

Leeches and their microbiota: naturally simple symbiosis models

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Strictly blood-feeding leeches and their limited microbiota provide natural and powerful model systems to examine symbiosis. Blood is devoid of essential nutrients and it is thought that symbiotic bacteria synthesize these for the host. In this review, three distinct leech-microbe associations are described: (i) the mycetome, which is the large symbiont-containing organ associated with the esophagus; (ii) the nephridia and bladders that form the excretory system; and (iii) the digestive tract, where two bacterial species dominate the microbiota. The current knowledge and features of leech biology that promote the investigation of interspecific interactions (host-microbe and microbe-microbe) and their evolution are highlighted.

Model systems for bacteria-animal symbioses

Symbiosis forms a pivotal component in the existence of many animals and plants by providing a multitude of indispensable biological functions [1]. The complexity and intimacy of the majority of these relationships present difficulties in examining how symbiosis arises, identifying the mechanisms that contribute to specificity and elucidating the functional roles of each partner [2]. Many established model systems are either monospecific (in which the host maintains relations with a single microbial species), artificially reconstituted from far more complex associations, or too multifaceted to reveal underlying mechanisms [3,4]. The study of these pioneering systems has resulted in exciting discoveries but to assess how widely applicable these findings are, a comparative approach using a wide range of model systems is required. The medicinal leech is a promising, bona fide model for symbiotic associations and has a microbial community of limited complexity to facilitate examination of fundamental aspects of interspecific relations (Box 1). In this review, we describe three distinct microbe-leech associations found in different leech species: mycetome, nephridia and bladders, and digestive-tract symbioses. Aspects of leech biology that promote its application as a powerful model system for the study of host-microbe and microbe-microbe interactions will also be highlighted.

Hirudinids: taxonomy, natural history and medicinal applications

Leeches are fascinating animals that can evoke contradictory responses. One can observe with amazement the leech undulating elegantly while swimming or with horror

as its whole body contracts rhythmically while pumping the blood from an unsuspecting victim. Strictly blood-feeding leeches are found in the orders Rhynchobdellida, species of which feed using a tubular proboscis and have a bacterial-symbiont-containing organ (the mycetome) associated with the esophagus, and Arhynchobdellida, which feed using toothed jaws and lack a mycetome [5]. Recent molecular studies have shown that the medicinal leech, although usually marketed as *Hirudo medicinalis* (Hirudinea: Arhynchobdellida: Hirudinidae), probably consists of a complex of at least three species: *Hirudo orientalis*, the commonly sold *Hirudo verbana* and the rare *H. medicinalis* [6–9]. Hirudinids are hermaphrodites that deposit cocoons containing multiple eggs at the land-water interface [5]. Juvenile leeches reportedly consume their first blood meal from amphibians whereas successive meals can be obtained from amphibians, fish or mammals [5,10]. The ingested blood is quickly modified in the crop by the discharge of water and osmolytes through the multiple pairs of bladders that lie near the lateral ceca of the crop (Figure 1). The erythrocytes are stored apparently physically intact within the crop for up to six months. The actual digestion of the blood meal and absorption of nutrients is thought to occur in the much smaller intestine (located between the last pair of crop ceca), which combines some functions of the intestine and rectum (Figure 1).

The remarkable abilities of the medicinal leech to consume five to six times its body weight in a single blood meal and to release an array of potent chemicals with its saliva has led to an unexpected resurgence of the use of leeches in modern medicine [5]. Recently, the medicinal leech was approved as a medical device for its bloodletting capabilities by the Food and Drug Administration of the USA (http://www.fda.gov/fdac/features/2004/504_leech.html). In a manner that has yet to be reproduced by pharmaceuticals, the direct application of *H. medicinalis* to areas of acute venous congestion provides a cost-effective and reliable treatment to ameliorate the postoperative effects associated with reconstructive surgery [11–13]. Powerful vasodilators and anti-inflammatory and anticoagulation molecules have been isolated, characterized and patented from leech saliva [14,15]. From the microbiological perspective, an interesting observation originally made in the 1980s was the diagnosis of wound infections caused by *Aeromonas* in patients receiving leech therapy [12,16]. The use of antibiotics before bloodletting usually prevents these infections. Earlier studies had identified *Aeromonas* as the sole digestive-tract symbiont of *H. medicinalis*. The

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Box 1. Advantages of using the leech as a symbiosis model

- (i) Inexpensive and easily bred invertebrate host.
- (ii) Simple host morphology.
- (iii) Limited dietary intake (blood).
- (iv) Simple trinary association in the crop.
- (v) In the digestive tract, the dominant *Aeromonas* symbiont is culturable and amenable to genetic manipulations and reintroduction.
- (vi) *Aeromonas* is also a pathogen, which provides an opportunity to compare symbiosis and virulence factors in one organism.

detection of one culturable symbiont led us to pursue the feasibility of using the medicinal leech as a naturally occurring simple model for digestive-tract associations [17].

Symbiotic associations of leeches*Mycetome symbiosis*

The most extreme and intimate examples of interspecific relationships are intracellular symbioses. In these specialized associations, leech symbionts are usually harbored in the cytoplasm of mycetocytes. These are large specialized cells that typically aggregate into a large symbiotic organ, which, although it houses bacteria, is called a mycetome for historical reasons [1]. Important physiological functions occur within mycetomes, such as the provision of essential host nutrients (e.g. vitamins and amino acids). Obligate insect symbionts such as *Wigglesworthia* spp. in tsetse flies and *Buchnera* spp. in aphids are well characterized by genome sequencing and elegant functional assays, and currently represent

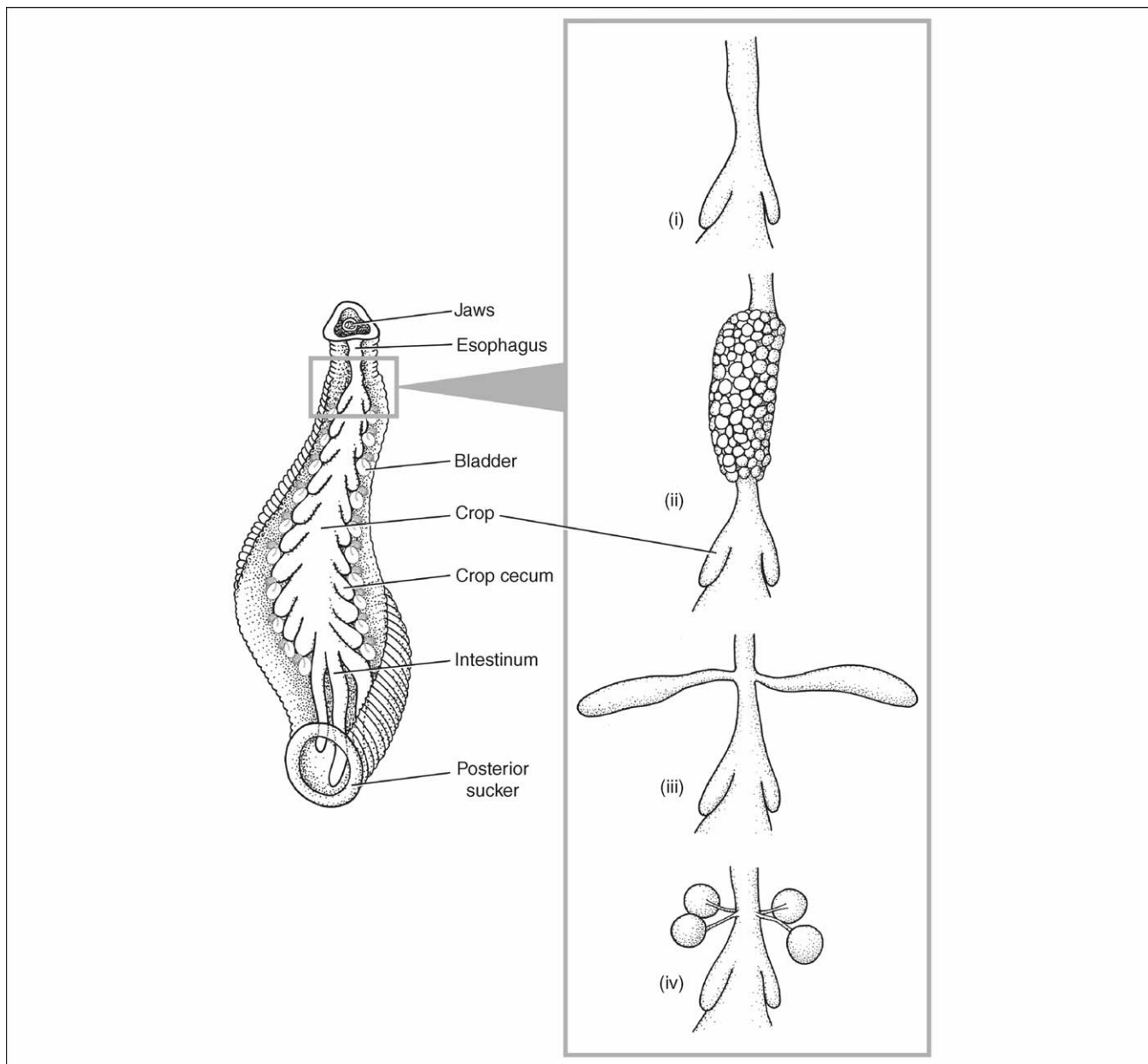


Figure 1. Leech internal morphology depicting the structural variety of mycetomes. Drawing of the digestive tract and excretory organs based on the medicinal leech. The inset shows schematic illustrations of mycetome morphological variations: (i) basic structure lacking mycetocytes in *Hirudo verbana*; (ii) large mycetocytes form a tube-like structure in *Placobdelloides* spp.; (iii) a pair of pear-shaped mycetomes join the esophagus at their narrowed ends in *Placobdella* spp.; (iv) two pairs of large bulbous sacs connected to the esophagus by narrow ducts comprise the mycetomes in *Haementeria* species. Figure redrawn, with permission, from Refs [21] and [43]. © (2006) American Society for Microbiology.

model systems for intracellular symbiosis in invertebrates [18].

In leeches, mycetomes occur in the order Rhynchobdellida (mainly in the family Glossiphoniidae [1,5]) but not in the order Arhynchobdellida, which includes the medicinal leech. Three distinct mycetome morphotypes have been described in glossiphoniid leeches [5]: large mycetocytes that surround the esophagus lumen in *Placobdelloides* spp. [Figure 1, part (ii)]; a pair of pear-shaped blind sacs in *Placobdella* spp. [Figure 1, part (iii)]; and two pairs of large bulbous sacs connected to the esophagus by narrow ducts in *Haementeria* spp. [Figure 1, part (iv)].

Recent molecular phylogenetic analyses based on 16S rRNA gene sequences have revealed that the symbionts of *Placobdelloides* spp. and *Haementeria* spp. belong to the γ -3 subdivision of the Proteobacteria, whereas the symbionts of *Placobdella* spp. belong to the Rhizobiaceae family in the α -Proteobacteria [19–21]. The γ -proteobacterial leech symbionts cluster with the insect symbionts *Buchnera* spp. and *Wigglesworthia* spp., although the symbionts of *Placobdelloides* spp. and *Haementeria* spp. do not form a monophyletic group (Figure 2) [21]. These phylogenetic relationships suggest that the evolution of mycetome symbioses in glossiphoniid leeches has occurred multiple times, which is also supported by the morphological diversity of this organ or, alternatively, suggests that the current symbionts replaced ancestral ones.

Rickettsia symbionts

In addition to the mycetome symbionts, intracellular symbionts that belong to the family Rickettsiaceae (α -Proteobacteria) were discovered in three Japanese glossiphoniid species [22,23]. The Rickettsiaceae are well-described parasitic and/or commensalistic bacteria that can be isolated from a wide range of animals [24]. Unlike the mycetome symbionts, rickettsial symbionts exhibit wider tropism: they are detected in various leech tissues such as the epidermis, esophagus and salivary glands and exhibit a heterogeneous distribution within host populations [22].

Intracellular symbiont transmission

Although it remains unclear whether the leech intracellular symbionts are transovarially transmitted, multiple observations support a transmission from parent to offspring through the egg. In *Placobdelloides* spp., the same microbial species found in adult mycetomes were also detected in 100% of examined eggs [19]. Rickettsial symbionts were also consistently detected in the eggs of infected leeches [22]. Finally, *Placobdella parasitica* juveniles that had never received a blood meal were shown to harbor already large symbiont populations in their mycetomes [20]. The inability to culture the mycetome symbionts suggests specialization towards an intracellular lifestyle, possibly because of their stable inheritance through host lineages and associated genome reduction (reviewed in Ref. [25]). The stability of these associations supports their indispensable roles in host biology.

Nephridia and bladder symbiosis

The seminal paper by Büsing *et al.* [26] described the presence of two morphologically distinct bacteria associated

within the nephridia and bladders, the excretory organs of *H. medicinalis* (Figure 1). The nephridia serve to remove waste products from the haemocoelomic fluid and recover salts from the primary urine. The urine and nitrogenous waste in the form of ammonia are stored in the bladder until released [5]. 16S rRNA gene sequencing was used to identify the symbionts residing in the nephridia and bladders as the intracellular *Ochrobactrum*, an α -proteobacterium related to *Sinorhizobium* (Figure 2), and an extracellular *Flavobacterium*, a member of the Bacteroidetes.*† Furthermore, bacteria were detected microscopically in the bladder of embryos, which strongly supports the hypothesis of vertical transmission [27]. Although their functional roles remain uncertain, experimental evidence suggests that the bacteria contribute to the degradation of nitrogenous waste [26].

Digestive-tract symbiosis

The digestive-tract microbiota of hirudinid leeches is of particular interest because of the medicinal application of these leeches and their lack of mycetomes (unlike the glossiphoniid leeches, in which the hallmark mycetomes are presumed to perform an essential function for the host). The first microbiologists who examined the hirudinid digestive-tract microbiota in the 1950s reported the surprising presence of a single, β -hemolytic bacterial species that released many proteolytic enzymes [26]. Combined with an apparent lack of host digestive enzymes in the crop, these early investigators posed three possible functions for the gut symbiont: (i) aiding in the digestion of the blood meal; (ii) providing essential nutrients; and (iii) ‘colonization resistance’, in which they function to prevent colonization by other potentially harmful microorganisms. More recent studies demonstrated the presence of host-produced proteases in the intestine, which cast some doubt on the importance of the bacterial-produced proteases in digestion [28]. However, the lack of direct experimental evidence has not ruled out any of these hypotheses (reviewed in Ref. [29]).

The symbiont was identified as *Aeromonas* using current taxonomy but the species identity differed between research groups [30–33]. Although initially reported as *Aeromonas hydrophila* by several investigators, our group identified the symbionts as *Aeromonas veronii* biovar *sobria* using biochemical tests and 16S rRNA gene sequences [31]. It is interesting to note that *Aeromonas culicicola*, which was isolated from the midgut of female *Culex quinquefasciatus* and *Aedes aegyptii* mosquitoes [34], has recently been renamed *A. veronii* [35], suggesting a propensity of this species to colonize the gastrointestinal tract of blood-feeding organisms. The differing species identifications probably reflect the dynamic and complex taxonomy of *Aeromonas*.

Culture-independent characterization

A limitation of the previous characterizations of digestive-tract microbiota was that the studies were purely culture-based. It is widely recognized that ~99% of microbes are

* A. Schramm *et al.*, abstract 505, 101st General Meeting of the American Society for Microbiology, Orlando, USA, 2001.

† J. Graf *et al.*, abstract 478, 100th General Meeting of the American Society for Microbiology, Los Angeles, USA, 2000.

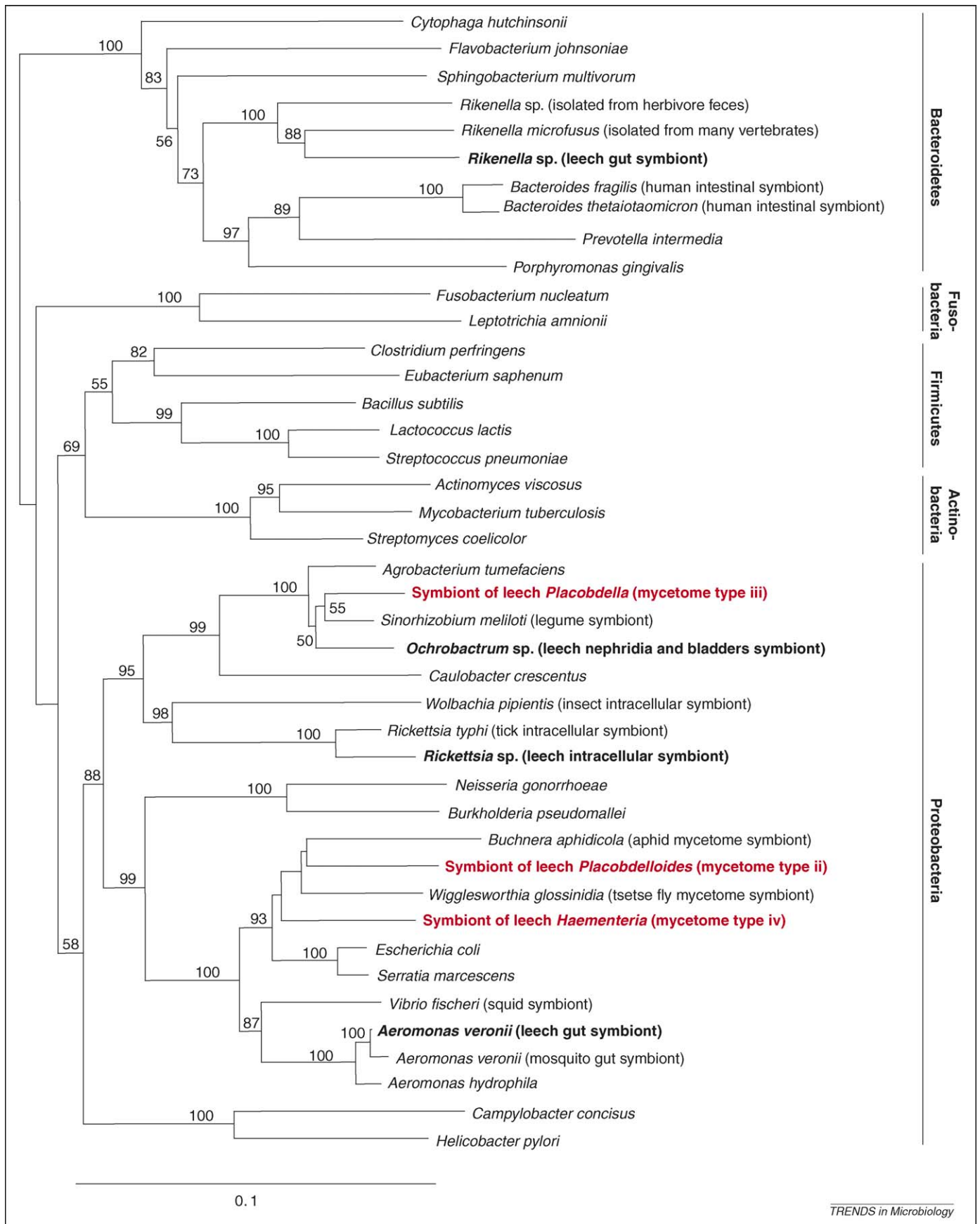


Figure 2. Phylogenetic tree based on the sequence of the 16S rRNA gene (neighbor-joining analysis with a Kimura's correction; aligned 1050 bp). Bacterial phyla are shown on the right. Leech symbionts are represented in bold and red text and their location within the host is stated in parentheses. The mycetome types in red text correspond to those in Figure 1. Bootstrap values higher than 50% are depicted at the nodes. The scale bar represents 0.1 changes per base.

presently unable to be cultivated [36,37]. Technological developments in the culture-independent profiling of microbial community complexity and diversity have revealed a plethora of novel cohabiting microorganisms that far outnumber the culturable organisms. These developments have also greatly advanced our understanding of the residential microbiota of digestive tracts, to which essential roles in host biology have been attributed, such as the provision of essential nutrients (reviewed in Ref. [3]), development [38], energy balance [39] and the priming of immunity [40,41]. Interesting parallels in the digestive-tract microbial composition among various host species have been described [39,42], which raises the question of whether their functional roles are universal or tailored to the different hosts.

A recent culture-independent study discovered the presence of a second symbiont in the crop that we have been unable to cultivate in the laboratory [43]. The 16S rRNA gene sequence indicates that it is a relative of *Rikenella*, members of the Bacteroidetes that have been found in several different digestive tracts. An exciting aspect about the discovery of a second symbiont is the natural occurrence of a restricted – but not monospecific – digestive-tract microbiota, which will enable us not only to investigate microbe–host interactions but also to investigate the interaction between different microbial species. Relevant features of the *Aeromonas* and *Rikenella* species that comprise the basic two-member microbial community in the crop are discussed here.

Aeromonadaceae

Aeromonas species are motile, Gram-negative rods that belong to the family Aeromonadaceae [44]. A widely noted characteristic of *Aeromonas* spp. is the production of a large number of exported hydrolytic enzymes that could aid in the breakdown of nutrients inside the digestive tract of animals. This family currently consists of 17 facultatively anaerobic species that occupy a spectrum of niches ranging from free-living occupants of freshwater to opportunistic pathogens of fish, amphibians and humans (reviewed in Ref. [45]), and to the digestive tract symbionts of a variety of blood feeders including mosquitoes, the medicinal leech and the vampire bat [17,31,34,46]. Three *Aeromonas* species including *A. veronii* are associated with a range of maladies including wound infection, septicemia and diarrhea in humans [45]. Therefore, *A. veronii* seems to have an innate ability to infect the digestive tracts of multiple host species where manifestations of infection span from pathogenesis to cooperative.

Rikenellaceae

The recurring identification of 16S rRNA gene sequences that belong to the Rikenellaceae from a wide range of digestive tracts is suggestive of both evolutionary adaptation and physiological contributions towards digestive-tract ecosystems (Figure 2). All of the isolates or sequences were obtained from a variety of gastrointestinal environments including goat rumen, termite gut, murine cecum and the human colon [39,47,48]. Knowledge of the *Rikenella* genus is further obscured because of their fastidious growth and obligate anaerobic requirements.

A novel *Rikenella* species, related to *Rikenella microfusus* isolated from the cecal and fecal samples of Japanese fowl [49], has been identified as one of two dominant residents of the medicinal leech crop [43]. An intriguing question is whether the leech crop is sufficiently anaerobic to support the growth of the *Rikenella* symbiont or if *A. veronii* has to remove residual oxygen from the ingested blood meal to prime the microenvironment for the *Rikenella* symbiont.

The presence of a basic two-member microbial community in the leech digestive crop provides an exciting and unique opportunity to further extend knowledge of *Rikenella* species, albeit indirectly. For example, differential antibiotic regimens might be used selectively to clear the *Aeromonas* or *Rikenella* symbiont. The reintroduction of various concentrations of *A. veronii* and/or isogenic mutants into the host can reveal whether spatial or quantitative alterations of the *Rikenella* population occur by employing techniques such as fluorescence *in situ* hybridization and quantitative PCR. Host fitness assays after differential antibiotic treatments to examine classical life history traits such as reproductive output, growth rate and viability could also prove valuable towards the elucidation of microbial functional roles.

Factors that contribute to a limited microbial complexity

Factors that contribute to the unusual simplicity of the leech digestive symbiosis could be derived from three sources: the ingested blood, the host and/or the symbiotic bacteria [17,50,51]. The complement system of vertebrate blood contains powerful antimicrobial properties [52]. Two lines of evidence suggest that the ingested complement system remains active for some time inside the leech and contributes to the specificity of the microbiota. Heat-inactivation of the blood before feeding enables colonization by some bacterial species that were unable to colonize when fed to the leech in fresh blood [50]. Furthermore, the importance of the *Aeromonas* lipopolysaccharide (LPS) layer in protecting against the antimicrobial properties of the complement system has been demonstrated [53] by observing that serum-sensitive *Aeromonas* mutants with a defect in their LPS had a dramatically reduced ability to colonize the leech [51].

Other bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* were tested for their ability to colonize the leech digestive tract and were able to persist inside it but had a dramatically reduced ability to grow, independent of the activity of the complement system, which suggests the presence of a second layer of defense [50]. The discovery of the *Aeromonas* symbiont led to speculation that this symbiont might release antimicrobial compounds [26]. As part of a culture-independent characterization of the leech digestive system, the microbiota of the intestine in which the actual digestion of the blood occurs was also characterized. The intestine harbored a more diverse microbial community with an average of eight species detected [43]. The microbial community of the intestine, similar to the crop, was dominated by the *Rikenella* and *Aeromonas* symbionts. The presence of a more diverse microbiota despite the presence of the crop

Box 2. Future directions

Mycetome symbiosis: The possibility of three distinct origins of this symbiosis in the Rhynchobdellid leeches makes for exciting evolutionary developmental biology ('evo-devo') studies. The ability to dissect out these organs and isolate large numbers of symbionts enables the implementation of genomic and proteomic tools to gain an insight into their functional significance.

Nephridia and bladders symbiosis: This symbiosis has potential for being used in comparative physiological studies with the *Acidovorax*-like symbionts that are harbored within the nephridia of earthworms, a terrestrial annelid [54,55]. It would be interesting to observe whether the functional roles of these phylogenetically distinct symbionts are similar.

Digestive-tract symbiosis: The combination of molecular genetic tools for *Aeromonas*, fluorescence *in situ* hybridization for the localization of both *Aeromonas* and *Rikenella* and the ability to remove symbionts from juvenile hosts will provide new understanding about molecular interactions, functional analyses and symbiont population dynamics.

symbionts suggests that these two species are not responsible for inhibiting the growth of microorganisms in the crop unless this activity is specifically downregulated within the intestinal environment.

Concluding remarks

Symbiosis is an important driving force of metazoan evolution. The association of an animal with microorganisms provides the host animal with new metabolic capabilities – for example, enabling animals to feed exclusively on blood. Whereas intracellular symbioses presumably require the tightest coordination between microbe and host, extracellular digestive-tract associations are more prevalent and usually involve more complex microbial communities. This complexity not only makes understanding the molecular interactions between symbionts and host difficult but also complicates the dissection of those interactions between the bacterial symbionts. Although the general behavior of bacteria belonging to one species is well understood, we are still at the early beginnings of understanding how different species of bacteria interact in a microbial community. Digestive tracts are an important environment where microbial interactions are likely to have an important role. The digestive-tract symbiosis of the medicinal leech with the two dominant extracellular *A. veronii* and *Rikenella*-like symbionts provides a unique opportunity to investigate not only microbe–host but also microbe–microbe interactions in a naturally simple system (Box 2).

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