Common trends in mutualism revealed by model associations between invertebrates and bacteria

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Abstract
Mutually beneficial interactions between microorganisms 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 microorganisms. Here, we review several invertebrate model systems that contribute 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.

Introduction
Mutually beneficial symbiotic associations between microorganisms 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 allows 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 microorganism–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 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 provide insights into how microorganisms move between host species, how symbiotic partners adapt to each other, and the evolutionary factors that drive the emergence and maintenance of cooperative microbial–host associations. Because 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 > 5600 taxa in the intestine alone (Hooper et al., 1998; Bäckhed et al., 2005; Dethlefsen et al., 2008), making it challenging to assign specific symbiotic functions to individual organisms. The relatively low diversity of microorganisms in invertebrate symbiotic associations simplifies the task of teasing apart apart complex molecular and cellular interactions between the symbiont and the host (McFall-Ngai, 2002; Ruby, 2008). Furthermore, because animal evolution has been continuously influenced by symbiosis with microorganisms, fundamental molecular features of the animal–microorganism 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 reveal common mechanisms of symbiotic transmission, recognition, and specificity (Table 1).

Table 1. Selected experimental models of animal–microbe mutualism

<table>
<thead>
<tr>
<th>Host/habitat</th>
<th>Symbiont</th>
<th>Host association structure</th>
<th>Host-derived fitness benefit</th>
<th>Transmission strategy</th>
<th>Presence of lectin-binding residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steinernema carpocapsae terrestrial</td>
<td>Xenorhabdus nematophila</td>
<td>Receptacle/intestine</td>
<td>Insect pathogenicity, nutrition/reproduction</td>
<td>Horizontal (within insect) oligo-initiated colonization</td>
<td>WGA intravesicular structure inside receptacle</td>
</tr>
<tr>
<td>Heterorhabditis bacteriophora terrestrial</td>
<td>Photobacter luminescens</td>
<td>Intestine</td>
<td>Insect pathogenicity, nutrition/reproduction</td>
<td>Vertical (within nematode mother) oligo-initiated colonization</td>
<td>NT</td>
</tr>
<tr>
<td>Hirudo verbena freshwater</td>
<td>Aeromonas veronii biovar sobria</td>
<td>Crop</td>
<td>Putative: nutrition, passive or active exclusion</td>
<td>Likely vertical, poly-initiated colonization</td>
<td>WGA-5 Microcolonies embedded in intraluminal fluid</td>
</tr>
<tr>
<td>Eisenia foetida terrestrial</td>
<td>Verminephrobacter eiseniae</td>
<td>Nephridia</td>
<td>Putative: protein degradation or N-cycling Nutrition</td>
<td>Vertical colonization (egg case)</td>
<td>NT</td>
</tr>
<tr>
<td>Riftia pachyptila deep sea marine</td>
<td>‘Candidatus Endoriftia persephone’</td>
<td>Trophosome</td>
<td></td>
<td>Horizontal oligo-initiated colonization</td>
<td>NT</td>
</tr>
<tr>
<td>Laxus oneistus shallow marine</td>
<td>Gamma-proteobacterium (a single phylotype)</td>
<td>Cuticle</td>
<td>Nutrition</td>
<td>Likely Horizontal</td>
<td>WGA, ConA bacterial surface</td>
</tr>
<tr>
<td>Euprymna scolopes shallow marine</td>
<td>Vibrio fischeri</td>
<td>Light organ</td>
<td>Counterillumination</td>
<td>Horizontal oligo-initiated colonization</td>
<td>WGA, sophora japonica</td>
</tr>
<tr>
<td>Hydra viridis freshwater</td>
<td>Chlorella sp.</td>
<td>Intracellular vesicles</td>
<td>Nutrition</td>
<td>Vertical oligo-initiated colonization Horizontal colonization also occurs</td>
<td>WGA, sophora japonica Mucous at pores of light organ Concanavalin A</td>
</tr>
</tbody>
</table>

WGA, wheat-germ agglutinin; NT, not tested.

The role of transmission and partner recognition in the 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. Because 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 (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. Because 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 noncheating 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. Fulfilment 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 elucidated in a number of horizontally acquired invertebrate animal–microorganism mutualisms yield 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. Because 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 five 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 microorganisms to community fitness (Broderick et al., 2004, 2006; Dillon & Dillon, 2004; Brinkmann et al., 2008). For example, investigations of insect systems are revealing 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 microorganisms. The relative simplicity of such systems has facilitated experimental progress in understanding the biology, genetics, and physiology of host–microorganism 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 the advances such systems have provided 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 the 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 *Gammaproteobacteria*. 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 nonfeeding 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 Steinhernema 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 that 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 two to three generations until high nematode density and low nutrition cue development of progeny into the infective stage (Popiel et al., 1989), which emigrates to infect a new insect host. Development into the nonfeeding 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 Steinhernema spp. and Heterorhabditis spp. exhibit specificity for their symbiont at this stage (Akhurst, 1983; Han & Ehlers, 1998; Ciche & Ensign, 2003; Sicard et al., 2003, 2004, 2005; Cowles & Goodrich-Blair, 2008).

Entomopathogens provide a rare opportunity to elucidate bacterial traits that contribute to mutualism and pathogenesis, because Xenorhabdus and Photorhabdus, while mutualists of their nematode hosts, are also 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, 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, 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 yet been demonstrated, 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).

Intergenerational 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 multispecies 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 $10^6$ (Aeromonas) and $10^{10}$ (Rikenella) bacteria per milliliter (Kikuchi & Graf, 2007). The mammalian host parasitized by the leech contributes to the specificity of the leech–symbiont association.
because *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).

Because *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 microorganisms 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 fetida* and the betaproteobacterium *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 before 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 to conserve nitrogen compounds otherwise lost during excretion (Schramm et al., 2003).

The symbionts have been identified within developing earthworm eggs, suggesting vertical transmission (Davidson & Stahl, 2006, 2008), although for a brief period, the eggs are unsealed outside of the body of the parent, allowing environmental bacteria to gain access to the egg before 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, before 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 $3 \times 10^5$ CFU (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, pers. commun.). 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 symbioses have 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 chemosymbiosis is the microbrial 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 chemosymbiotic 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 gammaproteobacterial 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.

*Riftia 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 toward the coelom of the tubeworm. The bacteriocytes constitute 50–70% of the trophosome, and ‘C. Endoriftia persephone’ alone accounts for approximately 25% of the total volume of the trophosome (Jones & Gardiner, 1988; Bright & Sorgo, 2003). *Riftia 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 chemosynthetic bacteria colonize the entire *L. oneistus* body surface, except the head. Colonization is likely an early event in the life history of the nematode, because 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 phyloype, 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 microorganisms that colonize the light organs of numerous marine fishes and squid (Dunlap, 1985; Dunlap *et al.*, 2004, 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 bilobed organ with a pair of three pores on each lobe that allow 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 the 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 toward the pores. Once localized at the pores, *V. fischeri* travels down a canal, and approaches the
crypts. One or two cells initiate colonization of each of the six crypts (Wollenberg & Ruby, 2009) and reproduce to form the large population (∼10⁵ 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 entire 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, 2006), light production (Bose et al., 2008), motility (Graf et al., 1994; Millikan & Ruby, 2002, 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 microorganisms provides beneficial activities (nutrition and/or defense) to the host. These microorganisms 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 one to two 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 were typically colonized by only one strain or at most two (Martens et al., 2003a). A 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 monoclonized animals, and inoculation with higher concentrations led to polyclonized 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 the individual crypts. This clonality applies only to the bacterial populations within individual crypts; within an 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, Oligobrachia masuikoi, 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,
one to three *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, and 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 nonsymbiont 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 because 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 (∼10⁵ individuals), the symbiont population as a 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 intersymbiont 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 the transmission route, mutualistic microbial symbionts tend to be targeted and restricted to specific tissues within their hosts. This localization is likely 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 ‘intravesicular structure’ (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 coincubating *N*-acetyl-n-galactosamine with *V. fischeri* inhibited its hemagglutination of erythrocytes (Vdyryakova, 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 noninvading cells, before 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, before 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 yet been determined. The two known leech crop symbionts *Rikenella* and *A. veronii* form micro-colonies embedded in an extracellular matrix that reacts with wheat-germ agglutinin (but not 12 other lectins that were tested) (Kikuchi & Graf, 2007). This material also reacted with succinylated wheat-germ agglutinin, which has specificity for *N*-acytelo glucosamine relative to *N*-acetyleneuraminic acid. However, these studies do not reveal whether the extracellular matrix is derived from the microorganism 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 (Bulgaresi 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 (Bulgaresi et al., 2006; Zhang et al., 2006). The bacterial epibionts are predicted to utilize surface-exposed β-mannose and α-rhamnose residues to bind to the host lectin (Nussbaumer et al., 2004).

*N*-acytelo glucosamine, 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 microorganism 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 the 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–microorganism 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). Because chitin is a conserved feature of invertebrates generally, and especially arthropods, it can be considered as an HAMP that may be a common binding motif for microbial association factors. HAMP-binding microbial factors may function to nonspecifically initiate interactions that then progress toward specificity using distinct processes. The latter scenario appears to be the case for *E. scolopes* squid, which nonspecifically 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 the glycan structure and the lectin-binding capacity. HAMPs such as chitin may also serve as host–symbiont signaling molecules, as in plant–microorganism 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 nonperforming, 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 *Verninephrobacter* bacteria distribution among earthworms suggests that a highly selective specificity exists between the host and the 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 a 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 *Aeromonas 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 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 a 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). The 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* at 18 °C (Nishiguchi, 2000).

Individual *Steinernema* nematode species are found to be associated with a single *Xenorhabdus* species in nature, 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 *Steinernema scapterisci*. Each was shown to be colonized only by its cognate bacterial symbiont, *X. nematophil* and *Xenorhabdus innexi*, respectively: infective-stage juvenile nematodes that develop in the presence of non-native species of *Xenorhabdus* have uncolonized intestinal receptacles (Akhurst, 1983; Sicard *et al*., 2004, 2005; Cowles & Goodrich-Blair, 2008). *Heterorhabditis* nematode specificity appears to be strain specific: *Photorhabdus luminescens* symbionts of *H. bacteriophora* and *Heterorhabditis indica* are able to colonize only their respective host species, and not the others (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 *nod* gene to form a regulatory complex that stimulates the 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 varies depending on the species that produces it. Nod factors consist of linked *N*-acylglucosamine subunits with a long acyl chain attached to the terminal subunit. Variations depend on the number of subunits or modifications to the subunits, and on the length and saturation of the acyl chain, which 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, intraspecies 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–microorganism associations: *Steinernema–Xenorhabdus* and *Euprymna–Vibrio*.

Species specificity in the association between *S. carpocapsae* nematodes and *X. nematophilia* bacteria was recently shown to be mediated by two *X. nematophilia* 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, 2006, 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 noncognate *Xenorhabdus* species, *Xenorhabdus bovienii* and *Xenorhabdus 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 in 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 the 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, is necessary for activation of 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, 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- 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 the presence of rsCS 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 drastic expansion in our understanding of symbiont selection and specificity in diverse animal–microorganism 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 in each generation. Therefore, it is unclear whether 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, 2006; 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 the parent to the offspring, while the native species was transmitted with nearly 100% efficiency (Sakamoto et al., 2005), suggesting that insects can select certain endosymbiont bacterial species with adaptations that promote intergenerational 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 that is thought to help control endosymbiont populations and tissue localization (Anselme et al., 2006, 2008). Whether or not these observations represent a general role for partner recognition and molecular patterns in endosymbioses will be revealed by further studies.

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 principles presented apply across domains in the tree of life. Hydra can obtain their algal symbionts, from which they derive photosynthetic nutrients, either vertically through the egg, or as the hydra grows to
maturity (Muscatine & Lenhoff, 1963; Muscatine, 1965; Thorton 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 monoclonized 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 before 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, because 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–microorganism mutualisms, particularly those of invertebrates. These models individually provide fascinating insights into symbiotic processes, and together reveal common themes by which animals and microorganisms 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–microorganism 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–microorganism 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–microorganism 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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