Gut Microbiota of an Invasive Subcortical Beetle, *Agrilus planipennis* Fairmaire, Across Various Life Stages

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ABSTRACT We characterized gut microbial communities in the emerald ash borer, *Agrilus planipennis* Fairmaire, an invasive phloem-feeding and wood-boring beetle that has caused extensive mortality to urban and forest ash trees. Analyses included both 16S rRNA gene–based and culture-based approaches. We estimated that the emerald ash borer gut harbors 44, 71, and 49 operational taxonomic units ($OTUs_{0.03}$) in the larval, prepupal, and adult stages, respectively, and a total of 132 $OTUs_{0.03}$ when data from the three stages are pooled. The larval gut community shared all its $OTUs_{0.03}$ with either the adult or the prepupal gut community, and the adult and prepupal gut communities shared 27 $OTUs_{0.03}$. Twenty-two $OTUs_{0.03}$ were shared among the three life stages. Rarefaction analyses suggest that these gut microbial communities are close to being completely sampled at the phylum level. Culture-independent techniques yielded a higher diversity of bacteria than did culturing. Three species of bacteria inhabiting guts of emerald ash borer showed cellulolytic activity. The diverse, dynamic, and presumably multifunctional microbial community associated with emerald ash borer guts suggests that invasive insects should be viewed as multispecies complexes and that such an interpretation can improve our ability to develop more effective management approaches.

KEY WORDS forest insect, emerald ash borer, invasive species, symbiont, insect gut

It is widely recognized that invasive insects pose significant challenges to environmental quality and human welfare (Chornesky et al. 2005). However, it is less widely appreciated that these species often derive increased opportunities for establishment and cause additional environmental and economic injury because of their associations with symbiotic microorganisms. The potential threats arising from symbionts of invasive insects are three-fold. First, symbionts may contribute to the damage caused by their insect host. Some prominent examples include *Scolytus multistriatus* (Marsham) (Coleoptera: Curculionidae), which vectors the pathogen causing Dutch Elm Disease, and *Sirex noctilio* F. (Hymenoptera: Siricidae), a vector of several phytopathogenic fungi. Second, symbionts may "escape" from their host, thus beginning a subsequent invasion. For example, the pinewood nematode, a commensal organism tightly associated with *Monochamus* species (Coleoptera: Cerambycidae) and innocuous to native pines in North America, became a virulent pathogen of Japanese and Portuguese pines after acquiring native *Monochamus* hosts there (Mota et al. 2006). Third, and perhaps least understood, symbionts play important roles in the survival and reproduction of their host insects (Leadbetter et al. 1999, Dillon et al. 2000, Lilburn et al. 2001, Moran et al. 2005, Cardoza et al. 2006). Although this increases their invasive potential, it also provides potential targets for control.

Overall, we have a greater understanding of external than internal and fungal than bacterial symbionts. There have been some studies of gut bacteria in invasive insects such as the gypsy moth (Broderick et al. 2004, 2006), the subterranean Formosan termite (Higashiguchi et al. 2006), and the Asian longhorned beetle (Schloss et al. 2006). However, little is known about the diversity, physiology, and ecology of microorganisms associated with guts of beetles that develop within the wood and bark of trees (Moore 1972, Brand et al. 1975, Bridges 1981, Bridges et al. 1984, Delalibera et al. 2005, 2007, Schloss et al. 2006, Vasanthakumar et al. 2006). These insects are particularly amenable to accidental introduction because of their cryptic life history, slow development time, and association with degraded wood commonly used for packing materials, pallets, and donnage (Hanks 1999, Haack 2002, Cappaert et al. 2007).

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Table 1. Lifestage, date, and location of live emerald ash borers collected in Michigan and total no. of insect samples analyzed from each site and time

Stage	No. individuals sampled					
	Lower Huron Metro Park, Belleville 42°09′47″ N, 83°24′43″ W (July 2004)	Matthaei Botanical Gardens, Ann Arbor 42°17′59″ N, 83°39′59″ W (Sept. 2004)	Willoughby Park, Lansing 42°39′17″ N, 84°32′41″ W (June 2005)	County Farm Park, Ann Arbor 42°15′28″ N, 83°42′4″ W (Oct. 2005)	Willoughby Park, Lansing 42°39′17″ N, 84°32′41″ W (Oct. 2005)	Total
Larvae	25	10	0	0	20	55
Prepupae	6	10	0	0	0	16
Male adults	10	7	7	10	0	34
Female adults	10	14	7	10	0	41

Samples collected in 2004 and June 2005 were frozen at −80°C before analysis; samples collected in Oct. 2005 were not subjected to freezing.

The emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), has caused extensive mortality to both urban and forest ash trees (*Fraxinus* sp.), resulting in severe economic and environmental losses (Poland and McCullough 2006, McCullough and Siegert 2007). Millions of ash trees have been killed in the United States during the last 6 yr. The impact to homeowners, the nursery and forest industries, and governments is estimated in the billions of dollars (USDA–APHIS, http://www.aphis.usda.gov/). In natural forests, mortality to ash results in a variety of adverse ecological impacts, such as disturbance that may foster invasive plant species (Poland and McCullough 2006). Various approaches to responding to this threat are underway but all have elements of scientific uncertainty, high economic cost, and political contention.

Adult *A. planipennis* beetles lay eggs in crevices on the bark of ash trees. The larvae burrow into the bark immediately after hatching and begin feeding on the cambium and phloem. Larvae are the most destructive stage of this pest, because their feeding galleries disrupt the phloem and flow of nutrients, resulting in death of the tree (Poland and McCullough 2006, Cappaert et al. 2007). After the fourth instar, the larvae stop feeding and excavate shallow chambers in the xylem in which they overwinter. This stage is commonly called the prepupal stage and is followed by pupation in the spring or summer. Adults emerge in late spring and fly to new host trees. Adults feed on ash foliage throughout their lives but cause little damage.

The purpose of this research was to characterize the gut microbial communities associated with the emerald ash borer. We examined fourth-instar larvae, prepupae, and newly emerged adults using both culture-based and culture-independent methods. We also made comparisons among fore-, mid-, and hindguts of selected beetles. Furthermore, we evaluated gut bacteria for cellulolytic activity.

Materials and Methods

Insect Collection and Gut Dissection. *Agrilus planipennis* adults and larvae were collected from ash trees in Michigan and shipped on dry ice to the forest entomology laboratory in Wisconsin (Table 1). The following day, they were surface sterilized in 70% ethanol for 1 min, rinsed in sterile water, and dissected in 10 mM sterile phosphate-buffered saline (PBS) in a sterile laminar flow hood using dissection scissors and fine-tipped forceps. The head and last abdominal segment of each larva were severed, and larvae were dissected open through the middle. 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 body but still attached. The gut was separated from the body by cutting its extremities in a drop of sterilized PBS. Bacteria were isolated from both entire guts as well as gut sections. Of the 41 adult beetles, guts from seven males and seven females were separated into foregut, midgut, and hindgut samples before being washed. Guts and gut sections were washed in PBS and transferred individually to 1.5-ml microfuge tubes containing 500, 600, or 200 μl of PBS. The guts were sonicated (50/60 Hz, 117 V, 1.0 Amps; Branson Ultrasonics, Danbury CT) for 30 s.

Isolation of Bacteria. Each gut was placed in 500–600 μl of 10 mM PBS. Each gut section was placed in 200 μl of 10 mM PBS. Four or five serial 10-fold dilutions were spread on duplicate plates of one-tenth strength TSA (3 g/liter tryptic soy broth; Difco Laboratories, Franklin Lakes, NJ; 15 g/liter agar, pH 7.0). Plates were incubated in a growth chamber at 28°C for 3–4 wk. Bacterial colonies were categorized based on morphology and counted across all plates on the three lowest countable dilutions. At least two bacterial isolates representative of each morphology and isolated from at least two different insects (if not unique to a single insect) were chosen for sequencing (see below). Clones to be sequenced were chosen randomly.

DNA Extraction from Cultured Bacteria and Total Gut Extracts. 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. Bacterial pellets were washed and resuspended in 800 μl of sterile distilled water and combined with ≈100 μl of 0.5-mm zirconia-silica beads (BioSpec Products, Bartlesville, OK) and mixed vigorously using a vortex mixer for 10–40 min. DNA from bacterial isolates was extracted using 200 μl of InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's directions. Two hundred microliters of matrix was combined with gut supernatant, 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 and centrifuged at high speed to separate the matrix from the gut supernatant. Twenty microliters of the resulting DNA was used in a 50-μl polymerase chain reaction (PCR) reaction.

Individual guts from larvae and prepupae and pooled guts from larvae and adult beetles were placed in 1.5-ml tubes containing 500 μl PBS and maintained at −20°C until DNA extraction. A previously described protocol was used to extract total DNA from guts (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.

PCR Amplification and 16S rRNA Gene Libraries. DNA extracted from individual cultured bacterial colonies was either diluted (1:10–1:20) or used directly in PCR. The 16S rRNA genes were amplified using primers 27f and 1492r (Broderick et al. 2004). PCR products were purified using either QIAquick PCR purification Kit (Qiagen, Valencia, CA) or a mixture of Exonuclease I (10 U/μl) and shrimp alkaline phosphatase (1 U/μl). This treatment removes small DNA fragments that might hinder further processes.

16S rRNA genes were amplified by PCR from total DNA isolated from gut extracts, as described above. PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen) and cloned into a pGEM-T vector (Promega) according to the manufacturer's directions. Ninety-six clones from each sample were transferred to plates of Luria-Bertani agar amended with 50 mg/liter ampicillin and incubated at 37°C for 24 h. Colonies were directly used in PCR for amplification of the insert using M13 vector primers (Broderick et al. 2004). PCR products were purified using a mixture of Exonuclease I (10 U/μl) and shrimp alkaline phosphatase (1 U/μl), as described above. Seven 16S rRNA gene libraries, including three larval, two adult, and two prepupal libraries, were analyzed. One larval library and the two adult libraries consisted of pooled guts (larvae: L41, four guts; adults: A-M5 and A-F6, two guts each). All the other libraries (two larval and two prepupal) were constructed from single guts.

Sequencing Cultured Bacterial Isolates and Clones and Sequence Analyses. Bacterial isolates and clones with inserts of the right size were sequenced using 16S rRNA gene primers 27F and 787R (Broderick et al. 2004). Sequencing reactions were performed using BigDye (Perkin-Elmer, Wellesley, MA). Sequenced products were purified using CleanSEQ magnetic beads (Agencourt Bioscience, Beverly, MA). Sequences were determined on an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA) at the University of Wisconsin-Madison Biotechnology Center.

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).

Sequences from each library were aligned in ARB (Ludwig et al. 2004) using the RDP 7.1 phylogenetic tree (Cole et al. 2005) as comparison. The sequences were automatically aligned in the ARB sequence editor by using FastAligner. Alignments were manually corrected if necessary. Aligned sequences were added to the phylogenetic tree using a maximum parsimony method. 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 (distance-based OTU and richness determination) to calculate operational taxonomic units (OTUs) and construct rarefaction curves at distance levels of 30, 20, 10, 5, 3, and 1% (Schloss and Handelsman 2005). These cut-offs are considered to correspond to division, phylum, class, genus, species, and strain levels (Schloss and Handelsman 2004). 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 (Chao 1984). Terminal Chao1 richness estimates are reported for 3% difference between sequences.

We also used SONS (shared OTUs and similarity) (Schloss and Handelsman 2006) to compare community structure and overlap of community membership. Briefly, an output file from DOTUR containing the list of sequences in each OTU and a file containing the names of all the sequences and their corresponding library names were used to run SONS. The output files from SONS contain data to construct collectors' curves for the shared Chao1 richness estimator (Chao et al. 2006). Data from comparisons of two or three libraries in a community and the three separate communities (larvae, prepupae, and adults) were assembled into Venn diagrams to easily visualize the membership overlap. The Jaccard similarity index, which estimates the fraction of sequences that belong to shared OTUs, is also reported. In addition, a nonparametric estimator for community structure overlap, theta (θ) was also calculated in SONS and is presented here (Yue and Clayton 2005).

Nucleotide sequences were deposited in GenBank under accession numbers EU148613-EU149259 and EU153061-EU153099.

Cellulolytic Activity. We tested a subset of bacterial isolates for celluloytic activity in vitro on culture media containing carboxymethylcellulose (CMC), using the method of Delalibera et al. (2005) as modified from Teather and Wood (1982). Briefly, colonies were spotted onto CMC media and allowed to grow at 28°C for at least 48 h. Colonies were flooded with Congo Red, a dye that binds CMC. After this step, zones of clearing where bacteria had digested CMC were visible as clear halos, whereas the rest of the medium on the plate was dyed red. The diameters of the colonies

Table 2. Phylotype distribution in larvae (L41, L4I6, L4I1), prepupae (PP6, PP4), and adults (A-M5, A-F6) of the emerald ash borer

Phylum	L41 (n = 74)	L4I6 (n = 85)	L4I1 (n = 44)	PP6 (n = 130)	PP4 (n = 75)	A-M5 (n = 76)	A-F6 (n = 86)	Total (n = 570)
Acidobacteria	1	1	0	0	0	0	3	5
Actinobacteria	6	7	1	28	10	5	2	59
Alphaproteobacteria	1	15	10	44	7	12	22	111
Bacteroidetes	0	1	0	8	0	2	0	11
Betaproteobacteria	1	12	10	18	10	8	11	70
Deinococcus-Thermus	5	0	0	0	0	0	4	9
Deltaproteobacteria	0	0	0	1	0	0	0	1
Firmicutes	12	5	7	10	32	37	20	123
Gammaproteobacteria	48	44	15	19	14	12	23	175
Nitrospirae	0	0	1	0	0	0	0	1
OP10	0	0	0	1	0	0	0	1
Planctomycetes	0	0	0	1	0	0	1	2
TM6	0	0	0	0	2	0	0	2

Five hundred seventy clones from seven 16S rRNA gene libraries were analyzed and assigned taxonomic descriptions based on their positions in a phylogenetic tree. For larvae, two libraries are from individual guts and one is from a pooled sample of four guts. For prepuape, both libraries are from individual guts. For adults, both libraries are made from pooled samples of two guts. "n" refers to the total no. of clones in each library.

alone and the colonies plus the halos were measured. At least two separate measurements were taken. The assays were performed a minimum of two times on each isolate.

Results

Phylotype Distribution in 16S rRNA Gene Libraries. Sequences similar to members of the Acidobacteria, Actinobacteria, Bacteroidetes, Deinococcus-Thermus, Firmicutes, OP10, Planctomycetes, Proteobacteria, Nitrospirae, and TM6 were represented in 16S rRNA gene libraries from larvae, prepupae, and adults (Table 2). The γ-Proteobacteria dominated the larval gut libraries (53% of sequences in the pooled dataset, $n = 203$), whereas the Firmicutes and α-Proteobacteria were abundant in the adult and prepupal gut libraries, respectively (35%, pooled $n = 162$; 25%, pooled $n = 205$). Members of the Actinobacteria, Bacteroidetes, Firmicutes, and the classes α-, β-, and γ-Proteobateria were represented in all three life stages.

Below the phylum level, 55 OTUs from 42 genera were obtained using culture-independent methods (Table 3). Forty-four percent of the sequences from larval guts were similar to representatives of the *Pseudomonas* and *Acinetobacter* spp. (γ-Proteobacteria; Table 3). Sequences similar to *Leuconostoc* spp. (Firmicutes) accounted for 34% of clones in the two adult gut libraries. Sequences in the prepupal gut libraries were somewhat distributed among various divisions.

The clone libraries also contained sequences from less well-characterized phyla. Two larval gut libraries and one adult gut library contained members of the Acidobacteria (Tables 2 and 3), which are common in soils and have few cultured representatives. Sequences similar to the *Isosphaera* spp. in the Planctomycetes were represented in one prepupal gut and one adult gut. The TM6 candidate division was represented in one prepupal gut library, and one sequence similar to members of the OP10 candidate division was found in the other prepupal gut library. Sequences similar to *Meiothermus silvanus* (Deinococcus-Thermus) were found in one larval gut library and one adult gut library. Members of the Bacteroidetes were found in a prepupal gut library. The same library also contained sequences similar to Sphingobacteriales and *Hymenobacter* spp. (Bacteroidetes), of which the latter was also represented in a larval gut library. Sequences similar to *Chryseobacterium* spp. were found in an adult gut library.

Rarefaction Analyses. Rarefaction curves of individual stages and pooled data are shown in Fig. 1. These indicate that the $OTU_{0.20}$ larval gut biodiversity has been adequately sampled (Fig. 1a). Results of analyses of libraries from prepupal guts varied with taxonomic level (Fig. 1b). At the 1 and 3% difference levels, substantially more species remain to be sampled, but at 10 and 20%, the curves approach asymptotes. Analysis of the adult gut libraries were generally similar to those of the larvae, indicating that adult gut biodiversity was adequately sampled (Fig. 1c). Analysis of the pooled data set of all 570 sequences from the seven 16S rRNA gene libraries indicates that the emerald ash borer gut microbial communities in larvae, prepupae, and adults are close to being completely sampled at 10% difference among sequences (Fig. 1d). The rarefaction curves at 1 and 3% differences among sequences were still rising, but with relatively low slopes.

Comparison of Community Membership and Structure Across Life Stages. The results of SONS analyses are presented in a Venn diagram to show the shared membership among larval, prepupal, and adult gut communities at 3% difference among sequences (Fig. 2). A core membership of 22 OTUs was shared among all three communities. The larval gut community was a subset of the adult and prepupal gut communities (Fig. 2). Larval guts shared 29 and 43 OTUs with prepupal guts and adult guts, respectively. The shared richness estimate between prepupal and adult guts was 27 OTUs. The terminal Chao1 richness estimates were 44, 71, and 49, for larval, prepupal, and adult gut communities, respectively, and 132 for the pooled data.

Table 3. Taxonomic placement and abundance of sequences obtained using culture-independent analyses

Phylogenetic affiliation	Larvae			Prepupae		Adults		Total
	L4I1	L4I6	L4I1	PP6	PP4	AM5	AF6	
Acidobacteria								
Uncultured Acidobacteria	1	1	0	0	0	0	3	5
Actinobacteria								
Arthrobacter spp. (including *A. protophormia*)	0	0	0	0	4	0	0	4
Uncultured *Corynebacterium* sp.	0	0	0	0	2	0	0	2
Microbacterium spp.	0	3	1	2	0	0	0	6
Propionibacterium spp. (including *P. acnes, P. granulosum*)	6	4	0	20	2	1	0	33
Rothia amarae	0	0	0	4	0	3	1	8
Streptomyces spp. (including *S. negayawaensis, S, ipomea*)	0	0	0	0	3	1	0	4
Rhodococcus fascians	0	0	0	2	0	0	0	2
Unidentified Actinobacteria	0	0	0	0	0	0	1	1
Bacteroidetes								
Chryseobacterium sp.	0	0	0	0	0	2	0	2
Unclassified Bacteroidetes	0	0	0	5	0	0	0	5
Uncultured *Hymenobacter* spp.	0	1	0	1	0	0	0	2
Uncultured Sphingobacteriales	0	0	0	2	0	0	0	2
Firmicutes								
Ammoniphilus oxaloaceticus	0	0	0	0	0	0	1	1
Bacillus spp. (including *B. cereus, B. thuringiensis* and uncultured *Bacillus* sp.)	0	0	0	6	0	0	1	7
Lactococcus spp. (including *Lactococcus lactis*)	3	0	0	0	0	0	0	3
Lactobacillus acidophilus	0	0	0	0	3	0	0	3
Leuconostoc spp. (including *L. gelidum* and uncultured *Leuconostoc* spp.)	9	0	2	0	0	37	18	66
Enterococcus sp.	0	0	0	2	0	0	0	2
Staphylococcus sp.	0	0	0	2	0	0	0	2
Streptococcus spp. (including *S. infantis, S. mitis, S. sanguinis, S. thermophilus* and uncultured *Streptococcus* spp.)	0	5	5	0	28	0	0	38
Deinococcus-Thermus								
Meiothermus silvanus	5	0	0	0	0	0	4	9
Nitrospira								
Nitrospira sp.	0	0	1	0	0	0	0	1
OP10								
Uncultured OP10	0	0	0	1	0	0	0	1
Planctomycetes								
Uncultured *Isosphaera* group	0	0	0	1	0	0	1	2
Proteobacteria								
Alphaproteobacteria								
Acetobacteraceae bacteria	0	0	0	0	1	3	1	5
Blastomonas sp.	0	0	2	0	0	0	0	2
Caulobacter sp. (including uncultured Caulobacteraceae)	0	10	4	19	2	1	6	42
Bradyrhizobium japonicum	0	2	3	1	0	0	0	6
Novosphingobium spp.	0	0	0	6	0	0	0	6
Sphingobium spp.	0	0	1	0	1	1	0	3
Sphingomonas spp. (including *S. rhizogenes*)	0	1	0	7	0	2	4	14
Hyphomicrobium spp. (including *H. zavarzanii*)	1	0	0	0	1	4	8	14
Rhizobium spp. (including *R. tropici*)	0	0	0	2	0	0	0	2
Methylobacterium spp. (including *M. extorquens*)	0	0	0	0	1	1	0	2
Uncultured Methylobacteriaceae bacteria	0	0	0	6	0	0	0	6
Unclassified Alphaproteobacteria	0	2	0	1	0	0	2	5
Rhodobacter sp.	0	0	0	0	1	0	0	1
Rhodopseudomonas sp.	0	0	0	2	0	0	1	3
Betaproteobacteria								
Ralstonia sp.	0	5	0	0	0	0	0	5
Burkholderia spp. (including *B. cepacia*)	0	4	5	0	0	0	0	9
Commamonadaceae bacteria	0	3	3	11	5	3	3	28
Oxalobacteraceae bacterium	0	0	0	1	0	0	0	1
Variovorax spp.	0	0	0	7	0	2	1	10
Tepidimonas ignava	1	0	0	0	0	1	2	4
Unclassified Betaproteobacteria	0	0	2	1	5	2	5	15
Deltaproteobacteria								
Uncultured Deltaproteobacterium	0	0	0	1	0	0	0	1
Gammaproteobacteria								
Acinetobacter spp. (includes *A. junii, A. johnsonii, A. calcoaceticus* and uncultured *Acinetobacter* spp.)	0	29	11	4	10	1	0	55
Pseudomonas spp. (incl. *P. nitroreducens, P. pseudoalcaligenes, P. alcaliphila, P. mendocina, P. poae* and uncultured *Pseudomonas* spp.)	43	6	1	0	2	11	23	86
Moraxella spp. (includes *M. osloensis*)	0	9	3	0	1	0	0	13
Pantoea agglomerans	3	0	0	2	1	0	0	6
Erwinia persicinus	1	0	0	0	0	0	0	1
Stenotrophomonas maltophila	1	0	0	0	0	0	0	1
Klebsiella spp. (includes *K. ornithinolytica*)	0	0	0	11	0	0	0	11
TM6								
Uncultured TM6 bacterium	0	0	0	0	2	0	0	2

PCR-amplified 16S rRNA gene libraries were constructed from larval, prepupal, and adult guts of the emerald ash borer. The no. of clones is indicated in each column. Library designations are L41, L4I6, L4I1 (larvae), PP6, PP4 (prepupae), and A-M5, A-F6 (adults).

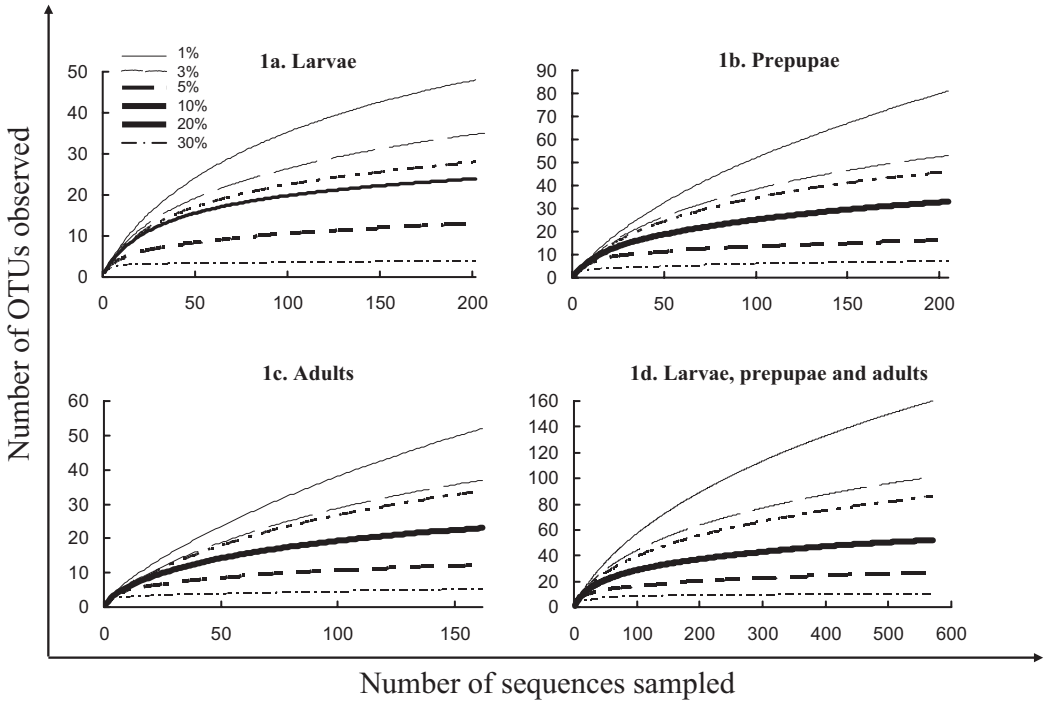

Fig. 1. Rarefaction analyses of 16S rRNA gene libraries constructed from larval, prepupal, and adult guts. Rarefaction curves were constructed based on DOTUR analyses using the furthest neighbor assignment algorithm. A total of 570 sequences were analyzed. Rarefaction curves are shown for 1, 3, 5, 10, 20, and 30% difference among sequences. Rarefaction curves were constructed from pooled data from larvae (a), prepupae (b), adults (c), and the entire pooled dataset (d).

The abundance-based Jaccard similarity indices (J_{abund}) for the pairwise comparisons between larvae and prepupae, larvae and adults, and prepupae and adults were 0.45 ± 0.12, 0.66 ± 0.30, and 0.14 ± 0.10 (SE), respectively. All three were significantly different from 1.0. However, the J_{abund} values for the larval-prepupal and larval-adult comparisons were relatively high, indicating that the probability of finding a sequence that belonged to a shared OTU was higher than it was for the prepupal-adult comparison.

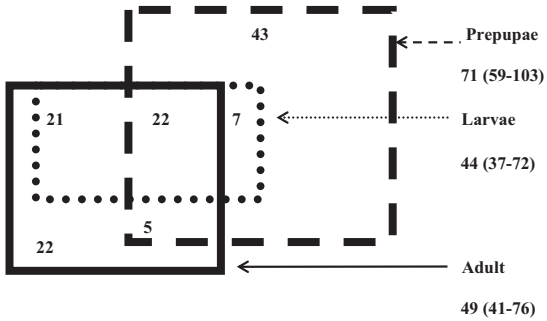

Fig. 2. Venn Diagram showing the membership (at 3% difference between sequences) of emerald ash borer gut microbiota in the larval, prepupal and adult stages. Each life stage was separately analyzed and then pooled for this analysis. The terminal Chao1 richness estimate of each community along with the 95% confidence interval is listed next to each rectangle. The Chao1 richness estimate of the pooled communities was 132 (C.I. = 115–169). The three communities shared a core membership of 22 OTUs. Pairwise Chao1 richness estimates were Larvae-Prepupae = 29, Larvae-Adult = 43 and Prepupae-Adult = 27. Shared Chao1 richness estimates at $OTU_{0.03}$ estimated by pooling two communities and estimating the number of OTUs shared with the third one were as follows: LarvaePrepupae-Adult = 27; PrepupaeAdult-Larvae = 50; LarvaeAdult-Prepupae = 28.

The community structure similarity indices (θ) for the pairwise comparisons between larvae and prepupae, larvae and adults, and prepupae and adults were 0.163 ± 0.035, 0.111 ± 0.029, and 0.014 ± 0.005 (SE), respectively, indicating that these three communities were significantly different from each other. Although the larval gut community was a subset of the adult and the prepupal communities in membership, its structure was not very similar to either of the other two communities.

The terminal Chao1 richness estimates of the three larval libraries were similar (20, 16, and 17). However, the three libraries shared only two OTUs at 3% difference among sequences. The θ values for pairwise comparisons of the three libraries (0.56, 0.011, and 0.010) reinforce that, although the community structures of two are moderately similar to one another, the third is significantly different from them.

The two libraries from prepupal guts had terminal Chao1 richness estimates of 44 and 27 at 3% difference among sequences, with a shared Chao1 richness estimate of only 4 OTUs. The J_{abund} value of 0.12 ± 0.07

Table 4. Taxonomic placement of bacteria isolated from guts of larvae, prepupae, and adults of the emerald ash borer

Taxonomic affiliation	Larvae	Prepupae	Adults
α-Proteobacteria			
Agrobacterium tumefaciens	−	−	+
Chelatococcus asaccharovorans	−	−	+
Methylobacterium spp.	−	−	+
Ochrobactrum sp.	−	−	+
Sphingomonadales bacteria	−	+	−
β-Proteobacteria			
Alcaligenes sp.	−	+	−
Burkholderia cepacia	−	−	+
γ-Proteobacteria			
Enterobacter and Enterobacterales	++[a]	+	−
Pseudomonas spp.	+	+	+
Firmicutes			
Bacillus spp.	+	++	++
Paenibacillus spp.	−	+	++
Staphylococcus spp.	+[a]	+	+
Actinobacteria			
Arthrobacter spp.	+	–	+
Cellulomonas sp.	−	++	+
Curtobacterium sp.	−	−	+
Frigoribacterium sp.	−	+	−
Kocuria rhizophila	−	−	+
Microbacterium spp.	+	−	+
Micrococcus luteus	−	+	+
Mycobacterium sp.	−	−	+
Nocardiopsis alba	−	−	+
Ornithinimicrobium sp.	−	+	−
Rhodococcus sp.	+[a]	+	+
Streptomyces spp.	++	++	++

Isolations were made on 1/10th strength tryptic soy agar. Bacteria were identified by 16S rRNA gene sequencing.

[a] Isolated only from samples that were not frozen.

+, presence in the sample; ++, frequently found genus in the sample (approximately one half or more of the total no. of isolates sequenced were similar by 16S rRNA gene sequence).

suggests that only a small fraction of sequences is shared between these two libraries. The community similarity index, θ, was 0.042 ± 0.019, suggesting that the two libraries are significantly different from each other in structure at 0.03 difference. The two libraries from adult guts had terminal Chao1 richness estimates of 31 and 32 and a shared Chao1 richness estimate of 26 when sequences differed by 3%. The Jaccard similarity index (J_{abund}) was 0.77 ± 0.17, and although different from 1.0, was high enough to suggest that high-abundance members were shared between the two libraries.

Culturable Bacteria. Bacterial isolates from emerald ash borer guts had 16S rRNA gene sequences that matched sequences from α-, β-, and γ-Proteobacteria, Actinobacteria, and Firmicutes (Table 4). All of these classes/phyla were also represented in the culture-independent analyses, but some of the genera obtained by culturing were different. *Pseudomonas*, *Bacillus*, *Staphylococcus*, *Rhodococcus*, and *Streptomyces* spp. were isolated from all three life stages. No α-Proteobacteria were isolated from larvae, even though they were represented in clone libraries from larval guts. *Cellulomonas* spp. were isolated from prepupae and adults and were dominant in the former.

Twelve different morphologies were observed among bacterial isolates from the three gut sections, of which four were most commonly found. Isolates similar to *Staphylococcus* spp., *Bacillus* sp., and *Burkholderia cepacia* (identified by 16S rRNA gene sequencing) were isolated from the foregut. In contrast, the midgut and the hindgut contained only *Staphylococcus* sp. and *Burkholderia cepacia*, respectively.

Table 5. Cellulolytic enzyme activity among bacteria isolated from the emerald ash borer

Bacterial isolate	Source	Enzyme activity
Streptomyces sp.	Larva	2.9 ± 0.15
Erwinia sp.	Adult	2.0 ± 0.27
Burkholderia cepacia	Adult	2.4 ± 0.71

A subset of bacteria was tested. Enzyme activity was calculated as the ratio of the diameter of the clearing zone (including the colony) to the diameter of the colony alone. At least two separate measurements were taken. The assays were performed at least twice on each isolate. Bacterial isolates were identified by 16S rRNA gene sequencing.

Because insects had to be frozen at −80°C before being shipped to Wisconsin to comply with quarantine regulations, we also performed isolations from guts of live emerald ash borer larvae and adults reared from infested ash logs in the laboratory in East Lansing, MI. Bacteria similar to Enterobacteriales and *Staphylococcus* spp. were isolated from larval guts only in the unfrozen samples. The Enterobacteriales were dominant in the fresh insects, whereas Actinobacteria dominated the frozen samples.

Cellulolytic Activity. Bacterial isolates similar to *Streptomyces* spp., *Erwinia* sp., and *Burkholderia cepacia* digested CMC in vitro and showed relatively high enzyme activity (Table 5).

Discussion

The emerald ash borer harbors a diverse community of gut bacteria. This community undergoes changes as insect progresses through different life stages, but a group of 22 OTUs was shared among all life stages tested. We isolated *Pseudomonas*, *Bacillus*, *Staphylococcus*, *Rhodococcus*, and *Streptomyces* spp. from larvae, prepupae, and adults. Other OTUs were limited to specific stages. The Chao1 richness estimates in the three developmental stages ranged from 44 to 71 OTUs at 3% difference between sequences, a cut-off commonly used for species level delineation. Larval gut OTUs were a subset of either the prepupal or the adult gut OTUs.

This study is one of only a few to analyze gut microbial communities of different life stages in a holometabolous insect. Overall, the prepupal and adult gut communities were less similar to each other than those of larvae and adults. The larval gut community was a subset of either the adult or the prepupal gut communities. This suggests that larvae retain some of their symbionts after they stop feeding. In the wood-feeding *Odontotaenius disjunctus* (Illiger) (Coleoptera Passalidae), there is likewise substantial conservation of larval microbes in adults and structural similarities

between larval and adult hindguts (Nardi et al. 2006). Similarly, *Pantoea* and *Strenotrophomonas* spp. were found across larvae, pupae, and adults of *Ips pini* (Say) (Delalibera et al. 2007). In addition to stage-specific variation, some bacterial types were only obtained from certain parts of guts. This may reflect the different physical and chemical conditions that exist in these different microhabitats (Lemke et al. 2003). Although we use the term symbiont in its broadest sense to denote gut-associated microorganisms in this study, we cannot conclude that these microorganisms are persistent in, or how frequently they are associated with, emerald ash borer guts. More information is needed before we can verify how commonly these microorganisms are associated with the host insects.

Compared with the few other wood-feeding beetles whose guts have been studied (Schloss et al. 2006, Vasanthakumar et al. 2006, Delalibera et al. 2007), the microbiota of the emerald ash borer seems somewhat more complex. However, compared with another wood-feeding order, Isoptera, the emerald ash-borer's microbiota seems less species rich. For example, *Reticulitermes speratus* contains up to 700 species (Nakajima et al. 2005). Compared with a leaf-feeding lepidopteran, the gypsy moth *Lymantria dispar* L. (Broderick et al. 2004), the emerald ash borer seems to have higher overall gut microbiota complexity.

The emerald ash borer gut contained some clone sequences that have been found either infrequently or not at all in insect guts. These sequences matched *Meiothermus silvanus* (Deinococcus-Thermus), unclassified Bacteroidetes (<90% similarity to sequences in the databases), and mostly uncultured members of the TM6, OP10, and Planctomycetes. Most sequences in culture-independent analyses matched uncultured members of bacteria in the databases. In addition, many sequences of cultured bacteria from larval guts were similar to sequences of uncultured Enterobacteriales in the databases, indicating that the emerald ash borer gut communities might contain previously uncultured bacteria.

Although all the bacterial phyla represented in our culture-based study were also found in culture-independent analyses, we found that some bacterial genera could be cultured on growth media but not found among clones from emerald ash borer guts. We found similar results with the southern pine beetle, *Dendroctonus frontalis* Zimmermann (Vasanthakumar et al. 2006), and this has also been shown in other environments such as soil (Dunbar et al.1999). In addition, we found some differences in the types of bacteria isolated from fresh versus frozen insects. Therefore, we caution against relying on preserved specimens alone for culture-based analyses.

Three bacteria, similar to *Streptomyces* spp., *Erwinia* sp., and *Burkholderia cepacia*, seem capable of digesting carboxymethylcellulose in vitro, with their enzyme activity being higher than that observed for isolates from another wood-boring beetle, the linden borer *Saperda vestita* Say (Coleoptera: Cerambycidae) (Delalibera et al. 2005). Likewise, the presence of *Cellulomonas* spp. in prepupal and adult guts suggests that these bacteria might digest cellulose and related bacteria are widely known to perform this function (Cazemier et al. 2003). Bacteria such as those found here that thrive at mesophilic/thermophilic or psychrophilic temperatures include species that degrade phytochemicals (Martin et al. 1999), raising the possibility that symbionts assist emerald ash borers in detoxifying tree defense compounds. Conversely, many species of *Paenibacillus* are pathogenic to insects (Enright and Griffin 2004), and therefore these might be sources of biological control.

The diverse, dynamic, and presumably multifunctional microbial community with emerald ash borer guts suggests that this and other invasive insects should be viewed as multispecies complexes. Such an interpretation can improve our ability to access risk and develop more effective management approaches. Substantial work remains to fully characterize the members and roles of gut communities in the emerald ash borer. In particular, future research should quantify sources of variation among individuals and populations, determine mechanisms of passage and loss among life stages and gut sections, and determine processes by which bacteria affect host plant utilization.

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