First Impressions in a Glowing Host-Microbe Partnership

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Despite the clear significance of beneficial animal-microbe associations, mechanisms underlying their initiation and establishment are rarely understood. In this issue of Cell Host & Microbe, Kremer et al. (2013) reveal that first contact within the squid-vibrio symbiosis triggers profound molecular and chemical changes that are crucial for bacterial colonization.

With a three billion year head start on macroscopic life, microbes formed the planet that we inhabit. It’s now widely appreciated that all visible life, including our own species, evolved amidst diverse microbial communities. It’s no surprise, then, that host-microbe alliances abound in nature and fundamentally shape how species function in their environment today. Animal species house an astonishingly diverse array of friendly microbial associates, ranging from exclusive one-on-one partnerships to complex communities (McFall-Ngai et al., 2013). The remarkable diversity and significance of beneficial microbiota have sparked efforts to understand not only who’s there and what they’re doing, but also how such associations are established and maintained. What host and microbial signals ensure colonization of friendly microbes, while excluding potential interlopers?

Key experimental model systems offer the power to dissect signals underlying host-microbe crosstalk. Such models include diverse invertebrate hosts representing deep animal lineages, such as sponges, cnidarians, mollusks, arthropods, and nematodes (Chaston and Goodrich-Blair, 2010; Nyholm and Graf, 2012). Practical advantages of these invertebrate-microbe models often include the ease of rearing abundant hosts in the lab and the ability to investigate microbial colonization via the natural inoculation route. Additionally, the identity and functions of the microbial residents of invertebrate models are often relatively well characterized, particularly so in binary interactions between one host species and its exclusive microbial symbiont. Once viewed as “simple” interactions, these one-on-one associations rely on intricate steps involving complex host-microbe communication. In fact, given that animal hosts are bathed in complex microbial communities filled with potential trespassers, such binary interactions have much to teach us about how the right resident is selected to successfully colonize the host.

The bioluminescent symbiosis between the Hawaiian bobtail squid (Euprymna scolopes) and the marine bacterium Vibrio fischeri exemplifies each of the advantages noted above (Figure 1). Since its characterization more than 20 years ago (McFall-Ngai and Ruby, 1991), this experimentally tractable mutualism has shed light on the molecular “language” of animal-microbe interactions (reviewed by McFall-Ngai et al., 2012; Nyholm and McFall-Ngai, 2004). Elegant work has also discovered key bacterial signals in the symbiosis, including peptidoglycan derivatives that function as toxins in related Vibrio species (reviewed in Nyholm and McFall-Ngai, 2004) and the bioluminescent signal itself (reviewed in Miyashiro and Ruby, 2012). Clearly, even within a binary association, the reciprocal dialog between host and microbe is remarkably complex.

Within this rich body of knowledge, the groundbreaking experiments of Kremer et al. (2013) reported in this issue of Cell Host & Microbe make important strides in clarifying the very first steps in this animal-microbe conversation. The authors address a key question that is relevant not only for this marine model, but also for advancing our understanding animal-microbe interactions more broadly. Given that animals live in environments teeming with diverse microbes, how are specific—even exclusive—microbial associations established, particularly at epithelial surfaces? Kremer et al. (2013) demonstrate that the answer is intricate yet crystal clear: first contact with V. fischeri impacts gene expression of the squid host, which above pores leading to ducts and eventually the larger crypts of the light organ. Successful colonization relies on physical forces (beating of cilia to concentrate particles near the pores), chemical factors (reactive oxygen and nitrogen species in mucus shed by the epithelial surface), innate immune responses (e.g., hemocytes and pattern recognition receptors), and bacteria-induced developmental transformations (loss of these ciliated fields and the full maturation of the light organ) (for overviews, see McFall-Ngai et al., 2012; Nyholm and McFall-Ngai, 2004).
leads to key changes in the epithelial mucus. These changes, in turn, influence the bacterial symbiont in ways that are crucial for successful colonization.

The mechanisms discovered by Kremer et al. (2013) put a spotlight on chitin, a modified polysaccharide that is abundant in nature. Chitin forms an integral structural component of many invertebrate endo- and exoskeletons, may function in the invertebrate immune system, and offers an important food source for chitinolytic bacteria. In fact, earlier work showed that the squid provides chitin as a nutrient to V. fischeri (Wier et al., 2010). In addition to this nutritional role, chitin byproducts—chitobiase—form a chemoattractive gradient that guides V. fischeri into the pore of the light organ (Mandel et al., 2012). More specifically, V. fischeri cells attach to the cilia and aggregate above the pores, appear to pause as they are “primed” for later encounters with other host tissues, and then use chemotaxis to follow a chitobiase gradient into the pore. Thus, the existing abilities of chitin production by the squid, and chemotaxis toward chitin byproducts by V. fischeri, have been co-opted within this mutualism.

Kremer et al. (2013) dissect these initial steps of the encounter—from V. fischeri’s aggregation to its navigation into the pore—with astounding precision. Their results illuminate the underlying mechanisms responsible, while also uncovering two broadly significant phenomena: animal host “readiness” and microbial “priming.” Briefly, their analysis of the squid transcriptome points to an apparent readiness for its very first V. fischeri encounter. Initial contact, when V. fischeri cells attach to cilia and aggregate above the pores, triggers rapid transcriptional responses in the squid host. Upregulated host genes include proteases and hydrolases directed at sugar bonds, including chitin. The hair-trigger nature of this transcriptional response suggests that squid hatch well prepared for the encounter, before being exposed to microbes or V. fischeri specifically.

Most notable among the genes upregulated upon contact with V. fischeri is the host chitotriosidase, which breaks down chitin and releases chitobiase. The authors demonstrate its catalytic activity, which is optimal in the acidic pH of the mucus. The resulting release of chitobiase is crucial for the symbiosis, as it is responsible for generating the chemoattractant gradient known to guide V. fischeri’s migration into pores. Thus, a key contribution of Kremer et al. (2013) is demonstrating the specific mechanism and timing of the production of this essential gradient.

In addition, the results of Kremer et al. (2013) suggest a new dimension of the “priming” of V. fischeri, or physiological changes that occur in preparation for subsequent encounters with chemically distinct host tissues. Outside of squid hosts, V. fischeri exposed to chitobiase in culture medium exhibit increased migration to chitobiase in a capillary tube assay. This result suggests that, within the symbiosis, early exposure of aggregated V. fischeri cells to chitobiase may make them more sensitive to the crucially important chemoattractive gradient leading bacteria into the pore. This potential significance of chitin priming underscores the value of investigating a natural inoculation route, which allows for the fact that microbes navigate through, and are fundamentally impacted by, the distinct local chemistries of the host.

Insights from the squid-vibrio model have broad implications for understanding animal-bacterial communication at the epithelial interface. Squid are certainly not alone in their need to ensure colonization of epithelial surfaces with specific beneficial microbes, while simultaneously living amid diverse microbe communities. All animal species, including our own, face this challenge to varying degrees. With attention to these broader parallels, Kremer et al. (2013) note that mucociliary membranes of nascent light organ have structural and chemical similarities to mammalian epithelial surfaces. In both host groups, the epithelium is not just a simple physical barrier, but rather the mucus contains a wealth of complex antimicrobial compounds that shape microbiota and prevent pathogen proliferation. Further, the phenomenon of host readiness, or preparation for rapid and profound physiological shifts upon first exposure to microbes, is broadly significant. Kremer et al. (2013) remind us that, in mammals, communication across the placenta...
prepares the host immune system for subsequent exposure to microbes. Finally, from the bacterial perspective, members of the mammalian gut flora are known to undergo priming, whereby early exposure of bacteria to particular chemical signals can affect their performance in subsequent steps of an interaction.

These broad parallels across invertebrate and vertebrate systems underscore the very ancient nature of processes that mediate animal-bacterial interactions. Clearly, the glowing squid-vibrio model has much more yet to teach us, not only about the underpinnings of a fascinating marine mutualism, but also about the fundamental molecular strategies that hosts and microbial residents employ to find each other, initiate encounters, and establish successful interactions upon which all animals depend.

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REFERENCES

Initial Symbiont Contact Orchestrates Host-Organ-wide Transcriptional Changes that Prime Tissue Colonization

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SUMMARY

Upon transit to colonization sites, bacteria often experience critical priming that prepares them for subsequent, specific interactions with the host; however, the underlying mechanisms are poorly described. During initiation of the symbiosis between the bacterium Vibrio fischeri and its squid host, which can be observed directly and in real time, approximately five V. fischeri cells aggregate along the mucociliary membranes of a superficial epithelium prior to entering host tissues. Here, we show that these few early host-associated symbionts specifically induce robust changes in host gene expression that are critical to subsequent colonization steps. This exquisitely sensitive response to the host’s specific symbiotic partner includes the upregulation of a host endochitinase, whose activity hydrolyzes polymeric chitin in the mucus into chitooligosaccharides, thereby priming the symbiont and also producing a chemoattractant gradient that promotes V. fischeri migration into host tissues. Thus, the host responds transcriptionally upon initial symbiont contact, which facilitates subsequent colonization.

INTRODUCTION

Bacterial partners, whether beneficial or pathogenic, often travel great distances from the initial site of contact to their eventual location of sustained colonization within the animal host. For example, the journey of a bacterium through the mammalian digestive tract to its target tissues in the hindgut is equivalent to the host animal migrating hundreds of kilometers. During such a journey, the microbes necessarily pass through a series of chemically and physically distinct environments that, in the case of some pathogens, primes them for colonization of a specific tissue (Krukonis and DiRita, 2003; Alvarez-Ordóñez et al., 2011). For example, exposure and subsequent response to acid stress of the stomach render Vibrio cholerae more effective at colonizing the intestine (Merrell et al., 2002); similarly, intracellular passage through a protist in the rumen enhances the pathogenicity of some Salmonella enterica strains (Rasmussen et al., 2005; Carlson et al., 2007). However, because symbiotic initiation is difficult to observe under natural conditions and many models bypass the normal inoculation route, questions about in vivo site-specific colonization by a microbial species have remained largely unexplored, such as how does the passage through various host environments affect their ability to colonize a specific site, and what is the molecular nature of the host-symbiont dialog underlying the process?

The acquisition of the bacterial symbiont Vibrio fischeri by its squid host Euprymna scolopes offers the rare opportunity to probe colonization by specific bacteria directly and in real time in an intact, natural symbiosis using imaging technology. Although the process occurs across a distance of only ~100 μm, it is highly complex. In the established association, in which the symbiont population resides along the apical surfaces of microvillous epithelia within the crypts of the light organ, V. fischeri is the exclusive partner; i.e., in its absence, no other bacteria can colonize the organ.

Confocal microscopy has revealed that during passage to this site, V. fischeri cells interact first with a patch of juvenile-specific mucociliary epithelial surfaces on the light organ’s exterior (Figure 1A), which are concomitantly exposed to thousands of other environmental bacteria. A series of events in the first 3 hr following hatching results in the specific enrichment of the symbiont along these surfaces. At environmentally relevant concentrations of V. fischeri (~5,000 cells/ml of seawater), only 3–5 V. fischeri cells eventually attach to the cilia (Altura et al., 2013) and aggregate above the pores on the organ’s surface (Yip et al., 2006). Once aggregated, this population migrates to and through the pores, and then down the ducts to a physical bottleneck where one or two bacteria squeeze into each crypt and proliferate, filling the spaces. Only V. fischeri can negotiate this journey beyond the pores (McFall-Ngai and Ruby, 1991).

Studies of the system indicate that the chemistry of the mucus is an important determinant of the events leading to colonization. During aggregation, other bacterial species are excluded as V. fischeri becomes primed for their colonization process. In brief, within seconds of hatching from the egg, the animal begins ventilating seawater through its body cavity and shedding mucus from the epithelial surface of the nascent light organ as a nonspecific response to cell-wall (peptidoglycan) derivatives.
released by environmental bacteria (Nyholm et al., 2002). The mucus matrix contains antimicrobial biomolecules (Troll et al., 2010) and vesicles containing nitric oxide (NO) (Davidson et al., 2004). As in other host-symbiont interactions, including the highly complex mammalian-microbiota alliances (Duerkop et al., 2009), these antimicrobials provisioned into the mucus may create a selective “cocktail” that functions in specificity and stability of the associations.

V. fischeri responds to this specific environment presented by the host during aggregation; e.g., the low concentration of NO present in the mucus upregulates NO-detoxification mechanisms (Wang et al., 2010), which protect the symbiont against the more acute NO stress subsequently encountered in the ducts. Additionally, chemotaxis by V. fischeri mediates migration into the host tissues in response to signals like the dimeric breakdown product of chitin, chitobiose (di-N-acetyl-glucosamine) (Mandel et al., 2012). Thus, bacterial symbionts respond both transcriptionally and behaviorally to the host-derived conditions they encounter during initiation.

Here we show that, although exposed to the myriad of other bacteria in the seawater, host tissues respond to the few attaching V. fischeri cells with significant changes in gene expression, most notably in genes encoding proteins that alter the environment to favor symbiont colonization. To explore the impact of symbiont-induced host transcription on the environment presented to the symbiont, we examined in depth one upregulated gene and its encoded protein, a chitotriosidase. We provide evidence that symbiont-induced upregulation of the host chitotriosidase gene results in two downstream events: a priming of V. fischeri by shaping of the biochemistry, wherein they aggregate before entering host tissues, an activity that primes the symbiont for responses to chitin polymers; and the production of a gradient of chitobiose that mediates effective symbiont migration into host tissues. These studies provide a detailed window into the first minutes to hours of interaction between the tissues of a host animal and its symbiotic partner.

RESULTS

The Transcriptome of the Nascent Organ Is Diverse

To determine whether changes in gene expression accompany early symbiotic events, we first constructed a reference
transcriptome (Figure 1). Libraries built from extracted organs included (1) “hatchling,” <15 min after hatching from the egg; (2) “aposymbiotic,” 3 hr after hatching and incubation in Hawaiian offshore seawater (HOSW), which contains environmental bacteria (~10⁶/ml) but is devoid of V. fischeri; and (3) “symbiotic,” the “aposymbiotic” condition, but with wild-type V. fischeri added at a typical, field-relevant concentration of ~5,000 cells/ml (Figure 1B).

This database of early-stage symbiosis increased the number of known transcripts for the E. scolopes light-organ transcriptome by ~40% over a previously constructed Expressed Sequence Tags database (Chun et al., 2006). Pyrosequencing yielded about 2 × 10⁶ reads, from which 79% were assembled, with an inferred read error of 1.5%–2.4% (Figure 1C; Figure S1C available online). Assembly yielded >20,000 isogroups, i.e., unique sequence assemblies potentially representing gene loci, with a median length of 700 bp (Figures 1C and 1D) and mean coverage of 12X. These isogroups were constituted on average by 1.23 isotigs, alternative transcripts associated with a single locus. About 47% of the isotigs could be annotated by Blast2GO software (Figure 1E) with relatively high similarity and low e-values (Figures S1D and S1E). These annotations were inferred principally from UniProt Knowledgebase by electronic annotation against several animal species (Figures S1F–S1H). Each annotated isotig was assigned an inferred read error of 4.8 Gene Ontology (GO) terms (Figures 1E and S1I–S1K). Assignment to Cluster of Orthologous Groups revealed enrichment of clusters related to cellular processes and signaling (39%) compared with information storage and processing (21%) and metabolism (19%); 21% were poorly characterized (Figure S1L).

Only a Few V. fischeri Cells Are Needed for Inducing Specific Changes in Host Gene Expression

We compared the transcriptome of symbiotic organs during the aggregation of the group of approximately five V. fischeri cells with that of aposymbiotic organs. It is important to note that these aggregating V. fischeri cells were in contact with only two to three host cells, although the libraries were generated with entire juvenile organs, which have approximately 10⁴ eukaryotic cells each. As such, any changes in the inductive signal from V. fischeri must be either highly upregulated in the two to three interacting host cells or spread across the entire organ. Recent studies also indicate that, at low bacterial inoculum, V. fischeri products that probably induce host responses would be in concentrations too low to cause these widespread effects (Altura et al., 2013).

We first analyzed a subset of the 3 hr symbiotic animals, obtained concurrently with tissue harvested for the production of the reference transcriptome, to confirm that an average of 5.2 ± 0.8 V. fischeri cells directly interact with squid tissues after a 3 hr incubation (Altura et al., 2013; Figures 2A, S1A, and S1B). Examination of the 454-sequencing libraries identified 84 isotigs (0.31% of the total) differentially represented between the aposymbiotic and symbiotic conditions, of which 72% had annotations (Figure 2B; Table S1). Functional enrichment analysis of these differentially expressed isotigs revealed two major predicted molecular signatures of first contact between symbiotic partners: protease and hydrolase activities directed at sugar bonds, especially those associated with chitin metabolism (Figure 2C). None of these transcripts were differentially regulated in the hatching compared to the aposymbiotic condition (Table S1).

To reveal changes in gene expression that would result from exposure to nonspecific environmental microbes and/or postembryonic development over the first 3 hr posthatch, we also compared the transcriptomes of hatching light organs to those of the aposymbiotic condition. Based on functional enrichment analysis, we detected a significant differential representation of transcripts only in GO terms associated with the ribosomal machinery (Table S1). This differential expression is also detectable between hatchling and symbiotic light organs (Table S1).

Focusing on molecular signatures highlighted by functional analysis in the symbiotic condition, we confirmed the RNA sequencing (RNA-seq) results by determining expression of isotigs encoding proteins associated with protease activity (chymotrypsin protease, cathepsin L, and legumain) and chitin metabolism (chitotriosidase) by quantitative RT-PCR (qRT-PCR) (Figures 2D and 2E). Additionally, we tested differentially expressed isotigs encoding proteins involved in immune responses to bacteria (ferritin and lysozyme). We assayed squid light organs from multiple egg clutches (seven individual clutches laid by different captive females, thus with different genetic backgrounds) to control for genetic variability that may influence development and responsiveness to a symbiotic infection. Five of the seven clutches responded to contact with V. fischeri by upregulating a set of several isotigs encoding, in order of highest to lowest fold change, chymotrypsin protease, lipase, chitotriosidase, legumain, cathepsin L, lysozyme, and ferritin. The overall within-clutch patterns were consistent; i.e., all of these genes were either upregulated or not regulated in the organs of a given clutch. Despite this biological variability, these data demonstrate that the ciliated epithelial cells are able to sense the few associating V. fischeri cells, in a background of 10⁶ nonsymbiotic bacterial cells, and induce specific transcriptional changes in the juvenile light organ.

A Catalytically Active Chitotriosidase Localizes to the Apical Surfaces of Epithelial Cells, Pores, and Ducts of the Organ Surface and Is Secreted into the Associated Acidic Mucus Matrix

Because chitin and its breakdown products play major roles in both the establishment and maintenance of the symbiosis (Wier et al., 2010; Heath-Heckman and McFall-Ngai, 2011; Miyashiro et al., 2011; Mandel et al., 2012), as well as in the pathogenesis of other Vibrio species (Meibom et al., 2005; Blokesch and Schoolnik, 2007), we chose to focus on an in-depth characterization of the symbiont-induced chitotriosidase gene and its encoded protein.

Analysis of the derived amino-acid sequence of this putative chitotriosidase supported its role in chitin degradation. Based on the full-length sequence of EsChitotriosidase obtained by rapid amplification of complementary DNA (cDNA) ends (RACE), the encoded protein was similar in sequence (Figure S2) and tertiary structure (Figure 3A) to a highly conserved animal endochitinase. Chitotriosidase catalyzes the random hydrolytic cleavage of β-1,4 glycosidic bonds of the chitin polymer; as an
endochitinase, it releases chitobiose and larger multimers, but not the monomer, GlcNAc. It contains a signal peptide for secretion, a glycosyl hydrolase 18 (GH18)-type catalytic domain with the critical DxxDxDxE residues essential for activity (Fusetti et al., 2002) (SWISS-MODEL Z score = 2.53 against human chitotriosidase complexed with chitobiose; Benkert et al., 2011) and two chitin-binding domains with conserved cysteine residues critical for substrate binding. As such, it is predicted to be a secreted, catalytically active protein. We confirmed its catalytic activity using affinity-purified EsChitotriosidase (Figures 3B and 3C).

With microscopy, we localized the encoding transcript and the protein in the light organ. Whole-mount in situ hybridization (ISH) revealed abundant transcript in the superficial ciliated epithelium and the cells surrounding the light-organ pores (Figure 3D). Confocal immunocytochemistry localized the protein...
Figure 3. Localization of Chitotriosidase in the Squid Light Organ and Protein Characterization

(A) Predicted three-dimensional structure of the catalytic chitotriosidase (EsChit) GH18 domain. Swiss-Prot model: crystal structure of human chitotriosidase in complex with chitobiose (1LG1; Fusetti et al., 2002). Although the protein sequences are only 46% identical, the structure of EsChit is predicted to be similar to that of human chitotriosidase (Z score = −2.53), especially in the proximity of the chitobiose-binding site (cc, catalytic center). The gradient from cold to warm colors corresponds to residue error compared to the human chitotriosidase structure.

(B) SDS-PAGE gel of purified EsChit (SYPRO Ruby staining).

(C) Chitotriosidase activity of EsChit purified using chitin-magnetic beads (mean ± SE; measurements done in triplicate). The activity was measured in assay buffer (Sigma). Chitinase (100 ng) from the fungus Trichoderma viride (T. viride; Sigma) was used as a positive control for the effect of binding to beads on chitinase activity.

(D) ISH of EsChit in a symbiotic light organ (antisense probe). Purple staining, corresponding to the RNA expression of EsChit, is particularly high in the proximity of the pores (triangles, inset), in the epithelium of the appendages, and in the hindgut.

(E) Coomassie staining of an SDS-PAGE gel of squid-tissue soluble-protein extract (left) and its associated western blot against EsChit (right, expected size: 59.2 kDa without signal peptide).

(F–I) ICC of EsChit in the light organ (F). The antigen labeling is particularly bright in the ciliated field and the cytosol of the appendage epithelium (G) and in the pores and ducts region (H). Colored boxes in (F) show representative locations of pictures zoomed in panels (G) and (H). Green, α-EsChit; red, rhodamine phalloidin (f-actin); blue, TOTO-3 (nuclei). (I) ICC of EsChit in the mucus coating the appendage epithelium. Triangles indicate foci of the protein in the mucus. The inset is a higher magnification of the region highlighted by the box. Green, α-EsChit; blue, Alexa 633 WGA (mucus-binding lectin WGA). Negative controls for ISH (sense probe) and ICC, as well as the RACE sequence of EsChit and its predicted domains and functional residues, are presented in Figure S2.
to intracellular regions along the apical surfaces of the cells of the ciliated field, pores and ducts of the organ (Figures 3E–3H). In addition, the antibody recognized the secreted protein in the mucus matrix overlying the light-organ ciliated surface (Figure 3I). These data demonstrate that the gene is transcribed and the protein produced and secreted in locations where V. fischeri aggregates and establishes dominance over other seawater bacteria.

**The EsChitotriosidase Occurs in Sites with an Optimal pH for Its Activity**

Using a chitinase activity assay, we determined that the pH optimum for in vitro endochitinase activity (i.e., the conversion of chitotriose to chitobiose) of the EsChitotriosidase is between pH 5.0 and 7.5 (Figure 4A), typical of chitinotriosidases (e.g., Xu and Zhang, 2012). Because the pH of the seawater surrounding the squid in nature (~8.2) is well above this range, we hypothesized that the environment created by the host in the location where V. fischeri aggregates is more acidic. To determine the pH of the ciliary-mucus environment, we constructed a pH-sensitive probe coupled to wheat-germ agglutinin (WGA), a lectin known to bind the mucus (Nyholt et al., 2002). The pH in the mucus along the organ’s ciliated appendages was approximately 6.4, and it was 5.9 in the region of the pores (Figures 4B and 4C). Thus, the mucus matrix has a pH range different from that of the seawater passing over these tissues but compatible with the optimal pH for EsChitotriosidase activity. No difference in pH was observed in these regions over the first 3 hr following hatching under any condition; i.e., interaction of host tissues with V. fischeri cells did not influence the pH.

**Chitin Derivatives Are Presented in the Mucus and Ducts where the EsChitotriosidase Occurs**

To present V. fischeri with chitobiase for either priming or the creation of a chemotaxinattractant gradient, the host chitotriosidase must have the substrate chitin, the production of which has been shown to be dependent on chitin synthase in other systems. A chitin synthase localized mainly to the apical surfaces of the ciliated epithelia and to regions of the pores and ducts (Figure 5A). For chitin, we used three different labeling methods. A previous study with a chitin-binding protein (CBP; New England Biolabs) that detects polymers larger than hexamers showed no labeling at the pores or in the ducts (Heath-Heckman and McFall-Ngai, 2011); this study did not examine the mucus. Using a fix to preserve the mucus, the CBP labeled chitin polymers in the mucus matrix (Figure 5B). In animal tissues, calcofluor white binds to polysaccharides. This reagent labeled strongly in regions of the pores and ducts (Figure 5C). To characterize the type of polysaccharides present in these regions, we performed a complementary staining method capable of differentiating GlcNAc and small chitin breakdown products from sialic acid. GlcNAc residues were present on the organ surface and to ~10–12 μm into the ducts; in contrast, only sialic acid residues were present farther down in the ducts (Figures 5G–5I). These results suggest a relative enrichment of small polymers of chitin surrounding the pore and superficial duct regions, where V. fischeri senses a chitobiase gradient.

**Degradation of Chitin Promotes V. fischeri Chemoattraction and Efficient Host Colonization**

Although V. fischeri cells exhibit chemotaxis toward chitobiase during early symbiotic initiation, laboratory studies directly assaying this behavior suggest that, when grown under the conditions used for squid colonization experiments, these bacteria exhibit minimal chemotaxis toward chitobiase (Figure S3). Given that EsChitotriosidase is localized not only close to the pores, but also in the mucus along the ciliated field (Figure 3I), we hypothesized that (1) the EsChitotriosidase activity would produce low levels of chitin breakdown products in the mucosal environment; and (2) during aggregation, V. fischeri cells would be
presented with these products, priming these cells to subsequently sense the chitin-based chemoattractant gradient that mediates migration into the host ducts. To test whether pre-exposure to chitin breakdown products does in fact increase chemotactic recognition of chitobiose by *V. fischeri*, we preincubated cells in culture medium either with or without added CBP. (A and D) Immunocytochemical localization of *E. scolopes* chitin synthase (EsCS). (A) Expression of EsCS in duct cells. (D) The negative control using IgY instead of the antibody, because the EsCS antibody was generated in chicken. Green, α-EsCS (A) or α-IgY (D); red, rhodamine phalloidin (f-actin); blue, TOTO-3 (nuclei). (B and E) Localization of chitin in the mucus surrounding the ciliated appendages. (B) Chitin residues in the mucus labeled with CBP. (E) Negative control, without added CBP. Inset: Magnification of the boxed region. Green, FITC-CBP (chitin); blue, WGA (mucus). (C and F) Localization of polysaccharides in the light organ. (C) Calcofluor staining in the pores and ducts. (F) Negative control, no calcofluor added. Insets: Higher magnification of the pores and ducts region. (G–I) Confocal images of WGA labeling at different levels, from the pores (blue star) to the ducts. Inset: Illustration showing the location of the section (arrow) along the light organ. Red, Alexa 633 WGA (GlcNAc and sialic-acid residues); green, FITC-succinylated WGA (GlcNAc residues only); blue, CellTracker Orange (animal cells).
chitobiose and measured their subsequent chemotaxis toward chitobiose using capillary assays (Figure 6A). *V. fischeri* cells that were previously exposed to chitobiose showed increased migration toward chitobiose. These data suggest that exposure of *V. fischeri* to chitin in the aggregate primes the symbionts to later respond to a chitobiose gradient during transit through the pores.

To determine whether EsChitotriosidase played a role in chitobiose priming for symbiont chemotaxis, we measured the effect of the chitotriosidase inhibition on colonization efficiency by adding the α-chitotriosidase antibody (20 µg/ml) to the surrounding seawater as symbionts were initiating colonization. Antibody binding significantly decreased chitotriosidase activity in vitro (ANOVA, treatment: F = 21.7, p = 0.007; replicate: F = 18.4, p = 0.009; comparison between anti-EsChitotriosidase (α-EsChit) immunoglobulin G (IgG) and control IgG: p adj = 0.01). In vivo efficiency of symbiosis initiation, as measured by the proportion of *V. fischeri*-exposed animals that had successfully colonized one day after inoculation, was statistically lower in animals that had been exposed to the antibody during initiation (Figure 6B), suggesting that antibody binding of the EsChitotriosidase compromises the ability of the symbiont to colonize normally.

**DISCUSSION**

In this study, we demonstrate that, during the initiation phase of an animal-bacterial association, partner interactions create an environment that facilitates the species-specific colonization of distant host tissues. In the squid-vibrio symbiosis, the initiation of colonization occurs as a progression of events that cover a distance of only a few tens of microns during the first ~3 hr after the host hatches from its egg. These discrete events have allowed a fine-scale spatiotemporal resolution of the colonization process, leading to the construction of a model describing the host’s progression from a state of readiness to one of selective responsiveness (Figure 7). This model illustrates two major findings of our study: (1) the remarkable sensitivity of host gene expression to the presence of just three to five cells of the specific symbiont, and (2) the link between these changes in expression and the symbionts’ successful migration into distant tissues.

Much as in the newborn mammal, the juvenile squid hatches from an essentially sterile environment that surrounds the embryo; however, its first postembryonic ventilations bring myriad environmental microbes into the juvenile’s body cavity. Perhaps one of the most remarkable observations in the present study is that as few as three to five *V. fischeri* cells are sufficient to induce a specific transcriptional expression pattern in light-organ tissues, suggesting that the animal hatches from the egg in a state of readiness, but is highly selective in its response (Figure 7A). In an analogous manner, although transcriptomic studies of the initial hours of host-microbe interaction have not been conducted in mammals, normal embryonic development and communication across the placenta result in the production of specific cells (e.g., RORγt+ cells; Eberl, 2012) and biomolecules (e.g., maternal antibodies; Blümer et al., 2007) that ready the host’s immune system for subsequent early postembryonic exposure to microbes.

The mucociliary membranes of the nascent light organ, where the first steps of colonization occur, have a structure, activity, and biochemistry similar to those of epithelial surfaces of the airway, excretory, and reproductive systems of mammals, as well as of the peritrophic membranes that line the midgut of insects (Dinglasan et al., 2009; Hegedus et al., 2009). For example, in both the squid (Davidson et al., 2004; Troll et al., 2010) and mammalian (Duerkop et al., 2009) model systems, antimicrobial compounds incorporated into the mucus influence the composition of the native microbiota, as well as the successful colonization by potential pathogens. Such symbiont-partner selection has also been explored at the cellular and molecular levels in basal metazoans. Specifically, in certain cnidarian species, the secretion of mucus containing antimicrobial peptides along the apical surfaces of the superficial epithelia is critical for the establishment of the normal community composition of the native microbiota (Fraune et al., 2010). Our data suggest that the mucus matrix of the host squid changes as the nascent tissues modulate their gene expression in response to interactions with *V. fischeri*. It is tempting to speculate that the regulation of squid genes, such as those encoding potentially antimicrobial acidic proteases, iron-sequestering proteins, and chitinases, which all have homologs associated with general inflammatory responses (Cho et al., 2002; Conus and Simon, 2010; Lee et al., 2011), is a
mechanism through which proper symbionts are selected. Interestingly, many of these proteins are regulated in response to microbe-associated molecular patterns in other systems (Ong et al., 2005; Badariotti et al., 2007; Liu et al., 2009) and have activities with optima at a low pH, like that found in the light-organ mucus (Figure 4). Thus, it can be postulated that the matrix on the surface of epithelia is responsible for a type of “ecosystems management,” wherein fluid dynamics (e.g., ciliary activity and peristalsis) function together with the biochemistry of the mucus to control microbial associations.

In what ways might such changes in host gene expression also control symbiont specificity and colonization efficiency? In this study, we considered in depth one such V. fischeri-induced host gene, encoding EsChitotriosidase, and tested the hypothesis that its degradation of chitin to chitobiose might increase the efficiency of symbiont colonization. Between aggregation and migration, V. fischeri cells “pause,” a behavior that has been associated with preparation for the upcoming oxidative environment of the duct (Wang et al., 2010) and that may also similarly prime them for other conditions, such as a host-derived chemotactic gradient. Many species of the Vibrionaceae, including V. fischeri, are chemotactic toward chitooligosaccharides (Bassler et al., 1991; Hirano et al., 2011), and chitobiose specifically induces a subset of V. fischeri genes, including several encoding certain chemotaxis proteins (A. Schaefer, personal communication). In addition, a recent study (Mandel et al., 2012) showed that V. fischeri uses chemotaxis toward chitobiose to navigate into host tissues; however, when and how this chemoattractant gradient is produced was not addressed.

We showed that the initial host-symbiont contact results in an...
upregulation of \( \text{EsChitotriosidase} \), encoding a protein whose catalytic function releases chitobiose. The data suggest that this activity establishes a chitobiose gradient in the vicinity of the pores and primes the symbionts to induce chitin-responsive gene transcription in the aggregates. As a result, pre-exposure to chitin breakdown products enhances the ability of symbiotic \( V. \ fischeri \) cells to subsequently sense a chemoattractant gradient toward chitobiose (Figure 6). Taken together, these results suggest that symbiont induction of host \( \text{EsChitotriosidase} \) shapes the local chemical environment in a way that favors the eventual migration of the symbionts through the pores, and thus efficient establishment of a symbiotic colonization. Chitobiose may not be the only chemoattractant in the system; other chemoattractants may be responsible for bringing the bacteria to their final residence, the crypt spaces. This priming behavior is not unlike that of the human symbiont \( \text{Bacteroides thetaiotaomicron} \), which responds to host-derived fucose residues by inducing enterocyte glycosylation (Bry et al., 1996). Interestingly, a pathogenic \( \text{Escherichia coli} \) strain has recently been shown to also sense \( \text{Bacteroides} \)-released fucose and, as a result, use it as a cue to induce expression of virulence genes (Pacheco et al., 2012). Whether other hosts produce (oligo)saccharide signals that are used by bacteria to establish a persistent association remains to be determined, but the existing examples already point to the role of such signaling in interdomain communication between coevolved partners (Oldroyd, 2013; McFall-Ngai et al., 2013).

Surprisingly, our results suggest the possibility that interaction with only a few symbiont cells results in the propagation of a transcriptional signal for \( \text{EsChitotriosidase} \) across the \( \sim 10^5 \) cells of the entire organ; i.e., the pattern revealed by ISH provides evidence that the induction of gene expression is not confined to the few host cells directly interacting with the symbiont aggregate (Figure 2A), but is shared by much of the ciliated surface epithelium. These results contrast with a previous study of whole light organs in which ISH indicated that a symbiont-induced increase in expression of the \( \text{C8} \) subunit of the proteasome was confined to one cell type in the ciliated fields (Kimbell et al., 2006). The results of the present study suggest that localized signaling by \( V. \ fischeri \) cells not only promotes specificity on the ciliated surface and migration into host tissues, but also may prepare distant tissues (e.g., the crypt epithelium) for eventual colonization by the symbiont. More refined techniques, such as laser-capture microdissection (Hooper, 2004), will be required to determine the types and amounts of transcripts that change in those host cells that directly bind symbionts, compared to adjacent epithelial cells. Also, future studies will determine whether the observed changes in gene expression are either transient, and thus specific to these early events, or part of a longer-term or permanent change in response to the symbiont. Sequential waves of transcriptomic changes have been detected following induction of different immune responses in influenza-infected lungs (Pommerenke et al., 2012), a kinetic pattern that may also occur during the subsequent 3–5 hr transit of \( V. \ fischeri \) cells from the surface to the deep crypts.

In summary, the squid-vibrio system provides an ideal candidate for testing recent models of epithelial selection, which predict parameters that favor particular bacterial species and stabilize a host-bacteria mutualism (Schluter and Foster, 2012). This study has afforded a window into initial animal-host responses to a bacterial partner, and the rapid development of new sequencing technologies promises to provide robust methods for the study of single-cell transcriptomics for bacteria such as \( V. \ fischeri \). These horizons will present the opportunity to eavesdrop into the very first conversations of an animal host with its coevolved partner.

**EXPERIMENTAL PROCEDURES**

Adult \( E. \ scolopes \) were collected in Oahu (Hawaii) and bred in the laboratory. All experiments conform to the relevant regulatory standards established by the University of Wisconsin-Madison.

**Transcriptomic Database Using 454 Pyrosequencing**

Juvenile animals were collected within 15 min of hatching and randomly segregated into various experimental conditions in HOSW, which contains \( \sim 10^6 \) environmental bacteria/ml but undetectable \( V. \ fischeri \): hatching, apsymbiotic, and symbiotic (with \( \sim 5,000 \) cells/ml of strain ES114 added to the HOSW). Light organs were dissected and placed into RNA later (Ambion) (\( n = 400 \) per condition for 454 sequencing) and frozen at \( -80^\circ \)C until RNA extraction (RNeasy kit, QIAGEN). cDNA was generated (SMART cDNA synthesis kit, Clontech Laboratories), and its quality was checked using a 2100 Bioanalyzer (Agilent Technologies). Samples were prepared for the 454 sequencing and sequenced on a GS FLX system with Titanium chemistry (Roche Life Sciences). Sequences were processed and assembled through Newbler, annotated for BLAST results and GO by Blast2GO, and clustered in orthologous groups. Reads were quantified (Rpackage, R software), and their differential expression was estimated using Stelke’s method (\( R > 3 \) were differentially expressed) and Fatigo software. For details and references, see Supplemental Experimental Procedures.

**Expression of Candidate Genes**

Squid were collected as for the 454 sequencing, with an additional “sterile” condition in filter-sterilized Instant Ocean (FSIO). qRT-PCR procedures conform to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009) for light-organ collection (20 squid per replicate), RNA preparation, cDNA preparation, qPCR amplification, expression normalization against three housekeeping genes, and data analysis.

Full-length sequence was confirmed by RACE. In situ probes were synthesized from cDNA using a T7 polymerase (Promega) and primers containing the T7-binding site at the 5’ end. Whole-mount ISHs were performed as described in Lee et al. (2009) with incubations at 60°C. For details, see Supplemental Experimental Procedures.

**Immunocytochemistry and Protein Biochemistry**

Polyclonal antibodies against the squid chitotriosidase and chitin synthase were generated from synthetic peptides made to unique regions in the protein (GenScript) and were determined to be highly specific by western blot when used against squid protein extracts. For immunocytochemistry (ICC), the fixation, permeabilization, blocking, and washing procedures were modified from Troll et al., 2010 (see Supplemental Experimental Procedures). For ICC experiments involving mucus staining, the procedure was identical to classical ICCs, except that squid were fixed in Bouin’s solution for 3 hr and permeabilized for 2 hr, and the mucus was counterstained with 10 \( \mu \)g/ml Alexa 633-conjugated WGA.

The chitotriosidase was purified by affinity chromatography: squid protein extract was incubated for 1 hr at 4°C with 50 \( \mu \)l of prewashed chitin-bound to magnetic beads (New England Biolabs). Unbound proteins were washed, and purified proteins were loaded (after beads were boiled for 10 min in 1X TCEP-loading buffer) into a NuPAGE 4%–12% Bis-Tris gel and stained with SYPRO Ruby (Life Technologies). Chitotriosidase activity was tested using a chitinase assay kit (Sigma-Aldrich) on the bead-protein complex against 50 \( \mu \)g of 4-methylumbelliferyl)-\( \beta \)-D-\( N,N \),\( N \)-triacetylcitritriose, which fluoresces at 335 nm after cleavage by chitotriosidase (for details, see Supplemental Experimental Procedures).
Determination of Mucus pH

WGA (Vector Laboratories), which labels host mucus, was mixed with the dual-emission pH probe SNARF-4F 5-(and 6-)carboxylic acid (SNARF; Molecular Probes) at a 1:10 ratio for 15 min with agitation. SNARF was covalently linked to WGA by incubating EDAC at a 1:10 ratio with the protein overnight at 4°C, a reaction that was quenched by adding 1 M glycine. Free SNARF was rinsed from the protein by filtration through Amicon Ultra-4 Centrifugal Filter Units (Millipore) with mPBS. A calibration curve of SNARF, at a working concentration of 10 μM in mPBS at pH values from 5.5 to 6, was generated after excitation at 588 nm. Squid were incubated for 30 min in 10 μg/ml WGA-SNARF in FSIO and washed three times in FSIO before anesthesia. The signal intensity in 100 μm² was measured at 580 nm and 650 nm emissions.

Localization of Chitin Derivatives

The 3 hr squid were incubated with a mix of 20 μg/ml of Alexa 633 WGA, which stains GlcNAc residues and sialic acid; 20 μg/ml of fluorescein isothiocyanate (FITC)-sucinylated WGA, which stains GlcNAc residues only; and 10 mM CellTracker Orange in FSIO for 30 min. Squid were washed three times in FSIO and anesthetized before mounting. To localize chitin in the light organ, squid were incubated in 1% calcofluor white, which intercalates into chitin and cellulose, in FSIO for 5 min following the manufacturer’s instructions (Sigma-Aldrich). For FITC-conjugated chitin-binding protein (CBP; New England Biolabs) staining, squid were fixed and permeabilized as for the ICC protocol in mucus, and CBP was added at a dilution of 1:250 in mPBS for 3 days.

Capillary Assays for Chemoattraction

Chemotaxis toward 10 mM chitobiose was measured in 1 μl capillary tubes after growth in seawater tryptone medium (SWT) supplemented or not with 1 mM chitobiose (GlcNAc2). Nonprimed cells showed chemotaxis toward GlcNAc (Figure S3), which indicated that they were both motile and chemotactic. To further control for any indirect effects during growth in the presence of a sugar, we measured chemotaxis toward chitobiose by V. fischeri cells that were pre-exposed to either 100 nM or 1 mM GlcNAc and observed no response (fewer than 4,000 colony-forming units [cfu] per capillary, data not shown). For details, see Supplemental Experimental Procedures.

Squid Colonization after Chitotriosidase Inhibition

Squid were preincubated in 12-well plates for 2 hr in 2 ml FSIO+IgG, i.e., FSIO containing either α-EsChitotriosidase antibody (20 μg/ml) to bind specific sites or rabbit IgG (20 μg/ml, GenScript) as a control (six squid per well, three wells per condition). Squid were then transferred for 3 hr into FSIO+IgG containing either 3,000 or 5,000 V. fischeri per ml. Squid were quickly rinsed to stop colonization, transferred into FSIO+IgG, and incubated overnight. Luminiscence was scored after 18 hr in a luminometer (Turner Designs), and bacterial density was checked at the end of the experiment to ensure a similar inoculation for both conditions. Because colonization data follow a binomial distribution, statistics were calculated using the Fisher’s exact test (R software, version 2.14.1). Control with 20 μg/ml of bovine serum albumin (BSA) was also performed to rule out any potential effect of the IgG (Fisher’s exact test between IgG and BSA; 3,000 cfu/ml, p = 0.71; 5,000 cfu/ml, p = 1.00). Reduction of chitotriosidase activity against 4-methylumbelliferyl-D-N,N′-diacetylchitotriose was tested in vitro as described above, with no addition, addition of 40 μg of α-EsChit antibody, or addition of rabbit IgG (n = 3 replicates per condition).

ACCESSION NUMBERS

Sequences were deposited in the Sequence Read Archive under the biological project PRJNA205147. The GenBank accession number of EsChitotriosidase is KFO15222.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.07.006.

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