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Recognition between symbiotic *Vibrio fischeri* and the haemocytes of *Euprymna scolopes*

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Summary

The light organ crypts of the squid *Euprymna scolopes* permit colonization exclusively by the luminous bacterium *Vibrio fischeri.* Because the crypt interior remains in contact with seawater, the squid must not only foster the specific symbiosis, but also continue to exclude other bacteria. Investigation of the role of the innate immune system in these processes revealed that macrophage-like haemocytes isolated from *E. scolopes* recognized and phagocytosed *V. fischeri* less than other closely related bacterial species common to the host's environment. Interestingly, phagocytes isolated from hosts that had been cured of their symbionts bound five times more *V. fischeri* cells than those from uncured hosts. No such change in the ability to bind other species of bacteria was observed, suggesting that the host adapts specifically to *V. fischeri*. Deletion of the gene encoding OmpU, the major outer membrane protein of *V. fischeri*, increased binding by haemocytes from uncured animals to the level observed for haemocytes from cured animals. Co-incubation with wild-type *V. fischeri* reduced this binding, suggesting that they produce a factor that complements the mutant's defect. Analyses of the phagocytosis of bound cells by fluorescence-activated cell sorting indicated that once binding to haemocytes had occurred, *V. fischeri* cells are phagocytosed as effectively as other bacteria. Thus, discrimination by this component of the squid immune system occurs at the level of haemocyte binding, and this response: (i) is modified by previous exposure to the symbiont and (ii) relies on outer membrane and/or secreted components of the symbionts. These data suggest that regulation of host haemocyte binding by the symbiont may be one of many factors that contribute to specificity in this association.

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Introduction

A wide variety of both aquatic and terrestrial animals serve as the environment for life-long beneficial symbioses with a complement of extracellular bacteria that associate with their epithelial surfaces (McFall-Ngai, 2002; Dale and Moran, 2006). Most of these animal–microbe interactions are formed anew with each host generation by ambient bacteria that trade soil or seawater for a nutrient-rich environment of animal tissue. Because many of these associations are species-specific, an effective communication must occur between the bacterium and its host, mediating both the harvesting of potential symbionts, and the establishment of a stable, long-term association. Increasing evidence suggests that in both invertebrates and vertebrates, interactions between the bacteria and the host immune system play a critical role in this communication (Pruzzo *et al*., 2005; Burge *et al*., 2007; Pham *et al*., 2007). For example, in the healthy human gut, a system that has undergone intensive investigation, immune defence mechanisms are modulated in response to the normal microbiota (Hooper *et al*., 2001; Noverr and Huffnagle, 2004; Mazmanian *et al*., 2005; Cash *et al*., 2006; Liu *et al*., 2008). This effect is often referred to as the development of 'tolerance'. However, recent studies suggest that the factors controlling beneficial bacteria–animal associations involve complex, active processes that promote dynamic interactions between the bacterium and its host, and are not a simple insensitivity to their presence (e.g. Rakoff-Nahoum *et al*., 2004; Peterson *et al*., 2007; Salzman *et al*., 2007; Fujiwara *et al*., 2008).

The innate immune system appears to play a critical role in the conversation between beneficial microbes and their hosts (e.g. Rakoff-Nahoum *et al*., 2004; Iwasaki, 2007; Vaishnava *et al*., 2008). Much of this interaction is mediated through signalling at the cell surfaces of the partners. In Gram-negative bacteria, microbe-associated molecular patterns (MAMPs), consisting of cell surface components such as capsule, lipopolysaccharide and

outer membrane proteins (OMPs), play an important role in this mediation (Ofek and Doyle, 1994; Aeckersberg *et al*., 2001). In the host, recent studies of the role of innate immune responses in symbiosis have demonstrated that the underlying cellular and molecular mechanisms are well conserved throughout the animal kingdom, and the simplicity of invertebrate models has proven valuable in understanding the mechanisms of these responses (e.g. Weis *et al*., 1996; Anselme *et al*., 2006; Silver *et al*., 2007; Ryu *et al*., 2008). During pathogenesis, the host's cellular response aims to kill invading microorganisms principally through phagocytosis by blood cells, a process that is mediated in the vertebrates by polymorphonuclear leucocytes, monocytes and tissue macrophages, and in the invertebrates by macrophage-like haemocytes (Irving *et al*., 2005; Pruzzo *et al*., 2005). The stages of this process, recognition, binding and internalization of microorganisms, are conserved in all systems studied (Canesi *et al*., 2002; Hoffmann *et al*., 2002; Lavine and Strand, 2002; Govind and Nehm, 2004; Magnadottir, 2006). The realization that significant numbers of beneficial bacteria occur in association with animals begs the question: At what point(s) do these bacteria avoid phagocytosis, and does this avoidance involve a reciprocal interplay between host and symbiont?

The binary association between the Hawaiian squid *Euprymna scolopes* and the marine luminous bacterium *Vibrio fischeri* offers an experimentally accessible model for defining the role of the host innate immune system in the dynamics between beneficial bacteria and animal tissue. The juvenile squid obtains its bacteria from the seawater each generation, yet the association formed is highly specific; i.e. only *V. fischeri* can colonize the tissues of a nascent light-emitting organ (McFall-Ngai and Ruby, 1991). The symbiont population, which is maintained throughout the life of the host, grows within polarized epithelium-lined crypt spaces of the highly vascularized, light organ (Fig. 1). Several aspects of the innate immune system have been implicated in the control of this association, but the role of the blood cells has remained unexplored.

Unlike other invertebrates, which may have several types of blood cells in their circulatory system, cephalopods (i.e. squid, octopus and their relatives) have a single type of haemocyte. *E. scolopes* haemocytes traverse the epithelium into the crypt spaces where the symbionts reside and appear to 'sample' these spaces, not unlike the way that mammalian blood cells sample the enteric microbiota (Rescigno *et al*., 2001; Niess *et al*., 2005). Previous studies of cephalopod haemocytes have indicated that they behave like vertebrate macrophages, binding, engulfing and killing bacteria (Cowden and Curtis, 1981; Schipp *et al*., 1990). Within the crypts of newly colonized juvenile *E. scolopes*, haemocytes have been observed

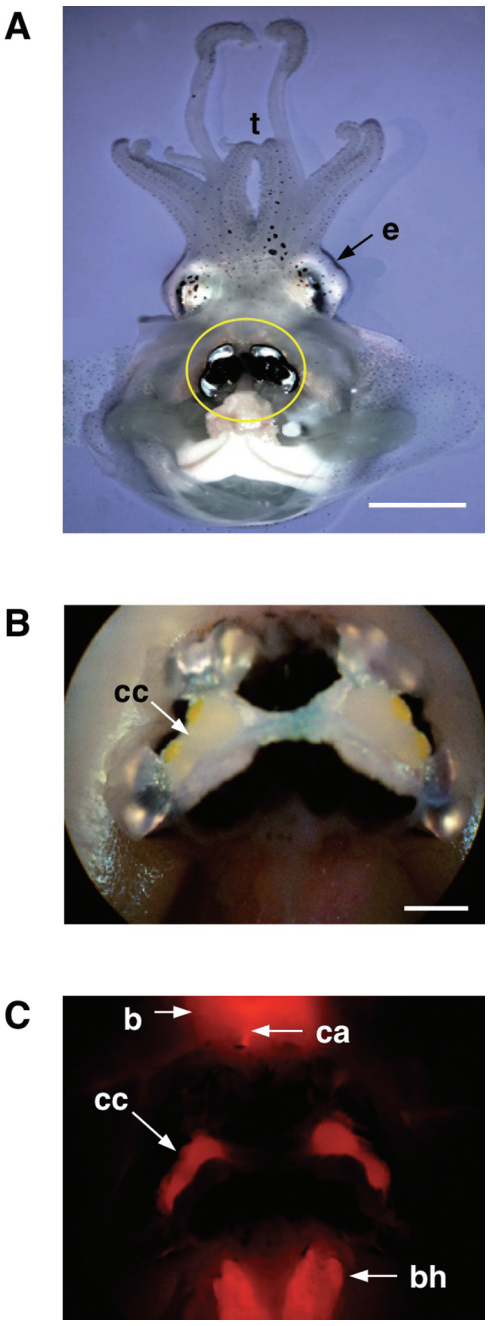

Fig. 1. Vascularization of the *E. scolopes* light organ.
A. Ventral dissection of an adult *E. scolopes*, revealing the bilobed light organ (circled in yellow). Eye (e) and tentacles (t) are also indicated. Scale bar = 1 cm.
B. Higher-magnification view of the light organ, dissected further to reveal the symbiont-containing central core (cc) tissue. Scale bar (for both B and C) = 2 mm.
C. View of the same light organ, in which the circulatory system has been visualized by injecting the fluorochrome CellTracker Orange (Molecular Probes) through the cephalic artery (c), located at the mantle/head interface. Fluorescent excitation reveals a high degree of vascularization of the central core (cc) tissue. Other highly vascularized organs in the vicinity include the brain (b) and the brachial hearts (bh).

with internalized bacterial cells (Nyholm and McFall-Ngai, 1998). However, within the crypts of adult squid, haemocytes have never been observed with engulfed bacteria although host haemocytes are entirely surrounded by *V. fischeri* cells. These preliminary observations suggested that the squid's haemocytes change or mature in response to the persistent presence of the symbionts, perhaps as part of the complex developmental programme induced in the host by *V. fischeri* (Nyholm and McFall-Ngai, 2004). Such observations, along with the discovery that certain *V. fischeri* genes are required for normal colonization (Visick *et al*., 2000; Aeckersberg *et al*., 2001; Lupp and Ruby, 2005; Visick and Ruby, 2006; Whistler *et al*., 2007), have provided evidence that both the host and the symbiont participate in ensuring an exclusive, persistent partnership.

To gain insight into the nature of the interaction between squid haemocytes and bacteria during the establishment and maintenance of the light–organ association, we compared the responses of these host cells to *V. fischeri* with those to other bacterial species common to the marine environment of the squid. We report evidence that the host haemocytes are 'educated' specifically to the presence of symbionts in the light organ, and that binding of *V. fischeri* prior to phagocytosis is inhibited both by host determinants and by a specific bacterial OMP.

Results

Binding of bacterial cells to E. scolopes *haemocytes*

In vitro binding assays demonstrated that host haemocytes that had been isolated from colonized adult animals recognized and bound the cells of four marine bacterial species (Table 1) to different degrees (Fig. 2). Specifically, host cells bound significantly more cells of *Vibrio harveyi* and *Photobacterium leiognathi* than of *V. fischeri* and *Vibrio parahaemolyticus*. Control experiments using unlabelled bacterial or host cells yielded identical results to those using GFP-expressing or -stained cells (data not shown).

To examine whether haemocyte behaviour was affected by whether they were isolated from an animal that had *V. fischeri* in its light organ, the symbiont population was eliminated from a subset of animals by antibiotic treatment prior to the collection of the haemocytes. In control experiments, after 24 h of antibiotic exposure, no *V. fischeri* colony-forming units (cfu) were present in homogenates of the central-core tissue of treated light organs. A cohort of cured animals was maintained in the cured state for an additional 4 days, during which time bacteria-binding efficiency was determined for haemocytes isolated either from cured (naïve haemocytes) or symbiotic (normal haemocytes) animals (Fig. 3). No change was detected in the ability of the normal haemocytes to bind any of the three bacterial strains over the 5 day experiment; however, by day 4 the ability of the naïve haemocytes to bind *V. fischeri* cells had become significantly greater, increasing to fivefold by 5 days (Fig. 3). This increased activity towards *V. fischeri* cells was not the result of a general enhancement of binding by haemocytes of cured animals; binding to *V. parahaemolyticus* or *V. harveyi*, which are bound with low and high efficiency, respectively, was unchanged over the course of the experiment.

V. fischeri *avoidance of binding to host haemocytes*

To determine whether the avoidance of symbiotic haemocyte adherence by *V. fischeri* is mediated by bacterial surface components, we examined mutant strains of *V. fischeri* that were defective in the synthesis of flagella (Millikan and Ruby, 2004), type-4 pili (Stabb and Ruby,

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics	Reference
Strain		
Vibrio fischeri		
ES114	Wild-type isolate from *E. scolopes* light organ	Boettcher and Ruby (1990)
ESR1	Spontaneous Rr^r ES114 derivative	Aeckersberg *et al*. (2001)
OM3	ESR1 deletion mutant of *ompU*	Aeckersberg *et al*. (2001)
Vibrio harveyi		
B392	Seawater isolate	Reichelt and Baumann (1973)
Vibrio parahaemolyticus		
KNH1	Seawater isolate	Nyholm *et al*. (2000)
Photobacterium leiognathi		
KNH6	Seawater isolate	E. Stabb, pers. comm.
Plasmid		
pVO8	*V. fischeri* cloning vector; Cm^r	Visick and Ruby (1998)
pFA9	pVO8 bearing intact ES114 *ompU*	Aeckersberg *et al*. (2001)
pKV111	pVO8 bearing the gene encoding green fluorescent protein	Nyholm *et al*. (2000)

Rr^r, rifampicin resistance; Cm^r, chloramphenicol resistance.

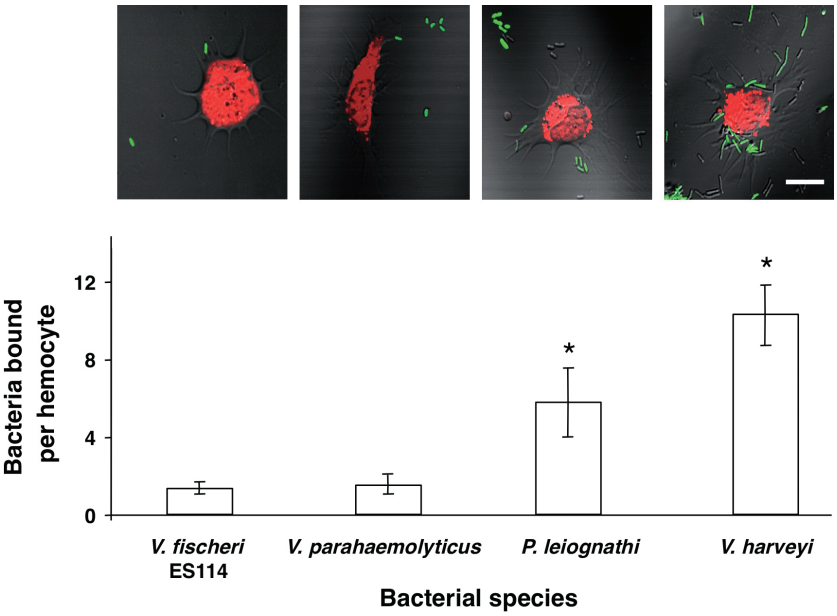

Fig. 2. Differential binding of bacteria by squid haemocytes. Above: representative confocal microscopy images of isolated squid haemocytes stained with CellTracker Orange. Differential interference contrast views of the cells are superimposed to reveal the extended pseudopodia. The extent to which different species of bacteria (green) bound to the haemocytes' external surfaces was easily visualized and enumerated. Scale bar = 10 μm. Below: mean number of bacteria bound to each haemocyte was determined for at least 10 haemocytes per microscopic field ($n = 5$) in three replicate experiments. Error bars indicate SEM; asterisks denote levels of binding that were significantly different ($P < 0.05$) from that of *V. fischeri* ES114, as determined by ANOVA pair-wise analysis.

2003) or a major OMP (Aeckersberg *et al*., 2001). Only strain OM3 (Table 1), which is unable to produce the OMP OmpU, showed a significant effect on haemocyte binding. When strain OM3 was exposed to adult haemocytes from symbiotic animals, its level of adherence was five times higher than that of wild-type *V. fischeri* (Fig. 4). Complementing this mutation *in trans* with a wild-type copy of *ompU* that restores the OmpU protein (Aeckersberg *et al*., 2001) resulted in a specific decrease in haemocyte binding to the levels observed for wild-type *V. fischeri*. These data suggest that the ability of *V. fischeri* to avoid haemocyte recognition is due, at least in part, to the presence or activity of a bacterial OMP.

One possible explanation for this result is that *V. fischeri* may produce a secreted signal that passes though the OmpU channel and contributes to bacterial evasion of the haemocyte defences of the host. If this hypothesis were correct, one would predict that the presence of *V. fischeri* cells might influence how the haemocytes bind other bacterial species as well. To test this hypothesis, we conducted binding assays with GFP-labelled *V. fischeri* cells co-incubated with unlabelled cells of either *V. parahaemolyticus*, *V. harveyi* or *P. leiognathi*. A combination of both fluorescence and differential interference contrast microscopy allowed the differentiation of the number of cells of each type of bacteria that were bound by the haemocytes. Incubation of *V. fischeri* with any of these other species resulted in a reproducible, but not statistically significant, increase in binding of *V. fischeri* cells by haemocytes (Fig. 5A). Conversely, the presence of wild-type *V. fischeri* cells did not cause any decrease in the binding of *V. parahaemolyticus*, *V. harveyi* or *P. leiognathi* to host haemocytes (Fig. 5B). In fact, the presence of *V. fischeri* resulted in a significant increase in binding of the *V. parahaemolyticus* and *P. leiognathi* to host haemocytes (Fig. 5B). However, co-incubation with wild-type *V. fischeri* cells reduced OM3 binding to the level of wild type, further supporting the notion that OmpU provides an activity or factor that complements the defect of the *ompU* mutant (Fig. 5C). This complementation required the presence of viable wild-type *V. fischeri*: neither heat-killed nor azide-killed cells protected the mutant from binding. These data suggest the involvement of a secreted signal whose activity provides a protective mechanism that is specific for *V. fischeri*.

Bacterial uptake by haemocytes

Fluorescence-activated cell sorting (FACS) analysis was used to determine whether a difference could be detected in the ability of the squid haemocytes to engulf cells of either *V. fischeri* or *V. parahaemolyticus*, two strains of bacteria isolated from the natural environment of the squid, which are bound at relatively low levels. Our results show that the haemocytes are capable of engulfing bound bacteria regardless of the strain that was used (Fig. 6). Specifically, haemocyte engulfment of *V. fischeri* and *V. parahaemolyticus* was compared. Engulfment was determined by whether or not bacterial cell fluorescence was susceptible to quenching. The fluorescence of cells not phagocytosed was quenched, whereas the fluorescence of engulfed cells was not affected. The cells were sorted into these two categories by FACS analysis. No measurable differences could be detected between the two bacterial species in the rates of phagocytic activity between 30 and 180 min after exposure (Fig. 6C). The

fact that the haemocytes did not appear to discriminate between these two bacterial species during phagocytosis supports the idea that the critical event in evading removal by the haemocytes is associated with recognition and/or initial binding. No bacterial uptake was detected for any strain in the presence of the phagocytosis inhibitor cytochalasin D (data not shown).

Discussion

The major findings of this study offer insight into the role of innate immunity in the adaptation of bacteria to an environment whose importance is becoming increasingly recognized: the tissues of their specific animal host. In the squid–vibrio association both host and symbiont cells have characters that mediate this exclusive interplay. The ability to avoid adherence to haemocytes of *E. scolopes* varied among related bacterial species, and the haemocyte's response was specifically altered by previous exposure of the host to *V. fischeri*. These data provide evidence that host haemocytes become conditioned to colonization or 'educated' by the presence of their co-evolved bacterial symbiont. A coarse level of recognition has recently been shown in the innate immune response of *Drosophila* to challenge by bacterial pathogens (Pham *et al.*, 2007); however, the ability of the innate immune system to distinguish between closely related members of one bacterial family, coupled with a mechanism(s) for immune education to a specific bacterial symbiont, are unprecedented among the invertebrates. In addition, we show that *V. fischeri* with mutations in the gene encoding the OMP OmpU are bound to host haemocytes at significantly higher levels than wild type. The data further showed that the critical step in haemocyte specificity in the squid–vibrio system is binding itself as, once bound, both symbiotic and non-symbiotic bacteria are phagocytosed by host haemocytes equally well.

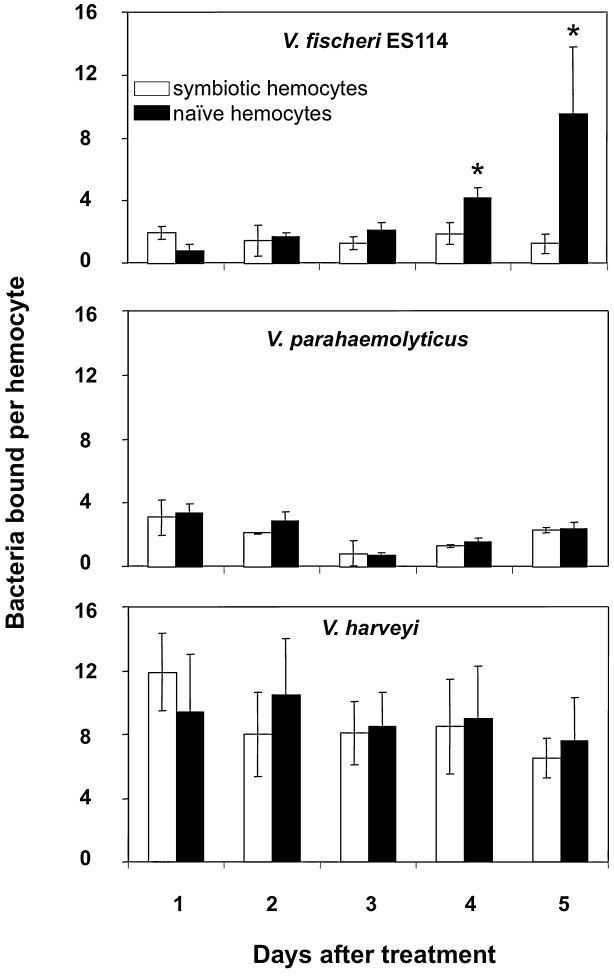

Fig. 3. Effect of curing squid of their symbiont population on haemocyte binding to different bacteria. Mean number of bacteria bound to haemocytes isolated from animals at different times after initiating antibiotic treatment to remove symbiotic *V. fischeri*. Adult squids were maintained in sterile seawater either in the normal symbiotic state or under antibiotic conditions that cured the light organ. Haemocytes were removed from either the symbiotic (normal) or cured (naïve) animals over 5 days for adherence assays (see *Experimental procedures*). The mean numbers of bacteria bound by symbiotic (white bars) or naïve (black bars) haemocytes were determined in three independent experiments. Error bars indicate SEM; asterisks denote levels of binding that were significantly different ($P < 0.05$) from those measured on day 1, as determined by ANOVA pair-wise analysis.

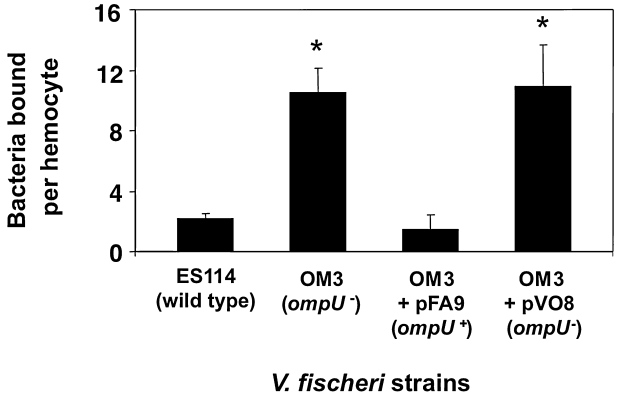

Fig. 4. Effect of an *ompU* mutation on *V. fischeri* evasion of haemocyte recognition and binding. The mean numbers of wild-type (ES114) or *ompU* (OM3) *V. fischeri* cells that were bound by isolated haemocytes was determined in three independent experiments. Restoration of an intact copy of *ompU* on pFA9 eliminated the elevated level of haemocyte binding of strain OM3. Addition of the *V. fischeri* cloning vector pV08 had no significant effect on OM3 binding. Error bars indicate SEM; asterisks denote levels of binding that were significantly different ($P < 0.05$) from those of *V. fischeri* ES114, as determined by ANOVA pair-wise analysis.

Differences in haemocyte binding of bacteria

In all systems described, adherence of a microorganism or particle to a phagocyte is an essential step (Aderem

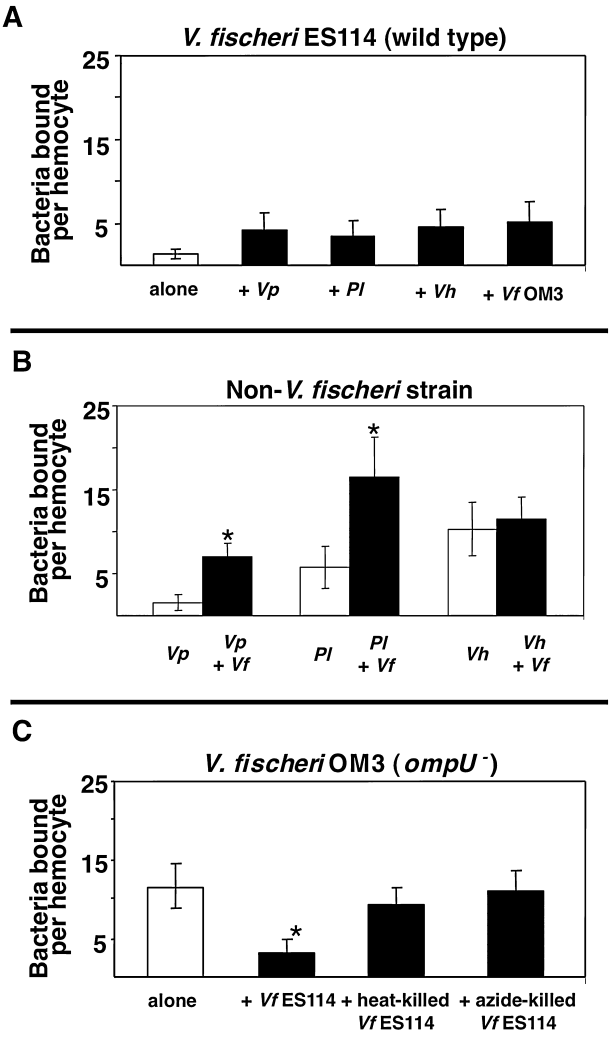

Fig. 5. Complementation of the *V. fischeri ompU* defect by viable, wild-type bacteria.
A. Haemocyte binding of wild-type *V. fischeri* ES114 incubated either alone or in combination with cells of *V. harveyi*, *V. parahaemolyticus*, *P. leiognathi* or *V. fischeri* OM3.
B. Haemocyte binding of *V. parahaemolyticus*, *P. leiognathi* and *V. harveyi* in either the absence (white bars) or presence (black bars) of wild-type *V. fischeri* ES114. Error bars indicate SEM; asterisks denote levels of binding that were significantly different ($P < 0.05$) in the presence of *V. fischeri* ES114, as determined by ANOVA pair-wise analysis.
C. Haemocyte binding of *V. fischeri* OM3 (*ompU*) either alone (white bar) or in the presence of wild-type *V. fischeri* ES114 cells (black bars). Values are the mean number of bacteria adhering to 10 haemocytes in three independent experiments. Error bars indicate SEM; asterisk denotes levels of binding that were significantly different ($P < 0.05$) from those of *V. fischeri* ES114, as determined by ANOVA pair-wise analysis.

and Underhill, 1999). Characterizations of the initial onset of the symbiosis have implicated haemocytes in the determination of specificity and the induction of development (Nyholm and McFall-Ngai, 1998; Koropatnick *et al*., 2004). Given that a subpopulation of *E. scolopes* haemocytes enters the microenvironment of the light organ crypts (Nyholm and McFall-Ngai, 1998), the resistance of *V. fischeri* to adherence to host haemocytes may aid in the persistence of the symbiont in the light organ crypt spaces, and contribute to the long-term specificity of the association. The non-symbiont *V. parahaemolyticus* also did not adhere well to squid haemocytes. As these haemocytes circulate throughout the host's vasculature and serve as the only cellular component of the squid's innate defences, various environmental bacteria, including *V. parahaemolyticus*, may interact with the haemocytes in contexts outside the light organ, i.e. during infections of other tissues. However, previous studies using this strain have demonstrated that *V. parahaemolyticus* is unable to migrate through the ducts and colonize the light organ of uncolonized hatchling squid (Nyholm *et al*., 2000). Analysis of the juvenile host has revealed multiple mechanisms to ensure specificity, both biomechanical (e.g. ciliary currents along the ducts leading to the crypt spaces) (Nyholm and McFall-Ngai, 2004) and biochemical (e.g. lectin–glycan interactions and oxidative stress) (Weis *et al*., 1996; Visick and Ruby, 1998; Nyholm *et al*., 2000). Thus, the behaviour of the haemocytes is one of a set of specificity determinants in the system.

An apparent education of the haemocytes

The ability for the host haemocytes to react differently over time to *V. fischeri* may be analogous to vertebrate immune 'tolerance' involving features of both partners. Curing of the light organ only affected the binding of haemocytes to *V. fischeri* (Fig. 3), suggesting that some component of the host haemocytes changes after specific recognition and interaction with the symbiont. The mechanism behind this conditioning, however, remains unknown. Chronic infection by pathogenic bacteria can alter the response of the innate immune system over time. For example, tolerance of airway epithelia that are chronically exposed to *Pseudomonas aeruginosa* appears to be mediated by downregulation of the NF-kappaB pathway (Wu *et al*., 2005).

The fact that the extent of *V. fischeri* binding increased with time after curing suggests that changes in the haemocytes, or turnover in the haemocyte population, takes several days. The development of haemocytes in cephalopods takes place in the specialized white body located adjacent to the optic lobe (Cowden and Curtis, 1981). However, the rate at which these cells develop and

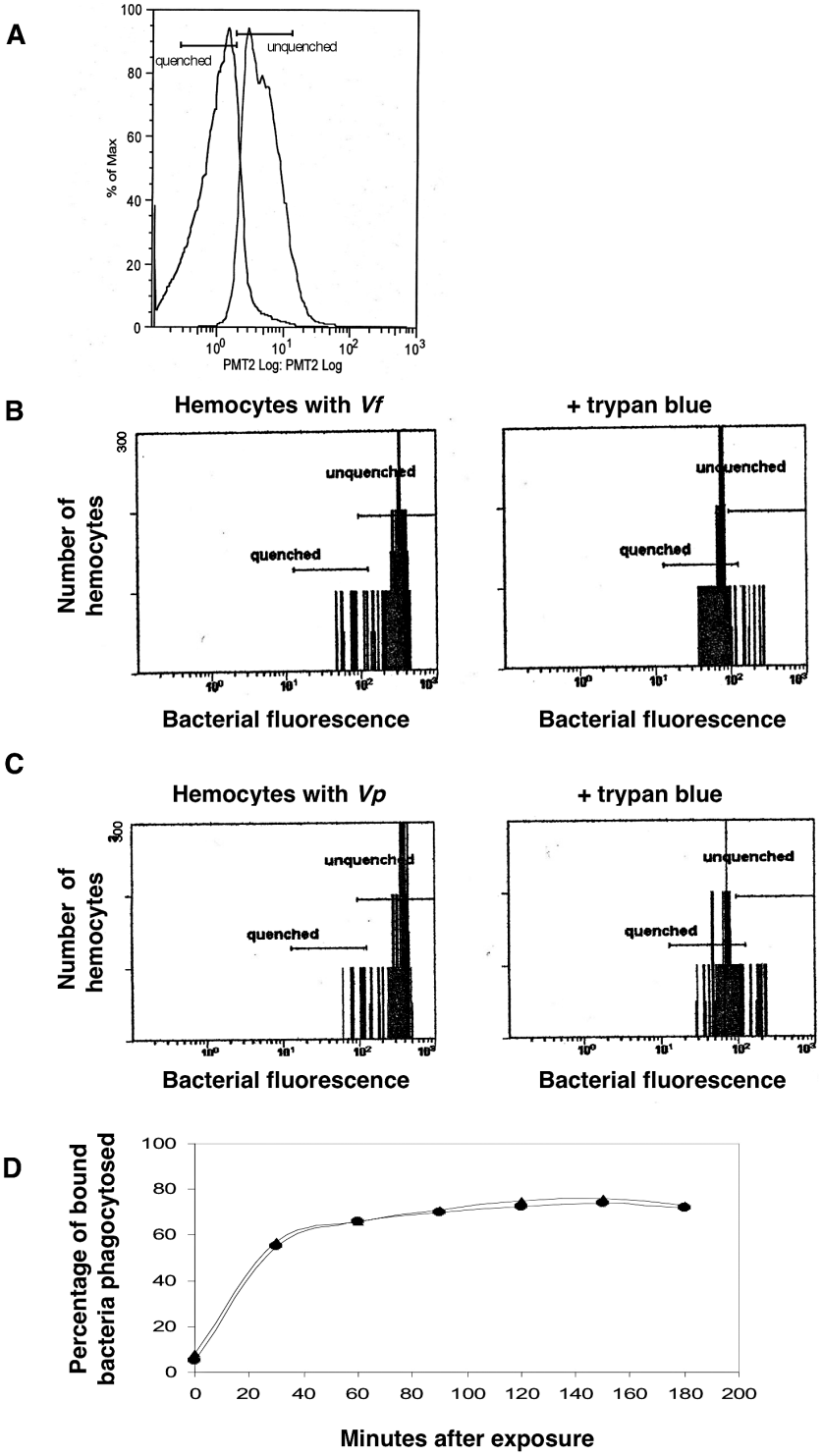

Fig. 6. Rates of phagocytosis of bacteria bound by haemocytes.
A. Representative flow cytometry plot recorded 30 min after the start of the phagocytosis assay. The fluorescence of haemocytes exposed to TAMRA-labelled cells of *V. fischeri* ES114 was assessed by flow cytometry before (left panel) and after (right panel) the addition of trypan blue, which quenches the fluorescence of cells not phagocytosed.
B. The fluorescence of haemocytes exposed to TAMRA-labelled cells of *V. parahaemolyticus* was assessed as described above.
C. The extent of reduction in the mean fluorescence after the addition of the trypan blue (A and B) corresponded to the degree of internalization of the bound bacteria. This value was determined every 30 min after exposure of the bacteria to haemocytes, and was a measure of the relative rates of phagocytosis of *V. fischeri* (closed circle) and *V. parahaemolyticus* (closed triangle) cells.

their duration in the circulatory system remain unknown. Because the light organ is highly vascularized, haemocytes may be exchanged between the circulatory system and the crypt spaces on a regular basis. A previous study has shown that exposure to different pathogens can alter the expression of MAMP receptors during the maturation of innate immune cells (Kokkinopoulos *et al.*, 2005). In the squid–vibrio association, the host's haemolymph may contain bacterial products from the symbionts that are carried to the white body and somehow alter the development of pattern recognition receptors on nascent haemocytes.

The importance of OmpU

This study provides evidence that the *V. fischeri* outer membrane porin OmpU plays a role in preventing symbiont adhesion to host haemocytes. An opposite role for OMPs, i.e. the mediating of adherence to immune cells, has been documented in bacterial pathogenesis (Negm and Pistole, 1999; Soulas *et al.*, 2000; Biswas *et al.*, 2001). The finding that co-incubation of wild type with *ompU* mutants rescues the mutant phenotype suggests that ompU is acting as a porin, i.e. transporting something into the medium-recognized *V. fischeri* cells. The finding that co-incubation with wild-type V. fischeri does not rescue non-symbiont strains suggests an exclusivity to the response. *V. cholerae* OMPs have been shown to function in a channel capacity, facilitating the movement of proteins into and out of the cell (Chakrabarti *et al.*, 1996; Provenzano *et al.*, 2001).

Unlike the non-symbiont marine species (*V. parahaemolyticus*, *P. leiognathi* and *V. harveyi*) used for comparison in the previously described experiments, *V. fischeri* strain OM3 does enter the light organ crypts of *E. scolopes.* However, this mutant has been shown to be defective in interacting with the host *E. scolopes* during the onset of the symbiosis compared with wild-type *V. fischeri* (Aeckersberg *et al.*, 2001). The strain OM3 was tested for its ability to resist adherence by haemocytes from both symbiotic and cured squid. Because this mutant is more easily recognized and bound by host haemocytes, this phenomenon may explain why this mutant is defective in interacting with the squid host. Also, the fact that the *ompU* mutant could resist adherence by haemocytes *in vitro* when co-incubated with wild-type *V. fischeri* may also explain the complementation of this mutant's defect during animal colonization when co-infected with wild-type *V. fischeri.* How OmpU mediates resistance to binding host haemocytes and the identity of other symbiont molecules involved in adhesion to these cells are important points that remain to be determined. Interestingly, the related pathogen, *V. vulnificus* has been shown to require the presence of its homologous OmpU protein to bind a fibronectin-coated surface (Goo *et al.*, 2006); however, similar analyses comparing the *V. fischeri ompU* mutant and its parent indicated that both attached equally well to fibronectin (data not shown).

Conclusions

Innate immunity is often cited solely for its role as an effector system in dealing with potential pathogens, the molecular basis of which may involve broad recognition of MAMPs. However, the role of this ubiquitous metazoan function in mediating very specific and selective communications with mutualistic microorganisms is poorly understood. Here we show that squid host haemocytes are capable of altering their response to symbiotic and non-symbiotic bacteria. This study lays the groundwork for using the *E. scolopes*/*V. fischeri* association for studying the interactions between a host's innate immune system and its beneficial bacterial symbionts.

Experimental procedures

General procedures

Adult *E. scolopes* were collected from the shallow reef flats of Oahu, Hawaii and maintained in running seawater tables as described previously (Nyholm and McFall-Ngai, 1998). All chemicals were obtained from Sigma-Aldrich, unless otherwise stated.

Isolation of host haemocytes

Animals were anaesthetized by placing them in a 2% solution of ethanol in seawater. For experiments using isolated haemocytes, haemolymph was withdrawn from the cephalic artery located between the eyes (Fig. 1), using a sterile 1 ml syringe with a 28-gauge needle. An average of ~5000 haemocytes μl^{-1} of haemolymph were obtained using this method; animals served as donors multiple times, and a total of approximately 10^6 haemocytes were obtained from each individual. Freshly collected haemocytes were washed and re-suspended in Squid Ringer's solution (S-Ringers; 530 mM NaCl, 10 mM KCl, 25 mM $MgCl_2$, 10 mM $CaCl_2$ and 10 mM HEPES buffer, pH 7.5). Haemocyte concentrations were determined by haemocytometer, and approximately 10 000 cells were added to chamber slides, and allowed to adhere to the glass for 10 min at room temperature. At this density, the haemocytes form a uniform monolayer on the glass slide surface.

Curing squid of symbiotic bacteria

To cure adult *E. scolopes* of their population of *V. fischeri* symbionts, squid were maintained individually in 5 gal tanks containing Instant Ocean artificial seawater (Aquarium Systems). For one set of animals chloramphenicol (Cm) and gentamicin were added to the seawater to a final concentration of 20 $\mu g\ ml^{-1}$ of each. The concentration of antibiotics used in these experiments effectively eliminates *V. fischeri* symbionts from the light organ without compromising the health of the host in any detectable way. The animals were transferred daily into fresh Instant Ocean, either with or without antibiotics, for 5 days, and samples of haemolymph were removed at noon each day as described above. The resulting two sets of haemocytes were designated either 'normal' (untreated/symbiotic) or 'naïve' (treated/cured).

Twenty-four hours after initiating the treatment, the effectiveness of the antibiotics was determined by sacrificing a subset of animals, dissecting out and homogenizing the central core of the light organ (which normally contains the symbionts), and plating an aliquot of the homogenate on seawater nutrient medium (SWT) agar. Untreated adult light

organs contain more than 10^8 *V. fischeri* cfu (Ruby and Asato, 1993); the absence of cfu in the treated light organs was considered evidence of curing.

Preparation of bacterial cultures

Bacterial strains (Table 1) were cultured in a SWT as previously described (Nyholm *et al*., 2000). The growth kinetics and yields of the different bacteria were roughly the same in this medium. The optical density at 600 nm (OD_{600}) was taken periodically and, at an OD_{600} of 0.7 (i.e. mid-exponential growth phase), the culture was centrifuged at 8000 *g* to pellet the bacterial cells. Bacterial cell number, as determined by optical density, was confirmed by the plating of serial dilutions of cultures and determination of cfu. The pellet was washed by re-suspension in S-Ringer's solution and re-centrifugation a total of three times. The bacteria were finally re-suspended to a concentration of 2.0×10^8 cells ml^{-1} of S-Ringer's.

Suspensions of non-viable *V. fischeri* were produced by preparing the cells as described above, and exposing them either to 42°C for 20 min, or to 0.01% NaN_3 for 20 min. Treated bacteria were then washed three times with S-Ringer's solution, and the loss of viability was confirmed both by microscopy (the bacteria were stained with propidium iodine, which is excluded from living cells) and by spreading the cell suspension on SWT agar to observe cfu.

Strains harbouring the GFP-encoding plasmid pKV111 (Table 1) were used to visualize and quantify bacterial binding to squid haemocytes using fluorescent microscopy (Nyholm *et al*., 2000). During initial culturing, 2.5 μg of Cm was added per ml of SWT to maintain pKV111 in the bacteria; however, no Cm was added to the S-Ringer's solution used during the haemocyte binding assays.

Haemocyte/bacteria binding assay

To determine the extent of haemocyte binding, bacteria were added either as individual strains, or as pairs of strains at a ratio of 1:1. In all cases a total of 50 bacteria were added per haemocyte; bacterial cell numbers were confirmed by plating and quantification of cfu. The haemocyte/bacteria mixtures were incubated in S-Ringer's solution at 25°C for 1 h, a time determined to yield the maximum level of binding (data not shown). The cytoplasm of the haemocytes was then stained with 0.005% CellTracker Orange (Molecular Probes) to visualize the cells. Bacteria associated with individual haemocytes were viewed by fluorescence and differential interference contrast using a Zeiss LSM 510 laser-scanning confocal microscope, and enumerated over the entire surface of the animal cell.

Quantification of haemocytic phagocytosis of bacteria

To detect bacteria with a fluorescence-activated cell sorter, cells were labelled by incubation for 20 min in a solution of 1.0 mg tetramethylrhodamine carboxylic acid (TAMRA, Molecular Probes) per ml of S-Ringer's solution. The cells were then washed three times, and re-suspended in S-Ringer's solution. To determine the efficiency of trypan-blue quenching, the fluorescence level of labelled cells of *V. fischeri* ES114 or *V. parahaemolyticus* KNH1 (unquenched) was determined by flow cytometry. Trypan blue was then added to the suspension and the fluorescence of the labelled bacteria (quenched) was measured again. For both strains the reduction in mean fluorescence levels indicated a quenching of 95% of the initial fluorescence (data not shown).

An aliquot of 2.0×10^5 TAMRA-labelled *V. fischeri* ES114 or *V. parahaemolyticus* KNH1 cells was added to 500 μl of Squid Ringer's solution containing 10 000 haemocytes. The mixture was incubated for 30–60 min at 25°C to allow binding. Phagocytosis was then measured using an Altra fluorescence-activated cell sorter (Beckman-Coulter) equipped with an argon laser operating at excitation wavelengths of 488/630 nm. The results were analysed using PC-compatible Expo32 MultiComp software (Beckman-Coulter), as well as Macintosh-compatible FlowJo software (Tree Star). Cells were gated using forward- and side-light scatter to discriminate between eukaryotic cells and bacteria. The mean fluorescence of at least 10 000 haemocytes was determined using excitation and emission filters appropriate for the TAMRA dye, and fluorescent emission was monitored using the FL2 channel. To quench the fluorescence of extracellular bacteria, 0.2% trypan blue (Merck and Co.) in Squid Ringer's solution was added to each tube, and the fluorescence was immediately re-counted. The difference in mean fluorescence between the untreated sample and the sample treated with trypan blue was a measure of the level of phagocytosis; the fluorescence of intracellular, or engulfed, bacteria will not be quenched by the dye, whereas the fluorescence of cells not engulfed would be susceptible to quenching. Phagocytic ability was defined as the percentage of haemocyte cells with one or more ingested bacteria (TAMRA fluorescent cells) within the total cell population (10 000 cells). In control experiments, phagocytosis was blocked by pre-incubating the haemocytes with cytocholasin D (10 μg ml^{-1}) for 30 min at 25°C before inoculation.

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