Bacterial Communities within Digestive Tracts of Ground Beetles (Coleoptera: Carabidae)

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ABSTRACT We identified the bacterial communities within the alimentary tracts of two granivorous ground beetles as a first step in the exploration of bacteria–ground beetle symbioses. Terminal-restriction fragment length polymorphism analyses of bacterial rRNA extracted from the guts of field-collected individuals of *Harpalus pensylvanicus* (DeGeer) and *Anisodactylus sanctaecrucis* (F.) (Coleoptera: Carabidae) revealed that gut-associated bacterial communities were of low diversity. Individuals from the same beetle species possessed similar bacterial community profiles, but the two species exhibited unique profiles. Bacterial 16S rRNA clone libraries constructed for the two beetle species showed that *H. pensylvanicus* had a more diverse community (six operational taxonomic units [OTUs]) compared with *A. sanctaecrucis* (three OTUs). Only one OTU, closely related to *Hafnia alvei*, was common between the two beetle species. Cloned partial 16S rRNA sequences for each OTU were most closely matched to the following cultivated bacteria: *Serratia* sp., *Burkholderia fungorum*, and *H. alvei* and *Phenylbacterium* sp., *Caedibacter* sp., *Spiroplasma* sp., *Enterobacter* strain B-14, and *Weissella viridescens*, representing the divisions Alpha-, Beta- and Gammaproteobacteria, Mollicutes, and Bacilli. Some, but not all of these organisms have been previously associated with insects. The identification of bacteria uniquely and consistently associated with these ground beetles provides the basis for further investigation of species-specific functional roles.

KEY WORDS biological control, endosymbiont, granivore, insect

Mutualistic symbiotic associations with microorganisms are phylogenetically widespread in insects, and the microbes serve an array of functions for their insect hosts, including nutritional services. Microbes provide deficient nutrients, or building blocks and enzymes requisite for their biosynthesis, essential to insect development and reproduction. Specific nutrients produced by microbes include essential amino acids (Prosser and Douglas 1991, Douglas and Prosser 1992, Shigenobu et al. 2000, Gil et al. 2003), precursors or enzymes essential to the generation of vitamins and micronutrients (Wicker 1983, Nakabachi and Ishikawa 1999, Aksoy 2000, Akman et al. 2002), and sterols (Norris et al. 1969, Wetzel et al. 1992, Morales-Ramos et al. 2000). Metabolic processes related to insect nutrition that are assisted by mutualistic bacteria include the storage and recycling of nitrogen (Potrikus 1981, Cochran 1985, Sasaki et al. 1996, Douglas 1998, Lauzon et al. 2000, Gil et al. 2003), cellulose digestion (Breznak and Brune 1994), sulfate assimilation (Douglas 1988, Shigenobu et al. 2000, Gil et al. 2003), and augmentation of existing host metabolic processes (Heddi et al. 1993, 1999). Ultimately, the effects of these nutritional symbionts are manifested in the development, fecundity, and survivorship of their insect hosts (Norris and Baker 1967, Douglas 1998, Nakabachi and Ishikawa 1999, Aksoy 2000).

Many of the bacterial symbionts known to play a role in insect nutrition belong to Enterobacteriaceae within the Gammaproteobacteria and less frequently to other bacterial families (Douglas 1998, Moran and Baumann 2000). These bacteria can live intracellularly within specialized tissues named bacteriocytes or bacteriotomes, or they can live extracellularly within the gut or other tissues. Intracellular bacteria are often obligate symbionts with reduced genomes that rely in part on their insect hosts for several gene products in exchange for supplying their host with some nutritional function (Gil et al. 2003, Tamas and Andersson 2003, Schaber et al. 2005). Many extracellular gut bacteria have been described previously (Ohkuma and Kudo 1996, Harada et al. 1997, Dillon et al. 2002, Broderick et al. 2004), and these species can be abundant within the guts of some insects (Cazemier et al. 1997). Compared with intracellular species, the roles of extracellular gut bacteria in host nutrition are not well understood and deserve additional study, particularly for economically important insect species.

Ground beetles (Coleoptera: Carabidae) are an important group of beneficial insects that consume a range of different foods. Most species are polyphagous carnivores of arthropods, and this group has long been

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appreciated as a source of nonchemical pest management of insect pests in cropland (Brust et al. 1986, Lovei and Sunderland 1996, Lundgren 2005a). Granivory is also widespread within Carabidae, occurring most frequently within the tribes Harpalini and Zabrini (Zhavoronkova 1969, Zetto Brandmayr 1990). Granivorous insects, including ground beetles, play an important role in shaping the density and dispersion of weed communities within agricultural systems (Cavers 1983, Crawley 2000). The bacterial symbionts that influence the feeding behavior and nutrition of ground beetles undoubtedly affect their utility as biological control agents of insect pests and weed seed banks.

Harpalus pensylvanicus (DeGeer) and *Anisodactylus sanctaecrucis* (F.) are ground beetles that occur throughout much of North America (Bousquet and Larochelle 1993), are commonly encountered in cropland (Kirk 1973, 1977; Brust et al. 1986; Ellsbury et al. 1998; Lundgren et al. 2006), and consume both insect pests (Brust et al. 1986) and weed seeds (Brust and House 1988, Lundgren 2005b). Preliminary experiments revealed that feeding *A. sanctaecrucis* and *H. pensylvanicus* diet-incorporated tetracycline hydrochloride and sorbic acid reduced consumption of weed seeds (*Chenopodium album* L.) by 40% in the laboratory relative to those fed diet without antibiotics. We hypothesize that there is a microbial contribution to seed digestion in these ground beetles.

Here, we report the first in a series of articles on the role of microbial endosymbionts in the nutrition of ground beetles. Specifically, we conducted analyses of gut DNA from individuals of *A. sanctaecrucis* and *H. pensylvanicus* by using terminal restriction fragment length polymorphism (T-RFLP) analyses of bacterial rRNA to gauge the complexity of the gut bacterial communities and to evaluate both intra- and interspecific variation of the gut communities on a small number of beetles. Also, we constructed 16S rRNA clone libraries to determine the identity of these gut bacteria. To our knowledge, there are no previous culture-independent studies of the gut bacterial communities from Carabidae.

Materials and Methods

Adults of *H. pensylvanicus* and *A. sanctaecrucis* were collected from organic farmland in Champaign, IL, during October 2004. Individuals were maintained in the laboratory before the experiment in unsterilized soil (Fer-Til, GreenGro Products, Jackson, WI) and were fed cat food (Iam's Original formula, The Iam's Company, Dayton, OH).

DNA Extraction. *A. sanctaecrucis* ($n = 6$; 17% male) and *H. pensylvanicus* ($n = 4$; 50% male) adults were collected, and the digestive tracts were dissected within 24 h. The entire alimentary canal, excluding the Malpighian tubules, was dissected in a Ringer's solution (0.75 g of NaCl, 0.35 g of KCl, and 0.28 g of $CaCl_2$ in 1 liter of water). Guts were stored at −20°C for 48 h before DNA extraction.

DNA from all gut samples was extracted using modified bead beating procedures to optimize recovery of nucleic acids. Briefly, samples were washed once with 1× phosphate-buffered saline (0.12 M KH_2PO_4 and 0.15 M NaCl, pH 8), and the biomass was resuspended in fresh buffer. Cell lysis was accomplished using mixtures of zirconia/silica beads (0.1–2.5 mm), and lysis efficiencies were assessed microscopically. The samples were shaken on a platform (MO BIO Laboratories, Inc., Carlsbad, CA) attached to a standard vortex on high speed for 2 min. Additional cell disruption procedures were applied as needed, including the addition of 750 μl of lysis buffer (0.1 M NaCl and 0.5 M Tris, pH 8) or additional grinding with a sterile stainless steel rod. Samples were split equally to recover DNA either through chemical extraction or solid phase extraction by using commercially available columns according to manufacturer's instructions (MO BIO Laboratories, Inc.) following cell lysis procedures. For chemical extraction, an equal volume of phenol/chloroform/isoamyl alcohol (24:24:1) (Sigma-Aldrich, St. Louis, MO) was added to the cell lysate, mixed well, and DNA from the recovered aqueous fraction was precipitated overnight at −80°C in 3 M sodium acetate, pH 5.2, and an equal volume of cold isopropanol (−4°C). The DNA pellet was washed once with 70% cold ethanol, air-dried, and resuspended in TE buffer (10 mM Tris and 1 mM EDTA, pH 8). The pool of DNA from each sample was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA quantities were in the range of 5–211 ng/μl.

T-RFLP Analysis. T-RFLP analysis was applied to assess the complexity of the bacterial communities residing in *A. sanctaecrucis* and *H. pensylvanicus* guts, particularly noting the intra- and interspecific variation in the guts of these beetles. The 16S rRNA genes were amplified using bacterial primers F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) labeled at the 5′ end with 6-carboxyfluorescein and 1492R (5′-GGT TACCTTGTTACGACTT-3′) (Operon, Huntsville, AL). Amplification reactions were performed in 100-μl reactions containing 50 ng of DNA, 2.5 U of *TaKaRa Ex Taq* (Takara Mirus Bio, Madison, WI), 10 μl of 10× *Ex Taq* buffer, 10 μl of dNTP mixture (2.5 mM each), 2.5 μg of T4 gene 32 protein (Roche Diagnostics, Indianapolis, IN), and 2 μl of each primer (40 pmol/μl). The samples were amplified in a DNA Engine PTC-200 Thermal Cycler (MJ Research, Waltham, MA) by using the following conditions: 94°C for 5 min, followed by 25 cycles consisting of 94°C for 1.5 min, 55°C for 1.5 min, 72°C for 1.5 min, and a final extension at 72°C for 7 min. One sample from *A. sanctaecrucis* and three samples of *H. pensylvanicus* required an additional nested reaction of 20 cycles by using 0.5–1 μl of the primary polymerase chain reaction (PCR) product under the conditions described above.

The correct size of the amplified DNA product was confirmed by gel electrophoresis, and 70–95-μl volumes were cleaned using the QIAquick PCR purification kit (QIAGEN, Valencia, CA) and eluted in 30 μl of buffer EB (QIAGEN). Purified DNA was quan-

tified spectrophotometrically, and ≈450 ng of each sample was digested overnight in separate 12-μl reactions with 10 U of HhaI (New England Biolabs, Beverly, MA) at 37°C. Fragments were analyzed at the University of Illinois W. M. Keck Center for Comparative and Functional Genomics (Urbana-Champaign, IL) with an ABI 377 genetic analyzer (Applied Biosystems, Foster City, CA) by using a mixture of 3 μl of digested PCR product and 0.5 μl of a 35-mer TET internal standard, along with Genescan-2500 5-carboxytetramethylrhodamine size standard (Applied Biosystems). Fragments were analyzed using GeneScan Analysis version 2.1 (Applied Biosystems), and peaks above a threshold value of 80 fluorescent units were determined to be above background relative to blank samples.

Cloning and Sequencing. The T-RFLP analyses indicated that the composition of gut microbial communities from both species was fairly simple and relatively consistent intraspecifically but that species-specific differences may be present. The DNA was pooled from the six *A. sanctaecrucis* and four *H. pensylvanicus* individuals used for the T-RFLP analysis. Two 16S rRNA gene clone libraries were generated to identify the populations within the gut microbiota representative of each insect species. Based on these findings, we constructed two 16S rRNA gene clone libraries from the DNA extracts described above.

Partial (≈560 bases) 16S rRNA gene sequences were amplified in a T-Gradient thermal cycler (Biometra, Goettingen, Germany) from the extracted DNA by using oligonucleotide primers 338 F (5′-ACT CCT ACG GGA GGC AGC-3′) (Amann et al. 1990) and 907R (5′-CCG TCA ATT CMT TTR AGT TT-3′) (Lane et al. 1985) (Invitrogen, Carlsbad, CA) in 50-μl reactions composed of 0.4 mg/liter bovine serum albumin (BSA) (Roche Diagnostics. Indianapolis, IN), 1× PCR buffer (Promega, Madison, WI), 2 mM $MgCl_2$, 0.5 μM each primer, 1.25 U of *Taq*DNA polymerase (Promega), 0.2 mM each dNTP (Promega), 1 μl of template DNA (≈100 ng of DNA), and molecular grade water (Promega). PCR amplification was performed using the following conditions: 94°C for 4 min; 30 cycles of 94°C for 0.5 min, 55°C for 0.5 min, 72°C for 0.5 min, and a final elongation at 72°C for 3 min. PCR products were examined with positive (*Escherichia coli* DNA) and negative controls (reagents only) in a 1.2% agarose gel to confirm specificity of the amplification reactions.

Purified PCR products (Wizard PCR prep; Promega) were cloned into *E. coli* JM109 competent cells by using the pGEM-T Easy Vector System II (Promega) per manufacturer's instructions. Inserts from randomly selected transformed colonies (47 for each beetle species library) were reamplified using the same PCR protocol modified by an initial lysing step (15 min; 99°C). Clone sequences were screened by RFLP analysis with restriction enzymes RsaI (10 U) and MspI (10 U) (New England Biolabs), 1× buffer (New England Biolabs), 1 mg/ml BSA (Roche Diagnostics), molecular grade water (Promega), and 10 μl of DNA template in 20-μl reactions at 37°C (90 min) with products resolved on 4% Metaphor agarose (Cambrex Corp., East Rutherford, NJ) gels. Plasmid minipreps (Wizardplus Minipreps, Promega) were performed on several representatives of each RFLP type, and sequencing reactions (2× coverage) were conducted using M13 F/R (sequencing performed at the Iowa State University Sequencing Facility, Ames, IA). Consensus sequences (≈550 bases) were determined based on alignments and editing performed with Bioedit 7.5 (http://www.mbio.ncsu.edu/BioEdit/page2.html). Clones representing the same RFLP pattern were grouped under a representative sequenced clone, and these groupings were further consolidated by considering all representative sequences >97% similar as the same (Speksnijder et al. 2001). Sequences representative of each clone grouping were compared against the GenBank database by using BLASTn (Altschul et al. 1997) to determine the closest database match. Chimeric sequences that were identified after screening with Chimera_Check version 2.7 RDP8.1 and Bellerophon (Huber et al. 2004) were removed from further consideration. The coverages of our clone libraries were estimated using the following equation from Marchesi et al. (2001):

$$\text{clone coverage} = 1 - (n/N) \times 100$$

where n is the number of clones only occurring once, and N is the total number of clones in the library. Unique 16S rRNA gene sequences representing the clones reported in Table 1 were deposited in GenBank under accession numbers EF154420–EF154427, EF198466.

Results

T-RFLP Analysis. T-RFLP profiles revealed that the bacterial communities existing in the beetle guts were not diverse and that profiles were relatively similar among individuals of the same species (Fig. 1). Interspecific differences in the bacterial diversity within the digestive systems of *A. sanctaecrucis* and *H. pensylvanicus* were observable. Two fragments, 369 and 379 bp, were similar between the two beetle species. Two operational taxonomic units (OTUs) could be identified from *A. sanctaecrucis*, and six OTUs were identified from *H. pensylvanicus.*

Cloning and Sequencing. Forty-five of the 47 clones from *A. sanctaecrucis* gave useable sequence information. These clones formed three groups that are closely related (>99%) to previously cultivated organisms (Table 1). Seven clones were closely related to *Burkholderia* species, and the remaining 38 clones were affiliated with the Enterobacteriaceae. The estimated coverage of this clone library was 100%.

All 47 clones from *H. pensylvanicus* gave useable sequence information. At the 97% level of sequence similarity, these clones formed six groups that are relatively closely related, with one exception, to previously cultivated organisms (Table 1). There were three groups with only a single representative, of which two were Alphaproteobacteria. The third loner was only 89% similar to a cultured database entry,

Table 1. Bacterial residents of the digestive tracts of the seed-feeding ground beetles *A. sanctaecrucis* and *H. pensylvanicus*, based on the closest cultured matches to generated sequences of the 16S rRNA gene fragment

Clone no.	Clone relative abundance	Closest cultured database match (% similarity, ≈550 bases)	Taxonomic affiliation	Accession no.[a]
A. sanctaecrucis (45 clones)				
AS-13	7	*B. fungorum* (>99%)	Betaproteobacteria	AF215706
AS-25	23	*H. alvei* (>99%)	Gammaproteobacteria (Enterobacteriaceae)	AY572428
AS-41	15	*Serratia* sp. (>99%)	Gammaproteobacteria (Enterobacteraceae)	AF286869
H. pensylvanicus (47 clones)				
HP-1	1	*Phenylobacterium koreense* (97%)	Alphaproteobacteria (Caulobacteraceae)	AB166881
HP-6	1	*Caedibacter caryophilus* (92%)	Alphaproteobacteria (Rickettsiales)	X71837
HP-7	1	*Spiroplasma* sp. BIUS-1 (89%)	Mollicutes	AY189319
HP-10	5	*Enterobacter* sp. B-14 (100%)	Gammaproteobacteria (Enterobacteriaceae)	AJ639856
HP-43	11	*W. viridescens* (97%)	Bacilli (Lactobacillales)	M23040
AS-25	28	*H. alvei* (>99%)	Gammaproteobacteria (Enterobacteriacae)	AY572428

[a] Closest cultured match in Genbank.

Spiroplasma sp. BIUS-1. Eleven of the clones were genetically similar with another gram-positive entry, *Weisella viridescens.* The remaining majority of clones were split between two groups (28 and five clones) of Enterobacteriaceae. The estimated coverage of this clone library was 94%.

The clone library from *H. pensylvanicus* had a greater taxonomic richness (six OTUs) compared with that of *A. sanctaecrucis* (three OTUs). *H. pensylvanicus* was represented by four taxonomic classes, compared with two for *A. sanctaecrucis.* Only one class, Gammaproteobacteria, was common between the two libraries, and this class was the dominant class in both species. Only one cloned OTU was shared between the two beetles, indicating a 13% similarity in the species compositions between the two libraries (Σ

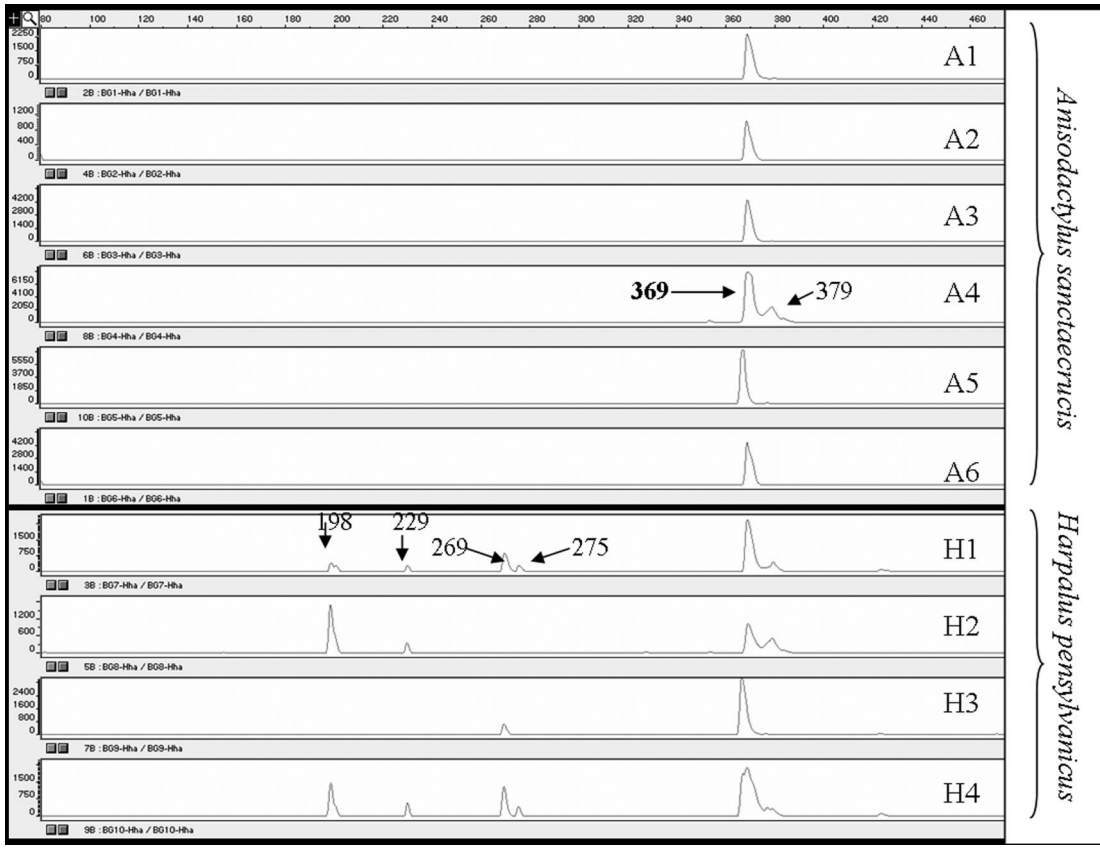

Fig. 1. T-RFLP profiles for the gut communities of the granivorous ground beetles *H. pensylvanicus* and *A. sanctaecrucis.* Each profile represents an individual beetle gut, and amplified 16S rRNA bacterial gene fragments were digested using the HhaI restriction enzyme.

shared OTU/Σ total OTU). However, this dominant OTU represented ≈50% of the total clones from each library (23/45 for *A. sanctaecrucis;* 28/47 for *H. pensylvanicus*).

Discussion

DNA-based analyses of the gut bacteria indicate the presence of beetle-specific, indigenous bacterial communities. The T-RFLP profiles revealed simple gut communities that were relatively reproducible in individuals from the same beetle species, although only a small number of insects were analyzed in each species. Clonal analyses confirmed that the bacterial communities within *H. pensylvanicus* were more diverse than in *A. sanctaecrucis*, and both beetle species possessed unique microorganisms. 16S rRNA clone libraries showed that both beetle species share a single bacterium, identified as *H. alvei* that was numerically dominant in both libraries (Table 1). An in silico digest of the T-RFLP profiles supports the assertion that the dominant peak (369 bp; Fig. 1) is likely *H. alvei* (J.C.-S., unpublished data). It is remarkable that common soil bacteria were not well represented in the guts and that the bacterial communities present in the alimentary system of ground beetles were primarily made up of species that have been retrieved from animals and plants. The sum of the observations indicates that these gut bacteria have a close association with the beetles and that they are not simply transient organisms consumed with the beetle's food.

Many of the bacteria identified from the ground beetle guts are commonly associated with animals and plants, and several of the species are related to cultivated organisms or clones previously associated with insects. *Burkholderia fungorum* has only recently been named (Coenye et al. 2001), and in addition to its association with humans, it may have a symbiotic relationship with fungi (Coenye et al. 2001). Its congener, *Burkholderia cepacia* has been isolated from the gut of a bee, *Osmia bicornis* Lividict (Mohr and Tebbe 2005). *Serratia marcescens* is known to associate with a broad range of insects that include gypsy moths (Broderick et al. 2004), field crickets (Adamo 2004), house flies (Cooke et al. 2003), termites (Adams and Boopathy 2005), locusts (Dillon et al. 2002), curculionids (McNeill et al. 2000), aphids (Saguez et al. 2005), and apple maggots (Lauzon et al. 2003). Caulobacteriaceae are frequently ingested by aquatic insects (Thanabalu et al. 1992); the closest cloned sequence to ours was isolated from the gut of the mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) (Dunn and Stabb 2005). *Spiroplasma* spp. are common residents of insect guts and hemocoel (Klein et al. 2002, Williamson et al. 1998, Hurst et al. 2003, Anbutsu and Fukatsu 2003). Although *Weisella* is otherwise not frequently associated with insects, one similar clone to our HP-47 (*Weisella viridescens*) was found in the guts of honey bees, *Apis mellifera* L. (Mohr and Tebbe 2005).

Three clones isolated in our study have not been previously associated with insects: *Hafnia alvei* (AS-25), *Caedibacter* sp. (HP-6), and *Enterobacter* B-14 (HP-10). *Caedibacter* is a polyphyletic genus related through their intracellular lifestyle within paramecia. Congeners closely related to *C. caryophilus* tend to be intracellular symbionts of amoebas and paramecia (Beier et al. 2002). Although *Hafnia alvei* and *Enterobacter* B-14 have not previously been found in associated with insects, many other Enterobactereaceae are frequently associated with insect guts (Moran and Baumann 2000, Delalibera et al. 2005, Dunn and Stabb 2005), so it is not surprising to find these species as abundant bacteria within the alimentary canals of ground beetles.

Based on our previous knowledge of the microorganisms found within the guts of ground beetles, these gut bacteria could be serving many functions, such as causing or preventing disease, degrading insecticides, and directly or indirectly contributing to food digestion. Many of the bacteria identified here are potential pathogens, such as *B. fungorum* (Gerrits et al. 2005), *H. alvei* (Sakazaki 2005), and *Serratia* spp. (especially *S. marcescens*). At subpathogenic levels, *S. marcescens* may be important in regulating the dynamics of the bacterial community within the insect gut. For example, within the Formosan termite, *S. marcescens* is a facultative anaerobe that aids in consuming oxygen at the periphery of the insect stomach, thereby maintaining a habitable gut for the strict anaerobes that digest cellulose (Adams and Boopathy 2005).

Microbes associated with insects are known to function in degrading plant defensive chemicals and insecticides (Berenbaum 1988). Several bacterial species present in ground beetle guts are known to catabolize aromatic hydrocarbons, which are commonly found in insecticidal chemicals. *Enterobacter* strain B-14 degrades chlorpyrifos and organophosphate insecticides (Singh et al. 2004), and although it has not previously been found within insect guts, it is a candidate for catabolizing the insecticides frequently encountered by these beetles in agroecosystems. Also, *B. fungorum* (Bodour et al. 2003) and a close genetic match to our *H. alvei* clone (Laramee et al. 2000) are constituents of polyaromatic hydrocarbon-degrading consortiums. Finally, Caulobactereaceae are known to degrade aromatic compounds, which are often found in some pesticides.

Microbes are frequently associated with nutritional functions in insects, and the bacterial species found in ground beetle guts may assist in digestion. Many of the Enterobacteriaceae, which constituted the majority of clones in our beetle guts, produce digestive enzymes, and have a demonstrated role in insect nutrition. For example, *Enterobacter* spp. assist nitrogen recycling in *Rhagoletis pomonella* (Walsh) by producing uricases (Lauzon et al. 2000), and *S. marcescens* and *H. alvei* are known to produce chitinases (Whitaker et al. 2004, Ruiz-Sanchez et al. 2005), which may be useful in digesting insect prey or fungi. Given the original observations of reduced seed consumption in "cured" ground beetles, this is a symbiotic function that merits additional exploration.

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