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Shaping the microenvironment: evidence for the influence of a host galaxin on symbiont acquisition and maintenance in the squid-vibrio symbiosis

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Summary

Most bacterial species make transitions between habitats, such as switching from free living to symbiotic niches. We provide evidence that a galaxin protein, EsGal1, of the squid *Euprymna scolopes* participates in both: (i) selection of the specific partner *Vibrio fischeri* from the bacterioplankton during symbiosis onset and, (ii) modulation of *V. fischeri* growth in symbiotic maintenance. We identified two galaxins in transcriptomic databases and showed by quantitative reverse-transcriptase polymerase chain reaction that one (*esgal1*) was dominant in the light organ. Further, *esgal1* expression was upregulated by symbiosis, a response that was partially achieved with exposure to symbiont cell-envelope molecules. Confocal immunocytochemistry of juvenile animals localized EsGal1 to the apical surfaces of light-organ epithelia and surrounding mucus, the environment in which *V. fischeri* cells aggregate before migration into the organ. Growth assays revealed that one repeat of EsGal1 arrested growth of Gram-positive bacterial cells, which represent the cell type first 'winnowed' during initial selection of the symbiont. The EsGal1-derived peptide also significantly decreased the growth rate of *V. fischeri* in culture. Further, when animals were exposed to an anti-EsGal1 antibody, symbiont population growth was significantly increased. These data provide a window into how hosts select symbionts from a rich environment and govern their growth in symbiosis.

Received 11 December, 2013; revised 13 February, 2014; accepted 24 February, 2014. *For correspondence. E-mail mjmcfallngai@wisc.edu; Tel. (+1) 608 262 2393; Fax (+1) 608 262 8418. †Present address: Zoological Institute, Christian-Albrechts University of Kiel, 24098 Kiel, Germany. ‡These authors contributed equally to this work. Author contributions: EACH, AAG and MXG performed the experiments, and EACH performed the data analysis. EACH, AAG, RA and MJM-N designed the experiments. WEG contributed reagents. EACH and MJM-N prepared the manuscript, with help from, and editing by, all co-authors.

Introduction

A current interest in ecology focuses on how microbial communities are structured in natural habitats, and how microbes within those communities make transitions between different niches (Fuhrman, 2009; Needham *et al.*, 2013). One common niche transition occurs when marine microbes move from planktonic habitats to persistent associations with animal partners (Gallo and Hooper, 2012; Bulgarelli *et al.*, 2013). The light-organ symbiosis between *Euprymna scolopes*, the Hawaiian bobtail squid, and *Vibrio fischeri*, a luminescent Gram-negative marine bacterium, is a horizontally transmitted association, i.e. acquired each generation (for review, see Stabb and Visick, 2013). The symbionts colonize deep light-organ tissues, or crypts, within hours of the host's hatching, taking up residence along the apical surfaces of the crypt epithelia. The host responds to a variety of cues presented by *V. fischeri* in the crypts, including luminescence and microbe-associated molecular patterns (MAMPs), notably cell envelope molecules (for review, see McFall-Ngai *et al.*, 2010, and McFall-Ngai *et al.*, 2012). Transitions of *V. fischeri* between membership in the bacterioplankton to the symbiotic niche occur both at the onset of the symbiosis, when *V. fischeri* is recruited *into* animal tissues, and each day of the association with the venting of ~90% of the symbiont population *out of* the light organ at dawn, when *V. fischeri* cells cycle into the planktonic niche.

How the bacterioplankton is winnowed in the selection of the specific symbiotic partner at onset of the association has been a continuing focus of studies of the squid-vibrio system. Soon after hatching, juvenile *E. scolopes* begin to ventilate, bringing environmental microbial communities, as well as the products they release, over superficial ciliated epithelial fields that are specific to the juvenile light organ. The epithelia begin to shed mucus

within minutes in response to environmental peptidoglycan (PGN) (Nyholm *et al*., 2002). *Vibrio fischeri* is then enriched along these tissues by first attaching to the cilia and then aggregating (Altura *et al*., 2013). Whereas Gram-positive environmental bacteria appear not to associate with this ciliated field, all tested Gram-negative bacterial cells have the capacity to adhere (Nyholm and McFall-Ngai, 2003; Altura *et al*., 2013). Through the recruitment process, however, all non-specific Gram-negative bacteria are gradually lost from the aggregating cells such that, by about 3 h, only a small population of *V. fischeri* cells remains (Altura *et al*., 2013).

While this stepwise dominance of *V. fischeri* on the ciliated surface is not well understood, recent research has provided some insight. The shed mucus is acidic (Kremer *et al*., 2013) and contains abundant biomolecules typically associated with 'antimicrobial' activity, including nitric oxide (Davidson *et al*., 2004) and a peptidoglycan recognition protein that has an amidase activity that breaks down PGN (Troll *et al*., 2010). In addition, a recent study revealed robust changes in host light-organ transcript abundance after only 3 h of exposure to the symbiont (Kremer *et al*., 2013). The data suggest the possibility that some of the upregulated genes encode proteins with activities that shape the chemical environment of the mucus to favour *V. fischeri*. A role for host biomolecules as critical determinants of host–symbiont interactions is not without precedent; they have been widely studied as features that can structure and modulate behaviours of host-associated microbial communities (de Oliveira *et al*., 2012; Shnit-Orland *et al*., 2012). For example, host factors function in symbiont attraction in the legume–rhizobia symbioses (Redmond *et al*., 1986), in the structuring of symbiont communities in the mammalian gut (Cash *et al*., 2006), and in maintaining specificity in nematode–microbe associations (Bulgheresi *et al*., 2011).

In this study, we have explored the possible roles of another host biomolecule, a protein known as galaxin, in shaping the transition to a symbiotic habitat by *V. fischeri*. Galaxins were first reported in other symbiotic associations. For example, several studies on corals have correlated galaxin activity with the process of calcification of the exoskeleton (Fukuda *et al*., 2003; Watanabe *et al*., 2003), and galaxin transcripts have also been identified in the body wall of *Riftia pachyptila* (Sanchez *et al*., 2007), although their function remains unexplored. In the squid-vibrio system, previous studies of symbiont-induced changes in host gene expression showed that transcripts encoding putative galaxin proteins are upregulated at first colonization of the light organ by the symbionts (Chun *et al*., 2008), and are regulated over the day/night cycle in the adult light organ (Wier *et al*., 2010). Here we characterize in depth one of the host-squid galaxins, EsGalaxin1 (EsGal1), the prominent isoform in the host light organ, during the selection of the symbiont in onset of the partnership and during the maintenance of the mature association as a balanced system.

Results

Features of the esgal *genes and of* EsGalaxin1 *(*EsGal1*), the principal galaxin of the light organ*

We identified two *esgal* sequences, *esgal1* and *esgal2*, in the *E. scolopes* transcriptomic databases. Using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) to examine expression in different tissues of the animal, we confirmed that the *esgal1* gene is the most highly expressed galaxin gene in both the juvenile and adult light organs. Thus, for this study, we explored aspects of *esgal1* gene expression and the EsGal1 protein patterns in the squid-vibrio symbiosis. An unexpected finding was that *esgal1* expression is ~250× higher in the light organ than in the accessory nidamental gland (ANG), another symbiotic organ, but one that is less well studied than the light organ. The ANG contains a consortium of bacteria thought to play a role in protection of the eggs during host development (Collins *et al*., 2012). The expression of *esgal2* showed the opposite pattern in that it was ~250× higher in the ANG than in the light organ (Fig. S1).

To examine the biochemical characteristics of the EsGal1 protein more fully, we verified the full-length coding sequence for EsGal1 by 5′RACE. *esgal1* encodes a 13.1 kD protein with a isoelectric point (pI) of ~10. RADAR analysis of the sequence predicted that the protein has three tandem repeats (Fig. 1A) anchored by dicysteine pairs. SignalP analysis predicted a signal peptide, suggesting that the protein is secreted. The high predicted pI, repeat structure and abundant cysteines (12.3%) provide evidence that the protein is antimicrobial (Fedders and Leippe, 2008).

BLAST analysis (NCBI) of the derived amino-acid sequence of the protein revealed 25 proteins with some similarity to EsGal1 (Table S2), with the closest match being to a galaxin occurring in the coral *Acropora millepora* (E-value = $4e^{-13}$). Although *A. millepora* galaxin was the closest BLAST hit to EsGal1, we used the *Acropora palmata* galaxin sequence for alignment and biochemical analysis; *A. palmata* galaxin was chosen because its repeats were predicted to have a higher antimicrobial activity. Examination of full-length sequences revealed a protein family with an array of sizes, from 116 to 828 amino acids (Table S2). Our alignment with portions of other galaxin proteins (Figs. 1B and S2) revealed that EsGal1 shares with the other family

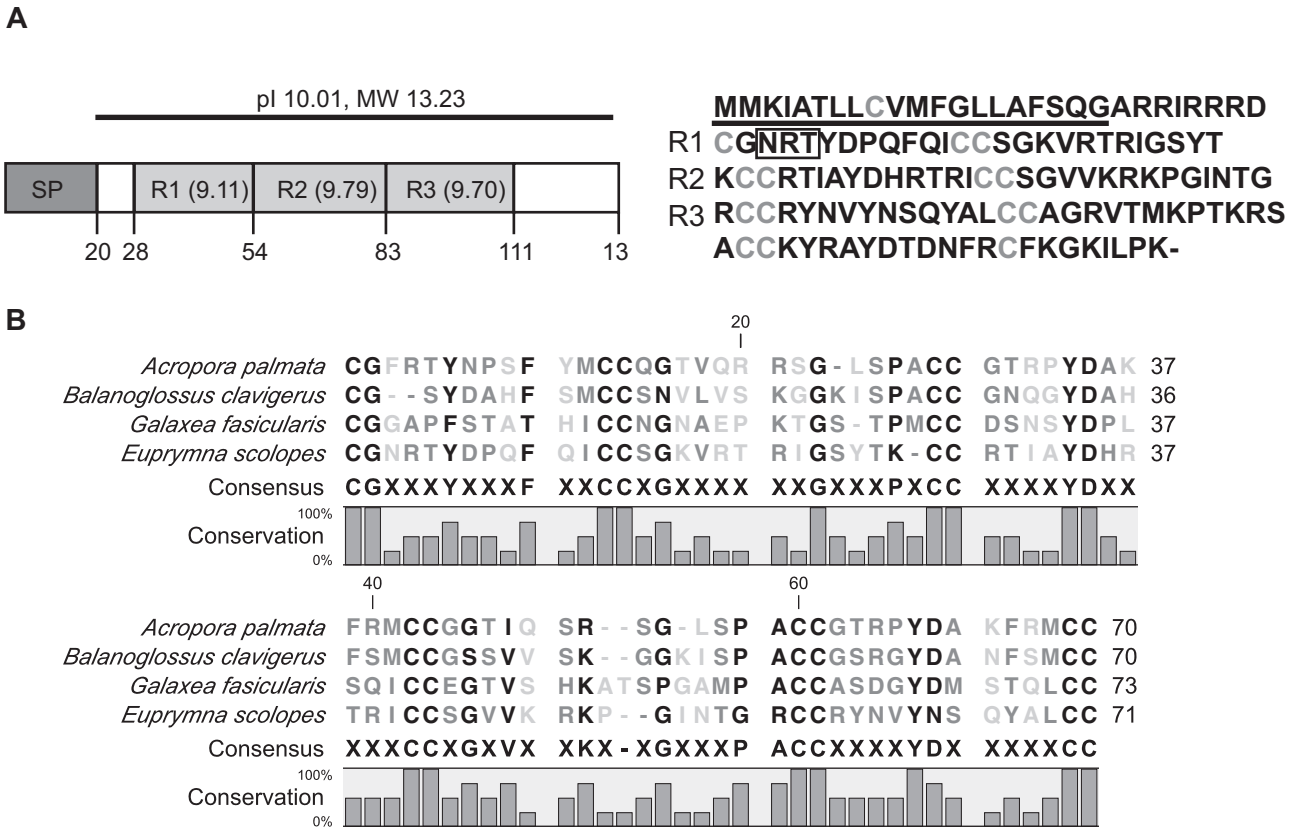

Fig. 1. Biochemical properties of the EsGal1 protein.
A. The structure of the protein. Left, major regions, with amino acid position indicated by the numbers below. M_r, molecular mass; pI, predicted isoelectric point; R1-3, predicted repeats and their pIs; SP, predicted signal peptide. Right, the derived amino acid sequence of EsGal1 R1–R3, with the RADAR-predicted repeat structure; predicted signal peptide, underlined; predicted N-glycosylation site, boxed; cysteines, grey.
B. MUSCLE alignment of portions of galaxin sequences from *Acropora palmata, Balanoglossus clavigerus, E. scolopes* and *Galaxea fascicularis*, with the consensus sequence and graphical representation of conservation at each amino acid residue shown below the alignment. Grey shading denotes the classes of the labelled amino acids.

members the conserved repeat structure and a number of residues, which suggests that these regions of the protein are critical to structure and function of galaxins.

Patterns of esgal1 expression

To characterize the regulation of expression of *esgal1* over early light-organ development, we performed qRT-PCR on both aposymbiotic and symbiotic light organs collected at six time points over the first 2 days of colonization of the juvenile squid (Fig. 2A). These points were chosen to determine when the regulation of *esgal1* begins after colonization and then how long after colonization the regulation persists. The *esgal1* mRNA levels were higher in the symbiotic than the aposymbiotic light organs; significant upregulation began at 10 h post-hatching and persisted over the first 2 days of the symbiosis. In addition, symbiotic light-organ expression of *esgal1* increased twofold between 10 and 14 h post-hatching, whereas we observed no difference in expression in aposymbiotic light organs, suggesting that symbiosis is increasing expression rather than expression being downregulated in aposymbiotic animals. To determine if galaxin expression is more prevalent in symbiotic tissues than other portions of the squid, we analysed *esgal1* expression in six different tissues of the adult squid and found that relative expression of *esgal1* is 100–1000-fold higher in *V. fischeri*-containing tissue than in any other tissue sampled (Fig. 2B).

In the squid-vibrio symbiosis, certain symbiont-induced host phenotypes are reversible upon depletion of symbionts from a colonized organ after 12 h, i.e. they require persistent interaction with the symbiont (Lamarcq and McFall-Ngai, 1998; Nyholm *et al.*, 2002), while some are irreversibly triggered by a 12 h colonization (Doino and McFall-Ngai, 1995; Davidson *et al.*, 2004). To

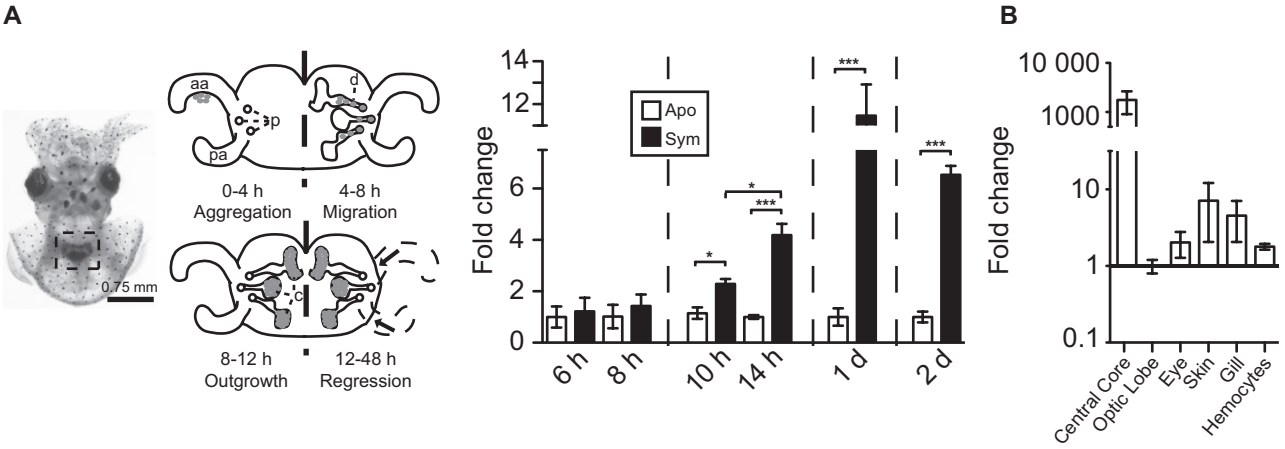

Fig. 2. Patterns of *esgal1* expression.
A. Top, a ventral view of the juvenile animal, with relevant features. Left, the light organ (box), seen through translucent ventral mantle tissue. Right, stages of early symbiosis development. The acquisition of symbionts from the seawater is facilitated by anterior (aa) and posterior (pa) epithelial 'appendages' of a superficial ciliated epithelium. Symbionts (grey) accumulate along these epithelia in the first hours after exposure (Aggregation). They then migrate into pores (p), through the ducts (d) into the crypt spaces (c) (migration). In the crypts, the symbionts proliferate to fill the spaces (Outgrowth). At 12 h, the symbionts deliver an irreversible morphogenic signal that leads to the eventual loss of the ciliated epithelia (regression). Bottom, the expression of *esgal1* in the light organ at time points over the first 2 days in aposymbiotic and symbiotic animals. Significant differences (*, < 0.05; ***, < 0.001) between conditions by an ANOVA followed by a Tukey's pairwise comparison.
B. Expression of *esgal1* in six tissues of adult animals. Relative qRT-PCR values (± SEM). All data are normalized to the condition of lowest level of expression; $n = 3$ biological replicates and 2 technical replicates per condition.

address whether persistent colonization of the light organ by symbionts was necessary for *esgal1* regulation, we depleted colonized animals of their symbionts with antibiotics at 24 h post-hatching and then compared *esgal1* expression levels in these depleted light organs 24 h later, i.e., at 48 h post-hatching, to those of symbiotic and aposymbiotic animals. Depletion of symbionts did not significantly alter the expression of the *esgal1* transcript from the symbiotic level of expression under the conditions used (Fig. 3A), suggesting that the persistence of viable symbionts in the light organ is not necessary for the regulation of *esgal1,* or that any change in expression requires significantly longer than other reversible phenotypes.

We explored signals from *V. fischeri* that might regulate *esgal1* expression. Because the bacterial products lipid A and tracheal cytotoxin (TCT) induce many host phenotypes in the squid-vibrio system (reviewed in McFall-Ngai *et al*., 2010), we determined whether these products are also responsible for the regulation of *esgal1*. While exposure to TCT and lipid A increased expression of *esgal1* in the light organ on average ~1.75-fold (Fig. 3B), the response was only ~20% of the increase observed in symbiotic relative to aposymbiotic organs. One other inducer of host phenotypes is symbiont light production (McFall-Ngai *et al*., 2012). However, *V. fischeri* mutants defective in light production showed no defect in *esgal1* upregulation (Fig. 3B).

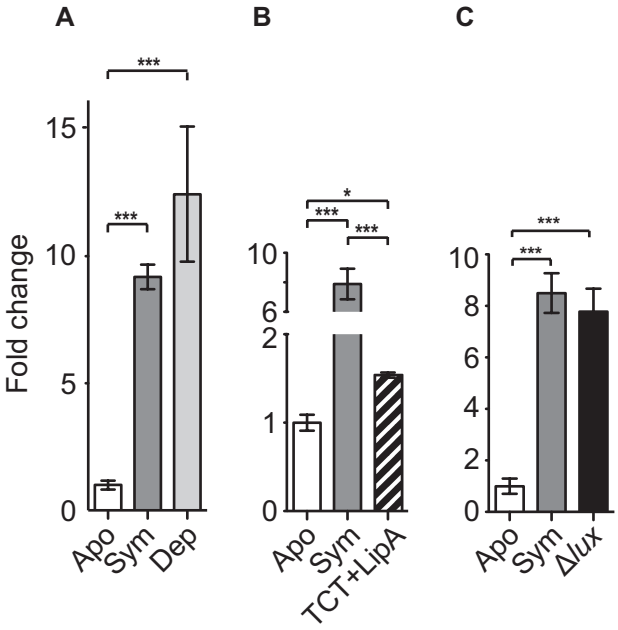

Fig. 3. The influence of experimental manipulation on *esgal1* expression. Expression, as measured by qRT-PCR, at 48 h in the light organ of animals remaining uncolonized (Apo) or colonized with *V. fischeri* strain ES114 (Sym) compared with: (A) animals colonized with *V. fischeri* for 24 h and then antibiotically depleted of their symbionts (Dep); (B) treated with the bacterial products TCT and lipid A (TCT + LipA) (see *Experimental procedures* for details); (C) animals colonized with mutants defective in light production (*Δlux*). Data are normalized to the time point of lowest expression. Values +/– SEM; Significant differences (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$) between conditions by an ANOVA followed by a Tukey's pairwise comparison; $n = 3$ to 4 biological replicates and 2 technical replicates per condition.

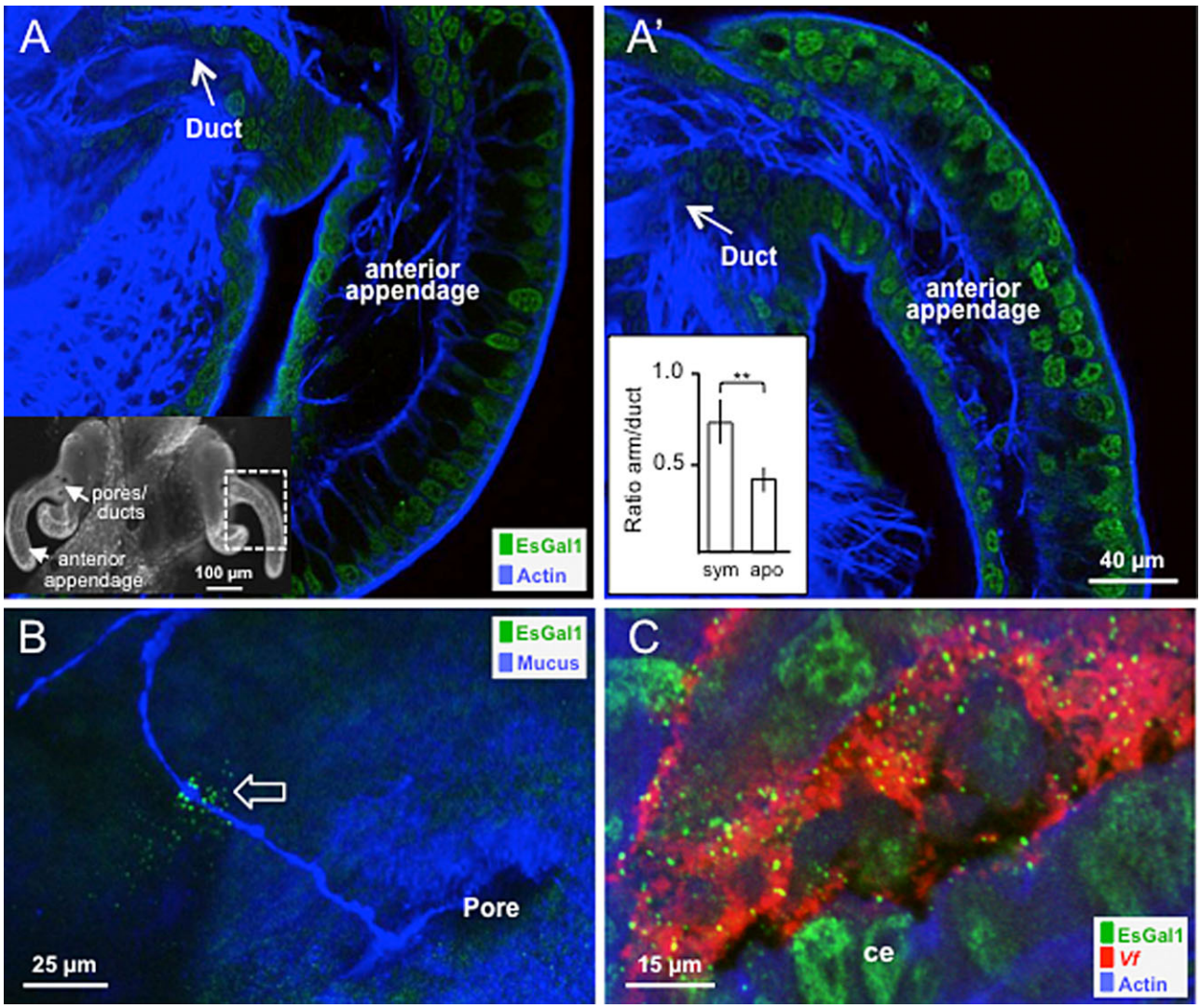

Fig. 4. Localization and abundance of EsGal1 in the juvenile light organ. Light organs from 24 h uncolonized (A) and colonized (A') juveniles probed with the EsGal1 antibody, showing the relative abundance of EsGal1 in each condition. Labelling of tissue actin (phalloidin) was used as a counterstain. Inset in A, confocal micrograph of the entire organ showing regions explored by ICC. Inset graph in A', relative fluorescent intensity; ** = $P < 0.01$ by an unpaired t-test after log transformation to ensure normality. (B) EsGal1 localization to the mucus outside of the juvenile light organ. Open arrow, EsGal1 in contact with a mucus strand outside of the light-organ pore. (C) EsGal1 secretion into crypt spaces of the colonized organ. ce, crypt epithelium; Vf, *Vibrio fischeri.*

Localization and abundance of EsGal1 *in the juvenile animal*

The polyclonal antibody to two EsGal1 peptides recognized apical cytoplasmic and perinuclear sites in the epithelia of the light organ, including the appendages and ducts, but did not label internal musculature (Fig. 4A and A'). The apical localization, in conjunction with the presence of a signal peptide in the *esgal1*-derived amino acid sequence, suggested that the protein is secreted, which was confirmed by localization of EsGal1 to the mucus outside of the light organ (Fig. 4B). In quantification of labelling of EsGal1 antibody in aposymbiotic and symbiotic animals, although the two conditions had the same signal intensity in the duct cells, the epithelial appendages of symbiotic light organs had about twice the signal intensity (Fig. 4A', inset). We also observed antibody cross reactivity in the symbiont-containing crypts (Fig. 4C). It localized to perinuclear sites inside of the epithelial cells and to the extracellular crypt spaces where the symbionts reside, suggesting secretion of the protein into symbiont-containing regions. We observed no staining in samples exposed to rabbit IgG as a negative control (Fig. S3A–C). When we performed a western blot analysis on squid proteins using the EsGal1 antibody, we observed several specific bands, one of which

corresponded to the predicted molecular weight (Fig. S3D). The existence of multiple bands may be due to the putative N-glycosylation site on the EsGal1 protein (Fig. 1A), the modification of which could alter the migration of the protein on an SDS gel, or due to the abundant dicysteine bonds that may not have been fully reduced before protein preparation.

To determine whether epithelial localization of EsGal1 protein was unique to the light organ, we performed immunocytochemistry (ICC) with the antibody on other host tissues (Fig. S4). The antibody recognized cytoplasmic and perinuclear sites in all epithelial tissues exposed to seawater, including the tentacles and gills, but did not label internal tissues, such as the white body (the haematopoietic organ of the animal) or musculature of the tentacles. Symbiotic state of the light organ did not detectably alter the localization of EsGal1 in any non-symbiotic tissue.

Evidence for antimicrobial activity of EsGal1

Due to the small size, basic pI, presence of a secretion signal and numerous cysteines in EsGal1, we hypothesized that the protein acts as an antimicrobial effector, a class of molecules that tend to share all of these properties (Fedders and Leippe, 2008). To determine whether the protein might have such activity, we synthesized one of the three repeats present in the EsGal1 protein (EsGal1R3, Table 1) that, by *in silico* analysis, was predicted to be the most likely region of the protein to be antimicrobial, and characterized its ability to inhibit bacterial growth. The third repeat of EsGal1 was chosen because of its high net charge and hydrophobic residue ratio, as well as its significant sequence similarity to a known oyster defensin, all determined with the APD2 antimicrobial peptide calculator and predictor (see *Experimental procedures*). EsGal1R3 affected the growth of Gram-positive bacteria at concentrations as low as 27 nM (Table 1), but was about 10–100 times more effective against non-marine species under the assay conditions used. However, EsGal1R3 did not inhibit the growth of Gram-negative marine species, including *V. fischeri*, in minimum inhibitory concentration (MIC) assays. To determine whether antimicrobial activity was a shared characteristic of galaxin proteins from other species, we also synthesized a peptide corresponding to the predicted repeat 2 from *A. palmata* galaxin (ApGalR2), which we chose for its basic pI and predicted antimicrobial capabilities, and determined its ability to inhibit bacterial growth under the same conditions used for assays with EsGal1R3. ApGalR2 did not exhibit any capacity to inhibit the growth of marine bacteria and was, at most effective, 10-fold less active than EsGal1R3 against non-marine strains.

Because of the influence of day/night cycles on the symbiont growth in the association (Nyholm and McFall-Ngai, 2004; Wier *et al.*, 2010) and the potential that EsGal1 has to modulate bacterial growth, we also measured *esgal1* gene expression at three time points over the day/night cycle in both juvenile light organs and adult central cores, i.e. the epithelial tissue of the adult light organ that supports the symbiont. In both sample groups, we found an upregulation of the *esgal1* gene 2 h after dawn (Fig. 5), at a time point coincident with the rapid symbiont growth in the organ that immediately follows the venting of 90% of the symbionts (Graf and Ruby, 1998; Nyholm and McFall-Ngai, 1998). These data suggest that in both juvenile and adult animals, *esgal1* is

Table 1. Antimicrobial activity of EsGal1R3 and ApGal R2.

Strain characteristics and strain	EsGal1 R3 MIC (μM) – RCCRYNVYNSQYALCCAGRVTMKPTKRS	ApGal R2 MIC (μM) – VQRRSGLSPACCGTRPYDAKFRMCCGGT	Source
Gram-positive			
Non-marine			
B. megaterium	0.027	0.287	This Study
B. subtilis	0.5375	4.76	This Study
Marine			
B. algicola CNJ 803	4.3	> 152.29	(Gontang *et al.*, 2007)
B. megaterium-like CNJ 778	6.88	> 152.29	(Gontang *et al.*, 2007)
E. aestuarii CNJ 771	3.44	> 152.59	(Gontang *et al.*, 2007)
Gram-negative			
Non-marine			
E. coli	1.72	76.145	(Blattner *et al.*, 1997)
Marine			
P. leiognathi	> 137.5	> 152.29	(Dunlap, 1985)
V. fischeri ES114	34.4	> 152.29	(Boettcher and Ruby, 1990)
V. parahaemolyticus	137.5	> 152.29	(Nyholm *et al.*, 2000)

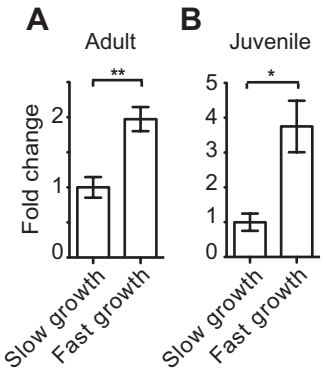

Fig. 5. Daily changes in *esgal1* expression with variation in light-organ symbiont growth rate. *esgal1* expression in wild-caught adult (A) and juvenile (B) light organs during periods of slow and rapid bacterial growth, approximately 12–14 h before dawn and 2 h after dawn respectively. All data are normalized to the condition of lowest expression. Error bars denote the standard error of the mean. * = $P < 0.05$ and ** = $P < 0.01$ by an unpaired t-test after log transformation to ensure normality.

upregulated when the bacterial symbionts are rapidly dividing in the light organ.

Although the MIC assay showed that EsGal1R3 does not inhibit growth of *V. fischeri* except at high concentrations, we hypothesized that in lower concentrations, the peptide could modulate the rate of bacterial growth in the light organ. We first tested the growth response of *V. fischeri* to subinhibitory concentrations of the peptide under *in vitro* culture conditions. We observed that exposure to EsGal1R3 over 18 h both delayed the start of exponential growth of *V. fischeri* by approximately 2 h and decreased its growth rate 1.5-fold once in exponential phase growth (Fig. 6A and B). While the most robust result was obtained using 17.4 μM EsGal1R3 (half of the concentration determined to be inhibitory), the peptide showed a dose-dependent ability to modulate the growth of *V. fischeri* at a concentration as low as 8.2 μM (Fig. S5).

The growth curve (Fig. 6A) had a higher terminal optical density (OD) for the EsGal1R3 exposed cells, which suggested that either smaller cells or more numerous cells had resulted from the conditions. Measurements of the cells showed an average decrease in their length of about 20% (Fig. 6C), similar to the decrease in size noted in *V. fischeri* cells adapted to the crypts of the light organ (Ruby and Asato, 1993).

To determine whether EsGal1 might affect *V. fischeri* growth during onset of the association, we co-incubated the animals during colonization with the EsGal1 antibody to absorb the protein from the environment, a proven approach to the study of interactions of the symbiont with host biomolecules (Aeckersberg *et al.*, 2001; Kremer *et al.*, 2013). In the treated hatchlings, we observed a 2.5-fold increase in symbiont luminescence (Fig. 6D) and a 1.6-fold increase in bacterial density (Fig. 6E) at 8 h post-bacterial exposure as compared with the negative control. In experiments with addition of the peptide, we

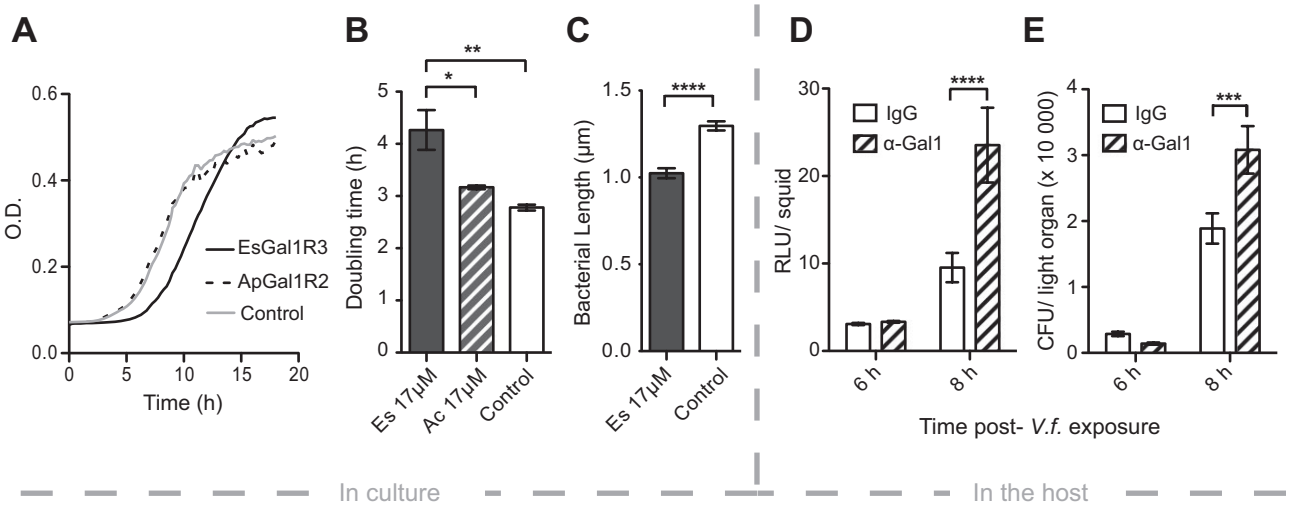

Fig. 6. The effect of EsGal1R3 on *V. fischeri* growth *in vitro* and *in vivo*. (A) Growth curve of *V. fischeri* cells exposed to 17.4 μM EsGal1R3 (solid line), 17.4 μM ApGal1R2 (dashed line), or to no peptide (grey line). (B) Quantification of *V. fischeri* doubling times in experiment shown in (A). Experiment in (A) and (B) was performed with three biological replicates and two technical replicates. (C) Length of *V. fischeri* cells exposed to 17.4 μM EsGal1R3 (grey bar) or no peptide (open bar). N = 117 and 124 respectively. (D) Effect of α-EsGal1 antibody or rabbit IgG exposure on animal luminescence at 6 or 8 h after exposure to bacteria. (E) Effect of α-EsGal1 antibody or rabbit IgG exposure on the number of colony-forming units (cfu) in the juvenile light organ at 6 and 8 h after exposure to *V. fischeri*. N = 23 to 27 for each condition. Error bars denote the standard error of the mean. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$ by an ANOVA with a post-hoc Tukey's pairwise comparison.

found no evidence for a depression of *V. fischeri* growth in the crypts, suggesting that EsGal1 activity reaches an asymptote during symbiosis initiation. Due to the lack of an ANG in juvenile animals, we were unable to assess the effect of the EsGal1R3 peptide on the ANG-associated bacterial community.

Discussion

In this study, we investigated the role of a host protein, EsGal1, in the habitat transition of *V. fischeri* from the seawater to the light organ of the squid *E. scolopes.* We provide evidence for the activity of this protein in the selection of *V. fischeri* from the complex bacterioplankton, as well as in the long-term maintenance of this symbiont in a cooperative, binary association.

EsGal1 *in* V. fischeri *habitat transition*

In marine habitats, a given microbial species such as *V. fischeri* can be found in a wide variety of environments; for example, they can occur as members of the bacterioplankton, in marine snow, sediments and symbiotic associations (Fuhrman, 2009; Stabb and Visick, 2013). As such, habitat transitions are often a critical feature of their ecology. Because the association between *V. fischeri* and *E. scolopes* is a horizontally transmitted symbiosis (Bright and Bulgheresi, 2010), the microbial partner must be capable of a robust response both to environmental stressors in the planktonic environment and to the selective pressures imposed by the host niche (Wang *et al*., 2010). The host, in turn, must provide conditions that: (i) promote selection of the coevolved symbiont from thousands of microbial species in the bacterioplankton during symbiosis onset; and (ii) once the symbiosis is established, mediate the maintenance of the association so that it persists throughout the life of the animal (Visick and Ruby, 1996; Nyholm *et al*., 2002).

Over the 3–4 h following initial exposure to seawater with natural levels of environmental bacteria, including the symbiont, *V. fischeri* cells become the competitive dominant among bacterial cells associating with the epithelial cell surface of the host's light organ. The first step in this process of 'winnowing' is the elimination of Gram-positive bacteria (for review, see Nyholm and McFall-Ngai, 2004). We found that the host protein EsGal1 is produced on the surface of the epithelial field and secreted into the mucus (Fig. 4C), the environment where *V. fischeri* attaches and then aggregates (Altura *et al*., 2013). The lack of symbiont-dependent regulation of *esgal1* transcription during time points coincident with aggregation demonstrates that provision of EsGal1 stores is 'hard-wired' during embryogenesis to be in place, ready for secretion when the animal hatches from the egg (Fig. 2A). This provision does not appear to be relegated to the light organ in juvenile animals, as all epithelia tested that had contact with the seawater also produced EsGal1 (Fig. S4), suggesting that the protein may be used as a growth modulator in other sites on the squid's body. Further, a peptide predicted to occur on the protein surface, generated to a single repeat of EsGal1, was active against Gram-positive bacteria in *in vitro* assays. While the peptide shows robust activity, we recognize that the whole protein may behave differently. The protein itself has three repeats predicted to have antimicrobial activity, so it is possible that the whole protein is more antimicrobial than the derived peptide. However, we feel it is necessary to exercise caution in interpreting how the intact galaxin protein behaves in the symbiosis. Earlier studies provided evidence for the involvement of other proteins that are active against Gram-positives, specifically the findings that a peptidoglycan recognition protein (EsPGRP2, Troll *et al*., 2010) is also secreted into the mucus and the gene encoding a lysozyme isoform is upregulated during initial interactions with *V. fischeri* (Kremer *et al*., 2013). In addition, other antimicrobials, such as nitric oxide (Davidson *et al*., 2004), occur in abundance in the mucus. From these studies, a scenario is emerging in which the host provides a selective environment that *V. fischeri* can withstand and to which it can adapt, and that the symbiont cells participate in the creation of this environment through signal exchange with the host.

Growth modulation by host molecules

Our data also provide evidence that EsGal1 modulates the growth of symbiont populations that have already colonized the host. An earlier study demonstrated that colonization of the crypts causes a significant increase in expression of the gene encoding EsGal1 (Chun *et al*., 2008) at about 18 h following colonization. In the present study, we showed that this upregulation is detectable as early as 10 h, during the initial symbiont outgrowth to fill the crypt spaces. We confirmed that EsGal1 is secreted into the crypts, where it can act directly on symbiont cells, and co-incubation of juvenile squid during initial colonization with the EsGal1 antibody increased the growth rate of *V. fischeri,* such that the crypts are colonized more quickly. Once the symbiosis is established, we show that EsGal1 gene expression changes over the day/night cycle, being highest during the rapid growth of *V. fischeri* following the daily venting of cells into the environment in response to the dawn light cue (Graf and Ruby, 1998). The upregulation of *esgal1* during bacterial growth in the light organ is reminiscent of the provision of the antimicrobial coleoptericin by weevils into regions where their

intracellular symbionts occur. This protein controls the growth and limits the location of the symbionts to specific host cells, or bacteriocytes (Login *et al*., 2011). Taken together, these data suggest that the symbiont participates in creating an environment that imposes a governor on symbiont population growth.

The precise mechanisms by which the symbiont signals the host are not fully understood. Although our data show that bacterial cell-envelope molecules may play a role (Fig. 3B), they could not induce the same responses as the presence of the symbiont, suggesting that they may work in synergy with other factors. For some squid-host responses, light works in synergy with these molecules (Heath-Heckman *et al*., 2013). However, in this study, Δ*lux* mutants had no defect in inducing normal expression of *esgal1*, so the evidence suggests another factor. Alternatively, under normal conditions, these molecules may induce these host responses, but the cell envelope molecules, when added as pharmacological agents, are not presented to host tissue in the same manner as the intact bacterial cell (e.g. concentration, configuration); or, direct interactions with the bacterial cell enable host responses, as has been noted before for other host responses (Chun *et al*., 2008; Heath-Heckman *et al*., 2013).

The selection of symbionts by host biomolecules in the squid-vibrio system has parallels in other mutualisms. For example, in *Hydra* spp., overexpression of periculin, a secreted antimicrobial protein, changes the microbial community density along the host surface and the composition of the community in mature polyps (Fraune *et al*., 2010). Additionally, *Hydra* spp. lacking the capability to express antimicrobial peptides, such as arminin, lose the capacity to select for a species-specific microbiota (Franzenburg *et al*., 2013). In the mammalian gut, exposure of the host to Gram-negative bacteria during initial gut colonization induces the secretion of RegIIIγ, a lectin that is anti-Gram-positive (Cash *et al*., 2006). This protein alters microbial community structure in the gut by creating a zone of exclusion between host cells and the microbiota through its bactericidal activity. In so doing, it reduces potential symbiont-mediated inflammatory processes in the intestine (Cash *et al*., 2006; Vaishnava *et al*., 2011). The mammalian gut also expresses α-defensin, an antimicrobial peptide that has been shown to structure the microbial community to promote gut homeostasis (Salzman *et al*., 2010). Collectively, these studies suggest that the bacteriostatic property of EsGal1 against *V. fischeri* may benefit the host by restricting symbionts to the light organ, and maintaining immune homeostasis. Further, our finding that *esgal1* and *esgal2* have opposite patterns of expression in the adult light organ and ANG suggests that galaxins are a family of molecules that can be used to manage different microbial communities in the same animal. Further studies will be required to explore this possibility. Taken together, these examples demonstrate that antimicrobial peptide expression by animal hosts perform a conserved function of population or community structuring across multiple stages of symbioses.

Galaxin structure and function

Our *in silico* analysis of galaxin sequences in available databases identifies proteins with sequence similarity to EsGal1 in all major groups of the animal kingdom, except the arthropods and vertebrates (Table S2). Selection and alignment of a subset of widely divergent representatives showed that certain conserved residues are maintained through evolution (Fig. 1B). The conserved residues may serve as a structural scaffold for the protein with the remaining amino acids diverging to confer alternate functions within an animal or in different species. The wide range of sizes and pIs of galaxin proteins supports the hypothesis of other functions for the various members of the protein family.

The mode of action of EsGal1R3 was not determined in the present study, but the results provide some clues about its molecular behaviour. Antimicrobial peptides often compromise the integrity of bacterial membranes. The finding that EsGal1R3 was more inhibitory to Gram-positive species suggests that it targets the inner bacterial membrane or that it has a mode of action other than attack of the bacterial membrane (Epand and Epand, 2011). EsGal1 treatment reduced the size of *V. fischeri* cells, but it did not induce filamentation, suggesting that the peptide does not directly affect septation, as antimicrobial peptides do in some bacteria (Login *et al*., 2011).

Few functional studies of galaxins are available. Although BLAST analysis revealed other family members, our study is only the second outside of the Cnidaria to report a sequence as belonging to the galaxin family of proteins, the first being the identification of a partial sequence in the vent tubeworm *R. pachyptila* (Sanchez *et al*., 2007). Three in-depth studies of galaxins in corals (Phylum Cnidaria) have localized the proteins to the calcium carbonate exoskeleton, and larvae of the coral *A. millepora* express galaxins and galaxin-like transcripts in portions of the larva that secrete the exoskeleton during settlement (Reyes-Bermudez *et al*., 2009). The close association of the galaxins to the coral exoskeleton, their high abundance as well as the timing of their production, has led researchers to link the proteins with biomineralization of the exoskeleton. However, galaxins were not found to bind calcium, and direct involvement of galaxins with production of the coral exoskeleton has not been dem-

onstrated. Interestingly, galaxin proteins were not found in the exoskeleton of sun corals (*Tubastrea* spp.), which coincidentally do not host the photosynthetic partner of most corals, the zooxanthellae (Watanabe *et al*., 2003). These data do not preclude the involvement of galaxins in exoskeletal formation in some corals, but do suggest alternative functions. The pattern of expression in coral larvae mirrors the symbiont-dependent developmental upregulation and pan-epithelial localization we observed in the juvenile squid, and suggests that coral galaxins may be involved in the first contact between host and microbe, as corals associate with both algal and bacterial symbionts (Krediet *et al*., 2013). Although the peptide derived from *A. palmata* was not antimicrobial under the conditions of our experiments, the protein in its native conformation, or with the addition or subtraction of additional amino acids on either end, may be active or may serve another function in this species. Alternatively, these data suggest that the ability to modulate bacterial growth through potent antimicrobial activity is not conserved among all galaxin proteins; rather it is a character restricted to particular animals or galaxin types. The various roles of galaxins in other animal species present a fruitful area of future research.

Finally, galaxins may also function as part of a general response to either biotic (e.g. symbiosis) or abiotic (e.g. temperature, heavy metals) stress. In a wide array of symbioses, including the squid-vibrio system, plant–legume associations and coral–zooxanthellae partnership, various stress responses (e.g. oxidative, nitrosylative) are a part of the normal activity of the symbiotic state. In support of this possibility, transcription is downregulated by copper exposure in coral *Montastraea franksi*, and galaxin small nucleotide polymorphism variation is correlated with water temperature in *A. millepora* (Schwarz *et al*., 2012; Lundgren *et al*., 2013). Since corals also undergo profound biochemical and physiological changes due to their symbionts (Davy *et al*., 2012), it is possible that galaxin transcription may instead be due to a stressor's effect on algal endosymbionts.

In conclusion, our study proposes a new function for galaxin proteins as antimicrobial agents used by the squid host to select and maintain a specific symbiont, and identifies a new taxonomic group in which galaxin proteins occur in abundance. Future studies will determine whether the biochemical role of galaxins in the squid-vibrio system is a widespread attribute of these proteins across animal taxa. In the greater context of host–microbe interactions, our data support the growing paradigm that antimicrobial proteins do not serve to simply exclude bacteria from sites on an animal's body. Rather, they are selective forces imposed by hosts on microbial communities to favour the acquisition and maintenance of coevolved symbiotic partnerships.

Experimental procedures

General methods

Adult *E. scolopes* were collected and maintained as previously described (Wollenberg and Ruby, 2012). Juveniles from the breeding colony were collected within 15 min of hatching. Aposymbiotic (Apo) animals were maintained in *V. fischeri*-free, unfiltered seawater, while other juveniles were exposed to ~5000 colony-forming units (cfu) ml^{-1} of the *V. fischeri* strain ES114 (Boettcher and Ruby, 1990) overnight to produce the symbiotic (Sym) condition. Colonization of the symbiotic juvenile squid was determined by measuring luminescence output of the symbionts with a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, USA); aposymbiotic squid were also analysed to ensure that their light organs had not been colonized. In experiments with *V. fischeri* surface molecules, the lipid A and the peptidoglycan monomer (also called TCT) were prepared as previously described (Foster *et al*., 2000; Koropatnick *et al*., 2004) and exposed to animals at 10 ng ml^{-1} and 10 μM, respectively, for 48 h with the water + MAMPs replaced every 24 h. Symbiont depletion from the light organ was performed with antibiotics as previously described (Doino and McFall-Ngai, 1995). In experiments examining the role of symbiont light production in *esgal1* upregulation, animals were colonized with the light-deficient *V. fischeri* strain EVS102 (Δlux) (Bose *et al*., 2008) as described above and a subset of the exposed squid were plated to ensure colonization. All reagents were obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise noted. All animal experiments conform to the relevant regulatory standards established by the University of Wisconsin – Madison.

Identification of galaxin gene sequences from the EST database and subsequent analyses

Two galaxin gene sequences were identified by a tblastn search against the expressed sequence tag (EST) database of the juvenile-host light organ (Chun *et al*., 2006) using *Galaxea fascicularis* galaxin as the query sequence (Fukuda *et al*., 2003). The identified sequences in the EST database sequence were used for primer design for subsequent rapid amplification of cDNA ends (RACE) to obtain the full sequence of the open-reading frame (for details, see Appendix S1).

Sequences obtained by RACE were assembled into contigs using the CAP3 sequence assembly program (http://pbil.univ-lyon1.fr/cap3.php). The resulting full-length sequence was analysed by BLAST searches of GenBank using the default parameters. The cDNA sequence was translated using the ExPASy Translate tool (http://web.expasy.org/translate/), and the resulting derived amino acid sequence was examined for a putative repeat structure using RADAR (Rapid Automatic Detection and Alignment of Repeats), http://www.ebi.ac.uk/Tools/pfa/radar/). Signal peptide prediction was performed using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/), and a potential N-glycosylation site was identified using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Potential for antimicrobial activity was assayed *in silico* with the APD2 antimicrobial peptide predictor (http://aps.unmc.edu/AP/

prediction/prediction_main.php). The *G. fascicularis*, *A. palmata* and *Balanoglossus clavigerus* sequences used in this study were derived from the NCBI protein database. Amino acids from the first three putative repeats of the proteins (shown in Fig. S1) were aligned using MUSCLE (Edgar, 2004) and the subsequent alignment was visualized with the CLC Sequence viewer (CLC Bio, Cambridge, MA, USA, http://clcbio.com/index.php?id=28).

RNA and cDNA preparation and qRT-PCR

Whole juvenile animals were stored in RNALater RNA stabilization reagent (Qiagen, Valencia, CA, USA) for 24 h at 4°C and then transferred to –80°C for extended storage until RNA extraction. Extracted RNA was prepared by standard methods (for details, see Appendix S1).

All qRT-PCR assays were performed in compliance with the MIQE guidelines (Bustin *et al*., 2009). Gene-specific primers were designed for *esgal1* and *2*, and for the *E. scolopes* 40S ribosomal RNA sequence, which was used as a control for equal well loading (Table S1) (for details, see Appendix S1). Data were analysed according to Pfaffl (2001).

Western blotting and ICC

A polyclonal antibody to EsGal1 was produced in rabbit (Genscript, Piscataway, NJ, USA) to two unique peptides within the EsGal1 sequence (GNRTYDPQFQIC and CKYRAYDTDNFR), chosen for their predicted antigenicity, surface exposure and lack of similarity to other known, predicted *E. scolopes* or *V. fischeri* proteins. Protein samples for western blotting were prepared as described previously (Troll *et al*., 2010) (for details, see Appendix S1).

For ICC, light organs were fixed, permeabilized and blocked as described previously (Troll *et al*., 2009). A 1:500 dilution of the EsGal1 antibody was used in these experiments (for details, see Appendix S1). The light organs were then counterstained with rhodamine phalloidin as previously described (Troll *et al*., 2009), mounted on glass slides and examined on a Zeiss LSM 510 confocal microscope. For mucus secretion assays, samples were prepared as previously described (Kremer *et al*., 2013). Host mucus was stained with Alexa-633 conjugated wheat germ agglutinin (Life Technologies, Carlsbad, CA, USA) (for details, see Appendix S1). Fluorescence intensities were quantified using the Zeiss LSM 510 software.

MIC assays

To determine the minimal (growth) inhibitory concentration for the galaxin repeat-derived peptides, we performed microdilution susceptibility assays (Fedders and Leippe, 2008). Briefly, a twofold serial dilution of each peptide was carried out in 96-well plates in 10 mM sodium phosphate buffer, with the addition of 345 mM NaCl for marine strains, pH 8.0. One hundred colony-forming units of a log-phase bacterial culture were added to each peptide dilution and incubated overnight at 28°C for marine bacteria and 37°C for non-marine strains. After incubation, the MIC was defined as the peptide dilution where no visible bacterial growth was detected after incubation. The values are expressed as the median of at least two experiments, each performed in duplicate, with a divergence of not more than one dilution step. The following bacterial strains were used: *Bacillus subtilis* 1009, *Bacillus megaterium* 1006, *Escherichia coli* MG1665 K12 (Blattner *et al*., 1997), CNJ 771 (*Exiguobacterium aestuarii*-like) (Gontang *et al*., 2007), CNJ 778 (*B. megaterium*-like) (Gontang *et al*., 2007), CNJ 803 (*Bacillus algicola*-like) (Gontang *et al*., 2007), *V. fischeri* ES114 (Boettcher and Ruby, 1990), *Photobacterium leiognathi* Ln1a (Dunlap, 1985) and *Vibrio parahaemolyticus* KNH1 (Nyholm *et al*., 2000). Marine bacteria were grown in SWT medium (Boettcher and Ruby, 1990), whereas *B. subtilis*, *B. megaterium* and *E. coli* were grown in Luria–Bertani (LB) medium with (wt vol^{-1}) 1% tryptone, 0.5% yeast extract and 1% NaCl.

Bacterial growth assays

Overnight cultures of *V. fischeri* strain ES114 (Boettcher and Ruby, 1990) were diluted 1:500 and then grown to OD_{600} 0.2 at room temperature with shaking in LBS (LB with sodium chloride) medium with (wt vol^{-1}) 1% tryptone, 0.5% yeast extract, and 2% NaCl, and 50 mM Tris-HCl, pH 7.5. The cultures were then diluted with LBS 1:9 to reach an OD_{600} of about 0.02. Ten microlitres of the bacterial culture were then added to 190 μl of 10% LBS medium in a 96-well microtiter plate containing 17 μM EsGal1R3 or ApGalR2 (Genscript, sequences can be found in Table 1), or no addition as a control. The plate was then placed in a Tecan Genios Pro plate reader (Tecan Group, Männedorf, Switzerland) and incubated at 27°C with shaking for 18 h, with OD_{600} readings taken every 15 min. Sample wells were also run without added bacteria to ensure that no contamination occurred. Bacterial doubling times were calculated using the equation $G = [\log(N–n)/\log(2)]*(T–t)$ where N is the OD_{600} at time T (final) and n is the OD_{600} at time t (initial).

Measurement of bacterial length

Cultures of red fluorescent protein (RFP)-expressing *V. fischeri* [strain ES114 containing the plasmid pVSV208 (Dunn *et al*., 2006)] were grown as for the bacterial growth assays for 18 h, and were then visualized using a Zeiss AxioImager.M2 epifluorescence microscope. Bacterial length was then measured using Zeiss software.

In vivo antibody adsorption and peptide supplementation

Newly hatched juvenile squid were placed in seawater containing 5000 cfu ml^{-1} *V. fischeri* strain ES114 cells ml^{-1} filter-sterilized Instant Ocean (FSIO) for 3 h, and were then washed three times in FSIO to remove any non-attached bacteria. The juvenile squid were then either placed into FSIO containing a 1:500 dilution of the α-EsGal1 antibody or FSIO containing an equal concentration of purified rabbit IgG (Genscript) and then animal luminescence was measured and light organ bacterial density was determined by dilution plating of light organ homogenates on LBS agar at 6 and 8 h

post-bacterial exposure. For experiments in which EsGal1R3 was added during colonization, the above conditions were used in the absence of the antibody with the addition of 34.4 μM EsGal1R3. This concentration was chosen based on the activity of the peptide in *in vitro* analyses.

Statistics

All qRT-PCR data were log transformed to provide a normally distributed data set and then analysed in R [version 2.12.1; R Foundation for Statistical Computing, Vienna, Austria (http://R=project.org)] by one-way ANOVA followed by a Tukey's pairwise comparison. Shapiro-Wilk and Levene tests were used to ensure the normal distribution and homoscedasticity of the residuals respectively.

Nucleotide accession numbers

EsGal1 has been submitted to Genbank with the submission ID 1681541.

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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. Expression of *esgal1* and *esgal2* in symbiotic organs of adult squid. Expression, as measured by qRT-PCR, of *esgal1* (A) and *esgal2* (B) in the bacteria-containing epithelia of the adult light organ, or central core (CC) and the female-specific accessory nidamental gland (ANG). Data are normalized to the condition of lowest expression. Values ± SEM, and $n = 3$ biological replicates and 2 technical replicates per condition.
Fig. S2. Sequences of the Galaxin proteins used in the alignment in Fig. 1. Full-length protein sequences of the proteins (with Genbank identifying numbers in parentheses) are given, with the repeats used for the alignment highlighted in yellow.
Fig. S3. Controls for antibody staining. (A–C) Juvenile squid light organs stained with IgG as a negative control for antibody in the animals shown in Fig. 4. Shown are portions of the light organ including (A) the anterior appendage, (B) the colonized crypt spaces, and (C) the mucus outside of the light organ. No IgG staining (green) is shown, suggesting that the anti-EsGal1 staining in Figs. 4 and S4 is specific. (D), Western blot performed with the anti-EsGal1 antibody on aqueous soluble (S) and SDS-soluble, or membrane, (M) fractions. The arrow denotes a band at the predicted molecular weight. Molecular weight standards in kDa are shown at left.
Fig. S4. Localization of EsGal1 in whole juvenile squid. Top, diagram of a juvenile *E. scolopes*, showing the tissues examined in the remainder of the figure. Multiple tissues of 24 h *E. scolopes* that were examined for production of the EsGal1 protein by confocal immunocytochemistry, including the tentacles, the gills, and the main haematopoietic organ known as the white body. Day-old juvenile *E. scolopes* were exposed to the anti-EsGal1 antibody (green), and counterstained with phalloidin (red, actin cytoskeleton) and TOTO-3 (blue, nuclei). The anti-EsGal1 antibody stained the nuclei and apical portions of epithelial cells most brightly (white arrowheads). Aa, anterior appendage; d, ducts; ge, gill epithelium; pa, posterior appendage; te, tentacle epithelium; tm, tentacle musculature; wbe, white body epithelium.
Fig. S5. Dose-response of *V.* fischeri growth to exposure to EsGal1R3. Growth curve of *V. fischeri* cells exposed to 17.4 μM EsGal1R3 (orange line), 8.2 μM EsGal1R3 (red line), 4.1 μM EsGal1R3 (pink line), or to no peptide (black line). The experiment was performed with 3 biological replicates (except for the 17.4 μM condition, which had 2) and 2 technical replicates.
Table S1. Primers used in this study.
Table S2. Galaxin sequences and their relevant features, including predicted repeat structure and antimicrobial activity.
Appendix S1. Supplementary experimental procedures.