TSETSE FLY AND MEDICINAL LEECH SYMBIOSES: PROVIDING INSIGHTS INTO MICROBIAL SPECIES INTERACTIONS WITHIN GASTROINTESTINAL SYSTEMS

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TSETSE FLY AND MEDICINAL LEECH SYMBIOSES: PROVIDING INSIGHTS INTO MICROBIAL SPECIES INTERACTIONS WITHIN GASTROINTESTINAL SYSTEMS

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MICROBIAL INTERACTION
SYMBIOSIS
MEDICINAL LEECH
TSETSE FLY

ABSTRACT. – A wealth of information remains to be discovered on the functional and evolutionary significance of microbial interactions within hosts. In this review, the simple digestive tract symbioses of the tsetse fly (Diptera: Glossinidae) and the European medicinal leech (Hirudinea: Hirudidae) are described and promoted as powerful model systems for the examination of interspecific microbial relations. The comparative analyses of these two systems can highlight universal themes of microbial associations or particularities outfitted for a specific symbiotic system. This review concludes with areas of future research where the examination of concerted actions between microbial species may prove fruitful, such as in the exchange and breakdown of chemicals as well as in the regulation of microbial density. A brief description of the microanalytical tools and techniques that are available to advance our understanding of how different microorganisms interact within a host ecosystem is also included.

INTRODUCTION

Ecosystems are composed of symbiotic networks that often prompt ecological innovations. Despite the omnipresence of interspecific relations, variations in their intimacy, frequency, co-evolutionary history, and impact on biological fitness are vastly apparent (Buchner 1965). Major milestones in microbial cultivation, genetic manipulation techniques, and genome technology, as well as innovative culture-independent profiling have advanced our understanding of the species composition as well as the molecular and ecological cues that interface to drive the specificity, acquisition, progression and persistence of symbiotic systems.

A plethora of contributory roles benefiting host biology can be attributed to residential microbial communities. These include nutritional provisioning and energy balance (Ley *et al*. 2005, Sonnenburg *et al*. 2005, Backhed *et al*. 2004), the broadening of host food range (Buchner 1965), increased tolerance towards stress and infections (Scarborough *et al*. 2005, Oliver *et al*. 2003), and dramatic morphogenesis effects particularly towards the development of the immune (Mazmanian *et al*. 2005, Noverr & Huffnagle 2004, Rakoff-Nahoum *et al*. 2004, Hooper *et al*. 2003, Umesaki *et al*. 1999), vascular (Stappenback *et al*. 2002), and gastrointestinal systems (Bates *et al*. 2006, Bry *et al*. 1996). In an analogous manner, microbial species composition and density are spatially and temporally influenced by biotic and abiotic factors such as host genotype, immunity, diet and perturbations of the external host environment (Dethlefsen *et al*. 2006, Rio *et al*. 2006, Braschler *et al*. 2003, Graf 1999). Although we have made considerable progress in understanding host-microbe relations, it is now imperative to foster a more holistic approach that includes the examination of interactions between different microorganisms within a given host environment.

Individuals within microbial communities help determine neighboring species composition through environmentally modifying phenomena such as cooperation, competition, the alteration of host gene expression, quorum sensing, and interference mechanisms such as the production of bacteriocins and other toxins (Guan *et al*. 2007, Guarner & Malagelada 2003, Hooper & Gordon 2001, Ducluzeau 1993). As such, colonizers contribute towards the selection pressure to which they and future generations are exposed through feedback generated in the evolutionary process (Day *et al*. 2003). Initial areas of examination where microbial interactions within hosts can prove fruitful include interdependency in the exchange and breakdown of various metabolites and substrates (Wu *et al*. 2006) and microbial density regulation. Both of these activities, if indeed involving the concerted action of multiple microbial species, would require integrated molecular communication presumably through coordinated gene expression and translational processes. These integrative processes should ultimately result in further optimization of the symbiotic system contributing towards its stability and retention.

The present review commences with a description of two unique, albeit complementary, model systems for examining gastrointestinal associations: the tsetse fly (Diptera: Glossinidae) and the European medicinal leech (Hirudinea: Hirudidae). In contrast to the complex digestive tracts of omnivorous animals which, often contain hundreds of microbial species (Eckburg *et al*. 2005), both of these invertebrate hosts are exclusive blood feeders

that house limited microbial communities within simple guts. The simplicity of these symbiotic models in terms of limited dietary intake, immunological capabilities (i.e. invertebrate immunity consists solely of an innate component), resident microbial composition, and host gut morphology, along with the feasibility of genetically manipulating the microbial symbionts and hosts, provides natural and experimentally practical sources to examine fundamental aspects of microbial interactions. Furthermore, comparative analyses of the interactions within these systems can highlight universal themes of microbial associations or particularities outfitted for a specific symbiotic system. This review concludes with a brief discussion of areas of future research and a description of the microanalytical tools and techniques that are available to advance our understanding of how different microorganisms interact within a host microenvironment.

Tsetse fly

Tsetse flies are the sole vectors of flagellated protozoan *Trypanosoma* spp. (Kinetoplastida: Trypanosomatidae), the causative agents of sleeping sickness in humans and nagana in other animals. These zoonotic diseases are endemic to tsetse's sub-Saharan Africa geographic range and threaten more than sixty million people in an area of approximately 11 million km^2 (WHO 2001). Given that male and female adults are exclusively hematophagous, both sexes may serve as disease vectors if encountering an infectious meal.

Tsetse flies have a viviparous reproductive biology in that an adult female produces a single egg per gonotrophic cycle that, following fertilization, hatches and develops *in utero* (Fig. 1). After a period of maturation and sequential molting within the mother (i.e. 1^{st}-3^{rd} instar develop *in utero*), a fully mature 3^{rd} instar larva is deposited and quickly pupates in the soil. Unlike most insects that typically produce many offspring on multiple occasions, a female can only deposit five to seven larvae during her three to four month life span (Leak 1999).

In addition to trypanosome infections, tsetse can harbour two different digestive tract symbionts representing diverse associations and co-evolutionary histories. The γ–proteobacteria enterics, **obligate** mutualist *Wigglesworthia glossinidia* (hereafter *Wigglesworthia*) and the beneficial *Sodalis glossinidius* (hereafter *Sodalis*) are **vertically transmitted** to the intrauterine larvae though nutritional milk gland secretions (Denlinger & Ma 1975, Ma & Denlinger 1974) in contrast to the **horizontally transmitted** trypanosomes. Tsetse may also be infected with a **facultative** parasitic α–proteobacteria *Wolbachia* sp. (closely related to *Wolbachia pipientis*), which infects the developing oocyte (Cheng *et al*. 2000, O'Neill *et al*. 1993 and reviewed in Rio *et al*. 2004). Given the unique reproductive biology of the tsetse fly, all three symbionts are maternally transmitted.

(P)rimary symbiont: Wigglesworthia

The tsetse fly-*Wigglesworthia* symbiosis is characterized as obligate for both partners, primarily due to the fact that *Wigglesworthia* can not be cultured '*in vitro*' and tsetse's inability to reproduce in the absence of this microorganism (Nogge & Ritz 1982). Phylogenetic analysis supports a concordant evolution of the tsetse **P-symbiont** with its host species dating back 50-80 million years (Chen *et al*. 1999).

Wigglesworthia resides freely within the cytosol of specialized host cells, known as bacteriocytes. These cells, which collectively form an organ referred to as the bacteriome, are found in the anterior midgut. The *Wigglesworthia* genome is 697 kb in size and encodes for 621 predicted protein-coding sequences (CDSs) (Akman *et al*. 2002). As confirmed by dietary supplementation experiments (Nogge 1978, Nogge 1976), strong selection has favored the retention of metabolic pathways believed to be pivotal to the mutualistic relationship-such as those involved in the synthesis of the B complex vitamins that are scarce in vertebrate blood. Retention of these pathways complements the host's strictly hematophagous lifestyle. A detailed review of *Wigglesworthia* metabolic adaptations, assembled through genome content analysis and in context with host biology, is described by Zientz *et al*. 2004.

One of the unusual findings in the *Wigglesworthia* genome is the absence of *dnaA*, an essential gene encoding a DNA replication initiation protein, an observation previously unprecedented in eubacteria. Following the annotation of the *Wigglesworthia* genome, the genomes of other closely related obligate insect symbionts such as *Blochmannia floridanus* from the carpenter ant (Gil *et al*. 2003) and *Baumannia cicadellinicola* from the sharpshooter (Wu *et al*. 2006) were also found to lack a *dnaA* locus. Hence, lack of robust DNA replication machinery in these obligate symbionts could be one of the mechanisms by which their hosts regulate symbiont density as well as their corresponding physiological contributions.

Another unanticipated finding is *Wigglesworthia*'s ability to synthesize a complete flagellar apparatus despite the fact that neither a flagellum nor motility has ever been observed for *Wigglesworthia* within adult bacteriocytes. Nonetheless, approximately 6% of the *Wigglesworthia* genome is reserved for genes that encode this structure (Akman *et al*. 2002).

(S)econdary symbiont: Sodalis

Sodalis (which means companion in Latin) is harbored both intra- and extracellularly, primarily in gut tissue but also in muscle, fat body, hemolymph, milk gland and in the salivary gland tissue of some tsetse species (Cheng & Aksoy 1999). The specific elimination of the tsetse **S-symbiont** via antibiotic treatments results in reduced

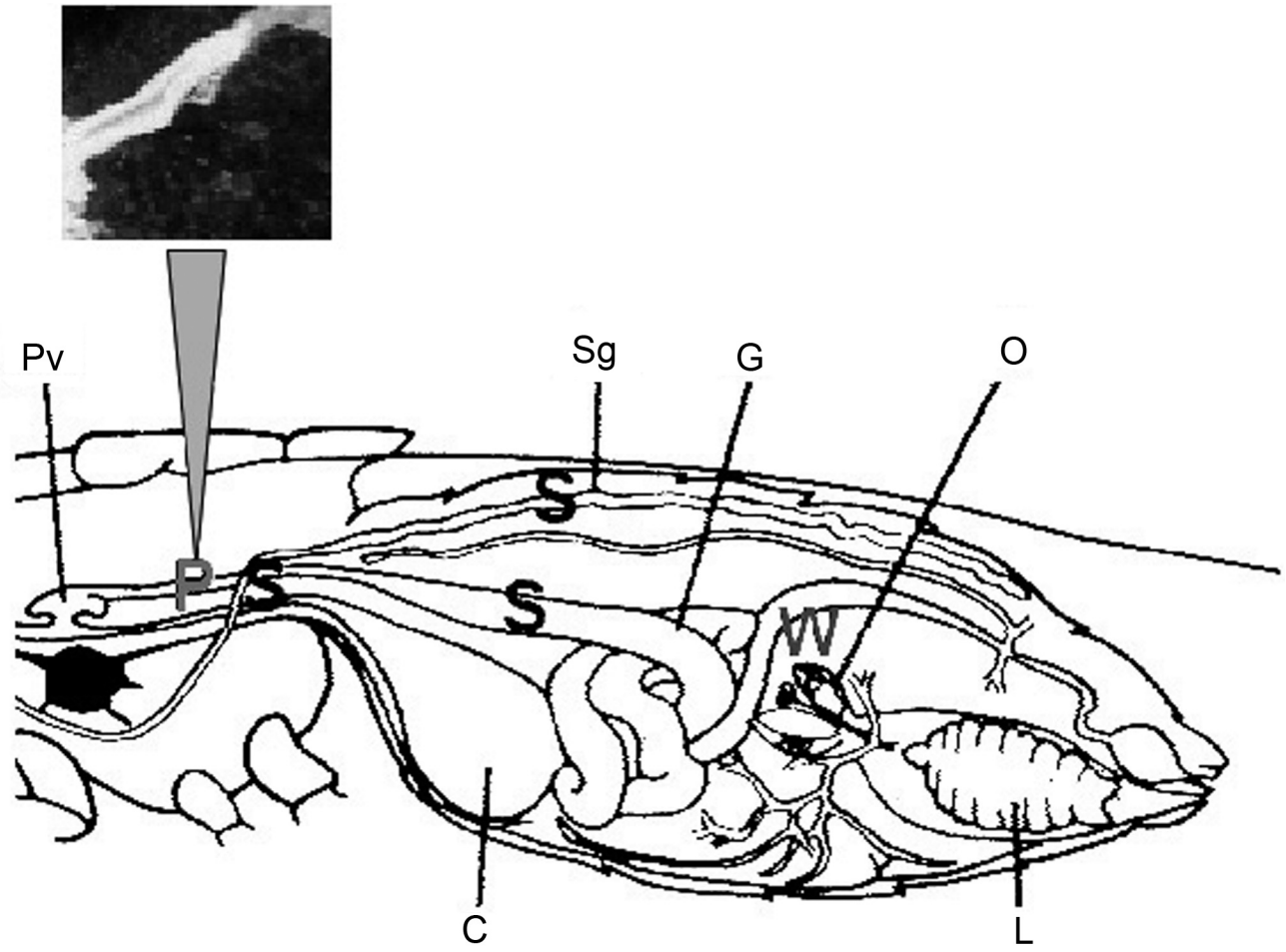

Fig. 1. – Drawing of tsetse thorax and abdomen depicting symbiont localization. Abbreviations: C, crop; G, gut; L, intrauterine larva; O, ovary, P, primary symbiont *Wigglesworthia*; Pv, proventriculus; S, secondary symbiont *Sodalis*; Sg, salivary gland; W, *Wolbachia*. The inset demonstrates the U-shaped, opaque bacteriome organ that houses *Wigglesworthia* within bacteriocytes.

tsetse longevity but does not affect host fecundity (Dale & Welburn, 2001), suggestive of a beneficial role towards host biology. Significant phylogenetic differences in 16S rDNA (Aksoy *et al*. 1997) and *fts*Z (Weiss *et al*. 2006) are lacking among *Sodalis* isolated from different tsetse species. However, amplified fragment length polymorphism (AFLP) markers have detected some genetic diversity, most likely reflecting some degree of host-driven selective pressure (Geiger *et al*. 2005).

Further support for the recent acquisition of Sodalis by tsetse includes the ability to culture this symbiont 'in vitro' (one of the few insect symbionts amenable to cultivation) (Beard *et al*. 1993, Welburn *et al*. 1987), the identification of genomic prophage sequences (thus far devoid in the genomes of ancient insect P-symbionts) (Clark *et al*. 2007), a lack of adenine-thymine bias within its genome (Clark *et al*. 2007, Toh *et al*. 2006) and the successful repopulation of tsetse by *Sodalis* isolated from distant tsetse species without any observable fitness effect (Weiss et al. 2006). Phylogenetic reconstructions reveal close relations between *Sodalis* and the symbionts of *Sitophilus* weevils (Rio *et al*. 2003, Heddi *et al*. 1998), the aphid *Acrythosiphon pisum*, (Fukatsu *et al*. 2000), the bloodsucking fly *Craterina melbae* (Novakova & Hypsa 2007), and the slender pigeon louse *Columbicola columbae* (Fukatsu *et al*. 2007). These data indicate that this group of microorganisms shares a recent common ancestor within the Enterobacteriaceae.

The sequencing of the *Sodalis* genome (Toh *et al*. 2006) revealed that this microbe has a 4.2 Mb chromosome, which is large relative to that of *Wigglesworthia* and other ancient insect P-symbiont genomes. Despite its size, the genome has a reduced coding capacity of only 51%; this is due largely to the presence of a significant number of pseudogenes. The majority of these pseudogenes are homologs of known proteins that, in other bacterial species, have functions related to defense or the transport and metabolism of carbohydrates and inorganic ions. The diminution of genetic components dedicated to defense may have arisen due to the strong fidelity of maternal transmission and constant host habitation, which provides protection from host defenses (Rio *et al*. 2006).

Interestingly, *Sodalis* has a higher resistance to the tsetse antimicrobial proteins Attacin and Diptericin in comparison to *Escherichia coli* (Hu & Aksoy 2005, Hao *et al*. 2001). The retention of multiple stress responses by *Sodalis* is likely indicative of the environmental pressures it encounters, such as reactive oxygen species within the host midgut. The erosion of *Sodalis* loci involved in carbohydrate and inorganic ion transport epitomizes the genome streamlining that complements utilization of the high iron-low carbohydrate makeup of the host blood diet.

Three phylogenetically distinct gene clusters with similarities to the type-III secretion systems (TTSS) of some virulent bacteria are encoded within the *Sodalis* chromosome. These *Sodalis* Symbiosis Regions (SSR), SSR-1, SSR-2, and SSR-3, share homology with *ysa* of *Yersinia enterolitica,* SPI-1 of *Salmonella* and the Mix-Spa of the *Shigella* virulence plasmid, and the SPI-2 of *Salmonella* and the chromosomally encoded pathogenicity island of *Y. pestis*, respectively (Toh *et al*. 2006, Dale *et al*. 2005, Dale *et al*. 2001). Evolutionary analyses support accelerated rates of amino acid divergence in the extracellular TTSS components, including the needle structure and effector proteins of SSR-1 and SSR-2 (Dale & Moran 2006), similar to what has been previously demonstrated for pathogenic microbes (Boyd *et al*. 1997, Li *et al*. 1995). Although critical components of these SSRs appear absent or under relaxed selection (Toh *et al*. 2006, Dale *et al*. 2005), SSR-1 is necessary for intracellular colonization and SSR-2 is essential for subsequent microbial proliferation. These divergent roles presumably arose through gene duplication and neofunctionalization events (Dale *et al*. 2001). The transcriptional activity of representative SSR loci are up regulated during the development of tsetse larval and early pupal stages (Toh *et al*. 2006), periods that correspond to *Sodalis* vertical transmission and population proliferation (Rio *et al*. 2006).

Similar to *Wigglesworthia*, the *Sodalis* genome is also capable of synthesizing a complete flagellum. Although the functional role of this apparatus in these symbionts remain unknown, flagella may be important for their transmission and establishment within the intra-uterine progeny (Toh *et al*. 2006) or for a more general role such as the invasion of suitable host cells or use as export machinery. The *Sodalis* chromosome is undergoing active gene decay and is being molded by slow erosion at individual loci (Toh *et al*. 2006), resulting in the degradation of pathways no longer necessary in the restricted host environment. As such, the *Sodalis* genome is thought to represent an evolutionary intermediate transitioning from a free-living to exclusively mutualistic lifestyle.

Tsetse symbiont population dynamics

Factors that influence the regulation of microbial symbiont density within their eukaryotic hosts remain largely unknown. In order to shed light on these factors, the population dynamics of tsetse's bacterial symbionts were recently examined throughout host development and during potentially disruptive physiological and ecological events including host immune challenge, the presence of third parties (such as African trypanosomes) and environmental perturbations (Rio *et al*. 2006). The mutualists *Wigglesworthia* and *Sodalis* exhibited well-regulated density profiles over different host developmental stages, thus supporting their integral symbiotic roles. Furthermore, host immune status and the presence of trypanosome infections did not impact the steady-state density levels observed for *Wigglesworthia* and *Sodalis*. Contrarily, the density of parasitic *Wolbachia* infections did vary among tsetse individuals throughout host development and fluctuated in response to host immune status and the presence of trypanosomes. Interestingly, perturbation of the maternal environment (via altering humidity levels) resulted in the deposition of progeny with greater overall symbiont loads. This compensation may represent a mechanism that acts to ensure the persistence of these symbioses in future host generations. The regulation of *Wigglesworthia* and *Sodalis* densities is believed to drive the interspecific relations to ensure their competitive survival and to further promote specialization of these associations. It is also tempting to suggest that similarities in *Wigglesworthia* and *Sodalis* population growth dynamics during metabolically intensive host development periods may indicate their overlapping contributions to host biology.

European medicinal leech

The European medicinal leech, usually marketed as *Hirudo medicinalis*, consists of a complex of at least three sister species: *H. orientalis*, the commonly sold *H. verbana*, and the rare *H. medicinalis* (Siddall *et al*. 2007). The medicinal leech is found in fresh water habitats through Europe and western Asia, depending on the particular species. The hermaphroditic leech exchanges sperm through copulation. Gravid leeches deposit cocoons containing multiple eggs at the water-land interface in a watertight, nutritious yolk filled cocoon (i.e. leech juveniles develop '*ex utero*' in contrast to the '*in utero*' development of tsetse larvae) (Sawyer 1986). Following an extensive maturation period within the cocoon, juveniles emerge by perforating the cocoon wall through a process known as eclosion (Reynolds *et al*. 1998).

The leech digestive tract consists of two main compartments; the crop and the intestinum (Fig. 2). In the wild, the medicinal leech feeds every two to eight weeks and is capable of acquiring a blood meal over five times its unfed body mass. Water and osmolytes are quickly expelled from ingested blood through a nephridial network connected to the excretory bladders, located adjacent to the lateral crop ceca. This creates a viscous intra-

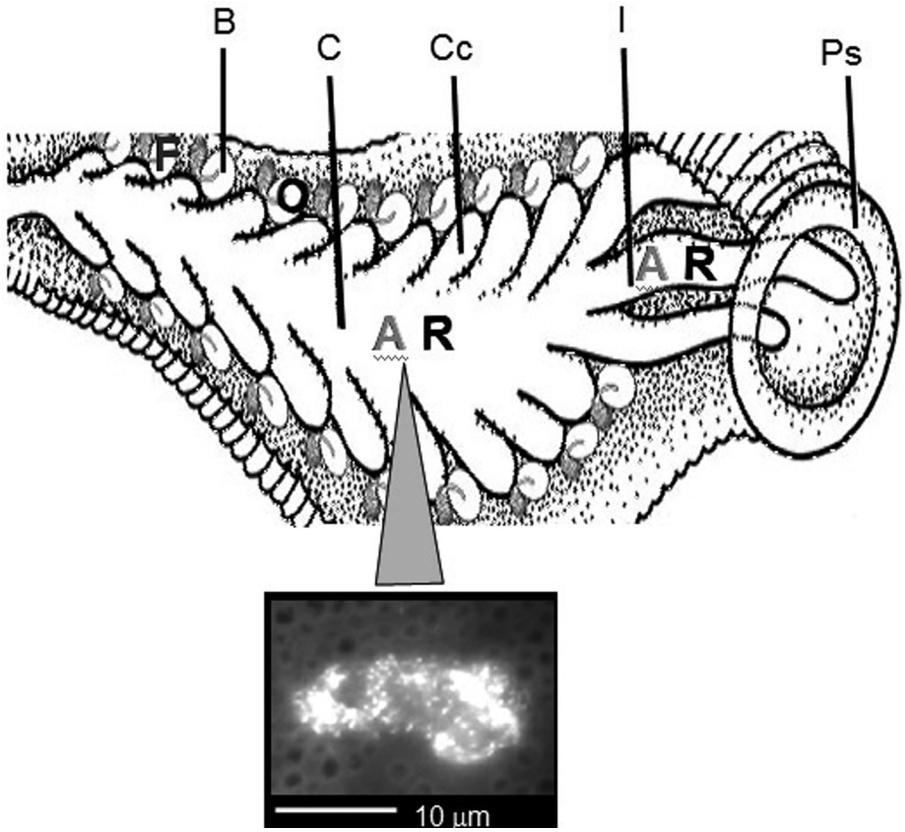

Fig. 2. – Drawing of digestive tract and excretory organs of the medicinal leech depicting symbiont localization. Abbreviations: A, *A. veronii*; B, bladder; C, crop; CC, crop cecum; F, *Flavobacterium*; I, intestinum; O, *Ochrobactrum*; PS, posterior sucker; R, *Rikenella*. Inset is a microscopic fluorescence *in situ* hybridization image of *A. veronii* (green) and *Rikenella* (red) microcolonies following host feeding (used with permission from Kikuchi & Graf 2007).

luminal fluid (ILF) consisting primarily of physically intact erythrocytes (Graf 1999). The processed blood meal can be stored for months within the crop until transported into the intestinum, the site of erythrocyte lysis and nutrient absorption.

Historically, medicinal leeches were believed to be involved in a monospecific digestive tract association with a member of the *Aeromonas* genus (Busing *et al*. 1953, Hornbostel 1942). Using a battery of biochemical tests and 16S rRNA sequencing (Graf 1999), the facultative anaerobe *A. veronii* biovar sobria was identified as a consistent and dominant extracellular microbial resident of the medicinal leech digestive tract. Recently, culture independent profiling has revealed the presence of an extracellular *Rikenella*-like bacterium coexisting alongside *A. veronii* in the leech crop, (Worthen *et al*. 2006). Concurrently, the intestinum was shown to contain not only the two core *A. veronii* and *Rikenella* sp., but also various transient organisms (Worthen *et al*. 2006). Furthermore, an intracellular *Ochrobactrum*, an α–proteobacteria related to *Sinorhizobium*, and an extracellular *Flavobacterium*, a member of the Bacteroidetes, have been identified in the nephridia and bladder organs (reviewed in Graf *et al*. 2006).

***Digestive tract symbiont:* A. veronii**

A. veronii is also present in the midguts of female *Culex quinquefasciatus* and *Aedes aegypti* mosquitoes (Huys *et al*. 2005), suggesting an inclination for establishment within the digestive tracts of various hematophagous species. A range of maladies in humans, including wound infection, septicemia, and diarrhea, are also associated with *A. veronii* infections (reviewed in Janda & Abbott 1998). The contrast in *A. veronii* infection outcomes between humans and hematophagous organisms provides an opportunity to compare symbiosis and virulence factors from a single microbial species. In the leech crop motile *A. veronii* are found primarily in the ILF as individual cells. However, these bacteria can also be associated with *Rikenella* microcolonies following blood meals (Fig. 2 inset) (Kikuchi & Graf 2007). Putative functional roles for the *A. veronii* symbiont within the leech host include aiding in digestion, provisioning essential nutrients that are scarce in blood or that the host is incapable of synthesizing (Sawyer 1986), and preventing the establishment of unwanted microbes (Busing 1951, Weiler 1949). Another potential function of *A. veronii* is syntrophy, such as priming the leech digestive tract to enable

the establishment and persistence of the obligate anaerobe *Rikenella* (Kikuchi & Graf 2007).

The medicinal leech cocoon fluid contains *A. veronii* and juveniles harbour infections upon eclosion, suggesting a vertical transmission mode for this symbiont (Rio and Graf, unpublished results). However, the specific timing of establishment and proliferation during host development remains to be determined. Several factors may contribute to symbiont establishment, specificity, and persistence, including the complement system of ingested vertebrate blood (i.e. the membrane attack complex) (Indergrand & Graf 2000), macrophage-like leech hemocytes (de Eguileor *et al*. 1999, 2000) that patrol the gut and remove sensitive bacteria (Silver *et al*. 2007a), and the *A. veronii* lipopolysaccharide surface structure which is required for resistance to the complement system (Braschler *et al*. 2003). A signature-tagged mutagenesis approach has identified additional gene loci involved in cell membrane structure and stabilization, including Braun's major outer membrane lipoprotein, gene expression regulation, nutrient transport, and host interactions to be paramount to *A. veronii* colonization of the leech host (Silver *et al*. 2007b).

Similar to *Sodalis*, *A. veronii* relies on a TTSS during host interactions. Within the leech crop, the *A. veronii* TTSS inhibits phagocytosis by leech hemocytes (Silver *et al*. 2007a). Interestingly, this same TTSS is also instrumental towards facilitating *A. veronii* virulence in mammals, a functional role quite contrary to that observed in the leech. Despite being required for symbiotic colonization of the leech crop, the *A. veronii* TTSS promotes macrophage destruction in mice. The importance of a functional TTSS in mouse septicemia was demonstrated by the strikingly lower lethality rate of a TTSS mutant in comparison to the wild type *A. veronii* (30% versus 100% mortality, respectively) (Silver *et al*. 2007a).

Digestive tract symbiont: **Rikenella** *sp.*

Rikenella species, members of the Bacteroidetes, have been identified in a wide spectrum of digestive tracts ranging from that of the termite gut (Ohkuma *et al*. 2002) to the human colon (Eckburg *et al*. 2005). The consistent identification of *Rikenella* spp. within digestive tracts is suggestive of physiological adaptations and functional roles within these environments. In addition to the ILF, where tight associations with *A. veronii* can occur, *Rikenella*-only microcolonies are also found in association with the luminal side of the leech crop epithelium (Kikuchi & Graf 2007).

Medicinal leech symbiont population dynamics

In the ILF, planktonic mixed microcolonies consisting of *A. veronii* and *Rikenella* are often embedded within a thick mucus-like substance consisting of *N*-acetylglucosamine. These microcolonies form floating, granular or erythrocyte-associated biofilms (Kikuchi & Graf 2007). Another common occurrence in multiple symbiotic systems (e.g. leech, nematodes, zebrafish, squid, etc.) is the presence of this mucus-like substance in or near microbial colonization sites. This substance can enable the sectoring of symbiont colonies, minimize exposure to stressful elements, or be a nutritional source for growth (reviewed in Cheeseman & Guillemin 2007).

Despite their mixing, *A. veronii* and *Rikenella* demonstrate different population dynamics. While both species proliferate rapidly during the first 3 days following feeding, *A. veronii* decreases in abundance while *Rikenella* symbionts remain at relatively higher population levels thereafter (Kikuchi & Graf 2007). The decreased growth rate of *A. veronii* may occur after the removal of a key nutrient or the depletion of oxygen (Kikuchi & Graf 2007). Notably, the number of cells within mixed microcolonies was consistently greater than that within monospecific microcolonies, suggestive of **synergy** between *A. veronii* and *Rikenella*. A spatial-temporal model depicting the two-species maturation-dispersion cycle within the leech digestive tract, reminiscent of many biofilm developmental patterns found in nature (Hall-Stoodley *et al*. 2004), has been described relative to host feeding (Kikuchi & Graf 2007).

The importance of comparative analyses of symbioses

The reoccurrence of colonization factors within various microbial-host relations, such as use of TTSS and bacterial communities within mucus-like substrates, suggests evolutionary conservation of these features regardless of association outcome (reviewed in Hentschel & Steinert 2001). Each symbiotic system may have also evolved specific genetic and regulatory elements that mediate their relations. For example, an *A. veronii* catalase mutant was able to colonize and persist within the leech digestive tract at densities comparable to wild type individuals (Rio *et al*. 2007). However, similar mutations result in significant colonization defects in the symbioses of *Sinorhizobium meliloti* and leguminous plants, as well as with *Vibrio fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes* (reviewed by Ruby & McFall-Ngai 1999). Comparative analyses of microbial interactions within different symbioses, such as the tsetse fly and medicinal leech, can also highlight universal themes of microbial associations or particularities outfitted for a specific symbiotic system.

Examining microbe interactions-revelations made and yet to be made

Potential complementarity in biosynthetic capabilities between microbial symbiont species, presumably through correlated genome evolution and reduction, has been pro-

posed for the dual bacterial symbiosis of the glassy-winged sharpshooter, *Homalodisca coagulata* (Wu *et al*. 2006). To enable survival on a diet of nutrient poor xylem, two unrelated microbes (the γ–proteobacterium *Baumannia cicadellinicola* and the Bacteroidetes species *Sulcia muelleri*) are believed to provide the sharpshooter with indispensable nutrients. The *Baumannia* genome is dedicated to the synthesis of vitamins, cofactors, and prosthetic groups, while *Sulcia* can produce most if not all of the essential amino acids (Wu *et al*. 2006).

The availability of complete *Sodalis* and *Wigglesworthia* genome sequences enables the investigation into perhaps even more intimate associations where genome interdependency and cooperative activity within single metabolic pathways may have materialized. For example, although *Wigglesworthia* is believed to provide B complex vitamins lacking in the blood diet to tsetse, some of these pathways are incomplete and require the coupling of biosynthesis genes and enzymatic activity that are encoded on the *Sodalis* chromosome. Interestingly, *Sodalis* (with the majority of genes involved in thiamine biosynthesis eroded), possesses the genetic machinery that encodes for a thiamine (B1) ABC transporter, which enables the ATP-driven uptake of thiamine to the cytosol (Toh *et al*. 2006). Transport of thiamine into *Sodalis* may provide a form of feedback regulation for the expression of respective biosynthetic genes. Other experiments demonstrate that simple genome mutations in one free-living microbial species prompted adaptation to the presence of a neighboring species, thus forming an intimate and specialized association where the derived community was more stable and productive than the ancestral state (Hansen *et al*. 2007). A similar situation may be occurring within the tsetse fly, where deficiencies arising from drastic genome erosion have been offset through adaptations involving complementation by the other symbiont's genome.

A working hypothesis for the functional role of *A. veronii* is the alteration of the host environment favoring establishment of *Rikenella* (reviewed in Graf *et al*. 2006). As a result of cellular metabolism, increases in host body temperature and respiration rate can arise following blood meal acquisition, resulting in higher levels of dissolved O_2 and potentially different levels of dissolved CO_2 (Hyde *et al*. 2007). These environmental conditions would prove detrimental to *Rikenella*, a non-motile obligate anaerobe. Further investigations are necessary to determine *A. veronii*'s role in modulating O_2 levels in the leech digestive tract following feeding. A mutual dependence for compounds and metabolic intermediates between the microbes may act as selective force for their retention (Wu *et al*. 2006), and potentially reduce the detrimental costs associated with competition in a limited host arena.

The role of microbial interactions in the density regulation of corresponding communities is of particular interest because concerted metabolic activity would seem to require similarities in population growth dynamics. One intriguing question is whether cell populations have to reach a certain threshold for species interactions to occur. The role of density sensing mechanisms (i.e. putative quorum sensing and biofilm production genes) in the maintenance of bacterial load and functional homeostasis within the symbiotic system should be examined. Recently, a gypsy moth midgut metagenomic library was screened for the identification of quorum-sensing inducing compounds that may be used for interspecific bacterial signaling (Guan *et al*. 2007). In addition, the selective elimination of symbionts through the use of specific antibiotics, followed by the reintroduction of density sensing mutants [genetic mutagenesis has been established for *Sodalis* (Dale *et al*. 2005) and *A. veronii* (Rio *et al*. 2007)] can, through the use of techniques such as fluorescence *in situ* hybridization and real-time quantitative PCR, reveal whether spatial, quantitative or gene expression alterations occur within the microbial consortia.

Recent advancements in culture- independent micro-analytical technology will enable us to further develop our understanding of microbial interactions and their roles in the specificity, acquisition, progression and persistence of symbiotic systems. Metagenomics is enabling the comprehensive characterization of microbial communities within a wide array of hosts including the human distal gut (Gill *et al*. 2006), ecologically impacted coral reefs (Dinsdale *et al*. 2008), and serious insect pests such as the gypsy moth (Broderick *et al*. 2004) and termites (Warnecke *et al*. 2007). Laser microdissection (reviewed in Hooper 2004) that can isolate particular cells of interest within complex tissues coupled with global expression studies can reveal valuable information on the shaping and activity of microbial communities through host development. Of interest is whether principles of ecological theory, originally created with much larger ecosystems in mind, will also prove applicable towards host-associated microsystems. Examination of how different microbial species interact will surely provide new insights into a broad range of topics including metazoan evolution, community organization, and the streamlining of biosynthetic pathways.

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GLOSSARY

Obligate symbiont

- Absolutely necessary for at least one partner, often for survival or reproduction
- Often significant co-evolution

Facultative symbiont

- Relationship is established if the opportunity arises

(P)rimary symbiont

- Reside within specialized host cells (bacteriocytes)
- Deep congruent host-symbiont phylogenies, often obligate
- Typically have essential nutritional roles towards host biology
- Reduced genomes

(S)econdary symbiont

- Wider tissue tropism relative to P-symbionts
- Can be facultative
- Wider range of functional roles
- Necessity may vary temporally and geographically
- Genomes can be transitioning from free-living to host-associated

Transmission*

Vertical

- Parental transmission via infection of reproductive tissue
- Often involves bottlenecks that result in small effective population size (Ne) that tends to promote symbiont genetic uniformity.
- Can promote attenuated relations when pure due to strict alliance

Horizontal

- Transmission *via* surrounding habitat
- New infection established at each generation
- Also known as environmental transmission
- Tends to maintain or increase symbiont genetic diversity

* Both modes can be utilized by a species concurrently. For example, during early symbiotic establishment both modes of transmission may be exploited.

Syntrophy

- Inter-specific activity where metabolic capabilities are combined to catabolise substances that can not be catabolised by either species alone
- Promotes mutual dependence

Synergy

- A cooperation that results in an enhanced combined effect greater than the sum of predicted individual effects