Symbiosis
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A peptidoglycan-recognition protein orchestrates the first steps of symbiont recruitment in the squid-vibrio symbiosis

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Abstract
In symbioses established through horizontal transmission, evolution has selected for mechanisms that promote the recruitment of symbionts from the environment. Using the binary association between the Hawaiian bobtail squid, *Euprymna scolopes*, and its symbiont, *Vibrio fischeri*, we explored the first step of symbiont enrichment around sites where *V. fischeri* cells will enter host tissues. Earlier studies of the system had shown that, within minutes of hatching in natural seawater, ciliated epithelia of the nascent symbiotic tissue secrete a layer of mucus in response to exposure to the cell-wall biomolecule peptidoglycan (PGN) from non-specific bacterioplankton. We hypothesized that a peptidoglycan recognition protein, EsPGRP4, is the receptor that mediates host mucus secretion by sensing the environmental PGN; earlier studies of this protein family had shown that this is the only member predicted to behave as a membrane receptor. Immunocytochemistry localized EsPGRP4 to the superficial ciliated fields of the juvenile organ. We found that production of EsPGRP4 increased over the first 48 h after hatching if the light organ remained uncolonized. When colonized by *V. fischeri*, the levels of the protein in light-organ tissue remained similar to that of hatchling organs. Pharmacologically curing the initially colonized light organ with antibiotics resulted in return of EsPGRP4 production to levels similar to light organs that had remained uncolonized since hatching. Furthermore, we found that preincubation of the tissues with an EsPGRP4 antibody decreased light organ mucus production and colonization. These findings provide evidence of an innate mechanism that underlies a crucial first step in the horizontal recruitment of bacterial symbionts.

Keywords *Vibrio fischeri* · *Euprymna scolopes* · Horizontal transmission · Peptidoglycan

1 Introduction

In systems where a host animal engages its bacterial symbiont anew each generation, the host must actively employ strategies for detecting, recruiting, and fostering environmental symbionts. A system that has motivated studies of these strategies is the binary mutualistic partnership between *Euprymna scolopes* and *Vibrio fischeri* (for reviews see Nyholm and McFall-Ngai 2021; Visick et al. 2021). This association, the 'squid-vibrio' symbiosis, is established by a cascade of events that takes place at the site of recruitment, the symbiotic light organ (Fig. 1). On either side of the nascent light organ is a ciliated epithelial field with two pairs of arm-like appendages. These pairs consist of one longer anterior appendage and a shorter posterior appendage that extend outward and appose to form a ring. Situated at the base of each appendage pair are three pores, each leading to a separate crypt. These crypt spaces house the symbionts following colonization of the organ.

The epithelial surfaces associated with the appendage pairs and surrounding the pores are coated in dense ciliated fields. Studies of the fluid environment at this interface have revealed two distinct ciliary arrays on these superficial fields, one of longer cilia that entrain bacteria in the mantle cavity of the squid and another set of shorter cilia that mix the local fluid environment near the pores (Nawroth et al. 2017). The ciliary activity of these arrays works simultaneously with mucus secretion to focus bacteria-sized particles within the shielded region of the appendages above the pores (Nyholm et al. 2000; Altura et al. 2013; Nawroth

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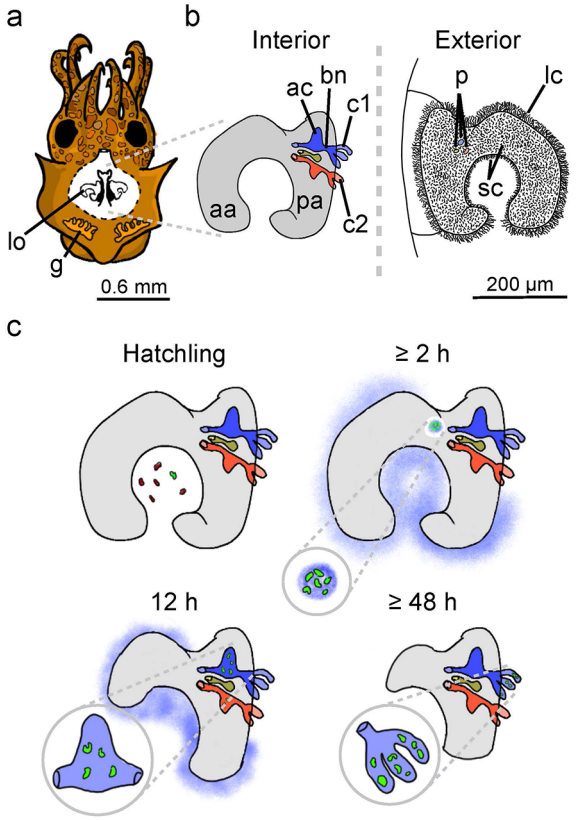

Fig. 1 Light organ system of a hatchling bobtail squid. (a) Ventral view revealing the light organ (lo) and gills (g) in the mantle cavity. (b) Ventral view of the light organ surface showing internal (shaded) and external structures necessary for symbiotic recruitment and colonization. The light organ has two pairs of appendages each consisting of one anterior (aa) and one posterior (pa) appendage. At the base of these structures are three pores (p) leading to a cavity called the antechamber (ac). Each antechamber leads to a crypt (c) connected by the bottleneck (bn). Currents coordinated by the long cilia (lc) and short cilia (sc) suspend the symbionts above the pores in the first steps of recruitment. (c) A timeline of events within the first 48 h of hatching. When exposed to peptidoglycan, the light organ epithelia begin to shed mucus. This mucus forms an aggregate of bacteria suspended above the pores by ciliary motion. *V. fischeri* is enriched in this aggregate and migrates through the migration path of the light organ. Within 12 h, the anterior appendage of the light organ undergoes visible apoptosis. After 48 h, mucus shedding ceases.

et al. 2017). Over several hours in this matrix, *V. fischeri* is enriched, and eventually chemotaxes into the light organ following a host-generated chitobiose gradient (Nyholm and McFall-Ngai 2003; Kremer et al. 2013). The remarkable stepwise selection performed by the light organ tissues results in host colonization solely by *V. fischeri*. In the days following colonization, the ciliated appendages undergo a symbiont-induced regression, reflecting the end of the symbiont-harvesting stage (Fig. 1c) (Montgomery and McFall-Ngai 1994).

The release of host-derived signals, the subsequent morphogenesis following colonization, and alterations of gene expression in the light organ are principally mediated by the interactions of bacteria-specific products called microbe-associated molecular patterns (MAMPs) and the pattern recognition receptors (PRRs) of the host innate immune system (for reviews, see Nyholm and McFall-Ngai et al. 2021; Visick et al. 2021). Peptidoglycan and its tetrapeptide monomer, tracheal cytotoxin (TCT), have been implicated as important MAMPs in host symbiont-induced development (Koropatnick et al. 2004; Troll et al. 2009, 2010).

Non-specific peptidoglycan (PGN) from environmental bacterioplankton drives the shedding of mucus from the ciliated epithelia of the light organ (Nyholm et al. 2002). This initial release of host mucus, within minutes of hatching, is the first step in symbiont recruitment. After 48 h of colonization of the crypt, mucus shedding ends. However, if the light organ remains uncolonized, mucus secretion continues, and the regression of the ciliated field does not occur. Further, if the colonized light organ is treated with an antibiotic, effectively removing the population of symbionts, the mucus shedding response begins anew (Nyholm et al. 2000).

We hypothesized that the molecular response to PGN exposure, and the resulting mucus shedding, is mediated by a PRR in the family of animal proteins, the peptidoglycan recognition proteins (PGRPs; Royet et al. 2011). Five PGRPs have been identified in *E. scolopes* (EsPGRP1-5; Goodson et al. 2005; Collins et al. 2012). Four of these proteins, EsPGRP1-3 and 5 are cytosolic and/or secreted and have an amino acid essential for a putative N-acetylmuramoyl-l-alanine amidase activity, which detoxifies PGN derivatives (Troll et al. 2010). EsPGRP4 lacks this residue and has two predicted transmembrane domains (Goodson et al. 2005). These characteristics are typical of other PGRPs that function as signal transduction receptors (Werner et al. 2000; Choe et al. 2002). These features provided a rationale to investigate EsPGRP4 as the receptor associated with PGN-induced mucus shedding shortly after the host's hatching.

In this study, using confocal microscopy and experimental manipulation of the symbiosis, we characterized the temporal and spatial expression of EsPGRP4 protein in the juvenile host. We observed the timing of EsPGRP4 production and defined the correlation of its production with the mucus shedding response over the trajectory of early host development. We also found that exposure to an anti-EsPGRP4 antibody attenuated normal mucus production and colonization rates. Together, we present evidence that EsPGRP4 mediates the first signal transduction event involved in the recruitment of the specific symbiont from the environment.

2 Materials and methods

2.1 Study system

Adult *E. scolopes* were collected on the shallow sand flats of O'ahu, Hawai'i and maintained in a flow-through seawater system at the Kewalo Marine Laboratory, University of Hawai'i at Mānoa. After mating, egg clutches from collected adult females were transferred to a second flow-through system on a 12-h day-night cycle. Squids were either sampled within 3 h of hatching, representing the "hatchling" time point, or exposed to a 16–18 h inoculum of the wild-type strain *V. fischeri* ES114 (SYM) or kept 'aposymbiotic' (APO), i.e., in unfiltered seawater (USW) containing no natural population of *V. fischeri*. In experiments where the light organ was treated with chloramphenicol (Cm), inoculations of ES114 + PVSV105 (Cmr; a strain carrying a plasmid that confers Cm resistance) were also used as a control for the effects of the antibiotic. Bacterial cultures were grown in a seawater tryptone medium overnight at 25 °C to mid-logarithmic phase and diluted to a concentration of 1,000 to 3,000 CFU ml^{-1} (Boettcher and Ruby 1990). Concentrations were confirmed spectrophotometrically by measuring optical density at 600 nm and by counting CFUs on an LBS medium grown for 24 h. Following the addition of an inoculum, the presence or absence of colonization was monitored using a TD20/20 luminometer (Turner Designs, Sunnyvale, CA), which provides a relative reading of animal luminescence. For juveniles raised for 24 and 48 h, individuals were placed in scintillation vials and 70–80% of the water in each vial was changed every 18–24 h. In experiments where the light organ was treated with chloramphenicol at 24 h post-hatching, seawater with 20 µg ml^{-1} of chloramphenicol was used instead. All experiments were replicated within a clutch using 5–18 juveniles per treatment. Each experiment was also replicated using three different clutches to account for inter-clutch genetic diversity.

2.2 Immunocytochemistry and western blotting

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and fluorescent dyes from Thermo Fischer Scientific (Waltham, MA) unless stated otherwise. Squids were fixed either immediately following hatching (hatchlings) or 24- or 48 h post-hatching in 4% paraformaldehyde in marine phosphate-buffered saline (mPBS; 50 mM sodium phosphate buffer, 450 mM NaCl, pH 7.4) for 24 h and stored in mPBS at 4 °C prior to tissue dissection. The following steps were carried out while shaking at 4 °C. Dissected light organs, or whole-body juveniles with exposed mantle cavities, were permeabilized with 1% Triton X-100 in mPBS (mPBST) for 48 h. Samples were then blocked for 24 h using a solution of 1% normal goat serum and 0.5% bovine serum albumin (BSA) in mPBST. Light organ samples were then exposed to a dilution of anti-EsPGRP4 antibody in block solution for 24 h or 7 d for experiments using a 1:50 or 1:500 dilution of the antibody, respectively. The rabbit polyclonal antibody used to image EsPGRP4 was generated from synthetic peptides from the epitope, SWDHYLVRRNCHIEPGMYK, conjugated to ovalbumin (Harlan Biosciences). This peptide sequence was chosen because it is unique to the EsPGRP4 sequence; i.e., the squid has five EsPGRPs, four in the soluble fraction; the EsPGRP4 antibody is the only antibody to these proteins that recognizes a protein in the membrane. Preimmune serum was used as a negative control for non-specific labeling and was used in place of primary antibody. Following 1° antibody incubation or incubation in preimmune serum, samples were washed three times for 15 min with mPBST and blocked again overnight. Samples were then exposed to a 1:25 goat anti-rabbit 2° antibody conjugated to fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch, Westgrove, PA) for 24 h. Excess 2° antibody was washed with four 30 min washes in mPBST. To label cilia, we used a monoclonal anti-acetylated α tubulin antibody produced in mouse (1 mg ml^{-1} stock; 1:500 dilution in block solution) followed by a 1:25 goat anti-mouse 2° antibody conjugated to tetramethylrhodamine isothiocyanate (TRITC). Nuclei and F-actin were labeled using a 48-h incubation of TOPRO-3 (1:1000 dilution) and rhodamine phalloidin (1:40 dilution), respectively. The samples were washed with mPBST for 15 min three times followed by a final 15-min wash in mPBS. Samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA) prior to imaging and were visualized using a Zeiss LSM 710 confocal microscope (Carl Zeiss AG, Jena, Germany) at the Kewalo Marine Laboratory. Confocal laser intensities were set to the preimmune negative control for each replicate.

To confirm the specificity of the antibody, we performed a western blot using the EsPGRP4 antibody at a 1:1000 dilution. We first extracted the soluble protein fraction from whole-body hatchling juveniles by homogenizing 5 individuals in 100 µL of homogenization buffer (phosphate buffered saline (PBS) and proteinase inhibitor cocktail) (Halt 100X Protease Inhibitor Single-Use Cocktail, Thermo Fischer Scientific, Waltham, MA). The lysate was transferred to a 2 mL tube and centrifuged for 10 min at 800 x g and 4 °C to spin down the intact solid material. After the supernatant of the fully homogenized tissue was transferred to a new tube, it was centrifuged for 15 min at 21,000 x g and 4 °C. The supernatant collected following the second spin produced the juvenile squid soluble fraction. The pellet was resuspended and spun down six times with the homogenization buffer to wash the pellet and remove residual soluble protein. To obtain the membrane fraction, the cleaned pellet

was subjected to 2% SDS in the homogenization buffer for 30 min at room temperature. The lysate was spun down again, and the supernatant collected produced the whole-body insoluble fraction. Thirty µg from each of the juvenile squid lysates were run on a 12.5% SDS-polyacrylamide gel and transferred onto immunoblot PVDF membrane (Bio-Rad, CA) using the Bio-Rad mini trans blot cell (Bio-Rad, CA). Instead of being transferred, the companion gel was stained with coomassie blue and imaged to indicate the protein lysate fractions loaded into the gel. The blots were blocked overnight at 4 °C with 0.5% BSA and 1% normal goat serum in Tris-buffered saline (TBS). Primary antibody and the associated preimmune serum were added at a 1:1000 dilution to the block solution and incubated overnight at 4 °C with gentle agitation. The following morning, the blots were washed with TBS with 0.1% Tween-20 before the goat anti-rabbit IgG secondary antibody (IRDye 800CW, Li-Cor Biosciences, NE) was added at a 1:10,000 dilution in block solution. The secondary antibody was incubated for 1 h at room temperature before the blots were imaged using a Licor Odyssey CLx imaging system (Li-Cor Biosciences, NE).

2.3 In vivo EsPGRP4 inhibition

To determine the effects of EsPGRP4 inhibition on light organ mucus secretion, *E. scolopes* hatchlings were transferred to artificial seawater (Instant Ocean®; 35 ppt) immediately after hatching to prevent uncontrolled exposure to environmental PGN. These samples were then pre-incubated for 1 h in a 1:100 dilution of the anti-EsPGRP4 antibody in artificial seawater. Squids were pre-incubated in identical dilutions of the preimmune serum as a negative control to determine if other components, besides the anti-EsPGRP4 antibody, inhibited the mucus shedding response. The animals were then inoculated for 2 h with either lysozyme-treated PGN isolated from *S. aureus* (InvivoGen, CA) at 200 µg ml^{-1} or live GFP-labeled *V. fischeri* ES114 grown in a seawater tryptone medium at 3,000 CFU ml^{-1}. To visualize mucus secretion, animals were exposed to fluorescently labeled wheat germ agglutinin (WGA) (Alexa Fluor 633; Molecular Probes, OR), which binds sialic acid residues in the mucus. Squids were incubated in 10 µg ml^{-1} WGA diluted in filter-sterilized seawater (FSSW) for 30 min, rinsed once in FSSW for 5 min, and anesthetized in 2% ethanol in FSSW for 2 min. Animals were then dissected and imaged by confocal microscopy.

To determine whether inhibition of EsPGRP4 resulted in decreased rates of colonization by *V. fischeri*, juveniles were exposed to a 1:100 dilution of the anti-EsPGRP4 antibody in artificial seawater for 2 h immediately after hatching. Squids were pre-incubated in identical dilutions of the preimmune serum as a negative control or an anti-rabbit EsPGRP1 polyclonal antibody in serum to control for added nutrients. Squids were then inoculated with a culture of *V. fischeri* ES114 grown in a seawater tryptone medium and diluted to 4,000 CFU ml^{-1} for 3 h. Squids were then transferred through three 5 min rinses of artificial seawater to remove particles not attached to the mucus aggregate. Squids were then raised for 48 h in artificial seawater, with water changes and luminescence readings every 24 h. Each squid was then anesthetized on ice and homogenized to be plated on an LBS medium and grown for 24 h to estimate the CFUs present in the light organ.

2.4 Image analyses and statistical methods

Image analysis was performed using Fiji (ImageJ) (Schindelin et al. 2012). The integrated intensity was used as a proxy for protein expression in each region of interest. For comparisons of relative fluorescence along the inner and outer superficial cell surface, these regions were designated by selecting the 5 epithelial cells closest to a bisect of the anterior appendage. Similarly, to quantify the degree of mucus secretion from live animals, the integrated intensity of the Alexa Fluor-WGA conjugate in the region of the anterior appendage, including aggregates of mucus formed by ciliary activity, was measured.

All graphing and statistical analyses were performed using R (v4.0.2; R Core Team 2020) and RStudio (v1.3.959; RStudio Team 2020). Data were imported using the readxl package (v1.3.1; Wickham and Bryan 2022) and were organized using the dplyr package (v1.0.8; Wickham et al. 2022). All experiments were replicated with three independent clutches laid by different females and the mean of each arm on the light organ was computed to treat each light organ as a biological replicate. Data normality and homoscedasticity were assessed using the car package's Shapiro-Wilk tests (v3.0.13; Fox and Weisberg 2019) and base R's Levene's test respectively. Log transformations were performed to normalize data if necessary. For data that showed normal distributions and equal variance, an analysis of variance (ANOVA), followed by Tukey's pairwise comparison, both performed using the car package, were used to compare the mean values across treatments for each experiment. In the experiment assessing colonization rates following antibody exposure, squid with relative luminescence readings greater than 7 light units were considered colonized. The luminescence-colonization data were fitted to a generalized linear model using a binomial distribution "family". Firth's logistic regression was applied by setting the "method" to brglmFit using the brglm2 package (v0.8.2; Kosmidis 2021) and was added to account for the negative control having no colonized squid. Post-hoc analysis of the model was done

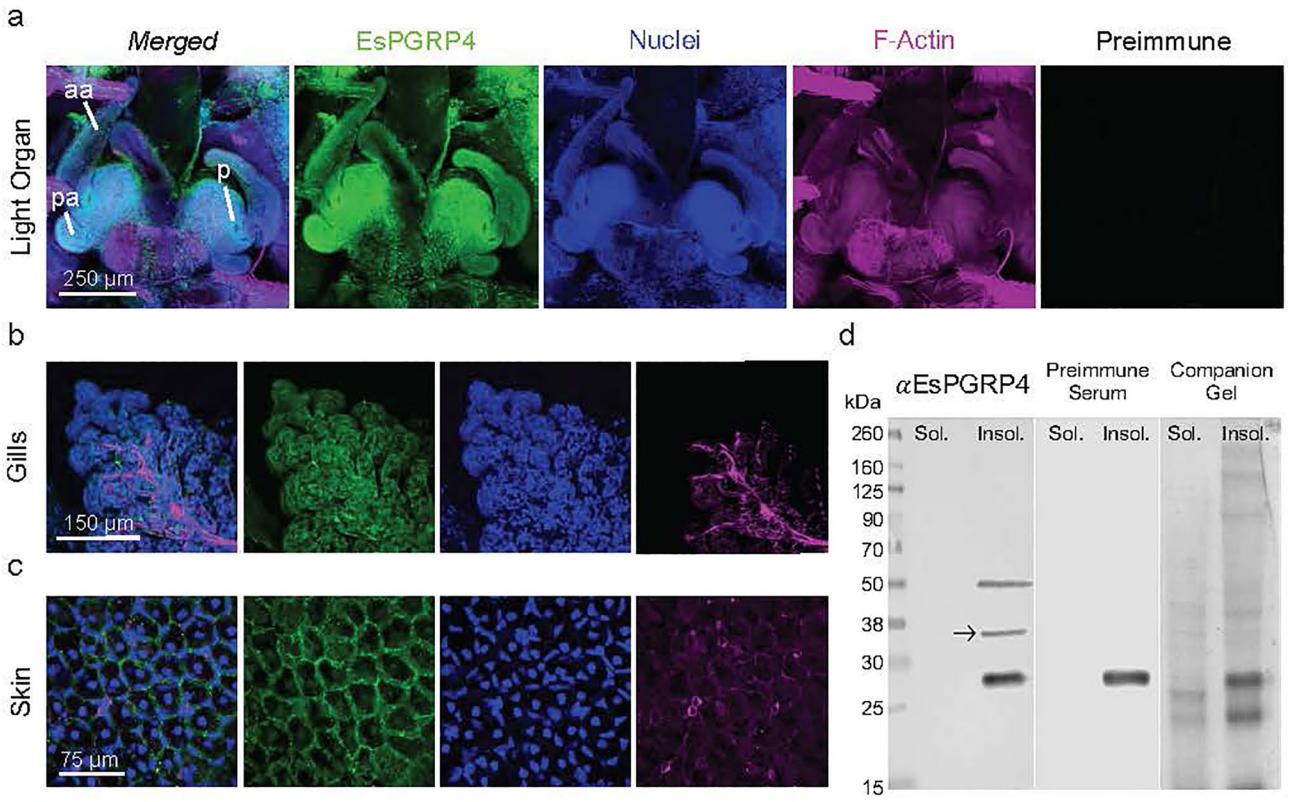

Fig. 2 Immunocytochemistry (ICC) and western-blot analyses at host hatching, i.e., within 3 h of emergence from the egg. (a) Confocal micrographs of immunocytochemistry reveal EsPGRP4 production on the surface of the light organ. Strong labeling is seen on the epithelial surface of the anterior (aa) and posterior (pa) appendages and around the pores (p). Confocal lasers were set relative to tissue incubated in preimmune serum (negative control). (b) The anti-EsPGRP4 antibody also labels the nuclei and epithelial surfaces of the gills. (c) Cross reactivity is strong on the cell junctions of the skin of the mantle. (d) Western blotting indicates cross reactivity of the EsPGRP4 antibody with whole-body lysate; right, coomassie-stained, companion gel (30 µg protein for all lanes).

using the multcomp package (v1.4.17; Hothorn et al. 2008) and applying Tukey all-pair comparisons. The multcomp package and the multcomview package (v0.1.8; Graves et al. 2019) were used to provide letters representing significant pairwise comparisons. The alpha level was set to 0.05 for all statistical analyses. Graphs were produced using the ggplot2 library (v3.3.6; Wickham 2016), ggpubr (v0.4.0; Kassambara 2020), ggtext (v0.1.1; Wilke 2022), and the extrafont library (v0.18; Chang 2022).

3 Results

3.1 Localization of the PRR EsPGRP4

Confocal immunocytochemistry of hatchling animals not only localized EsPGRP4 to the epithelial cells of the ciliated light-organ surface, but also to other tissues, including the gills and the skin of the mantle (Fig. 2), demonstrating that it is not a light-organ specific PRR. Labeling occurred in the apical cytoplasmic regions of cells and along the cell junctions of these tissues. The labeling within epithelial cells differed among the organs; stronger labeling occurred within the cytoplasm and nucleus of the light organ and gills than in those regions of the skin. Specifically, nuclei of light-organ epithelial cells that surround the pores and those of the medial portions of the ciliated fields stained avidly (Fig. 2a), whereas nuclei of the gills labeled less strongly (Fig. 2b), and no labeling in the nuclei of the skin cells was detectable (Fig. 2c). At higher magnification (Fig. 3), antibody cross reactivity was detected along portions of the migratory path, most notably at the pores (Fig. 3b) and at the entrances to the crypt spaces just beyond the bottleneck (Fig. 3c). Colocalization of the anti-EsPGRP4 antibody with anti-acetylated α tubulin antibody, which labels cilia, confirmed the localization of EsPGRP4 to the ciliary arrays of the migration path. These data provide evidence that EsPGRP4 is not only involved in the host's response to environmental PGN from bacterioplankton, but also in responses to PGN shed from *V. fischeri* entering the crypt spaces.

Western blotting with the whole-body juvenile lysate confirmed cross reactivity of the EsPGRP4 polyclonal

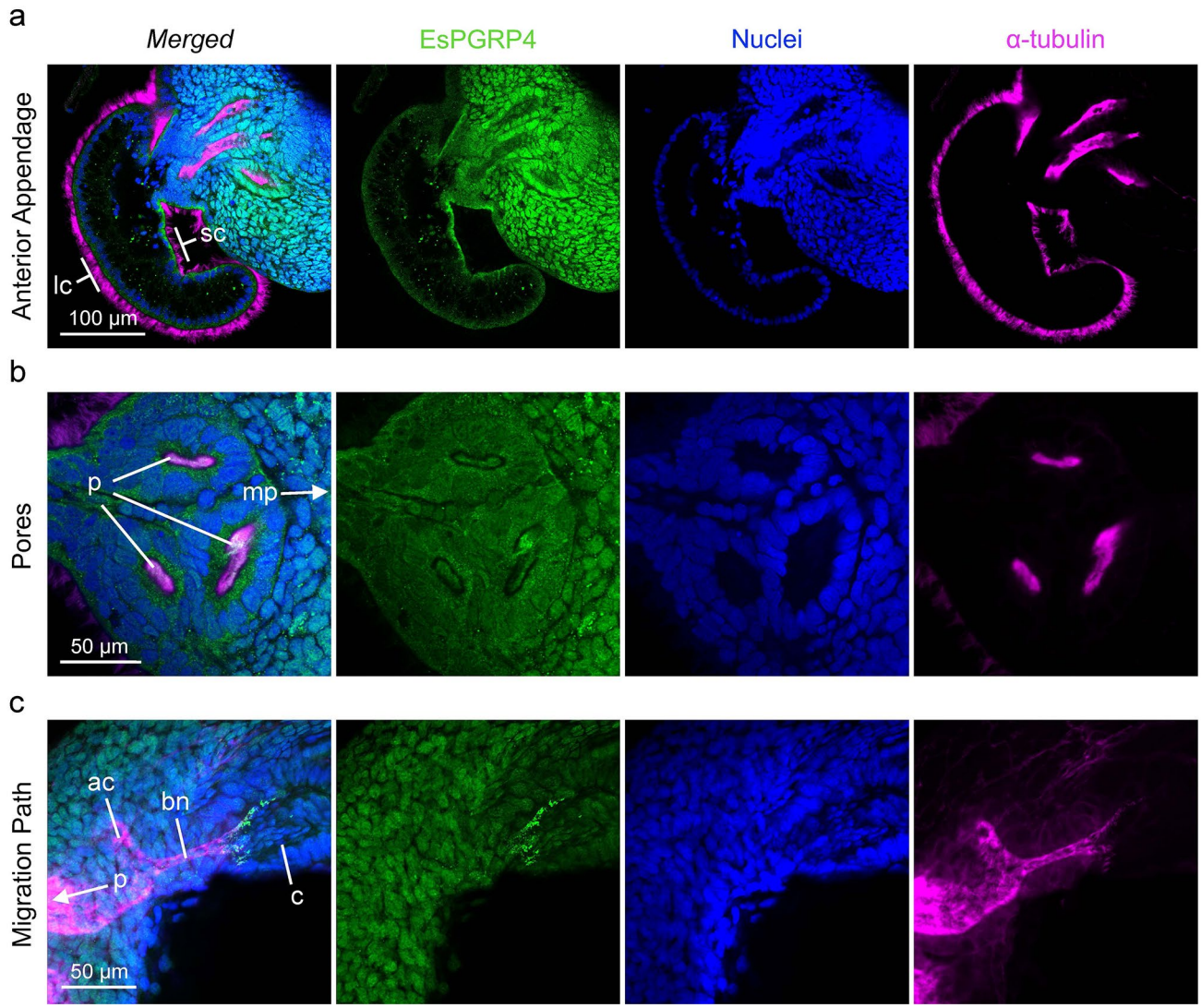

Fig. 3 Confocal micrographs showing localization of EsPGRP4 on the features of the hatchling light organ, i.e., within 3 h of emergence from the egg. (a) Labeling occurs along the epithelial surface of the anterior appendage and ciliated field. (b) Tissue surrounding the pores and cilia at the entrance to the pores are also labeled. (c) In the migration path, EsPGRP4 localizes to the entrance to the crypt spaces. Labeling of EsPGRP4 is weaker in the antechamber. ac, antechamber; bn, bottleneck; c, crypt; lc, long cilia; mp, migration path; p, pore; sc, short cilia.

antibody (Fig. 2d). As expected, the transmembrane EsPGRP4 protein did not cross react with the soluble protein fraction of the lysate. Against the complex mixture of proteins presented to the antibody, the cross reactivity was relatively specific. Protein bands of three different sizes (~27 kDa, ~35 kDa, ~50 kDa) from the insoluble membrane fraction cross reacted with the EsPGRP4 antibody. The ~35 kDa band is similar to the predicted molecular mass of 31.1 kDa. The ~27 kDa protein cross reacted with the preimmune serum as well, suggesting an innate predisposition to this protein in the rabbit itself and supporting the use of preimmune serum as a control for future experiments. The identity of the ~50 kDa subunit was not determined, but it is interesting to note that the antibody to *Drosophila melanogaster* membrane-associated PGRP, PGRP-LC, also shows similar cross reactivity in two bands, at 30–35 and at 50 kDa (Choe et al. 2005).

As the activity of the ciliated appendages is critical for mucus shedding and symbiont aggregation, we focused upon determining the patterns of EsPGRP4 labeling in the larger anterior appendage (Fig. 3a). The antibody labeled the apical cytoplasm of all cells; however, unlike the other cells of the ciliated fields, the appendage cell nuclei did not have detectable antibody cross reactivity. The intensity of cytoplasmic labeling in the inner and outer portions of the appendage differed (Fig. 4a). The relative fluorescence of the epithelial cells lining the inner appendage surface was ~50% greater than those along the outer tissue surface,

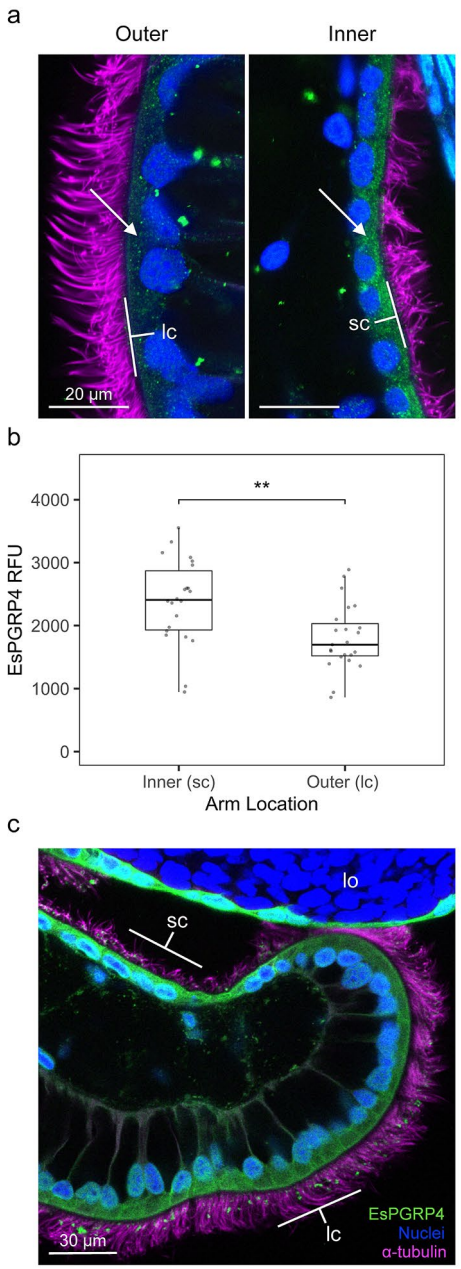

Fig. 4 Confocal microscopy capturing variation in EsPGRP4 labeling between cells of the long and short ciliary arrays in hatchlings. (a) Confocal micrographs of the inner and outer anterior appendage surface in hatchling light organs. (b) Plot of relative fluorescence of EsPGRP4-labeled cells lining the inner and outer surfaces of the anterior appendage (n = 22 light organs per location). (c) Confocal micrograph showing labeling of the anterior appendage of a 24-h APO light organ (lo) at a 1:50 dilution of the primary antibody, illustrating both the strong difference between labeling of the epithelial cells supporting the region of short cilia (sc) compared with those supporting the long cilia (lc). **, $p < 0.01$

reflecting significantly heightened stores of EsPGRP4 in the epithelia with shorter cilia (Fig. 4b; $F_{(1, 43)} = 10.51$, $p = 0.002$, n = 22 light organs per location). Lower dilution of the antibody revealed cross-reactive sites along the cilia of 24-h APO light organs (Fig. 4c). Labeling of the cilia was not detected in hatchlings (Fig. 5a). These data suggest that the protein is made and stored in the apical surfaces of the appendage-cell epithelia and then transported out to the cilia where environmental PGN might be detected. The labeling of the cilia showed no reproducible difference in the inner and outer faces of the appendages.

3.2 The influence of colonization on the patterns of EsPGRP4 in light organ tissues

To investigate the regulation of EsPGRP4 in response to successful colonization, host light organs were imaged at hatching, and at 24- and 48 h post-hatching in APO and SYM animals (Fig. 5). The migration path of SYM light organs had lower cross reactivity to the EsPGRP4 antibody over the first 24 h (Fig. 5b). Similarly, post-inoculation time and symbiont presence significantly impacted the reactivity to EsPGRP4 in the anterior appendage (Fig. 5c, d; $F_{(1,144)} = 12.079$, $p < 0.001$, n = 26–40 light organs per time point). While labeling of EsPGRP4 at the anterior appendage was identical between APO and SYM over the first 24 h, at 48 h, post-hoc analysis showed that labeling of the APO light organs was significantly increased ($p < 0.05$), while that of SYM juveniles at 48 h remained constant.

The constitutive production of EsPGRP4 in SYM light organs was dependent on persistent colonization of the organ (Fig. 6; $F_{(4, 130)} = 17.007$, $p < 0.001$, n = 21–30 light organs per treatment). Chloramphenicol treatment (Cm) in the absence of symbiont colonization did not significantly influence EsPGRP4 relative fluorescence relative to APO ($p = 0.92$), demonstrating that the antibiotic itself had no influence on EsPGRP4 production. Juveniles at 48 h following inoculation with wild-type *V. fischeri* or a chloramphenicol resistant strain (Cmr) showed equivalent labeling and had reduced EsPGRP4 labeling relative to APO (both $p < 0.001$). However, when the light organ was pharmacologically cured with Cm of its established wild-type symbiont at 24-h-post inoculation, EsPGRP4 labeling increased, with antibody cross reactivity identical to that of 48-h APO juveniles ($p = 0.99$).

3.3 Evidence for environmental PGN interactions with EsPGRP4

To correlate host mucus secretion to EsPGRP4 PGN recognition, variation in the abundance of PGN- and symbiont-induced mucus release were studied following a preincubation of the animal in anti-EsPGRP4 antibody to block the putative receptor. Preincubations with the anti-EsPGRP4 antibody significantly impacted the extent of mucus

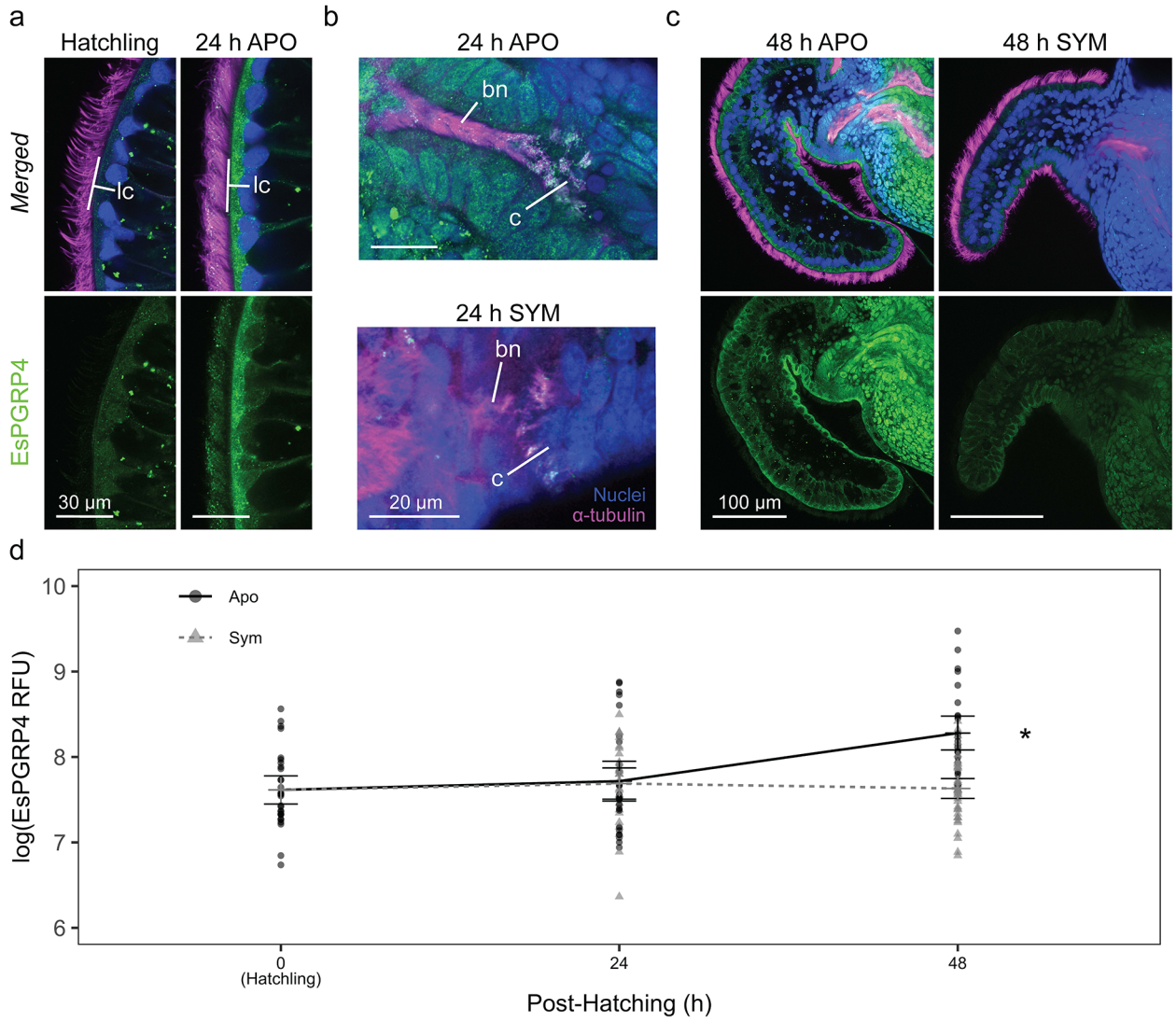

Fig. 5 Confocal microscopy capturing symbiont-induced regulation of EsPGRP4. (a) Confocal micrographs of the hatchling and 24-h APO surface of the anterior appendage supporting the long cilia (lc). After 24 h without colonization, PGRP4 begins localizing to the cilia along the appendage. (The micrograph used to demonstrate labeling in the hatchling epithelia is the same used in Fig. 4a.) (b) Micrographs of the bottleneck (bn) and crypt (c) of colonized and uncolonized juveniles. At 24 h, EsPGRP4 labeling decreases along the epithelial cell surface and the cilia of the colonized light organ. (c) Micrographs of the anterior appendage of colonized and uncolonized light organs. At 48 h, EsPGRP4 labeling is stronger in appendages from uncolonized juveniles. (d) Plot of relative fluorescence of EsPGRP4-labeled light organs. Relative intensities indicate an upregulation of EsPGRP4 in the absence of symbionts (n = 26–40 light organs per time point). *, $p < 0.05$

secretion upon exposure to PGN or a live inoculum of *V. fischeri* (Fig. 7; $F_{(4,98)=}14.58$, $p < 0.001$ and $F_{(4,88)=}12.54$, $p < 0.001$ respectively, n = 14–25 light organs per treatment). Control squid incubated with the EsPGRP4 antibody and not exposed to bacterial products showed no statistically significant difference in mucus production from juveniles in filter-sterilized artificial seawater ($p = 0.88$). When either symbionts, or isolated PGN, were added to water, squids showed significant increases in mucus production ($p < 0.001$, $p = 0.002$, respectively). However, bacterial products did not elicit the host mucus response when squids were preincubated in the anti-EsPGRP4 antibody (cells: $p = 0.72$; PGN: $p = 0.08$). In control experiments, preincubation in preimmune serum did not have the same inhibitory effect as the antibody, as squids showed increased mucus production relative to APO (cells: $p < 0.001$; PGN: $p = 0.01$). The reduced mucus production, due to blocking of the EsPGRP4, also affected the formation of gfp-labeled bacterial aggregates (Fig. 7b). Prior to entering the light organ, *V. fischeri* successfully aggregated adjacent to the light organ pores when juveniles were pre-incubated in artificial seawater or preimmune serum. When juveniles were preincubated

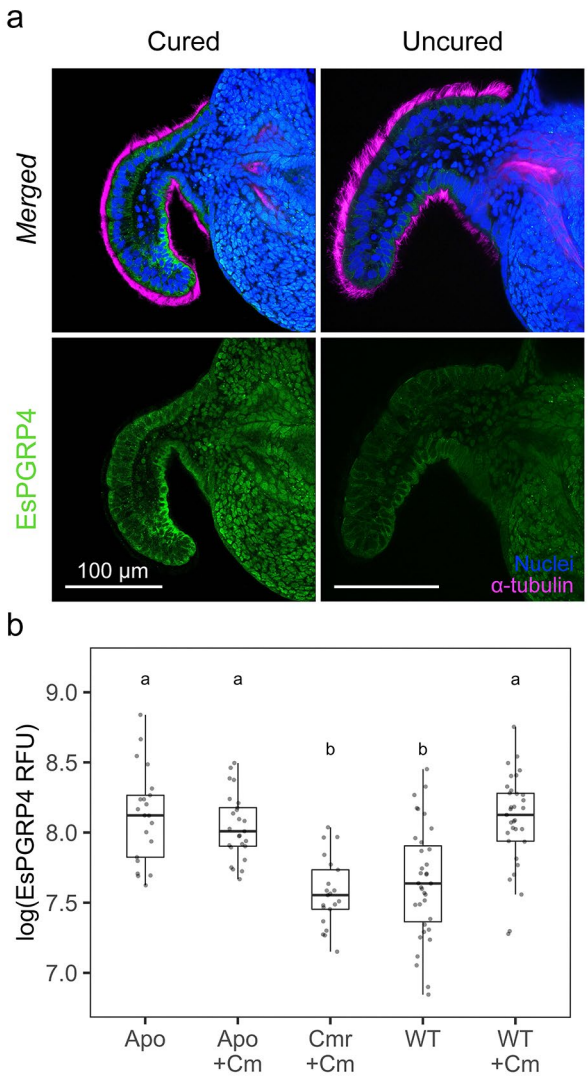

Fig. 6 Regulation of EsPGRP4 following an antibiotic treatment curing the light organ of its symbionts. (a) Micrographs of 48 h light organs with and without chloramphenicol treatment 24 h following inoculation with *V. fischeri*. (The micrograph used to demonstrate labeling in the uncured colonized light organ is the same used in Fig. 5c.) (b) Plot showing relative fluorescence of EsPGRP4-labeled light organs. Relative intensities indicate an upregulation of EsPGRP4 in response to curing the light organ of its symbiont (n = 21–30 light organs per treatment).

in EsPGRP4 antibody, no aggregates were found. These results suggest that the EsPGRP4 antigenic peptide is exposed on the host surfaces, as exposure to the polyclonal antibody alone was sufficient to block PGN recognition.

To determine the role that EsPGRP4-mediated responses may play in initiating colonization, colonization efficiency was quantified under different preincubation conditions (Fig. 8). Host colonization was significantly attenuated by preincubation treatment with the EsPGRP4 antibody ($\chi^2_{(3)} = 31.92$, $p < 0.001$, n = 43–48 animals per treatment), with no statistically significant difference in colonization compared to APO squid ($p = 0.43$). Significantly greater colonization was seen in control experiments, in which squids were preincubated in either preimmune serum or an anti-EsPGRP1 antibody prior to symbiont exposure ($p = 0.045$, $p = 0.02$, respectively). CFU data were in concordance with luminescence values (data not shown). EsPGRP1 is another PRR of the PGRP family active in symbiont-induced phenotypes in the juvenile light organ (Troll et al. 2009). These data suggest that the anti-EsPGRP4 blocking is specific and that EsPGRP4 plays an early functional role in the potentiation of symbiosis establishment.

4 Discussion

This contribution uses the *E. scolopes*-*V. fischeri* model association to describe the first step of bacterial-partner recruitment in a horizontally transmitted symbiosis. The data provide evidence that this initial engagement results from a receptor-ligand interaction that takes place along ciliated fields of the newly hatched host; specifically, an *E. scolopes* PRR of the peptidoglycan recognition protein family, EsPGRP4, binds a MAMP, PGN, that is shed by bacterioplankton in the host's environment. The findings further suggest that, by recognizing this conserved bacterial molecule, the host senses that it has hatched into bacteria-rich seawater, where it may begin the process of specific symbiont recruitment.

4.1 Patterns of EsPGRP4 localization

Although the labeling of the superficial ciliated epithelial cells of the juvenile light organ with an EsPGRP4 antibody was predicted by previous work on the system (Nyholm et al. 2002; Goodson et al. 2005), the patterns within and between cells had not been determined. The data presented here suggest that the juvenile animal stores abundant EsPGRP4 in the apical surfaces of epithelia. Similar patterns of localization can be seen in other PGRPs of the animal kingdom, such as the membrane-bound receptor, PGRP-LC, which occurs in the apical surfaces of the fat body epithelia of *Drosophila* (Neyen et al. 2012). The difference in labeling between tissues is intriguing. The occurrence in the cytoplasm of the light organ and gills suggests the possibility that the protein could be stored as a non-functional precursor protein.

Analyses of this localization in the anterior appendages of the juvenile *E. scolopes* light organ showed more avid antibody labeling in the apical cytoplasm of smaller cells with shorter cilia, which face the pores and beat randomly, than in the apical cytoplasm of the larger cells with longer

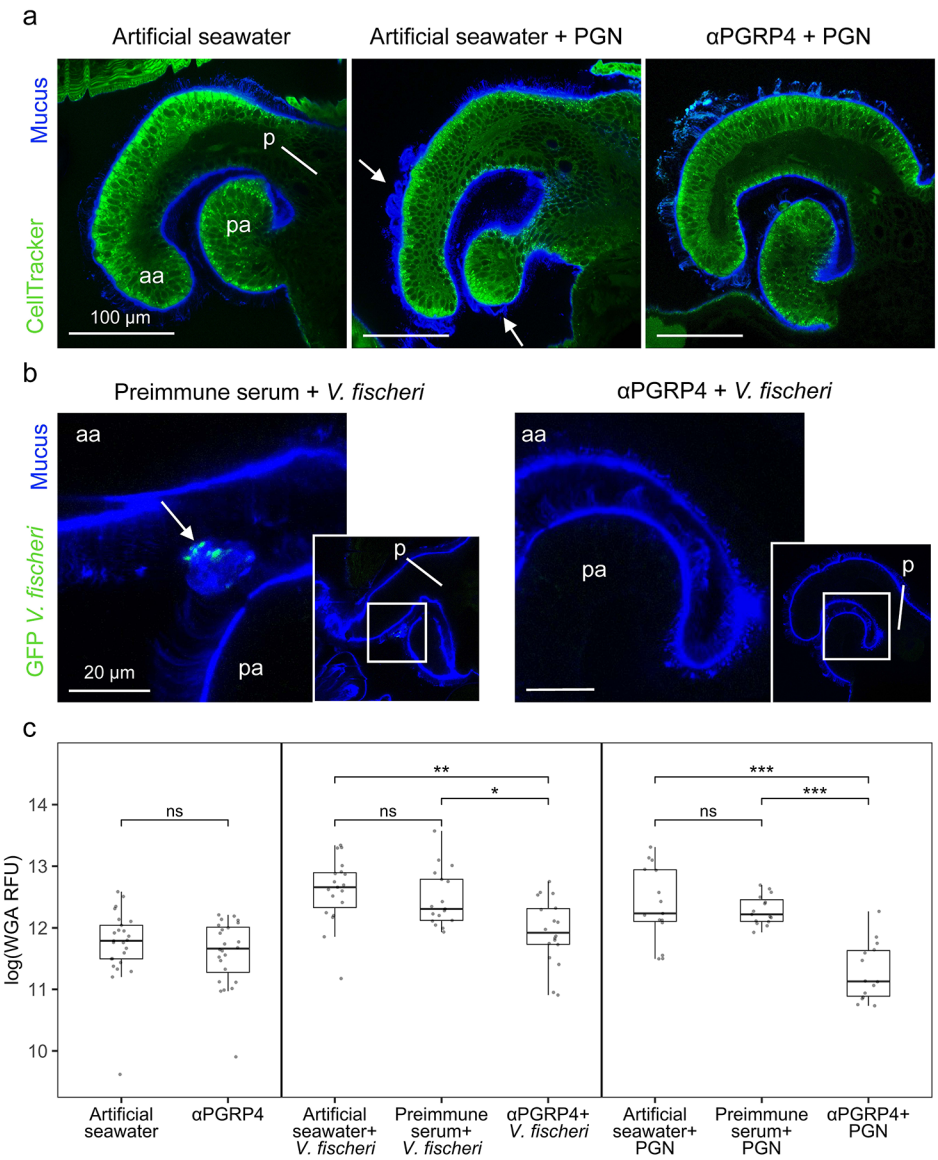

Fig. 7 Inhibition of the mucus shedding immune response caused by antibody blocking of EsPGRP4. (a) Micrographs of the light organ appendage stained with WGA. Mucus shedding in live squids is visible on the light organ epithelia within 2 h of exposure to purified peptidoglycan (left). Pre-incubating squids for 1 h in a solution of anti-EsPGPR4 antibody prior to peptidoglycan exposure inhibits the mucus shedding response (right). (b) Micrograph of an aggregate formed after pre-incubation of the squid in preimmune serum followed by inoculation. The area magnified is indicated by the white box. Preimmune alone does not inhibit the mucus secretion response. (c) Plot showing relative fluorescence of WGA stain, as a proxy of mucus secretion, in response to peptidoglycan and live symbiotic cells with pre-incubations of preimmune serum and anti-EsPGRP4 antibody. Pre-incubation with anti-EsPGRP4 antibody prior to treatment with peptidoglycan or live symbiotic cells results in decreased levels of mucus secretion. Controls without added bacterial products are not statistically different from anti-EsPGRP4 antibody treatments (n = 14–25 light organs per treatment). aa, anterior appendage; p, pores; pa, posterior appendage. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

cilia, which face away from the pores and beat metachronally (Nawroth et al. 2017). In this study, we did not resolve the basis of this pattern or determine which of these surfaces is more active in EsPGRP4 presentation. The cells of the inner face of the appendage may produce more EsPGRP4 or may store more of the protein. Protein production, turnover, and export may differ between these two populations of cells.

Labeling in the cilia along the anterior appendages was low in hatchling light organs, but blocking initial mucus shedding with the EsPGRP4 antibody suggested that sufficient receptors for environmental PGN must be present at that time. The detectable labeling of the cilia in the 24-h APO light organ is implicated in the observed increase in mucus shedding in APO animals (Nyholm et al. 2002). Motile cilia have been reported to serve not only biomechanical, but

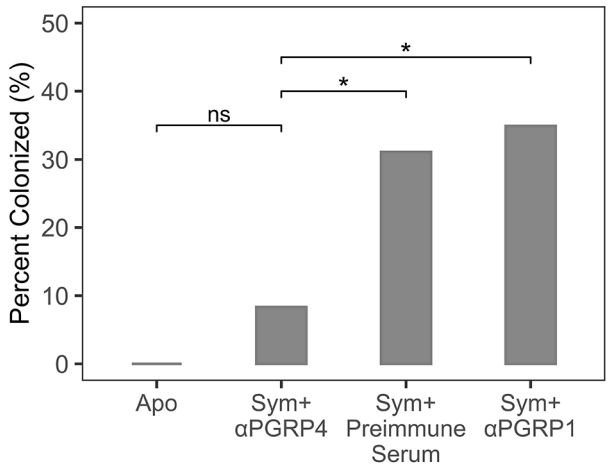

Fig. 8 Inhibition of colonization by competitive binding of PGN to EsPGRP4. Incubating the animals in anti-EsPGRP4 antibody prior to inoculation with *V. fischeri* resulted in reduced colonization rates comparable to APO animals; (n = 43–48 animals per treatment). *, $p < 0.05$

also sensory functions. This study suggests a novel sensory function in motile cilia, one that promotes the onset of post-embryonic symbiont recruitment.

The finding of EsPGRP4 inside of the crypts at the entrance beyond the bottleneck suggests PGN recognition at the crypt entrances during colonization by *V. fischeri*. Another study of the system demonstrated that the cells of the crypt entrances are ciliated in hatchling animals (Essock-Burns et al. 2020). These cells lose their cilia following colonization, taking on the microvillous-surface characteristic of the rest of the crypt epithelial cells. The timing of presence and loss of EsPGRP4 labeling of this region, being similar to the timing of cilia presence and loss, suggests a relationship between these features. While here we focused on the role of non-specific PGN from the bacterioplankton in the initial steps of symbiont recruitment, these observations of EsPGRP4 in the crypts presents an interesting direction for future studies.

The cross reactivity of EsPGRP4 antibody in the nuclei of some host cells was not entirely unexpected. The EsPGRP1 antibody localizes to the host nucleus of the appendages of the superficial ciliated field, and this signal is lost as the cells enter mid- to late-stage apoptosis at 18 to 24 h following colonization (Troll et al. 2009). In contrast, EsPGRP4 labeling was not found in the nuclei of the appendages, but on other superficial ciliated cells, as well as in cells of the migratory path. Two other studies of the squid-vibrio system suggest the possibility that fragments of PGN enter the nucleus. One describes the finding that fragments of this molecule occur in the outer membrane vesicles shed from the surface of *V. fischeri* cells in the crypt space (Aschtgen et al. 2016). These OMVs with PGN cargo are taken up by the host cells. Further, analyses by nanoSIMS (nano secondary-ion mass spectrometry) of ^{15}N-labeled *V. fischeri* cells, or OMVs derived from these cells, demonstrated that the principal ^{15}N signal occurs in the nucleus, with the strongest labeling in the nucleolus and the euchromatin (Cohen et al. 2020). The fate of OMV-trafficked PGN molecules within the host cells remains to be determined, but the presence of the receptor EsPGRP4 in host nuclei may signal recognition of that molecule in this subcellular region. Taken together, the presence in apical surfaces, both cytoplasmic and along cilia, and in the nuclei suggest two different functions for EsPGRP4.

4.2 EsPGRP4 with early development

The presence of EsPGRP4 in the epithelia of hatchlings suggests that evolution has selected for its production during embryonic development to ready the host to respond to environmental PGN immediately upon hatching. Our data provide evidence that the mucus shedding results from PGN binding to the EsPGRP4 receptor at this time. While PGN derivatives have been reported to induce mucus shedding from epithelia in mammals (Petersson et al. 2011; Kessie et al. 2021), to our knowledge, PGRPs have not been previously implicated as receptors for PGN ligands to mediate mucus shedding. In our experiments with manipulation of host colonization, our data show that these molecular interactions are essential for efficient colonization of the host. Once the host is colonized, EsPGRP4 labeling no longer increases, but this is reversible with antibiotic-mediated clearing of symbionts from the organ. The timing and pattern of these events correlates with the studies of the behavior of mucus shedding (Nyholm et al. 2002). Overall, these findings indicate a complex coordination across the organ during juvenile development that ensures a successful and persistent symbiosis.

4.3 Conclusions

A PGRP's involvement in establishing symbiotic partnerships is not unprecedented. This study details the patterns of occurrence and the influence on symbiosis of a single EsPGRP isoform in *E. scolopes*. However, the array of PGRP functions across the animal kingdom (Harris et al. 2015, Iasenko et al., 2016, Maire et al. 2019) and the common occurrence of persistent beneficial alliances between animals and microbes suggest that PGRPs play a role in mediating horizontal recruitment in other systems. This first study on EsPGRP4 presents a horizon for future studies. Potential areas of research on EsPGRP4-PGN interactions include, for example: (i) exploring the downstream mechanisms of signal transduction to the host cells; (ii) determining how mucus shedding is triggered; and, (iii) defining its

role in the regulation and feedback loops associated with persistent colonization throughout ontogeny and over day-night cycles.

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