

# Models and approaches to dissect host–symbiont specificity

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**Animals are symbiotic superorganisms, composed of eukaryotic cells and specific microbial residents that perform essential functions for their host. As humans, we are beginning to appreciate the diversity and function of our own microbiota, but model systems are leading the field in illustrating the molecular mechanisms that allow specific relationships to be recapitulated during each host generation. This review focuses on models in which genetic screens, coupled with genomics, imaging, phylogenetics and population biology, have begun to allow a remarkably detailed investigation into the molecular dissection of the evolution of host specificity in animal symbionts.**

## Specificity in symbiotic bacteria

Many bacterial symbiotic associations exhibit a pattern in which the partners are nonrandom, and the same collaborations are repeatedly reconvened [1]. Patterns that dictate host range or host specificity are observed in both beneficial (mutualistic) and detrimental (pathogenic) symbiotic associations (see Glossary). Some bacterial species colonize a single host, and in some cases strong evidence has been collected to suggest a basis for this restriction. For instance, host-restricted pathogenic symbionts have been shown to be capable of using an essential nutrient from their co-adapted host. *Neisseria gonorrhoea* (pathogen of humans), *Mannheimia haemolytica* (cows) and *Actinobacillus pleuropneumoniae* (pigs) each use transferrin only from their cognate host as an iron source [2]. In most cases, however, the mechanisms governing the patterns of host–symbiont associations are largely unknown.

To understand the general trends that govern host and symbiont specificity, it is necessary to first understand the specific host and symbiont genes, proteins, and pathways at play. Possible topics for study include the relative roles played by the microbe versus those played by the host; the effects of microbe–microbe competition on bacterial symbiotic capability and host fitness, and the importance of physical or environmental factors in the outcome of symbiotic initiation. In this article, I review recent literature that has provided novel insights into this fundamental question of specific transmission of symbiotic microbes.

## Transmission of symbiotic microbes

Specificity and selection dictate the acquisition and maintenance of symbiotic partners in beneficial relationships between eukaryotic hosts and their microbial symbionts. Two primary methods of symbiont acquisition have been

described: vertical and horizontal. Vertical transmission describes a situation in which microbial partners are passed from parent to offspring directly. Often, vertically transmitted symbionts reside within the cells of an animal host (as endosymbionts); for example the association between aphids and their endosymbiotic microbiota [4]. Many vertically transmitted symbionts do not have a corresponding environmental niche, and have consequently undergone significant genome reduction and are entirely codependent with their animal hosts. Hosts of endosymbionts have evolved organs to house and support the growth of the endosymbionts in these conditions, and the symbiont might be involved in influencing its transmission [4,5].

By contrast, during horizontal transmission, there is an environmental stage between successive host generations. For example, human infants are born aposymbiotically (lacking symbionts) [6], and begin at birth to obtain their symbiotic microorganisms. For animals that acquire their symbionts horizontally, each generation raises the same challenge for both the host and the microbe: how can the

## Glossary

**Aposymbiotic:** without symbionts, differs from axenic in that microbes might still be present, but not the specific symbiont, or not in the symbiotic organ.

**Axenic:** germ-free.

**CFU (colony forming units):** the number of culturable bacteria in a sample, as can be enumerated by counting on agar plates.

**Entomopathogenic:** parasitic on insects.

**Gnotobiotic:** from the Greek *gnostos* and *bios* (known life), axenic animals are exposed to specific symbionts or conditions.

**Mutualism:** symbiosis in which benefits are conferred to all partners.

**Pathogen:** the partner that causes harm in pathogenesis.

**Pathogenesis:** symbiosis in which at least one of the partners is harmed by the association.

**Specificity factor:** symbiosis factor that is both necessary and sufficient for associating with a host.

**Symbiosis:** close, prolonged association between two or more different organisms of different species, which might, but does not necessarily, benefit both members. From the Greek *symbioun*, to live together (adapted from [3]).

**Symbiosis factor:** a symbiotic molecule that is necessary for normal symbiotic development.

**Symbiosis specificity:** processes during symbiotic development that contribute to naturally reproducible patterns of symbiotic association.

***Vibrio fischeri* ES114 (VF<sub>ES114</sub>):** mutualist of Hawaiian bobtail squid *Euprymna scolopes*.

***Vibrio fischeri* MJ11 (VF<sub>MJ11</sub>):** mutualist of Japanese pinecone fish *Monocentris japonica*.

**Virulence factor:** a symbiotic molecule that is necessary for pathogenic development.

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### Box 1. Reciprocal experiments in vertebrates reveal host constraint

A study published in 2006 began to examine the mechanisms underlying the patterns of specificity that are observed in vertebrate symbionts [8]. In particular, the study sought to address whether the reproducible microbiota in a given vertebrate could be attributable to history (legacy effects) or to selection (gut habitat effects). The authors characterized the gut microbiota of zebrafish and mice, and identified six divisions (Proteobacteria, Firmicutes, Bacteroidetes, Verrucomicrobia, Actinobacteria and TM7) that are shared between the two animals. Importantly, the relative proportion of each division was distinct between fish and mouse, and the individual constituents of each division differed. This allowed the authors not only to track the abundance of divisions that are shared in the two organisms, but also to measure the contribution of the host in influencing the individuals that flourished after the manipulations [8].

The experimental design proceeded with reciprocal gnotobiotic transplantations: gut microbiota from zebrafish were transplanted into germ-free mice, and gut microbiota from mice were transplanted into germ-free zebrafish. The resulting communities were then analyzed by performing 16S rRNA sequencing on cecal contents [8].

The transplanted communities shared an evolutionary history with their original populations, which is an important control. However, the relative abundance of the above six divisions had changed significantly in the new hosts. Zebrafish typically have >75% of their gut community as Proteobacteria and <1% as Firmicutes; however, when this community was transplanted into the mouse, the resulting communities contained >50% Firmicutes, which more strongly resembled the community structure in mice [8]. Similarly, mice typically have approximately 5% Proteobacteria, which expanded to 20% upon transplantation into zebrafish [8]. This study demonstrated that in these distantly related vertebrates, the host environment plays an important role in determining the microbial constituency. The results begin to explain how humans are reliably colonized by organisms that express similar functions [9].

correct relationships be recapitulated, and how can harmful or unhelpful relationships be avoided? The partners meet these challenges in spite of the obstacles involved, with bacterial and host factors playing roles to ensure fidelity during transmission.

The focus of this article is on the selection for the correct symbiont during a new host generation, and the challenges inherent in horizontally transmitted microorganisms. Selection for the correct symbiotic microbes (or from a microbial perspective, selection for the right host niche) extends deeper than the level of individual hosts as there is strong evidence of tissue tropism at the phylum level. Microbiome studies have revealed, for instance, that 95% of the bacteria in our colon are Firmicutes or Bacteroidetes, whereas 70% of the bacteria in our skin are Actinobacteria or Proteobacteria [7]. Transplantation studies by Rawls *et al.* [8] have provided clear evidence for a host role in shaping the microbial constituency of the complex gut communities in fish and mice (Box 1).

In many cases, host or symbiont factors that contribute to specificity have been identified. Hooper *et al.* [10] demonstrated that presentation of fucosylated sugars in early stages of mouse colonization is dependent upon specific bacterial colonization. However, studies that have directly demonstrated host specificity factors through expanding the host range of a bacterial taxon (species or strain) by transferring genes from a colonization-competent taxon have been extremely limited. Such a demonstration of sufficiency is crucial for defining a specificity factor, and relies on an appropriate study system in which to conduct

the experiments. Recently, factors that control host range specificity have been identified and characterized in this manner in two model organisms: the Gram-negative mutualists *Xenorhabdus nematophila* and *Vibrio fischeri*. The sections below detail the historical context and initial discovery of these factors in forward genetic screens, and the subsequent discoveries that these genes play important roles in determining host colonization specificity. Importantly, in both models, the factors involved have been shown to be both necessary and sufficient for specific host colonization, marking important advances in our understanding of the evolution of symbiotic development.

### Historical perspectives

Insights into the molecular basis of host specificity have been largely achieved through the study of beneficial microbes that colonize plant roots. Studies in the *Rhizobiaceae* have demonstrated the molecular basis of specific host association, and identified genetic factors that allow microbes to establish host preference, most notably the bacterially derived Nod factors (Box 2).

The ability of Nod factors to be both necessary for symbiotic colonization and sufficient to confer specificity for a given host garnered special distinction in the field of host-microbe interactions. Among the myriad virulence factors or symbiosis factors that are necessary for normal host interaction, most act in concert with other factors, whereas very few have been shown to be sufficient to confer the phenotype in a naïve, or nonspecific, symbiont. Studies in rhizobia set the paradigm for specificity in microbe-host interaction studies, and the following features make the associations between nodulating bacteria and their cognate hosts superbly amenable to studies of microbial transmission specificity.

- (i) Monospecificity. One symbiont and one host provided reductionism in which to analyze the key interactions.
- (ii) Natural hosts. Rather than creating a model system, the model systems were the natural partners themselves, allowing for the full range of signal transduction between host and microbe to be accessible to investigation.
- (iii) Diversity and phylogenetics. Multiple strains and species (of both host and symbiont) were examined, and the evolution of the signaling system informed the interpretation of the mechanism.
- (iv) Genetic approaches, complemented by biochemistry. After the genes were cloned, intensive efforts by multiple groups identified the compounds involved.
- (v) Study of both partners to reveal interdomain signaling. General host molecules elicit a specific bacterial response, which interacts with specific host receptors to trigger symbiotic development. These revelations were made possible only by coordinated studies on both partners.

### The *X. nematophila* NilABC locus confers specificity for *Steinernema carpocapsae* nematodes

A significant advance in understanding the molecular basis of specificity was gained through the study of the

### Box 2. Principles of bacterial–host specificity revealed by nodulating bacteria

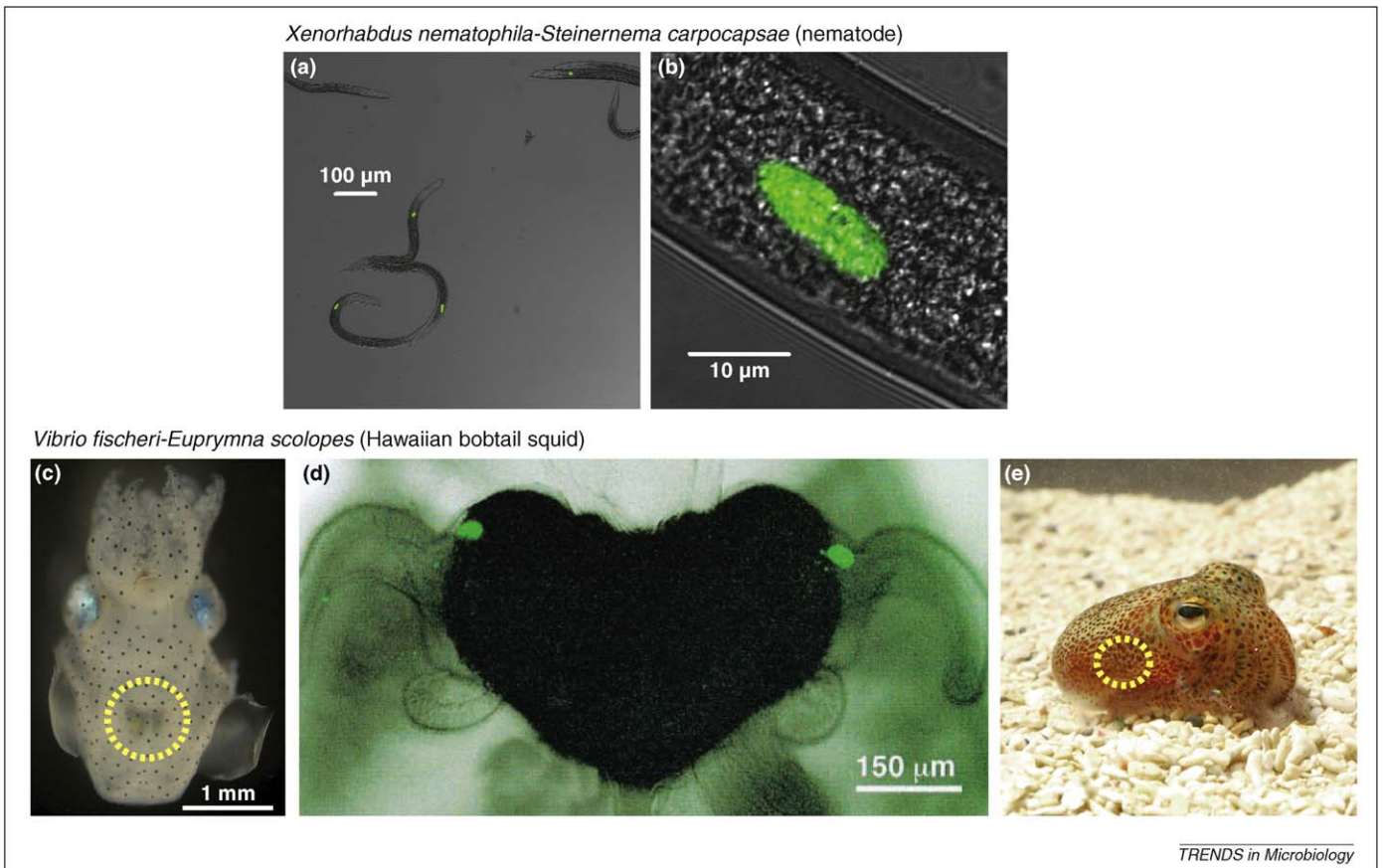
Host-derived flavonoids produced by legume roots induce production of bacterial Nod (nodulation) factors by symbiotic rhizobia. Nod factors are lipochito-oligosaccharides produced by the bacteria, which interact with corresponding host receptors to induce symbiotic development in a species and/or strain-specific fashion [11]. Bacterial NodD serves as a flavonoid receptor and as a transcriptional activator of *nodABC*, which synthesize the core Nod factor [12,13]. The Nod factor is specific to the symbiont, as modifications to the lipochito-oligosaccharide backbone modulate specificity. For example, when the specificity gene *nodH* is deleted from *Sinorhizobium meliloti*, its Nod factor is no longer 6-O-sulfated. This modification eliminates its ability to colonize its native legume host, alfalfa (*Medicago sativa*), but it confers the novel ability for *S. meliloti* to colonize vetch, *Vicia hirsute* [14,15]. In this manner, Nod factors are a common alphabet that is modified into different languages by different symbiont–host partnerships, with the bacterial modifications representing the vowels, accents and punctuation that create host-specific communication.

Once released, Nod factors interact with corresponding LysM-type receptors on the legume host [16–18]. Common downstream events are crucial for symbiosis, including calcium signaling in the host and subsequent nodule development, of which surface polysaccharide production by the bacteria is an important event [12].

It was recently demonstrated that rhizobia that colonize in a Nod factor-independent manner exist [19], making clear that we still have much to learn from nodulating bacteria.

symbiotic bacterium *X. nematophila*. *X. nematophila* lives a double life as a mutualist of entomopathogenic *Steinernema carpocapsae* nematodes and as a pathogen of lepidopteran insect hosts. A single life stage of the nematode, called the infective juvenile, carries *X. nematophila* in a specialized structure in the front of the nematode intestine called the receptacle (Figure 1a-b) [20]. The symbiotic nematode enters potential insect hosts through natural openings, where it releases its symbionts into the insect hemolymph, and the bacteria then kill the insect. The nematode feeds upon nutrition provided by the bacteria and reproduces within the insect, after which the nematode progeny reassociate with the bacteria, form infective juveniles, and leave the nutrient-depleted insect host in search of a new insect [21]. Typically, one bacterium seeds colonization in each nematode host before development of the infective juvenile [22], after which bacteria in the intestinal vesicle reproduce. After 1 week, the microcolony measures approximately 50 colony forming units (CFU) per infective juvenile [22]. Nutrients provided by the host, including *para*-aminobenzoate, pyridoxine and L-threonine, appear to be crucial for outgrowth and bacterial survival in the nematode [23].

There are many species of *Xenorhabdus*, each of which colonizes a specific *Steinernema* nematode host. There is functional specificity in the interactions, as laboratory cross-colonization experiments demonstrated that the



**Figure 1.** Sites of specificity in the models discussed. (a,b) Confocal micrographs of *S. carpocapsae* nematodes at the infective juvenile stage, showing green fluorescent protein (GFP)-expressing *X. nematophila* cells localized to the receptacle within each nematode. (c) Ventral view of paralarval *E. scolopes*, with the light organ/ink sac circled. (d) Confocal micrograph of paralarval *E. scolopes* with GFP-expressing *V. fischeri* localized in the squid mucus before entry into the internal anatomy of the light organ crypts. (e) An adult *E. scolopes*, with the approximate site of the light organ/ink sac circled. The light organ is on the ventral side of the animal, directing light downward. Part (d) is reprinted with permission from [48].

most successful symbioses are produced by the natural pairs [24].

Analysis of transposon-insertion mutants identified nine *X. nematophila* loci with important roles in nematode colonization [25], five of which (*aroA*, *serC*, *lrp*, *rpoE*, *rpoS*) were conserved in other bacteria and had known functions in metabolism or global gene regulation [25]. The role played by these genes during symbiosis has been investigated further to define important activities [23,26–28]. The remaining four loci defined by the signature-tagged mutagenesis screen were factors with no similarity to proteins of known function in online databases. They were named for their defects in nematode intestinal localization as *nilA*, *nilB*, *nilC* and *nilD* [25]. The mutation in *nilD* could not be assigned unambiguously to one open reading frame, and has not been studied further. *nilA*, *nilB* and *nilC* are located on a single genetic locus, and evidence suggests that they encode an integral inner membrane protein, an outer membrane beta-barrel protein and an outer membrane lipoprotein [29], respectively. These three structural *nil* genes are required for *S. carpocapsae* colonization, but the biochemical functions of the genes are still unknown. Of eight studied *Xenorhabdus* species tested [30], *X. nematophila* was found to be the only one with these three genes, which prompted further examination of the role of these genes in establishing host specificity between *X. nematophila* and *S. carpocapsae*.

Transfer of *nilABC* into two species of *Xenorhabdus* that do not colonize *S. carpocapsae* (*X. poinarii* and *X. bovienii*) was sufficient to allow these species to colonize *S. carpocapsae*. Neither species was able to colonize the nematode host without the *nil* transgenes [30]. With the *nil* genes, both species colonized *S. carpocapsae* at lower efficiency than *X. nematophila*, producing only ~1 CFU per nematode infective juvenile (CFU/IJ) for each of *X. bovienii-nilABC* and *X. poinarii-nilABC*, as opposed to >60 CFU/IJ for *X. nematophila* [30]. Despite colonizing at levels lower than the cognate species, these low levels of nematode colonization are still significant, as they reflect an increase of three orders of magnitude above the background limit of detection, and the bacteria localized to the receptacle that is specific for symbiotic colonization [30].

The discovery that NilABC mediated host specificity was a remarkable finding. By extending the principles of the rhizobial work into animal studies, characterization of NilABC as specificity factors demonstrated that the interaction of specific symbiont signals could be interpreted by an animal host to allow for the progression of symbiotic development. The finding that NilABC were sufficient for specificity was the first demonstration of a single locus in an animal symbiont that was both necessary and sufficient for determining initial colonization specificity. Distinct from rhizobia, however, was the presence of a core pathway that is modified in a species-specific manner. The *nilABC* genes in *X. nematophila* are absent from all other tested strains of *Xenorhabdus*, and the framework into which they fit remains to be determined. It has been suggested that the *nilABC* locus is part of a 20 kb island that was acquired by lateral gene transfer [30]. There is significant evidence of lateral gene transfer between *Xenorhabdus*

species [31]. Lateral transfer could explain the presence of this unique locus in only one species within *Xenorhabdus* and the corresponding benefit conferred with regard to *S. carpocapsae* colonization.

For *X. nematophila*–*S. carpocapsae*, additional stages of symbiotic development are important and contribute to specificity. These include bacterial replication and outgrowth, pathogenic ability, and bacterial-nematode reassociation after insect killing. Much of the activity during these stages is probably regulated independently of NilABC, as evidenced by the lack of outgrowth in the other *Xenorhabdus* species, even when they contained *nilABC*. Chapuis *et al.* [32] provide a detailed discussion of phylogeny influencing bacterial-nematode reassociation, and a number of recent studies have identified factors important for the pathogenic stage [28,33–37], including modulation of insect host immunity [38,39].

### The *V. fischeri* sensor kinase RscS confers specificity for *Euprymna scolopes* squid

*V. fischeri* is a luminous bacterial symbiont of fishes and squids worldwide. Distinct strains of *V. fischeri* colonize different species of bobtail squid in Hawaii (*Euprymna scolopes*), Japan (*Euprymna morsei*), Australia (*Euprymna tasmanica*) and the Mediterranean Sea (*Sepiolo robusta* and *Sepiolo affinis*), among others [40]. The relationship is mutually beneficial, as the host receives light produced by the bacteria [41]. This light provides the squid host with counterillumination; when foraging for prey in shallow waters at night, the squid, instead of casting a shadow in the moonlight, uses the ventrally directed light produced by the bacteria to camouflage itself to predators looking upward (Figure 1c–e) [42,43]. In return, *V. fischeri* is provided with a protected environment in which to grow, host-derived nutrients and oxygen, and a niche that is protected from predation [44]. For most squid hosts, *V. fischeri* is the only species in the light organ, a dedicated organ that forms ventral to the ink sac within the mantle cavity of the squid [45].

*E. scolopes* hatch as aposymbiotic paralarvae, and must recruit their symbionts from the environmental seawater each generation [46]. There are at most 1–2 culturable *V. fischeri* among the approximately  $10^6$  bacteria in each milliliter of ocean water [47]. In ocean habitats near squid populations, an additional 100–200 *V. fischeri* per milliliter might be present and competent for host colonization but might not be readily culturable [47]. The most generous estimate, then, suggests that only 1 of every 5,000 bacteria near the host is the correct symbiont, emphasizing the presence of a robust system that allows selection of only *V. fischeri* from this milieu to seed the monoculture inside the squid light organ.

Upon hatching, environmental peptidoglycan signals induce the host squid to release mucus, which it produces from ciliated epithelial fields that lie in the path of the water flow through the mantle [48,49]. As the bacterial-laden water is flushed through the mantle, at a rate of approximately 3  $\mu$ l per second, Gram-negative bacteria adhere to the squid-derived mucus. *V. fischeri* are competitively dominant over other Gram-negative bacteria [48], and they then enter through the pore of the light organ.

Flagellar motility and chemotaxis are crucial for the initial colonization [50–52]. As the association proceeds, the bacteria grow to high levels during the day and then produce light for the squid host at night in a cell density-dependent manner. At dawn, 90–95% of the bacterial cells are expelled into the environment, an occurrence that probably serves two purposes. First, the host tissue morphology has degraded from housing the high bacterial loads [53], and bacterial expulsion gives the host tissues an opportunity to recover. Second, bacterial release seeds the environment with *V. fischeri*, so that newly hatched paralarvae can be colonized by environmental *V. fischeri*, consistent with the observation that *V. fischeri* concentrations in the environment are highest where there are squid populations [54].

Bacterial genes that play a role in the symbiosis, including motility and nutrient-assimilating genes, were identified by a combination of forward and reverse genetics [44,50,52,55–60]. One class of transposon mutants stood out because the insertions abolished colonization but did not reduce motility or growth in minimal medium. These mutations mapped to either (i) a hybrid sensor kinase (named RscS for regulator of symbiotic colonization-sensor [57]), or (ii) to a locus of 18 genes that contribute to biofilm production [named *sypA-R* for symbiosis polysaccharide (*syp*) [59]]. Further genetic analysis revealed that the *syp* locus is activated by RscS [60] through the SypG response regulator that is encoded within the *syp* locus.

Overexpression of *rscS* in *V. fischeri* results in polysaccharide production, which results in wrinkled colonies, pellicles on the surface of liquid cultures, and extremely large aggregates of bacteria in the squid-derived mucus upon colonization of paralarval squid. These phenotypes were completely dependent on the *syp* gene cluster [60]. Together, these studies have established a model by which a predicted signal is perceived from the squid host by RscS, which autophosphorylates and then phosphorylates the response regulator and  $\sigma^{54}$ -dependent activator SypG. Activation of SypG leads to transcription of the *syp* genes from the *syp* promoters by  $\sigma^{54}$ , and the resulting gene products synthesize exopolysaccharide, which allows the cell to aggregate in a developmentally appropriate manner during colonization of *E. scolopes* [60]. Additional *syp* genes play roles in regulation, as described in a recent review [61].

The genome of a fish symbiotic *V. fischeri* strain (VF<sub>MJ11</sub>) was sequenced and compared with that of a squid symbiont (VF<sub>ES114</sub>). Both strains were found to have similar *syp* clusters but the fish symbiont lacked the regulator RscS [62]. Consistent with the absence of RscS, VF<sub>MJ11</sub> was unable to colonize *E. scolopes*, even if inoculated at concentrations 10-fold greater than VF<sub>ES114</sub>. Upon overexpression of *rscS* in VF<sub>MJ11</sub>, exopolysaccharide formation in culture was indistinguishable from that produced by the RscS-Syp pathway in VF<sub>ES114</sub>. Further, expression of *rscS* in VF<sub>MJ11</sub> was sufficient to allow VF<sub>MJ11</sub> to colonize *E. scolopes* [62].

This was the first demonstration that a single gene was sufficient to confer a novel host niche on an animal symbiont. Interestingly, it was not an adhesin or a toxin that distinguished the symbiont from the non-symbiont; the crucial difference was a regulatory gene that activated

capabilities already present, but not otherwise expressed, in the bacterium. It was further shown that ancestral *V. fischeri* strains lacked *rscS*, and that *rscS* was acquired once during *V. fischeri* evolution, leading to an *rscS*<sup>+</sup> clade of the species. By analyzing diverse isolates, it was shown that RscS was both necessary and sufficient for productive squid association with diverse isolates [62]. In addition, unlike the case with the *nil* genes, transgenic expression of RscS in the fish symbiont facilitated colonization to levels identical to those of the native squid symbiont [62]. These data suggested that once RscS facilitated entry, VF<sub>MJ11</sub> did not lack specificity factors necessary for outgrowth during the first 2 days.

RscS-Syp dependent initiation of colonization occurs during the first hours after exposure of the symbiont to the host. Similar to the Nil proteins in *X. nematophila*, absence of RscS results in a profound colonization defect, but there are additional facets of the *V. fischeri*–*E. scolopes* symbiosis that contribute to specificity beyond 48 hours from the perspective of innate immune regulation. *V. fischeri* must contend with these pressures and it is possible (but has not been demonstrated) that the genes required for host accommodation are shared by VF<sub>ES114</sub> and VF<sub>MJ11</sub>. For example, the squid ciliated appendages that produce mucus to attract *V. fischeri* undergo apoptosis and regression following bacterial colonization. This developmental program is triggered following successful colonization by *V. fischeri*. The bacteria release the peptidoglycan monomer, tracheal cytotoxin, which is interpreted by the host as part of a pro-apoptotic signaling cascade [63]. A second level of host immune recognition of the specific symbiont occurs as squid hemocytes bind and phagocytose Gram-negative bacteria. This response is specific to cells other than *V. fischeri* only when the host cells are isolated from animals that are successfully colonized with *V. fischeri* [64]. Finally, it has been suggested that host-produced nitric oxide is involved in mediating specificity in the host mucus [65]. Although some of these steps are downstream of RscS-Syp biofilm production, they probably play important contributory roles toward maintaining a productive, specific symbiosis.

### Performing mechanistic studies in a natural context yields unexpected benefits

As studies of host–microbe interactions have often focused on pathogenic interactions, particularly those of immediate medical relevance, we know a disproportionate amount about mechanisms of bacterial communication with human and mammalian cells and tissues. It is remarkable, then, that the recent crucial advances in understanding how specific interactions form during each generation have come from three beneficial host–microbe associations: rhizobia–plant, *X. nematophila*–nematode and *V. fischeri*–squid. These advances were facilitated not only by new genetic tools, but also by a concerted effort to study diverse natural isolates and to perform mechanistic assays in the context of natural associations [66].

An open question, therefore, is: have pathogen–host interactions evolved by similar mechanisms, which have

yet to be discovered, or is the specificity between hosts and bacteria established in a qualitatively distinct manner depending on whether the bacteria will lead to benefit versus harm for the host? A related question: to what extent do plants, invertebrates and vertebrates share mechanisms of symbiont acquisition and association, and to what extent do they differ? Vertebrates often house large multispecies consortia, in contrast to the limited number of microbial partners in invertebrates and it has been suggested that the presence of an adaptive immune system present only in vertebrates plays a role in these differences [67].

Complicating these questions is the fact that many bacteria cannot be simply defined as beneficial or as pathogenic symbionts, but rather the outcome of the interaction depends on the context of the engagement with the host. Many diseases of humans are caused by organisms that are constituents of our normal microbiota (e.g. *Staphylococcus aureus* [68]). It is important to study mechanisms underlying beneficial host–microbe interactions so that processes underlying specific colonization can be understood and disrupted. Furthermore, this might make it possible to target pathogens during nonpathogenic (and perhaps beneficial) phases of their lifecycle.

One example lies in the lifecycle of the causative agent of bubonic plague, *Yersinia pestis*. As it is apparent that rat flea vectors transmit *Y. pestis*, the flea is providing a natural environment for studying microbe–host interactions that affect human health. Not only must *Y. pestis* colonize the flea, but it is necessary for the bacteria to form a biofilm in the flea proventriculus so that the flea is inhibited from productive blood-feeding, thereby resulting in it repeatedly biting the rat host and transmitting small inocula of bacteria with each bite [69]. Studies on specificity have focused on this natural reservoir, comparing *Y. pestis* with its ancestor *Yersinia pseudotuberculosis*, which causes a much milder disease and is not transmitted through the flea vector. Initial work identified a candidate specificity factor, *Yersinia* murine toxin (Ymt) [70]. However, upon testing for sufficiency, it was demonstrated that this protein, a phospholipase D, enhanced survival in the flea but did not influence biofilm formation or proventriculus colonization [69,71]. Subsequent work has partially attributed gain of flea-specific biofilm formation to a loss of function mutation in the biofilm negative regulator *rcaA* during the evolution of *Y. pestis* [72]. There are parallels to the work in *Yersinia* in that described for beneficial symbionts; a regulatory change at one locus has far-reaching effects on the ability of a bacterium to interact with a host. It also provides a novel genetic mechanism, as it is the loss of function (of a negative regulator) that facilitates the transition, rather than the acquisition of new genetic material.

Together, these examples argue strongly for the consideration of ecology and natural history and for comparison of phenotypes across multiple strain backgrounds when analyzing microbe–host interactions. The recent advances made in our understanding of the molecular basis to host specificity in symbiotic microbes suggest that these complementary approaches all contribute to form a coherent picture of the relevant molecular interactions.

## Concluding remarks

Study of beneficial host–microbe associations is providing novel insights into the minimal requirements for host specificity in natural environments. There are many similarities to the work on Nod factors: interdomain signaling; bacterial-specific signals and perhaps host-specific receptors; the role for surface polysaccharides; lateral gene transfer; and the sufficiency of single loci to confer host specificity. There is also likely to be an important difference. Unlike the logic of the Nod factor, in which the alphabet was modified for each symbiosis to have its own language, the picture emerging for animal symbioses is that there are a large number of alphabets. Strains of *V. fischeri* did not make different Syp exopolysaccharides; they either made it or they did not. The Nil system also appears to be unique to *X. nematophila*, with no evidence as yet that it is acting in concert with a core specificity machinery that itself is conserved in other *Xenorhabdus* species. Additional work will be necessary to test these ideas, but the first glimpses of specificity in animal symbionts suggest that there will be a large variety of mechanisms at play, with more alphabets waiting to be discovered.

As model systems, these interactions have the potential to yield enormous insight into the interdomain signaling that allows aposymbiotic hosts to be colonized by the same microbiota generation after generation. These systems are also likely to provide information into the logic that allows pathogens to establish and maintain environmental reservoirs, and to colonize distinct sets of hosts. Modern genetic and genomic methods have boosted our ability to study symbiotic microbes in their natural habitats, and the approaches described here can be used for many natural host–microbe interaction systems, including the natural habitats of beneficial microbes, pathogens and so-called commensal microbes, which are known to be consistently present but whose roles remain to be established. Ideas for future research are listed in Box 3. Future work has the potential to inform how microbes make evolutionary transitions to new host species and what environmental and

### Box 3. Questions for future research

- How is specificity established in additional natural symbiotic systems?
- How do host immune systems influence the development of specificity, including protection from interlopers and pathogens?
- How do hosts sanction cheaters (correct symbionts who nonetheless avoid providing the full mutualistic benefit to the host)?
- What are the sources of mobile DNA that are transferred laterally to provide the raw material for leaps in host range? How are these genes transferred?
- What are the microevolutionary dynamics by which bacterial strains compete within a host?
- What are the ramifications to the host for being more, or less, specific? Do different specificity strategies accompany distinct relationships?
- Have pathogen–host interactions evolved by similar mechanisms or is the specificity between hosts and bacteria established in a qualitatively distinct manner depending on whether the bacteria will lead to benefit versus harm for the host?
- To what extent do plants, invertebrates and vertebrates share mechanisms of symbiont acquisition and association, and to what extent do they differ?

genetic factors contribute to this intriguing aspect of microbe–host interactions.

### Disclaimer statement

The author declares no conflict of interest.

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