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. PP05 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, PP05 was localized on external surfaces of *Artemia* and phyllosomas, and direct interaction with the pathogen was limited to the bacterioplankton. While PP05 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.

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 probiotic transconjugants were suitable biomarkers for short-term studies of bacteria–vector and bacteria–phyllosoma interactions.

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 ($1 \times 10^7$ cfu ml$^{-1}$ and $1 \times 10^6$ 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

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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 (monostain treatment), or with a combination of the two probions, or a combination of the two probions and the pathogen (multistain 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
of probiotic Vibrio sp. PP05[GFP or RFP] was transient or permanent. The probiont Pseudoalteromonas 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 anti-peristaltic 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 branch appendages (exopods; 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
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[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 (Verschueren et al., 2008; 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; Verschueren et al., 2008; 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 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 chemotaxtractant (dimethylsulfoxoniopropionate) 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 unoiled 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\(_{595 nm}\) 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\(_{595 nm}\) 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[GF], 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[GF]) and (iii) RFP pathogen transconjugant (*V. owensii* DY05[RFP]) and GFP probiont transconjugants (PP05[GF] and PP107[GF]).

Briefly, *Artemia* (200 nauplii ml\(^{-1}\)) were enriched through filter feeding with the probiotic combinations (1 \(\times 10^7\) 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 \(\times 10^6\) cfu ml\(^{-1}\)) together with the probiotic combination (1 \(\times 10^4\) 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 probiotic *Vibrio* sp. PP05[GF] fluoresce red and green, respectively, under blue light, while the wild type of probiotic *Pseudoalteromonas* PP107 has yellow pigmentation. Nauplii were enriched with monostrain or multistrain inoculums (1 \(\times 10^7\) cfu ml\(^{-1}\) for probionts; 1 \(\times 10^8\) cfu ml\(^{-1}\) for pathogen) for 2 h in cell culture flasks as described. The homogenization and plating protocol was modified from Hej and colleagues (2009). Briefly, 5 ml samples of nauplii culture (200 nauplii ml\(^{-1}\)) were transferred to sterile cell strainers (mesh size 40 \(\mu\)m; 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 (mono-strain 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 interference contrast (DIC) and fluorescence microscopy (AxioSkop 2 mt 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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**References**


Probiotic niche specialization in *P. ornatus* larvae


Goulden, E.F., Hall, M.R., Pereg, L.L., and Hoj, L. (2012b) Identification of an antagonistic probiotic combination pro-


**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.