbor cellulose-degrading microorganisms may also be capable of metabolizing cellulose on their own. For example, there is no evidence that an exo-β-1,4-glucanase (cellobiohydrolase) is needed for production of glucose from crystalline cellulose in wood-eating termites or cockroaches (Slaytor 1992, Scrivener and Slaytor 1994), and endogeneous cellulases from these insects consist of multiple endo-β-1,4-glucanase and β-1,4-glucosidase components (Slaytor 1992, Tokuda et al. 1999, Genta et al. 2003). Conversely, the presence of endocellulases in insects does not guarantee functionality on natural cellulose. For example, larval guts of the longhorn beetle, *Rhagium inquisitor* L, contain a number of enzyme activities necessary for the degradation of fibrous, hemicellulosic, and gum materials (Zverlova et al. 2003). Despite the presence of an endoglucanase and degradation of phosphoric-acid-swollen cellulose and CMC, hydrolysis of crystalline cellulose was not observed. Although this endoglucanase degrades β-1,4-glucans, it is inactive on crystalline, i.e., natural, cellulose. The possibility that insects possess redundant systems for metabolizing cellulose can likewise not be excluded. Therefore, whether *S. yanoikuyae* represents a valuable metabolic resource that enhances the ability of *S. vestita* to live on wood needs further consideration.

Acknowledgments

This research was supported by the USDA Forest Service North Central Research Station through L. Bauer, USDA Forest Service Southern Research Station through K. Klepzig, National Research Initiative of the USDA Cooperative State Research, Education and Extension Service Grant 2003-35302–13528, the Howard Hughes Medical Institute, the College of Agricultural and Life Sciences at the University of Wisconsin-Madison, and FAPESP (State of São Paulo Research Foundation). We thank the Milwaukee City Foresters for directing us to trees infested with linden borers and USDA FS personnel for shipping southern pine beetles. The laboratory assistance of B. Burwitz is greatly appreciated.

References Cited

Ausubel, F. M., B. Roger, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (eds.). 1994. Current protocols in molecular biology. Wiley, New York.

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. 1973. Reduction of progeny and development in the southern pine beetle following removal of symbiotic fungi. Can. Entomol. 105: 1295–1299.

Borde, X., B. Guieysse, O. Delgado, R. Muñoz, R. Hatti-Kaul, C. Nugier-Chauvin, H. Patin, and B. Mattiasson. 2003. Synergistic relationships in algal–bacterial microcosms for the treatment of aromatic pollutants. Bioresource Technol. 86: 3 293–300.

Breznak, J. A., and A. Brune. 1994. Role of microorganisms in the digestion of lignocellulose by termites. Annu. Rev. Entomol. 39: 453–87.

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

Cruden, D. L., and A. J. Markovetz. 1979. Carboxymethyl cellulose decomposition by intestinal bacteria of cockroaches. Appl. Environ. Microbiol. 38: 369–372.

Dillon, R. J., and V. M. Dillon. 2004. The gut bacteria of insects: nonpathogenic interactions. Annu. Rev. Entomol. 49: 71–92.

Genta, F. A., W. R. Terra, and C. Ferreira. 2003. Action pattern, specificity, lytic activities, and physiological role of five digestive beta-glucanases isolated from *Periplaneta americana.* Insect Biochem. Mol. Biol. 33: 1085–1097.

Gibson, D. T., R. L. Roberts, M. C. Wells, and V. M. Kobal. 1973. Oxidation of biphenyl by a *Beijerinckia* species. Biochem. Biophys. Res. Commun. 50: 211–219.

Haack, R. A., and T. M. Poland. 2001. Evolving management strategies for a recently discovered exotic forest pest: the pine shoot beetle, *Tomicus piniperda* (Coleoptera). Biological Invasions. 3: 307–322.

Hanks, L. M. 1999. Influence of the larval host plant on reproductive strategies of cerambycid beetles. Annu. Rev. Entomol. 44: 483–505.

Hoebeke, E. R. 1994. New records of immigrant bark beetles (Coleoptera: Scolytidae) in New York: attraction of conifer feeding species to ethanol-baited trap logs. Entomol. News. 105: 267–276.

Kang, Z., and H. Buchenauer. 2000. Ultrastructural and cytochemical studies on cellulose, xylan and pectin degradation in wheat spikes infected by *Fusarium culmorum.* J. Phytopathol. 148: 263–275.

Kang, Z., and H. Buchenauer. 2002. Studies on the infection process of *Fusarium culmorum* in wheat spikes: degradation of host cell wall components and localization of trichothecene toxins in infected tissue. Eur. J. Plant Pathol. 108: 653–660.

Karlsson, J., D. Momcilovic, B. Wittgren, M. Schülein, F. Tjerneld, and G. Brinkmalm. 2002. Enzymatic degradation of carboxymethyl cellulose hydrolyzed by the endoglucanases Cel5A, Cel7B, and Cel45A from *Humicola insolens* and Cel7B, Cel12A and Cel45Acore from *Trichoderma reesei.* Biopolymers. 63: 32–40.

Kim, E., and G. J. Zylstra. 1995. Molecular and biochemical characterization of two *meta*-cleavage dioxygenases involved in biphenyl and *m*-xylene degradation by *Beijerinckia* sp. strain B1. J. Bacteriol. 177: 3095–3103.

Kim, E., G. J. Zylstra, J. P. Freeman, T. M. Heinze, J. Deck, and C. E. Cerniglia. 1997. Evidence for the role of 2-hydroxychromene-2-carboxylate isomerase in the degradation of anthracene by *Sphingomonas yanoikuyae* B1. FEMS Microbiol. Lett. 153: 479–84.

Lane, D. J. 1991. 16S/23S rRNA sequencing, pp. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. Wiley, New York.

Logan, J. A., and J. A. Powell. 2001. Ghost forests, global warming, and the mountain pine beetle (Coleoptera: Scolytidae). Am. Entomol. 47: 160–173.

Manka, M. 1988. Cellulolytic activity of three *Fusarium culmorum* (W. G. Sm.) Sacc. isolates pathogenic towards wheat seedlings. J. Phytopathol. 122: 113–117.

Martin, M. M. 1983. Cellulose digestion in insects. Comp. Biochem. Physiol. 75: 3 313–324.

Moubasher, A. H., and M. B. Mazen. 1991. Assay of cellulolytic activity of cellulose-decomposing fungi isolated from Egyptian soils. J. Basic Microbiol. 31: 59–68.

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

Reese, E. T., R.G.H. Siu, and H. S. Levinson. 1950. The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J. Bacteriol. 59: 485–497.

Saxena, S., J. Bahadur, and A. Varma. 1993. Cellulose and hemicellulose degrading bacteria from termite gut and mound soils of India. Indian J. Microbiol. 33: 55–60.

Schäfer, A., R. Konrad, T. Kuhnigk, P. Kämpfer, H. Hertel, and H. König. 1996. Hemicellulose-degrading bacteria and yeasts from the termite gut. J. Appl. Bacteriol. 80: 471–478.

Schowalter, T. O., and G. Filip (eds.). 1993. Interactions among bark beetles, pathogens, and conifers in North American forests. Academic Press, San Diego, CA.

Scrivener, A. M., and M. Slaytor. 1994. Cellulose digestion in *Panesthia cribrata* Saussure: does fungal cellulase play a role? Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 107: 2 309–315.

Six, D. L., and T. D. Paine. 1998. Effects of mycangial fungi and host tree species on progeny survival and emergence of *Dendroctonus ponderosae* (Coleoptera : Scolytidae). Environ. Entomol. 27: 1393–1401.

Slaytor, M. 1992. Cellulose digestion in termites and cockroaches: what role do symbionts play? Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 103: 4 775–784.

Teather, R. M., and P. J. Wood. 1982. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol. 43: 777–780.

Tokuda, G., N. Lo, H. Watanabe, M. Slaytor, T. Matsumoto, and H. Noda. 1999. Metazoan cellulase genes from termites: intron/exon structures and sites of expression. Biochem. Biophys. Acta. 1447 146–159.

Varma, A., B. K. Kolli, J. Paul, S. Saxena, and H. König. 1994. Lignocellulose degradation by microorganisms from termite hills and termite guts: a survey on the present state of art. FEMS Microbiol. Rev. 15: 9–28.

Wenzel, M., I. Schonig, M. Berchtold, P. Kampfer, and H. Konig. 2002. Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite *Zootermopsis angusticollis.* J. Appl. Microbiol. 92: 32–40.

White, T. J., T. Bruns, S. Lee, and J. L. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315–322. *In* M. A. Innis, D. H. Gelfand, J. J. Sminsky, and T. J. White (eds.), PCR protocols: a guide to methods and application. Academic, San Diego, CA.

Zoberi, M. H., and J. K. Grace. 1990. Fungi associated with the subterranean termite, *Reticulitermes flavipes* in Ontario. Mycologia. 82: 289–294.

Zverlova, V. V., W. Höllb, and W. H. Schwarzc. 2003. Enzymes for digestion of cellulose and other polysaccharides in the gut of longhorn beetle larvae, *Rhagium inquisitor* L. (Col., Cerambycidae). Int. Biodeterioration Biodegradation. 51: 175–179.

Received for publication 11 October 2004; accepted 23 February 2005.