Characterization of the Cricket Hindgut Microbiota with Fluorescently Labeled rRNA-Targeted Oligonucleotide Probes

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Most cricket hindgut microorganisms (60 to 80%) were detected with a universal fluorescent rRNA-targeted probe and found to be eubacteria. Group-specific probes showed that the hindguts of five different cricket species harbor similar bacterial groups, although in different proportions, and that different diets shifted the structure of the hindgut microbial community. The *Bacteroides-Prevotella* probe, of the eight eubacterial probes tested, stained the largest percentage of cells in all crickets.

Although insect gut microbial communities play important roles in processes linked to global carbon cycling and production of greenhouse gases, only a few studies, predominantly with termites and cockroaches, have examined in detail the composition of insect gut microbiota (4, 6). As with most insects, knowledge regarding the cricket gut microbiota has been obtained primarily by conventional techniques that rely on the culturable status of the microorganisms (26). Since the insect gut harbors dozens of physiologically different microbial populations (4), some of which have not yet been cultured (21), the use of culturing techniques likely provides a biased picture of the structure and dynamics of these microbial communities (28). Alternatively, in situ hybridization studies with fluorescently labeled ribosomal probes can provide information regarding the composition of natural communities without relying on cumbersome culturing techniques (2). This approach has been used to quantify bacterial groups inhabiting several environments (1), including the earthworm gut (8). Due to the correlation between growth rate and ribosomal content, rRNA-targeting probes can also shed light on the in situ metabolic status of microorganisms in natural ecosystems (22).

Here, we report on studies using fluorescently labeled rRNA-targeted probes to assess the composition of the cricket hindgut microbial community. Phylogenetic probes with different levels of specificity were used to compare the microbial community structure of several cricket species and to examine how changes in diet affected the different microbial groups inhabiting the hindgut of *Acheta domesticus*.

Specimens of *Scapteriscus borelii*, *Scapteriscus vicinus*, and *Gryllus rubens* were collected in suburban grasslands in north-central Florida by T. Walker (University of Florida, Gainesville). Mole crickets were placed in glass vials containing moist sand, while specimens of *G. rubens* were placed collectively in a canister. These crickets were shipped overnight for analyses. Pieces of apple were included to serve as the water and food source until the crickets reached the laboratory. Specimens of *Gryllus pennsylvanicus* were collected in a similar habitat in central Michigan and immediately transported to the laboratory. House crickets (*A. domesticus*) were reared in the lab on Purina cricket chow and grown at 30°C in 60% relative humid-

ity under a 12-h light-dark cycle. For diet experiments, adult house crickets were switched to diets representing a wide range of nutritional levels likely to be encountered by omnivorous insects. These were ground alfalfa hay, ground pulp from sugar beet roots, or an artificial protein diet (40% casein 50% alpha-cell fiber) (19). The alfalfa and pulp diets were amended with salts and vitamins as in the protein diet (14). Crickets maintained on chow were used as controls. Water-soluble carbohydrates (7) were 50, 51, 20, and <5% of the total dry weight for the chow, pulp, alfalfa, and protein diets, respectively. Soluble carbohydrate/protein ratios were 5:1, 2:1, 1:1, and 1:5 for the pulp, chow, alfalfa, and protein diets, respectively. Crickets were sacrificed after 5 days on these diets to obtain hindguts.

Hindguts were surgically removed with fine forceps, immediately fixed in formaldehyde (3.7%)–phosphate-buffered saline (pH 7.2), and homogenized individually with sterile tissue grinders. Aliquots from homogenates were stained with 0.01% acridine orange for 10 min to estimate the acridine orange direct counts, following the method of Hobbie et al. (11). Hindgut homogenates were then transferred to microcentrifuge tubes, and eukaryotic tissue was removed by vigorously vortexing the samples for 10 s followed by quick centrifugation (1 s, 8,000 × g). The supernatant was collected and centrifuged for 1 min at 6,800 × g to recover microbial cells. The bacterial pellet was then resuspended in formaldehyde–phosphate-buffered saline and kept at 4°C until analysis. Aliquots (20 µl) of resuspended pellets were spread into wells of gelatin-coated toxoplasmosis slides (HTC-7; Cel-Line Associates, New Field, N.J.) prepared as described elsewhere (9), dried in an air-circulating oven at 37°C, and stored desiccated at room temperature (25 ± 2°C). Prior to hybridizations, slides were submerged sequentially in 50, 75, and 95% ethanol solutions (25). Cells were hybridized separately with each probe under the conditions and with hybridization solutions described elsewhere (references cited below) with minor modifications. Briefly, fluorescently labeled probes were suspended in prewarmed hybridization solution to a final concentration of approximately 100 ng/ml. Hybridizations were carried out for 16 h in prewarmed chambers (50-ml propylene tubes) containing a wet paper towel to preserve moisture and stained with 4,6-diamidino-2-phenylindole (DAPI) to determine the total number of microbial cells per microscopic field (29).

Fluorescently labeled oligonucleotide (rRNA-targeting) probes were used to determine the presence of the following phylogenetic groups: α-, β-, γ-, and δ-subgroups of the class

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FIG. 1. Direct counts of the hindgut microbial communities of different species of crickets as determined by the acridine orange direct count (AODC) method.

Proteobacteria (2, 17), high-G+C-content gram-positive bacteria (23), *Archaea* (25), *Bacteroides* or *Prevotella* spp. (16), *Acinetobacter* spp. (27), and *Pseudomonas* spp. (24). A universal probe (2) was used to determine the number of microbial cells that could be detected by in situ hybridizations, while a eubacterial probe (9) was used to determine the number of detectable cells that belong to the domain *Bacteria*. Phylogenetic probes were labeled with tetramethylrhodamine-5-(6)-isothiocyanate (TRITC) and purified by high-performance liquid chromatography (Genosys Biotechnologies Inc., The Woodlands, Tex.). Fluorescing cells were visualized with an Axioskop epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 75-W mercury light source and appropriate filter sets. A minimum of 500 DAPI-stained cells from 20 combined microscopical fields were counted for each individual hindgut. The percentage of each microbial group identified was determined as the number of TRITC-fluorescing cells from the total DAPI-stained cells. Mean numbers of cells detected with each probe were obtained from three independent hybridizations using one individual gut homogenate per hybridization. Only those cells that had hybridization sig-

nals stronger than any detectable background autofluorescence were counted as positive.

The number of microbial cells in the hindgut of *Scapteriscus* spp. and *Gryllus* spp. were more than an order of magnitude higher than in *A. domesticus* as determined by direct counts (Fig. 1). These differences correlate with the size of the hindgut, since the highest densities were observed for the cricket species with the largest hindguts (i.e., *Scapteriscus* spp.).

Hybridization studies with the universal probe showed that 62 to 81% of total hindgut bacteria in all crickets examined had the ribosomal content necessary to produce strong hybridization signals (Table 1; Fig. 2). Hence, these results suggest that the majority of the populations inhabiting the cricket hindgut are metabolically active. In addition, most hindgut microorganisms (i.e., at least 80 to 96%) that hybridized to the universal probe also hybridized to the eubacterial probe. Only hindgut microbial cells of *Scapteriscus* spp. hybridized to the *Archaea* probe. Methanogens are the only *Archaea* known to reside in the insect gut (3), although methanogenesis is thought to be absent in crickets (10). Nonetheless, methane evolution has been recently detected in mole crickets (14). Thus, the prokaryotes detected with the *Archaea* probe most likely belong to a methanogenic group. Recent studies with fluorescently labeled polyclonal antibodies have also suggested the presence of hindgut bacteria immunogenically related to methanogens (15).

Bacteroides or *Prevotella* spp. and the α -, γ -, and δ -subgroups of the *Proteobacteria* were detected in the hindguts of all crickets examined with group-specific probes. Bacteria from the β -subgroup of the *Proteobacteria* were detected in all cricket species with the exception of *S. vicinus*, while *Pseudomonas* spp. were detected in *A. domesticus* and *Gryllus* spp. but not in mole crickets. Additionally, high-G+C-content gram-positive bacteria were detected only in *Gryllus* spp. Thus, the coexistence of phylogenetically diverse eubacteria within the cricket hindgut was established. The group-specific probes, however, detected an average of 38, 33, and 20% of the hindgut bacteria of *Gryllus* spp., *A. domesticus*, and *Scapteriscus* spp., respectively (Table 1), indicating that approximately 60% of the hindgut bacteria could not be characterized with these probes. Differences in the relative abundance of several microbial groups were observed between the cricket groups. While it remains to be demonstrated if such differences play an ecolog-

TABLE 1. Results from in situ hybridization studies using fluorescently labeled rRNA-targeted probes

Probe type	% of microbial cells detected ^a in:				<i>A. domesticus</i> on a diet of:			
	<i>G. pennsylvanicus</i>	<i>G. rubens</i>	<i>S. borelii</i>	<i>S. vicinus</i>	Chow	Alfalfa	Pulp	Protein
Universal	79.2 (2.3)	81.5 (4.8)	69.4 (2.5)	62.3 (3.2)	73.3 (3.5)	63.9 (8.8)	68.1 (5.3)	71.2 (2.5)
Eubacterial	63.4 (5.2)	71.2 (3.1)	57.1 (3.2)	52.8 (2.8)	68.7 (4.2)	59.8 (5.3)	65.2 (2.8)	66.8 (6.8)
Archaeal	ND	ND	4.1 (1.2)	6.8 (2.3)	ND	ND	ND	ND
Proteobacterial subgroup								
α	3.1 (0.2)	7.5 (1.4)	1.1 (0.2)	1.9 (0.3)	ND	3.8 (1.2)	2.5 (0.5)	1.9 (0.9)
β	4.7 (1.1)	2.1 (0.4)	2.9 (1.0)	ND	0.4 (0.1)	0.1 (0.01)	7.9 (2.1)	9.7 (2.4)
γ	4.1 (2.1)	5.2 (0.5)	1.4 (0.2)	5.2 (0.9)	2.3 (0.3)	2.1 (0.2)	8.8 (1.4)	11.8 (1.9)
δ	6.9 (1.2)	8.2 (0.6)	2.1 (0.3)	1.3 (0.1)	8.0 (2.1)	5.3 (3.2)	ND	1.3 (0.4)
<i>Bacteroides-Prevotella</i>	11.2 (2.4)	12.9 (2.6)	8.2 (2.7)	5.2 (0.3)	14.5 (3.1)	15.9 (1.2)	13.7 (4.2)	16.2 (2.3)
High G+C content	2.3 (1.1)	1.9 (0.7)	ND	ND	ND	ND	ND	ND
<i>Pseudomonas</i>	2.4 (0.3)	4.1 (2.3)	ND	ND	4 (0.6)	ND	ND	ND
% Detected ^b	34.7	41.9	19.8	20.4	29.2	27.2	32.9	40.9

^a Values in parentheses are standard deviations from triplicate determinations. ND, not detected.

^b Values represent the percentage of microbial cells identified with group- and genus-specific probes. No cells stained with the *Acinetobacter* probe.

FIG. 2. Epifluorescence micrograph of hindgut microbial cells of *A. domesticus* hybridized with TRITC-labeled universal probe (B). Cells were stained with DAPI (A) to determine the total number of cells per field.

ical role in the host-symbiont interactions, differences in the hindgut microbial structure in the crickets examined could be attributable to differences in feeding behavior of these cricket genera. For instance, *A. domesticus* and *Gryllus* spp. are believed to be omnivorous although they predominantly feed on above-the-surface plant material (13, 18). In contrast, mole crickets are in more direct contact with soil microbial communities (20), a factor that might influence the composition and structure of their hindgut microbial community. On the other hand, anatomical differences might also influence the hindgut community structure. Again, mole crickets are different from other cricket groups in that their hindguts lack a peritrophic

membrane (20), allowing their microbiota to be in more direct contact with the ingested material, while in *A. domesticus* and *Gryllus* spp., hindgut microorganisms are predominantly exposed to soluble material that penetrates the pores of the peritrophic membrane.

Changes in diets alter the rates of volatile fatty acid (VFA) production and the ratios of VFAs produced by the cricket hindgut community without affecting significantly the microbial densities (14). The methods previously used to analyze the hindgut community have not established if changes in diet resulted in relevant changes in the hindgut community structure. In this study, hybridizations with group-specific probes indicated that dietary changes altered the structure of the hindgut community, although it did not change its predominantly eubacterial nature. The most notable changes were observed for members of the α -, β -, γ -, and δ -subgroups of the *Proteobacteria*. Although changes in community structure occurred, it has not been demonstrated whether a structurally flexible hindgut microbial community could benefit the host. It is conceivable that shifts in the ratio of predominant members due to changes in diet could maintain the pool of substrates (e.g., VFAs) for the host to absorb and thus use for growth or reproduction. This could be an incidental phenomenon in crickets, since germ-free colonies of *A. domesticus* have been successfully maintained in the laboratory (12). In contrast, in insects that strictly depend on their microbial symbionts (e.g., termites), having some level of flexibility in the hindgut community structure could be essential for their survival.

Although we could detect only up to 42% of cricket hindgut microorganisms with the group-specific probes, this represents a larger fraction of the community than can normally be detected by culturing techniques (i.e., <7%). Moreover, this study shows the usefulness of rRNA-targeting probes to study hindgut microbial groups cumbersome to detect via culturing methods (e.g., *Bacteroides* or *Prevotella* spp. and species of *Archaea*). In fact, the detection of some of these groups (e.g., *Bacteroides* or *Prevotella* spp. and possibly methanogens in mole crickets) suggests that the cricket hindgut is a highly reduced system. However, it should be noted that nearly 50% of the termite hindgut appears to contain oxygen (5), and thus, the presence of anaerobes in the insect gut does not indicate that the entire gut is anoxic. Additionally, considering that most hindgut microorganisms were metabolically active, our results suggest that rRNA-targeting probes via in situ hybridizations are useful for detecting and monitoring genera and species found in the cricket hindgut.

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