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Vibrio fischeri and its host: it takes two to tango

Karen L Visick[1] and Edward G Ruby[2]

The association of *Vibrio fischeri* and *Euprymna scolopes* provides insights into traits essential for symbiosis, and the signals and pathways of bacteria-induced host development. Recent studies have identified important bacterial colonization factors, including those involved in motility, bioluminescence and biofilm formation. Surprising links between symbiosis and pathogenesis have been revealed through discoveries that nitric oxide is a component of the host defense, and that *V. fischeri* uses a cytotoxin-like molecule to induce host development. Technological advances in this system include the genome sequence of *V. fischeri*, an expressed sequence tagged library for *E. scolopes* and the availability of dual-fluorescence markers and confocal microscopy to probe symbiotic structures and the dynamics of colonization.

Addresses
[1] Loyola University Chicago, Department of Microbiology and Immunology, Maywood, IL 60153, USA
[2] University of Wisconsin, Madison, Department of Medical Microbiology and Immunology, 1300 University Avenue, Madison, WI 53706, USA

Corresponding author: Ruby, Edward G (egruby@wisc.edu)

Current Opinion in Microbiology 2006, **9**:1–7

This review comes from a themed issue on
Prokaryotes
Edited by Judy Armitage and Joseph Heitman

1369-5274/$ – see front matter

DOI 10.1016/j.mib.2006.10.001

Introduction

Molecular approaches that identify and localize host-associated microorganisms demonstrate the existence of specific, predictable and, presumably, coevolved microbiota in many animals and plants [1]. Although they are a vital part of their host's life history, surprisingly little is known about how these microorganisms are selectively acquired from the environment, and how the resulting association develops into a stable, long-term symbiosis. Nevertheless, certain themes are emerging from recent studies of symbiotic development, most notably, an unexpected similarity in the bacterial signals and host responses that characterize both beneficial and pathogenic associations [2,3].

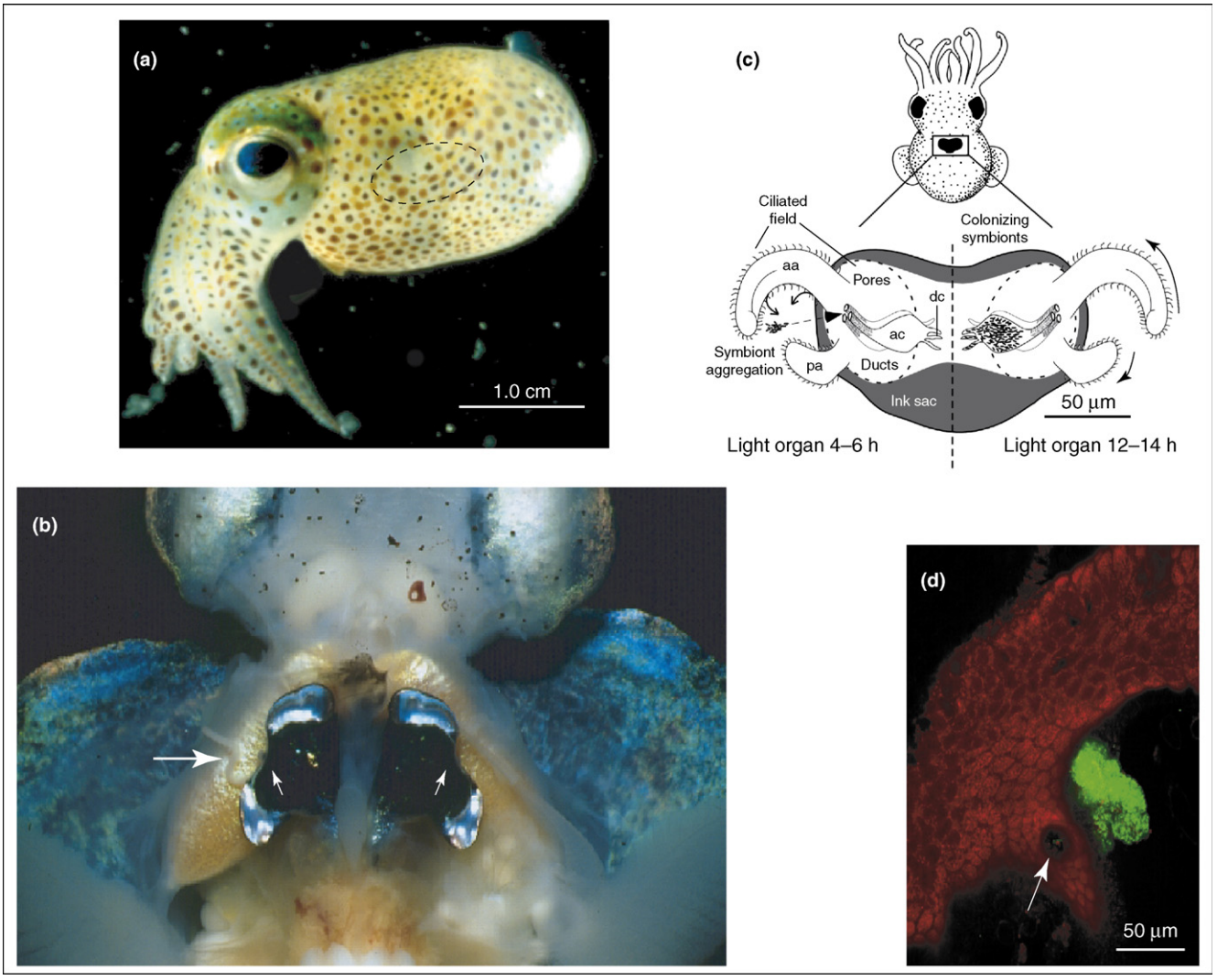
Multi-species bacterial consortia, the most common type of beneficial associations, are being examined using a variety of sophisticated phylogenetic, metagenomic, bioinformatics and gnotobiotic approaches [4–7]. In addition, symbioses consisting of only one or two bacterial species have proven particularly amenable to functional analysis using molecular genetics and confocal microscopy [8–11]; an example of the latter class is the association between the luminous bacterium *Vibrio fischeri* and its squid host, *Euprymna scolopes* [12]. Investigation of this natural symbiosis has been recently advanced by both the sequencing of the bacterium's genome [13••] and the development of an expressed sequence tagged (EST) gene set consisting of 14 000 unique members [14].

E. scolopes maintains a population of *V. fischeri* cells within a complex, bilobed organ (Figure 1a–c), and it uses the bioluminescence of this population at night in an anti-predatory behavior called 'counterillumination' [15]. The extracellular symbionts are housed deep within this light organ in epithelium-lined crypts that communicate directly with the seawater environment through external pores (Figure 1b). Juvenile squid are free of bacteria when they hatch, and must obtain an inoculum of the naturally occurring *V. fischeri* from the ambient seawater [16]. As a result of the activity of ciliated epithelial fields (CF) on the surface of the organ, the bacteria are harvested from the seawater, aggregating in host-derived mucus that accumulates around the pores on either side of the nascent structure (Figure 1c,d) [12]. Within 12 h, the *V. fischeri* cells in the aggregate have migrated through the mucus to the pores, made their way into the three crypts within each half of the light organ and proliferated to a population of approximately 10^6 cells that induce luminescence and presumably other symbiosis-related traits. Each day at dawn, the squid expels most of the crypt contents, including 90–95% of the bacterial population, out through the pores (Figure 1b); during the next 4–6 h the remaining symbionts proliferate, restoring the organ to a fully colonized state. The symbiosis is highly specific: only *V. fischeri* is capable of colonization, and their presence triggers a complex developmental program in the light organ, resulting in a pattern of stereotypic morphogenetic events [12]. Here we review recent studies of this process from the perspective of both partners, emphasizing bacterial behavior and gene regulation, and host biochemical signaling and development.

Regulation of behavior and gene expression in the bacterial symbiont

Mutational analysis of *V. fischeri* has established three stages of symbiotic colonization (Table 1) [16]: initiation (entering into and early multiplication in the light organ); accommodation (attaining high cell density); and persistence (continued regrowth to normal levels after each

Figure 1



The *V. fischeri–E. scolopes* light organ symbiosis. **(a)** An adult *E. scolopes*; the approximate position of the light organ, within the mantle cavity, is indicated by the dashed oval. **(b)** The large bilobed light organ of the adult squid is revealed by a ventral dissection. The smaller arrows indicate the position of the pores; the crypt contents are being expelled out of the pore on the left side (larger horizontal arrow). **(c)** Cut-away illustration of the juvenile light organ early (left) and late (right) in the initiation of symbiosis. The anterior (aa) and posterior (pa) appendages of the CF, as well as the antechamber (ac) and deep crypts (dc), and the position of the aggregation of *V. fischeri* cells, are indicated. Modified from [42]. **(d)** Confocal microscope image of GFP-labeled *V. fischeri* cells aggregating in mucus above a pore (white arrow) during the initiation of colonization.

venting). Early work established motility as a crucial behavior for initiation [17,18], very probably due, in part, to the importance of chemotaxis [19] (C DeLoney *et al.*, abstract N-244, 103rd General Meeting of the American Society for Microbiology, Washington DC, 18–22 May 2003). Recently, specific flagellar mutants have been constructed [20–22]. As in the case of the pathogen *Vibrio cholerae*, *V. fischeri* appears to control flagellar gene expression through a cascade of regulators that include σ^{54} and a σ^{54}-dependent master regulator, FlrA. Not surprisingly, mutations resulting in the loss of either σ^{54} or FlrA prevent initiation. In addition, a mutation in *flrA* reduced the ability of *V. fischeri* to aggregate outside the light organ. Surprisingly, complementation of the *flrA* mutation, whilst restoring motility and chemotactic ability, failed to promote a normal level of symbiotic colonization [20]. A clue to the reason behind this result might be that σ^{54} and FlrA also control expression of non-flagellar genes [20,23•] including, in the case of σ^{54}, those involved in biofilm formation [22,23•]; microarray-based studies of the FlrA regulon are underway.

In addition to these regulators, the roles of two of six *V. fischeri* flagellin genes have been described [21]. Whereas a mutation in FlaC failed to impact either motility or colonization, a disruption in FlaA caused several defects,

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Table 1

Important components of symbiosis development[a].

Bacterial regulator	Colonization stage	Symbiosis events affected	Refs
SypG	Initiation	Exopolysaccharide synthesis; (aggregation)	[23]
σ^{54}	Initiation	Motility; (other)	[22]
FlrA	Initiation and accommodation	Motility; (other)	[20]
GacA	Initiation and accommodation	Motility; colonization; growth	[36]
AinS	Initiation and persistence	Motility; luminescence; (other)	[29,38]
LuxS	(Accommodation)	(Colonization)	[34]
LuxI	Persistence	Luminescence	[29,38]
Signal/effector			
NOS/NO[b]	Initiation	Specificity; (signaling)	[42]
TCT[c]	Initiation	Hemocyte trafficking	[3]
Lipid A[c]	Initiation	Apoptosis of CF	[3,12]
p53[b]	Initiation and accommodation	Apoptosis and regression of CF	[51]
Proteasome[b]	Initiation and accommodation	Regression of CF	[52]
Actin[b]	Accommodation	Duct constriction	[43]
Reflectin[b]	Accommodation	Luminescence reflection	[46]
pES100[c]	(Persistence)	Genetic transfer	[26]

[a] Characteristics in parentheses are suggested, but not yet demonstrated.
[b] Host component.
[c] Bacterial component.

including reduced motility, a slow rate of initiation, failure to reach the high cell density achieved by the wild type strain, a substantial delay in colonization of crypt 3, and poor retention in the light organ following expulsion. These data support a model in which motility is necessary not only for entry, but also for reaching 'optimal' binding sites within the light organ. Such a model is particularly intriguing given that most symbiotic *V. fischeri* cells become aflagellate within 24 h of colonization [24], suggesting the presence of a temporal window during which flagellated wild type cells reach the putative preferred sites. Interestingly, high Mg^{2+} concentrations are required by *V. fischeri* for full flagellation [25]; such a dependence, coupled with the relatively low concentration of this ion in mollusk tissues, might contribute to the symbionts' aflagellate state in the light organ (see also Update).

Questions such as whether optimal colonization sites exist can now be addressed by employing two compatible fluorescent labels. Dunn *et al.* [26] constructed a set of *Vibrio* shuttle vectors based on pES213, one of several small *V. fischeri* plasmids that can be mobilized by a conjugative system encoded on pES100. Using GFP and RFP markers for complementation, tagging and gene expression analyses [27•], these workers revealed that light organs inoculated with two derivatives of the wild type (each carrying a different marker) contain both strains. Surprisingly, the two strains frequently occupied distinct zones within a crypt. Whereas there is as yet no explanation for this phenomenon, it is reminiscent of the localization of symbionts in the *Xenorhabdus nematophila* symbiosis [28]. In addition, use of these vectors as transcriptional reporters suggested that, even within a single crypt, distinct microenvironments exist that differentially influence *V. fischeri* gene expression [27•].

Luminescence, a behavior required for symbiotic persistence [29–31], is controlled both by a complex set of physiological conditions [32], and by genetic regulators that appear to play additional roles in symbiosis. In addition to the paradigm quorum sensing regulators, LuxI (a quorum signal synthase) and LuxR (the *lux* transcriptional activator), *V. fischeri* employs additional signal synthases (AinS and LuxS), two-component regulators (including the σ^{54}-dependent response regulator LuxO), and an activator of *luxR* transcription, LitR [29,33–35]. The resulting regulation is sequential: AinS appears to be the major regulator of bioluminescence at low cell densities, with LuxI as the primary regulator of bioluminescence in the high-density, symbiotic condition [29]. Both the *luxI* and *luxR* genes, as well as *luxA*, which encodes a subunit of luciferase, are required for normal symbiotic persistence [30]. It was unexpected that an *ainS* mutant exhibited a similar persistence defect as that of a *luxI* mutant, despite an almost normal level of symbiotic bioluminescence. The *ainS* mutant also failed to properly initiate colonization, a defect shared by *litR* and *luxO*, but not *luxIR* mutants. Taken together, these results indicate that the AinS pathway controls additional symbiosis determinants that affect both initiation and persistence. Microarray analysis revealed that several non-*lux* genes are controlled by AinS, including those required for motility. A connection between motility and bioluminescence has previously been reported for the symbiosis response regulator GacA [36]. A *gacA* mutant is normal for quorum signaling, but shows nutritional defects [36] and induces host development poorly [37]. The *gacA* mutant did not induce cessation of host mucus shedding, nor did it trigger apoptosis in the CF. In addition, animals colonized by a *gacA* mutant were susceptible to invasion by secondary *V. fischeri* colonizers, suggesting that because

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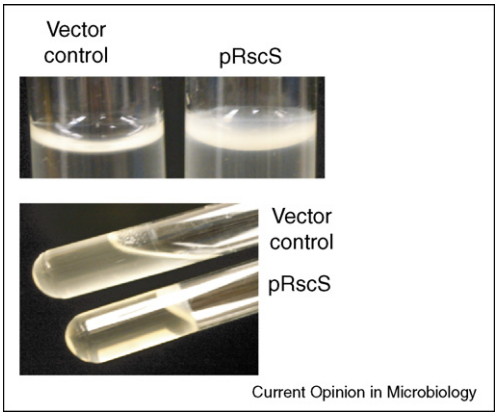
the *gacA* mutant is unable to signal the full program of host development, the light organ remains permissive to the recruitment of additional symbionts.

AinS also controls a putative exopolysaccharide synthesis cluster (VF0151–VF0201), and a set of genes (VFA1014, VFA1015 and VFA1017) linked to a second putative exopolysaccharide cluster (VFA1020–VFA1037), designated *syp*, that is essential for initiation of symbiosis [23•,38•]. No connection between AinS and *syp* has been established; however, both *ainS* and *litR* mutants exhibit altered colony morphology, a trait often associated with altered exopolysaccharide production [33,34]. Transcription of *syp* depends upon σ^{54} and a LuxO-like σ^{54}-dependent activator, SypG, encoded within the second exopolysaccharide cluster; cells that overexpress SypG exhibit a substantial increase in biofilm formation [23•]. Furthermore, a *syp*-dependent pellicle forms under conditions in which the symbiosis regulator RscS is overexpressed (Figure 2) [39] (Yip *et al.*, abstract N-105, 105th General Meeting of the American Society for Microbiology, Atlanta, GA, 5–9 June 2005). Thus, it is probable that exopolysaccharide production by *V. fischeri* enhances symbiotic initiation, either by promoting adherence or by providing protection against host defenses — or by doing both.

Symbiont-induced tissue development and signal pathways in the host

One of the key advantages of the squid–*Vibrio* association is the ability to observe how interaction with a specific bacterial symbiont triggers a pattern of distinctive changes in the developmental biology of the host [12]. As a result, there have been significant advances in understanding the mechanisms underlying both the biochemistry of the bacterium's signaling and the developmental responses of the host.

Figure 2



Pellicle formation by *V. fischeri* cells that overexpress the regulator, RscS. Cells carrying an *rscS*-overexpression plasmid (pRscS) form a thick pellicle, which is absent in the vector control, with sufficient tensile strength to retain the medium when the culture tube is inverted.

New details of the structure and development of the symbiotic light organ have been revealed in a recent confocal-microscopy study [40••]. Colonization by *V. fischeri* cells was known to involve passage through the external pores (Figure 1c), which communicate with the crypts through ducts [12]. Subsequent confocal examination has revealed that at the medial end of each duct there is a large antechamber that narrows into a region termed the 'bottleneck', before opening into the deep crypt. Bacterial symbionts are present only within the deep crypts (except during initial colonization and, briefly, during the daily expulsion). This specificity of localization might be a result of the inability of the bacteria to persist in the presence of the biochemical stresses found in the duct and the antechamber [41,42••]. Interestingly, the diameter of the bottleneck narrows from between 5–9 μm to 2–4 μm after symbiosis is established, apparently imposing an additional physical barrier to supernumerary colonization [40]. A colonization-induced narrowing was also noted in the duct itself, where the underlying mechanism involves both post-transcriptional control of actin synthesis and a restructuring of the actin in the polarized epithelium of the duct [43]. To date, such bacteria-induced remodeling of host actin has only been described as a response to bacterial toxins (e.g. *V. cholerae* repeats in toxin [RTX]) during pathogenic infections [44]. The presence of genes encoding two RTX homologs in the *V. fischeri* genome [13••] presents the possibility that a similar mechanism might play a role in a normal step in the development of symbiosis.

In the newly hatched juvenile, the three pairs of crypts on either side of the organ are at different stages of maturation, and initial colonization of the light organ produces location-specific responses [40••]. For instance, within the first 48 h only the most mature pair of crypts exhibits two previously reported symbiotic characteristics: colonization-induced swelling of the deep crypt epithelium [31], and efficient diurnal expulsion of the symbiont population [45]. It is probable that as the other less mature crypts continue to develop, they begin to express these functions as well. Another sign of maturity in the developing light organ is the thickening of a reflective tissue layer dorsal to the symbiont-containing crypts, which serves to direct the bioluminescence ventrally [12]. This layer is composed almost entirely of a single unique protein, termed 'reflectin', with remarkable biochemical properties [46].

The biochemical signaling between *V. fischeri* and its host has been further elucidated, revealing surprising parallels with pathogenesis. Both nitric oxide synthase (NOS) and NO, which are important components of innate immunity, were detected in the CFs, as well as the epithelia lining the ducts and antechambers [42••]. Interestingly, the levels of both NOS and NO were irreversibly down-regulated after symbiotic colonization, presumably as a

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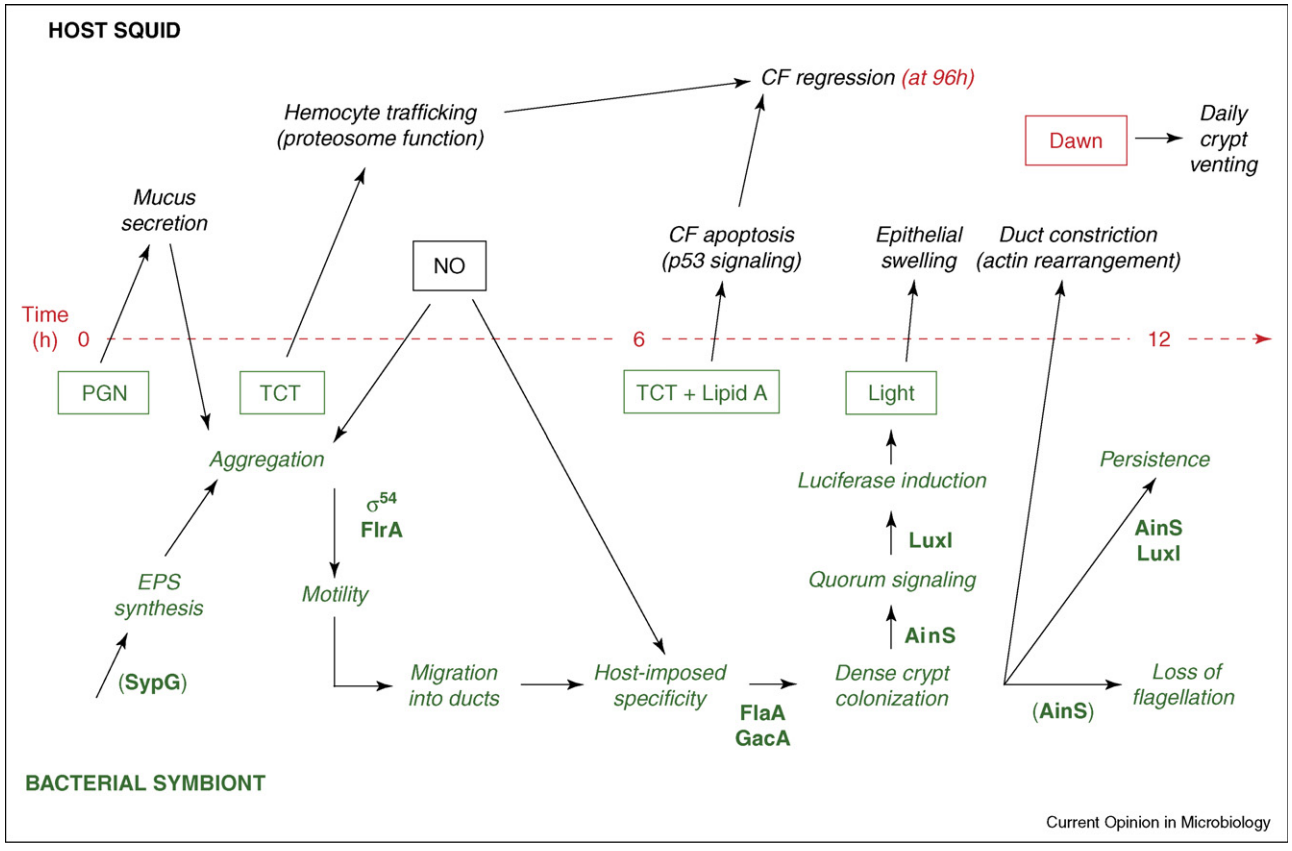
result of an as yet undescribed bacterial signal. The discovery of NOS in 1–5 μm vesicles embedded in the secreted mucus (a novel location for this enzyme), suggests that its activity might contribute to specificity as early as the aggregation stage of colonization [47].

Perhaps the most exciting development in signal identification was the discovery that the peptidoglycan tetrapeptide monomer — identical to the tracheal cytotoxin (TCT) of *Bordetella pertussis* [48] — is a morphogen that induces normal development of the juvenile squid [3]. Specifically, TCT secreted by *V. fischeri* during initiation of the symbiosis results in the trafficking of the host's phagocytic hemocytes into the sinus space of the CF and, in synergy with lipid A, induces the normal apoptosis and eventual regression of these structures. An analogous induction by lipid A of the development of zebrafish digestive function has also been reported [49]. Surprisingly, the target for TCT response in the squid is several cell layers distant from the colonizing bacteria (Figure 1c), suggesting that specific receptors and signal transduction pathways must serve an intermediary role [50]. These findings make it clear that bacterial products initially described as toxins can also trigger beneficial tissue development, and thus their function is highly context dependent. Similarly, the recent discovery of a crucial role for the normal mammalian microbiota in signaling the enteric immune system has suggested a need to re-evaluate the concept of 'tolerance' [2].

The study of pathways activated by these and other bacterial signals has been made possible by analysis of an EST-cDNA library of symbiotic squid tissue [14,50,51]. Specifically, homologs encoding at least 11 components of the NFκB (nuclear factor κB) pathway have been discovered, including a Toll-like receptor and four peptidoglycan receptors. The activity of this pathway, working through a proteasome-dependent degradation step [52], might link the TCT and lipid A signals described above to the host's biochemical (NO, halide peroxidase and mucus production) and cellular (macrophage trafficking and apoptosis) responses [12]. In addition, two homologs of the p53 family of apoptosis-inducing developmental regulators were found to be activated in the light organ CF in a symbiosis-dependent manner [51]. Pathogen-induced apoptosis also can function through the host's p53 pathway, suggesting parallels with *V. fischeri*-induced apoptosis of the CF [12,51]. Taken

Figure 3



Early colonization events and signals described in this review. The approximate timeline of events is indicated in red. The relationships linking the events (italics), signals (boxed) and bacterial gene products (bold) are indicated by arrows, and are associated with either *E. scolopes* (black) or *V. fischeri* (green).

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together, the similarity of signals and host responses in the squid–*Vibrio* association to those characteristic of pathogenic infections is striking, and further demonstrates that symbiosis and pathogenesis might use a similar language, but to different ends.

Conclusions

There has been progress toward understanding the events and signals underlying host–microbe symbioses, using the *E. scolopes*–*V. fischeri* association as a model (Figure 3). Recent work in several systems has addressed several central questions. First, how do bacteria sense their host, and how do their responses adapt them to this environment? Second, how is subsequent host development triggered, and what are the signals and/or pathways used? Finally, in what ways do beneficial and pathogenic associations share common signaling mechanisms? Future studies of microbial symbiosis will begin to focus on poorly understood emergent properties such as signal networks, metabolic interactions, and genetic diversification within symbiont populations. As we begin to recognize the crucial role beneficial microbes play in animal health and development, microbiology enters a new and exciting era of discovery.

Update

The requirement for magnesium in flagellation depends, at least in part, upon the activity of diguanylate cyclases, which produce the second messenger c-di-GMP (3′-5′-cyclic diguanylic acid) [53•]. Because this molecule has been shown in other systems to mediate the switch between motility and biofilm formation, it is a good candidate for playing a role in the symbiosis.

Acknowledgements

We thank MJ McFall-Ngai for helpful comments on the manuscript, and E Yip for the image in Figure 2. The authors are supported by grants from the National Science Foundation (IBN0517007), and the National Institutes of Health (NIH) (GM59690 and RR12294). The contents of this manuscript are solely the responsibility of the authors, and do not necessarily represent the official views of the National Center for Research Resources (NCRR) or the NIH.

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Please cite this article in press as: Visick KL, Ruby EG, *Vibrio fischeri* and its host: it takes two to tango, Curr Opin Microbiol (2006), doi:10.1016/j.mib.2006.10.001