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Common Trends in Mutualism Revealed by Model Associations Between Invertebrates and Bacteria

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Abstract

Mutually beneficial interactions between microbes and animals are a conserved and ubiquitous feature of biotic systems. In many instances animals, including humans, are dependent on their microbial associates for nutrition, defense, or development. To maintain these vital relationships animals have evolved processes that ensure faithful transmission of specific microbial symbionts between generations. Elucidating mechanisms of transmission and symbiont specificity has been aided by the study of experimentally tractable invertebrate animals with diverse and highly evolved associations with microbes. Here we review several invertebrate model systems that are contributing to our current understanding of symbiont transmission, recognition, and specificity. Although the details of transmission and symbiont selection vary among associations, comparisons of diverse mutualistic associations are revealing a number of common themes, including restriction of symbiont diversity during transmission and glycan-lectin interactions during partner selection and recruitment.

Keywords

symbiosis; cooperation; bottleneck; chitin; lectin; species specificity

Introduction

Mutually beneficial symbiotic associations between microbes and animals occur in every ecological niche and range from obligate to facultative dependence (Baumann, 2005; Pontes & Dale, 2006). The benefits derived from, and selecting for these interactions are diverse and include mutual influence on nutrition, defense, reproduction, and development (Currie, 2001; Vance, 2001; McFall-Ngai, 2002; Taylor *et al.*, 2005; Pais *et al.*, 2008). Indeed, microbial mutualism is the basis for the evolution of the eukaryotic cell and permits organisms to exploit otherwise inaccessible niches (Margulis, 1992; Minic & Herve, 2004; Sachs *et al.*, 2004; Moran, 2006; Janson *et al.*, 2008). For example, many marine invertebrates living near deep-sea hydrothermal vents have an “internal oasis” that allows them to survive in the nutrient-poor deep benthos: they cultivate sulfur-oxidizing mutualistic bacteria that can provide them with fixed carbon in return for access to oxygen and reduced inorganic compounds that are usually found in mutually exclusive environments (Cavanaugh *et al.*, 2006; Dubilier *et al.*, 2008).

Our current understanding of selective forces driving the evolution and maintenance of microbe-animal symbiosis, and the molecular underpinnings of these processes, has been expanded by the establishment of model systems (Ruby, 2008), the development of tools to

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probe uncultured symbiont gene function (Moya *et al.*, 2008) and metabolism (Nicholson *et al.*, 2005), and a broader appreciation of the role of symbionts in human health and disease (Dethlefsen *et al.*, 2007). Together these advances are providing insights into how microbes move between host species, how symbiotic partners adapt to each other, and what evolutionary factors drive the emergence and maintenance of cooperative microbial-host associations. Since microbial symbioses are integral to the function of every earth ecosystem, these advances undoubtedly will have impacts at all levels of science and society. For example, medical treatments may be selected based on their impacts on both the microbial and animal components of the patient (Nicholson *et al.*, 2005).

The invertebrates have played a particularly important role as models of microbial symbiosis because of their diversity, general experimental tractability, and tendency to associate with specific and relatively simple microbial communities. Although zebrafish and gnotobiotic mice have formed the bases of numerous elegant studies (Cheesman & Guillemin, 2007), most vertebrates, particularly humans, are difficult experimental subjects (Dethlefsen *et al.*, 2007). Also, their microbial communities are complex and variable, containing greater than 5,600 taxa in the intestine alone (Hooper *et al.*, 1998; Bäckhed *et al.*, 2005; Dethlefsen *et al.*, 2008), making it challenging to assign specific symbiotic function to individual organisms. The relatively low diversity of microbes in invertebrate symbiotic associations simplifies the task of teasing apart complex molecular and cellular interactions between symbiont and host (McFall-Ngai, 2002; Ruby, 2008). Furthermore, because animal evolution has been continuously influenced by symbiosis with microbes, fundamental molecular features of the animal-microbe interface are conserved among all animals, allowing knowledge from invertebrate symbiosis models to be applied broadly (Hooper *et al.*, 1998; Ruby, 1999; Scully & Bidochka, 2006; McFall-Ngai, 2008a). For example, invertebrates are being used to understand the molecular and cellular processes that promote fidelity of symbiotic associations between generations. Such studies will be integral to understanding how a single species can establish itself in a complex consortium, such as that within the vertebrate intestine (Hooper *et al.*, 1998).

Here we will describe several invertebrate models of symbiosis, review recent experimental advances in these models, and highlight how these findings are revealing common mechanisms of symbiont transmission, recognition, and specificity.

The role of transmission and partner recognition in maintenance of symbiosis

In cooperative relationships, each symbiont incurs a cost by providing goods and services to other members of the community, while receiving benefits (directly or indirectly) that balance this cost (Sachs *et al.*, 2004). Such interactions are subject to cheating behavior in which one of the partners receives the benefits but does not provide services. Since cheaters do not incur a cost, they are expected to be more fit than their cooperating counterparts, and therefore to have a selective advantage. Hardin (Hardin, 1968) articulated “the tragedy of the commons” – a situation where individuals sharing a common resource each have incentive to exploit the common resource in the short term, even though it is to the disadvantage of all the members of the population, including the exploiting individual, in the long term. Since microbial symbionts are thus predicted to exploit the common resources of their host, cooperation is not expected to be a stable evolutionary trend. However, there is ample evidence of mutually beneficial symbiotic interactions in nature. To help explain this conundrum, a sizeable body of literature has theorized upon the selective pressures that promote cooperative associations (Hammond & Axelrod, 2006). A key element of many theories is the faithful transmission of non-cheating symbionts between generations, in which specific partners that act cooperatively are selected within the available population

(Douglas, 2008). One model describing the evolution of altruism within a population revealed that costly altruism could emerge in a monophyletic group composed entirely of selfish individuals (those that will not reciprocate cooperative acts) if individuals within the population were able to recognize one another as "kin" (e.g. monophyletic) and if migration of individuals within the population was blocked (e.g. population dispersal was limited) (Hammond & Axelrod, 2006). Microbial populations have the capacity to recognize self from non-self, such as through quorum sensing (Diggle *et al.*, 2007; Sandoz *et al.*, 2007). Therefore, it is feasible that the first criterion could be met by symbionts within a host, where cheating (selfishness) is repressed and providing a benefit to the host (altruism) is promoted. Fulfillment of the latter criterion, limited migration, depends on the mode of transmission of the symbionts between hosts. Vertical transmission of mutualist symbionts directly from parent to offspring limits dispersal while horizontal transmission in which the symbiosis is initiated anew each generation, promotes dispersal. Both types of transmission, as well as intermediate variations are well represented in nature, indicating that limited dispersal is not necessarily essential for maintaining cooperation. The molecular details of transmission and partner recognition that are being elucidated in a number of horizontally acquired invertebrate animal-microbe mutualisms are yielding insights into the evolution and stability of cooperative associations.

Biology of Invertebrate Models of Microbial Symbiosis

An essential step in understanding any symbiotic association is identifying members of the symbiosis and how they interact with each other. Since the complexity of this task is inversely proportional to the complexity of the system, the investigation of gastrointestinal tract microbial communities of diverse animals poses a significant challenge that may be aided by invertebrate models. One of the best studied, and perhaps most complex insect gut communities is in termites comprising 10^2–10^3 bacterial phylotypes with specific intestinal niches (Hongoh *et al.*, 2005; Yang *et al.*, 2005; Ohkuma, 2008). The midguts of the Lepidoptera *Galleria mellonella* (Walsh & Webster, 2003; Gouge & Snyder, 2006), *Manduca sexta* (van der Hoeven *et al.*, 2008) and *Lymantria dispar* (Broderick *et al.*, 2004) as well as the Dipteran *Drosophila melanogaster* (Ryu *et al.*, 2008) harbor between 5 and 22 phylotypes each. Among these, *Serratia*, *Pseudomonas*, and *Enterococcus* are common isolates. The "simplicity" of insect consortia relative to those of vertebrates will help expedite investigations of niche heterogeneity within the gut ecosystem, fluctuations of community members in response to development, nutrition, drugs, and illness, and contributions of individual microbes to community fitness (Broderick *et al.*, 2004; Dillon & Dillon, 2004; Broderick *et al.*, 2006; Brinkmann *et al.*, 2008). For example, investigations of insect systems are lending insights into the dynamics of the host-consortium interface and its impact on disease resistance. In *D. melanogaster* a healthy microbiome is maintained by host-mediated suppression of immune antimicrobial peptides. In the absence of suppression, the composition of the microbiota shifts to one dominated by a pathogen that elicits cell apoptosis and fly death (Ryu *et al.*, 2008). In *M. sexta* and *L. dispar*, antibiotic treatment alters microbial community diversity (Broderick *et al.*, 2006; van der Hoeven *et al.*, 2008), and in *L. dispar* this change directly reduces larval susceptibility to infection by *Bacillus thuringiensis* (Broderick *et al.*, 2006). Thus, the gut microbiota can both synergize and prevent pathogenesis in insect larvae.

Another class of invertebrate symbioses comprises associations between a single host and only one or a few microbes. The relative simplicity of such systems has facilitated experimental progress in understanding the biology, genetics, and physiology of host-microbe communication and association, such that current studies in several systems are on the verge of describing the association at biochemical resolution. The focus of this review is to discuss advances such systems have contributed toward understanding symbiotic partner

selection and recognition. We therefore emphasize associations for which aspects of transmission and specificity have been experimentally characterized. To put these experimental advances in context we will briefly describe the life histories and biology of the systems discussed. Generally these associations feature exchange of adaptive benefits between microbial symbionts and animal hosts, ranging from nutritional to defensive services.

Entomopathogenic nematodes

Insects are exploited as a nutritional niche by a subset of terrestrial nematodes that are symbiotically associated with γ-proteobacteria. This evolution appears to have occurred at least two independent times, in the genera *Steinernema* and *Heterorhabditis* (Poinar, 1993; Boemare & Akhurst, 1994; Adams & Nguyen, 2002; Goodrich-Blair & Clarke, 2007). Although *Steinernema* spp. and *Heterorhabditis* spp. nematodes are not closely related (Blaxter *et al.*, 1998), they associate with bacteria, *Xenorhabdus* and *Photorhabdus*, respectively, which form a phylogenetic sister group (Suzuki *et al.*, 1996). A non-feeding soil-dwelling infective juvenile stage of the nematodes carries the bacterial symbionts between insect hosts that are subsequently killed and used as a nutrient source for reproduction. The bacterial symbiont contributes to virulence, immune modulation, and nematode reproductive fitness (Forst & Nealson, 1996; Forst *et al.*, 1997; Han & Ehlers, 2001; Sicard *et al.*, 2003; Eleftherianos *et al.*, 2007; Goodrich-Blair & Clarke, 2007; Held *et al.*, 2007; Herbert & Goodrich-Blair, 2007; Park *et al.*, 2007; Clarke, 2008). In both nematode genera approximately 30–500 symbiont bacteria (Cowles & Goodrich-Blair, 2004; Goetsch *et al.*, 2006; Snyder *et al.*, 2007; Ciche *et al.*, 2008) colonize the epithelial surface of the anterior intestine of the infective stage (Poinar, 1966; Bird & Akhurst, 1983; Ciche & Ensign, 2003; Martens *et al.*, 2003a; Snyder *et al.*, 2007). In *Steinernema spp.*, this region is an extracellular space, known as the receptacle, between two epithelial cells (Bird & Akhurst, 1983; Martens *et al.*, 2003a; Snyder *et al.*, 2007). The colonized intestinal region of *Heterorhabditis spp.* does not appear to be morphologically distinct from the rest of the intestine, although the *Photorhabdus* symbionts are clearly limited to a defined region, suggesting distinct biochemical or cellular features do occur (Poinar *et al.*, 1977; Ciche & Ensign, 2003; Ciche *et al.*, 2008). The infective juvenile nematodes ambush or hunt insect prey (Campbell & Gaugler, 1997) and penetrate the insect integument to infect the blood. The bacterial symbionts released from the infective stage nematode host into the insect blood (Ciche & Ensign, 2003; Martens *et al.*, 2004; Snyder *et al.*, 2007) help overcome insect immunity and kill the host. Within the insect cadaver, nematodes reproduce through 2–3 generations until high nematode density and low nutrition cue development of progeny into the infective stage (Popiel *et al.*, 1989) that emigrates to infect a new insect host. Development into the non-feeding infective stage is preceded by colonization of the bacterial symbiont, ensuring its transmission to the next host. This event is therefore a critical aspect of the maintenance of the symbiosis. Both *Steinernema spp.* and *Heterorhabditis spp.* exhibit specificity for their symbiont at this stage (Akhurst, 1983; Han & Ehlers, 1998; Ciche & Ensign, 2003; Sicard *et al.*, 2003; Sicard *et al.*, 2004; Sicard *et al.*, 2005; Cowles & Goodrich-Blair, 2008).

Entomopathogens provide a rare opportunity to elucidate bacterial traits that contribute to mutualism and pathogenesis, since *Xenorhabdus* and *Photorhabdus*, while mutualists of their nematode hosts, also are pathogens of insects (Herbert & Goodrich-Blair, 2007; Clarke, 2008). Furthermore, these systems allow examination of how the effects of pathogenic traits are avoided or neutralized by the mutualistic nematode host. Much progress has been made in both systems toward identifying and characterizing bacterial virulence and mutualism determinants. Mutualism determinants include factors involved in nutrition (Heungens *et al.*, 2002; Martens *et al.*, 2003b; Martens *et al.*, 2005; Orchard & Goodrich-Blair, 2005; Watson

et al., 2005), surface structure (Bennett & Clarke, 2005), gene regulation (Heungens *et al*., 2002; Cowles & Goodrich-Blair, 2006; Joyce *et al.*, 2006; Cowles *et al.*, 2007; Herbert *et al.*, 2007), and stress response (Vivas & Goodrich-Blair, 2001; Heungens *et al.*, 2002), as well as factors of unknown function (Heungens *et al*., 2002; Cowles & Goodrich-Blair, 2004; Cowles & Goodrich-Blair, 2008). Additionally, RNA interference has been successfully adapted for use in *Heterorhabditis bacteriophora* nematodes (Ciche & Sternberg, 2007) and the genome of this nematode is being sequenced (Ciche, 2007). This advance, combined with the utilization of the well-studied insect *D. melanogaster* as a model insect host (Hallem *et al*., 2007) sets the stage for genetic manipulation of each player in this tripartite symbiosis.

Medicinal leeches

Another model that allows comparative investigation of mechanisms underlying mutualism and pathogenesis is that between the medicinal leech, *Hirudo verbana*, and its bacterial symbionts. Leeches ingest vertebrate blood into a structure known as a crop that is colonized by two microbial symbionts: *Aeromonas veronii*, which is also a pathogen of mammals, and a recently described, but as yet uncultured *Rikenella*-like bacterium (Worthen *et al*., 2006). The leech harvests its main food source, erythrocytes, in the crop and digests them in another structure, the intestinum (Graf *et al.*, 2006). The intestinum acts as both an intestine and a rectum, and houses a microbial community including *Aeromonas*, the *Rikenella*-like symbiont, and other bacterial species (Graf *et al.*, 2006). The contributions of *A. veronii and Rikenella* to host fitness have not been demonstrated yet, but may be provision of nutrients or other metabolic activities, or exclusion of potentially harmful opportunistic colonizers, such as through the production of antibiotics (Graf *et al.*, 2006). It should be noted that bacterial associations with leeches other than Hirudinids occur in different host structures and may or may not make similar contributions to the health of the hosts. These include associations in the mycetome, bladder, and nephridia (Graf *et al.*, 2006).

Inter-generational transmission of *Rikenella* and *A. veronii* is predicted to be vertical through the egg capsule (Graf, 1999). Once localized inside the crop *Aeromonas* and *Rikenella* symbionts form aggregated microcolonies, which grow to a larger size when the two species associate with each other than when they are comprised of either species by itself, suggesting synergism between symbionts for growth. When not in multi-species microcolonies, the *Rikenella* species formed aggregates on the wall of the crop while *A. veronii* was usually found as single cells suspended in the crop fluid (Kikuchi & Graf, 2007). Symbiont populations within the crop are estimated as high as 10^8 (*Aeromonas*) and 10^{10} (*Rikenella*) bacteria per ml (Kikuchi & Graf, 2007). The mammalian host parasitized by the leech contributes to the specificity of the leech-symbiont association since *A. veronii* (and presumably the *Rikenella*-like symbiont) is not susceptible to active complement in the blood, in contrast to other species tested (Indergand & Graf, 2000). Furthermore, a complement-sensitive mutant of *A. veronii* is unable to colonize the leech crop (Braschler *et al.*, 2003).

Since *A. veronii* is amenable to culturing, the molecular mechanisms by which it associates mutualistically with leeches and pathogenically with mammalian cells is being investigated. These studies have revealed a requirement for type III secretion for both leech colonization and mammalian macrophage killing (Silver *et al.*, 2007b). Other *Aeromonas* genes required for leech colonization include those predicted to encode products involved in bacterial surface modification, gene regulation, and nutrition, or products with no currently predicted function (Silver *et al.*, 2007a). Continued characterization of these genes therefore has the potential to reveal both conserved and novel adaptations of a microbial symbiont to its host. Furthermore, the simple binary association between *A. veronii* and *Rikenella* provides an

opportunity to explore how microbial interactions with each other influence their community mutualism with the host.

Earthworms

Earthworms are an integral component of soil ecosystems, contributing to soil mixing and processing of organic and inorganic material. They house a consortium of gut microbes that are expected to aid in nutrient cycling (Toyota & Kimura, 2000; Furlong *et al.*, 2002; Horn *et al.*, 2003; Ihssen *et al.*, 2003; Egert *et al.*, 2004). In addition, recent work has focused on the symbiotic association between the earthworm *Eisenia foetida* and the β-proteobacterium *Verminephrobacter eiseniae* (formerly known as "*Acidovorax*-like") that colonizes a specific region of the nephridia (Schramm *et al.*, 2003; Pinel *et al.*, 2008). Nephridia are osmoregulatory excretory organs that absorb coelomic fluid and blood for subsequent release from the earthworm. They are found in pairs in each segment of the worm, with an internal opening into the digestive tract, and an external opening that allows release of fluids (e.g. coelomic fluids) into the environment (Ramsay, 1949). Fluid is passed through a series of three loops prior to excretion, and the bacteria are located near the tip of the second loop (Ramsay, 1949; Schramm *et al.*, 2003). Although experimental evidence of *V. eiseniae* contributions to host physiology are lacking, the symbionts are predicted to play a role in protein recycling during nitrogen excretion (Schramm *et al.*, 2003)

The symbionts have been identified within developing earthworm eggs, suggesting vertical transmission (Davidson & Stahl, 2006; Davidson & Stahl, 2008), though for a brief period the eggs are unsealed outside of the body of the parent, allowing environmental bacteria to gain access to the egg prior to the sealing of the egg coat. Whether or not this allows for horizontal transmission into the egg from environmental *Verminephrobacter* isolates is unknown (Davidson & Stahl, 2008). As the embryo develops, *Verminephrobacter* cells are the only bacteria observed within a series of canals, each of which leads to the nephridia of its respective segment, prior to colonization of the nephridia by the bacteria. This suggests that some selective pressures or recruitment mechanisms function to restrict entrance to the canal to only the specific symbiont. By the time of hatching and maturation of juveniles, each nephridium is colonized with a final bacterial population of approximately 3×10^5 colony forming units (Davidson & Stahl, 2008).

The bacterial symbiont can be cultured in the laboratory (Pinel *et al.*, 2008) and genetic approaches are underway to identify bacterial factors necessary for host association (S. Davidson, personal communication). Such studies hold promise for revealing molecular mechanisms of symbiont transmission and contributions to host physiology in this emerging model of symbiosis.

Marine chemoautotrophic symbioses

Microbial symbiosis has played a particularly important role in niche diversification and innovation in the nutrient poor marine environment. Indeed, six invertebrate phyla (Annelida, Arthropoda, Echinodermata, Mollusca, Nematoda, and Porifera) that inhabit a diverse variety of marine environments such as hydrothermal vents and coastal sediments are known to associate with bacteria (Cavanaugh *et al.*, 2006; Dubilier *et al.*, 2008). What distinguishes the habitats of these animals is that the normally nutrient poor ocean is enriched in chemical energy sources that bacterial symbionts can harness to provide nutrition for themselves and their animal hosts. One of the challenges posed by symbiont chemoautotrophy in the marine environment is the microbial requirement for both a source and a sink of electrons, which do not occur in the same microenvironments. The electron sources in these environments are reduced sulfur or methane compounds. As these are spontaneously oxidized in the presence of oxygen (Zhang & Millero, 1993), they are found

only in anoxic or microoxic zones (e.g. sediments). In contrast, oxygen, the electron sink, is obtained from the water column. Animal hosts of chemoautotrophic symbionts have met the challenge of acquiring both reduced compounds and oxygen for their microbial symbionts using behavioral, morphological, or metabolic adaptations. For example, the giant hydrothermal vent tubeworm *Riftia pachyptila* utilizes a specialized hemoglobin molecule that binds both oxygen and reduced sulfur compounds. The marine nematode *Laxus oneistus* furnishes substrates to its γ-proteobacterial epibionts by migrating repeatedly through the oxygen-sulfide gradient (Ott *et al.*, 1991; Polz *et al.*, 2000; Cavanaugh *et al.*, 2006). Both of these organisms are well-studied models of marine chemosynthetic symbiosis and will be used throughout this review as representatives of the diverse polyphyletic collection of organisms mentioned above.

R. pachyptila houses intracellular symbionts, recently named '*Candidatus* Endoriftia persephone' (Robidart *et al.*, 2008) in a unique structure called the trophosome, a distinct organ that fills the coelom of the tubeworm. The trophosome is organized into lobules that are each composed of a thick tissue of host cells (bacteriocytes) containing intracellular bacteria. This bacteriocyte tissue is organized around an axial central blood vessel that is immediately surrounded by a thin layer of non-symbiont containing host cells. On the outer surface the bacteriocyte tissue is surrounded by additional blood vessels and epithelial tissue that face out into the coelom of the tubeworm. The bacteriocytes constitute 50–70% of the trophosome, and '*Candidatus* E. persephone' alone account for approximately 25% of the total volume of the trophosome (Jones & Gardiner, 1988; Bright & Sorgo, 2003). *R. pachyptila* larvae differ from the mature tubeworm in that they are not sessile and do not have a trophosome or bacteriocytes and so do not carry symbiotic bacteria. Furthermore, larvae can travel away from the parents before settling and developing their symbiotic organs (Shank *et al.*, 1998; Marsh *et al.*, 2001). Thus, *R. pachyptila* acquire their symbionts horizontally from the environment. In an elegant study observing larvae at different stages of development Nussbaumer *et al.* (2006) showed that, contrary to the prevailing model that symbionts are acquired through feeding, symbionts accumulate within a secreted mucous layer on the outside surface of the animal and invade epithelial tissue. They then migrate toward the developing cells that will form the trophosome (Nussbaumer *et al.*, 2006).

Like *R. pachyptila*, the symbiont of the marine nematode *L. oneistus* is likely horizontally acquired. Epibiotic chemoautotrophic bacteria colonize the entire *L. oneistus* body surface, except the head. Colonization is likely an early event in the life history of the nematode, since it is rare to observe young juveniles in the wild without a complement of symbiotic bacteria. A recent study shows evidence that a lectin similar to human DC-SIGN is secreted by surface exposed glands of the nematode and participates in binding to surface exposed sugar residues on the bacterial surface. This lectin is predicted to contribute to the specificity of the association, which is highly specialized: individual species of nematodes are colonized by only a single bacterial phylotype, despite the presence of numerous other microbial species in the marine environment (Bulgheresi *et al.*, 2006).

As yet, genetic techniques have not been applied to the study of *Riftia* and *Laxus* symbionts due to challenges in culturing. Culture independent approaches such as enzyme assays and stable isotope signatures have been used extensively to predict the presence of active enzymes in the symbionts (Cavanaugh *et al.*, 2006). More recently, genomic and proteomic approaches have been utilized to further explore these symbioses (Woyke *et al.*, 2006; Kuwahara *et al.*, 2007; Markert *et al.*, 2007; Sanchez *et al.*, 2007; Robidart *et al.*, 2008). For example, subtractive suppressive hybridization was used to identify transcripts specifically expressed in the trophosome of *R. pachyptila*. This study suggested the participation of several genes in recognizing or responding to the symbiont partner including genes of

unknown function and genes predicted to encode a putative oxygen-binding protein and a protein with some similarity to a T-cell receptor (Sanchez *et al.*, 2007).

Marine bioluminescent symbioses

The examples of symbioses introduced thus far are based on nutritional benefits likely garnered by the host from their symbionts. However, another widespread basis for cooperation between species is defense against predators and pathogens. Such is the case for bioluminescent microbes that colonize the light organs of numerous marine fishes and squid (Dunlap, 1985; Dunlap *et al.*, 2004; Dunlap *et al.*, 2008; McFall-Ngai, 2008b). Among bioluminescent symbioses, the mutualism between the Hawaiian bobtail squid *Euprymna scolopes* and *Vibrio fischeri* is the best studied, and indeed represents one of the most developed model systems to study mutualism (McFall-Ngai, 2008b). In this system the microbial symbiont provides its host with light that can be used for predator avoidance (Jones & Nishiguchi, 2004). The squid utilizes the ventrally displayed bacterial bioluminescence to prevent casting a shadow on predators below.

The light organ is a bi-lobed organ with a pair of three pores on each lobe that permit entry of specific bacteria into six internal deep crypts (Nyholm *et al.*, 2000) where the bacteria reproduce and produce light. A juvenile squid hatches without bacteria localized within the nascent light organ. Soon after hatching, the juvenile squid acquires its symbiont from the ocean water that is drawn over the surface of the light organ by action of a large, ciliated appendage (Nyholm *et al.*, 2000). Near the pores the host secretes a mucus in response to the presence of bacterial peptidoglycan, and various gram-negative bacterial species accumulate in this substance (Nyholm *et al.*, 2000). Through an as yet unknown selective process *V. fischeri* cells become the dominant population within the mucus and are selectively recruited towards the pores. Once localized at the pores *V. fischeri* travels down a canal, and approaches the crypts. One or two cells initiates colonization of each of six crypts (Wollenberg & Ruby, 2009) and grows to form the large population (~10^5 cells in the entire juvenile light organ) that produces light. Bacterial colonization induces a developmental program that results in regression of the ciliated appendages as well as other morphological changes in the light organ crypts (Montgomery & McFall-Ngai, 1994; Doino & McFall-Ngai, 1995). Following these initial events, the light organ remains colonized for the life of the squid. Each day, after the bacteria have provided light for the squid during the night, ~95% of the bacterial population is expelled from the light organ, and the remaining bacterial population again grows to fill the light organ before the next evening (Ruby & Asato, 1993; Lee & Ruby, 1994; Ruby, 1996).

The biology of the squid-*Vibrio* association has been studied in detail at the physiological, molecular and genetic level. Many genes, behaviors, and molecules that contribute to or are required for the colonization of the squid have been identified, including those involved in direct host interaction (Aeckersberg *et al.*, 2001; Stabb & Ruby, 2003; Vydryakova, 2006), nutrition (Graf & Ruby, 1998; DeLoney-Marino *et al.*, 2003), gene regulation (Visick & Skoufos, 2001; Fidopiastis *et al.*, 2002; Millikan & Ruby, 2003; Whistler & Ruby, 2003; Wolfe *et al.*, 2004; Bose *et al.*, 2007; Hussa *et al.*, 2007; Whistler *et al.*, 2007) biofilm formation (Yip *et al.*, 2005; Yip *et al.*, 2006); light production (Bose *et al.*, 2008), motility (Graf *et al.*, 1994; Millikan & Ruby, 2002; Millikan & Ruby, 2004), transport of bacterial factors (Dunn & Stabb, 2008), and lipid modification (Adin *et al.*, 2008). Also, findings from this system have been successfully modeled onto vertebrate consortial systems (Hooper *et al.*, 1998), showing the broader applicability of invertebrate symbiosis research.

Transmission of symbionts

In each of the symbiotic systems described above, a specific set of microbes provides beneficial activities (nutrition and/or defense) to the host. These microbes occupy discrete locations within or on the host, and are acquired from the environment or maternally (from the egg or egg case). How do these hosts and symbionts initiate their association? How do the symbionts localize to the correct tissue of the host body? How are non-cooperative cheaters restricted from colonization? By elucidating the cellular and molecular processes underlying symbiont transmission between generations, the answers to these questions are beginning to be revealed.

Evolutionary theory predicts that a host will reap fewer benefits from a symbiotic association if its symbionts compete with each other for host goods (Frank, 1996). Therefore, selection should favor associations with limited microbial symbiont diversity and therefore reduced competition. This theory has been borne out experimentally in several model systems. As described above, entomopathogenic *Xenorhabdus* bacteria are transmitted between insect hosts (and therefore generations) by colonizing the intestine of the infective juvenile stage of *Steinernema* nematodes. The final bacterial population within an individual nematode ranges from between 30 and 500 cells, and is predominantly clonal (derived from 1–2 individual bacterial cells). This conclusion is based on experiments in which *Steinernema carpocapsae* nematodes were cultivated on mixed populations of differentially labeled, but otherwise isogenic strains of the bacterial symbiont. The progeny infective stage nematodes from these cultivations typically were colonized by only one strain, or at most two (Martens *et al.*, 2003a). Similar clone restriction may also occur in the squid light organ. Simultaneous inoculation of multiple *Vibrio* species showed that inoculation with lower concentrations of bacteria led to monocolonized animals, and inoculation with higher concentrations led to polycolonized squid, consistent with the idea that one or a few individuals initiate colonization, with increasing diversity correlated with inoculum size (McCann *et al.*, 2003). Recently, these experiments were extended by colonizing individual squid with isogenic strains expressing distinct fluorescent markers, and monitoring fluorescence within individual crypts (Wollenberg & Ruby, 2009). Mathematical modeling of the resulting data showed fewer than two bacteria initiated colonization of each of six individual crypts. This clonality applies only to the bacterial populations within individual crypts; within a entire light organ each of the six different crypts can be colonized by a differently labeled isogenic clone (Wollenberg & Ruby, 2009).

Similarly, in *R. pachyptila* fewer than 20 cells are found in the invading tissue in early stages but fewer than that number are predicted to actually initiate colonization within the trophosome (Nussbaumer *et al.*, 2006). Sequencing of highly variable internal transcribed spacers (ITS) regions of the symbionts obtained from three different individual tubeworms from the same location revealed that each tubeworm was predominantly colonized by symbionts with the same ITS variants, but which were different between the three worms. As above, this supports a model in which one or a few individual bacterial cells initiate colonization of a single *R. pachyptila* worm. Similarly, in another marine annelid, *Oligobrachiae mashikoi*, tubeworms collected from a single site collectively contained seven distinct bacterial phylotypes, but typically in any given worm only a single phylotype dominated the symbiont population (Kubota *et al.*, 2007).

Evidence also exists for symbiont restriction in vertically transmitted symbioses. The *Photorhabdus* symbionts of *Heterorhabditis* nematodes were only recently shown to be transmitted maternally (Ciche *et al.*, 2008). In this process, 1–3 *Photorhabdus* bacteria adhere to and colonize rectal gland cells within the mother, growing or accumulating to form a population of up to 50 cells. When juvenile nematodes hatch within the mother,

Photorhabdus are released into the maternal body cavity, and are available for colonization of the progeny nematodes. Microscopic imaging indicates that within the developing juvenile nematode, a single bacterium invades each of the two pharyngeal gland cells at the anterior of the nematode intestine. The bacteria replicate within this cell, then apparently emerge to colonize the intestinal lumen of the infective juvenile (Ciche *et al.*, 2008). Taken together, these data indicate that, as in *Xenorhabdus-Steinernema* associations, the colonization process serves to restrict the number of bacterial clones that occupy a single host niche.

Although the examples noted above provide a strong case for clone restriction during transmission between generations this may not be a universal trend among symbioses. For example, in the leech crop symbiosis, co-competition experiments between the wild-type symbiont and non-symbiont species (or mutants) of *Aeromonas* show that non-native (or mutant) species are always present in appreciable numbers in an individual crop along with wild type bacteria (Silver *et al.*, 2007a). This suggests that colonization of an individual crop is not limited to one or a few symbionts (Laufer *et al.*, 2008). However, the number of individual bacterial cells that initiate colonization or form microcolonies has not been investigated directly.

In general, restricted clonality of a population, such as for the symbiont populations discussed above, is predicted to have deleterious effects on host or symbiont fitness since symbiont bottlenecks should cause accumulation of deleterious mutations that eventually drive a population to extinction (i.e. Muller's ratchet (Muller, 1964; Felsenstein, 1974)). However, the experimental evidence summarized above indicates frequent occurrence of stable associations that exhibit clone restriction. One reason symbiont fitness in the examples above may not be reduced despite the expected susceptibility of each bottlenecked population to the ratchet is that selection on host populations plays a role in maintaining symbiont fitness. A recent model showed that if the effective host population size is sufficiently large (~ $>10^5$ individuals) the symbiont population as whole will not be driven to extinction, even though nested populations within a host individual may acquire some deleterious mutations (Pettersson & Berg, 2007). Thus, high population size in the wild may counter the deleterious effects of bottlenecking and Muller's ratchet, thereby allowing individual hosts to benefit from reduced inter-symbiont competition through clonal selection of symbionts.

The evidence reviewed above indicates that clonality of bacterial symbiont populations within an individual animal is a recurrent phenomenon, even in horizontally transmitted symbioses. This is contrary to the assumption adopted by some evolutionary models (e.g. (Foster & Kokko, 2006)) that horizontal transmission of bacterial symbionts is achieved by a population of bacteria, rather than individual clones. Thus, these comparative findings highlight an unanticipated commonality of symbiont clone restriction in both vertical and horizontal transmission routes.

Match-making: Host-Associated Molecular Patterns

Regardless of transmission route, mutualistic microbial symbionts tend to be targeted and restricted to specific tissues within their hosts. Such localization likely is determined in many symbioses by physical interactions between surface structures expressed by the appropriate host tissues and microbial symbionts. A very common, perhaps ubiquitous form of such physical interactions in both mutualistic and pathogenic relationships, is between surface sugars and protein lectins (Hooper & Gordon, 2001).

Current evidence reveals a striking recurrence among mutualistic associations of sugar-containing material at animal tissues involved in symbiont association or recruitment. For

example, within the bacterial colonization site of the infective stage of all *Steinernema spp.* examined to date there is a cluster of spherical bodies, termed the “intra-vesicular structure” (IVS) (Martens & Goodrich-Blair, 2005). In *S. carpocapsae*, *Xenorhabdus nematophila* symbiont bacteria can be seen adhering to this structure, which is itself associated with a mucus-like material comprised of *N*-acetylglucosamine or *N*-acetylneuraminic acid residues (based on reactivity with wheat germ agglutinin) (Martens & Goodrich-Blair, 2005). Similarly, at the site of bacterial recruitment *Euprymna* squid express a mucus-like material in which *V. fischeri* bacteria accumulate (Nyholm *et al.*, 2000). This material reacts with wheat germ agglutinin and *Sophora japonica* agglutinin, but not with succinylated wheat-germ agglutinin, *Ulex europeus* agglutinin-1, or concanavalin A, a reactivity profile that suggests the presence of *N*-acetylneuraminic acid and *N*-acetylgalactosamine (Nyholm *et al.*, 2000). Later studies also showed that co-incubating *N*-acetyl-D-galactosamine with *V. fischeri* inhibited its hemagglutination of erythrocytes (Vydryakova, 2006), implicating this sugar in mediating binding of *Vibrio* symbiont cells.

Although not understood in as much detail, *R. pachyptila* larvae also produce a surface mucus in which invading cells are embedded, along with non-invading cells, prior to the invasion of the epithelial layers and penetration into deeper tissue by the specific chemoautotrophic symbiont (Nussbaumer *et al.*, 2006). The authors predicted that this mucus is derived from the chitin-secreting pyriform glands (Gaill *et al.*, 1992; Shillito *et al.*, 1995; Chamoy *et al.*, 2001). In another deep-sea hydrothermal vent marine chemosynthetic mutualism, epibiotic bacteria adhere to the chitinous surfaces of *Kiwa hirsuta* Yeti crabs (Goffredi *et al.*, 2008). Also, symbiotic terrestrial earthworms secrete a thick mucus coat during mating into which the earthworm injects bacteria, sperm, and eggs, prior to the formation of a mature egg with a sealed, chitin-based protective coat (Davidson & Stahl, 2006). The saccharide constituents of this mucus have not yet been revealed.

In some cases where a glycan or other sugar is implicated in a symbiotic association the source of the sugar moiety is microbial, or has not been determined yet. The two known leech crop symbionts *Rikenella* and *A. veronii* form microcolonies embedded in an extracellular matrix that reacts with wheat-germ agglutinin (but not twelve other lectins that were tested) (Kikuchi & Graf, 2007). This material also reacted with succinylated wheat-germ agglutinin, which has specificity for *N*-acetylglucosamine relative to *N*-acetylneuraminic acid. However, these studies do not reveal if the extracellular matrix is derived from the microbe or the host. The marine nematode *L. oneistus* expresses a protein lectin in its surface-secreted mucus. This protein has a specific carbohydrate recognition domain that recognizes and recruits the nematode’s sulfur-oxidizing bacterial symbiont (Bulgheresi *et al.*, 2006). Surprisingly, this nematode receptor for a mutualistic symbiont is similar to the human dendritic cell-specific immunoreceptor, highlighting again how pathogenic and mutualistic relationships appear to be mediated by fundamentally conserved processes (Bulgheresi *et al.*, 2006; Zhang *et al.*, 2006). The bacterial epibionts are predicted to utilize surface exposed D-mannose and L-rhamnose residues to bind to the host lectin (Nussbaumer *et al.*, 2004).

N-acetylglucosamine, a primary reactant in many of the lectin-binding studies referenced above, polymerizes to form the macromolecule chitin. Chitin is a primary component of numerous eukaryotic organisms, including cell walls of fungi, exoskeletons of arthropods, specialized structures in mollusks and cephalopods, and nematodes (Weiner & Traub, 1984; Synowiecki & Al-Khateeb, 2003; Foster *et al.*, 2005; Zhang *et al.*, 2005). Therefore, chitin or its oligomers appear to be predominant surface molecules in invertebrates that may be specifically recognized by microbial symbionts, much as hosts recognize microbes through patterned surface molecules such as peptidoglycan (Guan & Mariuzza, 2007). While chitin is an unlikely molecular pattern of mammals, other types of molecules that are well

represented in mammalian cells may perform a similar function to elicit a bacterial symbiont response. For example, the vertebrate intestinal symbiont *Bacteroides thetaiotaomicron* initiates colonization of the small intestine in response to presentation of fucosylated glycans (Bry *et al.*, 1996; Hooper *et al.*, 2000).

The examples provided above highlight the striking ubiquity of oligosaccharides, particularly chitin and its derivatives, being localized at the sites of invertebrate host-microbe interactions. The observation that a common class of molecules is implicated in diverse symbiotic associations suggests that bacteria and hosts may recognize each other through conserved molecular patterns that are either host-associated (HAMPs) or, as is well established, microbially associated (MAMPs) (Didierlaurent *et al.*, 2002; Koropatnick *et al.*, 2004; Niedergang *et al.*, 2004). Since chitin is a conserved feature of invertebrates generally, and especially arthropods, it can be considered a HAMP that may be a common binding motif for microbial association factors. HAMP-binding microbial factors may function to non-specifically initiate interactions that then progress toward specificity using distinct processes. The latter scenario appears to be the case for *E. scolopes* squid, which non-specifically binds numerous bacteria in secreted mucus, but then specifically recruits *V. fischeri*, to colonize the deep crypts of its light organs. Alternatively, specificity in invertebrate mutualisms could be achieved through minor variations in glycan structure and lectin binding capacity. HAMPs such as chitin may also serve as host-symbiont signaling molecules, as they do in plant-microbe symbioses (Garg & Geetanjali, 2007), and the squid light-organ symbiosis (DeLoney-Marino *et al.*, 2003).

Symbiont Specificity

As discussed above, cooperative associations are maintained in evolutionary time through selection of cooperative symbionts, and potentially the exclusion of non-performing, or cheating symbionts (Douglas, 2008). This phenomenon can occur through “partner choice”: the selection of a specific symbiotic partner from among many potential partners based on its potential as a cooperator (Sachs *et al.*, 2004).

The relevance of mutualistic partner choice, and other mechanisms by which cooperation is promoted, is evident in the prevalence of experimentally demonstrated specificity in which a cognate symbiont is selected while other, even closely related, symbionts are excluded. For example, culture independent analysis of *Verminephrobacter* bacteria distribution among earthworms suggests that highly selective specificity exists between host and symbiont: symbionts isolated from the same host species at distant geographical locations are more similar than symbionts isolated from different host species at close locations (Schramm *et al.*, 2003; Pinel *et al.*, 2008). The degree of taxonomic specificity (i.e. genus, species, strain) varies among the associations. Furthermore, even within a single genus, some species will display more strict symbiont selectivity than others. This is exemplified by the leech crop symbiosis: *H. verbana* and *Macrobdella decora* specifically associate with *A. veronii* and *A. jandaei*, respectively. Both bacterial species are geographically ubiquitous, suggesting that preferential partner selectivity plays a role in determining which species associate with each host (Graf, 1999; Siddall *et al.*, 2007). In contrast, another leech species, *Hirudo orientalis*, associates with both *Aeromonas* species (Laufer *et al.*, 2008), highlighting that within an animal genus, specificity for symbionts can range from specialized to generalized associations.

Varying degrees of specificity are also observed among *Euprymna* and *Sepiola* squid light organ symbioses. For example, *E. scolopes* is naturally associated with *V. fischeri*, but can be colonized by *Vibrio logei*, a symbiont of *Sepiola* squid, albeit at a lower level (Fidopiastis *et al.*, 1998). However, *E. scolopes* can select certain *V. fischeri* strains over others. Some *V.*

fischeri strains that colonize the light organs of fish are unable to colonize squid (Mandel *et al.*, 2009). Furthermore, when non-native *V. fischeri* strains that can colonize *E. scolopes* light organs were competed against the native *V. fischeri* isolate, their colonization competency was in some cases correlated with their degree of relatedness to the native strain (Nishiguchi *et al.*, 1998). Thus, strains that are more closely related to the natural colonizer appear to have a competitive advantage over more distantly related strains. As in the leech crop symbiosis noted above, there appears to be variation among squid for their degree of specialization for specific light organ symbionts: In several species of *Sepiola* squid, the sister genus to *Euprymna*, the light organs are colonized by a mixture of two bacterial species, *V. fischeri* and *V. logei* (Fidopiastis *et al.*, 1998; Nishiguchi, 2000). Specificity of *Sepiola* for either of these two bacterial species is impacted by temperature, as *V. fischeri* is the predominant symbiont at 26° C and *V. logei* is at 18° C (Nishiguchi, 2000).

Individual *Steinernema* nematode species are found in nature to be associated with a single *Xenorhabdus* species, suggesting a specific association (Fischer-Le Saux *et al.*, 1998; Tailliez *et al.*, 2006). To date specificity has been experimentally examined only in two species of *Steinernema*, *S. carpocapsae* and *S. scapterisci.* Each was shown to be colonized only by its cognate bacterial symbiont, *X. nematophila* and *X. innexi* respectively: infective stage juvenile nematodes that develop in the presence of non-native species of *Xenorhabdus* have un-colonized intestinal receptacles (Akhurst, 1983; Sicard *et al.*, 2004; Sicard *et al.*, 2005; Cowles & Goodrich-Blair, 2008). *Heterorhabditis* nematode specificity appears to be strain specific: *Photorhabdus luminescens* symbionts of *H. bacteriophora* and *H. indica* are only able to colonize their respective host species, and not the other (Han & Ehlers, 1998).

Although evidence for specificity has been acquired in many mutualistic associations, as discussed above, until recently little was known regarding the molecular basis of specific recognition between symbiont partners, with the exception of the association between leguminous plants and nodule-forming nitrogen fixing bacteria (Rhizobia). In this system, specificity is determined by the identities of diffusible signals passed between hosts and symbionts. Host legumes secrete polycyclic aromatic small molecules called flavonoids into the soil. Specific modifications to a conserved core flavonoid structure results in a wide variety of flavonoid variants (over 4000 have been described) that are recognized by specific symbiotic Rhizobia. The specific flavonoid interacts with the gene product of the Rhizobial *nodD* gene to form a regulatory complex that stimulates production of other *nod* genes in Rhizobia. The products of the *nod* genes, called Nod factors, are small molecules that, like flavonoids, consist of a core structure that is varied depending on the species that produces it. Nod factors consist of linked *N*-acetylglucosamine subunits with a long acyl chain attached to the terminal subunit. Variations on the number of subunits or modifications to the subunits, and on the length and saturation of the acyl chain are recognized by specific host species. Within the plant, the nod factor elicits a complex gene expression cascade and physiological and morphological events that result in Rhizobia infection and nodule development (Garg & Geetanjali, 2007; Gibson *et al.*, 2008). Thus, intra-species-specificity between Rhizobia and leguminous plants is dictated by variations among conserved host-association molecules (flavonoids and Nod factors). The paradigm established by this groundbreaking work is that species specificity is mediated by minor variations in structure or motifs in core molecules conserved among symbiotic-competent members of the genus. However, the applicability of this model to other symbiotic systems awaits an in depth understanding of comparative examples of the molecular foundation of species specificity. Such investigations are just beginning to be pursued, and host-range specificity determinants have been identified to date in only two examples of mutualistic animal-microbe associations: *Steinernema-Xenorhabdus* and *Euprymna-Vibrio*.

Species specificity in the association between *S. carpocapsae* nematodes and *X. nematophila* bacteria recently was shown to be mediated by two *X. nematophila* genes, *nilB* and *nilC* (nematode intestine localization). These genes are divergently oriented on a 3.5-kb locus, and encode membrane-localized proteins that are each necessary for colonization (Heungens *et al.*, 2002; Cowles & Goodrich-Blair, 2004; Cowles & Goodrich-Blair, 2006; Cowles & Goodrich-Blair, 2008). While their function is unknown, they appear to have been horizontally acquired and are absent from other *Xenorhabdus* spp. based on Southern hybridization of DNA from 12 species (Cowles & Goodrich-Blair, 2008). Expressing *nilB* and *nilC* in non-cognate *Xenorhabdus* species, *X. bovienii* and *X. poinarii*, allows these strains to colonize *S. carpocapsae* nematodes, indicating that *nilB* and *nilC* are specificity determinants for this nematode (Cowles & Goodrich-Blair, 2008). These findings indicate that a species within the *Xenorhabdus* genus has evolved a host-range specificity determinant that is absent from other *Xenorhabdus* species, in contrast to the Rhizobia paradigm described above in which minor variations in genes or gene products present among all members of a given genus control host range (Gualtieri & Bisseling, 2000; Inatsuka *et al.*, 2005).

A recent study has also identified a species-specificity factor in the squid light-organ symbiosis (Mandel *et al.*, 2009). *rscS* (regulator of symbiotic colonization-sensor), a sensor kinase, activates genes that participate in biofilm formation by the bacteria, and is essential for colonization of the squid light-organ (Visick & Skoufos, 2001; Yip *et al.*, 2006). *rscS* acts upstream of *sypG* (symbiosis polysaccharide), a response regulator that in turn acts on a locus of 18 genes involved in exopolysaccharide production (*syp* genes) (Yip *et al.*, 2005; Yip *et al.*, 2006; Hussa *et al.*, 2008). Production of this exopolysaccharide is essential for aggregation of *V. fischeri* within host mucus layers during the early stages of light organ colonization, and mutants of the *syp* genes (including *sypG*) display host colonization deficiencies (Yip *et al.*, 2005; Hussa *et al.*, 2007). Different strains of *V. fischeri* have colonization specificity for either squid or pinecone fish. The *syp* genes are found in all tested strains of *V. fischeri*, while *rscS* is found in all squid symbionts but not in all fish-colonizing strains. Fish-colonizing *V. fischeri* strains that encode *rscS* are also able to colonize squid, while *rscS*-minus fish-derived strains do not colonize squid. Furthermore, *rscS* expression in those *V. fischeri* that lack this gene confers the ability to colonize squid. The evolutionary significance of *rscS* presence in some but not all fish-colonizing strains, and the role, if any, of the *syp* genes in fish colonization, are unknown. However, as in the *Steinernema-Xenorhabdus* model system, these findings reveal that the presence or absence of one or a few bacterial genes is sufficient to confer host-range specificity among bacterial strains within a single species (Cowles & Goodrich-Blair, 2008; Mandel *et al.*, 2009).

These early developments mark the beginning of what is likely to be a dramatic expansion in our understanding of symbiont selection and specificity in diverse animal-microbe associations. Identification of microbial specificity determinants in diverse mutualistic associations will reveal whether or not specificity is more commonly achieved through variations in conserved core structures such as in legume-Rhizobia symbioses, or through species-specific expression of novel surface or regulatory proteins. Furthermore, a critical component of this research area will be the identification of host factors that contribute to specificity, a task facilitated by the development of new technologies to manipulate host gene expression (Ciche & Sternberg, 2007; Hao *et al.*, 2008).

Broadening the Perspective

The identification of common symbiotic processes of colonization initiation and partner recognition among the model systems discussed above is tempered by the fact that these systems share a fundamental biological characteristic: they are each a mutualism between an

invertebrate host and one or a few bacterial symbionts selected from an external reservoir each generation. Therefore, it is unclear if these processes generally occur in other types of associations, such as maternally inherited intracellular symbioses (endosymbioses) (reviewed in (Baumann, 2005)). Invertebrates, particularly insects, engage in a diverse range of endosymbiotic associations. Insects can be obligately dependent on a primary, or P-endosymbiont, but also facultatively associated with other secondary S-endosymbionts. Primary and secondary symbionts differ in their evolutionary history with the host, mode of transfer within and between host species, site of residence within the host, contributions to host fitness, and genomic properties. Most insect endosymbionts cannot be cultured in the laboratory, yet a wealth of knowledge regarding their evolution and physiology has been gained using molecular and genomic tools (Moran *et al.*, 2008).

Current evidence indicates that in at least some instances, insect endosymbioses may engage in aspects of host-symbiont recognition discussed above. Secondary endosymbionts can be horizontally transmitted and therefore subject to selection (Moran & Dunbar; Moran *et al.*, 2008). When a foreign species of the endosymbiont *Wolbachia* was introduced into the adzuki bean borer moth it was not efficiently transmitted from parent to offspring, while the native species was transmitted with nearly 100% efficiency (Sakamoto *et al.*, 2005), suggesting insects can select certain endosymbiont bacterial species with adaptations that promote inter-generational transmission. Conserved molecular pattern molecules may be involved in mediating host-symbiont recognition in endosymbioses, an idea supported by the fact that weevil endosymbiont MAMPs can trigger a host immune response which is thought to help control endosymbiont populations and tissue localization (Anselme *et al.*, 2006; Anselme *et al.*, 2008). Whether or not these observations represent a general role for partner recognition and molecular patterns in endosymbioses will be revealed by additional inquiry.

Although the discussion of this review focused on bacterial symbioses, the common features highlighted also apply to a model symbiosis between two eukaryotic organisms, *Hydra* spp. and their algal symbionts *Chlorella* spp., raising the possibility that the presented principles apply across domains in the tree of life. Hydra can obtain their algal symbionts, from which they derive photosynthate nutrients, either vertically through the egg, or as the hydra grows to maturity (Muscatine & Lenhoff, 1963; Muscatine, 1965; Thorington *et al.*, 1979). Symbiont clonality is suggested by the fact that algae within hydra eggs are derived from as few as a single algal cell (Muscatine & Mcauley, 1982). Also, when aposymbiotic hydra were exposed to two competing algal species, most were mono-colonized by one species or the other, but very rarely both (Rahat, 1985). The idea that a sugar-containing substance is involved in the symbiosis comes from experiments in which incubation of either aposymbiotic hydra or algal symbionts with concanavalin A prior to mixing the two together inhibits the uptake of the algae by the hydra (Meints & Pardy, 1980). Finally, hydra exhibit permissive selectivity for their algal symbionts, since a number of different algal symbionts can associate with a given host, but native strains are preferred (Pardy, 1976; Rahat, 1985).

Concluding Remarks

During the past two decades there has been much progress in developing diverse experimental models of animal-microbe mutualisms, particularly those of invertebrates. These models are individually providing fascinating insights into symbiotic processes, and together are revealing common themes by which animals and microbes maintain long-term beneficial relationships (Ruby, 2008). Here we have discussed three themes that are apparent from a comparative study of several invertebrate symbiotic models: 1) clonality of colonizing symbiont populations within a host; 2) participation of sugar residues and lectins at the host-microbe interface based on conserved host (HAMPs) and microbial (MAMPs)

molecules; and 3) emerging principles of symbiont partner selectivity. Further study on developed and emerging models of animal-microbe symbiosis will help reveal how widely applicable these themes are among diverse associations. However, it is clear that the nascent and exciting field of animal-microbe mutualism is already revealing common themes applicable across diverse phyla. Therefore, the development of new model systems and the deeper investigation of established symbiosis models hold promise for understanding fundamental principles of symbiotic associations that occur within and around us.

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Table 1

Selected experimental models of animal-microbe mutualism

Host/Habitat	Symbiont	Host association structure	Host-Derived Fitness Benefit	Transmission Strategy	Presence of lectin-binding residues
Steinernema carpocapsae terrestrial	*Xenorhabdus nematophila*	Receptacle/Intestine	Insect pathogenicity, nutrition/reproduction	Horizontal (within insect) oligo-initiated colonization	WGA intravesicular structure inside receptacle
Heterorhabditis bacteriophora terrestrial	*Photorhabdus luminescens*	Intestine	Insect pathogenicity, nutrition/reproduction	Vertical (within nematode mother) oligo-initiated colonization	N.T.
Hirudo verbana freshwater	*Aeromonas veronii* biovar sobria Rikenella-like	Crop	Putative: nutrition, passive or active exclusion	Likely vertical, poly-initiated colonization	WGA-S Microcolonies embedded in intraluminal fluid
Eisenia foetida terrestrial	*Verminophrobacter eiseniae*	Nephridia	Putative: protein degradation or N-cycling	Vertical colonization (egg case)	N.T.
Riftia pachyptila deep sea marine	'*Candidatus* Endoriftia persephone'	Trophosome	Nutrition	Horizontal oligo-initiated colonization	N.T.
Laxus oneistus shallow marine	Gamma-proteobacterium (a single phylotype)	Cuticle	Nutrition	Likely Horizontal	WGA, conA bacterial surface
Euprymna scolopes shallow marine	*Vibrio fischeri*	Light organ	Counterillumination	Horizontal oligo-initiated colonization	WGA, sophora japonica Mucous at pores of light organ
Hydra viridis freshwater	*Chlorella* sp.	Intracellular vesicles	Nutrition	Vertical oligo-initiated colonization Horizontal colonization also occurs	Concanavalin A