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Probiont niche specialization contributes to additive protection against *Vibrio owensii* in spiny lobster larvae

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Summary

The development of efficient probiotic application protocols for use in marine larviculture relies on comprehensive understanding of pathogen–probiont–host interactions. The probiont combination of *Pseudoalteromonas* sp. PP107 and *Vibrio* sp. PP05 provides additive protection against vectored *Vibrio owensii* DY05 infection in larvae (phyllosomas) of ornate spiny lobster, *Panulirus ornatus*. Here, fluorescently tagged strains were used to demonstrate niche specialization of these probionts in both the live feed vector organism *Artemia* and in phyllosomas. The pathogen was vulnerable to direct interaction with PP05 in the bacterioplankton as well as in the *Artemia* gut and the phyllosoma foregut and midgut gland. In contrast, PP107 was localized on external surfaces of *Artemia* and phyllosomas, and direct interaction with the pathogen was limited to the bacterioplankton. While PP107 was the overall dominant ectobiont on the phyllosoma cephalothorax and inner leg segments, PP05 was the primary colonizer of outer leg segments, nutrient-rich locales that may promote ingestion during feeding. This study shows that niche specialization can contribute to the additive probiotic effect of a probiotic mixture and highlights that probiotic enrichment of *Artemia* cultures can intercept the infection cycle of *V. owensii* DY05 in early-stage *P. ornatus* phyllosomas.

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Introduction

Against a backdrop of stagnating or declining wild fishery supply of spiny lobsters (Palinuridae) (Jeffs, 2010), closed life cycle aquaculture of the economically important ornate spiny lobster (*Panulirus ornatus*) is on the verge of becoming a reality (Rogers *et al*., 2010). Nevertheless, a key challenge to refining *P. ornatus* hatchery technology is reducing the incidence of mass mortalities caused by bacterial disease (Bourne *et al*., 2004; 2007). *Vibrio owensii* DY05 is an emerging enteropathogen causing disease epizootics in the larviculture of *P. ornatus* (Cano-Gómez *et al*., 2010; Goulden *et al*., 2012a). Through the use of a green fluorescent protein (GFP)-expressing transconjugant of *V. owensii* DY05, we previously elucidated the niche preferences and infection cycle of *V. owensii* DY05 in *P. ornatus* phyllosomas, showing that soon after vectored transmission via the live feed organism *Artemia*, the pathogen invades the phyllosoma foregut (proventriculus) and midgut gland (hepatopancreas) tubules, preceding mass proliferation in the hepatopancreas lumen and ultimately systemic infection (Goulden *et al*., 2012a).

Combating epizootics and increasing hatchery production of *P. ornatus* will require the development of disease management strategies, including probiotics (Rogers *et al*., 2010). Earlier studies showed the addition of beneficial bacteria to larval-rearing systems of *Jasus edwardsii* phyllosomas led to increased survival and metamorphosis to puerulus stage (Igarashi *et al*., 1990), indicating that this approach could supersede current use of antibiotics in spiny lobster larviculture (Murakami *et al*., 2007). Recently, we showed that vectored administration of a combination of two probionts (*Vibrio* sp. PP05 and *Pseudoalteromonas* sp. PP107) by *Artemia* provides additive protection of stage 1 *P. ornatus* phyllosomas from experimental infection with *V. owensii* DY05, with survival not differing significantly from unchallenged controls (Goulden *et al*., 2012b). While each strain could individually reduce phyllosoma mortalities, their combined use provided more complete and more reproducible protection. It is clear that further understanding of probiont–pathogen–host interactions is required to develop efficient biocontrol strategies.

Probionts must function in the same ecological niche as the targeted pathogen (Verschuere *et al*., 2000a) as

differential niche specialization can render promising probionts incapable of protecting hosts against infection (Ruiz-Ponte *et al*., 1999; Gram *et al*., 2001; Spanggaard *et al*., 2001). Visualizing the niche specialization of live fluorescently labelled probionts *in situ* by non-destructive methods is an alluring possibility in transparent zooplankton forms (Goulden *et al*., 2012a). The purpose of the present study was to use FP-expressing strains to elucidate the respective niches of the two probiotic strains, *Vibrio* sp. PP05 and *Pseudoalteromonas* sp. PP107, which, when used in combination, efficiently interfere with the infection cycle of *V. owensii* DY05 in *P. ornatus* phyllosomas (Goulden *et al*., 2012b).

Results

Pathogen virulence and probiotic protection

Expression of FP had no significant effect on the virulence or probiotic properties of the bacterial transconjugant strains used in this work (Fig. 1). *Vibrio owensii* DY05[RFP] caused significant phyllosoma mortality (87%; Dunnett's test $P < 0.0001$) compared with the negative control, and did not statistically differ from the wild-type *V. owensii* DY05 (Dunnett's test $P > 0.05$). In all cases, probiotic-treated phyllosomas showed significantly enhanced survival by 79–82% over pathogen-only controls (*V. owensii* DY05 or DY05[RFP]; Dunnett's test $P < 0.0001$) and survival was not significantly different from unchallenged negative controls (Dunnett's test $P > 0.05$). Similarly, culture-based analysis showed that expression of FP did not affect the loading of bacteria in *Artemia* nauplii after enrichment (data not shown). These traits indicated that the pathogen and probiont transconjugants were suitable biomarkers for short-term studies of bacteria–vector and bacteria–phyllosoma interactions.

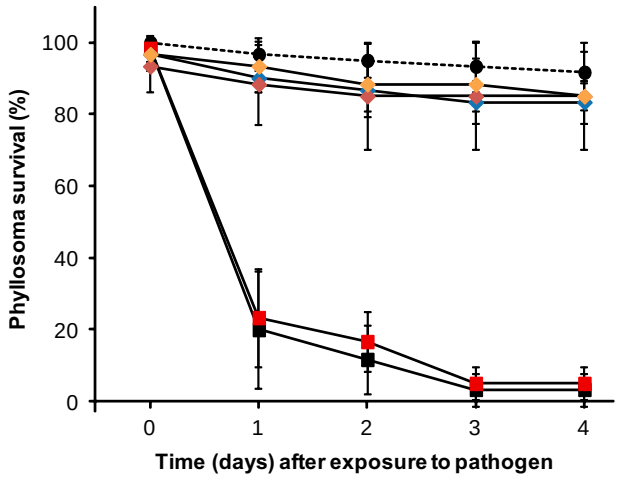

Fig. 1. Survival of *P. ornatus* phyllosomas after vectored challenge with bacteria-enriched *Artemia* nauplii. Non-enriched nauplii (negative control; ●), nauplii enriched with wild-type pathogen *V. owensii* DY05 (positive control; ■), RFP-labelled *V. owensii* DY05[RFP] (■), wild-type *V. owensii* DY05 and probionts (*Vibrio* sp. PP05 and *Pseudoalteromonas* sp. PP107) (◆), RFP-labelled *V. owensii* DY05[RFP] and GFP-labelled probionts (PP05[GFP] and PP107[GFP]) (◆), wild-type *V. owensii* DY05 and differently labelled probionts (PP05[RFP] and PP107[GFP]) (◆). Survival expressed as mean ± SD.

Pathogen–probiont–vector interactions

The localization and loading of bacterial strains in *Artemia* nauplii after enrichment with monostrain or multistrain suspensions were determined using fluorescence microscopy (Fig. 2) and spread plating of *Artemia* homogenates (Fig. S1). Repeated experiments showed that the overall bacterial loading varied between *Artemia* batches but major trends were confirmed as outlined below.

After monostrain enrichment, probiotic *Vibrio* sp. PP05[GFP or RFP] and pathogen *V. owensii* DY05[RFP] were concentrated in the gut but the degree of bioaccumulation varied between nauplii (Fig. 2a and b). In contrast, no *Pseudoalteromonas* sp. PP107[GFP] were internalized by *Artemia* nauplii following monostrain treatment, but ectobiotic attachment was observed (Fig. 2c). Monostrain enrichment resulted in a higher bacterial loading ($P < 0.005$) of the probiont *Vibrio* sp. PP05[GFP] as compared with *V. owensii* DY05[RFP] and *Pseudoalteromonas* sp. PP107 in three repeated experiments ($1.2–3.0 \times 10^3$, $1.9–4.9 \times 10^2$ and $0.9–4.0 \times 10^2$ cfu per nauplius respectively) (Fig. S1). This reflected that PP05 was loaded at a higher inoculum concentration than the pathogen (1×10^7 cfu ml^{-1} and 1×10^6 cfu ml^{-1} respectively) and was internalized by *Artemia* (in contrast to PP107).

Enrichment of *Artemia* nauplii with probionts PP05[RFP] and PP107[GFP] in combination showed that the multistrain application did not alter the preferred niche of the probionts, with *Vibrio* sp. PP05[RFP] confirmed as the dominant endobiont and no *Pseudoalteromonas* sp. PP107[GFP] cells seen transiting the *Artemia* gut (Fig. 2c). Interestingly, the presence of PP107 reduced the overall bacterial loading of *Artemia* by 49–66% compared with enrichment with PP05[GFP] alone (Fig. S1), although the trend was statistically significant ($P < 0.05$) in only two of three repeated experiments.

In *Artemia* nauplii cultures enriched with pathogen *V. owensii* DY05[RFP] in combination with both probionts (PP05[GFP] and PP107[GFP]), single pathogen cells were dispersed amidst a mass of green cells (presumably PP05[GFP]) in the nauplius gut (Fig. 2d). Additionally, very few *V. owensii* DY05[RFP] cells were visualized in the culture suspension surrounding nauplii in the three-strain enrichment. The loading of *Artemia* with the pathogen *V. owensii* DY05[RFP] was reduced during

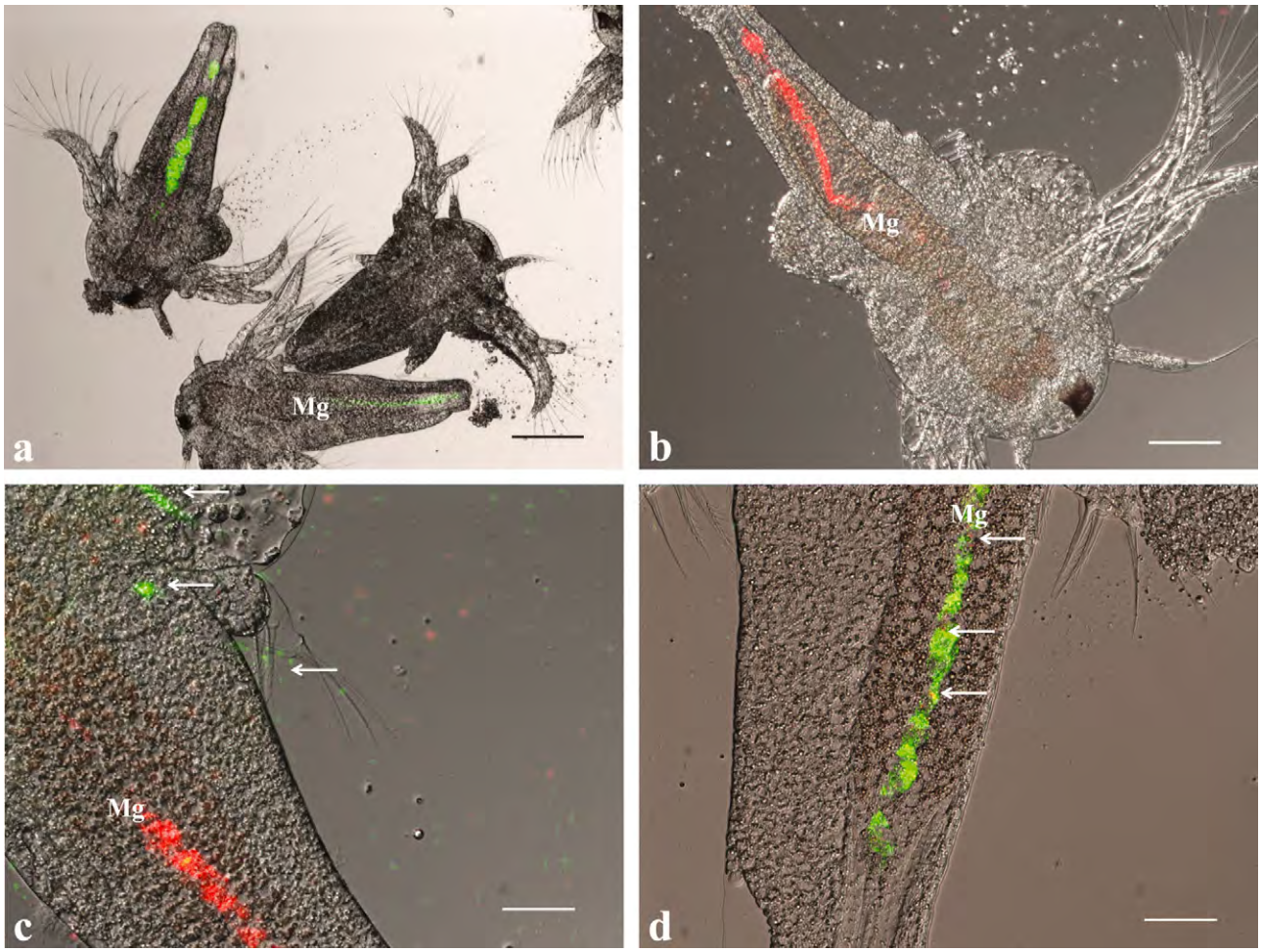

Fig. 2. Localization of FP-labelled probionts and pathogen in *Artemia* nauplii cultures after enrichment for 2 h.
a. Varied bioaccumulation of probiont *Vibrio* sp. PP05[GFP] in midgut after monostrain enrichment. Scale bar = 200 μm.
b. Concentration of pathogen *V. owensii* DY05[RFP] in posterior midgut after monostrain enrichment. Scale bar = 100 μm.
c. Endobiotic *Vibrio* sp. PP05[RFP] in gut and ectobiotic *Pseudoalteromonas* sp. PP107[GFP] confined to carapace and appendages (white arrows) after enrichment with the probiont combination. Scale bar = 50 μm.
d. Dense concentration of probiont (presumably PP05[GFP]) cells and single *V. owensii* DY05[RFP] cells (white arrows) in the gut after enrichment with *V. owensii* DY05[RFP], PP05[GFP] and PP107[GFP]. Note lack of red cells in the surrounding bacterioplankton. Scale bar = 50 μm.
Mg, midgut.

co-enrichment with the *Vibrio* probiont PP05[GFP] alone or with the probiont mixture (PP05[GFP] and PP107) ($P < 0.01$) (Fig. S1). With PP05 alone the pathogen load was reduced by 76–80%, while the probiont mix reduced the pathogen load by 89–97%. Co-enrichment with the *Pseudoalteromonas* probiont PP107 alone significantly reduced the pathogen loading in one trial by 64% ($P < 0.05$); however, in a repeated experiment the reduction was only 15%, which was not statistically significant ($P > 0.05$) (Fig. S1).

Pathogen–probiont–phyllosoma interactions

Spatiotemporal localization of FP-labelled probiont and pathogen strains in *P. ornatus* phyllosomas were monitored *in situ* after vectored challenge with *Artemia*. Treatments included *Artemia* enriched with a single strain (monostrain treatment), or with a combination of the two probionts, or a combination of the two probionts and the pathogen (multistrain treatments).

The spatiotemporal distribution of the probiont *Vibrio* sp. PP05[GFP or RFP] was the same in all treatments. Six hours after exposure, single or small clusters of cells were localized on setae of the phyllosoma foregut (proventriculus) and in the midgut gland (hepatopancreas), and cells were also observed transiting through the mid- and hindguts (Fig. 3a–c). Cells were retained in the proventriculus and hepatopancreas after 48 h, but faecal strands also showed massive colonization (Fig. 3d). Hence, it was not clear from this short-term study (48 h) whether residency

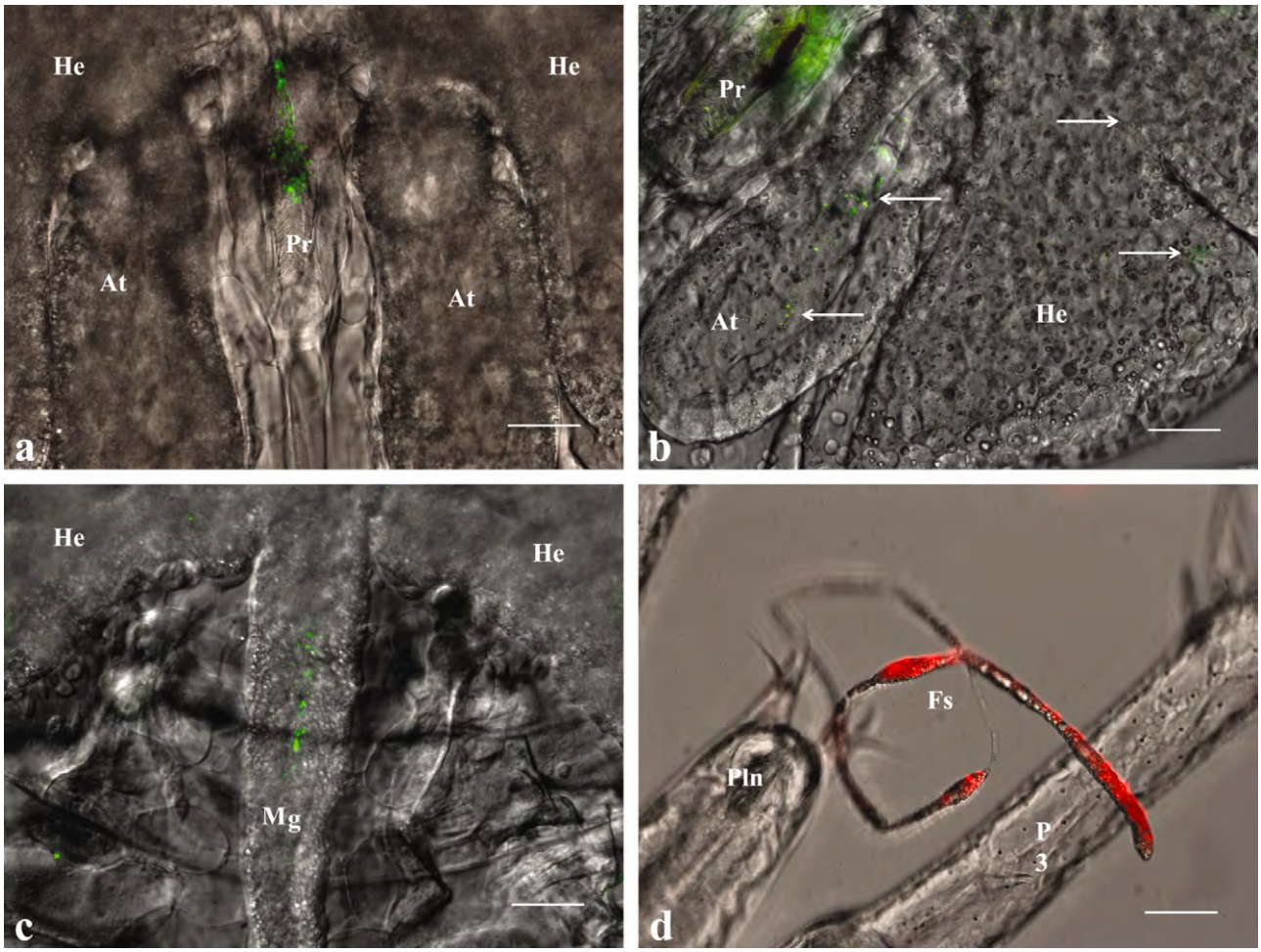

Fig. 3. Endobiotic localization of FP-labelled probiont *Vibrio* sp. PP05 in stage 1 *P. ornatus* phyllosomas after vectored challenge via *Artemia* nauplii enriched with PP05[GFP or RFP] only.
a. PP05[GFP] colonization of proventriculus (foregut) setae.
b. Single and aggregate cells of PP05[GFP] in hepatopancreas lobes (white arrows).
c. Transiting of PP05[GFP] cells through midgut.
d. Prolific colonization of faecal strand by PP05[RFP].
All scale bars, 50 μm. At, anterior tubule lumen of hepatopancreas; Fs, faecal strand; He, hepatopancreas (main lateral lobe); Mg, midgut; P, pereiopod (3rd); Pln, pleon; Pr, proventriculus.

of probiotic *Vibrio* sp. PP05[GFP or RFP] was transient or permanent. The probiont *Pseudoalteromona*s sp. PP107[GFP] mainly colonized external surfaces of phyllosomas in both monostrain and multistrain treatments and was not observed in the hepatopancreas (see below). However, a small number of endobiotic PP107[GFP] cells were seen in the lower hindgut, possibly indicating they were taken up with water by the anus during rectal antiperistaltic contractions (Fox, 1952). Endobiotic residency by the probionts did not inflict any visible structural damage to host tissues and organs.

The probiont *Pseudoalteromonas* sp. PP107[GFP] efficiently colonized phyllosoma external surfaces in both monostrain and multistrain treatments. The abundance of cells depended more on individual larvae than time; however, colonization overall appeared to be greater at $t = 30$ h just before transfer of phyllosomas to new 12-well plates. Attachment was often organized on spines, contours, fissures and depressions on the exocuticle but randomly dispersed cells were also observed. In multistrain treatments with *Vibrio* sp. PP05[RFP], clear zones of preferential ectobiotic colonization were observed on phyllosoma pereiopods (legs) and pereiopod branching appendages (expopods; Fig. 4a), although sporadic 'intrusions' into each other's preferential niche were frequently observed. *Pseudoalteromonas* sp. PP107[GFP] was the dominant ectobiont, colonizing the cephalothorax (Fig. 4b), eyestalks, eyes and inner segments of the pereiopods and maxillipeds (used to manipulate and shred food). In contrast, the *Vibrio* probiont (PP05) was

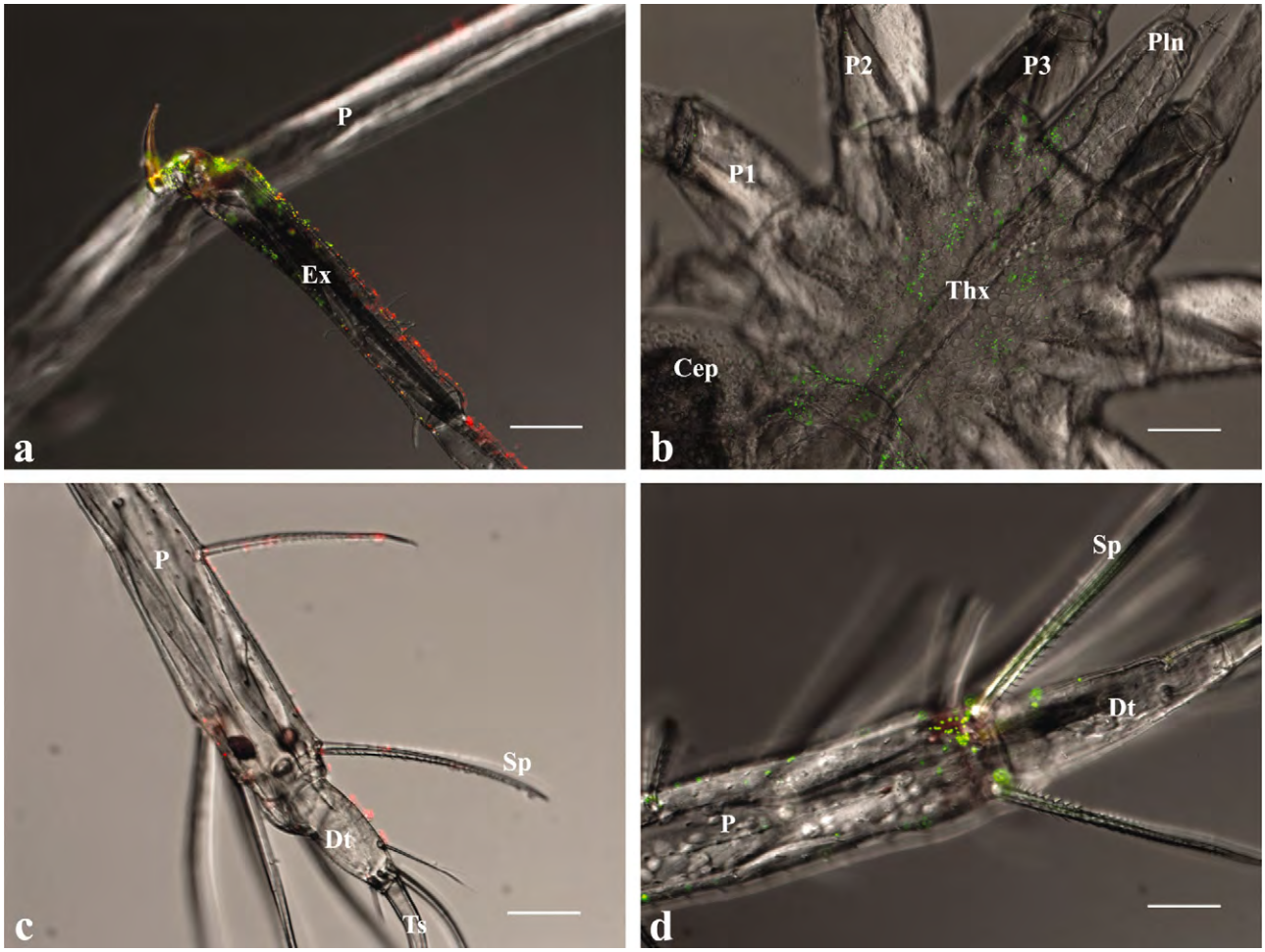

Fig. 4. Ectobiotic localization of FP-labelled probionts and pathogen on stage 1 *P. ornatus* phyllosomas after vectored challenge via bacteria-enriched *Artemia* nauplii.
a. Branching appendage (exopod) of pereiopod demonstrating preferential zones of colonization by probiont *Pseudoalteromonas* sp. PP107[GFP] and *Vibrio* sp. PP05[RFP] on inner and outer segments, respectively, after vectored challenge with PP107[GFP], PP05[RFP] and wild-type pathogen *V. owensii* DY05. Scale bar = 50 μm.
b. Prolific attachment of PP107[GFP] on cephalothorax after vectored challenge with PP107[GFP], PP05[RFP] and wild-type *V. owensii* DY05. Scale bar = 100 μm.
c. Attachment of PP05[RFP] clusters to dactylus and spines of pereiopod after vectored challenge with PP05[RFP] only. Scale bar = 100 μm.
d. Colonization of PP107[GFP] extending to dactylus of pereiopod after vectored challenge with PP107[GFP] only. Scale bar = 100 μm.
Cep = cephalic shield; Dt = dactylus; Ex = exopod; P = pereiopod (1–3); Pln = pleon; Sp = spine; Thx = Thorax; Ts = terminal spine.

the primary ectobiont on the outer pereiopod segments, with cell aggregates observed in particular on the terminal segment (dactylus) and its terminal spine, which is used to impale prey items (Fig. 4c). Interestingly, in monostrain treatments, *Pseudoalteromonas* sp. PP107[GFP] colonization extended to the phyllosoma pereiopod dactylus (Fig. 4d), while the spatial distribution of probiotic *Vibrio* sp. PP05[GFP or RFP] was conserved, with the cephalothorax and inner pereiopods remaining relatively devoid of attachment. Ectobiotic attachment by the probionts caused no apparent loss of exocuticle integrity within the monitoring period.

Phyllosoma challenge with the pathogen *V. owensii* DY05[RFP] without probiotic treatment resulted in similar spatiotemporal proliferative patterns as the GFP-expressing *V. owensii* DY05 transconjugant previously described (Goulden *et al*., 2012a). In contrast, 6 h after the phyllosoma were exposed to *Artemia* enriched with the pathogen and probiont combination, only one pathogen cell was detected in the proventriculus of a single phyllosoma and none were discovered in the hepatopancreas or other locations.

Discussion

Live *Artemia* are still an important diet component for aquaculture-reared early-stage lobster phyllosomas (Johnston *et al*., 2008; Smith *et al*., 2009a) and play

an important role in the infection cycle of pathogenic *V. owensii* DY05 in the *P. ornatus* hatchery (Goulden *et al*., 2012a). While addition of probionts to *Artemia* cultures has previously shown to reduce pathogenic *Vibrio* spp. populations (Verschuere *et al*., 2000b; Villamil *et al*., 2003), the present study showed that ecological niche specialization by two probionts likely contributed to their additive protective effect against *V. owensii* DY05 that was observed in an earlier study (Goulden *et al*., 2012b). The *Pseudoalteromonas* probiont PP107 was localized only on external surfaces of the vector organism (*Artemia*) and phyllosomas, while the *Vibrio* probiont PP05 was primarily localized in their respective digestive systems. This indicated that both strains could interact directly with planktonic *V. owensii* DY05, although the pathogen was exposed mainly to the inhibitory activity of PP05 once inside the gut.

Ambient planktonic pathogen cells in the *Artemia* cultures were reduced in the presence of probionts compared with pathogen-only controls, confirming that the probionts can inhibit planktonic growth of *V. owensii* DY05 (Goulden *et al*., 2012b). Our previous study showed that planktonic growth of *V. owensii* DY05 could be inhibited by either of the probiotic strains; however, PP107 was found to be more efficient at low densities (Goulden *et al*., 2012b). In the *Artemia* gut, the pathogen population was significantly reduced from high-density bioaccumulations in pathogen-only controls to isolated cells in *Artemia* enriched with pathogen and probionts, and spread plating confirmed a reduced pathogen load. Overall, this highlights the importance of the *Artemia* enrichment step to intercepting the infection cycle of *V. owensii* DY05 and providing the protective benefit to phyllosomas.

Although *Artemia* ingest free-living bacteria, the degree of bioaccumulation depends on the bacterial species and strain (Gomez-Gil *et al*., 1998; Makridis *et al*., 2000; Verschuere *et al*., 2000b; Soto-Rodriguez *et al*., 2003). In the present study, *Pseudoalteromonas* sp. PP107 was not internalized by *Artemia* nauplii and it is likely the cells produced chemical deterrents and were not grazed by *Artemia*. *Pseudoalteromonas* strains can produce several bioactive compounds (Bowman, 2007; Matz *et al*., 2008; Vynne *et al*., 2011), some of which can mediate grazing resistance from protozoa (Matz *et al*., 2008). Furthermore, it has been demonstrated that the bacterivorous nematode *Caenorhabditis elegans* do not graze on toxic bacterial clones of *Escherichia coli* expressing heterologous genomic fragments of *Pseudoalteromonas tunicata* (Ballestriero *et al*., 2010).

Interestingly, the presence of *Pseudoalteromonas* sp. PP107 was associated with decreases in the overall bacterial load of *Artemia* nauplii, including the load of the *Vibrio* probiont PP05. It is likely that PP107 inhibited the growth of *Vibrio* sp. PP05 to some degree, but it could also have caused physiological incapacitation of *Artemia* as shown previously for *Artemia* (Demaret *et al*., 1995) and copepods (Ives, 1987) exposed to certain toxic dinoflagellates. Further studies are necessary to clarify the mechanisms by which PP107 interferes with other bacterial populations and the *Artemia* host.

In both *Artemia* and phyllosomas, all observed *Pseudoalteromonas* cells were attached to external surfaces. Pseudoalteromonads have a propensity for surface colonization (Holmström and Kjelleberg, 1999) and recent genomic studies on epiphytic *P. tunicata* revealed the presence of numerous genes (curli, pili and capsular polysaccharide) that could mediate adherence to different surface textures and therefore potentially increase host range (Thomas *et al*., 2008). While *Pseudoalteromonas* sp. PP107 was isolated from arrow worms (*Chaetognatha*), a natural prey item of phyllosomas, we have isolated closely related *Pseudoalteromonas* strains from both wild and aquaculture-reared phyllosomas (Goulden *et al*., 2012b). It is uncertain whether pseudoalteromonads associated with phyllosomas simply use a hitchhiking strategy to facilitate their dispersal (Grossart *et al*., 2010) or whether they are true ectosymbionts. Surface associations are recognized as a complex coordination between bacterial and eukaryotic partners (Goffredi, 2010). For instance, phytoplankton are known to recruit symbiotic roseobacter partners by producing a chemoattractant (dimethylsulfoniopropionate) and in return, the roseobacters protect the host from epibiotic growth by producing a potent antibiotic, tropodithietic acid (Geng and Belas, 2010). Likewise, it is possible that *Pseudoalteromonas* sp. PP107 or similar ecotypes contribute to the unfouled condition of wild phyllosomas (Payne *et al*., 2008) by producing bioactive compounds (Egan *et al*., 2002; Holmström *et al*., 2002). If this were the case, there would be a potential additional benefit of using this probiont in the larviculture system where recalcitrant fouling by filamentous *Thiothrix* spp. occasionally leads to impaired larval feeding capacity and function (Bourne *et al*., 2007; Payne *et al*., 2007).

In contrast to *Pseudoalteromonas* sp. PP107, the *Vibrio* probiont (PP05) was mainly internalized in *Artemia* and phyllosomas. The gut microenvironment appears to be particularly competitive, where different *Vibrio* species inhabiting similar niches may produce distinctive bioactive compounds (Wietz *et al*., 2010) to enhance ecological success. This is supported by the isolation of many antagonistic and siderophore-producing vibrios from the gastrointestinal tracts of aquatic organisms (Makridis *et al*., 2005; Fjellheim *et al*., 2007; Sugita *et al*., 2012). It is well recognized that the establishment of probionts in the digestive tract is a possible means of controlling enteropathogen proliferation in invertebrates and fish (Sugita *et al*., 1998; Rengpipat *et al*., 2000; Balcázar and Rojas-Luna, 2007; Li *et al*., 2007; Avella *et al*., 2011; Boonthai *et al*., 2011).

The colonization of the phyllosoma proventriculus setae by PP05 could at least in theory have contributed to limiting the invasion of the hepatopancreas by *V. owensii* DY05. Furthermore, the residency of PP05 in the hepatopancreas could have provided the main source of its colonization of faecal pellets (Tang, 2005). Faecal matter can serve as microincubators to increase bacterial abundance and thereby re-inoculating ambient water (Beardsley *et al*., 2011). This strategy would permit cyclic reattachment of PP05 to the outer pereiopod segments of the phyllosomas (discussed below), and when captured prey items are brought to the oral cavity during mastication (Smith *et al*., 2009a), the presence of PP05 on the dactylus could promote ingestion during feeding.

A likely explanation for the non-random localization of *Vibrio* sp. PP05 on the outer pereiopod segments ascending to the dactyl spines is increased nutrient concentrations related to nutrient leaching during sloppy feeding events (Tang *et al*., 2010). Transient nutrient plumes generated by zooplankton (Lehman and Scavia, 1982) can become rapidly colonized by chemotactic bacteria (Stocker *et al*., 2008) and such conditions could select for PP05. Evidence in support of this is the finding that localization of PP05 was independent of the presence of *Pseudoalteromonas* sp. PP107. In contrast, *Pseudoalteromonas* sp. PP107 colonized also the outer limb segments in the absence of PP05, suggesting that PP05 either directly outcompeted PP107 for nutrients and/or inhibited the growth of PP107 by another mechanism such as the production of antibacterial compounds.

Conclusion

This study has demonstrated the usefulness of FP-based *in situ* biomonitoring systems to increase our understanding of probiont ecology in aquaculture systems. The niche specialization exhibited by *Vibrio* sp. PP05 and *Pseudoalteromonas* sp. PP107 likely contributes to their additive survival effect on *P. ornatus* phyllosomas challenged with *V. owensii* DY05. It was shown that preemptive conditioning of *Artemia* cultures with probionts represents a strong biocontrol method to avert pathogen ingestion and eliminate vector-mediated transmission during early-stage *P. ornatus* phyllosoma larviculture. Further studies of probiont behaviour and long-term phyllosoma health under commercial scale hatchery conditions are required along with the development of efficient probiont delivery protocols for early- and late-stage phyllosomas.

Experimental procedures

Larviculture

Maintenance of *P. ornatus* broodstock, production of larvae and larviculture were performed according to Smith and colleagues (2009b) at the Tropical Aquaculture Facility of the Australian Institute of Marine Science (AIMS), Townsville, Australia (lat. 16°17.728′S, long. 145°27.121′E). Only apparently healthy phyllosomas as assessed by photopositive response and active motility were used for experiments.

Bacterial strains, transconjugations and inoculum preparation

Bacterial strains used in this study are listed in Table S1. Plasmids carrying FP genes and selection markers were transferred from *E. coli* donor strains to *Vibrio* spp. or *Pseudoalteromonas* sp. by triparental conjugation as described previously (Dunn *et al*., 2006; Goulden *et al*., 2012a) using the helper strain CC118λpir or HB101 respectively. Briefly, wild-type *Vibrio* and *Pseudoalteromonas* strains were grown in LB20 broth (5 g l^{-1} yeast extract; 10 g l^{-1} neutralized peptone; 20 g l^{-1} NaCl) or half strength marine broth 2216 (Becton, Dickinson and Company) respectively. *Escherichia coli* strains were grown in LB broth (5 g l^{-1} yeast extract and 10 g l^{-1} neutralized peptone) supplemented with 40 μg ml^{-1} kanamycin or 15 μg ml^{-1} chloramphenicol, where appropriate. Agar (1%) was used to solidify media for plating and all incubations were performed at 30°C. To screen for FP expression in *Vibrio* spp., LB20 agar was supplemented with kanamycin or chloramphenicol in addition to 50 μg ml^{-1} colistin. To screen for GFP expression in *Pseudoalteromonas* sp., half strength marine agar was supplemented with chloramphenicol. Fluorescent transconjugant colonies were detected using a blue light transilluminator.

FP-expressing strains *V. owensii* DY05[RFP], *Vibrio* sp. PP05[GFP] and PP05[RFP], and *Pseudoalteromonas* sp. PP107[GFP] (Table S1) showed similar growth profiles compared with respective wild types in a microgrowth assay and the FP were stably expressed in *Vibrio* sp. PP05[GFP] (99%), PP05[RFP] (96%) and *V. owensii* DY05[RFP] (84%) after continuous subculture every 24 h for 7 days in non-selective medium (data not shown). In contrast, expression of GFP in PP107[GFP] dropped to below 79% after two subcultures, restricting its use to short-term (48 h) experiments.

Inocula were prepared by washing 20 h marine broth 2216 cultures using three cycles of centrifugation (10 min at 4650 r.p.m.; 10°C) and resuspension in 0.22 μm filtered seawater (FSW). Cell suspensions were adjusted to absorbance $OD_{600\,nm}$ 0.1 (Nanodrop ND1000). The corresponding total viable counts (expressed as cfu ml^{-1}) were determined for triplicate cultures of each inoculum in initial experiments using spiral plating (Eddy Jet; IUL) on marine agar (MA; Becton, Dickinson and Company) and enumeration by an automatic colony counter (Flash and Grow v1.2; IUL). This information was used to calculate the volume of each $OD_{600\,nm}$ 0.1 suspension needed to achieve the desired starting concentrations in the experiments described below.

Confirmation of virulence and probiotic effectiveness in vivo

To confirm the virulence of *V. owensii* DY05[RFP] and the probiotic protective benefits of PP05[GFP], PP05[RFP] and

PP107[GFP], stage 1 (2 day old) *P. ornatus* phyllosomas were experimentally infected using vectored challenge via instar II *Artemia* nauplii (Goulden *et al*., 2012a,b). Strain combinations were selected to correspond with the subsequent studies described below. Treatments included multistrain applications of: (i) wild-type strains (*V. owensii* DY05, PP05 and PP107), (ii) wild-type pathogen and two differently labelled probiotic transconjugants (PP05[RFP] and PP107[GFP]) and (iii) RFP pathogen transconjugant (*V. owensii* DY05[RFP]) and GFP probiont transconjugants (PP05[GFP] and PP107[GFP]).

Briefly, *Artemia* (200 nauplii ml^{-1}) were enriched through filter feeding with the probiotic combinations (1×10^7 cfu ml^{-1} of each strain) in tissue culture flasks for 2 h (28°C; 45 r.p.m.) and fed at a final concentration of 3 nauplii ml^{-1} to phyllosomas in 12-well cell culture plates at $t = 0$ h and $t = 30$ h. At $t = 24$ h, phyllosomas were vector-challenged for 6 h with *Artemia* enriched with the pathogen (1×10^6 cfu ml^{-1}) together with the probiont combination (1×10^7 cfu ml^{-1} of each strain). Phyllosomas were transferred to new cell culture plates following pathogen challenge ($t = 30$ h). Treatments were performed in quintuplicate ($n = 60$) and mortality was assessed every 24 h for 5 days. Phyllosomas that did not display any active movement after prolonged inspection were recorded as dead. Experimental controls consisted of a negative control (phyllosomas fed with non-enriched *Artemia*) and pathogen controls fed with *Artemia* enriched in *V. owensii* DY05 or DY05[RFP] only at $t = 24$ h, and non-enriched *Artemia* at $t = 0$ h and $t = 30$ h.

Enumeration of bacteria associated with Artemia *nauplii*

Culture-based enumeration of probionts and pathogen was performed with strains that could be identified on agar plates based on their respective colony coloration on MA. The pathogen *V. owensii* DY05[RFP] and the probiont *Vibrio* sp. PP05[GFP] fluoresce red and green, respectively, under blue light, while the wild type of probiont *Pseudoalteromonas* PP107 has yellow pigmentation. Nauplii were enriched with monostrain or multistrain inoculums (1×10^7 cfu ml^{-1} for probionts; 1×10^6 cfu ml^{-1} for pathogen) for 2 h in cell culture flasks as described. The homogenization and plating protocol was modified from Høj and colleagues (2009). Briefly, 5 ml samples of nauplii culture (200 nauplii ml^{-1}) were transferred to sterile cell strainers (mesh size 40 μm; Fisherbrand, Fisher Scientific) and washed in 20 ml of artificial seawater (ASW) (Instant Ocean; Spectrum Brands, Madison, WI, USA). The nauplii were resuspended in ASW and transferred to sterile screw-capped tubes containing glass beads (one 2 mm and two 3 mm) and the volume adjusted to 1 ml with ASW. Samples were homogenized for 30 s using a bead beater (BioSpec Products, Bartlesville, OK, USA) with a pre-chilled tube holder. Serial dilutions were spread-plated in triplicate on MA, incubated at 28°C and colony-forming units (expressed as cfu per nauplius) enumerated after 24 h.

Localization of pathogen and probionts in situ

A vector challenge experiment was performed as outlined above to investigate the spatiotemporal localization of the pathogen and probionts during interaction with the *Artemia* and phyllosoma hosts. Treatments consisted of *Artemia* enriched with suspensions of each FP-labelled strain (monostrain treatments) fed to *P. ornatus* phyllosomas at $t = 0$ h only, or *Artemia* enriched with combinations of FP-tagged probionts with or without the pathogen (wild-type or FP-tagged) using the administration regimes outlined above (multistrain treatments). For each treatment, enriched *Artemia* nauplii cultures and four vector-challenged *P. ornatus* phyllosomas were removed at 6 h intervals over a 48 h monitoring period, live mounted in FSW and viewed using differential inference contrast (DIC) and fluorescence microscopy (AxioSkop 2 mot plus; Carl Zeiss). Fluorescence was detected using a dual band filter set (59004; Chroma Technology Corp.) and images were captured by an AxioCam MRc5 camera (Carl Zeiss) directed by the multidimensional acquisition module of the AxioVision Rel. 4.8 software (Carl Zeiss).

Statistical analysis

Differences between survival curves were determined using the product limit (Kaplan-Meier) estimator and confirmed with an ANOVA. A *post hoc* Dunnett's test was used to compare multiple data sets with a defined control group. These analyses were performed using the statistical software package JMP®7 (SAS). Overall and strain-specific cfu counts for enriched *Artemia* were log-transformed and variation between means were tested by ANOVA and a *post hoc* Tukey's HSD test using the statistical software package STATISTICA 11 (StatSoft). A significance level of $\alpha = 0.05$ was used for all analyses.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Bacterial loading of *Artemia* after mono- or multistrain enrichment (2 h). Data from one replicate experiment are shown. Different upper case letters denote a significant difference in total bacterial loading, and different lower case letters denote a significant difference in the loading of pathogen *Vibrio owensii* DY05. The first three bars show monostrain enrichment with probiont *Pseudoalteromonas* sp. PP107, probiont *Vibrio* sp. PP05[GFP] or *V. owensii* DY05[RFP], respectively. The next two bars show enrichment with *V. owensii* DY05 [RFP] in combination with one probiont (PP107 or PP05 [GFP], respectively). The next bar shows enrichment with the probiont combination (PP107 and PP05 [GFP]). The last bar shows enrichment with *V. owensii* DY05[RFP] and the probiont combination (PP107 and PP05[GFP]).

Table S1. Bacterial strains used in this study.