THE WINNOWING: ESTABLISHING THE SQUID–*VIBRIO* SYMBIOSIS

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Most symbiotic associations between animals and microorganisms are horizontally transmitted — the microorganisms are acquired from the environment by each generation of the host. How are exclusive partnerships established in the context of the thousands of other microbial species that are present in the environment? Similar to winnowing during a harvest, the symbiosis between the squid *Euprymna scolopes* and its luminous bacterial symbiont *Vibrio fischeri* involves a step-wise elimination of potential interlopers that ensures separation of the 'grain' from the 'chaff'.

STOCHASTICALLY
Random interactions that are not predetermined.

Biologists are becoming increasingly aware that the formation of alliances between animals and microorganisms is a widespread phenomenon. Such relationships are often stable over the lifetime of an individual animal host, from generation to generation and over evolutionary time. The transmission of symbionts between generations is usually classified as either horizontal (environmental) or vertical (transovarian) (TABLE 1). In a horizontally transmitted symbiosis each new generation of the host acquires the symbiont from the surrounding environment, whereas in a vertically transmitted symbiosis the microbial partner is passed on to the next generation in, or on, the eggs.

Although both types of associations are widely distributed across phyla, horizontally transmitted symbioses in which the microbial partner(s) colonize extracellular apical surfaces of epithelial cells, such as the mammalian intestinal epithelia, are thought to be the most common type of symbioses that occur in animals. Such symbioses present challenges for each partner. The host must have mechanisms for the enrichment and harvesting of specific microorganisms, which are often scarce in the environmental microbiota. Once the symbiosis is established, there must be mechanisms to ensure that the association is stable so that the symbionts do not overgrow the host and the host does not eliminate the symbionts. Although this problem is not unique to horizontally transmitted associations, because the environmental niches that are involved in these associations are often open to the environment, there is an added layer of complexity. A stable association must be established with normal functioning of the immune system of the host, so that the host can retain the ability to remove other environmental, potentially pathogenic, microorganisms. The microbial partner, in turn, must be capable of switching niches. This facility demands that the microorganism must make the successful transition from a 'macroenvironment', in which it interacts STOCHASTICALLY with a variable assortment of micro- and macrobiota that are present, to a 'microenvironment', in which it occurs either as a monoculture or as a member of a more defined and limited community in a single host.

The association between the Hawaiian bobtail squid *Euprymna scolopes* and the luminous bacterium *Vibrio fischeri* has been studied for more than 15 years as a model for the establishment, development and maintenance of horizontally transmitted symbioses[1–3]. In this light-organ symbiosis, the host uses light that is produced by *V. fischeri* in counterillumination[4] to avoid predators during their nocturnal behaviour — the host emits luminescence from its ventral surface to match downwelling moonlight and starlight, thereby casting an obscured silhouette. Both partners can be cultured independently in the laboratory, which has allowed experimental manipulation of the partners both as individuals and as dual participants in the association. The female host lays clutches of hundreds of eggs that, after approximately 20 days of embryogenesis,

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doi:10.1038/nrmicro957

hatch almost synchronously at dusk, producing a large number of genetically similar juveniles for experiments. Establishment of the symbiosis takes place over a short period of time — within hours of the host hatching into the environment — so experiments are rapid. Unlike the mammalian digestive tract, in which infection by pathogens or other microbial interlopers can occur in tissues that are free of microorganisms or where the normal microbiota has been disrupted, the nascent squid light organ remains uncolonized by non-symbiotic bacteria, even in the absence of *V. fischeri*[1,2]. Therefore, light-organ colonization is specific to *V. fischeri*. Using the squid–*Vibrio* model, the development of symbiosis has been assigned several stages, including initiation, accommodation and persistence, which correspond with the emergence of, or requirement for, particular phenotypes in both the host animal and the bacterial symbiont[5]. This review summarizes the current knowledge of the development of this exclusive symbiotic association, which occurs by a winnowing process that specifically selects *V. fischeri* from the diverse seawater bacterioplankton to become the singular partner of the host squid.

Bringing the partners together

One of the main challenges for the partners of a horizontally transmitted symbiosis is the successful acquisition by the host of the microbial partner from the environment[6–9]. In many instances, such as in the hydrothermal vent and coral reef symbioses, the symbionts are scarce in their free-living niche and their location and abundance have remained a mystery[7,8]. Despite the difficulties that are associated with isolating or identifying horizontally transmitted symbionts in the environment outside the host, animal hosts are rarely, if ever, found without their symbiont partners, which are often crucial to host nutrition. So, it seems likely that the partners have mechanisms to ensure that the symbiosis establishes efficiently.

V. fischeri cells however, have been isolated from free-living populations in seawater, and studies of the ecology of this bacterial species, the morphology of the juvenile light organ and the squid colonization process have provided insights into how a horizontally transmitted symbiosis might be established in an aquatic environment. With the exception of coastal environments with large *E. scolopes* populations, where it is found at concentrations of 100–1,500 cells per ml of seawater among a background of ~10^6 total bacterial cells per ml (REFS 9,10), *V. fischeri* is generally a relatively rare constituent of Hawaiian seawaters. *V. fischeri* cells generally comprise <0.1% of the bacterioplankton population. Lee and Ruby showed that water samples that were taken at increasing distances from host populations had progressively fewer *V. fischeri* and were progressively less capable of infecting juveniles[11]. Taken together, these observations indicated that a behaviour of host populations results in seeding of the environment with potential symbionts.

Table 1 | Some examples of horizontally and vertically transmitted symbioses

Host	Symbiont	Transmission*	Type of association‡	References
Porifera				
Various sponges	Cyanobacteria (bacteriocytes)	Vertical	Binary	59
Aplysina cavernicola (mesohyl)	Bacteria	Horizontal	Consortial	60
Cnidaria				
Corals (gastrodermis)	*Symbiodinium* spp.	horizontal/vertical	Binary/consortial	61–64
Vestimentifera				
Riftia pachyptila (trophosome)	Sulphide-oxidizing bacteria	Horizontal	Binary	65
Mollusca				
Vesicomyid clams (gills)	Sulphide-oxidizing bacteria	Vertical	Binary	66
Bathymodiolus spp. (gills)	Sulphide-oxidizing bacteria	Horizontal	Binary/consortial	67,68
Euprymna scolopes (light organ)	*Vibrio fischeri*	Horizontal	Binary	69
Squids (accessory nidamental gland)	Bacteria	Horizontal	Consortial	32,70,71
Arthropoda				
Aphids (bacteriome)	*Buchnera spp*.	Vertical	Binary	72
Insects (reproductive tissues)	*Wolbachia* spp.	Vertical	Binary	73,74
Termites (hindgut)	Bacteria/protist	Horizontal	Consortial	75
Chordata				
Mus musculus (alimentary canal)	Bacteria/protist	Horizontal	Consortial	76
Bos spp. (cow rumen)	Bacteria/protist	Horizontal	Consortial	77
Homo sapiens (alimentary canal; skin)	Bacteria/protist	Horizontal	Consortial	78,79

*Horizontal, a symbiosis that is acquired anew each host generation; vertical, a symbiosis that is acquired by passage of the microbial partner in, or on, the eggs of the host. ‡Binary, an association between one host species and a population of a single microbial species; consortial, an association between one host species and a community, or communities, of mulitiple microbial species.

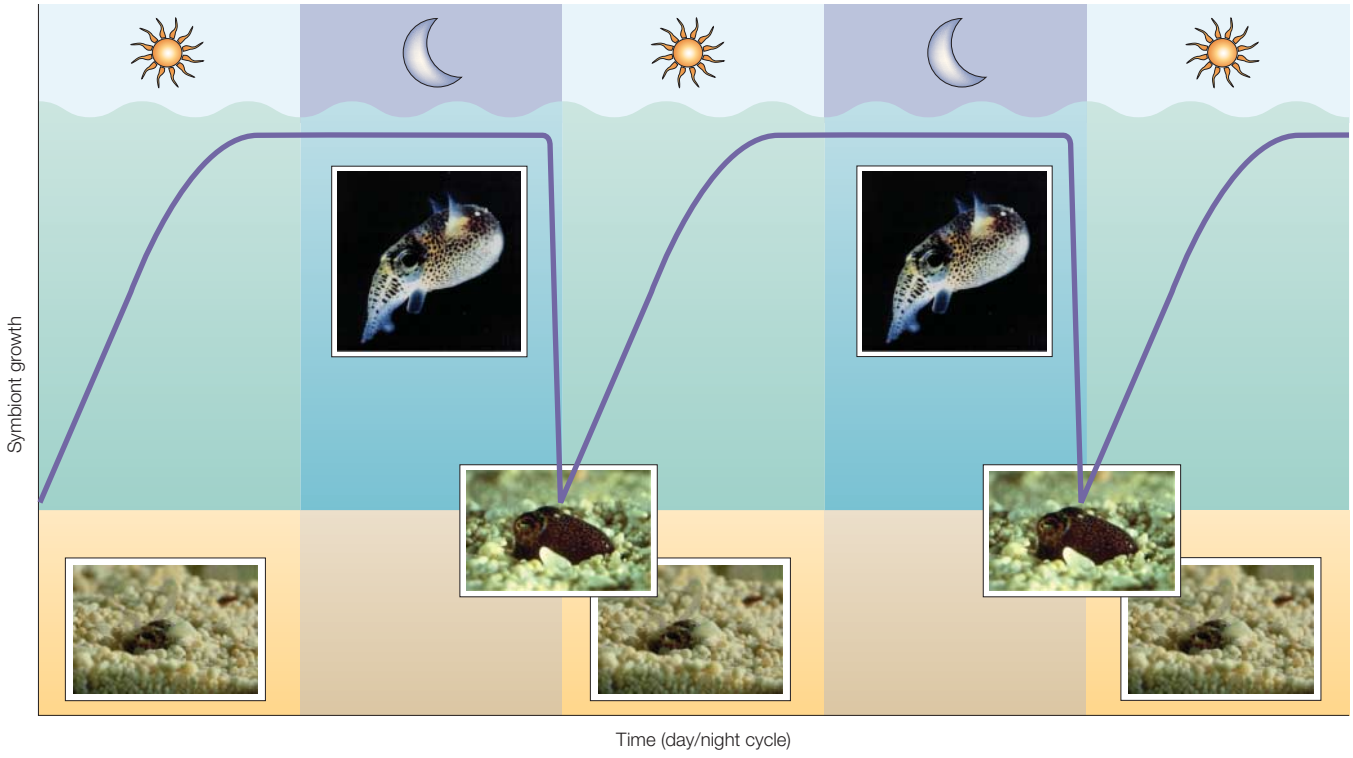


Figure 1 | **The diel pattern of host behaviour and the associated fluctuations in symbiont population density in the light organ.** The squid *Euprymna scolopes* is nocturnal and emerges at dusk to hunt. During this period the light organ is full of *Vibrio fischeri* and the bioluminescence from this bacterium is used by the squid to camouflage itself from potential predators. At dawn the host expels ~95% of the light-organ bacteria into the surrounding environment. The squid then buries beneath the sand, and during the day the remaining 5% of *V. fischeri* that remain in the light organ grow (indicated by the purple line) such that, by mid-afternoon, the light organ is again full of bacteria. Not only does this behaviour help maintain the symbiont population in the host, but it also seeds the environment with *V. fischeri* for colonization of the next generation of squid.

Subsequent research (FIG. 1) showed that each day at dawn, as the hosts bury into the sand for their daytime quiescence, they expel ~95% of their light-organ symbionts[12,13] together with a population of host macrophage-like blood cells, or haemocytes[13], into the surrounding environment (BOX 1). During the day, the bacteria that remain in the light organ multiply to repopulate it so that the animal has a full complement of symbionts when it emerges from the sand to forage in the water column at night. In common with other cephalopods (such as squids, octopuses and their relatives) but unlike many marine invertebrates, *E. scolopes* has no true dispersive larval stage — it hatches from the egg and remains in the vicinity of the parent population. So, this diel behaviour of expelling the symbionts provides a local enrichment of the environmental symbiont populations from which newly hatched juvenile hosts become colonized[9].

Experimental analyses of the onset of symbiosis confirmed the field observations that correlated the numbers of environmental *V. fischeri* with the success and timing of light-organ colonization[14,15]. Experiments using *V. fischeri* strains chromosomally tagged with mini-Tn*7* transposons carrying antibiotic resistance markers showed that the host has the ability to harvest the symbiont over a range of *V. fischeri* concentrations, from less than 10 cells ml^{-1} of seawater to more than 10,000 cells ml^{-1} of seawater[15]. At low cell concentrations (less than 100 cells ml^{-1} of seawater), the time to infection is protracted, that is, full colonization of the organ can take up to 48 hours. However, when environmental *V. fischeri* is present at concentrations of hundreds of cells or more per ml of seawater, the animal is infected within 8–10 hours[15].

The length of time over which the host can be infected by *V. fischeri* at concentrations of only a few hundred cells ml^{-1} of seawater presented a conundrum when the anatomy of the host animal was taken into consideration. Specifically, the nascent light organ lies in the centre of the mantle (body) cavity of the squid and environmental seawater is passed across the light organ during the ventilatory movements of the host (FIG. 2). With each ventilation, the animal brings in about 1 µl of seawater and the ventilation rate is about 2 per second. If the seawater contains 500 *V. fischeri* cells ml^{-1} (REF. 9), theoretically, each ventilation will bring in, on average, less than one potential symbiont. For ventilatory activity to be the sole mechanism mediating infection, the one bacterium that enters the mantle cavity with each inspiration would have to find and enter one of the six 15-µm pores on the surface of the light organ before it is returned back out to the environment with the expiration. Considering these probabilities, for the symbiosis to be established, it seemed likely that the animal would have to 'capture' the symbiont rather than expire it out.

The initial encounter

The ability to harvest symbionts efficiently is the result of the activity of tissues that are specific to the newly hatched juvenile (FIG. 2). During embryogenesis, the host develops two sets of elaborate fields of ciliated epithelia on the lateroventral surfaces of the nascent organ[1]. Each of these fields consists of two protruding appendages, the tips of which oppose to form a ring, and a base that surrounds three pores. It is through these pores that the symbionts enter, travel down long ciliated ducts and pass into crypt spaces that will become colonized for the lifetime of the host[16]. After colonization, the symbionts induce the loss of these ciliated fields that promote colonization[17].

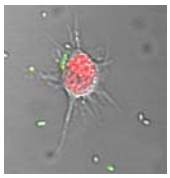
Detailed study of the activity of these ciliated fields and the timing of colonization[18,19] revealed that the process of symbiont harvesting begins almost immediately after hatching (FIG. 3). Within 10–20 seconds of the emergence of the host from the egg, the cilia of the surface epithelia begin to beat. Analyses of the activity of these cilia indicated that the pattern of their beat rapidly concentrates and sweeps particles into the ring that is created by the appendages, that is, above the pores (FIG. 2). Using fluorescently labelled bacterial cells or latex beads, it was shown that for the first ~30 minutes after hatching, nothing enters the light-organ crypts. However, between ~30 minutes and 1 hour after hatching, the light organ has a permissive period — if bacteria, either living or dead, or bacteria-sized particles are introduced into the seawater that contains the newly hatched juvenile, these bacteria or particles will enter the crypt spaces of the light organ. However, no cells or particles — including *V. fischeri* cells when they are presented as the inoculum — are detected in the crypt spaces between 1 and 2 hours, so the initial 'permissive' period only lasts for approximately 30 minutes. The mechanism by which these initial bacteria or particles pass through the ciliated ducts and whether the cells or particles that enter initially are subsequently destroyed by the chemical environment of the hatchling crypt spaces, cleared from these areas by the resident host haemocytes, or eliminated by another mechanism remains to be determined. After this restrictive period, a population of *V. fischeri* cells enters and grows in the crypt spaces (see below) and the colonization of these areas is then specific.

These descriptions of the anatomical arrangement of the tissues and the timing of events provided information for the stage at which the host initially interacts with symbiont cells, but did not reveal a mechanism for retention of the symbionts. This piece of the puzzle was obtained when GFP-labelled bacteria were shown to aggregate between 2 and 4 hours after inoculation in the mucus above the pores[18] (FIG. 4). Histological and histochemical analyses showed that, in addition to entraining materials into the vicinity of the pores, the ciliated epithelia are also responsible for secretion of the mucus in which the symbiont cells gather[19]. Studies of this behaviour showed that the shedding of mucus from these cells was the result of a nonspecific response to the bacterial envelope component peptidoglycan in the surrounding seawater[19]. So, this common bacterial cell-wall component triggers a mechanism by which the host detects that it has hatched into the environment and can initiate mucus shedding and the harvesting of symbionts.

Enriching for the specific symbiont

The next stages in the process begin the winnowing that leads to selection of the specific symbiont (FIG. 4). Although peptidoglycan from both Gram-positive and Gram-negative bacteria can induce mucus shedding by host cells, only live Gram-negative bacteria are capable of aggregating in the mucus[18,19]. However, Gram-negative bacteria are abundant in seawater and an assemblage of bacteria in the mucus is normally limited to a few hundreds cells, so if no enrichment for *V. fischeri* occurs during this period of aggregation, because symbiont cells generally represent <0.1% of the bacterial population, it would be unusual for a symbiont to be present in the aggregates. However, in experiments in which 200–1,000 GFP-labelled *V. fischeri* cells were added to natural seawater that contained 10^6 bacteria ml^{-1} of several species other than *V. fischeri*, the symbiont became the dominant cell in the

Box 1 | **The immune system of *Euprymna scolopes***



The invertebrate immune system has several important differences compared with the vertebrate immune system. Although invertebrates have an innate immune system, they lack adaptive immunity and immunological memory. The table compares vertebrate and invertebrate immune systems, specifically examining specific processes and gene homologues that are involved with either innate or adaptive immune responses[56–58]. The circulatory system of *Euprymna scolopes* has only a single blood-cell type, the haemocyte, that can participate in host defence against microorganisms. These haemocytes come into close contact with the highly vascularized crypt epithelium of the light organ (see the figure in which *Vibrio fischeri* cells (green) are shown adhering to a single squid haemocyte (red)) and are often observed migrating into the crypt spaces and interacting with the bacterial symbiont, *V. fischeri*.

Characteristic	Invertebrates	Vertebrates
Innate immunity		
Opsonization*	Present	Present
Phagocytosis*	Present	Present
Oxidative stress (nitric oxide, superoxide (O_2^-), H_2O_2)*	Present	Present
Acute phase-like proteins	Present	Present
Complement-related proteins*	Present	Present
Cytokine-like molecules/cytokine-like receptors*	Present	Present
Antimicrobial peptides*	Present	Present
MAMP receptors*	Present	Present
Toll-like receptors/NFκB pathway*	Present	Present
Immunoglobulin superfamily (Igsf) domains*	Present	Present
Adaptive immunity		
MHC (Class I/II)	Absent	Present
T-cell receptors	Absent	Present
Recombination-activating genes (RAG 1/2)	Absent	Present
Activation-induced cytidine deaminase (AID)	Absent	Present

*Evidence for these pathways or compounds in *E. scolopes*[13,25–27] (plus *E. scolopes* EST database; see the online links box). MAMP, microbial-associated molecular pattern.

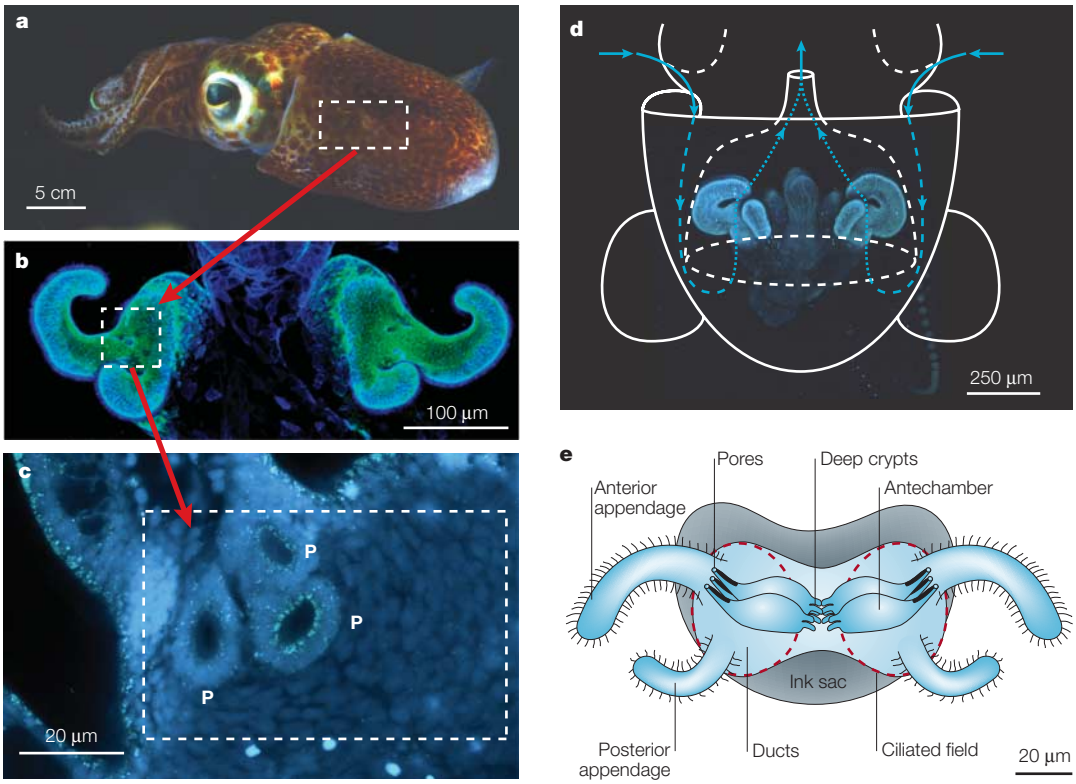


Figure 2 | **The juvenile light-organ system. a** | A host squid swimming in the water column. **b** | A confocal micrograph showing the complex ciliated epithelium on the surface of the juvenile light organ. **c** | A confocal micrograph showing the pores (P) on the surface of the organ through which the symbionts enter host tissues. **d** | The pattern of water flow through the mantle cavity. **e** | The internal components of the squid light organ at hatching. Part **d** is reproduced with permission from REF. 18 © (2000) National Academy of Sciences.

mucus aggregations within hours[20]. This was studied further under laboratory conditions to determine how *V. fischeri* establishes its dominance in the aggregations[20]. In competition experiments with non-symbiotic vibrios that, on their own, form robust aggregations in the presence of host mucus, *V. fischeri* was always the dominant member of the aggregates. Further experiments also showed that the symbiont does not multiply in the mucus, nor does it secrete substances that compromise the viability of the other congregating bacterial species. Although the mechanism underlying the phenomenon of dominance remains to be determined, these experiments showed that specificity — reducing the number of potential partners for the host — begins outside of the light organ even before *V. fischeri* interacts directly with host tissues. A recent study demonstrated *V. fischeri* chemotaxis towards *N*-acetylneuraminic acid, which is a component of squid mucus[21]. Further studies should focus on the possibility that *V. fischeri* might preferentially adhere to mucus, or alter the chemistry of mucus, to inhibit aggregation by non-symbiotic bacteria. The ability of microorganisms to adhere to and use mucus has a crucial role in the establishment of intestinal symbioses[22]. For example, strains of *Bacteroides thetaiotaomicron*, a prominent member of the human intestinal microbiota, that are deficient in processing certain mucopolysaccharides are at a competitive disadvantage in mice that are free of microorganisms[23,24].

The symbiont cells do not immediately migrate towards the host crypts after associating with secreted mucus. Instead, after a period of 2–4 hours in the aggregates they move through the pores, into the ducts and finally into the crypt spaces[18] — which requires both biophysical and biochemical obstacles to be overcome. Each duct is lined with dense cilia that beat in an outward direction[16] and bacteria are exposed to two types of oxidative stress. First, the cells of the ducts and the lateral portions of the crypts produce high concentrations of nitric oxide synthase (NOS), which results in toxic concentrations of nitric oxide (NO)[25]. Second, the symbionts face a second oxidative challenge in the deep crypts owing to the presence of a host halide peroxidase that can potentially generate bactericidal hypohalous acid[26,27]. Despite the ongoing threat of oxidative stress, the symbiont cells colonize the host epithelium of the deep crypts for the entire life of the host animal.

Symbiont-induced light-organ development

After colonization, *V. fischeri* induces a series of developmental events in the host light organ that transform the colonization morphology to a mature, functional light

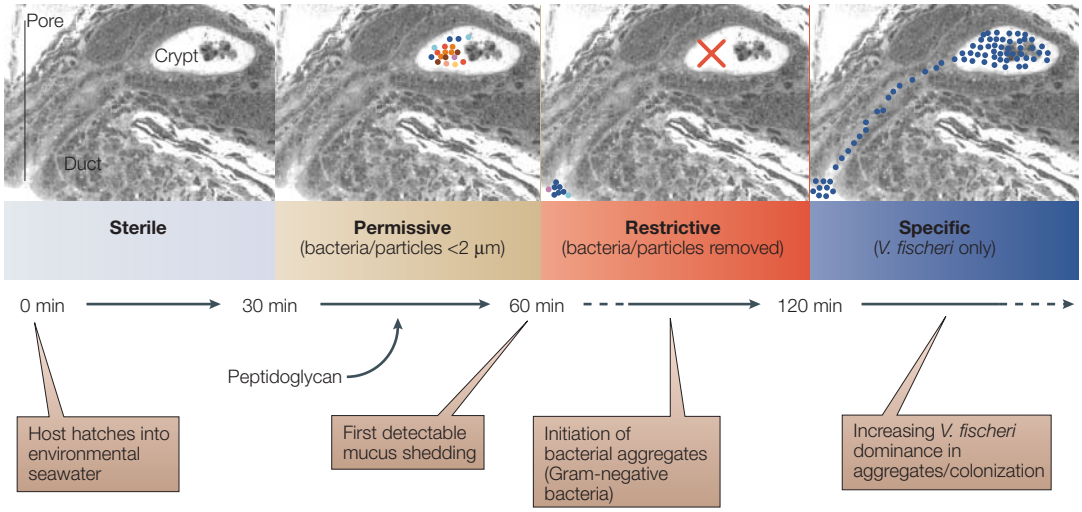


Figure 3 | **The initial interactions of the juvenile host with the environment.** Between 30 and 60 minutes after hatching, the light-organ crypts are open to small numbers (1–3) of bacterial cells or particles less than 2 μm in diameter. These initial entrants are later removed by an as-yet-unknown mechanism. After 1 hour, the host sheds mucus in response to bacterial peptidoglycan and by 2 hours, *V. fischeri* (~1 μm in diameter) begin to aggregate above the pore and then migrate through the duct before colonizing the crypt epithelium. At this point, the light organ transforms from a 'permissive' environment to an environment that is exclusive to the symbiont.

organ[16]. These changes occur both in the surface epithelial fields (FIG. 5) and ducts, which are remote from the colonizing symbionts in the crypts, and in the crypt epithelial cells that directly interact with the symbiont. Specifically, after the introduction of *V. fischeri* into the surrounding seawater, the symbionts induce: haemocyte trafficking into the blood sinuses of the ciliated epithelium (which takes place ~2 hours after the introduction of *V. fischeri*) and eventual apoptosis of these epithelial cells (after ~6 hours)[28]; attenuation of the NO signal in the ducts and crypt antechambers (after ~8 hours)[25]; constriction of the ducts (after ~6–12 hours)[29]; cell swelling and an increase in the microvillar density of the cells lining the deep crypts (after ~12 hours)[17,30]; mucus secretion by the crypt cells (after ~12–24 hours)[19]; cessation of mucus shedding from the ciliated epithelium and aggregation of symbionts (after 48 hours)[19]; and gradual loss of the surface ciliated epithelium (after ~96–120 hours) (FIG. 6)[17]. Some of these changes, specifically the constriction of the ducts, the cessation of mucus shedding and aggregation, and the loss of the surface epithelium, might discourage subsequent colonization by environmental symbionts, so the founding population of symbionts in the crypt spaces induces developmental changes that alter the morphological characteristics of the juvenile light organ that promote colonization.

Experimental studies were designed to identify the bacterial inducers of morphogenesis of the epithelial fields. The results of these studies revealed that haemocyte trafficking can be induced by bacterial peptidoglycan [28], apoptosis of the epithelial cells can be induced by lipopolysaccharide[31] and full regression of these cells can be induced by the synergistic activity of lipopolysaccharide and peptidoglycan[28]; so all of these important developmental events can be induced by two bacterial products that are not specific to *V. fischeri*. However, it was also shown that *V. fischeri* must enter the crypt

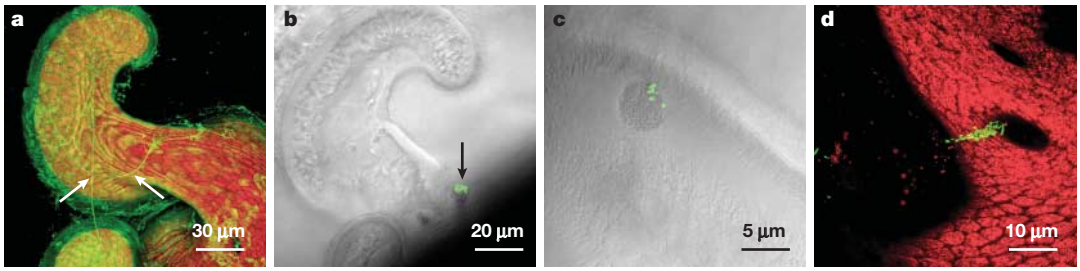


Figure 4 | **The harvesting of the specific symbiont: mucus secretion, aggregation, dominance and migration.** **a** | In response to bacterial peptidoglycan, the cells of the ciliated superficial epithelium begin to secrete mucus (indicated by arrows). **b** | This mucus is used as a substrate in which *Vibrio fischeri* (green) will form aggregations (indicated by an arrow). **c** | Although many viable Gram-negative bacteria will aggregate, *V. fischeri* out-competes other bacteria for space in the aggregations. Shown is a *V. fischeri*-dominated aggregation with non-symbiotic *V. parahaemolyticus* (green). **d** | After aggregating for 2–5 hours, *V. fischeri* (green) migrates through the pores and ducts and colonizes the host tissue.

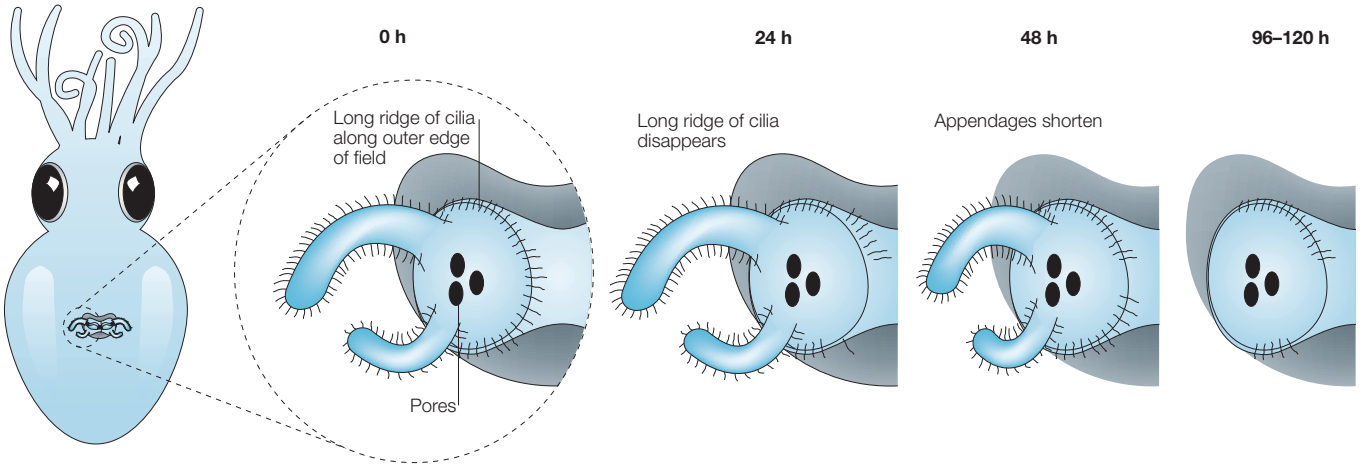


Figure 5 | **The gradual, symbiont-induced regression of the ciliated epithelium of the juvenile light organ.** Schematic depicting the loss of host ciliated epithelial fields after successful colonization by *Vibrio fischeri.*

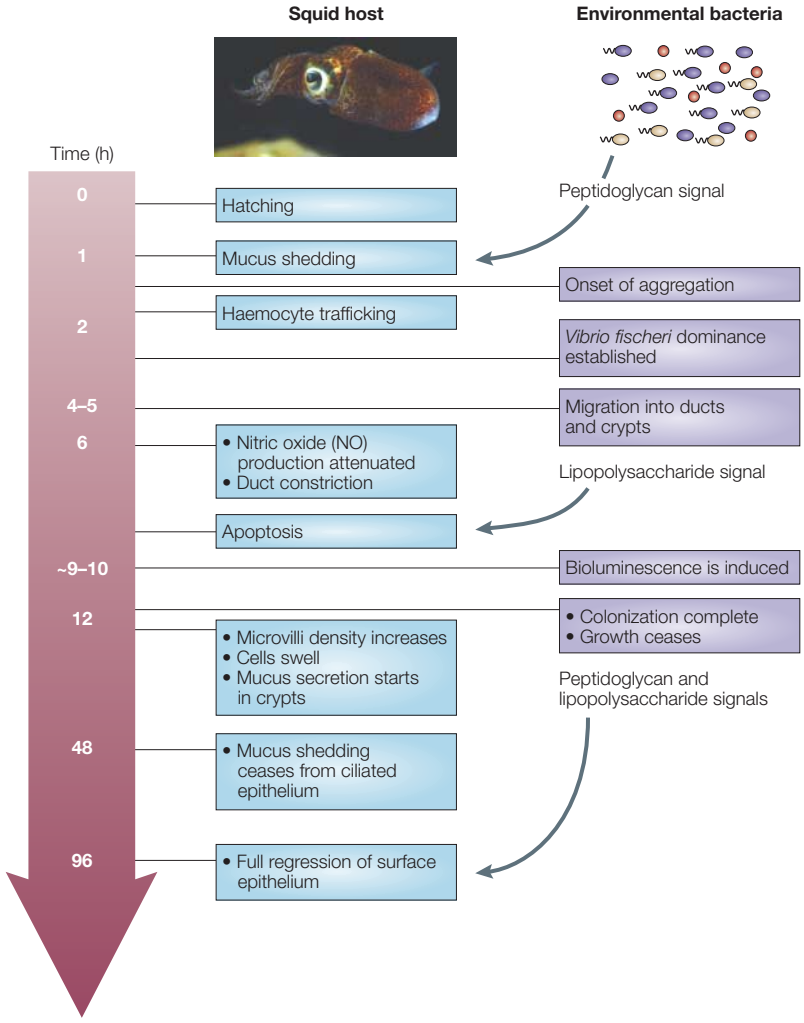


Figure 6 | **Timeline showing early events and signals during the onset of symbiosis.** Within 1 hour after hatching, the host responds to an increasing number of non-specific and specific bacterial signals. During the first 96 hours after hatching into environmental seawater, the symbiont induces host mucus shedding, haemocyte trafficking, nitric oxide (NO) attenuation and actin rearrangement, and apoptosis and regression of the superficial ciliated epithelium. In the crypts, *Vibrio fischeri* colonization leads to an increase of host crypt cell microvillar density and cell swelling.

spaces to induce full morphogenesis; only the peptidoglycan and lipopolysaccharide of the symbiont would be presented in a location that would trigger these developmental changes, so specificity of the response is conferred by the exclusive access of *V. fischeri* ligands to sensitive host receptors. Interestingly, low levels of haemocyte trafficking are induced even before the symbionts enter the light organ[28], which indicates that they might communicate with host tissues before directly contacting them, possibly through the mucus. The loss of ciliated structures associated with the tissues that are colonized by symbiotic bacteria is not unique to this light-organ association. This phenomenon has also been observed in the development of both the squid accessory nidamental gland[32] and the transient mouth of hydrothermal-vent tubeworms[33]. However, in these associations, possible bacterial triggers of these developmental events have yet to be identified.

Developmental changes in the duct include a decrease in NOS protein[25] and an increase in actin synthesis[29]. Although symbiosis reduces the NOS and NO signals to 20% of those of non-symbiotic animals, the concentrations of the enzyme and NO are still substantial. As the pores of the light organ remain open throughout the life of the host, and some crypts may experience vulnerability with the diel venting of symbionts, retaining some NO activity might provide continued protection against nonspecific colonization of the organ. This attenuation of NO might also promote the growth and viability of *V. fischeri*, which would in turn prevent interlopers from invading the light-organ crypts. The ability of bacteria to negatively affect host-cell oxidative burst is not unprecedented. Cells of *Pseudomonas aeruginosa* that infect the lungs of patients suffering from cystic fibrosis are capable of decreasing oxidative burst from host neutrophils[34]. The increase in actin synthesis correlates with an increase in filamentous actin in the apical regions of the epithelial cells lining the ducts. The deposition of this actin is concomitant with a constriction of the ducts, which effectively limits the diameter of the

entrance to the organ. The bacterial ligands that induce these particular changes are unknown. However, several bacterial toxins and compounds have significant effects on animal actin expression and the cytoskeleton in general[35]. An orthologue of *Vibrio cholerae* RTX[36], an actin cross-linking toxin, has been found in *V. fischeri* (see *V. fischeri* genome in the online links box) that warrants further study.

The use of antibiotics to eliminate symbionts at particular times during the programme of development have indicated that some of the symbiont-induced developmental changes are reversible and some are not[17,19,29,30,37]. If symbionts are eliminated from the crypts after the cessation of mucus secretion and its associated aggregation behaviour, the surface ciliated epithelium will once again begin to secrete mucus and gather symbionts[19]. Similarly, removal of symbionts from the light organ results in expansion of the duct to its pre-colonization diameter[29]. Furthermore, host phenotypes that are induced by direct interactions with symbiont cells — cell swelling[17] and an increase in the microvillar density of crypt epithelial cells[30] — are also reversible; these cells return to the microanatomy that is characteristic of hatchling animals or animals that have not been exposed to symbionts.

In contrast with these reversible symbiont-induced developmental changes, at ~6 hours after entry of the bacteria into the crypts (~12 hours after first exosure), *V. fischeri* cells deliver an irreversible signal to the host that results in the gradual loss of the surface ciliated epithelium over 4 days; if the light organ is cleared of symbionts in the first few hours of colonization, the epithelial field is not lost, but if it is cleared after that time, an irreversible developmental programme is triggered[37]. So, although the superficial field will begin to secrete mucus again after eliminating the symbionts from the crypts, because the field is becoming increasingly smaller with time, the harvesting of symbionts in mucus is likely to be restricted to the initial ~4 days[19]. The attenuation of NO in the ducts is also irreversible[25].

Symbiont responses to host-imposed hurdles

Characterization of the host light organ and of the colonization process indicates that *V. fischeri* has many characteristics that allow it to negotiate each CHECKPOINT presented by the host.

Genetic analyses of the bacterial symbiont. *V. fischeri* mutants have been isolated that are defective in one of each of the main stages of the encounter with the host[5,38–51] (FIG. 7). Initiation mutants are delayed in, or incapable of, establishing an association, ACCOMMODATION MUTANTS cannot fully colonize the host and persistence mutants initially colonize normally, but cannot remain in the symbiosis for more than a few days. The specific aspect of the colonization process for which each mutant is impaired is known for only some of these mutants. As the descriptions of the host phenotypes become refined, the bacterial geneticists can more precisely describe the nature of the defect of a given mutant strain.

CHECKPOINT
A hurdle imposed by the host that confers greater specificity to the symbiont during colonization.

ACCOMMODATION MUTANTS
Mutants of *Vibrio fischeri* that colonize the light organ of the host at lower numbers than wild-type symbionts.

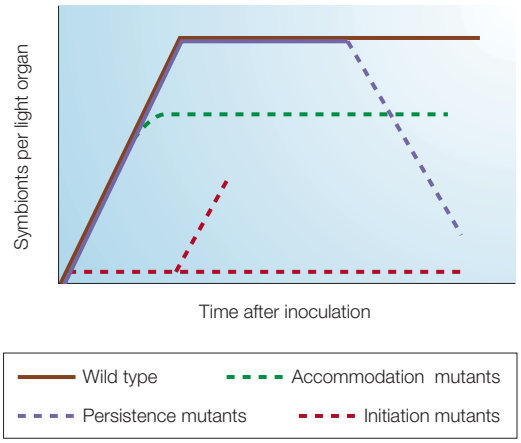


Figure 7 | **Initiation, accommodation and persistence mutants of *Vibrio fischeri*.** Isolation of bacterial mutants has identified several phenotypes that are necessary for successful colonization of the host. These mutants can be divided into three categories depending on the stage of the colonization process at which they are deficient. Initiation mutants are delayed in colonizing or fail to colonize the crypts and include motility mutants (non-flagellated, FlrA[39,40], and hyper-flagellated[41]), outer-membrane-protein mutant (OmpU[42]), gene-regulation mutants (GacA[43] and RscS[44]) and quorum-sensing mutants (AinS[45] and LitR; REF. 46 and E. Ruby, personal communication). Accommodation mutants successfully colonize the light organ, but at lower concentrations than wild-type *V. fischeri* and include amino-acid-synthesis mutants (LysA; REF. 47 and E. Ruby, personal communication), gene-regulation mutants (GacA[43]) and lipopolysaccharide mutants (Pgm[48]). Persistence mutants colonize at similar concentrations as wild-type bacteria but fail to maintain the association over extended periods of time and include luminescence mutants (LuxA[49]), quorum-sensing mutants (LuxIR[49] and AinS[45]), siderophore-production mutants (GlnD[50]) and catalase mutants (KatA[51]). Modified with permission from REF. 5 © (1996) Annual Reviews.

Motility. At present, perhaps the best-understood characteristic of *V. fischeri* that is important for the symbiosis is motility[39–41]. The microcurrents that are created by the duct cilia demand that *V. fischeri* is highly motile; symbiont cells that are defective in motility are incapable of negotiating the duct to colonize the organ. However, these mutants dominate normally in the aggregates, which shows that motility is not required for this stage of the colonization process[18,20]. Surprisingly, hypermotile mutants, which have supernumerary flagella, are incapable of aggregating normally[41]. These mutants are also delayed in colonization relative to the wild-type strain. Furthermore, these mutant strains are out-competed by wild-type *V. fischeri* in animal experiments in which they are present in equal numbers in the inoculum.

Oxidative stress defences. Characterization of the host phenotypes indicates that resistance to various oxidative stresses is important to the bacterial symbionts. Genes encoding putative aerobic and anaerobic NO-inactivating systems are present in the genome of *V. fischeri*[25]. Whether, and how, any of these genes are involved in responses or resistance to the NO that the

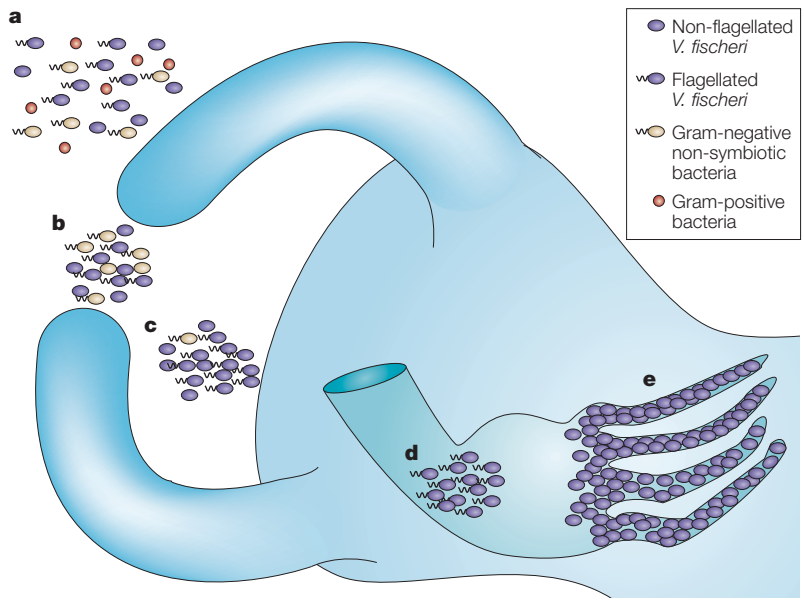


Figure 8 | **The winnowing.** This model depicts the progression of light-organ colonization as a series of steps, each more specific for symbiosis-competent *Vibrio fischeri*. **a** | In response to Gram-positive and Gram-negative bacteria (alive or dead) the bacterial peptidoglycan signal causes the cells of the ciliated surface epithelium to secrete mucus. **b** | Only viable Gram-negative bacteria form dense aggregations. **c** | Motile or non-motile *V. fischeri* out-compete other Gram-negative bacteria for space and become dominant in the aggregations. **d** | Viable and motile *V. fischeri* are the only bacteria that are able to migrate through the pores and into the ducts to colonize host tissue. **e** | Following successful colonization, symbiotic bacterial cells become non-motile and induce host-epithelial cell swelling. Only bioluminescent *V. fischeri* will sustain long-term colonization of the crypt epithelium.

host produces in the ducts and crypts awaits mutational analyses of the candidate genes. As mentioned above, in the deep crypt spaces, *V. fischeri* encounters a halide peroxidase, which converts hydrogen peroxide and halide anions to hypohalous acid, a product to which there is no known bacterial defence[26]. Therefore, *V. fischeri* might interfere with the activity of this enzyme by competing either for the hydrogen-peroxide substrate or for the upstream oxygen species from which this substrate is produced — either oxygen itself or a superoxide anion. Mutants that are deficient in the production of a periplasmic group III catalase, which could compete with the halide peroxidase for the hydrogen-peroxide substrate, are out-competed by wild-type bacteria[51].

Light production. The luminescence reaction consumes oxygen, and mutant *V. fischeri* strains that are defective in light production are incapable of persisting in the organ and are also out-competed by wild-type strains[49]. So, if the bacteria are not doing their 'job' for the host — producing luminescence — they are eliminated from the organ. Much work remains to be done to resolve how the light-emitting reaction ensures the persistence of the bacterial symbionts, but there are a few clues. *V. fischeri* strains with mutations in genes that are associated with light production — specifically *luxA*, *luxI* and *luxR* — are also incapable of inducing cell swelling of the epithelial cells of the deep crypts[49]. *luxA* encodes one of the subunits of bacterial luciferase, the enzyme that catalyzes the light-producing reaction, whereas the *luxIR* genes are associated with quorum-sensing and induction of the *lux* operon. As *luxA*$^-$ strains are only defective in light production, the host phenotype is likely to be due to an aspect of the light reaction itself and not the upstream quorum-sensing behaviour — the phenotype that is created by the *luxIR* mutants is due to their inability to induce the production of high concentrations of luciferase. Bacterial luciferase is a mixed function oxidase that converts a reduced flavin mononucleotide ($FMNH_2$) and a long-chain aldehyde to FMN and the corresponding long-chain fatty acid[52]. With the exception of oxygen, all substrates are recycled. So, it is likely that, in the case of crypt-cell swelling, the host cells sense either the oxygen consumption of the luciferase reaction, light production or both. Further analyses with bacterial mutants and studies of host responses to these mutants promise to reveal the mechanism that underlies this characteristic.

Genomic approaches to symbiosis

Perhaps the most exciting new developments in the squid–*Vibrio* symbiosis are those that are associated with the advent of genomic approaches. Sequencing of the *V. fischeri* genome has recently been completed and a host database of nearly 14,000 unique ESTs (expressed sequence tags) has been created for the light organ. These resources are presenting exciting potential candidates to study. For example, examination of the *V. fischeri* genome has revealed several homologues of important pathogenicity factors. Studies of these genes and their protein products will help reveal what role these disease-associated genes have in a beneficial association. On the host side, the EST database has revealed squid homologues to constituents of the Toll-receptor/NF-κB pathway, a response pathway to microbial-associated molecular patterns, such as lipopolysaccharide and peptidoglycan. In light of the findings that lipopolysaccharide and peptidoglycan have a role in inducing host responses it is likely that this response pathway is an important factor in the dynamics of the squid–*Vibrio* association. So far, this pathway has only been studied in pathogenic associations, so its regulation in a beneficial association will be of interest. Other parallels to pathogenic models can be drawn. For example, both *Helicobacter pylori* and *P. aeruginosa* can alter mucus secretion by the host cell during infection[53–55]. The squid–*Vibrio* symbiosis could provide another model to understand the effects of microbial factors on the regulation of animal mucus secretion.

Conclusions

The squid–*Vibrio* symbiosis has proven to be a useful model for investigating the establishment and maintenance of symbiosis. So far, the study of this system has focused on the description of the details of the onset and progression of the association and the development of molecular genetic approaches in the bacterial symbiont.

Most evidence indicates that the progression of colonization occurs in a series of stages, with each step conferring greater specificity between the host and the symbiont (FIG. 8). Although we liken this process to a winnowing in which the 'grain' (*V. fischeri*) is separated from the 'chaff' (non-symbiotic bacteria), it is clear that the 'grain' is not a passive player in this endeavour and that successful establishment relies on the cooperation of both partners. The results of host and symbiont genomics should allow a significant advance in our approaches to the study of this association. An understanding of this relatively simple model of the persistent colonization of animal epithelia by Gram-negative bacteria provides a valuable complement to studies of both beneficial and pathogenic consortial interactions, such as in the mammalian intestine, and chronic diseases that involve persistent colonization by Gram-negative bacteria, such as cystic fibrosis.

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Acknowledgements

We thank E. G. Ruby for helpful comments on the manuscript. Work on the squid–*Vibrio* system is funded by the National Science Foundation, the National Institutes of Health and the W. M. Keck Foundation.

Competing interests statement

The authors declare that they have no competing financial interests.

Online links

FURTHER INFORMATION
***V. fischeri* genome:**
http://www.integratedgenomica.com/ergo_light/
***E. scolopes* EST database:** http://trace.ensembl.org/
Margaret McFall-Ngai's laboratory:
http://www.medmicro.wisc/department/faculty/mcfall-ngai.html
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