REVIEW

Unseen Forces: The Influence of Bacteria on Animal Development

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The diversity of developmental programs present in animal phyla first evolved within the world's oceans, an aquatic environment teeming with an abundance of microbial life. All stages in the life histories of these early animals became adapted to microorganisms bathing their tissues, and countless examples of animal–bacterial associations have arisen as a result. Thus far, it has been difficult for biologists to design ways of determining the extent to which these associations have influenced the biology of animals, including their developmental patterns. The following review focuses on an emerging field, the goal of which is to understand the influence of bacteria on animal developmental programs. This integrative area of research is undergoing a revolution that has resulted from advances in technology and the development of suitable animal–bacterial systems for the study of these complex associations. In this contribution, the current status of the field is reviewed and the emerging research horizons are examined.   © 2002 Elsevier Science

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INTRODUCTION

As a discipline, animal developmental biology has principally focused on the intricate, conserved patterns of communication that occur among cells, transforming them from gametes to fully formed larval or juvenile animals through embryogenesis. Over the past 10 years, in a marriage of developmental and evolutionary biology, a growing interest has emerged in the diversity of animal body plans and the genetic regulation that underlies this diversity (for reviews, see Special Feature articles, April 25, 2000 issue of *Proceedings of the National Academy of Science USA*). Recently, in an elegant review for this journal, Gilbert (2001) highlighted the integration of yet another discipline into developmental biology, the field of ecology. As Gilbert points out, although biologists acknowledge that the environment is the ultimate driving force in the determination of developmental programs and the evolution of diverse body plans, relatively few recent studies have focused on the nature of these environmental influences. As a subset of

these influences, the impact of the constant presence of bacterial cells in an animal's environment has been little studied. Evidence is accumulating that interactions of animals with environmental microbes have resulted in the coordinate evolution of complex symbioses, both benign and pathogenic (McFall-Ngai, 1998; Henderson *et al.*, 1999; Hooper and Gordon, 2001), and that coevolved animal–bacterial partnerships represent a common, fundamental theme in the biology of animals. Thus, environmental bacteria present two types of potential influences on animal developmental programs: (1) the nonspecific influences of bacteria as ubiquitous and critical constituents of the environment, and (2) the specific influences of the bacterial cells that have coevolved with animals in tight associations that are maintained across generations. It is this latter category that is the subject of the following review.

The role of bacteria as coevolved partners in animal development is an issue not only of ecology but also of cell biology. The implicit assumption that has accompanied the study of animal development is that only "self" cells (i.e., those containing the host genome) communicate to induce developmental pathways. This viewpoint is understandable in light of the fact that embryogenesis often occurs in the absence of direct contact with bacteria. But even during

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embryogenesis, the imprint of the influence of bacteria can be seen in the formation of tissues that are destined to interact with coevolved microbial species (Falk *et al.*, 1998; Cebra, 1999; Umesaki and Setoyama, 2000; Visick and McFall-Ngai, 2000; McCracken and Lorenz, 2001). Once these tissues are "prepared" during an animal's embryonic period, essential interactions with its partner bacterial species ensue after hatching or birth. These interactions ensure the formation of healthy stable communities, which are composed of a single dominant eukaryotic cell genome and an array of microbial genomes, both eukaryotic and prokaryotic. In addition, the composition of the specific microbial communities associated with a host may vary in time and space, that is, through ontogeny and in different locations in the host, adding to the complexity of the system (e.g., Smith and Crabb, 1961; Rotimi and Duerden, 1981). For example, in mammals, the best characterized of the animals, 9 of the 10 organ systems (i.e., integumentary, digestive, respiratory, excretory, reproductive, immune, endocrine, circulatory, and nervous) have components that interact directly with the outside environment for their normal function (only the musculoskeletal system is completely internal). With the exception of the nervous system, strong evidence is available for a coevolved bacterial consortium either in direct, persistent association with or sampled by these organ systems (Tannock, 1999).

Until very recently, this overwhelming complexity had dissuaded biologists from integrating these communities into considerations of the various aspects of an animal's biology, including their developmental programs. However, huge strides have been made in certain areas of biotechnology during the last three to five years that are revolutionizing this field of study, opening up frontiers that heretofore were highly unapproachable. Specifically, the advent of high throughput DNA sequencing has provided the tools to characterize not only large animal genomes but also the genomic structure and function of entire microbial communities, including both the culturable and unculturable constituent species (see, e.g., Kroes *et al.*, 1999; Suau *et al.*, 1999; Marsh *et al.*, 2000; Rondon *et al.*, 2000). The availability of these resources permits the first complete characterizations of the species diversity of animal–bacterial communities that coevolve as single complex units (Barbieri *et al.*, 2001; Paster *et al.*, 2001). Recognizing the importance of such analyses, Relman and Falkow (2001) recently proposed the "second human genome project," which would aim to characterize the microbial partners of the human body. Similarly, with the advances occurring in bioinformatics, biologists studying the development of animal–bacterial communities are beginning to apply microarray technology to unravel the complex dialogue between the host and its bacterial partners (Akman and Aksoy, 2001; Hooper *et al.*, 2001). Going hand in hand with these technological advances has been the development of model animal–bacterial associations that are amenable to experimental manipulation (Goebel and Gross, 2001; McFall-Ngai, 2001) (Table 1). Only with the advent of the

appropriate technology and the availability of newly developed models for their application is it now possible to design approaches to determine how these phylogenetically disparate genomes communicate as a dynamic unit in all phases of the development of the community, from the birth of the host to host reproduction.

The understanding of animal–bacterial interactions is a field in its infancy; as such, this review is necessarily largely a horizon analysis of a frontier rather than a retrospective. I present the conceptual landscape and the nature of the questions, as well as a summary of the advances in our understanding that has resulted from the development of new technology and the development of model systems over the past decade. For purposes of this review, I use the classical, more inclusive definition of symbiosis as first suggested by de Bary (1879), that is, two dissimilar organisms living in close association; thus, *symbiosis* is viewed as an umbrella concept that applies to all types of associations of animals with bacteria, independent of the effects of the interaction on the fitness of the partners. The use of this term becomes more attractive all the time, not only because it is difficult to assess the impact of symbiosis on the fitness of the partners but also because it now appears that the spectrum of interactions is more correctly viewed as a continuum from pathogenic to beneficial (Hentschel *et al.*, 2000). The critical issue here is that animal developmental programs occur now, and did so historically, within the context of coevolved associations with microbes, and we are now in a position to address questions such as: what portions of an animal's life cycle are affected by interactions with coevolved bacterial partners? and, how are these interactions integrated into the developmental program of a given host animal species?

MAINTAINING THE COMMUNITY BETWEEN GENERATIONS

In the context of an animal's development, a principal challenge is to elaborate those features that will ensure maintenance of the complex community of the host and its specific bacterial partners with fidelity over the life history of a given animal, between generations of the species, and over evolutionary time. From a research point of view, the ultimate goal is to characterize the mechanisms by which this fidelity is achieved and to define the impact of these associations on the evolution of developmental patterns in animals. A useful and inclusive intellectual framework in which developmental biology might approach the vast and varied array of symbioses is to divide them based on mode of transmission between generations, which is perhaps the most important and defining character of the developmental program of an animal–bacterial community. Although the array of symbioses has been characterized in a variety of ways based on their mode of transmission, the degree to which the symbionts have the opportunity to influence the embryonic period is critical in a consideration of their

TABLE 1

Some Existing and Potential Systems for the Study of Animal–Bacterial Interactions during Host Development

Type of symbiosis	Specific system host–symbiont(s)	Culturability[a] H[c]	Culturability[a] S[d]	Molecular genetics available H	Molecular genetics available S	Genome sequence[b] H	Genome sequence[b] S	Selected references
Consortial								
	Vertebrate alimentary canal microbiota							
	Homo sapiens	(+)	+/−	−	+/−	+	+/−	Hooper and Gordon, 2001; Pasteur *et al.*, 2001
	Mus musculus (mouse)	(+)	+/−	+	+/−	+	+/−	Hooper *et al.*, 2001
	Bos spp. (cow rumen)	(+)	+/−	−	+/−	−	+/−	Russell and Rychlik, 2001
	Brachydanio rerio (zebrafish)	?	[+/−]	+	[+/−]	+	[+/−]	—
	Other fishes	?	+/−	−	[+/−]	−	+/−	Hansen and Olafson, 1999
	Termites–hindgut microbiota	−	+/−	−	−	−	−	Abe *et al.*, 2000
	Squids–accessory nidamental gland microbiota	?	+/−	−	−	−	−	Kaufman *et al.*, 1998; Grigioni *et al.*, 2000; Barbieri *et al.*, 2001
Monospecific								
	Euprymna scolopes (sepiolid squid)–*Vibrio fischeri*	+	+	−	+	−	+	McFall-Ngai, 1999; Claes and Dunlap, 2000
	Hirudo medicinalis (leech)–*Aeromonas veronii*	+	+	−	+	−	−	Graf, 1999, 2000
	Heterorhabditis bacteriophora–*Photorhabdus luminescens*	−	+	−	+	−	+[e]	Forst *et al.*, 1997; ffrench-Constant *et al.*, 2000; Ciche *et al.*, 2001
	Steinernema carpocapsae–*Xenorhabdus nematophilus*	+	+	−	+	−	−	Akhurst, 1983; Forst *et al.*, 1997; Vivas and Goodrich-Blair, 2001
	Caenorhabditis elegans–*Microbacterium nematophilum*	+	+	+	−	+	−	Hodgkin *et al.*, 2000
	Arthropods/nematodes–*Wolbachia* spp.							O'Neill *et al.*, 1997; Zimmer, 2001
	Drosophila melanogaster (fruit fly)	+	−	+	−	+	+	Bourtzis *et al.*, 1996; Hoffmann *et al.*, 1998
	Asobara tabida (parasitic wasp)	+	−	−	−	−	?	Dedeine *et al.*, 2001
	Onchocerca ochengi (filarial nematode)	+	−	−	−	−	?	Taylor and Hoerauf, 1999
	Insect–primary endosymbionts (bacteriome symbioses)							Douglas, 1989; Moran and Baumann, 2000
	Glossina spp. (tsetse fly)–*Wigglesworthia glossinidia*	(+)	−	−	−	−	+	Akman and Aksoy, 2001
	Aphids–*Buchnera aphidicola*	(+)	−	−	−	−	+	Baumann and Moran, 1997
	Sitophilus spp. (weevils)–undescribed enterobacter	(+)	−	−	−	−	−	Nardon and Grenier, 1988; Heddi *et al.*, 1999
	Insect–secondary endosymbionts							
	Glossina spp. (tsetse fly)–*Sodalis glossinidius*	(+)	+	−	+	−	−	Cheng and Aksoy, 1999; Dale *et al.*, 2001

[a] Ability to rear independently of the symbiotic state.

[b] In available databases or known to the author.

[c] Host; (+) = can survive curing, but with compromised health and/or fecundity.

[d] Symbiont(s); +/− = some species culturable/with genetics/with sequenced genome, and some species not; [+/−] = likelihood high.

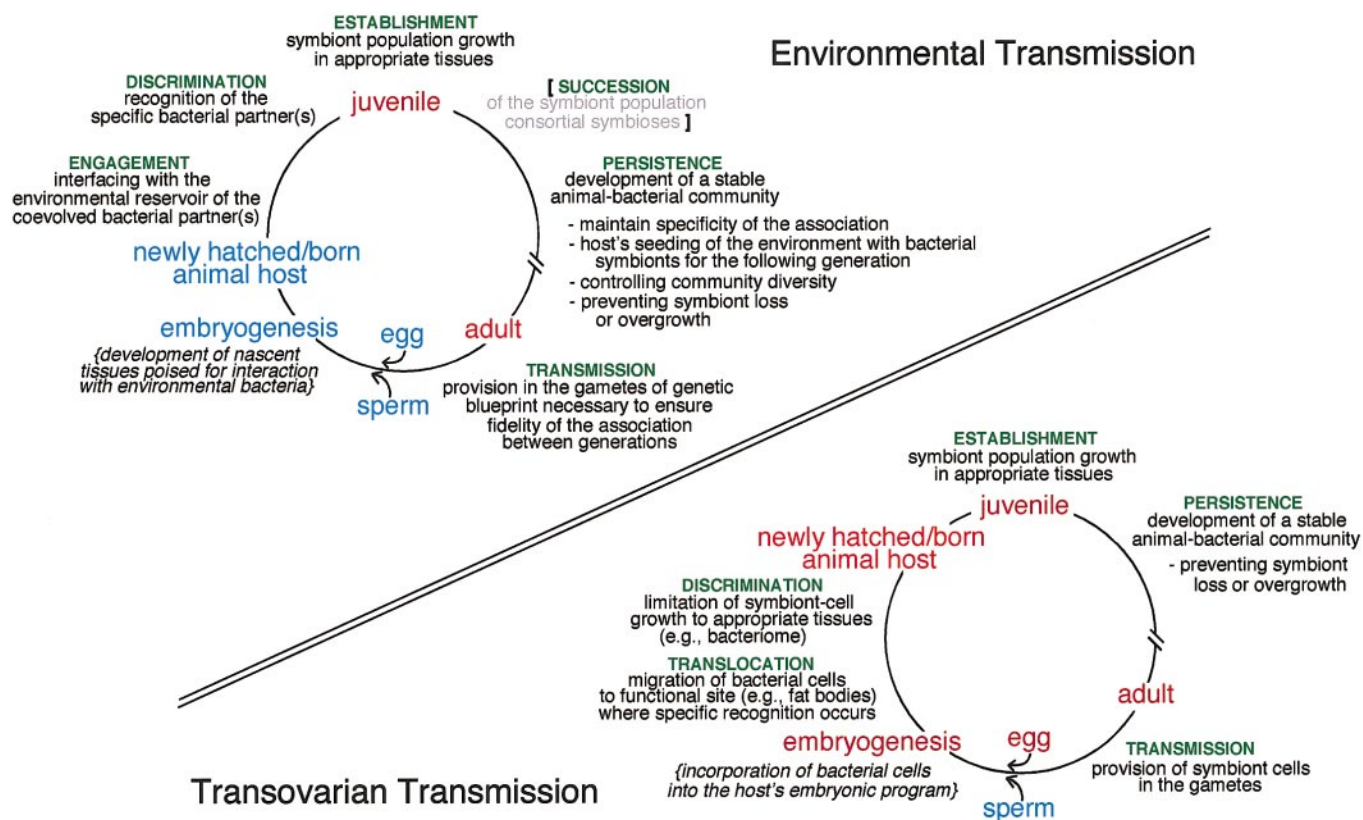[e] Partial.

The 'Life Cycles' of Animal Symbioses

FIG. 1. Environmental and transovarian transmission are the ends of the spectrum by which maintenance of animal–bacterial symbioses occurs between generations. The symbionts are associated with host cells over most of the life history in both types of associations (stages with symbionts in red). Specifically, in animals with transovarian transmission only the sperm cells are devoid of symbionts, whereas in animals with environmental transmission the symbionts are not associated with the germ line or the embryonic period (stages in the absence of symbionts in blue). The processes (green) that ensure development of the association through the life history of the animal are numerous and complex. Although many of the "challenges" are the same between these two modes of transmission, the mechanisms by which persistence of the association are achieved and maintained are markedly more complex in environmentally transmitted associations. In addition, only in some environmentally transmitted, consortial associations does a succession of the microbial community (gray) occur from initiation through the maturation stages of the relationship.

influence on host development. Thus, for the present discussion, I focus on communities that are maintained faithfully between generations by one of two mechanisms, environmental or transovarian transmission. This dichotomy describes most symbiotic associations and is one that serves to emphasize the effects of symbiosis on the patterns of development (Fig. 1).

In an environmentally transmitted association, the larval or juvenile host acquires its specific symbionts from the surrounding habitat with each generation and, thus, the microbial partners are not present to interact directly with host cells during embryogenesis. In these associations, the bacterial partners occur most often as extracellular consortia colonizing polarized epithelia, such as in the mamma-

lian alimentary canal (Falk *et al.,* 1998; Tannock, 1999; Kolenbrander, 2000) and termite hindgut (Abe *et al.,* 2000). However, some monospecific and/or intracellular alliances that exhibit environmental transmission do occur [e.g., the squid–vibrio relationships (McFall-Ngai and Ruby, 1991) and the associations between vent tube worms and their sulfur-oxidizing bacterial symbionts (Cary *et al.,* 1994)]. Fortunately, some of the bacterial partners in these associations can be cultured in the laboratory. In addition molecular genetics has been developed in many of these bacterial symbiont species and the genomes of a growing number of them have been sequenced (Table 1). In addition, some of the host animals have been raised independently of the symbiosis, which has allowed the experimental manipula-

tion of the associations during their development (see, e.g., Bry *et al.,* 1996; Graf, 1999, 2000; Ruby, 1999; Vivas and Goodrich-Blair, 2001).

In an association with transovarian transmission, the bacterial partners are provided in or on the gametes by the female parent (Douglas, 1994). Examples of well-characterized symbioses transmitted in this manner are the arthropod–bacteriome systems (Douglas, 1989; Moran and Baumann, 2000), the invertebrate–*Wolbachia* symbioses (O'Neill *et al.,* 1997), and the relationships between solemyid clams and their gill-associated sulfur-oxidizing bacterial symbionts (Krueger *et al.,* 1996). Interestingly, these types of associations are prevalent in, yet largely restricted to, the invertebrates, and the bacterial partners in such symbioses are most often intracellular constituents of organs located deep in the body cavity. In many of these symbioses, the bacterial partner can be isolated from the host and subjected to immediate analysis, although a bacterial symbiont from only one association with transovarian transmission has been successfully brought into culture (Cheng and Aksoy, 1999; Dale *et al.,* 2001). Only in a few cases has the host been cultured in the absence of its symbionts without severely affecting the health and fitness of the host (e.g., Brooks and Richards, 1955; Nardon, 1973; Douglas, 1989). Such technical problems have rendered the study of the development of symbioses with transovarian transmission difficult. However, these kinds of alliances are not only very common but they are also often ecologically and economically very important associations and have become the subjects of a rich literature (for review, see Douglas, 1989; O'Neill *et al.,* 1997). Thus, an understanding of these types of symbiosis is critical for a comprehensive picture of the influence of bacteria on animal development, particularly in invertebrates.

As with most categorizations of biological phenomena, the dichotomy of environmental or transovarian transmission is artificially discrete and actually describes the extremes of a spectrum. Some associations employ both strategies, such as in systems with intermediate insect vectors and terminal mammalian hosts (O'Neill *et al.,* 1997). Also, during the evolution of systems that have transovarian transmission, the bacterial partner must be presumed to have been acquired from the environment at some point, and only later did it become a permanent component of the host's germ line. It is important to note that, although such an evolutionary progression may take place within a given species, associations transmitted through the germ line are not necessarily more ancient or more specific than relationships that are environmentally transmitted. For example, *Wolbachia,* a common bacterial symbiont that is most noted for its role in sex-ratio distortion in arthropods, is generally passed through the germ line (O'Neill *et al.,* 1997). However, its widespread distribution and its pattern of phylogenetic occurrence among diverse invertebrate species suggest that transmission of this bacterium has occurred between taxa, and that some of these associations may have evolved relatively recently (Werren *et al.,* 1995). In contrast, experimental studies of symbiont recognition in sepiolid squids have demonstrated that these

environmentally transmitted associations are highly specific (Nishiguchi *et al.,* 1998) and that such symbioses often occur as shared, derived characters of ancient clades, suggesting they have been present throughout the evolution of that clade (McFall-Ngai and Toller, 1991).

Whether an association is transmitted by the environment, by transovarian transmission, or by an intermediate mode, the challenges for the development of the community of animal and microbial cells are numerous and diverse, both within the ontogeny of the individual host animal, between generations of a given host, and among the array of host species. Because this field is in its infancy, no complete picture is available for the entire ontogeny of any animal–bacterial association. The pieces of evidence for bacterial involvement in developmental processes are currently somewhat disparate. However, very recent studies of specific associations (e.g., the insect–bacterial, the mouse–intestinal consortial, and the squid–vibrio systems), some of which I describe below, are beginning to bring the scope of this field into focus. In particular, they are helping to clarify the critical questions that should be addressed to define the mechanisms by which host animals and their bacterial partners meet some of their developmental challenges.

ASSOCIATIONS OF COEVOLVED BACTERIA WITH EGGS AND EMBYROS

Bacteria are incorporated into the events of gametogenesis and embryogenesis of an animal host in symbioses with transovarian transmission, by definition (Douglas, 1989; Krueger *et al.,* 1996). In addition, bacteria are included as components of the extraembryonic layers of eggs of some species, where they can serve a protective function against potential pathogens (Gil-Turnes *et al.,* 1992; Hansen and Olafson, 1999; Barbieri, 2001). In many examples of transovarian transmission, the bacterial symbionts appear to have little effect on the activity of the egg as a gametic cell. Rather, they behave as passive passengers, either adhering to the surface of the egg or residing in its cytoplasm. However, in some cases, the symbionts can have profound effects on the cell biology of the eggs and can become incorporated into the normal reproductive biology of certain animal species.

Perhaps the best studied of such influences occurs in the association between arthropod hosts and their endosymbiont *Wolbachia.* The prevalence of *Wolbachia* is still controversial, although recent surveys of insect populations in some areas reported that over 70% of the resident species were carrying *Wolbachia* (Jiggins *et al.,* 2001; Zimmer, 2001). Depending on the host species, *Wolbachia* can exert a variety of effects with developmental consequences (O'Neill *et al.,* 1997). In a recent study of the parasitic wasp *Asobara tabida,* the presence of *Wolbachia* was shown to be essential for oogenesis, the first report of a symbiosis being critical for this process (Dedeine *et al.,* 2001). Often in

Wolbachia symbioses, reproduction favors the retention and spread of the association in host populations through a phenomenon called *cytoplasmic incompatibility* (CI) (Hoffmann and Turelli, 1997). In CI, the mating of a *Wolbachia*-infected male with an uninfected female yields no offspring, whereas the mating of an infected female with either an infected or uninfected male will produce progeny. Through this mechanism, infected females have a reproductive edge over those not harboring *Wolbachia* and, under certain conditions, *Wolbachia* can sweep through the species populations in which it has been introduced. For example, biologists studying the progression of this microbe in fruit fly populations in California have documented the spread of *Wolbachia* at a rate of 100 km/year (Turelli and Hoffmann, 1991). *Wolbachia* is also known to have an influence on other aspects of the reproductive system in invertebrate hosts, including sex determination, sex ratios, and gamete viability (O'Neill *et al.*, 1997).

The study of animal–*Wolbachia* associations has a promising future. Although *Wolbachia* symbionts have not been brought successfully into laboratory culture, the host can be cured of its symbionts under laboratory conditions. Thus, it has been, and continues to be, an elegant system for genetic studies of the host. In addition, while best studied in arthropods, *Wolbachia* also occurs in filarial nematodes (Taylor and Hoerauf, 1999), and perhaps other ecdysozoan phyla, or even other more distantly related invertebrate groups. The genomes of several *Wolbachia* species are currently the subject of sequencing projects (Zimmer, 2001), the data from which will provide great insight into the role of *Wolbachia* in host life cycles. These advances should also be of significant heuristic value to the development of strategies for the future studies of these associations.

The eggs of aquatic hosts are susceptible to overgrowth by environmental bacteria and fungi. Although they can be grown axenically under laboratory conditions without much effect on embryogenesis, they are unlikely to develop under natural conditions in the absence of protective mechanisms. In some cases, the adherent microbiota on eggs has been implicated in providing the first line of defense against pathogens. For example, a correlation has been made between species-specific differences of surface adhesion characteristics of fish eggs and a species-specific microbiota associated with the eggs (for review, see Hansen and Olafsen, 1999). In some aquatic invertebrates, strong experimental evidence indicates an essential role for specific bacteria in host egg protection. The eggs of the American lobster *Homarus americanus* and the caridean shrimp *Palaemon macrodactylus* require a monospecific association with a bacterium that produces an antifungal compound (Gil-Turnes *et al.*, 1989; Gil-Turnes and Fenical, 1992). Experimental manipulation of these associations has shown that when these eggs are devoid of their bacterial partner, they are quickly overgrown by fungal species resident in the ambient environment. Several squid and cuttlefish species have consortial symbioses in an organ

called the accessory nidamental gland (ANG), which occurs only in the females of the species and appears to be involved in elaboration of the egg capsule (Kaufman *et al.*, 1998; Grigioni *et al.*, 2000; Barbieri *et al.*, 2001). Studies have shown that the cephalopod species with ANG symbioses lay eggs that harbor bacteria within layers of the capsule. Characterization of the symbiont communities of the ANG and eggs has shown many of the bacterial phylotypes are shared between the two tissues, and biologists studying these associations have hypothesized that the bacteria provide an essential protective function to the eggs (Grigioni *et al.*, 2000; Barbieri *et al.*, 2001). How widespread such a protective function is among aquatic animals and whether such associations represent coevolved communities have been difficult questions to address, because many of these bacteria are unculturable and the communities adhering to eggs may be quite diverse. However, the application of recent technical advances in the study of the structure and function of bacterial populations promises to provide answers to such questions.

Although the eggs of hosts with environmentally transmitted associations may be protected by bacteria, the associations with their lifelong symbiotic partners are initiated immediately upon hatching or birth of the host, in contrast to hosts with transovarian transmission. Thus, it is the function of the embryonic program to include the elaboration of morphological, cellular, and molecular determinants that will promote establishment of the association with the appropriate environmental microbes, to the exclusion of nonspecific bacteria or potential pathogens. Such "preparation" of tissues for eventual postembryonic interactions has been studied very little; the major emphasis has instead focused on bacterial maturation of tissues postbirth or hatching, most notably in comparisons between germ-free and conventionally raised animals (see under "Postembryonic Development of the Association"). One reason for this scarcity of information is that, in many systems, it is difficult to decipher which components of the incipient colonized tissue are selected specifically for interaction with microbes. For example, the tissues of the alimentary canal are adapted for functions such as nutrient uptake in addition to maintenance of the bacterial consortium. However, in the association between the sepiolid squid *Euprymna scolopes* and its marine luminous bacterial partner *Vibrio fischeri*, the host has an embryonically elaborated set of tissues, whose sole function is to ensure colonization of the host with its symbiont (McFall-Ngai and Ruby, 1991; Montgomery and McFall-Ngai, 1993; see under "Postembryonic Development of the Association"). Because the only benefit to the host is the bacterial production of light, the cellular, biochemical, and molecular characters of these tissues of the hatchling squid can be presumed to have developed during embryogenesis solely to promote host–symbiont interaction. This relatively simple functional background should allow relatively accurate characterizations of the reciprocal interactions between the host and symbiont through biochemical and molecular

comparisons of symbiotic and aposymbiotic (not colonized by the symbiont) animals, as well as through the genetic manipulation of the bacteria.

FINDING RARE SYMBIONTS IN THE ENVIRONMENTAL "HAYSTACK"

Environmental transmission of symbionts requires a set of processes not essential in transovarian transmission, that is, to initiate the association, the bacterial partners must be brought into physical contact with the host, while nonsymbiotic species must be excluded. In terrestrial animals, the mechanism is usually relatively direct and several examples have been well characterized. In most such cases, either the parents or other members of the adult host population participate in transmission. For example, the microbe-containing feces of adult termites are fed to the newly hatched juveniles by workers in the colony (Abe *et al.,* 2000). In contrast, the mechanisms by which aquatic hosts with environmental transmission acquire their symbionts have remained largely a mystery. For example, even where hosts are highly abundant, such as in coral reefs or hydrothermal vents, highly sensitive molecular methods have failed to detect the free-living stage of the symbiont in samples obtained from the adult host habitat. The absence of evidence notwithstanding, the assumption has remained that the symbionts are shed by the adult host populations into the surrounding water, and that the juveniles somehow harvest these dispersed cells.

Recent studies with the squid–vibrio system have uncovered a mechanism for symbiont harvesting in one symbiosis, which may be an example of a more generally occurring phenomenon. Hatching of the juveniles into their microbe-rich seawater environment induces the incipient host to secrete mucus near the sites where colonization will take place (Nyholm *et al.,* 2000). The activity of an embryonically developed, ciliated epithelium maintains the mucus mass in a position above the sites of colonization and the symbionts, which represent less than 0.1% of the total bacterial population present in the ambient seawater, are gathered into aggregates. These aggregates eventually migrate to and colonize the epithelia-lined crypts that have also formed during embryogenesis. While the precise mechanisms by which other aquatic animals with environmentally transmitted symbioses acquire their symbionts remain largely undescribed, it is likely that the mode of symbiont harvesting that occurs in the squid–vibrio system is not unique; that is, ciliary-mucus currents are common mechanisms by which aquatic animals bring bacteria-rich environmental water across their surfaces. In some systems, such as in the vent tube worms, patches of cilia that are lost subsequent to inoculation with the symbiont have been noted near the sites of symbiont colonization (Southward, 1988; Jones and Gardiner, 1988).

POSTEMBRYONIC DEVELOPMENT OF THE ASSOCIATION

Over the past several decades, plant biologists studying the legume/nitrogen-fixing rhizobial symbioses have provided the only detailed insight into eukaryotic–prokaryotic interactions during development (for review, see Stougaerd, 2000). These studies have revealed that dozens of genes of both the host and symbiont orchestrate a complex developmental program, which results in the formation of a functional root–nodule symbiosis. In contrast, because of past technical difficulties, only two experimental animal systems, the germ-free mouse (Falk *et al.,* 1998) and the squid–vibrio association (McFall-Ngai, 1999), have provided significant progress in the characterization of the host–symbiont dialogue during the successful establishment of stable associations during postembryonic development. Early studies of germ-free (axenic) and gnotobiotic (with known colonizing microbes) mammals demonstrated that microbial colonization of tissues results in profound changes in their form and function (for review, see Hooper *et al.,* 1998; McFall-Ngai, 1998). In the mid-1990s, researchers began to examine the molecular basis for these normal changes using the genetically defined mouse model and a genetically engineered bacterial species *Bacteroides thetaiotaomicron* (Bry *et al.,* 1996), which is a member of the intestinal microbial community. These studies focused on the influence of the bacteria on the nature of the surface molecules on the intestinal epithelial cells. In the days before exposure to the symbionts (i.e., during the end of the weaning period), the host is preprogrammed to begin decorating the epithelium with fucosyl residues. Without bacterial interaction within a certain time frame, the fucosyl residues are lost from these surfaces. However, upon exposure to *B. thetaiotaomicron,* which uses fucose as a nutrient source, the host upregulates the production of fucosyl transferases. This activity leads to an increase in the production of fucosyl residues on the apical cell surfaces, which promotes the colonization of this bacterial species. Bacterial mutants that are defective in fucose utilization are incapable of inducing host cell fucosylation. The data demonstrated that the host tissues are poised for interaction with the symbiont, and interaction with the symbiont is essential for further normal development to ensue.

These integrative studies with the germ-free mouse model, which required the cooperation of bacteriologists and developmental biologists, were the beginnings of a revolution in the study of such complex animal–bacterial associations. The concomitant development of technology over the past five years has been timely in the progress of this revolution. Specifically, the combination of the availability of sequenced mammalian genomes, the ability to identify and characterize the several hundred to thousands of coevolved bacterial species in the alimentary canal, and the opportunity to study host and symbiont gene expression have paved the way for rigorous, detailed studies of consor-

tial interactions. Consequently, the community of biologists can look forward to a rapid proliferation in our knowledge of these areas.

The application of such technology formed the basis of a recent benchmark study using the germ-free mouse model, in which Hooper *et al.* (2001) performed the first in depth characterizations of the changes in gene expression in the intestinal epithelia that result from the interaction with indigenous bacteria. Using DNA microarrays and laser-capture microdissection, coupled with quantitative, real-time RT-PCR, they found that the coevolved microbiota induce a wide variety of genes in these intestinal epithelia cells, including genes associated with maturation of the intestine, nutrient processing, and mucosal immunity. With this system, they were also able to compare the effects of introducing single species with exposure to the entire community. Their data show different patterns of host gene expression when the host is exposed to one or a few bacteria than when it is exposed to a complex mixture of species from the native community. These experiments provided evidence that, in normal development of the host–symbiont community, the host is not engaging in a general (i.e., nonspecific) response to the presence of bacteria but, rather, is responding to overtures made by the specific coevolved community.

Because the complexity of consortial interactions presents specific difficulties with experimental manipulation and interpretation, as is a common practice in developmental biology, the study of a simpler model would be useful. The squid–vibrio model offers such a complementary system. This symbiosis represents the most common type of animal–bacterial association, that is, the interaction of extracellular Gram-negative bacteria with polarized host epithelia. The model has several experimental advantages for the study of the mechanisms by which such relationships develop. The host and symbiont can be raised independently of the association (McFall-Ngai and Ruby, 1991) and the bacterial symbionts can be genetically manipulated (Ruby, 1999; Stabb *et al.*, 2001). In addition, the time course of the dramatic bacteria-induced developmental changes is relatively short, that is, 0.5–48 h following first exposure to symbionts (Montgomery and McFall-Ngai, 1994; Lamarcq and McFall-Ngai, 1998; Visick *et al.*, 2000). Tens of thousands of host juveniles can be produced each year with a breeding colony of a dozen females and relevant host tissues are abundant enough so that stage-specific cDNA libraries are being created in symbiotic and aposymbiotic animals. The availability of abundant, quickly developing hosts has allowed for the screening of thousands of bacterial mutants (Visick and Skoufos, 2001). In addition, the *V. fischeri* genome is currently being sequenced, and a representation of its genes will be ordered on a microarray chip (E. Ruby, personal communication). Such an array can be used to determine the pattern and identity of bacterial responses to the initiation of a developmental dialogue with the host (Khodursky *et al.*, 2000; Akman and Aksoy, 2001). Taken together, the combination of all these features renders the squid–vibrio symbiosis a powerful model for the study of the interactions of animal tissue with extracellular, Gram-negative bacteria.

In the squid–vibrio association, both the host and symbiont undergo marked developmental changes in response to symbiosis (Visick and McFall-Ngai, 2000). The bacteria induce morphogenesis in the host light organ that transform it from a morphology that promotes inoculation of the tissues at hatching to one that promotes maintenance of the association throughout the life history of the host. The morphology of the light organ developed during embryogenesis that awaits the colonization process consists of: (1) a complex superficial field of ciliated cells involved in promoting the harvesting of symbionts; and (2) a series of deeply invaginated crypts lined by polarized columnar epithelia, the site of eventual symbiont colonization (Montgomery and McFall-Ngai, 1993). Once the bacteria are harvested into aggregates (see under "Finding Rare Symbionts in the Environmental 'Haystack'"), the aggregates move through long ducts into the crypt spaces. Within 12 h, the bacteria induce several changes in host light organ morphology, both in cells directly associated with the symbionts and in cells remote from the growing bacterial population. The most dramatic of these changes is the bacteria-induced irreversible morphogenesis of the remote, superficial field of ciliated epithelia. Around 12 h following first exposure to the symbionts, the bacteria signal the loss of this field, which requires a 4-day program (Montgomery and McFall-Ngai, 1994; Doino and McFall-Ngai, 1995). When the tissues are antibiotically cured of the symbionts after 12 h, but not before, the 4-day program continues unabated (Doino and McFall-Ngai, 1995).

Some of this morphogenetic program involves the triggering of cell death by the symbiont lipopolysaccharide, an abundant cell surface molecule (Foster and McFall-Ngai, 1998; Foster *et al.*, 2000). The bacteria also induce changes in the epithelia with which they interact. Specifically, these cells swell (Montgomery and McFall-Ngai, 1994; Visick *et al.*, 2000) and the density of the microvilli along their apical surfaces increases dramatically (Lamarcq and McFall-Ngai, 1998), but these changes are reversible with antibiotic curing of the tissues. Interestingly, strains of *V. fischeri* defective in light production do not induce cell swelling and are defective for persistence in the organ (Visick *et al.*, 2000). These findings suggest a mechanism, which is built into the host's developmental program, whereby the principal function of the symbiosis (i.e., light production) is maintained. Studies of the proteome of the light organ in symbiotic and aposymbiotic animals over the first four days of symbiosis have shown that these morphological changes correlate with significant changes in host protein production (Doino and McFall-Ngai, 2000). The further characterizations of the molecular basis to the host–symbiont dialogue are under way. The data derived from these studies, and their comparison to results from other research efforts, such as those in the germ-free mouse system, will make possible the determination of what components of the

squid–vibrio interactions are general to the reciprocal conversation between animal epithelial cells and their bacterial symbionts, and what components are unique to this system. In addition, it should provide insight into the similarities and differences in host response to bacterial interactions in vertebrates and invertebrates.

Studies of this bacterium have shown that it must also undergo morphological differentiation and changes in gene expression during initiation of the association (Ruby and Asato, 1993; Visick and Ruby, 1998). Although the range of cellular morphology is relatively limited in bacteria, recent advances in both genomics and proteomics are revealing the extent to which global activation and repression of bacterial genetic responses can influence bacteria–host communication through changes in surface components and exported signal molecules. For instance, recent studies have shown that pathogenic bacteria have the ability to manipulate the normal developmental program of a host cell by subverting the signal-transduction pathways controlling its cellular determination and differentiation (Finlay and Cossart, 1997; Ireton and Cossart, 1998). Recognition and elucidation of these phenomena have led to the founding of a new interdisciplinary field designated *cellular microbiology* (Henderson *et al.*, 1999; Cossart *et al.*, 2000). Continued examination of the squid–vibrio and other similar associations should provide insight into how Gram-negative bacteria like *V. fischeri* form cooperative alliances in some hosts or tissues, while initiating pathogenic ones in others (Small and McFall-Ngai, 1999; Mahajan-Miklos *et al.*, 2000; Vivas and Goodrich-Blair, 2001).

Although the germ-free mouse model and the squid–vibrio association have been, to date, the most exploited experimental models of animal–bacterial interaction during development of the association, the newly available technologies that can be applied to the study of such interactions are resulting in the development of new models and a renaissance of the study of other associations (Table 1).

KEEPING THE PEACE: PROMOTING PERSISTENCE AND EDUCATING THE DEVELOPING IMMUNE SYSTEM

Postembryonically, in all symbioses, the host and symbiont must initiate the development of features that will promote maintenance of a stable association, that is, one in which the host neither eliminates the bacterial partners nor allows them to overgrow the tissues. In the intracellular symbioses of invertebrates, the morphological and molecular characteristics of the host and symbiont cells suggest that the host severely limits symbiont proliferation (Buchner, 1965; Nardon, 1988; Stouthamer *et al.*, 1999), eliminates supernumerary cells (Milburn, 1966; Nardon and Grenier, 1988), and restricts the bacteria to specific tissues. For example, in the maturation of the trophosome (the symbiont-specific organ) of the hydrothermal vent tube worm *Riftia pachyptila*, a gradient of host and symbiont cell morphologies occurs. The bacteria-containing cells, or bacteriocytes, and their associated symbionts are created, mature, and senesce along a morphological cline from the center of the trophosome to the periphery (Bosch and Grasse, 1984).

The mechanisms by which bacteria are restricted to a given cell type or tissue in intracellular symbioses are not well understood. It has been hypothesized that the environment of the symbiotic cell protects the symbiont from host defenses; any escaping symbiont would be recognized and eliminated (Hinde, 1971; Brooks, 1975). While this hypothesis may predict the fate of a symbiont cell escaping from the symbiotic tissue, it does not account for the restriction of symbionts to a given cell type. Interestingly, recent biochemical studies of insect tissues have shown that some of the highest levels of antimicrobial peptides are in the very tissues (e.g., the fat bodies) that are symbiotic in some species (Hoffmann *et al.*, 1996). The molecular characteristics of the coevolved symbiont cell also reflect growth restriction in host tissues (Ochman and Moran, 2001). A large number of the intracellular symbionts have undergone extensive gene loss, reflecting their obligate alliance with host cells (Maniloff, 1996; Shigenobu *et al.*, 2000). While achieving an understanding of maturation of symbiotic tissues in animals with intracellular associations will continue to be technically challenging, new approaches are rapidly developing (e.g., Akman and Aksoy, 2001; Dale *et al.*, 2001) that promise to provide great insight into these widespread symbioses.

Most environmentally transmitted symbioses in animals, such as the alimentary canal and squid–vibrio associations, are extracellular and often remain open to the environment throughout the life history of the host. Thus, the host/symbiont community must not only develop mechanisms by which to achieve a balanced, functioning population ratio but it must also ensure specificity of the interaction from the inception of the relationship throughout its persistence. Available evidence suggests that such controls are mediated by: (1) the direct interaction of the bacterial cells with the host cells that are colonized; and (2) the immune system, both innate as well as adaptive (when present), which samples the population and keeps the host informed of the state of the interaction. Bacteria-induced changes in host cells in the systems that have been studied (Bry *et al.*, 1996; Lamarcq and McFall-Ngai, 1998; Visick *et al.*, 2000) suggest that some component of persistence results from signaling between the surfaces of the partners' cells. These processes include components that enhance persistence, such as the induction of symbiont nutrient provision by the host (Bry *et al.*, 1996), and components that limit the location of the growing symbiont population. For example, interactions with the gut microbiota induce the mammalian intestinal mucosa to produce mucins and alpha-defensins, which inhibit the symbionts from invading host tissues (Hooper *et al.*, 2001).

While an understanding of developmental changes in

colonized tissues is as yet poorly grasped, a significant body of data is available on the role of the coevolved microbiota in the maturation of the adaptive immune system of mammals. The immune system not only performs surveillance for the detection of dangerous antigens and pathogens but also "educates" the body about the state of the indigenous microbiota. The ability of the immune system to perform these complex functions results from a maturation process that is dependent on colonization of the mucosal surfaces with the proper symbionts (Umesaki *et al.,* 1995; Cebra, 1999; Lanning *et al.,* 2000; Umesaki and Setoyama, 2000; Boman, 2000). The organization and cellular composition of the gut-associated lymphoid tissue (GALT) are altered when the appropriate microbiota are not provided (Gordon *et al.,* 1997). Evidence also exists that in the absence of appropriate exposure to microorganisms, the balance of immune responses associated with T-helper 1 and 2 cells does not commence properly (Rook and Stanford, 1998). Further, in some cases, the microbiota have been shown to be responsible for induction of the production of MHC class II molecules (Cebra, 1999; Umesaki and Setoyama, 2000) and the diversification of the antibody repertoire (Lanning *et al.,* 2000). Evidence is growing that medical problems, such as allergies, asthma, and inflammatory bowel disease, may in some cases result from the lack of proper development of the interactions between the indigenous microbiota and host tissues (Rook and Stanford, 1998).

While the mechanisms underlying this maturation process are presently not well understood, evidence is accumulating that one critical facet of the process is dependent on the ability of the immune system to differentiate between the native resident microbiota and the "tourist" bacteria passing through. There is increasing evidence to suggest that one effect of colonization by normal microbiota is a "turn down" in the inflammatory response in the mucosa (Shroff *et al.,* 1995; Neish *et al.,* 2000). In a recent study, Neish and coworkers (2000) compared the response of human gut epithelial cells to interactions with virulent and avirulent *Salmonella* strains. Exposure of host cells to avirulent strains results in a decrease in the synthesis of inflammatory cytokines through inhibition of the NFκB pathway. Specifically, the activity of these strains blocks the ubiquination of IκB. Thus, NFκB remains bound to IκB and is prevented from entering the nucleus to carry out its function of upregulating the transcription of genes associated with the inflammatory response, such as those that encode cytokines and antimicrobial peptides. In addition, recent evidence suggests that IgA, the immunoglobulin that protects the mucosa from environmental microorganisms, responds differently to foreign bacteria than to the indigenous microbiota. Specifically, the induction of IgA secretion in response to the native symbionts occurs through a pathway that is independent of cooperative interactions with T cells and follicular lymphoid tissue, whereas responses to foreign microbes requires these interactions (Macpherson *et al.,* 2000).

Clues to the mechanisms by which the immune system distinguish cooperative bacteria from pathogens are not available for animals other than mammals. However, the immune system is likely to be a principal player in all animal–prokaryotic interactions. In invertebrates, such controls would be mediated by the innate immune system, through the activity of hemocytes and specific biochemical pathways in these cells and other host cells that interact directly with microbes. It is already known that the Toll-receptor/NFκB pathway, which is a critical developmental pathway, is pivotal to how both vertebrates and invertebrates respond to microbial cells. In the squid–vibrio system, for example, in addition to interacting with the epithelial cells lining the crypt spaces, the bacterial symbionts interact with a population of host hemocytes that sample the crypt spaces (Nyholm and McFall-Ngai, 1998). Whether the Toll-receptor/NFκB pathway is involved in host squid responses is under investigation.

FUTURE DIRECTIONS

A survey of our current state of knowledge reveals an array of fundamental questions that are relevant to understanding the developmental biology of animals. The questions include:

1. What is the nature of the conversation that occurs between host tissues and their symbiotic bacteria during development, and what types of molecular interactions underlie the patterns of development in tissues that are influenced by their associated microbiota?

A. To what extent have the bacterial partners been involved in the evolution of the tissues with which they associate? For example, were bacteria critical for the diversification of ruminates, or did bacteria participate in the selection pressure that formed early events in animal evolution, such as the advent of gastrulation?

B. How does the presence of microbes influence the development of the immune system in a given animal? How have interactions with microbes influenced the evolution of developmental patterns of the innate and adaptive immune systems?

C. What is the significance of the coincident signaling pathways, such as the Toll-receptor pathway, that are shared by developmental processes and the immune systems of both animals and plants? Did such pathways first arise as a language by which eukaryotic cells could interact with both benign and pathogenic bacteria, and which subsequently were incorporated into the developmental programs of animals?

2. How widespread are cooperative associations between animals and bacteria, and under what conditions does their presence influence animal developmental patterns?

A. What are the culturable and unculturable components of the complex, coevolved communities of microbes that associate with animal species, and what are their functions as members of these communities?

B. Is the presence of a set of coevolved consortia along the alimentary canal a shared character among all animals, or does this feature occur only in certain groups? If this character is general, how have these consortia impacted the developmental patterns of the surrounding tissues, both through the ontogeny of an animal and through its evolution?

3. What is the basis for developmental diversity in animal–bacterial associations?

A. What aspects of the development of animal–bacterial interactions do all animals share, and what features underlie the diversity of such associations?

B. Is transovarian transmission of cooperative animal–bacteria symbioses restricted to invertebrate groups and, if so, why? Is the germ line of invertebrates more susceptible than that of vertebrates to the incorporation of bacterial cells and, if so, what renders their germ line permissive? What aspects of the biology of vertebrates apparently preclude such incorporation? What renders some animal species susceptible to the promiscuous *Wolbachia*?

C. Why are cooperative monospecific symbioses rare in vertebrates and common in invertebrates? Similarly, why are intracellular cooperative associations rare in vertebrates and common in invertebrates?

The magnitude of the unknown in this field is daunting, but the technical means are now available to address many of these questions (for review, see McFall-Ngai, 2001) and the development of suitable model systems (Table 1) is occurring at a rapid pace. No single model will, by itself, provide a complete picture of these processes, but there are enough systems currently available to serve as nucleation sites for future exponential growth in this field.

An increasing awareness of how bacteria influence animal development has given rise to a new and rapidly growing discipline. However, the successful development and maturation of this field will require nothing less than a cultural change among biologists. Individuals in the fields of microbiology and developmental biology, disciplines that in the past have rarely shared either a common language or scientific goals, must bring their expertise to bear on the critical issues in this area. Without such an integration these two fields will run the risk of missing important clues to both the evolution of animal–bacterial interactions and the mechanisms underlying animal developmental processes. Several decades ago, ecologists realized that bacteria have in the geological past, as well as in the present day, dominated material and energy flow in the world's ecosystems. Subsequently, with the advent of molecular phylogenetic analyses, evolutionary biologists have come to recognize that bacteria are the most diverse of all organisms. In recent years an integration of bacterial genetics and physiology into host cell biology has produced a remarkable insight into the biochemical mechanisms that sustain the eukaryotic cell. This review argues that now is the time for the fields of microbiology and developmental biology to embrace a similar integration of thinking.

REFERENCES

Abe, T., Bignell, D. E., and Higashi, M., Eds. (2000). "Termites: Evolution, Sociality, Symbiosis, Ecology." Kluwer Academic, Dordrecht/Norwell, MA.

Akhurst, R. J. (1983). *Neoaplectana* species: Specificity of the association with bacteria of the genus *Xenorhabdus*. *Exp. Parasitol.* 55, 258–263.

Akman, L., and Aksoy, S. (2001). A novel application of gene arrays: *Escherichia coli* array provides insight into the biology of the obligate endosymbiont of tsetse flies. *Proc. Natl. Acad. Sci. USA* 98, 7546–7551.

Barbieri, E., Pasteur, B. J., Hughes, D., Zurek, L., Moser, D. P., Teske, A., and Sogin, M. L. (2001). Phylogenetic characterization of epibiotic bacteria in the accessory nidamental gland and egg capsules of the squid *Loligo pealei* (Cephalopoda:Loliginidae). *Environ. Microbiol.* 3, 151–167.

Baumann, P., and Moran, N. A. (1997). Non-cultivable microorganisms from symbiotic associations of insects and other hosts. *Antonie Van Leeuwenhoek* 72, 39–48.

Boman, H. G. (2000). Innate immunity and the normal microflora. *Immunol. Rev.* 173, 5–16.

Bosch, C., and Grasse, P. (1984). Cycle partiel des bacteries chimio-autotrophes symbiotiques et leurs rapports avec les bacteriocytes ches *Riftia pachyptila* Jones (Pogonophore Vestimentifere). I. Le trophosome et les bacteriocytes. *C. R. Acad. Sci. Paris* 299, 371–376.

Brooks, M. A. (1975). Symbiosis and attenuation. *Ann. N. Y. Acad. Sci.* 266, 166–172.

Brooks, M. A., and Richards, A. G. (1955). Intracellular symbiosis in cockroaches. I. Production of aposymbiotic cockroaches. *Biol. Bull.* 109, 22–39.

Bry, L., Falk, P. G., Midtvedt, T., and Gordon, J. I. (1996). A model of host–microbial interactions in an open mammalian ecosystem. *Science* 273, 1380–1383.

Buchner, P. (1965). "Endosymbiosis of Animals with Plant Microorganisms." Interscience, New York.

Cary, S. E., Warren, W., Anderson, E., and Giovannoni, S. J. (1994). Identification and localization of bacterial endosymbionts in hydrothermal vent taxa with symbiont specific polymerase chain reaction amplification and in situ hybridization techniques. *Mol. Mar. Biol. Biotechnol.* 2, 51–62.

Cebra, J. J. (1999). Influences of microbiota on intestinal immune system development. *Am. J. Clin. Nutr.* 69, 1046S–1051S.

Cheng, Q., and Aksoy, S. (1999). Tissue tropism, transmission and expression of foreign genes in vivo in midgut symbionts of tsetse flies. *Insect Mol. Biol.* 8, 125–132.

Claes, M., and Dunlap, P. V. (2000). Aposymbiotic culture of the sepiolid squid *Euprymna scolopes*: Role of the symbiotic bacterium Vibrio fischeri in host animal growth, development, and light organ morphogenesis. *J. Exp. Zool.* 286, 280–296.

Dale, C., Young, S. A., Haydon, D. T., and Welburn, S. C. (2001). The insect endosymbiont *Sodalis glossinidius* utilizes a type III secretion system for cell invasion. *Proc. Natl. Acad. Sci. USA* 98, 1883–1888.

de Bary, A. (1879). "Die Erscheinung der Symbiose." Verlag von Karl J. Trubner, Strassburg (Strasbourg), France.

Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M. E., and Bouletreau, M. (2001). Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proc. Natl. Acad. Sci. USA* 98, 6247–6252.

Doino, J. A., and McFall-Ngai, M. J. (1995). A transient exposure to symbiosis-competent bacteria induces light organ morphogenesis in the host squid. *Biol. Bull.* 189, 347–355.

Douglas, A. E. (1989). Mycetocyte symbiosis in insects. *Biol. Rev.* 64, 409–434.

Douglas, A. E. (1994). Oxford Univ. Press, Oxford.

Falk, P. G., Hooper, L. V., Midtvedt, T., and Gordon, J. I. (1998). Creating and maintaining the gastrointestinal ecosystem: What we know and need to know about gnotobiology. *Microbiol. Mol. Biol. Rev.* 62, 1157–1170.

ffrench-Constant, R. H., Waterfield, N., Burland, V., Perna, N. T., Daborn, P. J., Bowen, D., and Blattner, F. R. (2000). A genomic sample sequence of the entomopathogenic bacterium *Photorhabdus luminescens* W14: Potential implications for virulence. *Appl. Environ. Microbiol.* 66, 3310–3329.

Finlay, B. B., and Cossart, P. (1997). Exploitation of mammalian host cell functions by bacterial pathogens. *Science* 276, 718–725.

Foster, J. S., Apicella, M. A., and McFall-Ngai, M. J. (2000). *Vibrio fischeri* lipopolysaccharide induces developmental apoptosis, but not complete morphogenesis, of its host's ciliated epithelium. *Dev. Biol.* 226, 242–254.

Foster, J. S., and McFall-Ngai, M. J. (1998). Induction of apoptosis by cooperative bacteria in the morphogenesis of host epithelial tissues. *Dev. Genes Evol.* 208, 295–303.

Gilbert, S. (2001). Ecological developmental biology: Developmental biology meets the real world. *Dev. Biol.* 233, 1–12.

Gil-Turnes, M. S., and Fenical, W. (1992). Embryos of *Homarus americanus* are protected by epibiotic bacteria. *Biol. Bull.* 182, 105–108.

Gil-Turnes, M. S., Hay, M. E., and Fenical, W. (1989). Symbiotic marine bacteria chemically defend crustacean embryos from a pathogenic fungus. *Science* 246, 116–118.

Goebel, W., and Gross, R. (2001). Intracellular survival strategies of mutualistic and parasitic prokaryotes. *Trends Microbiol.* 6, 267–273.

Gordon, J. I., Hooper, L. V., McNevin, M. S., Wong, M., and Bry, L. (1997). Epithelial cell growth and differentiation. III. Promoting diversity in the intestine: Conversations between microflora, epithelium, and diffuse GALT. *Am. J. Physiol. Gastrointest. Liver Physiol.* 273, G565–G570.

Graf, J. (1999). Symbiosis of *Aeromonas veronii* biovar sobria and *Hirudo medicinalis*, the medicinal leech: A novel model for digestive tract associations. *Infect. Immun.* 67, 1–7.

Graf, J. (2000). Symbiosis of *Aeromonas* and *Hirudo medicinalis*, the medicinal leech. *ASM News* 66, 147–153.

Grigioni, S., Boucher-Rodoni, R., Demarta, A., Tonolla, M., and Peduzzi, R. (2000). Phylogenetic characterization of bacterial symbionts in the accessory nidamental gland of the sepiolid *Sepia officinalis* Cephalopoda:Decapoda. *Mar. Biol.* 136, 217–222.

Hansen, G. H., and Olafsen, J. A. (1999). Bacterial interactions in early life stages of marine cold water fish. *Microb. Ecol.* 38, 1–26.

Heddi, A., Grenier, A. M., Khatchadourian, C., Charles, H., and Nardon, P. (1999). Four intracellular genomes direct weevil biology: Nuclear, mitrochondrial, principal endosymbiont, and *Wolbachia. Proc. Natl. Acad. Sci. USA* 96, 6814–6819.

Henderson, B., Wilson, M., McNab, R., and Lax, A. J. (1999). "Cellular Microbiology: Bacteria–Host Interactions in Health and Disease." Wiley, New York/Chicester, UK.

Hentschel, U., Steinert, M., and Hacker, J. (2000). Common molecular mechanisms of symbiosis and pathogenesis. *Trends Microbiol.* 8, 226–231.

Hinde, R. (1971). The control of the mycetome symbiotes of the aphids *Brevicoryne brassicae, Myzus persicae* and *Macrosiphum rosae. J. Insect Physiol.* 17, 1791–1800.

Hoffmann, A. A., and Turelli, M. (1997). Cytoplasmic incompatibility in insects. *In* "Influential Passengers," (S. L. O'Neill, A. A. Hoffmann, and J. H. Werren, Eds.), pp. 42–80. Oxford Univ. Press, Oxford.

Hoffmann, J. A., Reichhart, J. M., and Hetru, C. (1996). Innate immunity in higher insects. *Curr. Opin. Immunol.* 8, 8–13.

Hooper, L. V., Bry, L., Falk, P. G., and Gordon, J. I. (1998). Host–microbial symbiosis in the mammalian intestine: Exploring an internal ecosystem. *BioEssays* 20, 336–343.

Hooper, L. V., and Gordon, J. I. (2001). Commensal host–bacterial relationships in the gut. *Science* 292, 1115–1118.

Hooper, L. V., Wong, M. H., Thelin, A., Hansson, L., Falk, P. G., and Gordon, J. I. (2001). Molecular analysis of commensal host–microbial relationships in the intestine. *Science* 291, 881–884.

Ireton, K., and Cossart, P. (1998). Interaction of invasive bacteria with host signaling pathways. *Curr. Opin. Cell Biol.* 10, 276–283.

Jiggins, F. M., Bentley, J. K., Majerus, M. E., and Hurst, G. D. (2001). How many species are infected with *Wolbachia*? Cryptic sex ratio disorders revealed to be common by intensive sampling. *Proc. R. Soc. Lond. B Biol. Sci.* 268, 1123–1126.

Jones, M. L., and Gardiner, S. L. (1988). Evidence for a transient digestive tract in vestimentifera. *Proc. Biol. Soc. Wash.* 101, 423–433.

Kaufman, M. R., Ikeda, Y., Patton, C., van Dykhuizen, G., and Epel, D. (1998). Bacterial symbionts colonize the accessory nidamental gland of the squid *Loligo opalescens* via horizontal transmission. *Biol. Bull.* 194, 36–43.

Khodursky, A. B., Peter, B. J., Cozzarelli, N. R., Botstein, D., Brown, P. O., and Yanofsky, C. (2000). DNA microarray analysis of gene expression in response to physiological and genetic changes that affect tryptophan metabolism in *Escherichia coli. Proc. Natl. Acad. Sci. USA* 97, 12170–12175.

Kolenbrander, P. E. (2000). Oral microbial communities: Biofilms, interactions and genetic systems. *Annu. Rev. Microbiol.* 54, 413–437.

Kroes, I., Lepp, P. W., and Relman, D. A. (1999). Bacterial diversity within the human subgingival crevice. *Proc. Natl. Acad. Sci. USA* 96, 14547–14552.

Krueger, D. M., Gustafson, R. G., and Cavanaugh, C. M. (1996). Vertical transmission of chemoautotrophic symbionts in the

bivalve *Solemya velum* (Bivalvia:Protobranchia). *Biol. Bull.* 190, 195–202.

Kurz, C. L., and Ewbank, J. J. (2000). *Caenorhabditis elegans* for the study of host–pathogen interactions. *Trends Microbiol.* 8, 142–144.

Lamarcq, L. H., and McFall-Ngai, M. J. (1998). Induction of gradual, reversible morphogenesis of its host's epithelial brush border by *Vibrio fischeri. Infect. Immun.* 66, 777–785.

Lanning, D., Sethupathi, P., Rhee, K. J., Zhai, S. K., and Knight, K. L. (2000). Intestinal microflora and diversification of the rabbit antibody repertoire. *J. Immunol.* 165, 2012–2019.

Lemus, J., and McFall-Ngai, M. J. (2000). Bacterial induction of changes in the host proteome in the squid–vibrio symbiosis. *Appl. Environ. Microbiol.* 66, 4091–4097.

Macpherson, A. J., Gatto, D., Sainsbury, E., Harriman, G. R., Hengartner, H., and Zinkernagel, R. M. (2000). A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* 288, 2222–2226.

Mahajan-Miklos, S., Rhame, L. G., and Ausubel, F. M. (2000). Elucidating the molecular mechanisms of bacterial virulence using non-mammalian models. *Mol. Microbiol.* 37, 981–988.

Maniloff, J. (1996). The minimal cell genome: "On being the right size." *Proc. Natl. Acad. Sci. USA* 93, 10004–10006.

Marsh, T. L., Saxman, P., Cole, J., and Tiedje, J. (2000). Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. *Appl. Environ. Microbiol.* 66, 3616–3620.

McCracken, V. J., and Lorenz, R. G. (2001). The gastrointestinal ecosystem: A precarious alliance among epithelium, immunity and microbiota. *Cell Microbiol.* 3, 1–11.

McFall-Ngai, M. J. (1998). The development of cooperative associations between animals and bacteria: Establishing détente among Domains. *Am. Zool.* 38, 593–608.

McFall-Ngai, M. J. (1999). Consequences of evolving with bacterial symbionts: Insights from the squid–vibrio associations. *Annu. Rev. Ecol. Syst.* 301, 235–256.

McFall-Ngai, M. J. (2001). Identifying "prime suspects": Symbioses and the evolution of multicellularity. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 129, 711–723.

McFall-Ngai, M. J., and Ruby, E. G. (1991). Symbiont recognition and subsequent morphogenesis as early events in an animal–bacterial mutualism. *Science* 254, 1491–1494.

McFall-Ngai, M. J., and Toller, W. W. (1991). Frontiers in the study of the biochemistry and molecular biology of vision and luminescence in fishes. *In* "The Molecular Biology and Biochemistry of Fishes" (P. Hochachka and T. Mommsen, Eds.), Vol. I, pp. 77–107. Elsevier, Amsterdam/New York.

Milburn, N. S. (1966). Fine structure of the pleomorphic bacteroids in the mycetocytes and ovaries of several genera of cockroaches. *J. Insect Physiol.* 12, 1245–1254.

Montgomery, M. K., and McFall-Ngai, M. J. (1993). Embryonic development of the light organ of the sepiolid squid *Euprymna scolopes* Berry. *Biol. Bull.* 184, 296–308.

Montgomery, M. K., and McFall-Ngai, M. J. (1994). Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Euprymna scolopes. Development* 120, 1719–1729.

Moran, N. A., and Baumann, P. (2000). Bacterial endosymbionts in animals. *Curr. Opin. Microbiol.* 3, 270–275.

Nardon, P. (1973). Obtention d'une souche asymbiotique chez le charaçon *Sitophilus sasakii* Tak: Différentes méthodes et comparaison avec la souche symbiotique d'origine. *C. R. Acad. Sci.* 277D, 981–994.

Nardon, P. (1988). Cell to cell interactions in insect endocytobiosis. *In* "Cell to Cell Signals in Plant, Animal and Microbial Symbioses" (S. Scannerini, D. Smith, P. Bonfante-Fasolo, and V. Gianinazzi-Peasron, Eds.), NATO Series, Vol. H17, pp. 85–100. Springer-Verlag, Berlin.

Nardon, P., and Grenier, A. M. (1988). Genetical and biochemical interactions between the host and its endocytobionts in the weevils Sitophilus (Coleoptera, Curculionidae) and other related species. *In* "Cell to Cell Signals in Plant, Animal and Microbial Symbioses" (S. Scannerini, D. Smith, P. Bonfante-Fasolo, and V. Gianinazzi-Peasron, Eds.), NATO Series, Vol. H17, pp. 255–270. Springer-Verlag, Berlin.

Neish, A. S., Gewirtz, A. T., Zeng, H., Young, A. N., Hobert, M. E., Karmali, V., Rao, A. S., and Madara, J. L. (2000). Prokaryotic regulation of epithelial responses by inhibition of IκB-α ubiquination. *Science* 289, 1560–1563.

Nishiguchi, M. K., Ruby, E. G., and McFall-Ngai, M. J. (1998). Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid–vibrio symbioses. *Appl. Environ. Microbiol.* 64, 3209–3213.

Nyholm, S. V., and McFall-Ngai, M. J. (1998). Sampling the microenvironment of the *Euprymna scolopes* light organ: Description of a population of host cells with the bacterial symbiont *Vibrio fischeri. Biol. Bull.* 195, 89–97.

Nyholm, S. V., Stabb, E. V., Ruby, E. G., and McFall-Ngai, M. J. (2000). Establishment of an animal–bacterial association: Recruiting symbiotic vibrios from the environment. *Proc. Natl. Acad. Sci. USA* 97, 10231–10235.

Ochman, H., and Moran, N. A. (2001). Genes lost and genes found: Evolution of bacterial pathogenesis and symbiosis. *Science* 292, 1096–1098.

O'Neill, S. L., Hoffmann, A. A., and Werren, J. H. (1997). "Influential Passengers: Inherited Microorganisms and Arthropod Reproduction." Oxford Univ. Press, New York.

Paster, B., Boches, S. K., Galvin, J. L., Ericson, R. E., Lau, C. N., Levanos, V. A., Sahasrabudhe, A., and Dewhirst, F. E. (2001). Bacterial diversity in human subgingival plaque. *J. Bacteriol.* 183, 3770–3783.

Relman, D. A., and Falkow, S. (2001). The meaning and impact of the human genome sequence for microbiology. *Trends Microbiol.* 9, 206–208.

Rondon, M. R., August, P. R., Bettermann, A. D., Brady, S. F., Grossman, T. H., Liles, M. R., Loiacono, K. A., Lynch, B. A., MacNeil, I. A., Minor, C., Tiong, C. L., Gilman, M., Osburne, M. S., Clardy, J., Handelsman, J., and Goodman, R. M. (2000). Cloning the soil metagenome: A strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Appl. Environ. Microbiol.* 66, 2541–2547.

Rook, G. A., and Stanford, J. L. (1998). Give us this day our daily germs. *Immunol. Today* 19, 113–116.

Rotimi, V. O., and Duerden, B. I. (1981). The development of the bacterial flora in normal neonates. *J. Med. Microbiol.* 14, 51–62.

Ruby, E. G. (1999). The *Euprymna scolopes–Vibrio fischeri* symbiosis: A biomedical model for the study of bacterial colonization of animal tissue. *J. Mol. Microbiol. Biotechnol.* 1, 13–21.

Russell, J. B., and Rychlik, J. L. (2001). Factors that alter rumen ecology. *Science* 292, 1119–1122.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., and Ishikawa, H. (2000). Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. *APS. Nature* 407, 81–86.

Shroff, K. E., Meslin, K., and Cebra, J. J. (1995). Commensal enteric bacteria engender a self-limiting humoral mucosal immune response while permanently colonizing the gut. *Infect. Immun.* 63, 3904–3913.

Small, A. L., and McFall-Ngai, M. J. (1999). A halide peroxidase in tissues that interact with bacteria in the host squid *Euprymna scolopes*. *J. Cell. Biochem.* 72, 445–457.

Smith, H. W., and Crabb, W. E. (1961). The faecal bacterial flora of animals and man: Its development in the young. *J. Pathol. Bacteriol.* 82, 53–66.

Southward, E. C. (1988). Development of the gut and segmentation of newly settled stages of *Ridgeia* (Vestimentifera): Implications for relationship between vestimentifera and pogonophora. *J. Mar. Biol. Assoc. UK* 68, 465–487.

Stabb, E. V., Reich, K. A., and Ruby, E. G. (2001). *Vibrio fischeri* genes *hvnA* and *hvnB* encode secreted NAD$^+$-glycohydrolases. *J. Bacteriol.* 183, 309–317.

Stougaerd, J. (2000). Regulators and regulation of legume root nodule development. *Plant Physiol.* 124, 531–539.

Stouthamer, R., Breeuwer, J. A., and Hurst, G. D. (1999). *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol.* 53, 71–102.

Suau, A., Bonnet, R., Sutren, M., Godon, J. J., Gibson, G. R., Collins, M. D., and Dore, J. (1999). Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl. Environ. Microbiol.* 65, 4799–4807.

Tannock, G. W. (1999). "Medical Importance of the Normal Microflora." Kluwer Academic, Dordrecht/Norwell, MA.

Taylor, M. J., and Hoerauf, A. (1999). *Wolbachia* bacteria of filarial nematodes. *Parasitol. Today* 15, 437–442.

Turelli, M., and Hoffmann, A. A. (1991). Rapid spread of an inherited incompatibility factor in California *Drosophila*. *Nature* 353, 440–442.

Umesaki, Y, Okada, Y., Matsumoto, S., Imaoka, A., and Setoyama, H. (1995). Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lympocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ-free mouse. *Microbiol. Immunol.* 39, 555–562.

Umesaki, Y., and Setoyama, H. (2000). Structure of the intestinal flora responsible for development of the gut immune system in a rodent model. *Microbes Infect.* 2, 1343–1351.

Visick, K., and McFall-Ngai, M. J. (2000). An exclusive contract: Specificity in the *Vibrio fischeri–Euprymna scolopes* partnership. *J. Bacteriol.* 182, 1779–1787.

Visick, K. L., and Ruby, E. G. (1998). Tn*luxAB* insertion mutants of *Vibrio fischeri* with symbiosis-regulated phenotypes. *Abstr. Gen. Meet. Am. Soc. Microbiol.* 98, 277.

Visick, K. L., and Skoufos, L. M. (2001). Two-component sensor required for normal symbiotic colonization of *Euprymna scolopes* by *Vibrio fischeri*. *J. Bacteriol.* 183, 835–842.

Vivas, E. I., and Goodrich-Blair, H. (2001). *Xenorhabdus nematophilus* as a model for host bacterium-interactions: rpoS is necessary for mutualism with nematodes. *J. Bacteriol.* 183, 4687–4693.

Werren, J. H., Windsor, D., and Guo, L. R. (1995). Distribution of *Wolbachia*: Reproductive parasites of arthropods. *Proc. R. Soc. Lond. B Biol. Sci.* 261, 55–63.

Zimmer, C. (2001). *Wolbachia*: A tale of sex and survival. *Science* 292, 1093–1095.