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HOST MICROBE INTERACTIONS

Bacterial Communities Associated with the Digestive Tract of the Predatory Ground Beetle, *Poecilus chalcites*, and Their Modification by Laboratory Rearing and Antibiotic Treatment

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Abstract Ground beetles such as *Poecilus chalcites* (Coleoptera: Carabidae) are beneficial insects in agricultural systems where they contribute to the control of insect and weed pests. We assessed the complexity of bacterial communities occurring in the digestive tracts of field-collected *P. chalcites* using terminal restriction fragment length polymorphism analyses of polymerase chain reaction-amplified 16S rRNA genes. Bacterial identification was performed by the construction of 16S rRNA gene clone libraries and sequence analysis. Intestinal bacteria in field-collected beetles were then compared to those from groups of beetles that were reared in the lab on an artificial diet with and without antibiotics. Direct cell counts estimated 1.5×10^8 bacteria per milliliter of gut. The digestive tract of field-collected *P. chalcites* produced an average of 4.8 terminal restriction fragments (tRF) for each beetle. The most abundant clones were affiliated with the genus *Lactobacillus*, followed by the taxa Enterobacteriaceae, Clostridia, and Bacteriodetes. The majority of the sequences recovered were closely related to those reported from other insect gastrointestinal tracts. Lab-reared beetles produced fewer tRF, an average of 3.1 per beetle, and a reduced number of taxa with a higher number of clones from the family Enterobacteriaceae compared to the field-collected beetles. Antibiotic treatment significantly ($p<0.05$) reduced the number of tRF per beetle and selected for a less diverse set of bacterial taxa. We conclude that the digestive tract of *P. chalcites* is colonized by a simple community of bacteria that possess autochthonous characteristics. Laboratory-reared beetles harbored the most common bacteria found in field-collected beetles, and these bacterial communities may be manipulated in the laboratory with the addition of antibiotics to the diet to allow study of functional roles.

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Introduction

Studies of associations between microorganisms and selected insects have sharpened appreciation for interspecies dependencies and revealed specific roles for some of the participants in these interactions [4]. In addition to the enrichment of fundamental ecological knowledge, studies of insect–microbe interactions have produced advancements in applications as diverse as biological control of insect pests [11, 29] and energy production strategies [35]. During the past decade, increased availability of culture-independent research tools has provided new perspectives on historically studied insect–microbe systems [3, 5, 13, 16, 30, 34] and stimulated microbiological studies of additional insects that have expanded known microbial diversity [6, 9, 12, 14, 22, 28, 33].

Ground beetles (Coleoptera: Carabidae) are beneficial insects in agricultural systems where they contribute to the control of insect and weed pests [18, 25, 32, 36, 39], and they are viewed as positive indicators of habitat quality [24, 31]. In an effort to extend understanding of beneficial

ground beetle ecology and the factors that may influence their abundance and fitness, we are investigating the bacterial communities inhabiting their digestive tracts. Previous research on nonpathogenic intestinal bacteria in insects has been reviewed [10], and there is little information regarding Carabidae and none using culture-independent approaches except our own study [28] that reported the composition of bacterial communities from the digestive tract of two preferentially granivorous ground beetles, *Anisodactylus sanctaecrucis* and *Harpalus pensylvanicus*.

A. sanctaecrucis and *H. pensylvanicus* are common in North American agricultural fields where their granivory results in the reduction of weed seed numbers [27, 36, 39]. In our prior study, we found that the digestive tracts of individual field-collected beetles within each of these two species had reproducible bacterial gut communities composed of three to six operational taxonomic units [28]. Preliminary evidence from trials to establish standard lab-rearing conditions for these two beetles showed that beetles receiving diet-incorporated tetracycline hydrochloride and sorbic acid consumed 40% less weed seeds compared to beetles not receiving antibiotics (J.G. Lundgren, unpublished observations). The addition of antibiotics with diet in lab-reared insects is common practice to preserve food and standardize rearing conditions. Among potential explanations, the association of antibiotics in the diet with reduced feeding suggests a nutritional role for the intestinal bacteria in these two ground beetles. To enable the testing of hypotheses regarding gut bacterial function in these two granivorous ground beetles and its potential relationship with diet, we employed a third ground beetle, *Poecilus chalcites*, that is strictly predaceous.

P. chalcites is a common predaceous beetle in North American agricultural settings and feeds on other arthropods and their eggs, such as cricket eggs. Standard rearing protocols for *P. chalcites* have been developed to facilitate its use for testing non-target effects for insecticides including genetically engineered, insect-resistant crops [26]. During that study, it was found that *P. chalcites* fed diet-incorporated antibiotics and reared in sterile substrate seldom reached adulthood versus those reared in unsterilized media. Since there are no data on the intestinal bacteria of *P. chalcites*, we initiated our studies with the following experimental objectives: (1) assess the complexity of bacterial communities in the intestines of field-collected single beetles and the variation in these bacterial communities among individual beetles using terminal restriction fragment length polymorphism (tRFLP) analysis of extracted and polymerase chain reaction (PCR)-amplified 16S rRNA genes, (2) assess the diversity and taxonomic affiliation of bacteria present within field-collected *P. chalcites* intestines using sequence analysis of amplified 16S rRNA genes, (3) determine the effect of laboratory beetle rearing on (1) and (2); and (4) determine the effect of antibiotic treatment on (1) and (2).

Materials and Methods

Beetle Collection and Handling

Fifty-eight *P. chalcites* were collected during a 2-week period in July–August of 2005 using dry pit fall traps in a soybean field in Brookings, SD, USA (latitude 44.35° N, longitude 96.81° W). Three groups of beetles were established: (1) "field-collected"—a group of 15 beetles that were freeze-killed and processed immediately following collection from the field, (2) "lab control"—a control group of 20 beetles that were fed an artificial diet [26] in the laboratory, and (3) "lab treatment"—a treatment group of 20 beetles that were fed the artificial diet with incorporated antibiotics (tetracycline, rifampycin, and sorbic acid, 0.04% *w*/*w*) that are commonly used to preserve artificial insect diets and to cure insects of selected microorganisms. The remaining three beetles were used to estimate total bacterial cell counts in the intestines as described below. All laboratory-reared beetles were sacrificed and processed after 2 weeks in the laboratory. All beetles were submersed in ethanol (70%) and aseptically dissected to remove the complete intestinal tract (fore-, mid-, and hindguts minus the Malpighian tubules) which was surface-sterilized (70% ethanol) and preserved in sterile Ringer's solution (0.75 g NaCl, 0.35 g KCl, 0.28 g $CaCl_2$ per liter, pH 7.4) at −80°C for direct DNA extraction. Five beetles were lost during lab rearing and dissections, leaving 18 and 17 beetles in the control and antibiotic-treated groups, respectively.

DNA Extraction

Frozen, excised whole intestines were thawed on ice, washed three times in sterile phosphate-buffered saline (PBS: 1.18 g Na_2HPO_4, 0.223 g $NaH_2PO_4{\cdot}H_20$, and 8.5 g NaCl per liter; pH 7.5) and macerated with a sterile polypropylene micropestle. DNA was extracted from each intestine using the BIO101 FastDNA SPIN kit (Qbiogene, Carlsbad, CA, USA) per manufacturer's instructions. Each set of DNA extractions were accompanied by a negative extraction control (no intestine), and results were screened on a 0.7% agarose gel (100 V, 25 min). Extracted DNA was purified with a Microcon YM-30 centrifugal filter (Millipore, Billerica, MA, USA) using the following conditions: 12,000×*g*, 20 min, 4°C.

Bacterial Cell Enumerations

The aseptically dissected intestinal tracts from three field-collected *P. chalcites* were fixed in ethanol (70%) and held

at −20°C for estimates of total bacterial cell counts. Each gut was washed in PBS (three times), mascerated with a sterile micropestle, vortexed with 1 ml 0.1% sodium pyrophosphate, sonicated (45 s, 125 W, 47 kHz) on ice, and re-vortexed prior to serial dilution in PBS. Aliquots of the gut suspension were filtered under vacuum onto 0.2-μm pore-size, black, polycarbonate membrane filters with cellulose-acetate support filters [17]. Cells concentrated on filters were stained with DAPI (0.01%, 3 min), washed, dried, and mounted in immersion oil (Cargille FF, Cargille Laboratories, Cedar Grove, NJ, USA) under a glass coverslip. Total bacterial cells were enumerated under epifluorescent illumination using a Leica DM LB2 microscope equipped with a ×100 objective, 100-W mercury bulb, and filter set for DAPI (Chroma #31000, Chroma Technology, Rockingham, VT, USA). A minimum of five fields and 200 cells were counted or 20 fields when 200 cells were not achieved. Counts were conducted in triplicate for each intestine and averaged.

Terminally Labeled Restriction Fragment Length Polymorphism

Nearly full-length 16S rRNA genes were PCR-amplified in triplicate from the purified DNA extracted from each gut using universal eubacterial primers 8F (5′-AGAGTTTGA TCCTGGCTCAG-3′) labeled with 6-carboxyfluorescein (FAM) at the 5′ terminus and 1492R (5′-GGTTACCTTGTT ACGACYT-3′) [21] for tRFLP analysis [23]. PCR reactions (50-μl) were composed of 0.4 mg/l bovine serum albumin (BSA; Roche Diagnostics, Indianapolis, IN, USA), 1× PCR buffer (Promega, Madison, WI, USA), 2 mM $MgCl_2$, 0.5 μM of each primer, 1.25 U Taq DNA polymerase (Promega), 0.2 mM each dNTP (Promega), 1 μl template DNA (approximately 25 ng DNA), and molecular-grade water (Promega). PCR amplification was performed in a T-Gradient thermal cycler (Biometra, Goettingen, Germany) using the following conditions: 94°C (4 min); 30 cycles of 94°C (0.5 min), 55°C (0.5 min), 72°C (0.5 min); and a final elongation at 72°C (3 min). PCR products were screened on 1.2% agarose gel (75 V, 45 min) for the expected size product along with a size ladder and positive (*Escherichia coli* DNA) and negative (reagents only) controls. Triplicate PCR products from each gut were then combined, purified (Wizard PCR preps, Promega), and quantified by absorbance at 260 nm. Combined, FAM-labeled PCR products for each gut were then restricted in triplicate (350 ng product; 10 U *Rsa*1 and 1× NEB1 buffer, New England Biolabs, Beverly, MA, USA; molecular-grade water, Promega) at 37°C (180 min) and terminated at 75°C (20 min). The triplicate digests with positive and negative controls were analyzed by capillary electrophoresis using a G5 filter and LIZ1200 size standards on an ABI Prism 3100 (Applied Biosystems, Foster, CA, USA) operated under ABI's recommended run parameters. The resulting electropherograms were analyzed with Genemarker 1.5 (SoftGenetics, State College, PA, USA) using the Local Southern method of size calling, a threshold of 40 RFU, a fragment range of 64–910 bases, and a peak window of 2 bp. Consensus terminal restriction fragment (tRF) profiles for each gut sample were prepared from the triplicate profiles using presence/absence and majority criteria.

16S rRNA Gene Clone Libraries

To determine the identity of bacterial populations existing within the digestive tracts of the beetle group, three 16S rRNA gene clone libraries were constructed from pooled DNA extracted from all the individual beetles within a group. Near full-length (approximately 1,450 bases) 16S rRNA gene sequences were amplified in triplicate from the pooled DNA extracts using primers 8F (unlabeled) and 1492R under the conditions and with the controls described previously for tRFLP. Triplicate PCR products (for each library) were combined, quantified (abs 260 nm), purified (Wizard PCR prep; Promega) and cloned into *E. coli* JM109 competent cells using the pGEM-T Easy Vector System II (Promega) per manufacturer's instructions. Inserts from randomly selected transformed colonies (95 for each library except the antibiotic treatment group which only yielded 40) were re-amplified by the same PCR protocol modified by an initial cell-lysing step (15 min, 99°C) and reduced number (25) of cycles. Clone sequences were screened by restriction fragment length polymorphism (RFLP) analysis using restriction enzymes *Rsa1* (10 U) and *Msp1* (10 U) (New England BioLabs), 1× buffer (New England BioLabs), 1 mg/ml BSA (Roche), molecular-grade water (Promega), and 10 μl DNA template in 20 μl reactions at 37°C (90 min) with products resolved on 4% Metaphor agarose (Cambrex) gels. For each RFLP type, several representative clones were selected, plasmid miniprep performed (Montage $Miniprep_{96}$, Millipore), and sequencing reactions were conducted using the primers M13F (5′-TGTAAAACGACGGCCAGT-3′), 530F (5′-GTG CCAGCMGCCGCGG-3′), and 1100F (5′-GCAACGAG CGCAACCC-3′) on an Applied Biosystems 3730xl DNA Analyzer. Nearly full-length sequences were edited and assembled within BioEdit 7.5 freeware (http://www.mbio.ncsu.edu/BioEdit/page2.html) and aligned with ClustalW. Clones representing the same RFLP pattern were grouped under a representative sequenced clone, and these groupings were further consolidated by considering all representative sequences more than 97% similar as within the same operational taxonomic unit ($OTU_{0.97}$) [37] using the program Grouper 1.0.3 (Andrew Shewmaker, Idaho National Laboratory). Chimeric sequences that were identified

following screening with Chimera_Check ver. 2.7, RDP8.1, Bellerophon, [20], and Mallard [2] were removed from further consideration. Unique, representative sequences for each $OTU_{0.97}$ were compared with entries in the GenBank database using BLASTn [1] to determine the closest database match. Unique sequences were deposited in GenBank under the following accession numbers: EF608508–EF608552. Calculations of diversity indices, the Chao1 estimator and rarefaction curves, were performed using FastGroupII [40]. The 32 representative clones were also each analyzed using the tRFLP procedures described above.

Results

Observations on Gut Structure

The digestive tract of *P. chalcites* consists of fore-, mid-, and hindgut compartments plus Malpighian tubules. The foregut is well-developed and contains brown fluid likely used during extra-oral digestion. The midgut is lined externally with numerous fingerlike caecae of unknown function. The hindgut is spiraled and is equal in length to the midgut. Malpighian tubules were excised from the dissected guts because they easily became removed during the dissection process and might have caused differential results among the beetles in the final analyses.

Terminally Labeled Restriction Fragment Length Polymorphism Analysis Performed on Individual Beetles

The number of terminal restriction fragments observed within each beetle gut was <10 per beetle for all of the 50 beetles analyzed (Fig. 1). The field-collected beetles had an average of 4.8 (±2.4, one standard deviation) tRF per gut. The lab control group had a lower average tRF per gut (3.1± 1.7) than the field-collected group, although this difference was not statistically significant (p=0.097, one-way analysis of variance of square-root transformed data, Tukey's post hoc test). Beetles from the lab treatment group averaged only 1.6 tRF per gut, which was significantly less than the field-collected (p<0.001) and lab control (p=0.040) groups. No tRF peaks were identified for three of the beetles receiving antibiotics.

A total of 23 unique tRF were observed to occur in at least one beetle within the field-collected group (Fig. 2). Of these 23 tRF, 11 were observed in only one beetle. From the remaining 12 tRF, there were three tRF (892, 311, and 417) that occurred in at least 50% of the beetles. The beetles in the lab control group had a total of 17 tRF that included the most abundant 12 tRF from the field-collected group with the exception of 911, only three of the 11 least abundant tRF, and five tRF were that had not been previously

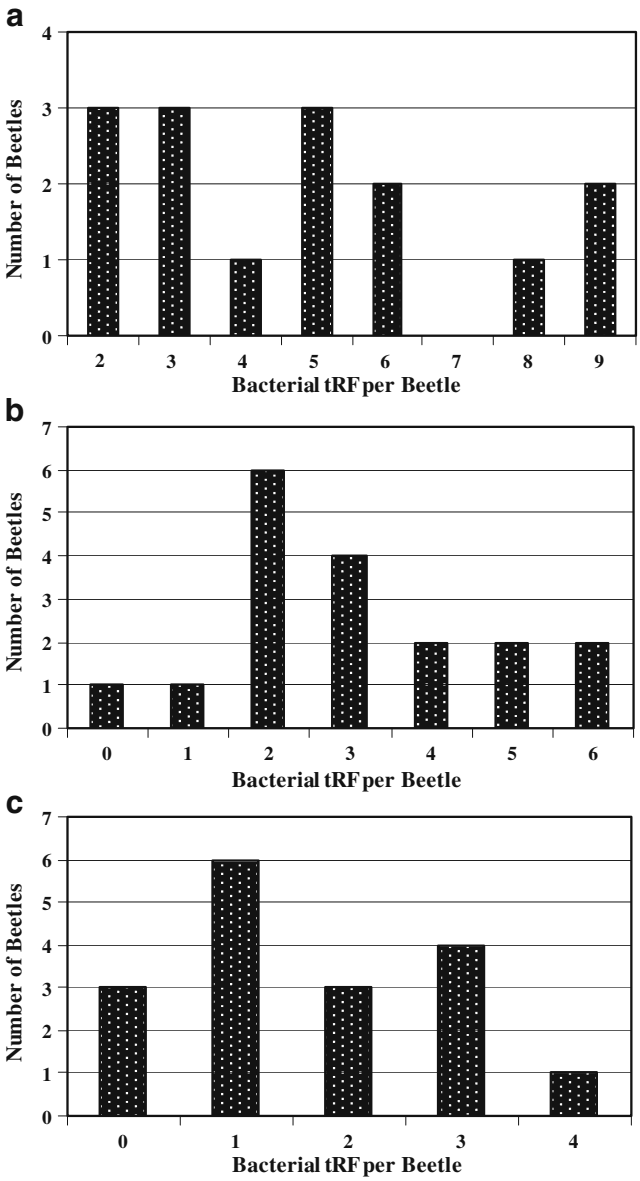

Figure 1 The distribution of bacterial terminal restriction fragments per beetle. **a** Field-collected beetles (n=15); **b** lab control (no antibiotic) beetles (n=18); and **c** lab treatment beetles (n=17)

observed. The three tRF (892, 311, and 417) that were most common in the field-collected group were still present in the lab control group; however, tRF 883 and 872 occurred in the highest proportion of these beetles that were reared in the laboratory. Only eight tRF were observed from all the beetles reared in the lab that received antibiotics with their diet (lab treatment). Of these eight tRF, tRF 417 and 883 were most abundant, and only one tRF had not been previously observed (475), and it occurred in only one beetle.

Using a Jaccard similarity coefficient to indicate the number of shared bacterial tRF between beetle groups, the highest similarity was between field-collected and lab

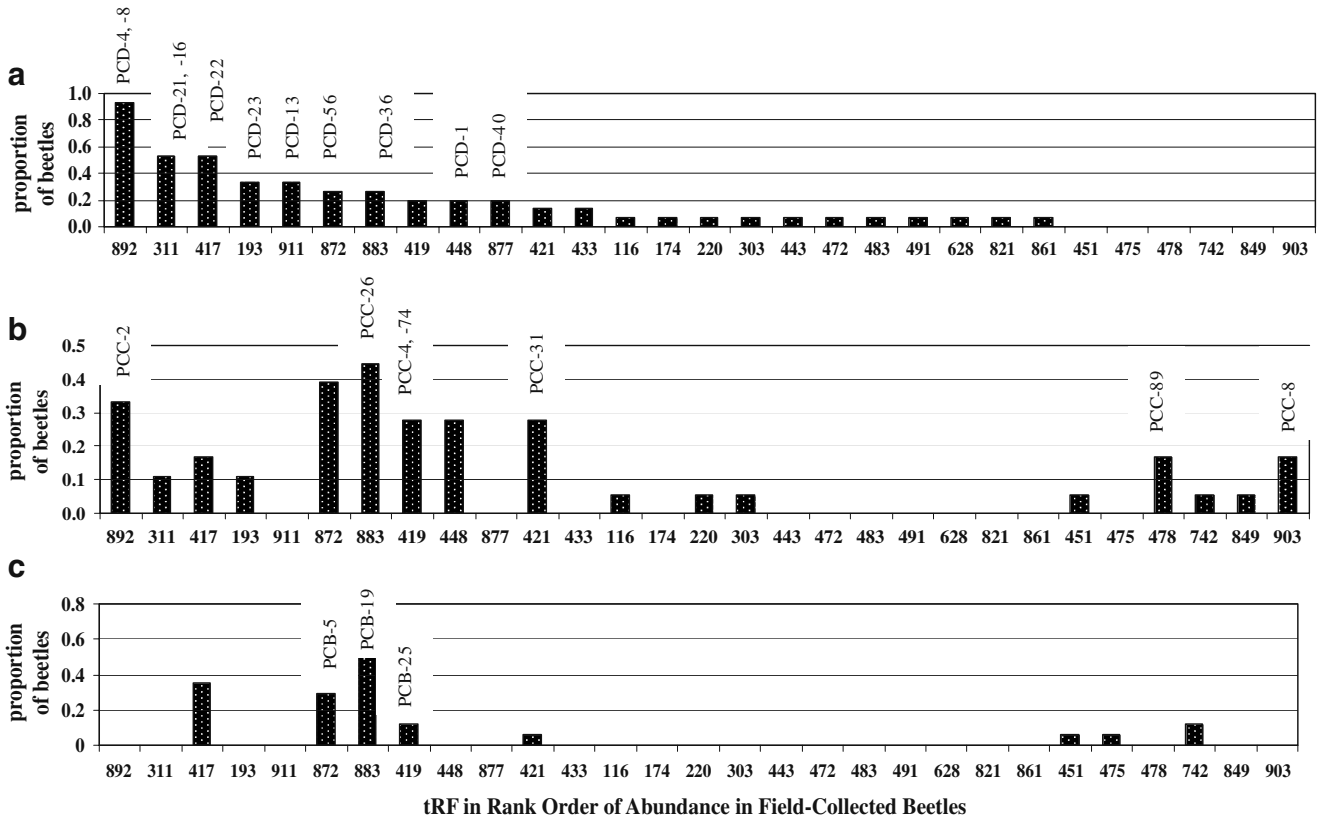

Figure 2 The proportion of beetles within each group containing each tRF. The tRF are arranged in rank order of abundance as they were observed in the field-collected beetles. tRF comprising >0.1 proportion of the beetles within each group are labeled with the clone from that group's library that had the same fragment length (if such a clone existed). **a** Field-collected beetles; **b** lab control (no antibiotic) beetles; and **c** lab treatment beetles

control beetles which shared 43% of the tRF that occurred in one of the two groups. There was similar homology (39%) between the lab control Group and the lab treatment group, but only 19% of the tRF were shared between the field-collected and lab treatment groups.

16S rRNA Gene Clone Libraries Constructed for the Three Groups of Beetles

Ninety-two of the 95 clones picked for the field-collected beetle group provided useful sequence information and formed the 19 $OTU_{0.97}$ that were affiliated with six taxonomic classes: Gammaproteobacteria, Bacilli, Clostridia, Bacteriodetes, Fusobacteria, and Alphaproteobacteria (Table 1). Most of the Bacilli and Gammaproteobacteria had strong matches to cultured entries in Genbank. In contrast, the Bacteriodetes, Clostridia, and lone Alphaproteobacteria had lower similarities to previously cultured organisms.

The top three most abundant clones in the library were affiliated with the class Bacilli of which two (PCD-4 and PCD-8) most closely matched lactic-acid-producing Enterococci that are found in insect (e.g., termite) intestinal tracts. Clones PCD-4 and -8 both had tRF of 892 bp, which was observed in the highest (93%) proportion of the field-collected beetles (Fig. 2). The other abundant Bacilli clone, PCD-13, was most similar to *Weissella cibaria* that are associated with lactic acid production in the food industry and have been isolated from humans. A fourth clone (PCD-36) affiliated with the class Bacilli most closely matched a *Lactococcus garvaeae* isolate (AF352166) which was "the predominant lactic-acid-producing bacteria in camels" and was also related (>99%) to a bacterium (AY946285) that was recovered from fire ant digestive tracts [22]. PCD-1, the fourth most abundant clone in the field-collected group, most closely matched *Sebaldella termitidis* (M58678), an acetate-producing bacterium isolated from termites. The closest cultured match to the Bacteriodetes-affiliated clones PCD-21 and PCD-74 was *Dysogomonas capnocytophagoides*; however, an uncultured bacterial sequence (AJ576338) recovered from the gut of a scarab beetle was a closer match [13]. Clones PCD-24, -27, -60, and -66 were affiliated with the Clostridia and have few close cultivated matches, one of which was *Sporobacter termitidis* (Z49863), an acetate-producing bacterium isolated from termites. PCD-66 most closely matched a Clostridia clone (AB088981) retrieved from the termite gut [19]. The best

Table 1 Clone identities for the field-collected group in order of relative abundance

Clone	Relative abundance (of 92)	Class affiliation	Closest cultured match, GenBank Accession number	Similarity (approximately 1,450 bases; %)	tRF (bases)
PCD-4	20	Bacilli	*Enterococcus casseliflavus*, AF039903	95.0	892
PCD-13	14	Bacilli	*Weissella cibaria*, AJ422031	95.9	911
PCD-8	13	Bacilli	*Enterococcus faecalis*, AF515223	99.9	892
PCD-1	8	Fusobacteria	*Sebaldella termitidis*, M58678	95.8	448
PCD-40	7	Gammaproteobacteria	*Xanthomonas* sp., DQ268763	97.1	877
PCD-56	6	Gammaproteobacteria	*Pragia fontium*, AJ233424	96.9	872
PCD-36	4	Bacilli	*Lactococcus garvieae*, AF352166	99.9	883
PCD-21	4	Bacteriodetes	*Dysgonomonas capnocytophagoides*, U41355	93.8	311
PCD-22	3	Gammaproteobacteria	*Citrobacter freundii*, AJ233408	99.7	417
PCD-24	2	Clostridia	*Lachnospiraceae* 19gly4, AF550610	94.7	457
PCD-27	2	Clostridia	*Anaeroarcus burkinensis*, AJ010961	91.7	491
PCD-70	2	Gammaproteobacteria	*Morganella* sp. JB-T16, AJ781005	95.8	869
PCD-23	1	Gammaproteobacteria	*Pseudomonas* sp., AF441203	93.2	193
PCD-16	1	Bacteriodetes	*Rikenella microfusus*, L16498	91.7	311
PCD-59	1	Bacteriodetes	*Alistipes massiliensis*, AY547271	93.3	67
PCD-60	1	Clostridia	*Clostridium saccharolyticum*, Y18185	93.1	486
PCD-66	1	Clostridia	None	n/a	465
PCD-68	1	Alphaproteobacteria	*Magnetospirillum magnetotacticum*, Y10110	86.8	821
PCD-74	1	Bacteriodetes	*Dysgonomonas capnocytophagoides*, U41355	91.7	872

cultured matches for clones PCD-16 and PCD-59 were affiliated with the genus *Rikenella* which is commonly isolated from gastrointestinal environments. PCD-23, a Gammaproteobacteria, only had a 93.2% match to a cultured database entry but a 96.9% match to a clone recently recovered from a honeybee [3]. PCD-68, the only Alphaproteobacteria found in any of the libraries, distantly (86.8%) matched the sequence of *Magnetospirillum magnetotacticum*.

The ninety-two clones (three clones did not produce useful sequence data) of the lab control group formed only nine $OTU_{0.97}$ (Table 2), and the top four most abundant clones were all Gammaproteobacteria. The clones PCC-26, -74, and -51 had the exact same closest cultured GenBank matches as $OTU_{0.97}$ in the field-collected library, and two other clones (PCC-4, -74) shared a high degree of similarity with counterparts in the field-collected library. The most abundant $OTU_{0.97}$ (PCC-89), a Coxiellaceae, comprised 28% of the lab control library and was most similar (98.8%) to an endosymbiont of a Collembola [9], an unclassified Coxiellaceae. No clones similar to PCC-89 were observed in the field-collected library. The closest cultured match to the abundant PCC-31 was *Halfnia alvei*, AY572428.

The 39 clones (one clone did not produce quality sequence) of the lab treatment library fell into four $OTU_{0.97}$ with a Gammaproteobacteria being most abundant followed by a Bacilli (PCB-19) that closely matched *Lactococcus garvieae*, which was also moderately abundant in the field-collected and lab control libraries (Table 3).

Based on diversity indices including the Chao1 estimator, the field-collected library was more diverse than the lab control, which was in turn more diverse than the lab treatment library (Table 4). Estimates of library coverage

Table 2 Clone identities for the lab control group in order of relative abundance

Clone	Relative Abundance (of 92)	Class Affiliation	Closest Cultured Match, GenBank Accession#	Similarity (approximately 1,450 bases; %)	tRF (bases)
PCC-89	26	Gammaproteobacteria	Unclassified *Coxiellaceae* AF327558	98.8	478
PCC-4	19	Gammaproteobacteria	*Citrobacter amalonaticus*, AF025370	99.7	419
PCC-31	16	Gammaproteobacteria	*Hafnia alvei*, AY572428	99.4	421
PCC-9	11	Gammaproteobacteria	*Serratia fonticola*, AF286869	96.7	220
PCC-8	8	Bacilli	*Lactobacillus letivazi*, AJ417738	99.9	903
PCC-26	6	Bacilli	*Lactococcus garvieae*, AF352166	99.7	883
PCC-74	3	Gammaproteobacteria	*Morganella* sp, AJ781005	91.7	419
PCC-2	2	Bacilli	*Enterococcus silesiacus*, AM039966	99.7	892
PCC-51	1	Bacilli	*Weissella cibaria*, AJ422031	99.8	911

Table 3 Clone identities for lab treatment group in order of relative abundance

Clone	Relative Abundance (of 39)	Class Affiliation	Closest Cultured Match, GenBank Accession#	Similarity (approximately 1,450 bases, %)	tRF (bases)
PCB-25	23	Gammaproteobacteria	*Enterobacter cloacae*, AJ251469	98.4	419
PCB-19	12	Bacilli	*Lactococcus garvieae*, AF352166	99.9	883
PCB-5	3	Gammaproteobacteria	*Serratia marscesens*, AF124040	99.7	872
PCB-9	1	Bacilli	*Bacillus fusiformis*, AY907676	99.9	448

and species rarefaction plots (Fig. 3) support the effectiveness of the sampling. In terms of specific compositional elements, the field-collected library was most complex of the three libraries, with equal numbers of $OTU_{0.97}$ from each of the four most abundant classes (Fig. 4a) and Bacilli comprising a majority (55.5%) of the total clones (Fig. 4b). The lab control and lab treatment libraries had a similar gross composition that was divided nearly equally between the classes Gammaproteobacteria and Bacilli in terms of number of $OTU_{0.97}$ and dominated by Gammaproteobacteria in terms of relative clone abundances. Statistical comparison of library composition using the Ribosomal Database Project Library Compare probability analysis [38] demonstrated that significant ($p \leq 0.05$) differences between the field-collected and lab control libraries were due to differences in the numbers of sequences affiliated with the family Enterobacteriaceae and in the presence/absence sequences from the family Coxiellaceae and the class Clostridia. No significant differences were observed between the composition of the lab control and lab treatment libraries.

tRF peaks for all 32 clones representing each $OTU_{0.97}$ were determined and presented with their taxonomic affiliations (Tables 1, 2 and 3) and also adjacent to the observed tRF abundance in the individual beetles (Fig. 2). Only four of the 32 clones had tRFs that were not represented in the tRF analyses performed on the individual beetles, and three of these were Clostrida (PCD-24, -60, -66). All tRFs that occurred in more than a single beetle could be matched with a clone from either the field-collected or lab control libraries, with two minor exceptions (tRF 433 appeared in two beetles and tRF 742 in three beetles). tRFs 417, 419, and 421 were maintained separately to uphold the 2 bp window used in data analysis; however, all clones that possessed these tRF were from closely related Gammaproteobacteria. Moreover, many of these Gammaproteobacteria with tRF peaks between 417 and 421 bp also produced a minor peak at 872 bp, which we also observed with our *E. coli* positive controls (data not shown).

Bacterial Cell Enumeration

An average of 5.92×10^6 ($\pm 3.54 \times 10^6$, one standard error, $n=3$) bacteria cells per gut were enumerated. Assuming a fresh gut weight of 40 mg and a density of one, then there were 1.48×10^8 bacteria ($\pm 8.85 \times 10^7$) per milliliter of gut.

Discussion

tRFLP analyses performed on individual beetles showed that wild *P. chalcites* gut bacterial communities were simple, producing an average of five tRF per beetle. Analysis of the clone library derived from the 15 wild beetles reinforced this conclusion, producing only 19 $OTU_{0.97}$ and a Shannon–Weiner diversity index of 2.46 compared to values between 3 and 4 reported for the hindgut of crane fly larvae [8]. The low complexity of *P. chalcites* gut communities were similar to that reported for intestines from the carabids *H. pensylvanicus* and *A. sanctaecrucis* [28], honeybees [3], aphids [16], ant lions

Table 4 Diversity indices for the 16S rRNA gene clone libraries using $OTU_{0.97}$

Beetle group	N[a]	S[b]	ChaoI[c]	Shannon–Weiner diversity index, H	Evenness, H/H_{max}[d]	Library Coverage[e]
Field-collected	92	19	27	2.46	0.84	0.93
Lab control	92	9	9	1.88	0.85	0.99
Lab treatment	39	4	NA	0.97	0.70	0.97

NA Not applicable (cannot be calculated because there were no doubletons)
[a] Number of clones
[b] Observed number of $OTU_{0.97}$ groups
[c] Chao1$=S+(n_1)^2/2n_2$, where n_1 is the number of singletons and n_2 is the number of doubletons
[d] $H_{max}=\ln(S)$
[e] Calculated using Good's equation: coverage$=1-(n_1/N)$

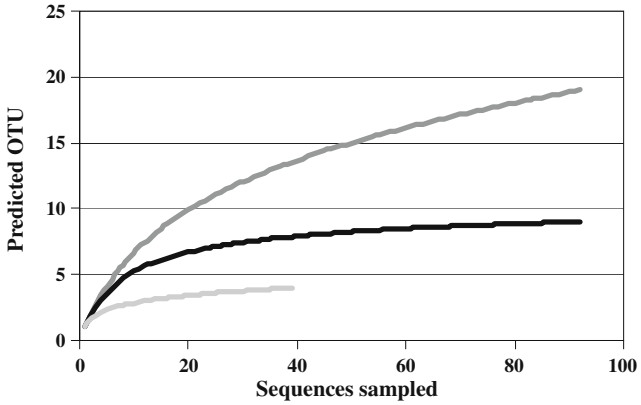

Figure 3 Rarefaction analysis of the bacterial 16S rRNA gene clone libraries from the three groups of beetles. The *darker gray line* represents the field-collected beetles; the *black line* represents the lab control beetles; and the *lighter gray line* represents the lab treatment beetles

[12], and gypsy moth larvae [6]. On the other hand, termites [34] and scarab beetle larvae [13] are reported to have much greater diversity of gut bacteria.

The use of tRFLP on low complexity communities has been shown to be particularly appropriate [15] and allows the assessment of variation among individual beetles. We found a distribution of specific tRF peaks across the individual *P. chalcites* with few of the tRF occurring at higher frequency within the continuum. This level of variability in the bacterial populations among individuals of the same species is similar to that observed within several bee species using single-strand conformation polymorphism (16S rRNA genes) analysis [30], within ant lions using clone abundances [12], within aphids using tRFLP profiles [16], or within honeybees using tRFLP [3]. In contrast, tRFLP profiles of individual field-collected granivorous carabids *H. pensylvanicus* and *A. sanctaecrucis* shared the majority of tRF peaks [28], and those of individual gypsy moth larvae were very uniform [6]. However, the gypsy moth larvae were raised from eggs under uniform laboratory conditions.

The taxa we found in the field-collected beetles Enterobacteriaceae and the other Gammaproteobacteria, Bacteriodetes, Lactobacillae, and Clostridia are consistently associated with animal hosts and frequently observed in clone libraries from arthropod digestive tracts [3, 6, 12, 13, 28, 30, 34]. The closest matches in the GenBank database for the majority of all clones have been isolated or cloned from insects, among other sources. Many of the clones are affiliated with taxa containing organisms that have facultative or obligate anaerobic physiologies and participate in organic acid production. No clones affiliated with Actinobacteria, common gram-positive soil bacteria, were found in any of the libraries. The number of bacteria per milliliter gut we estimated, 1.48×10^8 ($\pm 8.85 \times 10^7$), should be considered a minimum as the extraction and suspension of bacteria from the gut tissue were likely to be <100%. While the cell counts were only performed on three beetles, these values are within the range reported for the intestines of other arthropods in which bacterial roles have been studied [7, 12]. Together, these observations indicate that the bacteria are residents with potential functional relationships with their host.

We are only aware of one other study of carabid gut bacteria using culture-independent methods. A *Weissella* sp. found in the ground beetle *H. pensylvanicus* [28] was very similar to the *W. cibaria* clone (PCD-13) that was abundant in the field-collected group and also represented in the lab control group (PCC-51). The most abundant clone (*H. alvei*) reported in the carabids *H. pensylvanicus* and *A. sanctaecrucis* [28] was nearly identical to the clone PCC-31 that was abundant in the lab control library.

Laboratory rearing imposed a different and less diverse diet on the beetles than in the field. The microbial communities in other insects such as the gypsy moth larvae [6] and termites [5] respond to different diets. From the 11 tRF observed in only one of the field-collected beetles, eight were not observed in any lab-reared beetles, and five of these rare tRF were produced from the same beetle. These rare tRF may have been food-related, especially

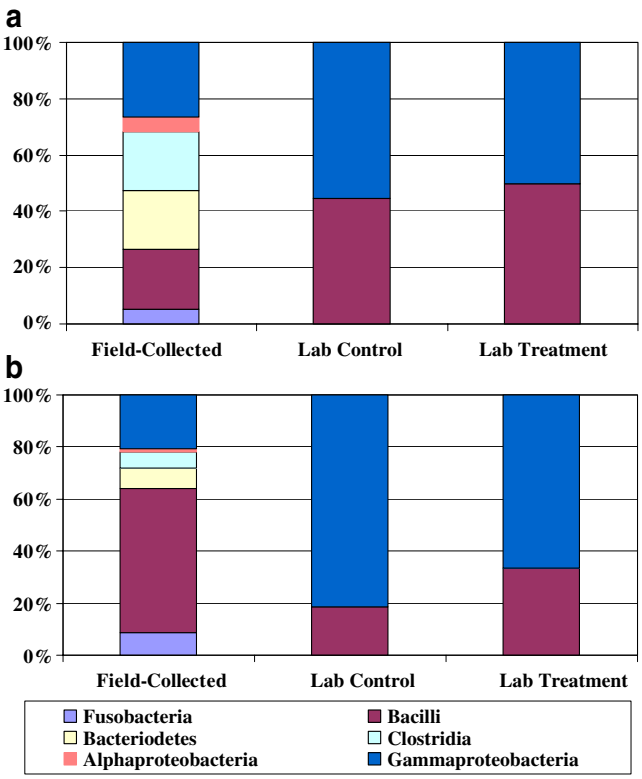

Figure 4 Class affiliation of clones for each of the three beetle groups. **a** As a percentage of the number of $OTU_{0.97}$ with in each library. **b** As a percentage of the total clones within each library

since there were no clones that possessed a matching tRF. Nonetheless, laboratory rearing only resulted in the loss of one relatively abundant peak (911) observed in the wild beetles; however, a clone from the lab control library (PCC-51) possessed this same-size tRF. In comparisons of lab-reared and wild aphids [16] or honeybees [3], little effect of lab rearing was detected on the bacterial intestinal populations using 16S rRNA gene analyses. The antibiotic-treated beetles clearly had a lower diversity of intestinal bacteria compared to their lab-reared controls, with an otherwise equivalent diet. Three of the 17 surviving beetles receiving antibiotics did not produce a single tRFLP peak. The ability to manipulate beetle gut bacteria with a combination of diet and antibiotics should provide a means to determine function of these bacteria.

In conclusion, we have found that digestive tract of the ground beetle, *P. chalcites*, is colonized by a simple bacterial community that possesses autochthonous characteristics. Based on the taxonomic affiliation of many of the clones, at least one possible function is nutritional—the production of organic acids from more complex substrates. The characterization of the gut bacteria of the predaceous ground beetle *P. chalcites* allows laboratory manipulative studies using diet and antibiotics to investigate the roles of gut microbes on a comparative basis with the two granivorous ground beetles, *H. pensylvanicus* and *A. sanctaecrucis*, that we have previously characterized. The bacterial 16S rRNA gene sequences generated during the current study permits analysis of in situ bacterial population abundances of individual gut compartments (fore-, mid-, and hindguts) using in situ hybridization or quantitative PCR methods.

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References

1. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 25:3389–3402
2. Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ, Weightman AJ (2006) New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. Appl Environ Microbiol 72:5734–5741
3. Babendreier D, Joller D, Romeis J, Bigler F, Widmer F (2007) Bacterial community structures in honeybee intestines and their response to two insecticidal proteins. FEMS Microbiol Ecol 59:600–610
4. Bourtzis K, Miller TA (2003) Insect Symbiosis. CRC, Boca Raton
5. Brauman A, Dore J, Eggleton P, Bignell D, Breznak JA, Kane MD (2001) Molecular phylogenetic profiling of prokaryotic communities in guts of termites with different feeding habits. FEMS Microbiol Ecol 35:27–36
6. Broderick NA, Raffa KF, Goodman RM, Handelsman J (2004) Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. Appl Environ Microbiol 70:293–300
7. Cazemier AE, Hackstein JHP, Op den Camp HJM, Rosenberg J, van der Drift C (1997) Bacteria in the intestinal tract of different species of Arthropods. Microb Ecol 33:189–197
8. Cook DM, Henricksen ED, Upchurch R, Peterson JBD (2007) Isolation of polymer-degrading bacteria and characterization of the hindgut bacterial community from the detritus-feeding larvae of *Tipula abdominalis* (Diptera: Tipulidae). Appl Environ Microbiol 73:5683–5686
9. Czarnetzki AB, Tebbe CC (2004) Diversity of bacteria associated with Collembola—cultivation-independent survey based on PCR-amplified 16S rRNA genes. FEMS Microbiol Ecol 49:217–227
10. Dillon RJ, Dillon VM (2004) The gut bacteria of insects: nonpathogenic interactions. Ann Rev Entomol 49:71–92
11. Douglas AE (2007) Symbiotic microorganisms: untapped resources for insect pest control. Trends Biotechnol 25:338–342
12. Dunn AK, Stabb EV (2005) Culture-independent characterization of the microbiota of the ant lion *Mymeleon mobilis* (Neuroptera: Mrymeleontidae). Appl Environ Microbiol 71:8784–8794
13. Egert M, Wagner B, Lemke T, Brune A, Friedrich MW (2003) Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Appl Environ Microbiol 69:6659–6668
14. Egert M, Stingl U, Bruun DL, Pommerenke B, Brune A, Friedrich MW (2005) Structure and topology of microbial communities in the major gut compartments of *Melolontha melolontha* larvae (Coleoptera: Scarabaeidae). Appl Environ Microbiol 71:4556–4566
15. Engebretson JJ, Moyer CL (2003) Fidelity of select restriction endonucleases in determining microbial diversity by terminal-restriction fragment length polymorphism. Appl Environ Microbiol 69:4823–4829
16. Haynes S, Darby AC, Daniell TJ, Webster G, van Veen FJF, Godfray HCJ, Prosser JI, Douglas AE (2003) Diversity of bacteria associated with natural aphid populations. Appl Environ Microbiol 69:7216–7223
17. Hobbie JE, Daley RJ, Jasper S (1977) Use of nuclepore filters for counting bacteria by fluorescent microscopy. Appl Environ Microbiol 33:1225–1228
18. Honek A, Martinkova Z, Saska P (2005) Post-dispersal predation of *Taraxacum officinale* (dandelion) seed. J Ecol 93:345–352
19. Hongoh Y, Ohkuma M, Kudo T (2003) Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). FEMS Microbiol Ecol 44:231–242
20. Huber T, Faulkner G, Hugenholtz P (2004) Bellerophon; a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 20:2317–2319
21. Lane DR (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 115–175
22. Li H, Medina F, Vinson SB, Coates CJ (2005) Isolation, characterization, and molecular identification of bacteria from the red imported fire ant (*Solenopsis invicta*) midgut. J Invertebr. Pathol 89:203–209
23. Liu W-T, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 63:4516–4522

24. Lovei GI, Sunderland KD (1996) Ecology and behavior of ground beetles (Coleoptera: Carabidae). Ann Rev Entomol 41:231–256
25. Lundgren JG (2005) Ground beetle (Coleoptera: Carabidae) ecology: their function and diversity within natural agricultural habitats. Am Entomol 51:218
26. Lundgren JG, Duan JJ, Paradise MS, Wiedenmann RN (2005) Rearing protocol and life history traits of *Poecilus chalcites* (Coleoptera: Carabidae) in the laboratory. J Entomol Sci 40:126–135
27. Lundgren JG, Shaw JT, Zaborski ER, Eastman CE (2006) The influence of organic transition systems on beneficial ground-dwelling arthropods and predation of insects and weed seeds. Renew Agric Food Sys 21:227–237
28. Lundgren JG, Lehman RM, Chee-Sanford JA (2007) Bacterial communities within digestive tracts of ground beetles (Coleptera: Carabidae). Annals Entomol Soc Am 100:275–282
29. Lundgren JG (2008) Relationships of natural enemies and non-prey foods. Springer, Dordrecht
30. Mohr KI, Tebbe CC (2005) Diversity and phylotype consistency of bacteria in the guts of three bee species (Apoidea) at an oilseed rape field. Environ Microbiol 8:258–272
31. Perner J (2003) Sample size and quality of indication—a case study using ground-dwelling arthropods as indicators in agricultural systems. Agric Ecosyst Environ 98:125–132
32. Saska P, van der Werf W, de Vries E, Westerman P (2008) Spatial and temporal patterns of carabid activity-density in cereals do not explain levels of predation on weed seeds. Bull Entomol Res 98:179–191
33. Schloss PD, Delalibera I, Handelsman J, Raffa KF (2006) Bacteria associated with the guts of two wood-boring beetles: *Anoplophora glabripennis* and *Saperda vestita* (Cerambycidae). Environ Entomol 35:625–629
34. Schmitt-Wagner D, Friedrich MW, Wagner B, Brune A (2003) Phylogenetic diversity, abundance, and axial distribution of bacteria in the intestinal tract of two soil-feeding termites (*Cubitermes* spp.). Appl Environ Microbiol 69:6007–6017
35. Schubert C (2006) Can biofuels finally take center stage? Nature Biotech 24:777–784
36. Shearin AF, Reberg-Horton SC, Gallandt ER (2007) Direct effects of tillage on the activity density of ground beetle (Coleoptera: Carabidae) weed seed predators. Environ Entomol 36:1140–1146
37. Speksnijder AGCL, Kowalchuk GA, DeJong S, Kline E, Stephen JR, Laanbroek HJ (2001) Microvariation artifacts introduced by PCR and cloning of closely related 16S rRNA gene sequences. Appl Environ Microbiol 67:469–472
38. Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267
39. White SS, Renner KA, Menalled FD, Landis DA (2007) Feeding preferences of weed seed predators and effect on weed emergence. Weed Sci 55:606–602
40. Yu Y, Breitbart M, McNairnie P, Rohwer F (2006) FastGroupII: a web-based bioinformatics platform for analyses of large 16S rDNA libraries. BMC Bioinformatics 7:57