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The first engagement of partners in the *Euprymna scolopes–Vibrio fischeri* symbiosis is a two-step process initiated by a few environmental symbiont cells

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Summary

We studied the *Euprymna scolopes–Vibrio fischeri* symbiosis to characterize, *in vivo* and in real time, the transition between the bacterial partner's free-living and symbiotic life styles. Previous studies using high inocula demonstrated that environmental *V. fischeri* cells aggregate during a 3 h period in host-shed mucus along the light organ's superficial ciliated epithelia. Under lower inoculum conditions, similar to the levels of symbiont cells in the environment, this interaction induces haemocyte trafficking into these tissues. Here, in experiments simulating natural conditions, microscopy revealed that at 3 h following first exposure, only ~ 5 *V. fischeri* cells aggregated on the organ surface. These cells associated with host cilia and induced haemocyte trafficking. Symbiont viability was essential and mutants defective in symbiosis initiation and/or production of certain surface features, including the Mam7 protein, which is implicated in host cell attachment of *V. cholerae*, associated normally with host cilia. Studies with exopolysaccharide mutants, which are defective in aggregation, suggest a two-step process of *V. fischeri* cell engagement: association with host cilia followed by aggregation, i.e. host cell–symbiont interaction with subsequent symbiont–symbiont cell interaction. Taken together, these data provide a new model of early partner engagement, a complex model of host–symbiont interaction with exquisite sensitivity.

Introduction

Many bacterial species occupy more than one niche. In symbiotic associations that are horizontally transmitted, i.e. acquired anew each host generation, the bacterial partner often has an extended free-living stage between periods as a symbiont. The transition of microbes from free-living to host-associated has been studied in a number of systems and is of interest to both environmental and medical microbiologists (Taylor *et al.*, 2005; Nelson *et al.*, 2009; Bright and Bulgheresi, 2010; Freeman *et al.*, 2010). Commonly, transitions occur as the bacterial partner first associates with the apical surfaces of epithelial cells of the host's mucociliary membranes. At these sites, motile cilia occur in dense fields where they work in concert with mucus to create 'mucociliary' currents that coordinate fluid flow across the tissue surface (Lee, 2011). Although these features are restricted to internal locations in terrestrial animals, they first evolved in marine organisms. They are widely distributed among aquatic animal taxa, where ciliated epithelia serve a variety of functions (Emlet, 1991; Riisgard and Larsen, 2001). Because cilia interface with the microbe-rich water column, they mediate not only colonization by symbionts, but also serve as reservoir sites for human pathogens such as *Vibrio parahaemolyticus* (Wang *et al.*, 2010a).

Recent evidence suggests that the cilia of animal mucus membranes not only function biomechanically to control the flow field, but can also sense and respond to foreign substances in the environment (Shah *et al.*, 2009). Thus, the initial harvesting of the partner along mucociliary membranes may also be the period of the first molecular interactions underlying partner recognition. Whereas the interface of beneficial or benign bacteria with host cilia is more common than the interaction with pathogenic microbes, the cilia–microbe relationship has been

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studied in depth principally in cell or tissue culture models of interactions with bacterial pathogens, such as *Pseudomonas aeruginosa* (Bajolet-Laudinat *et al*., 1994; Adam *et al*., 1997; Mewe *et al*., 2005), *Listeria monocytogenes* (Fadaee-Shohada *et al*., 2010), *Moraxella catarrhalis* (Balder *et al*., 2009) and *Bordetella* spp. (Soane *et al*., 2000; Groathouse *et al*., 2003; Anderton *et al*., 2004; Edwards *et al*., 2005). In such systems, a pathogen often binds to ciliary membranes and perturbs the coordinated behaviour of the ciliated field, although the mechanisms underlying these activities are not always well understood.

The symbiosis between the Hawaiian bobtail squid *Euprymna scolopes* and its Gram-negative luminous partner *Vibrio fischeri* offers an opportunity to study the interaction of bacteria with cilia in an intact, natural model. The first contact between the partners occurs along mucociliary membranes on the surface of the light organ (Fig. 1A). The light organ cilia, which develop during embryogenesis, are unique to newly hatched animals, and function to potentiate colonization of the organ (Montgomery and McFall-Ngai, 1993; 1994). *V. fischeri* cells aggregate outside the light organ prior to entry (Nyholm *et al*., 2000; Nyholm and McFall-Ngai, 2003). In these earlier studies, high numbers of *V. fischeri* cells (~ 10^6 ml^{-1} of seawater) were used to visualize the phenomenon. The data showed that bacterial aggregates which form during the first 2–3 h are associated with mucus, which is secreted by the light organ epithelial cells in response to environmental peptidoglycan (Nyholm *et al*., 2000). Then, at 4–5 h following hatching, the aggregated *V. fischeri* cells dissociate from their position on the surface, migrate to and enter three pores, and travel down ducts to the interior microvillus crypt spaces, where they proliferate and reside for the lifetime of the host [reviewed in McFall-Ngai (2008)]. Other Gram-negative bacteria are able to aggregate outside the light organ in the absence of *V. fischeri*; however, when *V. fischeri* is present, the symbiont is always the dominant bacterial species present at the end of the aggregation process (Nyholm and McFall-Ngai, 2003).

A subsequent, independent study of the early hours of the symbiosis under more natural conditions, with exposure to environmentally relevant numbers of *V. fischeri* (~ 5000 cells ml^{-1} of seawater), revealed that the animal is able to sense and respond to the symbiont during the period of its aggregation on the light organ's surface (Koropatnick *et al*., 2004; 2007). Specifically, as early as 2 h post-exposure to symbiont cells, a significant increase was observed in haemocyte trafficking into the blood sinus space underlying the ciliated epithelium, a behaviour induced by the peptidoglycan monomer tracheal cytotoxin (TCT) exported by symbiont cells. However, this work did not address how many bacteria are actually associating with host tissues at the inoculum size that induces haemocyte trafficking.

The present study was undertaken to characterize (i) the behaviour of *V. fischeri* cells, as well as their numbers, during the early hours of host-tissue engagement, (ii) the features of the cells that mediate these interactions and (iii) host responses to the bacterial partner. The data provide evidence that the squid interacts with and responds to the presence of very few bacteria, suggesting a highly sensitive recognition system, and that these bacteria are in direct contact with the host cilia as well as each other. The data further suggest that the response of the cilia in this beneficial symbiosis is similar to the responses of host animal cells to bacterial pathogens, providing yet another instance of convergence in the cellular language of these two different forms of symbiosis.

Results

A small number of host-associated V. fischeri *are sufficient to initiate symbiosis*

To characterize the initial events of host–symbiont interaction, we quantified, over time and with varying levels of

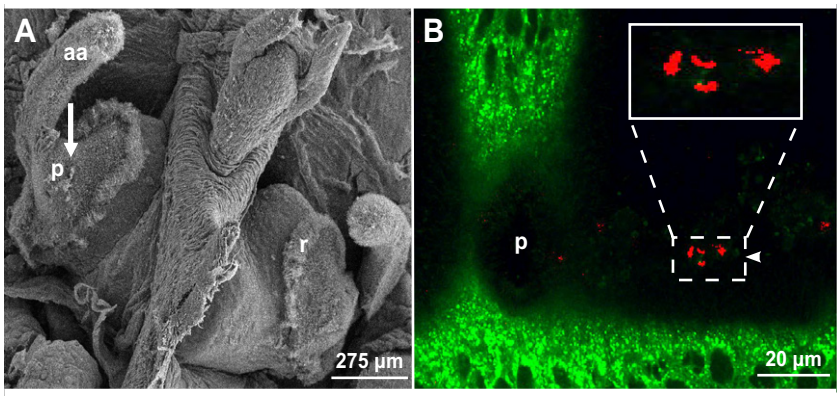


Fig. 1. The site of initial association of environmental *V. fischeri* with host tissues.
A. A SEM of the juvenile squid light organ. Ciliated epithelial fields, which occur on the lateral faces of the organ, are false-coloured in green; arrow, location in panel B of aggregating symbionts.
B. A confocal image of the ciliated epithelium (cytoplasm counterstained with CellTracker Green) of a living animal. An aggregate of 5 live RFP-labelled *V. fischeri* (red) associated with the light organ surface (not all are in this focal plane shown). White box, higher magnification of the associating *V. fischeri* bacteria. aa = anterior appendage, p = pore, r = ciliated ridge.

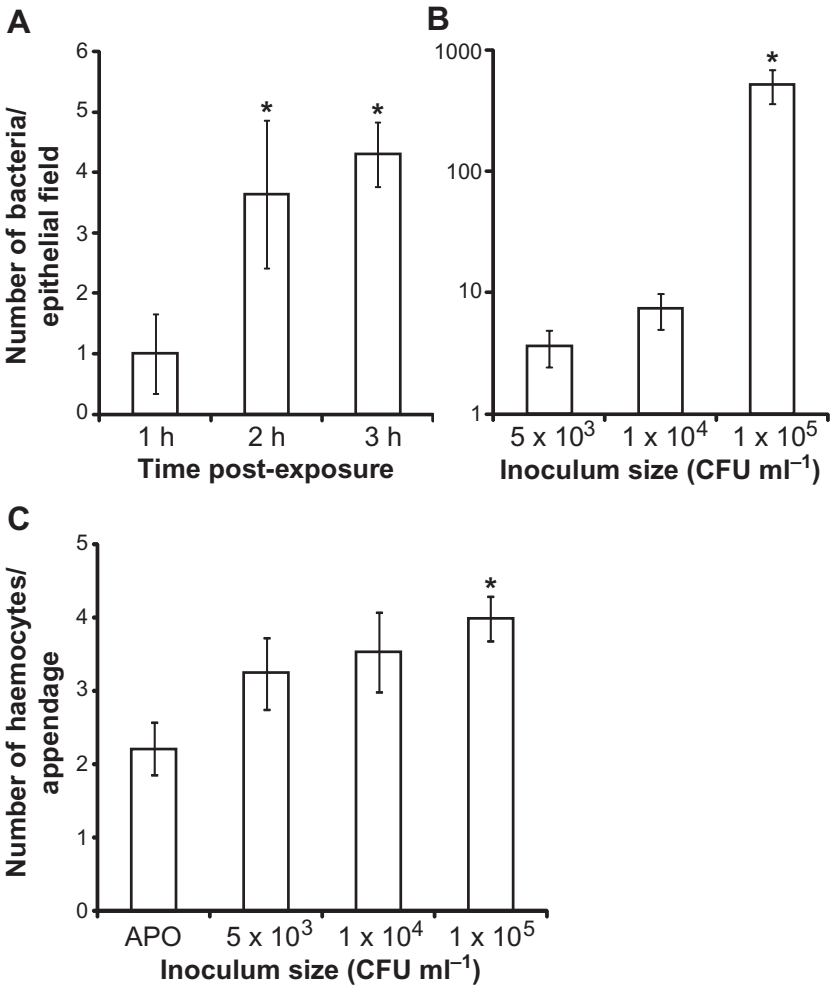


Fig. 2. Quantification of *V. fischeri* ES114 cells in ciliated epithelial fields of juvenile light organs. (Representative graphs; all experiments replicated at least twice).
A. The effect of varying exposure time (h = hours) on the number of wild-type *V. fischeri* per ciliated field after exposure to an environmentally relevant dose of 5×10^3 CFU ml^{-1}. The asterisk indicates data points that were significantly different from 1 h post-exposure, but not from one another (Mann–Whitney test with a Bonferroni correction for multiple comparisons; $n = 5$ independent sample animals for all conditions).
B. The effect of varying inoculum size on the number of *V. fischeri* cells per ciliated field at a constant exposure time of 3 h. The asterisk indicates data points that were significantly different from 5×10^3 CFU ml^{-1} (Mann–Whitney test with a Bonferroni correction for multiple comparisons; $n = 5$ independent sample animals for all conditions).
C. The effect of varying inoculum size on the average number of haemocytes trafficked to the blood sinus space underlying the ciliated epithelium of the light organ. The asterisk indicates data points that were significantly different from the aposymbiotic (Poisson *P*-value analysis; APO, $n = 9$; 5×10^3, $n = 8$; 1×10^4, $n = 11$; 1×10^5, $n = 11$). Bars, standard error.

inocula, fluorescently labelled environmental *V. fischeri* cells associating with the ciliated surface of the juvenile host's light organ. These experiments were performed in the background of natural seawater (~ 10^6 naturally occurring, non-specific marine bacteria per millilitre). To study the timing of association, we exposed the animals to levels of *V. fischeri* that the host typically encounters in the environment (~ 5×10^3 CFU ml^{-1} of seawater; Jones *et al.*, 2007), such doses typically result in 100% colonization and initiation of TCT-induced haemocyte trafficking (Koropatnick *et al.*, 2004). *V. fischeri* associated with the ciliated epithelium at low numbers beginning at 1 h post-exposure (Fig. 1); on average, one fluorescent bacterium per ciliated field was detected. The number of associated *V. fischeri* cells per field increased slightly, but significantly, over the first 3 h to ~ 4 fluorescent cells/ciliated field (Fig. 2A). Shortly after this time, migration into the pores began. With increasing inoculum size, we observed an increase in the number of bacteria associated with the ciliated field at 3 h post-exposure (Fig. 2B). When twice the bacterial dose was added (i.e. from 5000 to 10 000 CFU ml^{-1} of seawater), about twice as many *V. fischeri* cells associated with host tissues, although the result was not statistically significant. With a 20-fold increase in dose, however, we observed a significant 200-fold increase in the number of associating bacteria, which suggests cooperativity above a certain inoculum size. Taken together, these data provide evidence that a very small population of *V. fischeri* interacts with host tissues under normal conditions.

We also examined the effects of increased inoculum size on haemocyte trafficking to the blood sinus space underlying the ciliated epithelium of the light organ. Increased haemocyte trafficking was seen in response to the presence of the symbiont as was previously described (Koropatnick *et al.*, 2007). Further, an increase in trafficking was also observed in response to increasing inoculum size (Fig. 2C).

V. fischeri *cells intimately associate with cilia of the light organ's superficial epithelium*

Because only a few associating symbiont cells are capable of inducing a robust host cell phenotype, e.g. TCT-triggered haemocyte trafficking, we reasoned that the *V. fischeri* cells were not merely suspended above host tissues in host-secreted mucus as previously suggested by our work and the work of others (Nyholm *et al*., 2000; Yip *et al*., 2006), but are likely associated more closely with host cells. Examination of large aggregates of *V. fischeri* (i.e. those resulting from an exposure of the animal to 10^6 CFU ml^{-1}) by scanning electron microscopy (SEM) revealed multiple points of contact between *V. fischeri* and host cilia, as well as contact among the symbiont bacteria themselves (Fig. 3A).

Because the typical aggregates of a few symbiont cells were difficult to visualize by SEM and the fixation/dehydration steps for SEM may introduce artefacts, we also used confocal microscopy with live specimens to visualize the cilia–bacteria interactions. By staining the light organ of live animals with a fluorochrome that labels the abundant tubulin present in the cilia, we localized the fluorescent bacteria in close association with the cilia of the organ's surface. Although in the absence of the labelling of cilia, *V. fischeri* cells often appear to be at some distance from the surface (Nyholm *et al*., 2000), with the cilia labelled, all *V. fischeri* cells colocalized with the ciliary surfaces (Fig. 3B, C) whether the inoculum was at environmentally relevant (10^3 CFU ml^{-1}) or higher (10^5 CFU ml^{-1}) doses of bacteria. In areas that stained positively for mucus, but did not contain cilia, *V. fischeri* cells were not detected (Fig. 3C). These data suggest that *V. fischeri* cells at these initial stages are not suspended in a mucus matrix or biofilm of their making, but rather interact intimately with host cell surfaces. As such, even a small number of symbionts have the potential to deliver signals, such as TCT, directly to the host cells with which they associate.

Bacterial features affect normal interaction with cilia

We then sought to explore a general mechanism underlying *V. fischeri* attachment to host cells. In these experiments, to examine whether non-viable *V. fischeri* associate with host cilia, the bacteria were either heat- or azide-killed to disrupt cellular function. Both treatments led to bacteria that associated with the ciliated surface of the organ at statistically significantly lower levels (Fig. 4). When compared with treatment with azide, however, only one tenth as many heat-treated cells were able to interact ($P = 0.056$). These data suggest that while viability enables this host–bacterial association, a heat-labile cell-surface component likely also plays a role.

Early engagement of V. fischeri *cells is a two-step process: attachment to host cilia followed by bacterial-bacterial cell adhesion*

We used *V. fischeri* mutants defective in exopolysaccharide (EPS) production, which have phenotypes associated with early engagements with the host (Yip *et al*., 2006), as a tool by which to study initial partner–cell interactions. To assess the effect of symbiont EPS production on bacterial association with the ciliated field, we exposed squid to either *V. fischeri* mutants unable to produce EPS (*sypG*$^-$ and *rscS*$^-$), or a mutant that overexpresses EPS (*rscS*$^{++}$) (Table 1). We analysed the data by normalizing the counts to wild-type controls. The *sypG*$^-$ and *rscS*$^-$ mutants had consistently, but not always significantly, fewer bacterial cells associating with host cilia than wild type at all time points and inoculum levels assayed. This lower level of cell association was only statistically significant at high inocula of *sypG*$^-$ and *rscS*$^-$ (10^5 CFU ml^{-1}) at 3 h post-exposure and with short exposures (1 h) of 10^3 CFU ml^{-1} for *sypG*$^-$ (Fig. 5A). Although the bacteria associated with host cells at numbers close to those of wild type, a greater distance between individual *sypG*$^-$ mutant cells was detected, indicating the disruption of the aggregation phenotype (Fig. 5B), as had been previously described (Yip *et al*., 2006). In contrast, symbiont cells that overexpressed EPS (*rscS*$^{++}$) had significantly higher numbers of *V. fischeri* cells associating with the light organ surface compared with wild type at all time points and inocula (Fig. 5A), and were as closely associated with one another (Fig. 5C). The results of these experiments suggest that the *V. fischeri* EPS is not required for normal association with host cilia but is required for aggregation. As such, they provide evidence of a two-step process: attachment of *V. fischeri* to host cilia, followed by aggregation mediated by the EPS on the surface of these symbionts.

To determine which of these two steps, i.e. adherence to the cilia or EPS-mediated aggregation, induces the characteristic haemocyte trafficking into associated host tissues, we quantified haemocytes in the blood sinus underlying the superficial epithelium. Neither the underexpression of EPS (*sypG*$^-$) nor the overexpression of EPS (*rscS*$^{++}$) significantly affected the number of haemocytes that trafficked to the blood sinus space of the light organ anterior appendage (Fig. 5D).

We also examined four other mutants defective in outer envelope features (Table 1). The strains used included three mutants defective in early colonization: (i) a pilus mutant (Δ*pilA2*) (Stabb and Ruby, 2003), (ii) an outer membrane protein mutant (Δ*ompU*) (Aeckersberg *et al*., 2001) and (iii) a mutant defective in O-antigen production (*waaL*::Tn*erm*) (Post *et al*., 2012). We also examined a

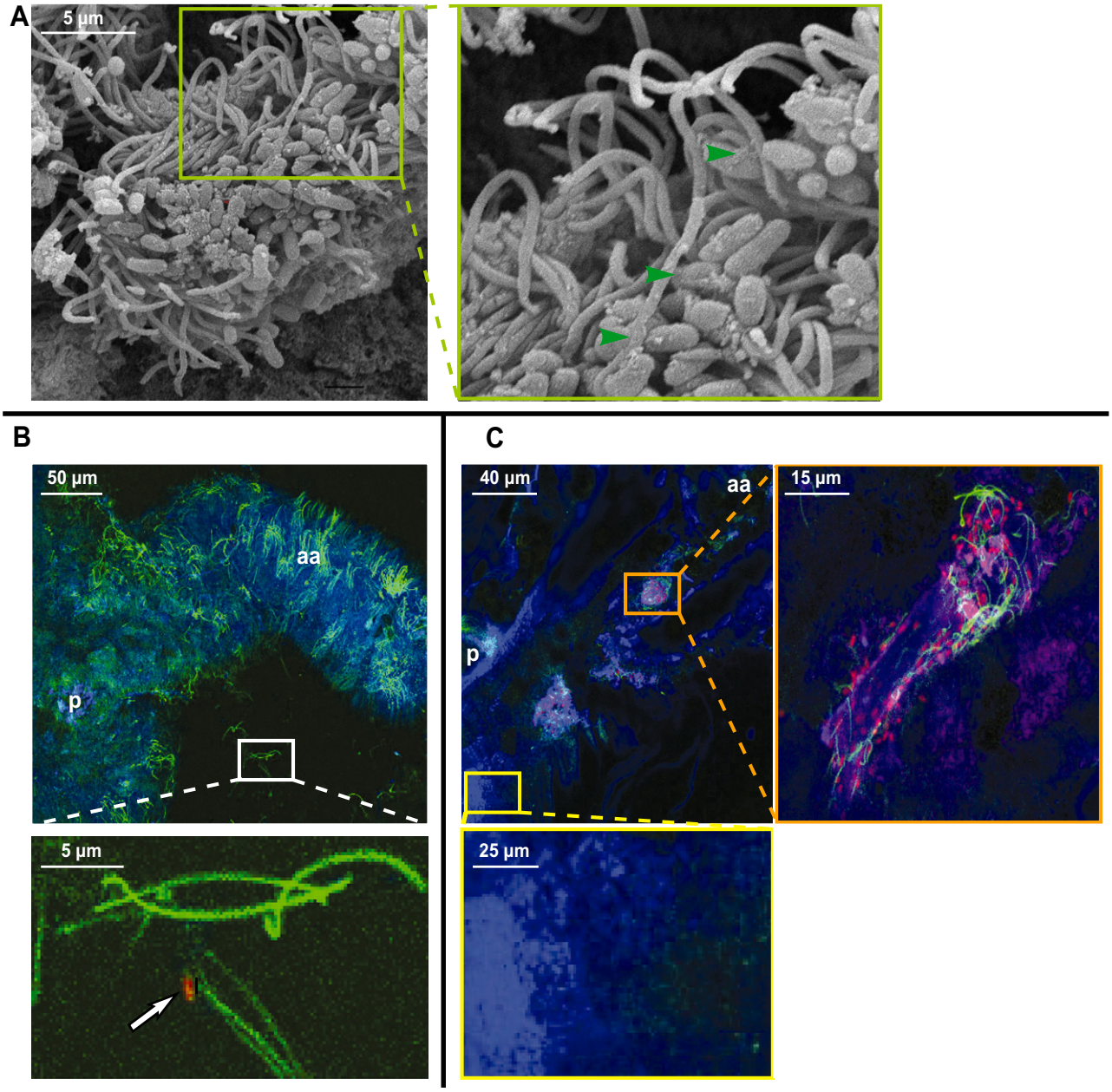


Fig. 3. Representative micrographs of *V. fischeri* associated with the ciliated epithelium of the juvenile light organ.
A. A SEM micrograph of *V. fischeri* cells (false coloured) associating with the organ surface of an animal exposed to 1×10^6 symbiont CFU ml^{-1} and then fixed. Green arrows indicate examples of bacterial-ciliary contacts.
B. Upper: confocal micrograph of a live animal specimen exposed to 5×10^3 CFU ml^{-1} *V. fischeri* (red) for 3 h. Box, area magnified in lower panel. Lower: RFP-expressing *V. fischeri* associating with a host cilium. [Counterstains: cilia, TubulinTracker (green); mucus, wheat germ agglutinin (blue); image is highly pixelated because of use of living tissue, which requires acquiring images at high scan speeds]
C. Upper left: confocal micrograph of a live animal specimen exposed to 1×10^5 CFU ml^{-1} *V. fischeri* (red) for 3 h. Orange box, area magnified in upper right. Yellow box, area magnified in lower panel. Upper right: higher magnification of RFP-expressing *V. fischeri* associating with host cilia. Lower: area of mucus without cilia, which contains no *V. fischeri*. [Counterstains: cilia, TubulinTracker (green); mucus, wheat germ agglutinin (blue)].

mutant in the *V. fischeri mam7* gene. The Mam7 protein was an attractive candidate for involvement in initial attachment, as in *V. cholerae*, the product encoded by this gene is involved in the early binding of *V. cholerae* to host microvilli (Krachler *et al*., 2011), and so its homologue in *V. fischeri* may mediate binding to host membranes. Further, the *V. fischeri* homologue has the same *in vitro* activity as the *V. cholerae* protein (Fig. S1). None of these

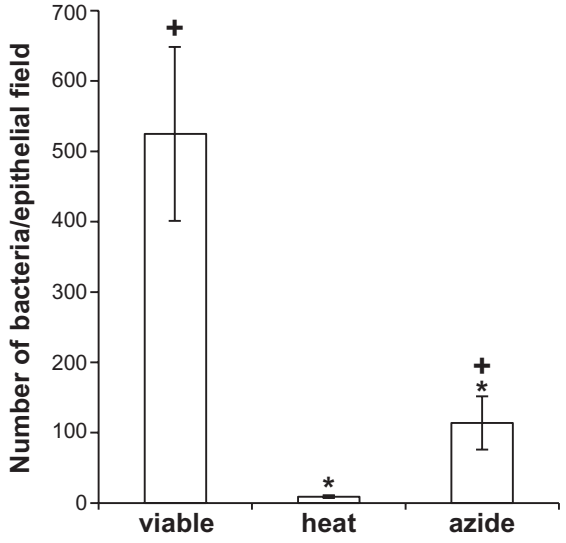


Fig. 4. The effect of *V. fischeri* viability on association with the ciliated epithelial field of the juvenile light organ. (Representative graph; all experiments replicated at least twice). Number of bacteria/ciliated field after 3 h exposure to 1×10^5 CFU ml^{-1} of viable, heat-killed, or azide-killed *V. fischeri*. The asterisk indicates data points that were significantly different from viable bacteria and +, data points that were significantly different from heat-killed bacteria, both according to a Mann–Whitney test with a Bonferroni correction for multiple comparisons ($n = 5$ independent sample animals for all conditions). Bars, standard error.

mutants had either an effect on attachment or an association defect under any condition (Fig. S2).

Association with host cilia is not specific to symbiotic V. fischeri

To examine the specificity of attachment to host cilia, we assessed the ability of a *V. fischeri* strain that is not symbiotic in the squid host to associate with light organ cilia (Table 1). We exposed the animal to *V. fischeri* MJ11, the symbiont of the Japanese pinecone fish *Monocentris japonica*, which is typically unable to colonize the squid (Mandel *et al.*, 2009). No significant difference was found in any of the six inoculum conditions tested (Fig. 6A). These data indicate that the defects in colonization of this strain are not likely due to defects in association of the cells with host cilia. MJ11 does not have the *rscS* gene (Mandel *et al.*, 2009), which renders them unable to aggregate, but similar to *rscS*$^-$ ES114, they show no defect in association with the cilia.

V. parahaemolyticus KNH1, an isolate from a nearshore habitat in Hawaii where both the host squid and free-living, symbiosis-competent *V. fischeri* co-occur, was also analysed. KNH1 forms aggregates outside the squid light organ in the absence of *V. fischeri* cells, but is unable to colonize the animal (Nyholm and McFall-Ngai, 2003); as the aggregate matures, *V. fischeri* outcompetes *V. parahaemolyticus*. Under the conditions of the experiments reported here, *V. parahaemolyticus* KNH1 cells

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Bacterial strains		
Vibrio fischeri		
ES114	Squid light organ symbiont from *Euprymna scolopes*	Boettcher and Ruby (1990)
MJ11	Fish light organ symbiont from *Monocentris japonica*,	Mandel *et al.* (2008)
rscS$^-$ (KV2787)	KV1323 (Δ*rscS*::erm) carrying pVSV208, Cam^R	Visick, unpublished
sypG$^-$ (KV2785)	KV1666 (*sypG*::pAIA4); carrying pVSV208, Cam^R	Hussa *et al.* (2007)
rscS^{++}	ES114 carrying pVSV208, Cam^R, and pKG11, Cam^R and Tet^R	Yip *et al.* (2006)
ΔompU	ES114, *ΔompU::camR*, carrying pVSV209, Kan^R	J. Graber and E. Ruby, unpublished
ΔpilA2	ES114, Δ*pilA2*::*kanR*, carrying pVSV208, Cam^R	Stabb and Ruby (2003)
waaL	ES114, *waaL*::Tn5*ermR*, carrying pVSV208, Cam^R	Post *et al.* (2012)
Δmam7	ES114, Δ*mam7*, carrying pVSV208, Cam^R	This study[a]
Vibrio parahaemolyticus		
KNH1	Environmental isolate from the coast of Oahu, HI	Nyholm *et al.* (2000)
Plasmids		
pVSV208	$oriV_{R6K\gamma}$, $oriT_{RP4}$,$oriV_{pES213}$, *rfp*-tagged, Cam^R,	Dunn *et al.* (2006)
pVSV209	$oriV_{R6K\gamma}$, $oriT_{RP4}$,$oriV_{pES213}$, KanR-constitutively expressed *rfp*, transcriptional terminators-(AvrII, SalI, StuI)-promoterless CamR and *gfp*	Dunn *et al.* (2006)
pKG11	pKV69 (SalI) + 3 kb SalI fragment from mutagenized pLMS33 containing *rscS1* allele; Cam^R, Tet^R	Yip *et al.* (2006)
pKV363	$oriV_{R6K\gamma}$, *oriT*RP4, *ccdB*-mediated suicide vector, Cam^R	Shibata and Visick (2012)

Cam^R, chloramphenicol resistance; Erm^R, erythromycin resistance; Kan^R, kanamycin resistance; Tet^R, tetracycline resistance; *gfp*, green-fluorescent-protein gene; *rfp*, red-fluorescent-protein gene.
a. See Supporting information Methods.

were able to associate with the cilia at levels statistically indistinguishable from the symbiont at all time points and inocula (Fig. 6A).

V. parahaemolyticus is known to be outcompeted in the aggregate by wild-type *V. fischeri* (Nyholm and McFall-Ngai, 2003). To determine whether association with the cilia played a role in dominance, we exposed the squid to *V. parahaemolyticus* and wild-type *V. fischeri* ES114, and counterstained the cilia to observe the extent of close association of these cells with host tissues. We detected no change in localization of *V. parahaemolyticus*. In addition, we observed that this non-symbiotic species continued to localize to the cilia, even in the presence of increasing levels of wild-type *V. fischeri* (Fig. 6B).

The factors that influence symbiont dominance are not well understood in the squid–vibrio system. To determine whether the EPS-induced, bacteria–bacteria associations described earlier influence dominance by wild-type *V. fischeri* over *V. parahaemolyticus*, we competed *V. parahaemolyticus* against either the wild-type *V. fischeri* or the *rscS*$^{-}$ mutant. The *rscS*$^{-}$ mutation in *V. fischeri* did not compromise its ability to dominate *V. parahaemolyticus* in the aggregate. However, no significant difference in the relative levels of association with the host's ciliated epithelium was detected at the inocula tested (Fig. 6C). These data provide evidence that dominance of *V. fischeri* occurs at a point downstream of both the attachment and aggregation steps.

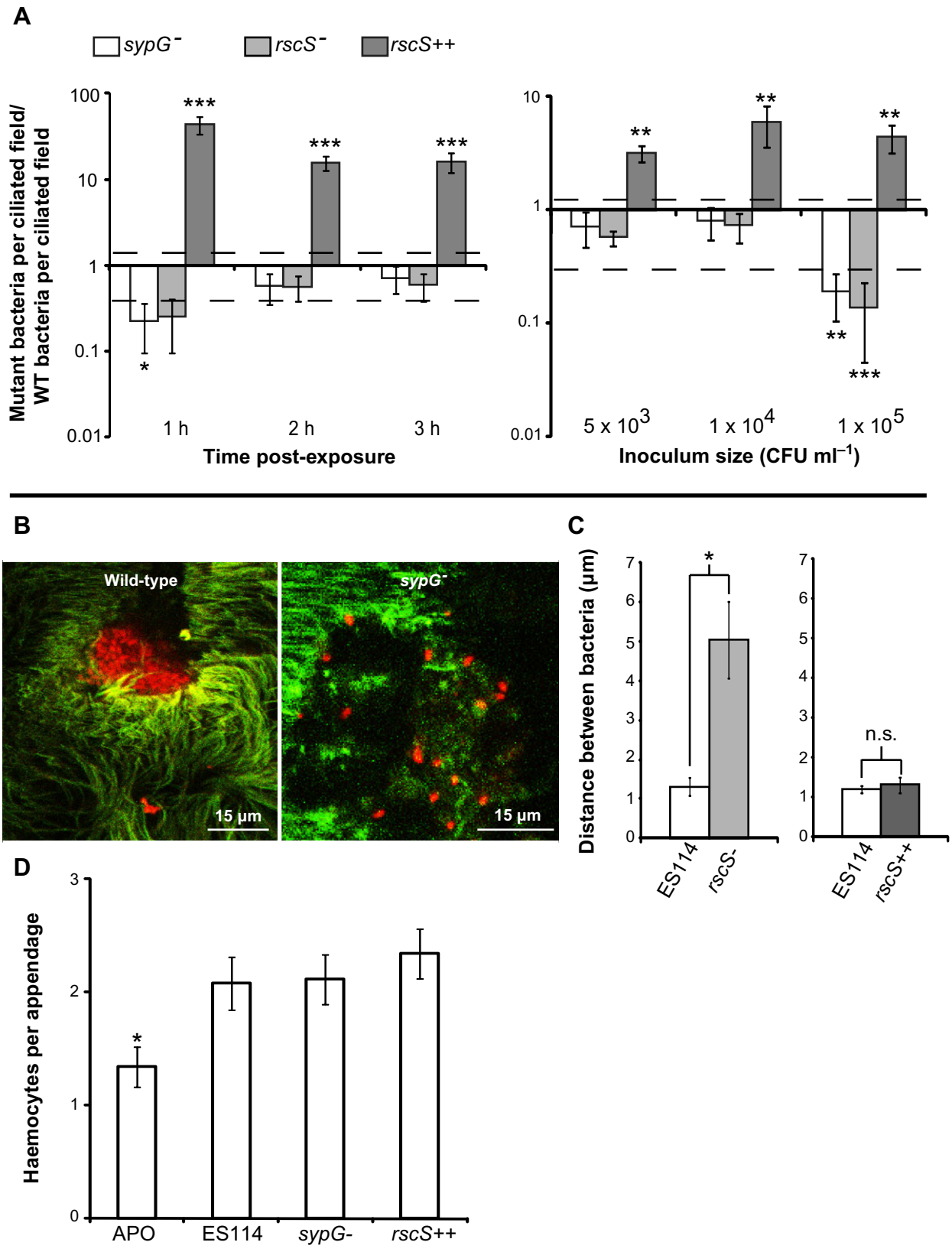
Discussion

Using the experimental squid–vibrio model of the colonization of animal tissues by bacterial symbionts, this study examined, *in vivo* and in real time, the interactions between host cilia and bacterial partners during the initial hours of bacterial transition from free-living to host-associated. Using the typical environmental conditions in which *V. fischeri* represents only ~ 0.5% of the ambient bacteria, we found that: (i) during the initial 2–3 h, only 3–5 candidate-symbiont cells are recruited into an aggregate that migrates into host tissues; (ii) these cells are in close contact with host cilia; (iii) their viability aids in association with host tissues; (iv) this recruitment phase progresses through at least two independent steps: initial association with cilia, followed by formation of small aggregates, the latter of which is EPS-mediated; and (v) these two steps are not responsible for the resolution of symbiont specificity, which also occurs before *V. fischeri* cells enter host tissues.

An exquisitely sensitive recognition system

The picture emerging from this and other studies indicates elaborate biomechanical and biochemical underpinnings that lead to the ultimate resolution of the exclusive partnership between *E. scolopes* and *V. fischeri*. While residing for hours on the light organ surface, 3–5 viable *V. fischeri* cells engage host cells first and then conspecific cells (Figs 3A and 4), a finding that suggests an active process with molecular and/or biochemical dialog between and among partnering cells. Recent data have provided support for this possibility. Genetic studies of *V. fischeri* have demonstrated that, before entering host tissues, they must be capable of adapting to nitric oxide in host-shed mucus on the surface of the organ (Davidson *et al*., 2004; Wang *et al*., 2010b; Altura *et al*., 2011). During these early hours, the host is also undergoing changes beyond the haemocyte trafficking described here. At first hatching, the light organ crypt spaces, where the symbionts will eventually reside, are permissive, allowing all environmental particles < 5 microns to enter into these spaces; however, no particles or cells, including any *V. fischeri* cells that might enter, persist. Then, during the ~ 2–3 h period of *V. fischeri* aggregation, the host crypts experience a restrictive period when nothing enters (Nyholm *et al*., 2002). After this 'restrictive' period, the light organ crypts exclude all cells other than those of *V. fischeri*. Taken together, these data provide several lines of evidence for an early period of adaptation by the partners, for which the host cilia–*V. fischeri* interactions described here may play a critical role.

The ability of the host to discriminate *V. fischeri* from most other environmental cells may result in the host's detection of exported peptidoglycan monomers (TCT) produced by the attaching cells. Haemocyte trafficking is a response to symbiont TCT (Koropatnick *et al*., 2004), and the onset of haemocyte trafficking correlates with *V. fischeri* attachment to the cilia. While other *Vibrio* spp. can induce host haemocyte trafficking (Koropatnick *et al*., 2007), *V. fischeri* is the dominant *Vibrio* spp. in the host environment (Jones *et al*., 2007) and, when it is present, it outcompetes other *Vibrio* spp. These features suggest that the response is specific to *V. fischeri* under normal conditions. One other possible explanation for haemocyte trafficking during this time is that the host may merely be responding to the presence of the 5000 *V. fischeri* CFU ml^{-1} in the seawater. Extrapolating from data on the export of TCT by culture-grown *V. fischeri* cells (Koropatnick *et al*., 2004), about 5000 CFU ml^{-1} in seawater would produce about 2 pM TCT, well below the detection limit of host cells (Koropatnick *et al*., 2004). This amount is likely an overestimate under the conditions of aggregation, as the cells are not growing in the seawater nor in the aggregates (Nyholm and McFall-Ngai, 2003). As such, peptidoglycan turnover and TCT export is likely to be attenuated. The finding that the host responds to 3–5 attaching *V. fischeri* cells provides evidence for very high


A
sypG⁻
rscS⁻
rscS++
Mutant bacteria per ciliated field/ WT bacteria per ciliated field
100
10
1
0.1
0.01
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1 h
2 h
3 h
Time post-exposure
10
1
0.1
0.01
**
**
**
**
***
5 x 10³
1 x 10⁴
1 x 10⁵
Inoculum size (CFU ml⁻¹)
B
Wild-type
sypG⁻
15 µm
15 µm
C
Distance between bacteria (µm)
7
6
5
4
3
2
1
0
*
n.s.
ES114
rscS-
ES114
rscS++
D
Haemocytes per appendage
3
2
1
0
*
APO
ES114
sypG-
rscS++

Fig. 5. The two-step process of the host's engagement of *V. fischeri* cells. (Representative graphs; all experiments replicated at least twice).
A. Left: The effect of varying time on the number of mutant *V. fischeri* bacteria/ciliated field after exposure to an environmentally relevant dose of 5×10^3 CFU ml^{-1}. Both strain and time post-exposure to bacteria affected variation in the RscS++ colonization data as tested by a two-way ANOVA, time, $P < 0.05$, and strain, $P < 0.0001$. Right: The effect of varying inoculum size on the number of mutant *V. fischeri* bacteria/ciliated field at a constant exposure time of 3 h ($n = 5$ independent sample animals for all conditions). For *sypG*$^-$ and *rscS*$^-$ strain experiments, all three factors (strain, inoculum and the interaction factor) were significant with $P < 0.05$ by a two-way ANOVA. For RscS++ strain experiments only strain accounted for variation in the data by a two-way ANOVA, $P < 0.0001$. Dashed line, standard error for wild-type controls. Significant differences from wild-type are denoted as follows: *, *P*-value < 0.05, **, *P*-value < 0.01, ***, *P*-value < 0.001 by a Dunn's post-hoc pairwise comparison within each strain's data set after a Bonferroni post-test for multiple comparisons.
B. Confocal micrographs of RFP-expressing *V. fischeri* wild type or *sypG*$^-$ bacteria in association with the ciliated epithelium of live animal specimens [Counterstain: cilia, TubulinTracker (green)].
C. The effect of altered exopolysaccharide production on the average distance between aggregating bacteria. ($n = 10$ pairs of bacteria from 3 independent sample animals for all conditions; inoculation, 1×10^5 CFU ml^{-1} seawater). The asterisk indicates *P*-value < 0.05, data points that were significantly different from WT/ES114 according to a Mann–Whitney test. Bars, standard error.
D. The amount of haemocyte trafficking to the blood sinus space of the light organ anterior appendage due to exposure to exopolysaccharide mutants. (APO, $n = 44$; ES114, $n = 38$; *sypG*$^-$, $n = 44$; *rscS*$^{++}$, $n = 47$; inoculation 10^4 CFU ml^{-1} of seawater) The asterisk indicates that the average of the two distributions is significantly different with a Poissonian *P*-value of < 0.05. Bars, standard error.

specific activity of TCT as well as for requirement for direct contact with host cells for delivery of this microbe-associated molecular pattern, or MAMP.

The role of symbiont features in initial host-symbiont engagement

The finding that heat-killed *V. fischeri* cells were more perturbed than azide-killed cells in their association with the host raises the possibility of a heat-labile surface component that is involved in the formation of the interactions of these cells with host cilia. Mutants in surface features known to be important in bacterial pathogenesis, including those encoding a pilin protein, a porin, LPS O-antigen and the Mam7 protein were normal in their ability both to associate with the host cilia and to aggregate (Fig. S1). Further analyses with these mutants in the squid–vibrio system will determine whether they mediate normal engagement and interactions with the microvillous surfaces of the crypt epithelia. While mutants defective in these molecular features of the cell envelope had no detectable defects in initial interactions with the host because the partners are interacting at this time with their surfaces, screens directed at production of mutants in outer membrane molecules (Kaufman and Taylor, 1994) promise to be a fruitful area of future research on this system.

EPS production by *V. fischeri* is a well-studied essential feature of early interactions in the squid–vibrio system (Visick, 2009). Mutations in symbiont genes encoding molecules that synthesize the EPS or regulate its production are defective in colonization of the host animal. Examination of mutants defective in EPS production in the present study suggests that the formation of bacterial–bacterial interactions is a distinct process from association with the host cilia, and that aggregate formation in part is EPS-mediated. A two-step process during initial host engagement may also occur in *Vibrio cholerae*, where attachment of *V. cholerae* cells to chitin and mucin is mediated by the colonization factor GbpA (Wong *et al*., 2012), and bacterial–bacterial interaction is controlled by the toxin-coregulated pilus (Kirn *et al*., 2000). Whereas EPS has a profound effect on the ability of the bacteria to aggregate, it does not appear to play a role in either dominance within the aggregate by symbiosis competent *V. fischeri* over other related bacteria or in the induction of host haemocyte trafficking (Figs 5D and 6C). The fact that EPS is non-essential to these processes indicates that bacterial aggregation is most critical for the effect it has on communication among *V. fischeri* cells participating in the aggregate. Additionally, the data indicate that the formation of host–bacterial, rather than bacterial–bacterial interactions, are likely to play a role in the haemocyte-trafficking and dominance phenotypes.

We also found that *V. fischeri* cells are not capable of outcompeting non-symbiotic *Vibrio* spp., specifically *V. fischeri* MJ11 and *V. parahaemolyticus* KNH1, for attachment sites on the cilia or for early aggregation. This capacity of non-specific Gram-negative cells may be related to the large number of sites available for attachment on the host's ciliated epithelial fields. These data also provide evidence that the dominance of *V. fischeri* observed in the mature aggregate (Nyholm and McFall-Ngai, 2003) is due to other factors, the production or presentation of which may be induced as downstream responses to earlier interactions of *V. fischeri* cells with host cilia.

A new model of early host–symbiont interaction in the squid–vibrio symbiosis

Earlier concepts of the squid–vibrio system postulated that host-shed mucus provides a sort of platform for aggregating symbiont bacteria. The data presented here on ciliary attachment, as well as earlier findings of abundant antimicrobials in the mucus matrix (Davidson *et al*., 2004; Troll *et al*., 2010), suggest that the mucus may be instead involved in the winnowing process, one in which

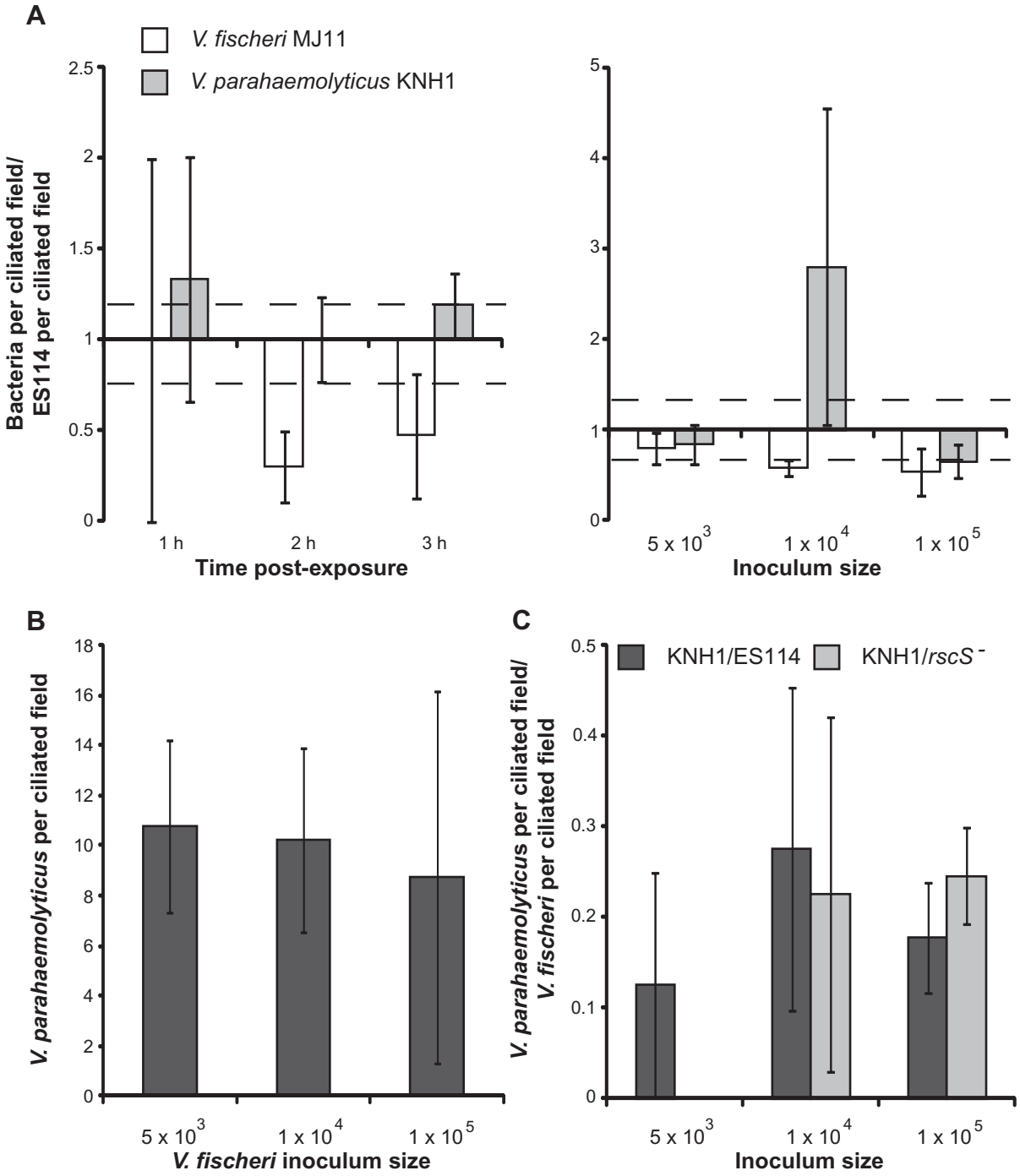

Fig. 6. The ability of non-symbiotic vibrios to associate with the ciliated epithelial field of the juvenile light organ. (Representative graphs; all experiments replicated at least twice).

A. Left: The effect of different lengths of exposure on the number of *V. fischeri* MJ11 and *V. parahaemolyticus* KNH1 bacteria/ciliated field after exposure to an inoculum of 5×10^3 CFU ml^{-1}. Right: The effect of inoculum size on the number of *V. fischeri* MJ11 or *V. parahaemolyticus* KNH1 cells per ciliated field after a constant exposure time of 3 h. Dashed line, standard error for *V. fischeri* ES114 controls. Data were analysed by a two-way ANOVA followed by a Bonferroni post-test for multiple comparisons, though no significant differences were found.

B. The effect of increasing *V. fischeri* ES114 inoculum size on the number of *V. parahaemolyticus* KNH1 bacteria/ciliated field. Exposure time, 3 h. KNH1 inoculum, 5×10^3 CFU ml^{-1}.

C. The effect of *V. fischeri* aggregation on the ability of *V. parahaemolyticus* KNH1 to compete with *V. fischeri*. Exposure time, 2 h. ($n = 5$ independent sample animals for all conditions). Bars, standard error.

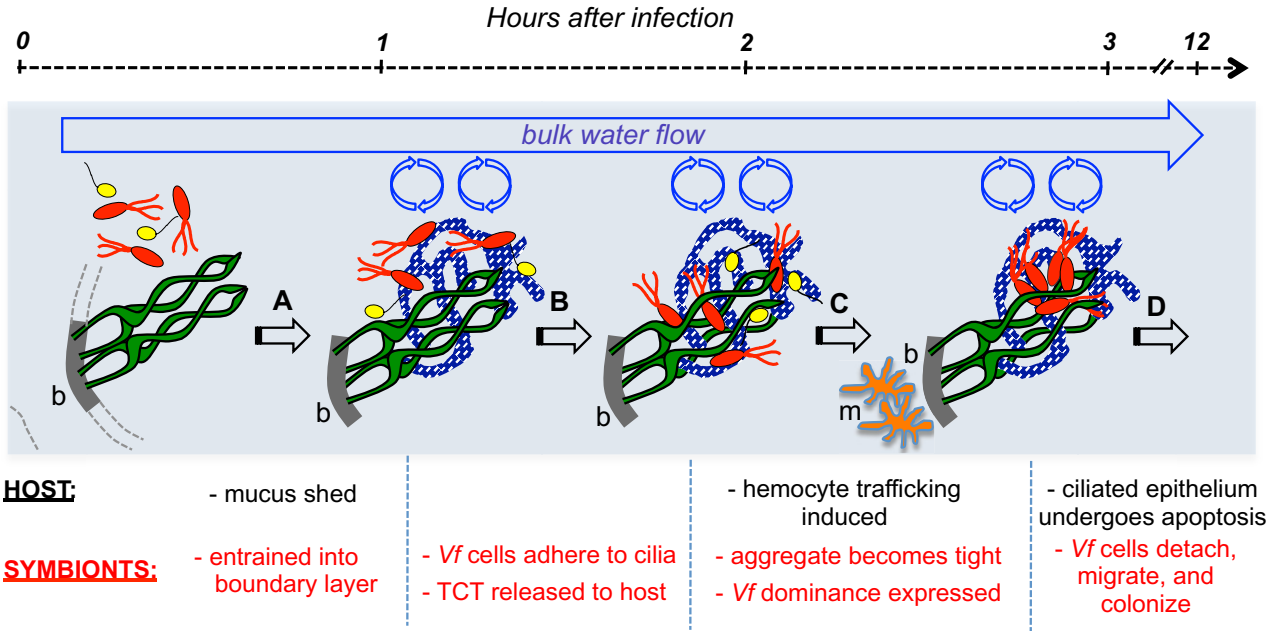

Fig. 7. A new model of the initial events of partner interaction in the squid–vibrio association. The early events can be defined as a series of transitions.
A–D. Immediately upon hatching, water harbouring environmental bacteria (red, *V. fischeri*; yellow, non-specific Gram-negative) is brought through the host's body cavity into the vicinity of the cilia (green) on the surface (thick grey line where cilia attach) of the nascent symbiotic organ. Mucus (blue mottling) is shed (transition A), Gram-negative bacteria then bind to the cilia (transition B), *V. fischeri* releases TCT and host responds with the trafficking of macrophage-like blood cells (m) into the blood sinus (b) under the ciliated epithelium (transition C), and finally *V. fischeri* migrates into host tissues and colonizes deep crypt spaces of the organ, where it induces host morphogenesis (transition D). (See text for details.)

V. fischeri is increasingly able to withstand biochemical challenges presented by the host. These data suggest a new model in which initial interactions between the host animal and *V. fischeri* cells involve a multi-step process (Fig. 7). Shortly after hatching, the host releases mucus stores from the light organ ciliated epithelia in response to environmental peptidoglycan (Nyholm *et al*., 2002). Then, environmental *V. fischeri* cells associate with host cilia, a behaviour that facilitates the delivery of TCT by a few cells to induce haemocyte trafficking into the blood sinus underlying the host ciliated epithelia [this paper, Koropatnick *et al*. (2007)]. Aggregation of *V. fischeri* occurs subsequently. This process, which is at least in part mediated by EPS (Visick, 2009), must involve migration of symbiont cells along the ciliated surface to affect cell–cell contact. During late aggregation, *V. fischeri* cells become competitively dominant in the aggregate and the cell populations localize near pores on the light organ surface, into which the cells migrate (Nyholm and McFall-Ngai, 2003). *V. fischeri* cells then disengage from the ciliated surface and migrate to these pores. There the cells sense chitobiose produced by the host, which they use for chemotaxis into host tissues (Mandel *et al*., 2012).

All horizontally transmitted symbioses undergo a habitat transition. The squid–vibrio system provides evidence of how intricate such a process can be. The findings presented here add pieces to the complex puzzle, but many questions remain unanswered. These questions include: What are the molecular mechanisms underlying attachment of *V. fischeri* cells to host cilia? Does attachment to the cilia induce changes in host gene expression and/or in the biochemistry of the environment around aggregating symbionts? How do the attached *V. fischeri* cells subsequently detach and move along the ciliated surface to form aggregates, eventually migrating to colonize host tissues? How is specificity in the mature aggregate of *V. fischeri* cells achieved? Although it is clear a good deal, more research must be done on the system before an accurate view of this process is achieved, the continued application of available methods, as well as the development of new technologies, such as single-cell transcriptomics and proteomics, promises to provide exciting avenues for future investigations of this system.

Experimental procedures

General methods

Adult *E. scolopes* were captured off the coast of Oahu and bred in aquaria as previously described (Montgomery and McFall-Ngai, 1993). Juveniles obtained from the breeding colony were collected within 15–20 min of hatching and

placed in unfiltered seawater (i.e. with ~ 10^6 environmental bacteria), which has no detectable *V. fischeri*, under the following conditions: (i) no additions, to generate the aposymbiotic condition or (ii) addition of various strains of symbiotic and non-symbiotic bacteria at varying doses and time periods indicated in the individual experiment. For inoculation, each strain was grown to mid-log phase and then diluted into seawater containing newly hatched animals grouped by treatment.

All *V. fischeri* and *V. parahaemolyticus* strains (Table 1) were grown shaking at 28°C in Luria-Bertani salt (LBS medium; Dunlap, 1989) or seawater-based tryptone media (Boettcher and Ruby, 1990) prior to inoculation of the animal. The numbers of cells added per millilitre of seawater was confirmed by determination of colony-forming units per millilitre (CFU ml^{-1}) on LBS-based agar plates. For antibiotic selection, chloramphenicol, tetracycline, erythromycin and kanamycin were used at concentrations of 5, 5, 5 and 50 μg ml^{-1} respectively.

Microscopy

To prepare specimens for SEM analyses, juvenile animals were exposed to *V. fischeri* cells at 10^6 CFU ml^{-1} of seawater for 2–3 h. To confirm aggregate formation within an experiment, a subset of live animals was examined by confocal microscopy. The remainder of the animals was dropped into 1% osmium tetroxide in marine phosphate-buffered saline (mPBS) (50 mM sodium phosphate, 0.45 M sodium chloride, pH 7.4) and incubated for 30 min at room temperature on a rotator. This unusual fixation procedure arrests ciliary beat on the ciliated surfaces of molluscan epithelia (Reed and Satir, 1986). Fixed animals were dehydrated in an ethanol series from 30% to 100%. Specimens were dried in a Tousimis Samdri 780 critical point dryer (Tousimis, Rockville, MD, USA) and mounted with colloidal silver onto stubs dorsal side down. The tissue covering the light organs was removed, and the specimens were sputter coated with gold in a SeeVac Auto Conductavac IV (SPI Supplies, West Chester, PA, USA). The samples were examined on a Hitachi S-570 LaB6 scanning electron microscope (Hitachi, Tarrytown, NY, USA).

To visualize the cilia on the surface of the organ by confocal microscopy, samples were exposed at 45 min prior to the conclusion of a time course to 250 nM TubulinTracker (Invitrogen Life Technologies, Grand Island, NY, USA) in the seawater containing the juvenile animal. Mucus shed from these host tissues was visualized by incubating the animal at 30 min prior to conclusion of a time course with 10 μg ml^{-1} Alexa633 wheat germ agglutinin (WGA), a fluorochrome that labels the sialic acid and N-acetylglucosaminyl residues of the mucus. At the end of the incubation period, the animals were anesthetized in 2% ethanol in seawater, and the tissues were dissected to reveal the light organ surface. Confocal experiments were performed on a Zeiss 510 laser scanning confocal microscope (Carl Zeiss, Munich, Germany); fluorochromes were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA).

To enumerate associating bacteria, we counted the number of individual RFP-expressing bacteria per ciliated field on one ciliated field per animal for five individual animals. When an aggregate was too large and dense to visualize individual cells, we estimated the number of cells present based on the volume of the aggregate. When various strains were characterized for their ability to associate with host tissues, the data were normalized to wild-type *V. fischeri* ES114.

For wild-type *V. fischeri* and *V. parahaemolyticus* co-infection assays, animals were exposed to both phylotypes simultaneously and coincubated throughout the duration of the experiment. In experiments where we visualized *V. parahaemolyticus* in association with the cilia, we used unlabelled *V. fischeri* and RFP-expressing *V. parahaemolyticus* and counterstained the cilia with TubulinTracker. In experiments where both phylotypes were visualized, we used an RFP-expressing *V. fischeri* strain with a GFP-expressing *V. parahaemolyticus*, and the mucus was counterstained with Alexa633 WGA as described earlier. In the competition experiments comparing relative numbers of *V. parahaemolyticus* in the presence of either wild-type or *rscS*$^-$ *V. fischeri*, the squid were exposed to equal inocula of each strain.

Assays with heat- or azide-killed V. fischeri

V. fischeri cells were grown as described earlier and then subjected to the following treatment for heat or metabolic inactivation. One hundred microlitre of *V. fischeri* culture at mid-log phase were transferred to an Eppendorf tube and incubated in a 50°C heat block for 1 h to heat inactivate the cells. To metabolically inactivate cells, they were handled similarly, but exposed to 0.1% sodium azide for 1 h at room temperature. Cells were checked for viability using propidium iodide staining as previously described (Nyholm and McFall-Ngai, 2003). For each of these conditions, we compared untreated to treated cultured bacteria by fluorescence microscopy to confirm that RFP fluorescence was not affected by the treatment.

Haemocyte trafficking assays

To determine numbers of haemocytes migrating into the blood sinus underlying the ciliated epithelium, animals were incubated with bacteria for 3 h and then fixed overnight in 4% paraformaldehyde in mPBS (50 mM sodium phosphate, 0.45 M sodium chloride, pH 7.4) at 4°C. Light organs were removed from the squid and permeabilized for 24 h in 1% Triton-X-100 (Sigma-Aldrich, St. Louis, MO, USA) in mPBS. To stain globular actin, which is highly abundant in haemocytes and thus used to localize haemocytes, the samples were incubated overnight in fluorescein isothiocyanate deoxyribonuclease 1, or FITC-DNAse I. The samples were counterstained with rhodamine phalloidin to localize the actin cytoskeleton, and with TOTO-3 to localize nuclei. Samples were mounted on glass slides as previously described (Altura *et al.*, 2011), and haemocytes were visualized and their abundance determined by confocal microscopy.

Statistics

All statistical analyses were performed in Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) as described in the figure legends.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Attachment to HeLa cells by *E. coli* BL21 cells expressing MAM7 homologues from *Vibrio* spp.

Fig S2. The effect of mutations in surface features on *V. fischeri* attachment to host cilia.