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ORIGINAL ARTICLE

# Microbial diversity of mid-stage Palinurid phyllosoma from Great Barrier Reef waters

M.S. Payne[1,2], L. Høj[1], M. Wietz[1,3,4], M.R. Hall[1], L. Sly[2] and D.G. Bourne[1]

1 The Australian Institute of Marine Science, Townsville, Australia
2 School of Molecular and Microbial Sciences, The University of Queensland, St Lucia, Australia
3 Max Planck Institute for Marine Microbiology, Bremen, Germany
4 University of Bremen, Bremen, Germany

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**Correspondence**
David G. Bourne, Australian Institute of Marine Science, Private Mail Bag 3, Townsville Mail Centre, Townsville, Queensland, Australia, 4810. E-mail: d.bourne@aims.gov.au

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## Abstract

**Aims:** This study aimed to determine the bacterial community associated with wild-caught, mid-stage larvae of spiny lobsters (*Palinuridae*) in their native oligotrophic marine environment, and to compare their diversity and composition with communities associated with aquaculture-reared larvae of the tropical rock lobster *Panulirus ornatus*.

**Methods and Results:** Bacterial clone libraries constructed from wild *P. ornatus* (two libraries) and *Panulirus penicillatus* (one library) larvae (phyllosoma) revealed a dominance of α-proteobacterial sequences, with *Sulfitobacter* spp.-affiliated sequences dominating both *P. ornatus* libraries and constituting a major portion of the *P. penicillatus* library. *Vibrio*-related sequences were rarely detected from wild phyllosoma clone libraries in contrast to similar studies of aquaculture-reared animals. Scanning electron microscopy analysis revealed low levels of bacterial colonization on the external carapace of wild phyllosoma, again in contrast to aquaculture-reared animals, which are often colonized with filamentous bacteria (mainly *Thiothrix* sp.) that compromise their health. Fluorescence *in situ* hybridization of sectioned wild phyllosoma tissue displayed low overall abundance of bacteria within the tissue and on external surfaces, with α-, β-, and γ-*Proteobacteria* being confirmed as members of this bacterial community.

**Conclusions:** The consistency in predominant clone sequences retrieved from the three libraries indicated a conserved microbiota associated with wild phyllosoma. In addition, the observed differences in the microbial composition and load of reared and wild phyllosoma are indicative of the different environments in which the animals live.

**Significance and Impact of the Study:** Bacterial disease during early larval stages is a major constraint currently hindering the development of an aquaculture industry for the ornate rock lobster *P. ornatus*. Knowledge of the microbial community associated with wild animals will be advantageous for the identification of bacteria that may promote animal health.

## Introduction

Lobsters of the family Palinuridae, commonly known as spiny lobsters or rock lobsters, are a valuable component of Australian marine fisheries (Phillips 1985). However, current supply levels cannot meet the high demand for the animals, and as a result, there is an interest in developing an aquaculture industry to bridge this gap and subsequently lower pressures on wild stocks. The potential for the culture of Palinurid rock lobsters in Australia has been discussed previously (Linton 1998; Bourne *et al.* 2004), with the species *Panulirus ornatus* being an obvious choice owing to its favourable growth characteristics and high market value (Linton 1998). Numerous

constraints affecting the culture of this species have however been highlighted (Linton 1998), in particular, disease-related problems associated with larval rearing (Bourne *et al.* 2004, 2006, 2007; Payne *et al.* 2006, 2007; Webster *et al.* 2006).

Few studies have examined the microbial flora associated with lobster larvae, commonly known as phyllosoma, and previous reports have focussed exclusively on the flora associated with reared phyllosoma and their aquaculture environment. For example, Bourne *et al.* (2004) described the microbial community dynamics within an experimental larval rearing system for *P. ornatus*, discussing both environmental and pathogenic bacteria. Other studies have focussed on defined microbial niches within the larval rearing environment of *P. ornatus*, including the water column (Payne *et al.* 2006) and the biofilm (Bourne *et al.* 2006). Payne *et al.* (2007) investigated the microbial community associated with live and dying phyllosoma reared within the aquaculture environment, describing both commensal and potentially pathogenic bacteria associated with the animals. Finally, Webster *et al.* (2006) described a *Vibrio* sp. infection within reared *P. ornatus* phyllosoma leading to mass mortalities.

The microbial flora associated with reared phyllosoma is expected to differ from that of their wild counterparts, owing largely to the differences in their growth environment. The culture environment will vary significantly from the larvae's natural environment, both in nutrient levels and increased stress owing to high stocking densities and handling. In addition, antibiotic and chemical treatments commonly used in aquaculture environments remove some, but not all, bacteria associated with phyllosoma, hence effectively selecting for certain organisms and accentuating the dynamics of the microbial community directly associated with phyllosoma (Bourne *et al.* 2004).

This study aimed to characterize the microbial community associated with wild Palinurid phyllosoma, with particular emphasis on the species, *P. ornatus*, the current identified candidate for aquaculture in Australia. A direct comparison is made between the results from this study and those from previous work elucidating the microbial flora of aquaculture-reared phyllosoma (Payne *et al.* 2007) with the aim of identifying differences in bacterial communities and potentially beneficial micro-organisms associated with wild phyllosoma.

## Materials and Methods

### Sample collection

Late stage (P8 to P10) phyllosoma were collected using a diamond mesh Isaac-Kidd mid-water trawl net with a relaxed mesh diameter of 5 mm. Trawls were conducted on the Great Barrier Reef, Queensland, Australia, between 18°34′102′ S, 146°27′659′ E and 12°01′861′ S, 143°57′615′ E at depths of 1–100 m. Phyllosoma were collected during the Austral Autumn month of May 2005 when late-stage (P8-10) larvae are known to be present in the Coral Sea (Dennis *et al.* 2004). Phyllosoma were identified to genus, and when possible, species level immediately upon collection using a dichotomous key (Baisre 1994). Before further processing, the animals were briefly washed in artificial sea water (ASW) to remove any loosely attached bacteria. Samples for DNA extraction were initially frozen at −20°C, and subsequently stored at −80°C upon return to the laboratory. Phyllosoma samples for histology, scanning electron microscopy (SEM) and fluorescence *in situ* hybridization (FISH) were fixed immediately as described subsequently.

### Extraction of genomic bacterial DNA

The total bacterial DNA associated with phyllosoma was extracted from two separately homogenized *P. ornatus* phyllosoma and one homogenized *Panulirus penicillatus* phyllosoma using the Qiagen DNeasy®Tissue Kit (Qiagen, Hilden, Germany), as per manufacturer's instructions for Gram-positive bacteria. DNA was quantified on a 1·5% agarose gel stained with ethidium bromide (0·5 $\mu$g ml$^{-1}$) and stored at −20°C.

### PCR amplification of 16S rRNA genes

The bacterial 16S rRNA gene was amplified from DNA extracted from phyllosoma using the primers 27f and 1492r (Lane 1991). All PCR reactions were performed in either an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) or a PE Applied Biosystems Geneamp PCR System 9700 (Perkin Elmer, MD, USA). PCR products were visualized on a 1·5% agarose gel stained with ethidium bromide (0·5 $\mu$g ml$^{-1}$) and stored at −20°C.

### Clone library analysis

PCR-amplified bacterial 16S rRNA gene fragments (27f/1492r) obtained from two separate *P. ornatus* phyllosoma and one *P. penicillatus* phyllosoma were cloned using a TOPO TA cloning® kit, Version K2 (Invitrogen, CA, USA) as per manufacturer's instructions. For each library, 100 clones were randomly selected and the cloned fragment re-amplified. Clones were screened by restriction fragment length polymorphism (RFLP) analysis of PCR products using restriction enzymes *Hha* I and *HAE* III (Promega, WI, USA). RFLP profiles were visualized on a 3% agarose gel stained with ethidium bromide

(1 $\mu$g ml$^{-1}$), and the clones were grouped into operational taxonomic units (OTUs) accordingly. Replicate clones from dominant OTUs representing 2% or more of the clone library were selected and grown in Luria-Bertani (LB)–ampicillin (50 $\mu$g ml$^{-1}$) broth overnight. Plasmid DNA was extracted using a QIAprep® Spin Miniprep Kit (Qiagen) as per manufacturer's instructions, quantified and directly sequenced. Similarly, nine OTUs represented by single clones were randomly selected from each library and sequenced.

### Sequencing and phylogenetic analysis

Partial and complete 16S rRNA gene sequences of bacterial clones were obtained using the primers 27f, 339f, 732f and 1492r (Lane 1991). Automated sequencing was performed using either Amersham DYEnamic ET-terminator sequencing dye (Amersham Biosciences) or ABI BigDye Terminator v3·1 sequencing dye (Applied Biosystems, CA, USA), as per manufacturer's instructions. Sequences were checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (Maidak *et al.* 1996). Sequence data were aligned to the closest relative using the BLAST database algorithm (Altschul *et al.* 1997), and further analysed with the ARB software package (Ludwig *et al.* 1998). The tree topologies were evaluated by reconstructing phylogenies using evolutionary distance (Phylip Distance Method with Jukes and Cantor model) analysis of aligned near full-length sequences (>1000 bp). Regions of ambiguous sequence were removed from the analysis. Aligned, partial 16S rRNA sequences (<1000 bp) were subsequently inserted without changing the overall tree topology using the parsimony tool available within ARB. Bootstrap values were obtained for branching patterns using the Phylip software package (version 3·65) (Retief 2000) and values ≥50% were included for the main nodes of the tree. The nucleotide sequence data of all phyllosoma clones appear in the GenBank nucleotide database under the accession numbers DQ985883–DQ985937.

### Histopathological analysis

*Panulirus ornatus* phyllosoma larvae were fixed in Davidson's fixative (Hasson *et al.* 1997) for 24 h and transferred to 70% ethanol until further processing. Samples were dehydrated in an ethanol series (2 × 60 min 70% ethanol; 60 min 80% ethanol; 60 min 90% ethanol; 3 × 60 min 96% ethanol), incubated in xylene for 2 × 60 min before embedding in fluid paraffin (2 × 120 min at 60°C under vacuum). Hardened paraffin blocks were sectioned (5 $\mu$m) and placed onto microscope slides and incubated at 60°C for 3 h for fixation. Parafin was removed in xylene for 3 min and the sample re-hydrated through a 96%/90%/80%/70% ethanol series to water (5 min each). Sections were stained with Mayer's hematoxylin and Young's eosin using routine histological procedures (Bancroft and Stevens 1990) and examined using light microscopy.

### Scanning electron microscopy

*Panulirus ornatus* phyllosoma were fixed in 3% glutaraldehyde diluted with 0·22 $\mu$m filtered ASW at 4°C. Prior to SEM analysis, samples underwent 3 × 10 min ASW washes, and dehydration in an ethanol series (50–100%) followed by a 1 : 1 v/v ethanol : hexamethyldisilazane (HMDS) and 3 × 100% HMDS series. Samples were sputter coated with platinum at 25 mA for 150 s in a Balzars MFD 020 sputter coating unit. Specimens were examined in a JEOL 6400F scanning electron microscope at 10 kV, with special attention given to mouth parts and the anus, which represent respectively high nutrient input and output regions of the animal.

### Fluorescence *in situ* hybridization

Phyllosoma were directly fixed in 4% paraformaldehyde suspended in phosphate-buffered saline (PBS) for 8–16 h at 4°C and transferred to 50% ethanol : 50% PBS and stored at −20°C until further processing. Fixed larvae were placed in histology cassettes, dehydrated through an ethanol series and embedded in paraffin. Tissue sections were cut on a microtome to a thickness of 5 $\mu$m (Zeiss Micron HM330) and transferred to adhesive glass slides (SuperFrost® Plus; Menzel-glaser, Braunschweig, Germany). Slides were incubated at 60°C for 3 h for fixation of sections. Prior to hybridization, paraffin was removed by incubation in xylene for 20 min and sections dehydrated by incubation in an ethanol series (50%, 75%, 85%, 96%) for 10 min at each step. 16S rRNA-targeted oligonucleotide probes used in this study and conditions for their use are presented in Table 1. Probes were labelled either with indocarbocyanine fluorochrome Cy3 or Cy5 (Thermo Hybaid, Ulm, Germany). Hybridization solution [18 $\mu$l of 0·9 mol l$^{-1}$ NaCl, 20 mmol l$^{-1}$ Tris-HCl (pH 7·2), 0·01% SDS, 20–35% (v/v) formamide (probe dependent)] was mixed with 1 $\mu$l of fluorescently labelled oligonucleotides (50 ng $\mu$l$^{-1}$), applied to sectioned phyllosoma and incubated in 50 ml polypropylene tubes at 46°C for 2 h. Probes BET42a, GAM42a, PLA886 and G123T were used with competitor oligonucleotides as described previously (Manz *et al.* 1992; Neef *et al.*; Kanawaga *et al.* 2000). After hybridization, slides were immersed in pre-warmed 20% wash buffer (20 mmol l$^{-1}$ Tris-HCl, 5 mmol l$^{-1}$ EDTA, 0·01% SDS, 225 mmol l$^{-1}$ NaCl) at 48°C for 10 min, rinsed with Milli-Q water to remove excess salts before being air-dried

**Table 1** Oligonucleotide fluorescent *in situ* hybridization probes used in this study

| Probe* | Specificity | Target site† | Sequence from 5′–3′ | FA‡ | Reference |
|---|---|---|---|---|---|
| EUB338 +§ | *Bacteria* | 16S rRNA, 338–355 | GCTGCCTCCCGTAGGAGT | 20–40¶ | Amann *et al.* (1990) |
| Non-EUB338 | Complementary to EUB338 | — | ACTCCTACGGGAGGCAGC | 35 | Amann *et al.* (1990) |
| ALF1b | *α-Proteobacteria* | 16S rRNA, 19–35 | CGTTCGYTCTGAGCCAG** | 20 | Manz *et al.* (1992) |
| BET42a | *β-Proteobacteria* | 23S rRNA, 1027–1043 | GCCTTCCCACTTCGTTT | 35 | Manz *et al.* (1992) |
| GAM42a | *γ-Proteobacteria* | 23S rRNA, 1027–1043 | GCCTTCCCACATCGTTT | 35 | Manz *et al.* (1992) |
| CF319a | *Cytophaga-Flavobacteria-Bacteroides* phylum | 16S rRNA, 319–336 | TGGTCCGTGTCTCAGTAC | 35 | Manz *et al.* (1992) |
| *Vibrio*-GV | *Vibrionaceae* | 16S rRNA, 822–841 | AGGCCACAACCTCCAAGTAG | 30 | Giuliano *et al.* (1999) |
| PLA886 | *Planctomycetes* | 16S rRNA, 886–904 | GCCTTGCGACCATACTCCC | 35 | Neef *et al.* (1998) |
| PLA46 | *Planctomycetes* | 16S rRNA, 46–63 | GACTTGCATGCCTAATCC | 35 | Neef *et al.* (1998) |
| G123T | *Thiothrix* spp. | 16S rRNA, 697–714 | CCTTCCGATCTCTACGCA | 40 | Kanagawa *et al.* (2000) |

*EUB338 + was labelled with Cy-3, all group-specific probes with Cy-5. BET42a, GAM42a, PLA886 and G123T were used in combination with equimolar amounts of their respective unlabelled competitor probes.
†*Escherichia coli* numbering of rRNA position.
‡Per cent formamide in hybridization buffer.
§EUB338 refers to a 1 : 1 : 1 mix of the probes EUB338, EUB338-II and EUB338-III (14).
¶FA (foramide) concentration was dependent on the stringency of the respective group-specific probe.
**Y – variable pyrimidine base.

and mounted in a fluorescence anti-fading gel (Biomedia; ProSciTech). FISH preparations were viewed and imaged on a BioRad MRC-024 confocal laser scanning microscope (CLSM). Replicate slides were processed for each sample and estimates of mean total bacterial counts from 10 fields of view were enumerated per slide.

### Statistical analysis

Various indices and models were used to analyse the variation of microbial diversity within clone libraries (Magurran 1988). These included the Shannon–Weaver diversity index (Shannon and Weaver 1963), the Fisher's Alpha log series richness index (Fisher *et al.* 1943) and Coverage (C) values (Good 1953). OTU groupings determined from RFLP analysis of clones within the libraries were used as input for these models. The diversity of clone libraries was further investigated by rarefaction analysis (Hurlbert 1971; Heck *et al.* 1975; Simberloff 1978). Rarefaction curves were produced by using the analytical approximation algorithm of Hurlbert (1971). Calculations were performed with the freeware program aRarefact Win (Holland 1988).

## Results

### Clone library analysis

Restriction fragment length polymorphism analysis of three clone libraries (100 clones each), two constructed

**Table 2** Calculated diversity indices/percentage of library coverage for cultured *Panulirus ornatus* and wild *Panulirus ornatus* and *Panulirus penicillatus* phyllosoma clone libraries

| Parameters | *P. ornatus* library 1 | *P. ornatus* library 2 | *P. penicillatus* library | Cultured *P. ornatus** |
|---|---|---|---|---|
| Number of clones analysed | 100 | 100 | 100 | 100 |
| Coverage of clone library (%) | 64 | 67 | 74 | 31 |
| Observed RFLP patterns | 36 | 33 | 26 | 70 |
| Shannon–Weaver diversity (H′) | 2·7 | 2·6 | 2·5 | 3·97 |
| Fisher's alpha (α) | 20·2 | 12·2 | 11·4 | 103·64 |
| Chao1 | 98 | 137 | 54 | 283·87 |

RFLP, restriction fragment length polymorphism.
*Cultured live phyllosoma data derived from Payne *et al.* (2007).

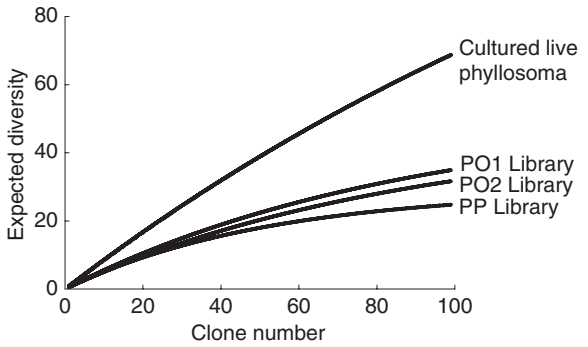
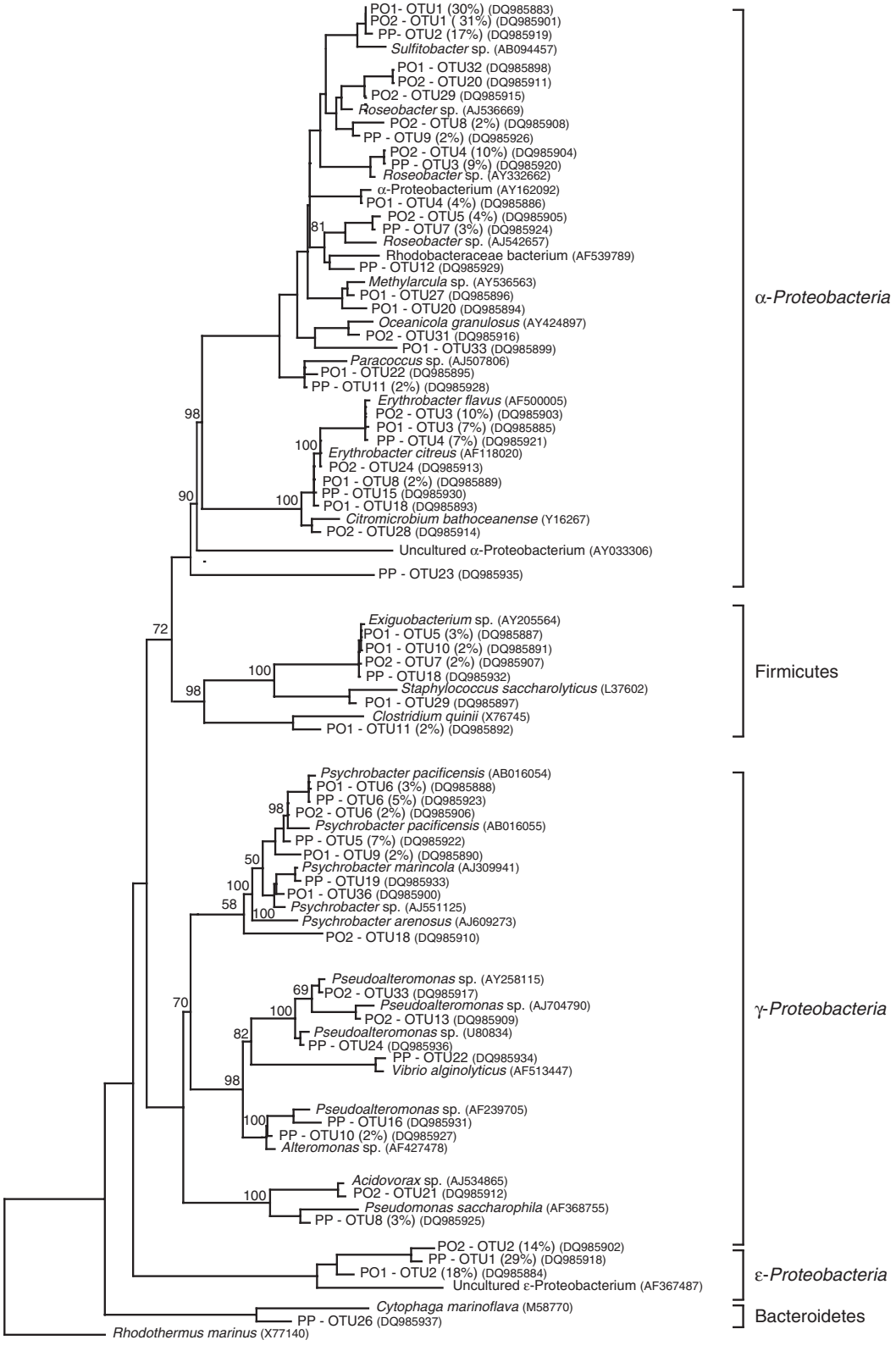



**Figure 1** Rarefaction curves for different restriction fragment length polymorphism patterns representing expected diversity of 16S rRNA gene clones in the wild phyllosoma clone libraries, *Panulirus ornatus* 1 (PO1), *P. ornatus* 2 (PO2), *Panulirus penicillatus* (PP) and cultured *P. ornatus* phyllosoma clone library (cultured live phyllosoma; data derived from Payne *et al.* 2007).





from mid-stage, wild *P. ornatus* phyllosoma (PO1 and PO2) and one from mid-stage, wild *P. penicillatus* phyllosoma (PP), identified 36, 33 and 26 independent OTUs, respectively. Calculated diversity indices (Table 2), library coverage (Table 2) and rarefaction analysis (Fig. 1) demonstrated higher bacterial diversity within the PO1 and PO2 clone libraries compared with the PP clone library. However, direct comparisons with similar studies of reared phyllosoma demonstrated a lower bacterial diversity associated with wild animals as compared with captive reared animals (Payne *et al.* 2007). Rarefaction curves of wild phyllosoma approached an asymptote in contrast to the curve of live reared animals (Fig. 1).

Phylogenetic affiliations for all clones sequenced within the PO1, PO2 and PP libraries are provided in Fig. 2 with most falling within the α-*Proteobacteria*, γ-*Proteobacteria* and *Firmicutes* families. Interestingly, sequence affiliations of the dominant OTU groups were consistent between the clone libraries (Table 3). Sequences affiliated with *Sulfitobacter* spp. constituted the largest OTU groups in both *P. ornatus* libraries (30% and 31% in PO1 and PO2, respectively), and the second largest OTU group in the PP library (17% in PP) (Table 3, Fig. 2). A sequence affiliated with an uncultured ϵ-proteobacterium constituted the largest OTU group in the PP library (29%) and the second largest OTU in the libraries PO1 and PO2 (18% and 14%, respectively). Sequences affiliated with the genera *Erythrobacter, Roseobacter* and *Psychrobacter* were also retrieved from all three libraries. Other sequence affiliations consistent between libraries included *Exiguobacterium*-related sequences in the PO1 (5%) and PO2 (2%) libraries. In contrast, sequences affiliated with *Clostridium* were only detected in the PO1 library (2%), and *Pseudomonas*, *Alteromonas* and *Paracoccus* affiliated sequences were dominant in the PP library only (3%, 2% and 2%, respectively).

A further nine clones from each library, representing individual OTUs, were randomly selected and sequenced to provide additional phylogenetic information on the microbial community associated with wild phyllosoma (Fig. 2). Additional α-*Proteobacteria* related sequences were retrieved from the libraries, including *Methylarcula* sp. (PO1-OTU 20 and 27), *Acidovorax* sp. (PO2-OTU 21), *Citromicrobium* sp. (PO2-OTU 28), *Oceanicola granulosus* (PO2-OTU 31) and *Cytophaga* sp. (PP-OTU 26). Within the PO1 library, one additional clone was affiliated with *Paracoccus homiensis* (PO1-OTU 22), while within the PO2 and PP libraries, additional clones were affiliated with *Pseudoalteromonas* sp. (PO2-OTU 13 and 33; PP-OTU 26). Interestingly, only one *Vibrio*-affiliated sequence was retrieved from analysis of the three libraries (PP-OTU 22), and this was closely related to *Vibrio parahaemolyticus* (Fig. 2).

### Microscopic analysis

Three separate late stage (P8-P10) *P. ornatus* wild phyllosoma were scanned by SEM, and all demonstrated minimal external bacterial colonization (Fig. 3). Histology showed that internal structures of wild phyllosoma were largely intact with no tissue lesions or signs of disease conditions observed within the intestinal tract (Fig. 4a). In addition, no large internal aggregations of bacteria were observed (Fig. 4b). FISH confirmed histological observations with low numbers of metabolically active bacteria detected within the intestinal tract of wild phyllosomas. The degree of colonization varied across the tissue sections, featuring areas without detectable bacterial cells, areas with low bacterial density, and occasional cell accumulations. Consequently, total bacterial numbers within a specimen were difficult to determine, but were estimated to not exceed $10^4$ bacteria per specimen.

Despite low total bacterial numbers within phyllosoma tissue, FISH analysis using group-specific probes showed that distinct numbers of α-*Proteobacteria* and γ-*Proteobacteria* were present (Fig. 4c,d). In addition, members of the *Bacteroidetes* group and cell accumulations of especially β-*Proteobacteria* were repeatedly recorded (Fig. 4e). *Vibrionaceae* were shown to be established within the intestinal tract, but at a very low prevalence (Fig. 4f). The other tested groups, *Planctomycetes* and *Thiothrix* sp., could not be observed within the tissue of wild phyllosoma. FISH analysis of section areas that included the carapace lining, detected minimal external bacterial attachment, supporting SEM analysis of the wild phyllosoma.

**Figure 2** Phylogenetic tree of 16S rRNA gene sequences recovered from clone libraries of wild *Panulirus* sp. phyllosoma obtained from Great Barrier Reef waters; *Panulirus ornatus* 1 (PO1 clones), *P. ornatus* 2 (PO2 clones), *Panulirus penicillatus* (PP clones). The scale bar represents 0·1 changes per nucleotide. GenBank accession numbers are provided for all retrieved clones and reference strain sequences. The proportion of each dominant clone type within the library is represented in brackets following the clone name (also summarized in Table 3). Clones without representative percentages represent single sequenced clones randomly selected from the libraries. Partial clone sequences were added to the phylogenetic tree using the parsimony algorithm tool in the ARB software package (Ludwig *et al.* 2004). *Rhodothermus marinus* was used as an outgroup for the analysis. Bootstrap values ≥50% are represented at the nodes of branching points.

**Table 3** Genus affiliation of dominant* bacterial sequences retrieved from 16S rRNA gene clone libraries derived from wild *Panulirus ornatus* (PO1 and PO2) and *Panulirus penicillatus* (PP) phyllosoma samples. The percentage of dominant sequences affiliated with each genus is presented, together with the corresponding operational taxonomic units (OTU) numbers in each respective library

| Genus† | PO1‡ | PO2 | PP |
|---|---|---|---|
| *Sulfitobacter* | 30% (OTU-1) | 31% (OTU-1) | 17% (OTU-2) |
| Uncultured $\epsilon$-proteobacterium | 18% (OTU-2) | 14% (OTU-2) | 29% (OTU-1) |
| *Erythrobacter* | 9% (OTU 3, 8) | 10% (OTU-3) | 7% (OTU-4) |
| *Roseobacter* | 4% (OTU-4) | 16% (OTU 4, 5, 8,) | 2% (OTU 3, 7, 9) |
| *Exiguobacterium* | 5% (OTU 5, 10) | 2% (OTU-7) | – |
| *Psychrobacter* | 5% (OTU 6, 9) | 2% (OTU-6) | 12% (OTU 5, 6) |
| *Clostridium* | 2% (OTU-11) | – | – |
| *Pseudomonas* | – | – | 3% (OTU-8) |
| *Alteromonas* | – | – | 2% (OTU-10) |
| *Paracoccus* | – | – | 2% (OTU-11) |

*Sequence affiliations are provided for OTUs represented by ≥2% of the total number of clones in the library.

†Sequences were aligned to the representative relative species using BLAST (Altschul *et al.* 1997).

‡OTU-7 was identified as a chimera and removed from further analysis.

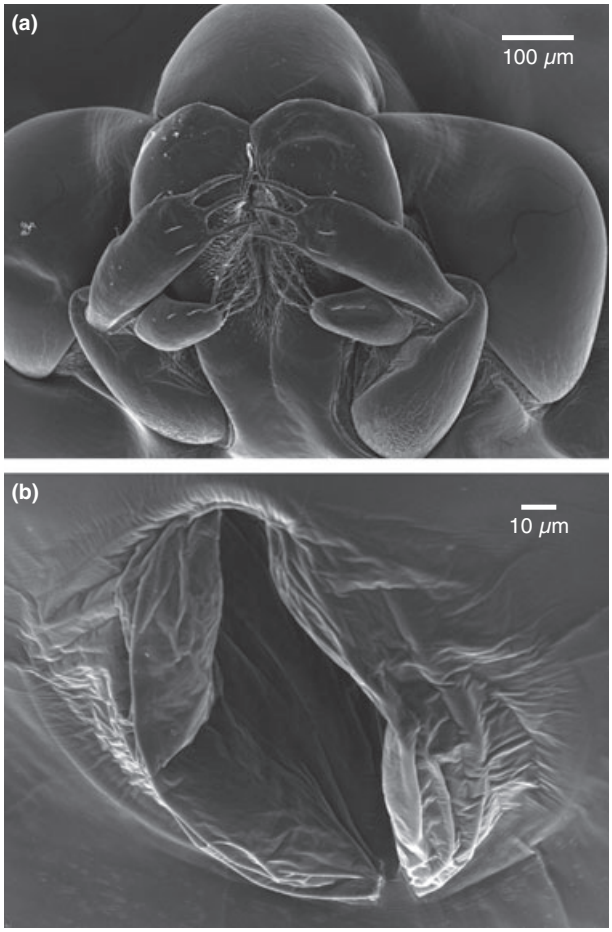



**Figure 3** Scanning electron micrographs of wild, P8-P10 *Panulirus ornatus* phyllosoma showing minimal bacterial colonization of the (a) mouthparts and (b) anus.

## Discussion

The microbial community associated with wild-caught, mid-stage (P8-P10) Palinurid phyllosoma differed substantially both in terms of diversity and bacterial loading when compared with similar analysis of aquaculture-reared early and mid-stage animals (Payne *et al.* 2007). Both SEM and FISH microscopy analyses of wild animals showed low levels of bacterial colonization on the external surface of wild phyllosoma. In contrast, reared phyllosoma are often extensively colonized by filamentous bacteria (predominantly *Thiothrix* sp.), in addition to rod- and coccoid-shaped bacteria (Bourne *et al.* 2004; Payne *et al.* 2007). In addition, histopathological analysis of wild phyllosoma showed no internal tissue lesions or bacterial aggregations, confirming that the studied wild-caught specimens were healthy. Again, this was in contrast to reared animals, where both development of lesions and proliferation of bacteria in the hepatopancreas tubule lumen have been observed (Bourne *et al.* 2004, 2007).

The diversity of the bacterial community associated with *P. ornatus* and *P. penicillatus* phyllosoma was similar, with many identical sequences retrieved from three independent clone libraries. Direct comparisons of diversity parameters derived from clone libraries demonstrated substantially higher bacterial diversity associated with captive reared phyllosoma as compared with wild phyllosoma. This is most likely attributed to the high stocking densities and complex nutrient microniches within aquaculture tanks that promote bacterial growth. It is unlikely that the dominant bacterial sequences retrieved from wild phyllosoma clone libraries were derived from sample contamination from the surrounding seawater, as the animals were rinsed in sterile ASW to removed loosely attached microbes.

Clone library analysis indicated that the distinct phyllosoma-associated bacterial community was dominated by *α-Proteobacteria*, and this group was also detected using a group-specific FISH probe. In the clone libraries, especially the *Roseobacter* spp. clade was predominant, and was represented by *Sulfitobacter* spp. and *Roseobacter* spp. affiliated sequences. This group has previously been shown to be an important constituent of the marine microbiota, and may account for up 40% of prokaryotic DNA from the ocean (Gonzalez *et al.* 2000; Selje *et al.* 2004; Bruhn *et al.* 2005). Previous investigations of aquaculture-reared phyllosoma only recovered a small

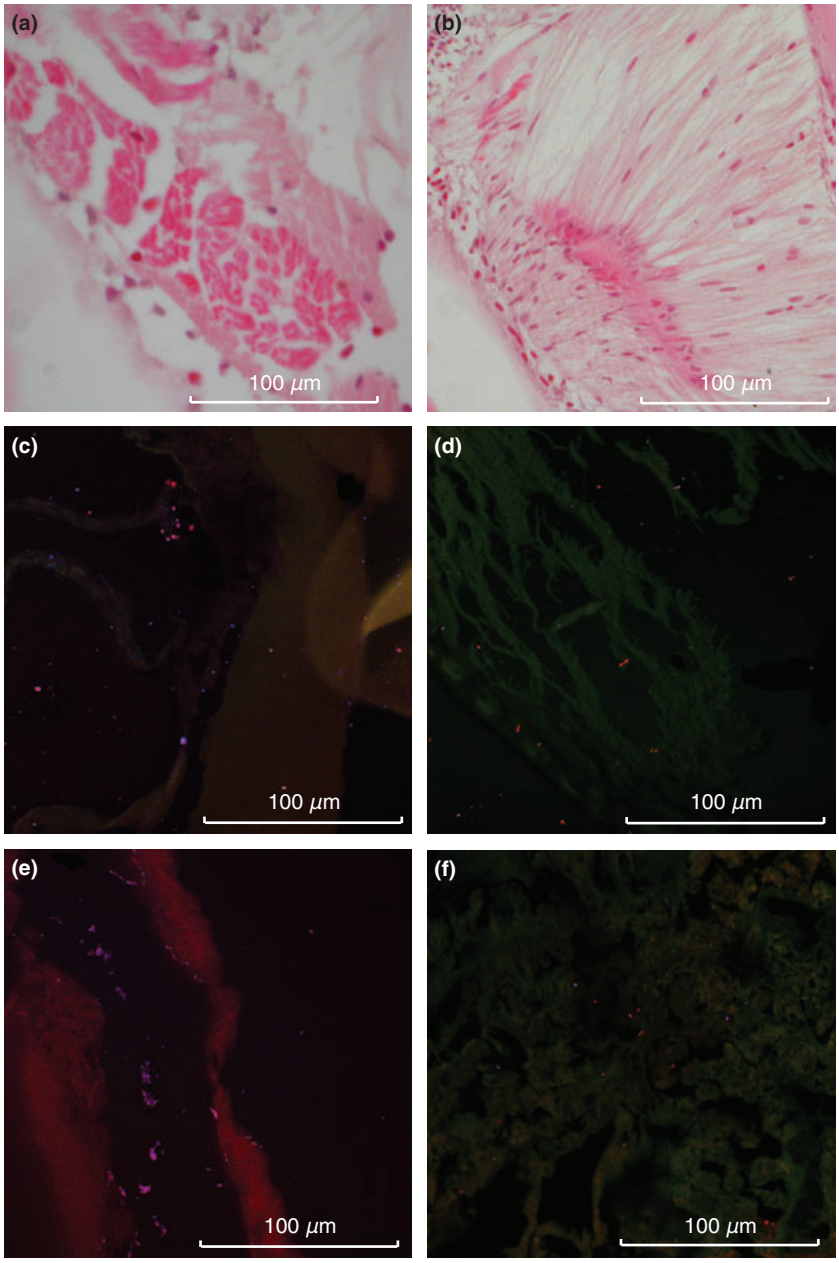



**Figure 4** Histopathological and FISH micrographs of wild, P8-P10 *P. ornatus* phyllosoma. Histopathology sections showed internal structures within (a) intact hepatopancreas tissue and (b) connective tissue. No tissue lesions or signs of disease were observed. FISH analyses of phyllosoma tissue sections were performed with a mixture of Cy-3 labelled *Bacteria* specific probes (EUB338+) and one of eight Cy-5 labelled group specific probes. Cells that appear magenta are positive for the respective group specific probe and those that appear red are other bacteria. (c) Phyllosoma tissue section hybridized with EUB338+ and an *α-Proteobacteria* specific probe (ALF1b). (d) Pyllosoma tissue section probed with EUB338+ and a *γ-Proteobacteria* specific probe (GAM42a). (e) Phyllosoma tissue section probed with EUB338+ and a *β-Proteobacteria* specific probe (BET42a). (f) Pyllosoma tissue section probed with EUB338+ and a *Vibrionaceae* specific probe (*Vibrio*-GV).

proportion (∼2%) of *Sulfitobacter* spp.-affiliated sequences from a clone library of apparently healthy animals (Payne *et al.* 2007). Similarly, *Roseobacter* spp.-affiliated sequences were found to be poorly represented in reared phyllosoma clone libraries (Payne *et al.* 2007).

Certain members of the Roseobacter clade are believed to play an important role in oceanic sulphur (Moran *et al.* 2003; Bruhn *et al.* 2005) and carbon cycling (Wagner-Dobler and Biebl 2006). Although the ecological role of the Roseobacter clade populations detected on wild phyllosoma is currently unknown, their dominance in the bacterial community suggests that they may possess attributes that promote phyllosoma health. In fact, the probiotic attributes of this clade is previously acknowledged, with some members shown to be successful in improving larval survival of fish and crustacean aquaculture species (Hjelm *et al.* 2004a,b; Bruhn *et al.* 2005, 2006; Makridis *et al.* 2005). Therefore, potential exists for the probiotic application of these genera to improve reared phyllosoma health, with preliminary studies currently underway to specifically isolate these groups from wild phyllosoma and apply to larval rearing trials.

Other sequences recovered from all three libraries included an uncultured organism of the $\epsilon$-*Proteobacteria* group, a group whose members potentially obtain energy by oxidation of reduced sulfur compounds (Moyer *et al.* 1994; Cary *et al.* 1997; Reysenbach *et al.* 2000; Corre *et al.* 2001; Longnecker and Reysenbach 2001; Takai *et al.* 2003) and *Erythrobacter flavus*, a newly described member of the genus *Erythrobacter* (Yoon *et al.* 2003). This genus is now recognized as a component of the marine microbial community that apparently plays a critical role in organic and inorganic carbon cycling in the ocean (Yoon *et al.* 2003).

Detection of only one *Vibrio* sp.-affiliated sequence in the three clone libraries indicated that this genus constituted a minor component of the wild phyllosoma bacterial community. Metabolically active *Vibrio* cells were nevertheless occasionally detected by FISH analysis, showing that this genus is indeed a part of the natural bacterial community also for wild phyllosoma. Previous culture-independent studies of both live and dying reared phyllosoma retrieved numerous *Vibrio* sp.-affiliated sequences, and vibrios are commonly associated with disease in crustacean aquaculture (Karunasager *et al.* 1994; Lee *et al.* 1996a,b; Liu *et al.* 1996; Vandenberghe *et al.* 1998; Sudheesh and Xu 2001; Bourne *et al.* 2006; Webster *et al.* 2006; Payne *et al.* 2007). Within our phyllosoma rearing facilities, Webster *et al.* (2006) demonstrated the progressive build up of *Vibrio* sp. cells within the hepatopancreas of reared live phyllosoma over a 22-day period, resulting in an explosion of *Vibrio* sp. cells on day 18, coinciding with mass larval mortalities.

The differences in the microbial composition of reared and wild phyllosoma are indicative of the different environments in which the animals live. The stresses stemming from high stocking densities and suboptimal water conditions in the culture environment appear to support the growth of a microbial community that is dominated by organisms capable of compromising the health of phyllosoma, such as *Vibrio* sp. In comparison, the phyllosoma's natural environment appears to support a microbial community that is largely dominated by $\alpha$-protebacterial organisms, such as *Sulfitobacter* spp., and with few organisms known to compromise phyllosoma health.

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