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ABSTRACT

Many monophagous animals have coevolutionary relationships with bacteria that provide unavailable nutrients to the host. Frequently, these microbial partners are vertically inherited and reside in specialized structures or tissues. Here we report three new lineages of bacterial symbionts of blood-feeding leeches, one from the giant Amazonian leech, *Haementeria ghilianii*, and two others from *Placobdelloides* species. These hosts each possess a different mycetome or esophageal organ morphology where the bacterial cells are located. DNA sequencing of the bacterial 16S rRNA genes and fluorescent in situ hybridization placed these symbionts in two separate clades in the subdivision gammaproteobacteria. We also conducted a broad phylogenetic analysis of the herein reported DNA sequences as well as others from bacterial symbionts reported elsewhere in the literature including alphaproteobacterial symbionts from the leech genus *Placobdella* as well as *Aeromonas veronii* from the medicinal leech, *Hirudo medicinalis*, and a *Rickettsia* sp. detected in *Hemiclepsis marginata*. Combined, these results indicate that bloodfeeding leeches have forged bacterial partnerships at least five times during their evolutionary history.

A wide variety of intimate bacterial partnerships with animals, particularly insects, have been described. The symbionts often allow their hosts to exploit an otherwise unavailable niche by supplying them with limiting or missing nutrients. Examples of these associations include *Buchnera* in aphids, *Wigglesworthia* in tsetse flies, and *Blochmannia* in carpenter ants in which the bacteria supplement monophagous diets of plant sap, vertebrate blood, and cellulose, respectively. Evidence of the
significance of the symbionts to their hosts is that the mutualist bacteria are frequently
directly transmitted from parent to offspring via vertical transmission and attempts to
“cure” the host of the symbiont typically result in death, sterility, or infertility (15,23).
Many of these obligate symbionts are found in specialized structures or organs,
variously termed bacteriocytes or mycetomes (7). Several of these bacterial/eukaryotic
partnerships have been intensively studied across coevolutionary (10, 30)
developmental (6) and, more recently, genomic (e.g. 1, 25) fronts.
Since the 1920's, it has been acknowledged that blood-feeding leeches in the
family Glossiphoniidae also possess bacterial symbionts that are housed in specialized
organs (mycetomes) associated with the esophagus (23). The morphology of these
mycetomes is highly variable, however. Species of Placobdella that feed on aquatic
reptiles and amphibians, for example, have mycetomes consisting of a pair of blind-end
sacs that extend laterally from the esophageal lumen (Fig. 1A), the endothelial cells of
which are packed with Gram-negative rods (27). Bacterial small ribosomal subunit
(16S) rRNA and large ribosomal subunit (23S) rRNA genes amplified from DNA
extracted from these sacs yielded single genotypes that grouped phylogenetically in the
alphaproteobacteria (27). Fluorescent in situ hybridization (FISH) of bacterial rRNA
showed strong signal exclusively within the mycetome epithelial cells. DNA isolates
from the mycetomes of three species of Placobdella collected in the same lake showed
distinct 16S sequences, but symbionts from within a given species of Placobdella
showed remarkable genetic homogeneity across continental geographic distances (27).
This distinct monophyletic clade of bacteria comprise the only known
alphaproteobacteria that are mutualistic in animals and has since been given the generic
name Reichenowia in honor of Eduard Reichenow who discovered the organs (27).
Other glossiphoniid leeches have different mycetome morphologies. Leeches of the genus *Placobdelloides* exhibit an "esophageal organ" consisting of a cluster of symbiont-bearing cells encircling the esophagus, just anterior to the gastric tissues (Fig. 1B). Kikuchi and Fukatsu (18) determined that the bacteria isolated from this organ in *Placobdella siamensis* and a *Parabdella* sp. were gammaproteobacteria, closely related to several of the well-described insect symbionts, including *Buchnera* and *Wigglesworthia*. A very different morphology for mycetomal organs, consisting of two pairs of globular sacs connected to the esophagus via thin tubules, is found in *Haementeria ghilianii*, the Giant Amazonian leech (Fig. 1C). Although it had been presumed that these sacs contain bacteria, it had never been confirmed.

In addition to these mycetome-associated symbionts, other bacterial lineages have been characterized in association with leeches. Kikuchi et al. (17) described a *Ricketssia* sp. found in various tissues of two species of Japanese glossiphoniid leeches. These bacteria were located intracellularly in epidermal, esophageal, and intestinal tissues, but were not present in all individuals sampled in one population. The medicinal leech, *Hirudo medicinalis*, which belongs to an entirely different suborder, the Rhyncobdellida, also maintains a specific bacterium, *Aeromonas veronii* in its gastric lumen (14). In this case, however, the bacteria appear to be acquired each generation from the environment (16).

Here we report three new isolates of mycetome-associated bacterial symbionts of leeches. One lineage comprises the bacterial symbiont found in the globular mycetomes of the Giant Amazonian Leech, *Haementeria ghilianii*. We also report new results obtained from two additional species of *Placobdelloides*, *Placobdelloides jaegerskioeldi*, the type species of the genus, and *P. multistriata*. Classification of these
bacteria to subdivision was performed with 16S rRNA sequencing and additionally confirmed with FISH. In an attempt to examine the overall evolutionary history of bacterial symbionts and leeches, we also conducted a broad phylogenetic analysis by combining our new DNA sequence data along with our previously published data from Reichenowia (27) and 16S rRNA sequences reported from the other leech bacteria (14, 17, 18).

Materials and Methods

Specimens of the Giant Amazon Leech were collected in the wild in French Guyana in January 2002 and also were obtained from a colony that had been laboratory-reared for over a decade (W. Wuttke, personal communication). The two Placobdelloides species were collected in South Africa in June 2003. Placobdelloides jaegerskioeldii individuals were removed from the rectum of a hippopotamus and P. multilineata was collected under rocks in a pond.

For transmission electron microscopy (TEM) of Haementeria ghilianii mycetomes, the structures were removed by dissection, fixed in 2.5% gluteraldehyde in 0.2 M phosphate buffer, washed in the same buffer, post-fixed in 1% osmium tetroxide in the same buffer, dehydrated through a graded ethanol series and embedded in Spurr’s (27) resin. Sections were cut on a Reichert ultramicrotome, collected on copper grids, stained in uranyl acetate and lead citrate and examined on a Zeiss LEO 902A transmission electron microscope. In light of having found only two adult specimens of P. jaegerskioeldi and one of P. multistriata, these specimens were devoted to molecular characterization via DNA sequencing and FISH as described below.

To perform symbiont DNA isolation, bacterial organs of leeches were dissected
aseptically and DNA was extracted with the DNeasy Extraction kit (QIAGEN, Valencia, Calif.), following the protocol for animal tissues except resolubilizing in only 50-100 µl of buffer. Bacterial rRNA sequences were amplified using bacterial universal primers BSF8 with BSR1541 (http://www.psb.ugent.be/rRNA/primers/index.html) and either AmpliTaq polymerase (Applied Biosystems, Foster City, Calif.) or PureTaq Ready-to-Go PCR Beads (Amersham Pharmacia, Piscataway, N. J.) and a cycling program of an initial denaturation at 94°C for 4 min, 35 cycles of 94°C for 15 sec, 55°C for 15 sec and 72°C for 60 sec and then a hold at 72°C for 7 min. Amplification products were purified with the QIAquick PCR Purification Kit (QIAGEN, Valencia, Calif.) and sequenced using the amplification primers as well as primers BSF517, BSR 534, and BSF1099 ((http://www.psb.ugent.be/rRNA/primers/index.html), BigDye™ terminator sequencing premix (Applied Biosystems, Foster City, Calif.) and an ABI 3700 automated capillary sequencer. Sequences in opposite directions were reconciled with Sequence Navigator (Applied Biosystems, Foster City, Calif.) or Sequencher (Gene Codes, Ann Arbor Mich.).

Sequences were aligned with Clustal W (31) and all phylogenetic analyses were performed using PAUP*4.0b4 (29). Unweighted parsimony using tree-bisection-reconnection (TBR) in a heuristic search was employed with thirty replicates of random addition sequences of taxa. Trees were rooted with Gram-positive taxa. Nodal support was determined via jackknife with 37% deletion of characters in each round under the full heuristic search with 30 random addition sequences. The following bacterial 16S sequences were obtained from GenBank: *Acrystosiphon pisum* P symbiont M27039; *Aeromonas hydrophila*, X74677; *Aeromonas veronii*, AF079299; *Agrobacterium tumifaciens*, ATU389908; *Bacillus anthracis*, AB116124; *Bartonella henselae*,

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Fluorescent in situ hybridizations were performed as previously described (27).

Briefly, leeches were fixed in paraformaldehyde, dehydrated in an ethanol series, embedded in Paraplast PLUS (Kendall Healthcare, Mansfield, Mass.) and sectioned. The 5-7 µM sections were then hybridized with eubacterial (3), alphaproteobacterial (20), or gammaproteobacterial (20) probes labelled with Cy3.

Results

As previously anticipated, TEM clearly revealed bacterial symbionts in the organs of the Giant Amazonian Leeches collected from French Guyana. However, unlike the rod-shaped symbionts found inside mycetomal cells in species of
*Placobdella*, the bacteria from *H. ghilianii* were pleomorphic and embedded in a collagenous extracellular matrix surrounding the periphery of the mature organ (Fig. 2). Amplification of the DNA extracted from the mycetomes of *H. ghilianii* with bacterial-specific 16S rRNA primers yielded a single sequence that was 93% identical to the *Providencia stuartii* 16S rRNA sequence in GenBank, clearly placing the symbiont in the gammaproteobacterial subdivision. The 16S rRNA sequences from separate DNA extractions performed on the anterior and posterior mycetome pairs isolated from the same leech were identical. Furthermore, the sequences from the symbionts of the wild-caught and lab-raised leeches were >99% identical.

The bacterial 16S sequences obtained form the esophageal organs of *Placobdelloides jaegerskioeldi* and *P. multristriata* were approximately 94% similar to the gammaproteobacterial symbionts previously reported from leeches in the same genus (18). FISH performed with eubacterial and gammaproteobacterial-specific probes supported these classifications and suggested relatively low concentrations of the symbiotic bacteria in *H. ghilianii* mycetomes, consistent with the TEM images (Fig. 3a), but large numbers of symbionts within each esophageal organ cell in *P. jaegerskioeldi* (Fig. 3b).

The phylogenetic analysis of the new leech mycetome-associated symbiont 16S sequences combined with those from the other leech symbionts and a set of broadly representative bacteria yielded two equally parsimonious trees; the strict consensus of these is illustrated in Figure 4. All traditional subdivisions of proteobacteria form monophyletic groups with 100% jackknife support. Leech bacterial symbionts appear as five distinct lineages in this phylogeny of proteobacteria. The *Reichenowia* bacteria here group with the human and animal pathogens, *Brucella* and *Bartonella* with a
moderate jackknife value (74%) on this node. The Placobdelloides symbionts comprise a monophyletic clade of gammaproteobacteria that, in turn, is part of a larger clade of gammaproteobacterial animal mutualists. Our new H. ghilianii symbiont lineage did not cluster with the Placobdelloides leech bacteria, but rather was basal to the clade containing these symbionts and the insect obligate symbionts. The alphaproteobacterial Rickettsia sp. from the Japanese glossiphoniid leeches clusters with the other Rickettsia, as expected, however we did not recover a close relationship with the Ixodes tick Rickettsia sp. as Kikuchi et al. (17) did. Finally, Aeromonas veronii from Hirudo medicinalis clusters predictably with A. hydrophila in a very basal gammaproteobacterial clade.

Discussion

The evolution of bacterial symbioses in the context of their leech hosts can be readily understood insofar as the phylogenetic relationships of leeches are well understood (4, 5, 26). Of the various symbiotic lineages identified above, most are concentrated in the family Glossiphoniidae (Fig. 5); that is, with the exception of Aeromonas veronii in Hirudo medicinalis in the very distantly related family Hirudinidae. On the whole, however, leeches in the remaining families have yet to be examined. In terms of associations in the Glossiphoniidae, only the non-bloodfeeding lineages (Helobdella and Glossiphonia) lack esophageal-associated symbionts. Species of Marsupiobdella, the most basal lineage in the group, possess esophageal organ structures identical to those seen in Placobdelloides, substantiating the notion that this is the ancestral condition for glossiphoniid leeches. From the ancestral state, there appears to have been two independent symbiont replacements, each correlated with a radically
different mycetome organ and occupied by phylogenetically independent bacterial symbionts. The ancestral *Placobdella* must have acquired the alphaproteobacterial symbionts in North America after Laurasia split off from Gondwana during the opening of the Tethys Sea about 250 million years ago (Mya). Meanwhile, in what would become South America, the ancestral *Haementeria* acquired a different gammaproteobacterium than that which was present in its predecessors, whereas the *Helobdella* lineage gave up bloodfeeding and the bacterial symbionts altogether (Fig. 5). The ultimate origin of the esophageal symbiosis can be inferred from our analyses to have originated in a freshwater context, probably in an amphibian-feeding leech sometime after the origin of freshwater tetrapods about 350 MYa (8).

Bacteria that provide scarce or unavailable nutrients to their hosts will evolve to become vertically transmitted as their presence is required for host survival and reproduction. It has been predicted that these symbionts eventually will resemble organelles such as mitochondria and chloroplasts and so their study can offer glimpses into what might have occurred in the evolution of these more ancient symbionts (13). Data from the complete *Buchnera* and *Wigglesworthia* genomes have shown that the symbiont genomes have undergone massive reduction in size, often as a result of many deletions, many of which are large and span several genes, as well as a pronounced nucleotide bias (expected to be toward A-T), a loss of RNA genes, and a loss of DNA repair pathways (21). These major genomic changes likely are the primary reason that obligate mutualist symbionts cannot be cultured independently of their host cells.

As of yet, it has not definitively shown that the bacteria found in leech mycetomes are vertically transmitted from hermaphroditic parent to its offspring, however multiple lines of evidence seem to support it. First, these bacteria are found
intracellularly, and in specialized structures, the presence of which has been termed a
“key lifestyle feature” of obligate, vertically transmitted symbionts (32). Second,
vertical transmission is expected to produce concordant phylogenies of symbiont and
host and we observed this pattern with Reichenowia and Placobdella. Placobdella
species from geographically isolated populations (in Ontario, Michigan, and Texas)
showed less genetic differentiation than those taken from different Placobdella species
collected from the same lake. Although much more sampling is clearly needed to
confirm this result, segregation by host leech species, consistent with vertical
transmission, is a likely explanation. Third, independent results have detected bacteria
in leech offspring at early stages. Kikuchi and Fukatsu (18), using PCR detected the
same bacteria as had been observed in the adult in 10 of 10 eggs removed from a P.
siamensis individual. In our previous studies, we used FISH to detect large populations
of Reichenowia in very young P. parasitica leeches that had never fed on blood and that
were removed from the ventral surface of their brooding parent (27). These results
suggest that the bacteria are not acquired from blood meals, though it is possible that
vertical transmission is effected by the parents regurgitating symbiotic bacteria when
depositing cocoons. Finally, the fact that the symbiont sequences from wild-caught
Haementeria ghilianii and those from the same species that had come from laboratory
colonies kept for over a decade were >99% identical, strongly suggest a pattern of
vertical transmission. Chen et al. (10) reported a very similar result in an analysis of
Wigglesworthia from colony-reared and field-collected tsetse flies.

It is not currently known what role any of these leech bacterial symbionts play.
Like the Wigglesworthia symbionts of tsetse flies, the symbionts, particularly those
associated with mycetomes or other specialized cells, may supply their hosts with B
vitamins. These nutrients are scarce in vertebrate blood and so those organisms that are hematophagous throughout their lives (in contrast to blood-feeders such as mosquitoes and fleas which as larvae are carnivores or detritvores) must obtain these nutrients from a symbiotic partner that has retained the metabolic capability to synthesize the nutrients (23). Traditionally, this question was answered with elaborate experiments involving treating the host animal with antibiotics to remove symbionts and then systematically augmenting the hosts’ diet with additional nutrients until comparable fitness to that of symbiont-possessing animals was obtained (12). Now, whole genomic sequencing of symbionts can provide this information, sometimes in a much more definitive way. Bacterial endosymbionts, over time, experience genome reduction, losing genes that are either redundant or code for products that can be provided by the host cell (13). Thus, the presence of complete biosynthetic pathways can suggest important roles in supplementing nutrients to the host. For example, the *Wigglesworthia* genome retains 62 genes involved in the synthesis of vitamins, including B vitamins (1). In contrast, the aphid symbiont, *Buchnera* has a significantly higher number of amino acid biosynthesis genes (1).

The role for the other bacterial species that have been found in leeches is also uncertain. There have been numerous suggestions as to what the role of the *Aeromonas* bacteria might play in medicinal leeches including not only synthesizing B vitamins, but also aiding in the digestion of blood and preventing the growth of other bacterial species in the digestive tract (16). It is possible that the *Rickettsia* reported from the glossophoniid leeches might be secondary symbionts, which are common in many of the insects with primary (obligate) symbionts, including aphids (9) and tsetse flies (11). These S symbionts are non-essential and may not be present in all host individuals or
populations, though they are typically vertically transmitted from parent to offspring (2, 13). Kikuchi et al. (17) provided some evidence that the *Rickettsia* found in *Torix tagoi* were vertically transmitted, however closely related leeches did not appear to be infected, thus the pattern is more reminiscent of S symbionts than obligate mutualists. The advantage to the host of possessing these S symbionts is not certain, however several suggestions have been made. One study found that the pea aphid secondary symbiont (PASS) was able to “rescue” its host from negative survival and fitness effects if the primary symbiont, *Buchnera* was lost (19). These authors propose that their results suggest a potential for repeated symbiont replacements and that bacterial partnerships could be “open for renewal and improvement over evolutionary time,” something that might help to explain the phylogenetic diversity of primary symbionts seen in closely related insects. Perhaps acquisitions of transient bacterial species by leeches, originally functioning as secondary symbionts and later establishing more obligate, primary roles in the host, is similar in this respect and can also help explain the rather unexpected high diversity of bacterial partners in these blood-feeding hosts as well.

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References


FIGURE CAPTIONS

**Figure 1.** Schematic illustration of basic morphology of bacterial organs from each of three groups of blood-feeding leeches in the family Glossophoniidae: A) *Placobdella* spp. with blind-end sacs; B) *Placobdelloides* spp. with small bacteriocytes encircling the leech esophagus; and C) *Haementeria* spp. with large, globular sacs joined to the esophagus via thin ducts.

**Figure 2.** Transmission electron micrograph showing pleomorphic bacterial cells separated by connective tissue in epidermal layer of *H. ghilianii* myctome.

**Figure 3.** Fluorescent in situ hybridizations (FISH) localizing bacterial cells in leech myctomes. A) *H. ghilianii* symbionts hybridized with gammaproteobacterial probe. M = myctome structure; D = duct. B) *P. jaegerskeoldi* esophageal organ symbionts hybridized with gammaproteobacterial probe. O = esophageal organ cell; EL = esophageal lumen. No fluorescence was observed with alphaproteobacterial probes on either of these leech species.

**Figure 4.** Phylogeny of 16S rRNA sequences of leech-associated symbionts in the context of other animal symbionts and various proteobacteria, rooted with Gram-positive taxa. The tree is a strict consensus of two equally parsimonious trees depicted as a phylogram. Jackknife support values as a percentage is indicated on major nodes. The five bacterial clades or lineages associated with blood-feeding leeches are outlined with dashed boxes.

**Figure 5.** Generalized phylogeny of the major groups of leeches. Losses of blood-feeding are depicted here with white branches leading to taxa. The date of divergence of *Placobdella* spp. is estimated to be 250 Mya at the point of separation of North and South America. The origin of the glossiphoniids is estimated to be ~350 Mya and the
ancestral morphology of bacteria-associated organs is hypothesized to be a cluster of small cells encircling the esophagus, as is seen in present-day Marsupiobdella and the Placobdelloides spp.