Symbiotic conversations are revealed under genetic interrogation

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Abstract | The recent development and application of molecular genetics to the symbionts of invertebrate animal species have advanced our knowledge of the biochemical communication that occurs between the host and its bacterial symbionts. In particular, the ability to manipulate these associations experimentally by introducing genetic variants of the symbionts into naive hosts has allowed the discovery of novel colonization mechanisms and factors. In addition, the role of the symbionts in inducing normal host development has been revealed, and its molecular basis described. In this Review, I discuss many of these developments, focusing on what has been discovered in five well-understood model systems.

Bioluminescence
The process by which some bacteria and other organisms produce light as the result of a chemical reaction. During symbiosis, this light can be used in behaviours such as counterillumination, in which the bioluminescence is used to eliminate the shadow of the host's silhouette.

Gnotobiotic
An animal that is born under aseptic conditions and is exposed only to experimentally introduced microorganisms. Gnotobiotic animals are used to investigate the symbiotic relationship between an animal and one or more of the consortia of interacting microbial species that normally inhabit its body.

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doi:10.1038/nrmicro1958

This is an exciting time for biologists, and for microbiologists in particular. We are at the convergence of two breakthroughs that are advancing our understanding of how animals and plants live with their microbiota. The first of these advances is the development of methods for dissecting the genetic mechanisms by which organisms signal and respond to each other. The second advance is conceptual: the recognition that higher organisms create a shared living space with a specific set of beneficial microorganisms. Together, these two developments have made it possible to begin to understand how animals and plants communicate with the many bacterial species that live in and on their tissues. Describing the genetic basis of this symbiotic conversation has become a new frontier of biology.

Recent research is expanding to identify and embrace the diversity of microbial symbioses in living systems (FIG. 1). This diversity includes associations in which bacteria perform conserved functions that are common to the needs of many host species (for example, digestive activities[1]), as well as those in which they perform unusual functions (for example, bioluminescence[2]) that have specifically evolved in a few host species. Included in this Focus issue are contributions that address a range of symbioses in depth and describe specific phylogenetic groups and metabolic processes. For example, Parniske[3] discusses plant–microorganism associations, whereas Werren and colleagues[4] concentrate on obligate intracellular partnerships and Ley and colleagues[5] discuss complex microbial consortia in vertebrates. By contrast, this Review focuses on a set of experimentally accessible, genetically developed systems that consist of a natural association between an invertebrate host and one or a few bacterial symbionts. These associations illustrate how the application of molecular genetics and genomics to a number of biologically diverse symbioses is revealing the nature of the conversation by which an animal and its microbiota initiate and maintain a shared existence.

The value of natural experimental models

An emerging awareness of the role of beneficial microorganisms in human health has led to a recent increase in interest in symbioses[6,7]. For example, there has been success in using gnotobiotic animals that were inoculated with specific combinations of microbial species to dissect the processes that underlie the complex enteric consortia of vertebrates[8]. These studies, which use constructed systems (FIG. 2) that are artificially simplified to focus on a specific set of events, have already revealed the role of specific bacteria in modulating such diverse and important health issues as obesity and immune dysfunction[9–12]. As a result of these discoveries, there has been a widespread re-evaluation of the extent to which microorganisms may influence other, as yet unrecognized, aspects of host physiology[9].

A second kind of model that is used in biology, natural systems, allows us to examine how interactions function in the context in which they evolved. In contrast to the genetically modified or inbred host lines that are developed for constructed systems, natural models purposely use a population of genetically heterogeneous hosts to provide insight into the natural range of responses that characterize normal animal populations. However, although these systems may be natural, they are most useful when they have experimentally

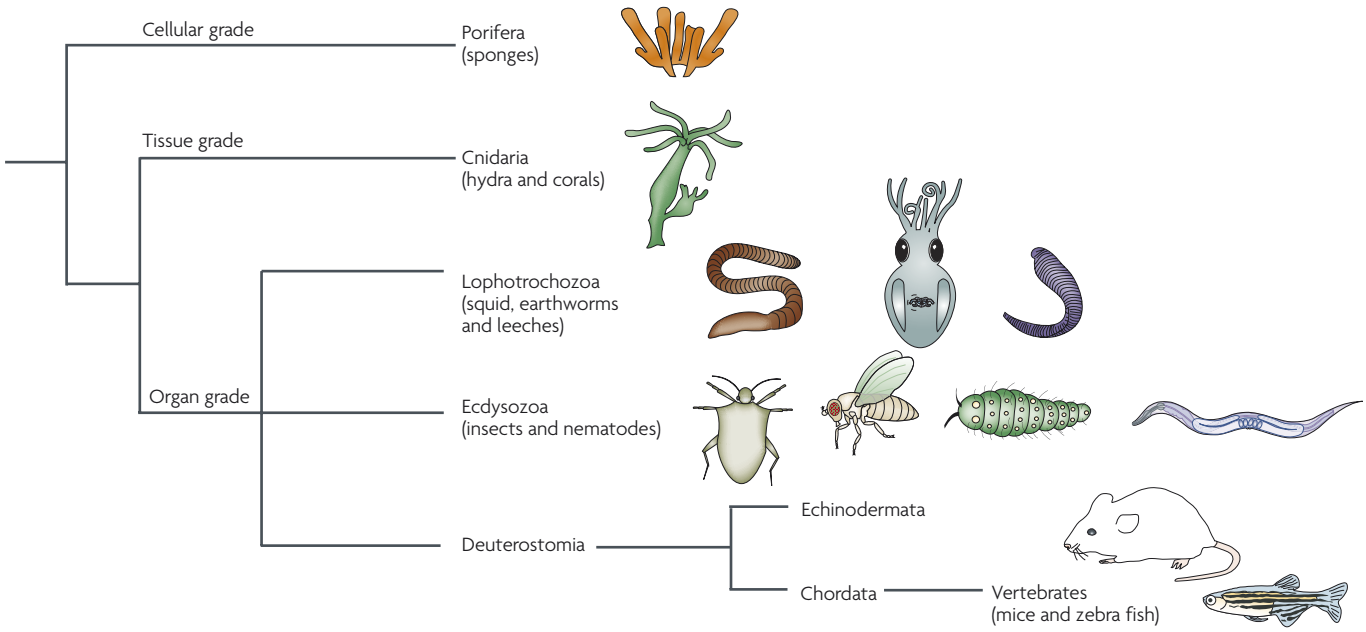

Figure 1 | **Microbial symbioses occur throughout the phylogeny of animals.** Experimentally accessible associations, including several that are described in this Review, occur in all the main phylogenetic groups. These associations span the breadth of animal diversity, and are represented in cellular-grade, tissue-grade and organ-grade levels of developmental and morphological complexity.

valuable characteristics, including culturable partners that can be maintained separately from each other. For some symbioses that are established through horizontal transfer, the newly hatched host can survive without its symbiont (aposymbiotic) and the symbiont (or symbionts) can be grown under axenic conditions. Such conditions present the opportunity to experimentally study the initiation of the association. Alternatively, in those cases in which mature hosts can be cured of their microbial symbionts and artificially reinfected with novel strains, the persistence of the association can be followed. In any event, if the association is binary (one host and one symbiont species) or consists of a simple bacterial consortium[13], it can be easier to focus on a specific set of relationships or events.

Numerous other attributes that allow for technological applications (BOX 1) have been exploited in a range of natural symbioses, but one that has recently begun to open many questions to experimental evaluation is the ability to genetically manipulate one or more of the symbiotic partners and subsequently reconstitute the association with these variants. This genetic level of manipulation has usually been developed in the bacterium, although there are exceptions[14]. However, in all cases, the desired result is to be able to build and interrogate testable models of the nature of the interaction.

Over the past 20 years a number of new systems have been developed to study animal–bacteria symbioses[15]. But why should we promote the development of so many different models? In addition to the ability of an individual model to reveal evolutionary novelties or clearly conserved mechanisms, each model allows a distinct set of difficult questions to be addressed, which when combined with other models provides opportunities that would not be available from any one system[6,16]. The importance of using a range of different models is illustrated in the field of animal development. More than a dozen species have been used to investigate how animals control the mechanisms by which an individual is built from a single cell. The number of different models that are available has been crucial to the advance of developmental biology: at least seven of these models have directly led to discoveries that were recognized by Nobel Prizes (TABLE 1). It is clear that each of these different animal models has provided a unique opportunity to better describe a fundamental process in the biology of animal development.

Examples of beneficial symbioses

With only a few known exceptions (for example, light-organ symbioses of marine fishes[2]), beneficial associations between vertebrates and bacteria exist as complex consortia of tens to hundreds of species[6]. By contrast, most beneficial symbioses in invertebrates are monospecific or constitute simple (<10 species) consortia, perhaps owing to the limited ability of their hosts to carry out immunological surveillance[17]. Although most beneficial symbionts of insects are obligately intracellular and are passed vertically through the maternal line[18], many bacteria that are associated with these and other invertebrates are passed horizontally, and therefore must be able to live in the external environment. It is therefore not surprising that most of the currently recognized, genetically tractable symbionts that can be readily cultured are horizontally passed, heterotrophic bacteria. Described below are five beneficial symbioses

Horizontal transfer
The process by which an animal or plant obtains its natural microbial constituents from the environment at each generation. By contrast, vertical transfer occurs when a young organism receives its microbiota from its parent, usually in or on the egg.

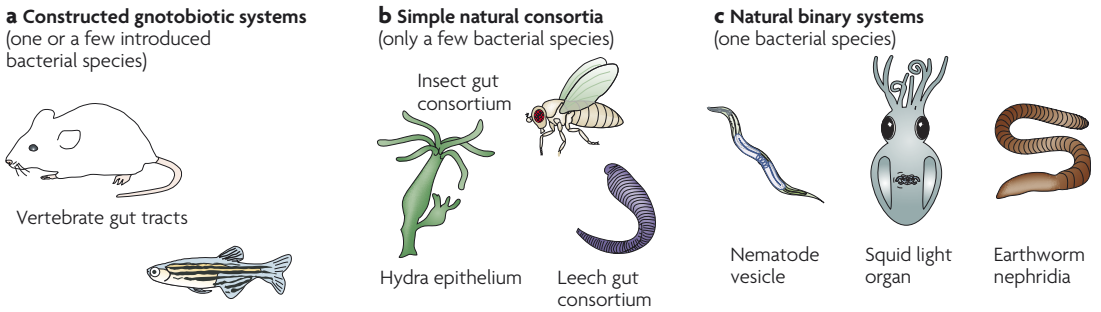

Figure 2 | **Classes of symbiosis models.** Experimental models of microbial symbioses can be characterized into three types. Gnotobiotic systems (**a**) have been useful for examining the interactions within the complex consortia that are normally present in vertebrate enteric tracts. In these systems, germ-free host animals are produced, and one or a few bacterial species are introduced to allow an examination of a simplified relationship. An alternative approach is to investigate consortia of invertebrates (**b**), which are often simpler in species composition. Finally, there are several natural animal models (**c**) in which only a single bacterial species is present.

of invertebrate hosts that have been examined by genetically manipulating at least one bacterial species. Three of these systems are normally found as binary associations of a monospecific symbiont population that grows within a specialized tissue of its host, whereas two consist of simple consortia that are found in an animal's enteric tract (FIG. 3).

Vibrio *and sepiolid squid.* Many species of marine animals are bioluminescent, and approximately half of these generate their light by developing associations with luminous bacteria in the genera *Vibrio* or *Photobacterium*[2,19]. The best studied of the light-emitting symbioses is that between *Vibrio fischeri* and the sepiolid squid *Euprymna scolopes*, although other species of sepiolids that are symbiotic with *V. fischeri* and/or the related *Vibrio logei* have also been described[20,21]. In these symbioses, the host is thought to use the luminescence in a behaviour called counterillumination[22]. The *E. scolopes* association is horizontally passed between generations of hosts[23], which must individually obtain their bacterial symbionts from environmental populations that live in the ambient sea water[24]. Thus, the newly hatched juvenile squid is aposymbiotic and, because bioluminescence is not a nutritional product, the host can be maintained in a free-living form in the laboratory for generations[25]. Similarly, *V. fischeri* cells are easily grown in culture, and can be genetically engineered using recently developed techniques (for example, REFS 26,27). In addition, the completion of two *V. fischeri* genomes[28,29] and associated microarrays[30,31], as well as a library of expressed sequence tags[32] and a microarray chip for the host squid[33], have ushered in an era of genomic-level analysis in both partners of this symbiosis.

Xenorhabdus–Photorhabdus *and nematode worms.* One of the best-developed systems for the study of beneficial symbioses is a series of specific associations that have evolved between two genera of nematode worms and one of two genera from the *Enterobacteriaceae*[16]. These associations share a common biological function: they allow the partnership to infect, kill and grow within insect larvae. As many of the infected insect species are crop pests, an understanding of the basis of bacteria–nematode symbiosis and its potential in biological control is of considerable interest in agriculture. Two pairs of bacteria–host genera are known: *Xenorhabdus–Steinernema*[34] and *Photorhabdus–Heterorhabditis*[35]. Although the interactions have distinct features, they share many developmental and genetic characteristics. Interestingly, in each interaction, the bacterium is highly specific for its particular nematode species, whereas the insect larvae from both taxa are susceptible to both species of nematode.

The nematodes exist in soil in a resting form, and carry dozens of symbionts in a region of their upper enteric tract. In *Xenorhabdus*, this region consists of a specialized structure called the vesicle. When the nematode invades a larval insect, it migrates into the blood system and begins to ingest blood. The nematode then expels its bacterial symbionts, which produce extracellular toxins and degradative enzymes, and proliferate, providing a food source that supports the reproduction of an increasing population of worms. When the insect carcass is depleted of nutrients, the worms revert back into the resting form, and the nematode vesicle becomes colonized by a few cells of the bacterial population[36]. The nematodes then escape into the soil, where they await the next insect host. Knowledge of the process by which the bacteria colonize their nematode host, and in particular the genetic basis of specificity and development in the bacterial symbionts, has increased markedly in recent years[37–39]. Although there is considerable interest in the genetic basis of the insect-parasitism stage of the bacteria–nematode life cycle, which has been aided by the development of a tripartite model system that targets larvae of the genetically facile fruit fly *Drosophila melanogaster*[35], the emphasis of this Review is on the beneficial association between the worm and its bacterial symbiont. Nevertheless, it should be noted that there is some overlap between the genetic requirements for pathogenic and beneficial symbiosis[39].

Expressed sequence tag (EST). One of a series of short nucleotide sequences which represent a pool of mRNAs that are expressed under a certain environmental or developmental condition. Libraries of ESTs can be used to identify gene transcripts in global expression studies.

Box 1 | **Valuable characteristics in a genetic model of symbiosis**

- An inexpensive and easily collected or bred host.
- A small host with a simple morphology.
- Easily imaged symbiotic structures.
- Known nutritional characteristics.
- A range of ecologically or evolutionarily distinct associations.
- An association that is economically important.
- An association that is representative of a general biological principle (or principles).
- A host and bacterium that can be cultured separately (grown in the laboratory).
- A host and bacterium for which genome sequences are available.
- An association in which one or both partners are amenable to genetic manipulation.

Sodalis glossinidius *and the tsetse fly.* Many, if not most, insect species maintain intracellular bacteria that provide essential metabolic and developmental activities for their hosts[40,41]. These organisms are classified as either primary symbionts (for example, species of *Buchnera* and *Wigglesworthia*), which are uniformly present in the host, but are not culturable in their free-living form, or secondary symbionts, which occur more sporadically and in several cases have been grown outside of the host[40,42]. Primary symbionts are always found in the specialized tissue called the bacteriome, whereas secondary symbionts can occur in several different tissues of the host. As for primary symbionts, secondary symbionts are generally inherited maternally. However, secondary symbionts retain their ability to pass to a new host from either the environment or another host[40]. *Sodalis glossinidius*, which is thought to provide a beneficial effect to its specific host, the tsetse fly, is of considerable epidemiological interest as a potential vector that can be engineered by paratransgenetics to artificially produce anti-trypanosomal proteins[43]. This symbiont is closely related to well-studied enteric species, and microarray-based analyses have shown that its genome is surprisingly similar to that of *Escherichia coli*[44]. In addition, because *S. glossinidius* is not essential to its host, it is possible to eliminate the native bacteria within a fly by antibiotic treatment and reinfect it with genetically modified strains[43].

Aeromonas, Rikenella *and the leech.* The diversity and specificity of invertebrate enteric microbiota have begun to be examined using culture-independent techniques, and, with the exception of specialized cellulosic species, such as termites[45], these communities seem to be generally composed of simple consortia of fewer than ten species[17,46]. One such invertebrate is the medicinal leech *Hirudo verbana*, the diet of which is restricted to vertebrate blood. Because of the initial activity of complement in the ingested blood[47], the ingestion of susceptible bacteria by leech haemocytes[48] and possibly other leech- or symbiont-produced antimicrobial compounds[49], only two species of bacteria (from the genera *Aeromonas* and *Rikenella*) are long-term inhabitants of the crop[50]. A higher number of species can be detected downstream of the crop in the intestinum, where the blood is digested, but even there *Aeromonas* and *Rikenella* remain the most abundant microbial species[13].

The role (or roles) of these symbionts in the leech remains unclear, but they might foster the nutrition and/or development of the host. *Aeromonas* spp. are easily cultured and produce many exoenzymes, whereas *Rikenella* spp. are fastidiously obligate anaerobes. Interestingly, when in the crop, cells of the two species associate tightly with each other, forming mixed microcolonies that are embedded in a polysaccharide matrix. In addition, the growth of one species is enhanced by the presence of the other[50]. Taken together, these observations suggest a synergistic interaction between the bacterial partners in this symbiosis.

Enterococcus *and the fruit fly.* Considerable interest has developed in determining the nature of the microorganisms that inhabit the enteric tracts of insects, and particularly the high number of species that are pests of environmentally and agriculturally important plants[51]. Surprisingly, it seems that despite the high number of different host species that have been examined species of the genus *Enterococcus* are typically among the natural members of the microbiota. This pattern is apparent even when specimens of *D. melanogaster* from laboratory-maintained stocks are compared with those from wild-caught populations[14]. When the anatomical distribution of these bacteria was determined in fruit flies, they were found to be restricted to portions of the foregut, midgut and hindgut. As for *Aeromonas* spp., native *Enterococcus* symbionts can be cured, and the consortium re-established using other genetically modified strains. Using this approach, Cox and Gilmore[14] recently showed that an *Enterococcus faecalis* strain which was engineered to produce a non-native haemolysin was lethal to its host.

Application of molecular genetics

What key mechanisms underlie the development of a beneficial symbiosis? Specifically, how have these mechanisms evolved to permit a host and its microbiota to effectively communicate during the initiation, accommodation and subsequent persistence of their symbiotic association (FIG. 3)? This is a dialogue in which the words and phrases are biochemical. However, the conversation as a whole is organized at the genetic and even genomic level. Approaches that apply molecular genetics to help us dissect and define the steps during a pathogenic infection have had a long history of success[52], and more recently have begun to provide a useful paradigm for studies of beneficial symbioses. For example, the application of signature-tagged mutagenesis has allowed us to identify symbiont genes that are involved in different steps of an infection, which has begun to reveal new colonization determinants[49,53] (FIG. 4).

Bacterial genetics has been most effectively applied to characterize the well-studied association between *V. fischeri* and its squid host. The application of bacterial genetics to this association is discussed below, together

Table 1 | **Examples of Nobel Prize awards in developmental biology***

Year‡	Recipient (or recipients)	Discovery	Model organism§
1935	H. Spemann	The 'organizer center' concept‖	Newt and frog
1995	E. Lewis, C. Nusslein–Volhard and E. Wieschaus	Homeobox genetic organization	Fruit fly and zebra fish
2001	L. Hartwell, T. Hunt and P. Nurse	Cyclin regulation of the cell cycle	Sea urchin and frog
2002	S. Brenner, H. Horvitz and J. Sulston	Programmed cell death	Roundworm
2006	A. Fire and C. Mello	RNA interference	Roundworm
2007	M. Capecchi, M. Evans and O. Smithies	Embryonic stem-cell development	Mouse

*Information obtained from Nobelprize.org (see Further information). ‡Of the past 13 Nobel Prizes awarded in Physiology or Medicine, 5 were from the area of developmental biology. §In several cases, results from more than one model system were specifically recognized. ‖The first Nobel Prize to be awarded in developmental mechanics (developmental biology).

with the similar and contrasting mechanisms of interaction that have been discovered in the other four experimental genetic associations. It is clear that each of the five systems described above have particular strengths owing both to the specific biology of the symbiosis and the current range of research activities (TABLE 2).

Surface structures and specificity of the association. A hallmark of all the symbioses considered in this Review is their exclusivity, and therefore the mechanisms that underlie this species specificity are of interest. Not surprisingly, surface-associated activities of both the host and the symbiont may be required. For example, mucus that is produced from the surface of the nascent light organ allows the juvenile squid to entrap planktonic cells of *V. fischeri* in aggregates. These aggregates form near surface pores that lead into the crypt spaces deep within the organ, where the bacteria grow and reside[54]. The ability of *V. fischeri* to aggregate in this mucus is dependent on signalling by the RscS two-component regulation system[55]. RscS controls production of an extracellular polysaccharide through its regulation of the *syp* operon[56], and strains with null mutations in either *rscS* or key *syp* genes become defective in colonization[57,58]. Similarly, genetic modification of *V. fischeri* lipopolysaccharide (LPS), either by deletion of *pgm*, which generates precursors for LPS glycosylation[59], or *htrB1*, which acylates the lipid A component of LPS[60], leads to a reduction in light-organ colonization. LPS modification also seems to be important to species of *Photorhabdus*: *pgbE1*, a homologue of a gene in the *pmr* locus of serovars of *Salmonella enterica*[61], is also thought to modify lipid A, and a *pgbE1* mutant loses the ability to colonize its nematode host[62]. In related bacteria, such modifications have also been linked to resistance to host cationic antimicrobial peptides[63]. Similarly, a normal LPS is important for *Aeromonas* spp. to overcome the activity of host complement and successfully colonize the leech[64].

Not surprisingly, other surface factors have also been shown to facilitate a bacterial interaction with host tissue during initiation of the association. In *V. fischeri,* mutation of either *ompU*, which encodes an outer membrane protein[65], or *pilA*[66], which encodes one of the ten type-IV pili of this bacterium[29], has only a small effect on colonization competence. Such defects become more apparent at a low inoculum that might more realistically mimic natural conditions[67,68]. More impressive effects follow loss of the *nil* genes of *Xenorhabdus* species. A *nilA* mutant strain is attenuated during colonization of the nematode host, whereas deletion of either *nilB* or *nilC*, which seem to encode membrane components, completely eliminates symbiosis competency[53]. The basis of this requirement is as yet unknown, but the similarity of NilB to homologues in mucosal pathogens has suggested it might be involved in an interaction with mucus in the nematode vesicle[34]. The *Xenorhabdus mrxA*-encoded pilin protein is another surface structure that has been proposed to function in colonization of the nematode host[69].

Bacterial behaviour and gene regulation. For horizontally acquired symbionts, which may exist for long periods in sea water or soil, a rapid and effective adaptation to the specific conditions inside their host can be crucial. In pathogenic associations, the expression of suites of genes that encode colonization factors is often triggered by the activities of specific transcriptional modulators, such as quorum sensing systems or two-component regulators[70,71]. Thus, many studies have targeted such transcription factors for mutagenesis as a way to affect their downstream regulons. In *V. fischeri*, there are two sets of quorum-sensing regulators, AinS–AinR and LuxI–LuxR, that work sequentially to induce distinct but partially overlapping regulons[30,31]. Mutations in either system lead to symbiosis defects. For example, an *ainS* mutant colonizes the squid light organ more slowly than the wild type, and when it does finally infect, it is unable to persist at normal levels[72]. Global expression analyses of *V. fischeri* revealed more than 280 genes that are differentially regulated in the mutant (E.G.R., unpublished observations), including those that encode the flagellar apparatus[31] and the central metabolic-switch enzyme acetyl-CoA synthetase (Acs)[73,74]. These microarray results led to construction of a *V. fischeri acs* mutant, which was found to be defective in colonization, indicating that acetate metabolism is important to the symbiont population.

V. fischeri LuxIR positively regulates the luminescence (or *lux*) genes, as well as a few other loci[75], in a cell-density-dependent manner[70]. Mutation of the *luxIR* genes or a gene that encodes luciferase (*luxA*), results in a symbiosis persistence defect that could relate to the inability of these mutants to produce normal light levels in the host[33,76]. However, in a separate

Two-component regulation system
A stimulus-response coupling mechanism that allows an organism to sense and respond to various changes in environmental conditions.

Lipopolysaccharide
A major component of the outer membrane of Gram-negative bacteria. The immune systems of animals generally sense and react to the presence of lipopolysaccharide.

Quorum sensing
A system by which bacteria respond to increased population density by coordinately controlling expression of a specific set of genes. By sensing the concentration of one of several continuously secreted signal molecules, including acyl homoserine lactones, peptides and autoinducer 2, the population can recognize when it reaches a 'quorum'.

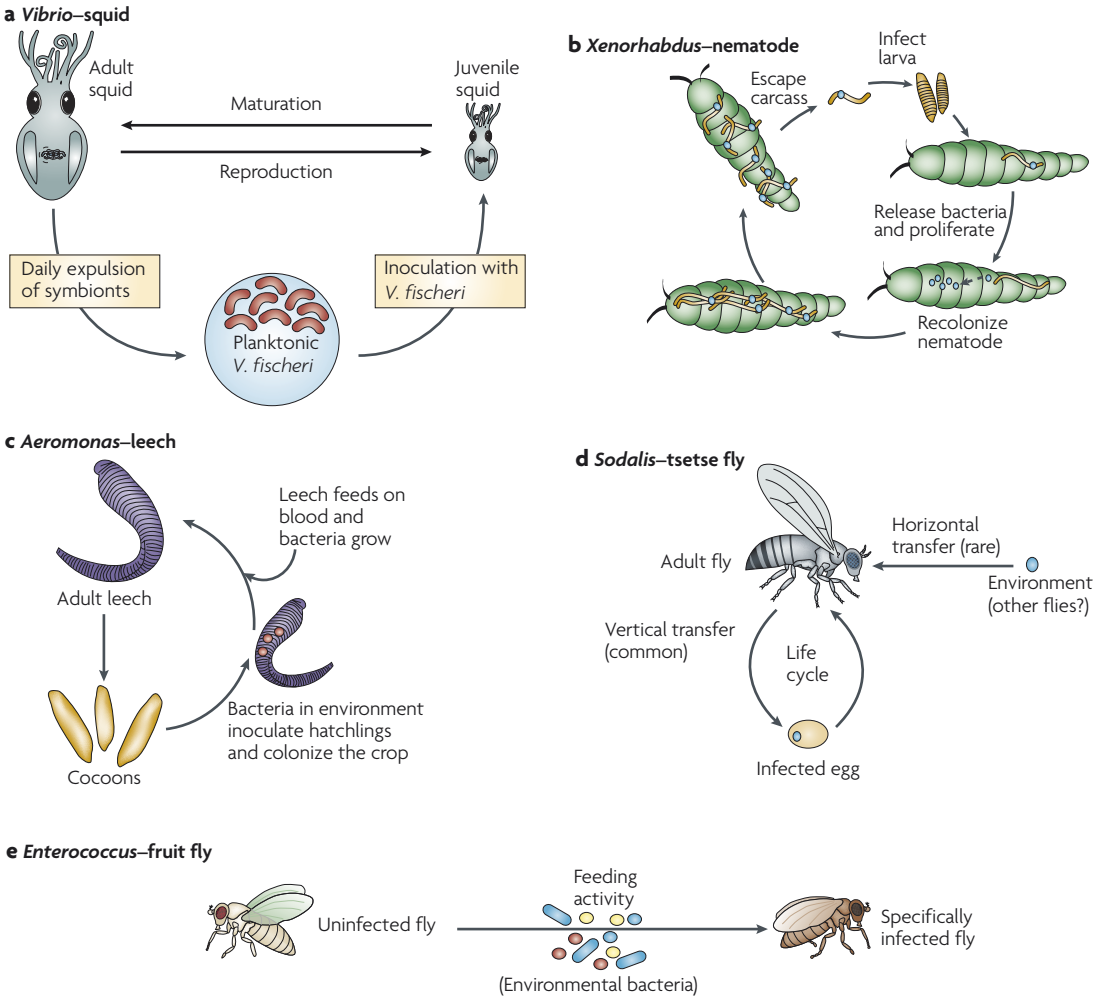

Figure 3 | **Simplified life cycles of five symbioses.** In each of the symbioses shown, the animal obtains a specific symbiont (or symbionts), which colonizes the host in a particular location. **a** | The squid obtains its symbionts from sea-water populations, which colonize the nascent light organ. **b** | The nematode brings its symbiont into the insect host, where both proliferate. The bacteria then recolonize the nematodes, which escape from the carcass. **c** | Juvenile leeches obtain symbionts after hatching from their cocoon (perhaps from the cocoon itself). They then take up residence in the crop, where they digest their blood meal. **d** | The tsetse fly can either pass the symbionts maternally to the eggs or pick up new strains from the environment. **e** | Specific symbionts on the food of the fruit fly colonize and persist in the enteric tract.

microarray-based study, 18 new genes were found to be regulated by the LuxIR system, and several of these were outer-membrane proteins or secreted proteases[30]. Thus, it remains possible that LuxIR, which fully induces its regulon only under the high cell densities that are achieved in the light organ[77], controls a number of different symbiosis factors, the activities of which are necessary for a long-term, stable colonization of the host. In any case, quorum signalling is a crucial activity in the squid–*Vibrio* association, as well as in certain plant–microorganism interactions[78]; the role of cell-density sensing in other beneficial bacteria–animal symbioses is less well defined.

Host-induced bacterial-gene expression is another way by which symbionts adjust to life in the tissue of an animal. Not surprisingly, two-component systems that detect specific environmental cues and modulate transcription output are important in this adjustment. In a pioneering effort, Hussa *et al.*[55] used a bioinformatics approach to identify all the putative response regulators that are encoded in the *V. fischeri* genome. Individual mutations were created in 35 of 40 of these genes, and the resulting strains were compared with the wild-type parent for colonization competence. Of these 35 genes, 12 had a symbiosis defect, although further examination of these individual regulators is needed to understand the basis of their effects. For example, in nematode symbioses, HexA, a LysR-like repressor that has been studied in species of both *Photorhabdus* and *Xenorhabdus*, seems to control nematode colonization in *Photorhabdus* but not *Xenorhabdus*[16]. Specifically, among the >100 genes that are repressed by HexA in *Photorhabdus* spp. are *cipA* and *cipB*, which encode small, crystal-forming proteins that promote nematode development when expressed *in trans* in *E. coli*[79]. Mutation of *hexA* results in a strain that supports the

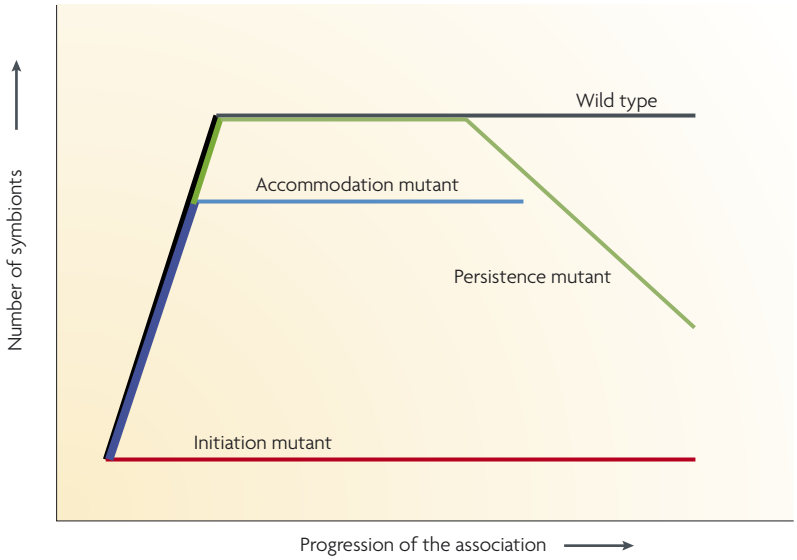

Figure 4 | **Categories of colonization mutants.** Microbial symbionts that are passed horizontally must negotiate several stages of the colonization process. Studies of genetically engineered mutant strains have revealed defects that can be placed in one of several classes. In this example, inoculation with a wild-type strain from the environment allows a few symbionts to colonize, which grow to a specific population size that is then stably maintained over time. Three broad classes of defects have been discovered in several symbiotic systems: initiation mutants, which are unable to inoculate the host; accommodation mutants, which fail to reach the usual population size; and persistence mutants, which at first colonize normally, but are unable to maintain themselves.

growth of a higher number of symbionts[80]. It seems that the primary function of HexA is to enhance bacterial virulence in its insect host, which indicates that there is a reciprocal relationship between those *Photorhabdus* genes that are required for pathogenesis and those that lead to beneficial colonization[39].

The importance of global changes in symbiont gene expression has also been revealed by mutating genes that encode sigma factors in *V. fischeri* (*rpoN*) and *Xenorhabdus* spp. (*rpoS* and *rpoE*), which leads to defects in flagellar motility[81] and resistance to oxidative stress[53], respectively, thereby indicating that these activities are required for symbiotic colonization. However, the fact that these transcription factors control the proper expression of dozens of genes makes it difficult to identify the individual contributions of the many downstream targets.

In addition to targeted mutagenesis of suspected symbiosis determinants, phenotypic screens of mutant libraries have provided a powerful tool for identifying both predicted and unexpected colonization factors and activities. For example, *V. fischeri* mutants that were created by random transposon insertion have been screened for strains that are defective in phenotypes such as flagellar motility and chemotaxis[82,83], siderophore production[84] and luminescence[85]. Subsequent colonization studies have revealed that these phenotypes are all required symbiosis factors. By contrast, unbiased screening of entire libraries in large-scale colonization studies has revealed the importance of new factors (for example, REFS 48,53,86,87). Finally, the transfer of genes that encode fluorescent proteins into different strains of bacteria has been applied not only to tag and localize different strains of symbionts within host tissues, but also to track their transcriptional activity[26,88].

Adaptation to host defences. Because all host animals must protect themselves against colonization by inappropriate or pathogenic microorganisms, a central theme in beneficial bacteria–host interactions is that the symbiont either avoids damage by the defences of the host or communicates with host cells to modulate them[89]. The ability to modulate host defences might therefore also contribute to symbiont specificity. Two ways in which invertebrates confront bacteria that enter their tissues are to produce oxidative or nitrosative stress molecules, or to initiate phagocytosis. Evidence for the avoidance of oxidative or nitrosative stress molecules by the symbiont came from the colonization defects of mutant strains that were unable to produce catalase[90] or autoinducer 2 (REF. 91). By contrast, *Aeromonas* spp. do not seem to require a functional catalase to colonize their leech host[92]. *V. fischeri* cells effectively avoid phagocytic haemocytes that are present in the light organ. Studies of these haemocytes in primary culture suggest that prior exposure to symbionts decreases the ability of the host cell to attach and engulf *V. fischeri*, but not other bacteria (E.G.R., unpublished observations). Mutation of *ompU* results in an increased susceptibility to phagocytosis, although the mechanism that underlies this phenomenon is unknown. In a related case, in which sensitivity to an immunity factor was encountered in the symbiosis, an *Aeromonas* LPS mutant was less able to escape killing by complement factors that were present in the blood meal of the leech[64].

A type III secretion system (T3SS) which exports effector proteins that moderate host defences by interfering with the antimicrobial function of the host is emerging as a common theme in several beneficial symbioses. Leech-associated *Aeromonas* spp. are the first extracellular animal symbionts to be shown to use a T3SS to avoid host phagocytosis[48]. Specifically, mutation and complementation analyses of *ascU*, a homologue of inner-membrane components in other T3SSs, have linked this gene to *Aeromonas* survival in the leech crop. Interestingly, wild-type cells did not rescue the T3SS mutant in co-infection experiments, indicating that the positive effect of the T3SS is restricted to those bacteria that can express it. In a similar manner, mutation of the *inv* (also known as *spa*) gene clusters that encode a *Sodalis* spp. T3SS eliminated the ability of this bacterium either to invade cells of the tsetse fly or even colonize cultured cells after introduction by microinjection[86]. Subsequent analysis revealed the presence of two separate T3SSs in the *Sodalis* genome that have sequential functions during host colonization[93]. *Photorhabdus* spp. also produce a functional T3SS that secretes a homologue of YopT, a protein effector that inhibits phagocytosis

Table 2 | **Genetic tools and resources for certain bacterial symbionts***

Partners	Year initiated‡	Clonality	Mutagenesis	Complementation	Screens	Sequenced genome	Microarray
Vibrio–sepiolid squid	1989	Yes	Yes	Yes	Yes	Yes	Yes
Xenorhabdus or Photorhabdus–nematode	1989	Yes	Yes	Yes	Yes	Yes	No
Aeromonas–medicinal leech	1999	Yes	Yes	Yes	Yes	No	No
Sodalis–tsetse fly	1995	Yes	Yes	No	Yes	Yes	No
Burkholderia–stink-bug	2005	Yes	Yes§	No	No	Yes§	No
Enterococcus–fruit fly	2007	Yes	Yes§	No	No	Yes§	Yes§
Acidovorax–earthworm	2006	Yes	No	No	No	Yes	No
Bacillus–gypsy moth	2006	Yes	No	No	No	Yes§	No
Endosymbionts– hydra	2007	No	No	No	No	No	No

*This list is rapidly becoming out of date, as these tools are being adapted on an ongoing basis in many of the systems. ‡Approximate year in which the association was first experimentally described. §Only found in strains from a non-symbiosis source so far.

of *Yersinia pestis* by host phagocytes[94]. However, it has not been definitively shown that this T3SS is important to *Photorhabdus* spp. in host colonization.

Induction of host development. One of the most exciting outcomes of recent studies of bacteria–host interactions is the recognition that symbionts can play a crucial part in triggering the development of their host. Often these effects lead to dramatic morphological changes that are required for the proper functioning of the association (for example, REFS 35,95,96). The bacteria in the *V. fischeri*–*E. scolopes* symbiosis have a remarkably important role in light-organ development[95]. Several of these developmental events have been linked to specific bacterial gene products through bacterial mutant analysis: infection by *lux* mutants fails to induce the oedemic swelling that is characteristic of the epithelial cells that line colonized crypts[76], which suggests that the host tissue normally responds to symbiont bioluminescence or to the hypoxia that is associated with luciferase activity[97]. Experiments that were designed to determine which of these factors triggers this developmental response will allow us to better understand the mechanisms that underlie this interaction.

The striking discovery that developmental regression of the ciliated surface of the light organ of the juvenile squid is induced by a bacterial cell-wall monomer[98], which was previously described as tracheal cytotoxin (TCT), shows how a normal bacteria–host signal can also serve a pathogenic function. Identification in the *V. fischeri* genome of homologues of genes that encode the activities which are necessary for TCT secretion has allowed these activities to be genetically linked to the developmental biology of this symbiosis. The role of GacA, a common bacterial transcriptional regulator, in squid development has also recently been reported[99]. Specifically, mucus secretion and ciliated-surface apoptosis are not induced in squid that are colonized by a *gacA* mutant. Because GacA affects the LPS structure of the bacterium, it has been suggested that the developmental defects are linked to outer-membrane modifications[100].

The proper development of nematode-infected juveniles has also been linked to the functions of different genes in species of *Xenorhabdus* and *Photorhabdus*. The *Xenorhabdus* global regulator Lrp is required for normal colonization and subsequent host maturation through both its repression (with NilR) of the *nil* genes and its induction of nematode development[16]. Microarray analyses of the Lrp regulon will help us identify genes that trigger nematode development. By contrast, the ability of *Photorhabdus* spp. to promote nematode growth and development seems to be largely due to the HexA repressor protein (discussed above). Nematodes that are infected by a *Photorhabdus ngrA* mutant are unable to develop from the infective juvenile stage to the self-fertile hermaphrodite stage[101]. Because NgrA is a homologue of a non-ribosomal peptide-synthesis complex, it has been suggested that the symbiont produces a peptide signal that induces nematode development[39]. Finally, although the *Photorhabdus* crystal-forming proteins CipA and CipB promote nematode development through an unexplained mechanism, it is not yet clear whether the analogous *Xenorhabdus* crystal protein that is encoded by *pixA* has an important role in the biology of the host[16].

Nutritional and metabolic accommodation. In a mutually successful symbiosis, both partners must obtain sufficient organic and inorganic nutrients to grow and sustain the relationship. Not surprisingly, virtually all symbiotic relationships include a transfer of such materials between the host and the bacterium, in at least one direction. Sometimes, the symbionts must be able to synthesize missing nutrients owing to the nature of the food, such as blood, that is provided by the host. The details of this nutritional dependency can often be revealed by following the colonization of genetic mutants that are defective either in specific biosynthetic or metabolic pathways[1], or in the ability to adjust to changing nutrient levels[102].

A central question in the *Vibrio*–squid symbiosis is: what does the host feed its bacterial symbionts and does this change over the course of the relationship?

Auxotroph
An organism, or mutant derivative, which is unable to synthesize a particular compound (for example, an amino acid) that is required as a building block for its growth.

Mutant library screening for auxotrophs of *V. fischeri* yielded a collection of strains that were defective in the biosynthesis of specific amino acids[103]. The colonization levels that were achieved by these mutants provided a relative measure of the extent to which the host provided each of these amino acids. The resulting pattern suggested that peptides of a typical protein composition were available to the symbiont population[103]. By contrast, a similar study of nematode colonization by *Xenorhabdus* auxotrophs indicated that two specific amino acids, methionine and threonine, were of limited availability in the vesicle[88]. Supporting this conclusion, the *Xenorhabdus* crystal protein PixA is methionine rich, and a *pixA* mutant out-competes its parent for growth in the vesicle, which suggests that excess methionine synthesis is a metabolic drain on the symbiont[104]. Because colonization by *Xenorhabdus* spp. requires some or all of the *isc–hsc–fdx* locus, which encodes activities that synthesize [Fe–S] centres, it is possible that this bacterium depends on some non-haem redox activity, such as anaerobic respiration, when in the host[105]. Interestingly, studies of the squid–*Vibrio* association also suggest that the generation of acetate[73] and metabolic pathways which require anaerobic respiration[106] are important to *V. fischeri* in the light organ.

As is typical for pathogenic infections[107], free iron seems to be the main inorganic nutrient that limits growth in beneficial animal symbioses. Several studies have focused on identifying the genetic determinants of iron acquisition that allow symbiotic bacteria to colonize their hosts. In *V. fischeri*, a mutation in the *glnD* gene results in pleiotropic phenotypes, including reduced production of at least one siderophore in culture[84]. When assayed for colonization, the *glnD* mutant failed to persist, but this defect was reversed when ferric chloride was added to the assay. Similarly, the addition of iron rescued colonization by a putative siderophore-uptake mutant (*exbD*) of a *Photorhabdus* species[108]. A putative iron-regulated promoter element has also been detected upstream of *ngrA*[39], a gene that could function to synthesize either a peptide signal or siderophore[109], which further indicates that iron levels are important to *Photorhabdus* species. Thus, the availability of iron can have both nutritional and regulatory implications.

Conclusions

The molecular genetics of symbiosis is a rapidly expanding field, and new systems are continually adding to its breadth and impact. It is important to continue to develop systems such as the earthworm nephridia–*Acidovorax* symbiosis[110,111], the caterpillar gut–*Enterococcus* association[51], the as-yet-uncultured epithelial symbionts of hydra[112] and the recently described louse fly–*Arsenophonus* relationship[113]. One particularly promising system is that between a *Burkholderia* species and its host, the broad-headed stink-bug[114]. In this association the bacterium is environmentally acquired at each generation, provides a clear benefit to the host and is extracellularly located[115]. A successful history of molecular studies of other *Burkholderia* species makes it highly likely that it will not be long before the symbiont will be amenable to molecular genetics.

The development of experimental strategies and technological approaches will be important to the continued growth of the field of symbiosis. Creating a community of researchers that can develop genomic (for example, genomes and microarrays) and molecular genetic (for example, mutants, vectors and methodology) resources, either within a single species, or more generally across the field, will quicken the pace of advance in all systems. Similarly, in addition to increasing the number of bacterial symbionts with sequenced genomes (TABLE 2), efforts to obtain genome sequences for different strains of the same symbiont species have proven valuable for comparative studies[28].

In most symbioses described here[14], the application of molecular genetics to the animal partner lags behind efforts with the bacterial symbiont. Nevertheless, in the past year there have been promising results in the host using a reverse-genetic RNA interference approach to silence specific genes[116] and with the application of microarray technology to determine the differential effect of colonization by mutant symbionts on host gene expression[33]. Future studies should focus on developing interactive genomics to track patterns of gene expression simultaneously in both the host and the symbiont. In this way we will not only be able to interrogate this conversation, but perhaps learn how to manipulate it.

1. Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A. & Gordon, J. I. Host–bacterial mutualism in the human intestine. *Science* **307**, 1915–1920 (2005).
2. Haygood, M. G. Light organ symbioses in fishes. *Crit. Rev. Microbiol.* **19**, 191–216 (1993).
3. Parniske, M. Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature Rev. Microbiol.* **6**, 763–775 (2008)
4. Werren, J. H., Baldo, L. & Clark, M. E. *Wolbachia*: master manipulators of invertebrate biology. *Nature Rev. Microbiol.* **6**, 741–751 (2008).
5. Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R. & Gordon, J. I. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nature Rev. Microbiol.* **6**, 776–788 (2008).
6. Dethlefsen, L., McFall-Ngai, M. & Relman, D. A. An ecological and evolutionary perspective on human–microbe mutualism and disease. *Nature* **449**, 811–818 (2007).
7. Ley, R. E., Peterson, D. A. & Gordon, J. I. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* **124**, 837–848 (2006).
8. Samuel, B. S. & Gordon, J. I. A humanized gnotobiotic mouse model of host–archaeal–bacterial mutualism. *Proc. Natl Acad. Sci. USA* **103**, 10011–10016 (2006).
9. Nicholson, J. K., Holmes, E. & Wilson, I. D. Gut microorganisms, mammalian metabolism and personal heath care. *Nature Rev. Microbiol.* **3**, 431–438 (2005).
10. Peterson, D. A., McNulty, N. P., Guruge, J. L. & Gordon, J. I. IgA response to symbiotic bacteria as a mediator of gut homeostasis. *Cell Host Microbe* **2**, 328–339 (2007).
11. Turnbaugh, P. J. *et al.* An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**, 1027–1031 (2006).
12. Vaishnava, S., Behrendt, C. L. & Hooper, L. V. Innate immune responses to commensal bacteria in the gut epithelium. *J. Pediatr. Gastroenterol. Nutr.* **46** (Suppl. 1), E10–E11 (2008).
13. Graf, J., Kikuchi, Y. & Rio, R. V. Leeches and their microbiota: naturally simple symbiosis models. *Trends Microbiol.* **14**, 365–371 (2006).
14. Cox, C. R. & Gilmore, M. S. Native microbial colonization of *Drosophila melanogaster* and its use as a model of *Enterococcus faecalis* pathogenesis. *Infect. Immun.* **75**, 1565–1576 (2007).
15. McFall-Ngai, M. J. & Gordon, J. I. in *Evolution of Microbial Virulence* (eds Seifert, H. & DiRita, V. J.) 147–166 (ASM, Washington DC, 2006).
16. Goodrich-Blair, H. & Clarke, D. J. Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: two roads to the same destination. *Mol. Microbiol.* **64**, 260–268 (2007).
17. McFall-Ngai, M. Adaptive immunity: care for the community. *Nature* **445**, 153 (2007).
18. Moran, N. A. Bacterial menageries inside insects. *Proc. Natl Acad. Sci. USA* **98**, 1338–1340 (2001).
19. Nealson, K. H. & Hastings, J. W. in *The Prokaryotes, a Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications* 2nd edn (eds Balows, A., Truper, H. G., Dworkin, M., Harder, W. & Schleifer, K. H.) 1332–1345 (Springer, Berlin, 1991).

20. Fidopiastis, P. M., von Boletzky, S. & Ruby, E. G. A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus *Sepiola*. *J. Bacteriol.* **180**, 59–64 (1998).
21. Nishiguchi, M. K. & Nair, V. S. Evolution of symbiosis in the Vibrionaceae: a combined approach using molecules and physiology. *Int. J. Syst. Evol. Microbiol.* **53**, 2019–2026 (2003).
22. Jones, B. W. & Nishiguchi, M. K. Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* Berry (Mollusca:Cephalopoda). *Mar. Biol.* **144**, 1151–1155 (2004).
23. Wei, S. L. & Young, R. E. Development of symbiotic bacterial luminescence in a nearshore cephalopod, *Euprymna scolopes*. *Mar. Biol.* **103**, 541–546 (1989).
24. Ruby, E. G. & Lee, K. H. The *Vibrio fischeri–Euprymna scolopes* light organ association: current ecological paradigms. *Appl. Environ. Microbiol.* **64**, 805–812 (1998).
25. Claes, M. F. & Dunlap, P. V. 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 (2000).
26. Dunn, A. K., Millikan, D. S., Adin, D. M., Bose, J. L. & Stabb, E. V. New *rfp*- and pES213-derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and *lux* expression *in situ*. *Appl. Environ. Microbiol.* **72**, 802–810 (2006).
27. Stabb, E. V. & Ruby, E. G. RP4-based plasmids for conjugation between *Escherichia coli* and members of the Vibrionaceae. *Methods Enzymol.* **358**, 413–426 (2002).
28. Mandel, M. J., Stabb, E. V. & Ruby, E. G. Comparative genomics-based investigation of resequencing targets in *Vibrio fischeri*: focus on point miscalls and artefactual expansions. *BMC Genomics* **9**, 138 (2008).
Introduced novel technological approaches to apply a comparative genomics approach to two strains of a beneficial bacterial symbiont.
29. Ruby, E. G. *et al.* Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. *Proc. Natl Acad. Sci. USA* **102**, 3004–3009 (2005).
Sequencing of the *V. fischeri* genome, which allowed molecular genetics to be applied to the squid–*Vibrio* system and opened up new approaches of genetic analysis.
30. Antunes, L. C. *et al.* Transcriptome analysis of the *Vibrio fischeri* LuxR–LuxI regulon. *J. Bacteriol.* **189**, 8387–8391 (2007).
31. Lupp, C. & Ruby, E. G. *Vibrio fischeri* uses two quorum-sensing systems for the regulation of early and late colonization factors. *J. Bacteriol.* **187**, 3620–3629 (2005).
32. Chun, C. K. *et al.* An annotated cDNA library of juvenile *Euprymna scolopes* with and without colonization by the symbiont *Vibrio fischeri*. *BMC Genomics* **7**, 154 (2006).
33. Chun, C. *et al.* Effects of colonization, luminescence, and autoinducer on host transcription during development of the squid–vibrio association. *Proc. Natl Acad. Sci. USA* 5 Aug 2008 (doi:10.1073/pnas.0802369105).
First large-scale transcriptional analysis of the host response to colonization by bacteria that possess mutations in their symbiosis genes.
34. Herbert, E. E. & Goodrich-Blair, H. Friend and foe: the two faces of *Xenorhabdus nematophila*. *Nature Rev. Microbiol.* **5**, 634–646 (2007).
35. Hallem, E. A., Rengarajan, M., Ciche, T. A. & Sternberg, P. W. Nematodes, bacteria, and flies: a tripartite model for nematode parasitism. *Curr. Biol.* **17**, 898–904 (2007).
36. Ciche, T. A., Kim, K. S., Kaufmann-Daszczuk, B., Nguyen, K. C. & Hall, D. H. Cell invasion and matricide during *Photorhabdus luminescens* transmission by *Heterorhabditis bacteriophora* nematodes. *Appl. Environ. Microbiol.* **74**, 2275–2287 (2008).
The first study to focus on development of the host during an association with a nematode.
37. Cowles, C. E. & Goodrich-Blair, H. The *Xenorhabdus nematophila nilABC* genes confer the ability of *Xenorhabdus* spp. to colonize *Steinernema carpocapsae* nematodes. *J. Bacteriol.* **190**, 4121–4128 (2008).
38. Goodrich-Blair, H. They've got a ticket to ride: *Xenorhabdus nematophila–Steinernema carpocapsae* symbiosis. *Curr. Opin. Microbiol.* **10**, 225–230 (2007).
39. Joyce, S. A., Watson, R. J. & Clarke, D. J. The regulation of pathogenicity and mutualism in *Photorhabdus*. *Curr. Opin. Microbiol.* **9**, 127–132 (2006).
40. Dale, C. & Moran, N. A. Molecular interactions between bacterial symbionts and their hosts. *Cell* **126**, 453–465 (2006).
41. Moran, N. A. Symbiosis. *Curr. Biol.* **16**, R866–R871 (2006).
42. Toh, H. *et al.* Massive genome erosion and functional adaptations provide insights into the symbiotic lifestyle of *Sodalis glossinidius* in the tsetse host. *Genome Res.* **16**, 149–156 (2006).
43. Weiss, B. L. *et al.* Interspecific transfer of bacterial endosymbionts between tsetse fly species: infection establishment and effect on host fitness. *Appl. Environ. Microbiol.* **72**, 7013–7021 (2006).
44. Rio, R. V., Lefevre, C., Heddi, A. & Aksoy, S. Comparative genomics of insect-symbiotic bacteria: influence of host environment on microbial genome composition. *Appl. Environ. Microbiol.* **69**, 6825–6832 (2003).
45. Warnecke, F. *et al.* Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature* **450**, 560–565 (2007).
46. Broderick, N. A., Raffa, K. F., Goodman, R. M. & Handelsman, J. Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Appl. Environ. Microbiol.* **70**, 293–300 (2004).
47. Indergand, S. & Graf, J. Ingested blood contributes to the specificity of the symbiosis of *Aeromonas veronii* biovar sobria and *Hirudo medicinalis*, the medicinal leech. *Appl. Environ. Microbiol.* **66**, 4735–4741 (2000).
48. Silver, A. C. *et al.* Interaction between innate immune cells and a bacterial type III secretion system in mutualistic and pathogenic associations. *Proc. Natl Acad. Sci. USA* **104**, 9481–9486 (2007).
Discovery of a T3SS in a beneficial bacteria–animal symbiosis, and described one of its functions.
49. Silver, A. C., Rabinowitz, N. M., Kuffer, S. & Graf, J. Identification of *Aeromonas veronii* genes required for colonization of the medicinal leech, *Hirudo verbana*. *J. Bacteriol.* **189**, 6763–6772 (2007).
50. Kikuchi, Y. & Graf, J. Spatial and temporal population dynamics of a naturally occurring two-species microbial community inside the digestive tract of the medicinal leech. *Appl. Environ. Microbiol.* **73**, 1984–1991 (2007).
51. Broderick, N. A., Raffa, K. F. & Handelsman, J. Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. *Proc. Natl Acad. Sci. USA* **103**, 15196–15199 (2006).
52. Falkow, S. Molecular Koch's postulates applied to bacterial pathogenicity — a personal recollection 15 years later. *Nature Rev. Microbiol.* **2**, 67–72 (2004).
53. Heungens, K., Cowles, C. E. & Goodrich-Blair, H. Identification of *Xenorhabdus nematophila* genes required for mutualistic colonization of *Steinernema carpocapsae* nematodes. *Mol. Microbiol.* **45**, 1337–1353 (2002).
One of the first applications of an advanced genetic screen for bacterial colonization factors in an animal symbiont.
54. Nyholm, S. V., Stabb, E. V., Ruby, E. G. & McFall-Ngai, M. J. Establishment of an animal–bacterial association: recruiting symbiotic vibrios from the environment. *Proc. Natl Acad. Sci. USA* **97**, 10231–10235 (2000).
55. Hussa, E. A., O'Shea, T. M., Darnell, C. L., Ruby, E. G. & Visick, K. L. Two-component response regulators of *Vibrio fischeri:* identification, mutagenesis, and characterization. *J. Bacteriol.* **189**, 5825–5838 (2007).
56. Yip, E. S., Geszvain, K., Deloney-Marino, C. R. & Visick, K. L. The symbiosis regulator RscS controls the *syp* gene locus, biofilm formation and symbiotic aggregation by *Vibrio fischeri*. *Mol. Microbiol.* **52**, 1586–1600 (2006).
Provided a breakthrough in our understanding of the regulation of genes that are involved in symbiosis initiation in the squid–*Vibrio* association.
57. Darnell, C. L., Hussa, E. A. & Visick, K. L. The putative hybrid sensor kinase SypF coordinates biofilm formation in *Vibrio fischeri* by acting upstream of two response regulators, SypG and VpsR. *J. Bacteriol.* **190**, 4941–4950 (2008).
58. Geszvain, K. & Visick, K. L. Roles of bacterial regulators in the symbiosis between *Vibrio fischeri* and *Euprymna scolopes*. *Prog. Mol. Subcell. Biol.* **41**, 277–290 (2006).
59. DeLoney, C. R., Bartley, T. M. & Visick, K. L. Role for phosphoglucomutase in *Vibrio fischeri–Euprymna scolopes* symbiosis. *J. Bacteriol.* **184**, 5121–5129 (2002).
60. Adin, D. M. *et al.* Characterization of *htrB* and *msbB* mutants of the light organ symbiont *Vibrio fischeri*. *Appl. Environ. Microbiol.* **74**, 633–644 (2007).
61. Gunn, J. S., Ryan, S. S., Van Velkinburgh, J. C., Ernst, R. K. & Miller, S. I. Genetic and functional analysis of a PmrA–PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar Typhimurium. *Infect. Immun.* **68**, 6139–6146 (2000).
62. Bennett, H. P. & Clarke, D. J. The *pbgPE* operon in *Photorhabdus luminescens* is required for pathogenicity and symbiosis. *J. Bacteriol.* **187**, 77–84 (2005).
63. Gunn, J. S. *et al.* PmrA–PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Mol. Microbiol.* **27**, 1171–1182 (1998).
64. Braschler, T. R., Merino, S., Tomas, J. M. & Graf, J. Complement resistance is essential for colonization of the digestive tract of *Hirudo medicinalis* by *Aeromonas* strains. *Appl. Environ. Microbiol.* **69**, 4268–4271 (2003).
65. Aeckersberg, F., Lupp, C., Feliciano, B. & Ruby, E. G. *Vibrio fischeri* outer membrane protein OmpU plays a role in normal symbiotic colonization. *J. Bacteriol.* **183**, 6590–6597 (2001).
66. Stabb, E. V. & Ruby, E. G. Contribution of *pilA* to competitive colonization of the squid *Euprymna scolopes* by *Vibrio fischeri*. *Appl. Environ. Microbiol.* **69**, 820–826 (2003).
67. Lee, K.-H. & Ruby, E. G. Detection of the light organ symbiont, *Vibrio fischeri*, in Hawaiian seawater by using *lux* gene probes. *Appl. Environ. Microbiol.* **58**, 942–947 (1992).
68. Lee, K.-H. & Ruby, E. G. Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. *Appl. Environ. Microbiol.* **60**, 1565–1571 (1994).
69. He, H., Snyder, H. A. & Forst, S. Unique organization and regulation of the *mrx* fimbrial operon in *Xenorhabdus nematophila*. *Microbiology* **150**, 1439–1446 (2004).
70. Fuqua, C. & Greenberg, E. P. Listening in on bacteria: acyl-homoserine lactone signalling. *Nature Rev. Mol. Cell Biol.* **3**, 685–695 (2002).
71. Alegado, R. A., Campbell, M. C., Chen, W. C., Slutz, S. S. & Tan, M. W. Characterization of mediators of microbial virulence and innate immunity using the *Caenorhabditis elegans* host–pathogen model. *Cell Microbiol.* **5**, 435–444 (2003).
72. Lupp, C. & Ruby, E. G. *Vibrio fischeri* LuxS and AinS: comparative study of two signal synthases. *J. Bacteriol.* **186**, 3873–3881 (2004).
73. Studer, S. V., Mandel, M. J. & Ruby, E. G. AinS quorum sensing regulates the *Vibrio fischeri* acetate switch. *J. Bacteriol.* **190**, 5915–5923 (2008).
74. Wolfe, A. J. The acetate switch. *Microbiol. Mol. Biol. Rev.* **69**, 12–50 (2005).
75. Callahan, S. M. & Dunlap, P. V. LuxR- and acyl-homoserine-lactone-controlled non-*lux* genes define a quorum-sensing regulon in *Vibrio fischeri*. *J. Bacteriol.* **182**, 2811–2822 (2000).
76. Visick, K. L., Foster, J., Doino, J., McFall-Ngai, M. & Ruby, E. G. *Vibrio fischeri lux* genes play an important role in colonization and development of the host light organ. *J. Bacteriol.* **182**, 4578–4586 (2000).
A key paper that links the production of luminescence to both the induction of normal host development and the capacity for persistent colonization.
77. Boettcher, K. J. & Ruby, E. G. Detection and quantification of *Vibrio fischeri* autoinducer from symbiotic squid light organs. *J. Bacteriol.* **177**, 1053–1058 (1995).
78. Sanchez-Contreras, M., Bauer, W. D., Gao, M., Robinson, J. B. & Allan Downie, J. Quorum-sensing regulation in rhizobia and its role in symbiotic interactions with legumes. *Philos. Trans. R. Soc. Lond. B* **362**, 1149–1163 (2007).

79. You, J., Liang, S., Cao, L., Liu, X. & Han, R. Nutritive significance of crystalline inclusion proteins of *Photorhabdus luminescens* in *Steinernema* nematodes. *FEMS Microbiol. Ecol.* **55**, 178–185 (2006).
80. Joyce, S. A. & Clarke, D. J. A *hexA* homologue from *Photorhabdus* regulates pathogenicity, symbiosis and phenotypic variation. *Mol. Microbiol.* **47**, 1445–1457 (2003).
81. Wolfe, A. J., Millikan, D. S., Campbell, J. M. & Visick, K. L. *Vibrio fischeri* σ^{54} controls motility, biofilm formation, luminescence, and colonization. *Appl. Environ. Microbiol.* **70**, 2520–2524 (2004).
82. DeLoney-Marino, C. R., Wolfe, A. J. & Visick, K. L. Chemoattraction of *Vibrio fischeri* to serine, nucleosides, and *N*-acetylneuraminic acid, a component of squid light-organ mucus. *Appl. Environ. Microbiol.* **69**, 7527–7530 (2003).
83. Graf, J., Dunlap, P. V. & Ruby, E. G. Effect of transposon-induced motility mutations on colonization of the host light organ by *Vibrio fischeri*. *J. Bacteriol.* **176**, 6986–6991 (1994).
84. Graf, J. & Ruby, E. G. Novel effects of a transposon insertion in the *Vibrio fischeri glnD* gene: defects in iron uptake and symbiotic persistence, as well as nitrogen utilization. *Mol. Microbiol.* **37**, 168–179 (2000).
85. Bose, J. L. *et al.* Bioluminescence in *Vibrio fischeri* is controlled by the redox-responsive regulator ArcA. *Mol. Microbiol.* **65**, 538–553 (2007).
86. Dale, C., Young, S. A., Haydon, D. T. & Welburn, S. C. The insect endosymbiont *Sodalis glossinidius* utilizes a type III secretion system for cell invasion. *Proc. Natl Acad. Sci. USA* **98**, 1883–1888 (2001).
87. Visick, K. L. & Ruby, E. G. Tn*luxAB* insertion mutants of *Vibrio fischeri* with symbiosis-regulated phenotypes. *Nova Acta Leopoldina* **333**, 93–100 (2003).
88. Martens, E. C., Russell, F. M. & Goodrich-Blair, H. Analysis of *Xenorhabdus nematophila* metabolic mutants yields insight into stages of *Steinernema carpocapsae* nematode intestinal colonization. *Mol. Microbiol.* **58**, 28–45 (2005).
89. Davidson, S. K., Koropatnick, T. A., Kossmehl, R., Sycuro, L. & McFall-Ngai, M. J. NO means 'yes' in the squid–vibrio symbiosis: nitric oxide (NO) during the initial stages of a beneficial association. *Cell. Microbiol.* **6**, 1139–1151 (2004).
90. Visick, K. L. & Ruby, E. G. The periplasmic, group III catalase of *Vibrio fischeri* is required for normal symbiotic competence and is induced both by oxidative stress and approach to stationary phase. *J. Bacteriol.* **180**, 2087–2092 (1998).
91. Krin, E. *et al.* Pleiotropic role of quorum-sensing autoinducer 2 in *Photorhabdus luminescens*. *Appl. Environ. Microbiol.* **72**, 6439–6451 (2006).
92. Rio, R. V., Anderegg, M. & Graf, J. Characterization of a catalase gene from *Aeromonas veronii*, the digestive-tract symbiont of the medicinal leech. *Microbiology* **153**, 1897–1906 (2007).
93. Dale, C., Jones, T. & Pontes, M. Degenerative evolution and functional diversification of type-III secretion systems in the insect endosymbiont *Sodalis glossinidius*. *Mol. Biol. Evol.* **22**, 758–766 (2005).
94. Brugirard-Ricaud, K. *et al.* Site-specific antiphagocytic function of the *Photorhabdus luminescens* type III secretion system during insect colonization. *Cell. Microbiol.* **7**, 363–371 (2005).
95. Nyholm, S. V. & McFall-Ngai, M. J. The winnowing: establishing the squid-*Vibrio* symbiosis. *Nature Rev. Microbiol.* **2**, 632–642 (2004).
96. Stappenbeck, T. S., Hooper, L. V. & Gordon, J. I. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proc. Natl Acad. Sci. USA* **99**, 15451–15455 (2002).
97. Ruby, E. G. & McFall-Ngai, M. J. Oxygen-utilizing reactions and symbiotic colonization of the squid light organ by *Vibrio fischeri*. *Trends Microbiol.* **7**, 414–420 (1999).
98. Koropatnick, T. A. *et al.* Microbial factor-mediated development in a host–bacterial mutualism. *Science* **306**, 1186–1188 (2004).
Developed a new paradigm by showing that a bacterial toxin serves as a required developmental signal compound in a beneficial host–microorganism association.
99. Whistler, C. A. & Ruby, E. G. GacA regulates symbiotic colonization traits of *Vibrio fischeri* and facilitates a beneficial association with an animal host. *J. Bacteriol.* **185**, 7202–7212 (2003).
100. Whistler, C. A., Koropatnick, T. A., Pollack, A., McFall-Ngai, M. J. & Ruby, E. G. The GacA global regulator of *Vibrio fischeri* is required for normal host tissue responses that limit subsequent bacterial colonization. *Cell. Microbiol.* **9**, 766–778 (2007).
101. Faraldo-Gomez, J. D. & Sansom, M. S. Acquisition of siderophores in Gram-negative bacteria. *Nature Rev. Mol. Cell Biol.* **4**, 105–116 (2003).
102. Cowles, K. N., Cowles, C. E., Richards, G. R., Martens, E. C. & Goodrich-Blair, H. The global regulator Lrp contributes to mutualism, pathogenesis and phenotypic variation in the bacterium *Xenorhabdus nematophila*. *Cell. Microbiol.* **9**, 1311–1323 (2007).
103. Graf, J. & Ruby, E. G. Host-derived amino acids support the proliferation of symbiotic bacteria. *Proc. Natl Acad. Sci. USA* **95**, 1818–1822 (1998).
104. Goetsch, M., Owen, H., Goldman, B. & Forst, S. Analysis of the PixA inclusion body protein of *Xenorhabdus nematophila*. *J. Bacteriol.* **188**, 2706–2710 (2006).
105. Martens, E. C. *et al. Xenorhabdus nematophila* requires an intact *iscRSUA–hscBA–fdx* operon to colonize *Steinernema carpocapsae* nematodes. *J. Bacteriol.* **185**, 3678–3682 (2003).
106. Dunn, A. K. & Stabb, E. V. The twin arginine translocation system contributes to symbiotic colonization of *Euprymna scolopes* by *Vibrio fischeri*. *FEMS Microbiol. Lett.* **279**, 251–258 (2008).
107. Schaible, U. E. & Kaufmann, S. H. Iron and microbial infection. *Nature Rev. Microbiol.* **2**, 946–953 (2004).
108. Watson, R. J., Joyce, S. A., Spencer, G. V. & Clarke, D. J. The *exbD* gene of *Photorhabdus temperata* is required for full virulence in insects and symbiosis with the nematode *Heterorhabditis*. *Mol. Microbiol.* **56**, 763–773 (2005).
109. Crosa, J. H. & Walsh, C. T. Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. *Microbiol. Mol. Biol. Rev.* **66**, 223–249 (2002).
110. Davidson, S. K. & Stahl, D. A. Transmission of nephridial bacteria of the earthworm *Eisenia fetida*. *Appl. Environ. Microbiol.* **72**, 769–775 (2006).
111. Davidson, S. K. & Stahl, D. A. Selective recruitment of bacteria during embryogenesis of an earthworm. *ISME J.* **2**, 510–518 (2008).
112. Fraune, S. & Bosch, T. C. Long-term maintenance of species-specific bacterial microbiota in the basal metazoan *Hydra*. *Proc. Natl Acad. Sci. USA* **104**, 13146–13151 (2007).
113. Dale, C., Beeton, M., Harbison, C., Jones, T. & Pontes, M. Isolation, pure culture, and characterization of "*Candidatus* Arsenophonus arthropodicus," an intracellular secondary endosymbiont from the hippoboscid louse fly *Pseudolynchia canariensis*. *Appl. Environ. Microbiol.* **72**, 2997–3004 (2006).
114. Kikuchi, Y., Meng, X. Y. & Fukatsu, T. Gut symbiotic bacteria of the genus *Burkholderia* in the broad-headed bugs *Riptortus clavatus* and *Leptocorisa chinensis* (Heteroptera: Alydidae). *Appl. Environ. Microbiol.* **71**, 4035–4043 (2005).
115. Kikuchi, Y., Hosokawa, T. & Fukatsu, T. Insect–microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. *Appl. Environ. Microbiol.* **73**, 4308–4316 (2007).
Introduced an emerging experimental symbiosis system that had intriguing parallels with the squid–*Vibrio* association.
116. Ciche, T. A. & Sternberg, P. W. Postembryonic RNAi in *Heterorhabditis bacteriophora*: a nematode insect parasite and host for insect pathogenic symbionts. *BMC Dev. Biol.* **7**, 101 (2007).

Acknowledgements
The author thanks M. McFall-Ngai for helpful discussions and ideas, and H. Goodrich-Blair, J. Graf and M. Mandel for reading parts of the manuscript. Support was provided by grants from the National Institutes of Health (grant number RR-12294) and the National Science Foundation (grant number IOB-0517007).

DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
acs | *ainS* | *gacA* | *glnD* | *htrB1* | *luxA* | *ompU* | *pgm* | *rpoN* | *rscS*
Entrez Genome Project: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj
Drosophila melanogaster | *Enterococcus faecalis* | *Escherichia coli* | *Sodalis glossinidius* | *Vibrio fischeri* | *Yersinia pestis*

FURTHER INFORMATION
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