Culture-Independent Characterization of the Digestive-Tract Microbiota of the Medicinal Leech Reveals a Tripartite Symbiosis

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Culture-based studies of the microbial community within the gut of the medicinal leech have typically been focused on various Aeromonas species, which were believed to be the sole symbiont of the leech digestive tract. In this study, analysis of 16S rRNA gene clone libraries confirmed the presence of Aeromonas veronii and revealed a second symbiont, clone PW3, a novel member of the Rikenellaceae, within the crop, a large compartment where ingested blood is stored prior to digestion. The diversity of the bacterial community in the leech intestine was determined, and additional symbionts were detected, including members of the α-, γ-, and δ-Proteobacteria, Fusobacteria, Firmicutes, and Bacteroidetes. The relative abundances of the clones suggested that A. veronii and the novel clone, PW3, also dominate the intestinum community, while other clones, representing transient organisms, were typically present in low numbers. The identities of these transients varied greatly between individual leeches. Neither time after feeding nor feeding on defibrinated blood caused a change in identity of the dominant members of the microbial communities. Terminal restriction fragment length polymorphism analysis was used to verify that the results from the clone libraries were representative of a larger data set. The presence of a two-member bacterial community in the crop provides a unique opportunity to investigate both symbiont-symbiont and symbiont-host interactions in a natural model of digestive-tract associations.

The digestive tracts of most animals are colonized by very complex microbial communities which provide important functions to the host, including the synthesis of essential nutrients, stimulation of the immune system, and resistance against the colonization of pathogens (17, 30). In hematophagous, blood-feeding invertebrates, these symbionts are thought to be critical for host fitness because of the need for blood-scarce nutrients (15, 32). Investigators have resorted to examining naturally occurring, monospecific associations, for example, Vibrio fischeri and the squid Euprymna scolopes, or simplified complex communities by using gnotobiotic animals, such as Bacteroidetes thetaiotaomicron and the mouse Mus musculus (18, 33). The use of such model systems has revealed an amazing molecular interplay between symbionts and hosts. However, these models are limited in their utility for investigating the interactions between different symbiont species that are likely to play an important role in most digestive-tract communities. In the characterization of the microbial communities associated with hematophagous animals, an additional interest is the potential risk these animals pose to human health by serving as vectors for infectious diseases, which is exemplified by wound infections following leech therapy in patients with venous congestion who do not receive preemptive antibiotics (9, 38).

The first detailed descriptions of the digestive-tract microbiota of medicinal leeches reported an unusual simplicity, as only one bacterium was cultured from the leech digestive tract (6, 19). Subsequent studies supported the presence of one dominant microbe, an Aeromonas species which was consistently present in medicinal leeches, but disagreed in two aspects, i.e., the identity of the Aeromonas species and the presence of other microbes in the leech midgut (11, 12, 14, 29). Case reports identified Aeromonas hydrophila infections associated with the clinical use of medicinal leeches, but these studies often used commercial phenotypic tests that do not accurately identify Aeromonas to the species level (1, 7, 29). A second controversial point has been the complexity of the midgut microbiota. A few studies implementing nonquantitative approaches reported the presence of additional bacterial species in the midgut (11), while other studies reported only Aeromonas spp. (14).

Recent studies of invertebrate midguts have utilized culture-independent methods to detect diverse gut microbial communities in a wide range of hosts, such as earthworms (21), gypsy moths (5), mosquitoes (28, 36), and termites (34, 42). These studies reported more complex microbial communities than culture-based studies, consistent with the proposal that well over 95% of microbes in the environment cannot be cultured in laboratory settings (3). The detection and phylogenetic analysis of these “unculturable” bacteria provide important information about the complexity of the microbial communities in invertebrate midguts and have led to the discovery of novel organisms. To our knowledge, culture-independent studies of leeches have been limited to the mycetomes, which are digestive-tract-associated organs that house symbiotic bacteria (24, 25, 40); however, mycetomes have not been detected in medicinal leeches (Hirudo spp.).

The midgut of the medicinal leech consists of two major components, the crop and the intestinum (Fig. 1). The crop is a large compartment that stores blood meals after ingestion, absorbs water and salts from the ingested blood, and is the...
location of the *Aeromonas* symbionts detected in previous studies (15b). The crop is further compartmentalized into 10 pairs of lateral caeca and 1 pair of elongated posterior caeca (Fig. 1). Directly adjacent to the lateral caeca are pairs of bladders that have been shown to house bacteria (6). From the crop, the ingested blood, or intraluminal fluid (ILF), passes into the intestinum, a short, tube-like structure, near the posterior caeca, where the blood meal is subsequently digested and nutrients are absorbed (Fig. 1). Prior to this report, no studies have specifically characterized the bacterial community within the intestinum of the medicinal leech. The goal of this study was to determine the composition of the bacterial community in the midgut of *Hirudo verbana* by using culture-independent methods, to compare the crop and intestinum microflora, to monitor the bacterial communities following feeding, and to determine the effects of diet on the microbiota.

**MATERIALS AND METHODS**

**Animals.** Medicinal leeches were obtained from Leeches USA (Westbury, NY). The animals were maintained prior to feeding in leech mobile homes (Leeches USA) containing local well water. The temperature was maintained at 25°C (±1°C) with a 14 h:10 h light-dark cycle. Leeches were identified morphologically as *Hirudo verbana*, which was verified by sequencing of multiple loci (M. Siddall, personal communication).

**Feeding.** Leeches were divided into two groups. The first group was fed 5 ml fresh blood (containing heparin [25 U/ml] [Sigma Chemical Co., Atlanta, GA]) obtained from sheep at the University of Connecticut’s School of Agriculture in accordance with an approved IACUC protocol (Storrs, CT), and the second was fed commercially available defibrinated whole sheep blood (QuadFive, Ryegate, MT). The animals were fed as described previously (14).

**Isolation of digestive-tract contents.** The leeches were rinsed, dried, and then disinfected by gently being washed with a 10% bleach solution. A 2- to 3-cm longitudinal incision was made along the ventral surface of each leech, beginning approximately 1 cm below the head. The skin and connective tissue were removed from the crop. The crop was then pierced, and the ILF of the crop was collected using a positive-pressure pipette. Following extraction of the ILF, a 1- to 2-cm longitudinal incision was made approximately 2 cm posterior to the first incision, and the intestinum of the animal was removed intact and placed in saline (0.85% [wt/vol] NaCl). The intestinum was rinsed three times with saline, homogenized, and vortexed.

**DNA extraction.** DNAs were isolated from the homogenized intestine and from sheep blood by using a DNeasy tissue kit (QIAGEN Inc., Valencia, CA) according to the manufacturer’s instructions for increased DNA yield from gram-negative bacteria. Due to the presence of a PCR inhibitor, DNA extraction from ILF was performed using a protocol for enhanced extraction of bacterial DNA from blood samples (31), with the only modification being that the ILF was diluted 1:1 with sterile saline before DNA extraction.

**PCR amplification of bacterial 16S rRNA genes.** 16S rRNA genes were amplified from DNA extracts by utilizing the primers 27F (5’-AGAGTTTGATCMTGCTCAG-3’) and 1492R (5’-TACGGYTACCTTGTTACGACTT-3’) (26). Amplification was conducted using a PTC-200 DNA engine (MJ Research, Waltham, MA) and the following thermal profile: 95°C for 5 min followed by 20 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 90 s, with a final step of 72°C for 10 min. Positive amplification of 16S rRNA genes was verified by visualization of a single 1.5-kb band. Negative controls were included in every set of reactions. PCR products were purified using a QIAquick PCR purification kit (QIAGEN). No PCR products were detected from sheep blood extracts.

**Construction of 16S rRNA gene clone library.** 16S rRNA gene amplicons were cloned into pCR2.1 and transformed into *Escherichia coli* TOP10 cells by using a TA cloning kit (Invitrogen Co., Carlsbad, CA) according to the manufacturer’s instructions. The presence of one 1.5-kb insert in each clone was verified by digesting purified plasmids with the restriction endonuclease EcoRI (New England Biolabs, Beverly, MA). Inserts were amplified using the 27F and 1492R primers described above and 5 ng of plasmid DNA. Libraries from the ILF of the crop and the intestinum were constructed from leeches 7 and 90 days after feeding. In addition, ILF and intestine libraries were constructed from a leech 7 days after it was fed defibrinated blood.

**Identification of symbionts.** Amplicons from individual clones were digested separately with the restriction endonucleases HaeIII and TaqI (New England Biolabs) to generate restriction profiles, which were visualized in a 2.0% MetaPhor agarose gel (Cambrex, East Rutherford, NJ) stained with ethidium bromide. Multiple clones for each representative restriction profile were sequenced using the following primers: 27F, 1492R, 530F (5’-GTCGCACGCTCGCCGCGG-3’), 519R (5’-GWATTCGGCCGGCCKGCTG-3’), 519R (5’-GWATTCGGCCGGCCKGCTG-3’), 519R (5’-GWATTCGGCCGGCCKGCTG-3’), 519R (5’-GWATTCGGCCGGCCKGCTG-3’), 907R (5’-CCGCTAATTCTTATGTTT-3’). Sequencing was carried out at the University of Connecticut Biotechnology Center, using a CEQ protocol (Beckman Coulter, Fullerton, CA), and at the Yale University Ecology and Evolutionary Biology Sequencing Center, using a Big Dye protocol (Applied Biosystems, Foster City, CA). The DNA sequences were aligned and assembled into contigs using ContigExpress in the Vector NTI software package (v3.1) (20) was generated with the same alignment, using the G model of evolution. The analysis was run in duplicate with four

FIG. 1. Drawing of the digestive tract and excretory organs of *H. verbana* (Modified from reference 15a with kind permission of Springer Science and Business Media.)
independent chains and for 100,000 generations, of which the initial 25,000 were discarded as burn-in. Treeview (35) was used to generate image files.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences generated in this study were deposited in the GenBank nucleotide sequence database under accession numbers DQ355170 to DQ355182.

RESULTS

Bacterial community in the ILF. An analysis of 16S rRNA gene clone libraries revealed the presence of a second bacterial symbiont in the ILF, clone PW3 (Table 1). The 16S rRNA gene sequences of clone PW3 most closely matched Rikenella microfusus (89.7% identity), a member of the Bacteroidetes (Fig. 2). In addition, clone PW1 corresponded to the Aeromonas symbiont, and its 16S rRNA sequence differed by only 1 bp from that of Aeromonas veronii, further supporting the identity of the symbiont as A. veronii. In the library from the animal sacrificed 90 days after being fed, clone PW2 was detected only twice, and it had >99% sequence identity with the 16S rRNA gene sequence from Morganella morganii, a widely distributed opportunistic pathogen (23).

The relative abundances of these sequences in our clone libraries suggested that Aeromonas and clone PW3 were the dominant members of the ILF community 7 and 90 days after leech feeding, together accounting for >98% of the cloned sequences. Good’s formula was used to determine the coverage of the 7- and 90-day libraries, which was >97.8% and >98.5%, respectively, indicating that all abundant sequences were likely sampled in our libraries.

Bacterial community of the intestinum. In contrast to the extremely simple community present in the ILF, the clone libraries from the intestinum suggested the presence of a more diverse community of bacteria (Table 1). The majority of plasmids (66.7%) from these libraries matched 16S rRNA genes from A. veronii and clone PW3. The remaining plasmids contained 16S rRNA genes corresponding to members of the α-Proteobacteria (2.4%), γ-Proteobacteria (12.7%), δ-Proteobacteria (1.6%), Bacteroidetes (4.8%), Fusobacteria (2.4%), and Firmicutes (9.5%). Many of the sequences detected (53.8%) were distinct from sequences in the databases (<97% match with any organism in the BLAST database). No more than nine different clones were detected in a single clone library, and plasmids corresponding to only three organisms, A. veronii, clone PW3, and M. morganii, were detected in intestinum clone libraries both 7 and 90 days after feeding. No apparent changes to the dominant members, overall number of species, or relative diversity of the community were detected between the 7- and 90-day libraries. Coverage of the 7- and 90-day libraries was >94.5% and >98.7%, respectively.

T-RFLP analysis of the bacterial communities in multiple leeches. T-RFLP analysis was used to determine whether the clone libraries were representative of the symbionts detected in leech populations. The median number of peaks detected in ILF samples obtained between 7 and 30 days after leech feeding was 2 (n = 11). Peaks corresponding to A. veronii and clone PW3 were detected in 45.5% and 100%, respectively, of T-RFLP samples from the ILF. The median number of peaks detected in intestinum samples from 1 to 30 days after leech feeding was 10 (n = 16), with at most 17 distinct peaks detected in one intestinum sample. In the intestinum, 36.7% of peaks matched the calculated peak locations for organisms detected in our clone libraries, and these peaks corresponded to 71.5% of the total peak height. This suggests that the peaks corresponding to the clone libraries accounted for most of the 16S rRNA genes present in the intestinum. Peaks corresponding to the core symbionts, A. veronii and clone PW3, were detected in 66% and 100%, respectively, of intestinum samples. The inability to detect Aeromonas in all samples could be due to several factors. The animals used in our study were maintained for medicinal use on humans and were starved for at least 3 months prior to shipment to reduce the number of

### TABLE 1. Bacterial phylotypes recovered from 16S rRNA gene clone libraries constructed from the digestive tract of the medicinal leech, H. verbana

<table>
<thead>
<tr>
<th>Source</th>
<th>Clone name</th>
<th>Bacterial division</th>
<th>Genus</th>
<th>Species</th>
<th>No. (%) of clones recovered after indicated time (days) of feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>ILF</td>
<td>PW1</td>
<td>γ-Proteobacteria</td>
<td>Aeromonas</td>
<td>A. veronii</td>
<td>11 (21.1) 29 (43.9)</td>
</tr>
<tr>
<td></td>
<td>PW2</td>
<td>γ-Proteobacteria</td>
<td>Morganella</td>
<td>M. morganii</td>
<td>0 2 (3.0)</td>
</tr>
<tr>
<td></td>
<td>PW3</td>
<td>Bacteroidetes</td>
<td></td>
<td></td>
<td>41 (78.8) 35 (53.0)</td>
</tr>
<tr>
<td>Intestinum</td>
<td>PW1</td>
<td>γ-Proteobacteria</td>
<td>Aeromonas</td>
<td>A. veronii</td>
<td>15 (27.2) 21 (29.5)</td>
</tr>
<tr>
<td></td>
<td>PW2</td>
<td>γ-Proteobacteria</td>
<td>Morganella</td>
<td>M. morganii</td>
<td>3 (5.5) 12 (16.9)</td>
</tr>
<tr>
<td></td>
<td>PW3</td>
<td>Bacteroidetes</td>
<td></td>
<td></td>
<td>27 (49.1) 21 (29.5)</td>
</tr>
<tr>
<td></td>
<td>PW4</td>
<td>α-Proteobacteria</td>
<td>Ochrobactrum</td>
<td></td>
<td>2 (3.6) 0</td>
</tr>
<tr>
<td></td>
<td>PW5</td>
<td>α-Proteobacteria</td>
<td>Sphingobacterium</td>
<td></td>
<td>1 (1.8) 0</td>
</tr>
<tr>
<td></td>
<td>PW6</td>
<td>γ-Proteobacteria</td>
<td>Pseudomonas</td>
<td></td>
<td>1 (1.8) 0</td>
</tr>
<tr>
<td></td>
<td>PW7</td>
<td>δ-Proteobacteria</td>
<td>Desulfovibrio</td>
<td></td>
<td>0 2 (2.8)</td>
</tr>
<tr>
<td></td>
<td>PW8</td>
<td>Bacteroidetes</td>
<td></td>
<td></td>
<td>0 5 (7.0)</td>
</tr>
<tr>
<td></td>
<td>PW9</td>
<td>Bacteroidetes</td>
<td>Chryseobacterium</td>
<td>C. meningosepticum</td>
<td>1 (1.8) 0</td>
</tr>
<tr>
<td></td>
<td>PW10</td>
<td>Fusobacteria</td>
<td>Fusobacterium</td>
<td>F. varium</td>
<td>3 (5.5) 0</td>
</tr>
<tr>
<td></td>
<td>PW11</td>
<td>Firmicutes</td>
<td>Clostridium</td>
<td></td>
<td>0 8 (11.2)</td>
</tr>
<tr>
<td></td>
<td>PW12</td>
<td>Firmicutes</td>
<td>Vagococcus</td>
<td>V. carniphilus</td>
<td>0 2 (2.8)</td>
</tr>
<tr>
<td></td>
<td>PW13</td>
<td>Firmicutes</td>
<td>Enterococcus</td>
<td>E. malodoratus</td>
<td>2 (3.6) 0</td>
</tr>
</tbody>
</table>

* a ≥93% identity.  
* b ≥98% identity.
FIG. 2. Phylogenetic tree of sequences detected in this study (shown in bold) and sequences from related organisms. The tree was constructed using a maximum likelihood analysis of 16S rRNA gene sequences with the HKY+I+G model of evolution. Support at nodes is shown with circles. Black circles indicate nodal support of >90% bootstrap support and a Bayesian posterior probability of >0.9; gray circles indicate >70% bootstrap support and a Bayesian posterior probability of >0.7; and white circles indicate >50% bootstrap support and a Bayesian posterior probability of >0.5.
**Aeromonas** organisms present. Alternatively, *A. veronii* was present at levels below the limit of detection or was not present in all members of the population investigated.

**Effect of blood meal on bacterial diversity.** Some commercial leech farms feed leeches defibrinated blood (M. Roth, personal communication), which may have reduced antimicrobial activity after storage compared to that of fresh blood. The bacterial communities in leeches who were fed defibrinated blood were characterized by constructing clone libraries and performing T-RFLP analysis. As expected, clone sequences corresponding to *A. veronii* and clone PW3 were abundant in both the ILF and intestina of leeches fed defibrinated blood. *M. morganii* was also detected at a lower level in both clone libraries. In addition, two novel organisms were detected within these libraries. Surprisingly, one of these, clone PWD1, accounted for 52.6% of the plasmids from the ILF library and 24.1% of plasmids from the intestina clone library. However, T-RFLP analysis of five additional leeches fed defibrinated blood failed to detect a peak corresponding to clone PWD1, indicating that this organism is not a common resident. This suggests that an infirmity allowed clone PWD1 to proliferate within this leech, leading to an inaccurate clone library, or that the defibrinated blood allowed PWD1 to proliferate. Phylogenetic analysis revealed that clone PWD1 is closely related to the genus *Clostridium*. The second novel sequence, clone PWD2, was detected in small numbers and confined solely to the intestinum library. Clone PWD2 clustered within the genus *Desulfovibrio*.

**DISCUSSION**

Previous studies relied on culturing techniques to characterize the digestive-tract microbiota of the medicinal leech, and several investigators (6, 19) as well as our previous report (22) concluded that the ILF of the crop contains a monocolulture of *Aeromonas*. The culture-independent analysis described in this report provides evidence of a second symbiont, clone PW3, residing in the crop. Sequence analysis identified clone PW3 as originating from an uncultured *Bacteroidetes* organism belonging to the *Rikenellaceae*, and T-RFLP analysis detected a peak corresponding to this clone in all samples. These data indicate that PW3 is a resident of the leech digestive tract and coexists with *A. veronii* in the ILF of the crop.

Very few *Rikenella* sp. isolates have been cultured, possibly due to their fastidious growth requirements and their low oxygen tolerance. It is also possible that culture-based studies of leeches were unable to detect PW3 due to a growth disadvantage when grown with *Aeromonas* under lab conditions or that PW3 requires the leech and/or *Aeromonas* for growth. It is tempting to speculate that *A. veronii*, a facultative anaerobe, lowers the local oxygen concentration, permitting PW3 to proliferate. We are currently employing anaerobic and microaerophilic culturing techniques to attempt to cultivate PW3 outside the leech.

The 16S rRNA gene phylogeny of clone PW3 indicates that it is clustered within a clade of organisms that is highly divergent from the other *Bacteroidetes* organisms (Fig. 2). The family *Rikenellaceae* consists of only a half-dozen described organisms, all of which were isolated from digestive systems or fecal matter. Recent PCR-based studies amplified sequences belonging to this clade from a wide variety of gut environments, including bacteria detected within the guts of termites (unpublished submissions to GenBank), the goat rumen (unpublished), the feces of swine (unpublished), mouse cecal samples (27), and human gut and fecal samples (10, 37). These findings strongly suggest an adaptation of these organisms to colonizing digestive systems of a wide range of animals, from invertebrates to mammals, including humans. This could indicate that organisms within this group are highly specialized for gut symbioses and that they may play an important role within the host digestive tract. Evaluating the symbiotic role of these *Rikenella*-like symbionts in digestive tracts in the crop of the leech, where only one additional bacterial species is present, may be feasible.

In contrast to the crop, the intestinum of the medicinal leech supports a more diverse bacterial community consisting of three core organisms, namely, *A. veronii*, PW3, and *M. morganii*, and several transient organisms. The core organisms were defined as present 7 days and 90 days after leech feeding, while transient organisms were detected at only one of these time points. We cannot exclude the possibility that the transient organisms are continuously present but below our detection level, which does not exclude them from possibly performing important functions. In addition, our study did not search for archaea, which could also be present in the crop and intestinum. As in the crop, *A. veronii* and PW3 were the dominant organisms, with each accounting for at least 25% of the plasmids analyzed. The abundance of these two organisms in the intestinum may be due to the continual inflow of crop content during the digestion of the blood meal.

Analogous to the situation in mammalian guts, a greater number of different organisms were detected further down the digestive tract. The intestinum contained a more diverse microbiota than the crop, despite the fact that the inhabitants of the intestinum presumably had to pass through the crop before reaching the intestine. We hypothesize that the presence of multiple antimicrobial factors in the crop contributes to this simplicity. One antimicrobial factor is the complement system of the ingested vertebrate blood, which remains active for some time inside the leech and can kill sensitive bacteria (4, 22). A previous study suggested another complement-independent mechanism that inhibited the proliferation of some bacteria in the crop yet allowed them to remain viable (22). This is consistent with the ability of some organisms to pass through the crop and into the less restrictive intestine. If the source of these bacteria is water, rock, sediment, or the skin of a prey animal, the arbitrary encounter of species that can survive the passage through the crop could result in the appearance of the transient organisms in the intestinum. An alternative but not exclusive explanation is that at different time points of the feeding cycle, some species increase in number while others decrease below the limit of detection. The overall composition of the bacterial community in the leech digestive system was stable during the host’s feeding cycle. Wild leeches feed approximately every 2 to 8 weeks (39), and our data suggest that the digestive-tract microbiota remains stable throughout this entire feeding cycle. We can only draw limited conclusions about the relative abundances of the different symbionts because the copy numbers of the ribosomal operons vary and T-RFLP is not quantitative.
One of the proposed functions of the symbionts is the production of antimicrobial compounds that enforce the simplicity of the crop microbiota (6). The presence of both *Aeromonas* and clone PW3 in the intestine, where the bacterial community is more complex, suggests that these symbionts are not responsible for creating antimicrobial activity in the leech digestive tract because one would expect to see a similar set of organisms present in both the crop and intestine. This evidence is indirect, and the observation could also be explained by down-regulation of the antimicrobial activity in the intestine. It seems likely that the antimicrobial activity is a result of the leech and the ingested blood and that either becomes inactive after a certain time or is inactivated in the intestine.

The core microbial symbionts are more likely to play a role in digestion and the formation of essential nutrients absent in the single food source, blood.

T-RFLP analysis indicated that feeding leeches defibrinated blood did not affect the composition of the digestive-tract microbiota. An interesting exception was the sudden appearance and dominance of organism PW1 in our clone library. However, clone PW1 was only isolated from the leech from which our clone library was created and was not detected by T-RFLP in other leeches. Thus, it is likely the result of an irregularity within that individual leech rather than an effect of the blood meal and shows the importance of using a second technique to increase the sample size.

To our knowledge, this study represents the first time the digestive-tract microbiota of the medicinal leech has been analyzed using PCR-based methods. Previous studies have suggested a monospecific digestive-tract symbiosis between *Aeromonas* bacteria and the leech. However, our results show that the crop has a bacterial community consisting of two dominant core symbionts and that the intestine has a more complex community consisting of the core symbionts and several transient organisms. We are currently pursuing the exact location of the symbionts and confirmation of their presence by using a PCR-independent approach. The simplicity of this tripartite association may allow us to determine the contributions of the individual partners and investigate interactions between the two symbionts as a model system for the study of complex digestive-tract symbioses between bacteria and their hosts.

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