

## Microbial Diversity within Early-Stage Cultured *Panulirus ornatus* Phyllosomas<sup>∇</sup>

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A thorough understanding of the microorganisms and pathogens associated with the larval stage of the tropical ornate rock lobster, *Panulirus ornatus*, is required to overcome disease outbreaks that currently block aquaculture attempts. This study used microscopy in addition to culture and molecularly based microbiological techniques to characterize the bacterial community associated with cultured, developmental stage PI to PII *P. ornatus* phyllosomas. Scanning electron microscopy demonstrated colonization of phyllosomas by filamentous, rod-shaped, and coccus-shaped bacteria. A clone library constructed from dead phyllosomas sampled from the larval rearing tank on day 10 was dominated by *Thiothrix*-affiliated sequences (56% of clones). A comparable library from live phyllosomas also contained *Thiothrix*-affiliated sequences, though these only represented 19% of clones within the library. Fluorescent *in situ* hybridization (FISH) confirmed identification of the filamentous bacteria as *Thiothrix* sp., being present on dead phyllosomas. FISH also identified *Leucothrix* sp. and *Vibrio* sp., as well as a range of other rod- and coccus-shaped bacteria, colonizing both live and dead phyllosomas. The development of the microbial community associated with phyllosomas was monitored through a standard larval rearing run using denaturing gradient gel electrophoresis (DGGE). *Vibrio* sp.-affiliated bands dominated the profiles of live animals through the rearing period and dead phyllosomas sampled on selected days. The population of *Vibrio* sp. associated with phyllosomas was monitored with culture-based analysis on selective media and demonstrated to increase significantly on day 7, coinciding with the beginning of the larval molt. An isolated *Vibrio harveyi* strain demonstrated an identical 16S rRNA sequence with retrieved DGGE and clone library sequences. Colonization of phyllosomas with filamentous bacterial species potentially hinders the ability of the animals to molt and, combined with the added stress of the molt process, likely results in reduced immune function, allowing opportunistic pathogenic *Vibrio* sp. to cause larval mortalities.

Market forces have demanded an increased research investment into the culture of the tropical ornate rock lobster, *Panulirus ornatus* (1). However, a number of constraints, including an extended larval phase (34), a lack of suitable live and artificial feeds, and disease (5, 58, 73) have prevented the establishment of this aquaculture industry. During larval development, *P. ornatus* typically undergoes 20 or more molts over the extended larval period of approximately 160 days (33). This period is far greater than that of other commonly cultured crustaceans such as penaeid prawns, which typically undergo 12 molts over approximately 15 days before reaching postlarval stages (30). The long larval cycle of phyllosomas accentuates difficulties associated with controlling disease. Phyllosoma, like most crustacean larvae, are particularly susceptible to microbial infection, especially during the molt process, prior to the new shell hardening and subsequently providing some protection against pathogen invasion (41, 74). In addition, the culture environment tends to expose larvae to a variety of stresses that can compromise the larvae's immune system (70). Antibiotic and chemical treatments commonly used in aquaculture environments remove some but not all bacteria associated with phyllosomas, effectively selecting for certain organisms and

accentuating the dynamics of the microbial community directly associated with phyllosomas (5).

Most studies examining the microbiological aspects of crustacean larval rearing have focused on the isolation and identification of pathogenic organisms, with *Vibrio* sp. being the most commonly reported pathogen (13, 21, 23, 24, 37, 39, 40, 51, 53, 62, 63). Previous research has questioned whether *Vibrio* spp. are in fact primary pathogens, instead opportunistically proliferating when the immune system of the host has become compromised as a result of other infectious agents or water quality issues (59, 61, 65, 68, 72). Particular species implicated in disease in larval and juvenile rock lobsters have included, *V. alginolyticus*, *V. anguillarum*, *V. harveyi*, and *V. tubiashii* (13, 23). Filamentous bacteria have also been associated with disease in aquaculture target species, including larval and juvenile rock lobsters. Bourne et al. (5) identified a *Thiothrix*-affiliated sequence in denaturing gradient gel electrophoresis (DGGE) analysis of early-stage *P. ornatus* phyllosomas. Diggle et al. (13) and Handlinger et al. (23) reported "*Leucothrix*-like" bacteria as disease causing organisms in *Jasus* sp. rock lobsters, while Johnson et al. (29) described an infestation of *Leucothrix mucor* on the surface of larvae of the lobster *Homarus americanus*.

Bourne et al. (5) examined the microbial diversity within a *P. ornatus* larval rearing system and reported a number of bacterial genera associated with phyllosoma larvae, including *Alteromonas* sp., *Desulfobulbus mediterraneus*, *Pirulella* sp., *Pseudoalteromonas* sp., an uncultured gammaproteobacte-

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rium clone, and a number of *Vibrio* sp. *Vibrios* were reported to be the dominant organisms with *Vibrio parahaemolyticus* being the dominant species. Further research reported the proliferation of *Vibrio* spp. within the hepatopancreas of *P. ornatus* phyllosomas and associated internal bacterial proliferation with larval mortality (73). In order to help establish *P. ornatus* as an aquaculture species, it is necessary to have a thorough understanding of the microbial composition and dynamics of the larval rearing system and how these impact on phyllosoma health. Such an understanding will allow a more effective microbial management regime to increase phyllosoma survival. The aim of the present study was to characterize the microbial community associated with cultured, early-stage *P. ornatus* phyllosomas and to determine how this microbial community changes over time in an attempt to identify bacteria that compromise the animal's health. The study focused on early-stage larval rearing since high mortality is frequently observed in this period.

#### MATERIALS AND METHODS

**Larval rearing technology.** The larval rearing process, including broodstock source, treatment and spawning, tank design, stocking methods, feeding regimes, and water recirculation and treatment regimes, was conducted according to the methods of Bourne et al. (5). During standard larval rearing trials, molting from phyllosoma stages PI to PII occurs between days 7 and 12. Mass larval mortalities are observed during this period and commonly peak on day 10, being characterized by a larger than normal number of dead larvae accumulating on the bottom of the larval rearing tank. Typical rearing attempts result in surviving phyllosomas falling below 1% of the original population within 30 days of commencing the trial (5).

**Sample collection.** Live phyllosomas were sampled from the top of the water column by focusing a light source into the larval rearing tank and collecting the attracted animals. Dead phyllosomas (1 to 4 h postmortem) were siphoned from "dead patches" on the bottom of the tank and sampled only on days where patches were observed. For clone library and fluorescence in situ hybridization (FISH) analysis, both live and dead samples were obtained on the day of a larval mass mortality event (day 10). For DGGE analysis, live phyllosomas were collected each day up until 5 days past a larval mass mortality event, and dead phyllosomas were collected on days where dead patches were observed. Live phyllosomas were collected 1 day prior to the PI-PII molt for scanning electron microscopy (SEM) and daily for culture-based analysis up until a larval mass mortality event, at which time dead phyllosomas were also collected. All collected phyllosomas were washed briefly in sterile artificial seawater (ASW) prior to sample processing.

**Culture-based microbial analysis.** *Vibrionaceae* numbers associated with live phyllosomas were monitored daily through a larval rearing trial by collecting triplicate samples, each containing three larvae. Samples were homogenized with a pestle, resuspended in 1 ml of ASW, and serially diluted, and 100  $\mu$ l from each dilution was inoculated onto thiosulfate-citrate-bile salt-sucrose (TCBS) agar (Difco) in triplicate. Plates were incubated overnight at 28°C, and individual colonies were counted, with the CFU counts standardized per phyllosoma. Plates affected by swarming cultures were not included in the analysis.

On the day of a larval mass mortality event (day 10), both live and dead phyllosomas were collected and processed separately as described above. Serial dilutions were inoculated onto Marine agar (Difco/ Becton Dickinson, Australia) and TCBS agar (Difco), followed by incubation overnight at 28°C. Morphologically distinct colonies were subcultured for later 16S rRNA analysis.

**Extraction of genomic bacterial DNA.** Genomic DNA was extracted from isolated bacterial cultures by using a QIAGEN DNeasy tissue kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions for gram-positive bacteria. Similarly, total bacterial DNA associated with phyllosomas was extracted from approximately 10 homogenized phyllosomas by using a QIAGEN DNeasy tissue kit. All DNA was quantified on a 1.5% agarose gel stained with ethidium bromide (0.5  $\mu$ g ml<sup>-1</sup>) and stored at -20°C.

**PCR amplification of 16S rRNA genes.** The bacterial 16S rRNA gene was amplified from both isolates and extracted DNA from phyllosomas by using the conserved primers 27f and 1492r as described by Lane (35). For DGGE analysis, PCR on extracted bacterial DNA from phyllosomas was conducted as described

by Ferris et al. (14), using the primers 1055f (conserved for domain *Bacteria*) and 1392r (universal conserved primer) (14). A 40-bp GC-clamp was attached to the 5' end of the 1392r primer (14, 55). These primers amplify a 323-bp section of the 16S rRNA gene of members of the domain *Bacteria*, including the highly variable V9 region. All PCRs were performed in either an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) or a PE Applied Biosystems GeneAmp PCR system 9700 (Perkin-Elmer, Maryland). PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide (0.5  $\mu$ g ml<sup>-1</sup>) and stored at -20°C.

**Clone library construction analysis.** Amplified bacterial 16S rRNA gene PCR products (27f/1492r) obtained from day 10 live and dead phyllosoma samples were cloned by using a TOPO TA cloning kit (version K2; Invitrogen, California) in accordance with the manufacturer's instructions. A total of 100 clones were randomly selected, and the 16S rRNA gene was reamplified. Clones were screened by restriction fragment length polymorphism (RFLP) analysis of PCR products using the restriction enzymes HhaI and HaeIII (Promega, Wisconsin). RFLP profiles were visualized on a 3% agarose gel and stained with ethidium bromide (1  $\mu$ g ml<sup>-1</sup>), and clones were grouped into operational taxonomic units (OTUs). Representative clones of each OTU were selected and grown in Luria-Bertani (LB) ampicillin (50  $\mu$ g ml<sup>-1</sup>) broth overnight. Plasmid DNA was extracted by using a QIAprep Spin Miniprep kit (QIAGEN) in accordance with the manufacturer's instructions, quantified, and directly sequenced.

**DGGE.** We performed DGGE by using an INGENY phorU-2 (Ingenuity International BV) DGGE system. PCR products were run on a 6.5% acrylamide gel with a 50 to 70% linear gradient of urea and formamide and electrophoresed at 60 V and 60°C for 20 h. Denaturing gradient gels were stained, destained, and photographed as described by Payne et al. (58). Distinct bands were excised, and DNA was eluted overnight at 4°C in 100  $\mu$ l of nuclease-free water (Ambion) and reamplified by PCR as previously described. DGGE was performed on reamplified bands to check the mobility against original excised bands, and original PCR products of correct mobility were purified and directly sequenced.

**Sequencing and phylogenetic analysis.** Partial and complete 16S rRNA gene sequences of bacterial clones were obtained by using the primers 27f, 339f, 732f, and 1492r (35). DGGE PCR products were sequenced with the primer 1055f (14). Automated sequencing was performed using either Amersham DYEnamic ET-terminator sequencing dye (Amersham Biosciences) or ABI BigDye terminator v3.1 sequencing dye (Applied Biosystems, California) in accordance with the manufacturer's instructions. Sequences were checked for chimera formation with the CHECK\_CHIMERA software of the Ribosomal Database Project (48). Sequence data were aligned to the most similar sequence by using the BLAST database algorithm (3) and then further analyzed with the ARB software package (43). Tree topologies were evaluated by reconstructing phylogenies using evolutionary distance (PHYLP distance method with the Jukes and Cantor model) analysis of aligned near full-length sequences (>1,000 bp) (42). Regions of ambiguous sequence were removed from the analysis. Aligned, partial 16S rRNA sequences (<1,000 bp) were subsequently inserted without changing the overall tree topology using the parsimony tool available within ARB. Bootstrap values were obtained for branching patterns by using the PHYLP software package (version 3.65) (60), and values of  $\geq 50\%$  were included for the main nodes of the tree. The nucleotide sequence data of all isolates, DGGE bands, and clones appear in the GenBank nucleotide database under the accession numbers DQ831086 to DQ831095 and DQ831116 to DQ831122, DQ831072 to DQ831085 and DQ831109 to DQ831114, and DQ831043 to DQ831071 and DQ831096 to DQ831108, respectively.

**SEM.** Day 9, PI phyllosomas were fixed in 3% glutaraldehyde diluted with 0.22- $\mu$ m-pore-size-filtered ASW and left overnight at 4°C. The glutaraldehyde solution was removed and replaced with ASW twice, and fixed larvae were stored at 4°C. Prior to SEM analysis, larvae were washed in ASW three times at 10 min for each wash. Larvae were then fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (2 h at 4°C), washed in Milli-Q (Millipore) water (3  $\times$  10 min) and dehydrated in an ethanol series (50% to 100%), followed by a 1:1 (vol/vol) ethanol-hexamethyldisilazane and 3  $\times$  100% ethanol-hexamethyldisilazane series. Larvae were sputter coated with platinum at 25 mA for 150 s in a Balzers MFD 020 sputter coating unit. Specimens were examined in a JEOL 6400F scanning electron microscope at 10 kV.

**FISH.** Day 10, PII phyllosomas were suspended in a 4% paraformaldehyde-phosphate-buffered saline solution and stored in darkness at 4°C overnight. After fixation, the larvae were washed three times in a 1:1 (vol/vol) phosphate-buffered saline-ethanol buffer and stored at 4°C until required. Larvae were placed onto a slide and hybridization solution containing the appropriate amount of formamide, and 50 ng of each probe was added, followed by incubation at 46°C for 2 to 3 h. Larvae were individually washed for 10 to 15 min in 50 ml of an appropriate wash buffer preheated to 48°C, rinsed with Milli-Q water, and filtered onto a 0.2  $\mu$ m GTTP filter (Millipore) for orientation. Larvae were



TABLE 1. Oligonucleotide probes used for FISH

Probe	Sequence	rRNA target site (position)	Specificity	% Formamide	Source or reference
EUB338	GCTGCCTCCCGTAGGAGT	16S (338–355)	Bacteria	20	3a
GV	AGGCCACAACCTCCAAGTAG	16S (841–822)	<i>Vibrionaceae</i>	30	18a
G123T	CCTTCCGATCTCTATGCA	16S (697–714)	<i>Thiothrix</i> sp.	40	31
LMU	CCCCTCTCCCAAACCTCTA	16S (652–669)	<i>Leucothrix mucor</i>	35	72a
NonEUB338	ACTCCTACGGGAGGCAGC	16S (338–355)	Bacteria (negative control probe)	20	3a

covered with fluorescence microscope oil (Zeiss), and a coverslip was placed on top and viewed and imaged on a Bio-Rad MRC-1024 confocal laser scanning microscope. The illumination source was a 15-mW argon-krypton laser (American Laser Corp.) with excitation peaks at 488 nm (blue), 568 nm (green), and 647 nm (red). The images were captured in three different photomultiplier tubes to distinguish between the probe conferred fluorescence and autofluorescence of the phyllosoma tissue. A 560-nm long-pass emission filter separated the green signal (fluorescein isothiocyanate) from the red signal, and a 640-nm short-pass emission filter separated the far-red signal (from Cy5) from the near-red signal (from Cy3). The confocal laser scanning microscope was controlled by an OS-2 PC running the Bio-Rad LaserSharp software package. Images were collected, and the final image evaluation was done in Adobe PhotoShop version 7.0. The green emission was presented in the green channel of the color image, the red emission was presented as the red channel and, by convention, the far-red emission (Cy5) was presented as the blue channel. The various probes, probe combinations, their respective formamide levels, and fluorochromes are provided in Table 1. The specificity of each probe was established by previous studies and checked against relevant controls with specificity references provided in Table 1.

**Statistical analysis.** Culture-based bacterial counts were analyzed by using a one-way analysis of variance in SPSS version 10. Homogeneity of variances for the data set was not assumed. The data were transformed by using a  $\log_{10}$  transformation, graphed on a histogram, and a normality curve was applied to check the normal distribution of the data. Where significant differences were recorded, data were further analyzed by using a Dunnett T3 post-hoc test.

Clone library data were analyzed by using various indices and models in order to give an indication of the variation of microbial diversity within the clone library (47). The freeware program, EstimateS, version 7.5 (10), was used to calculate the following indices: the Shannon-Weaver index (64), calculated as:

$$H' = - \sum_{i=1}^S p_i \ln(p_i)$$

where  $p_i$  is the proportion of clones belonging to the  $i$ th OTU and  $S$  is the total number of OTUs (52); the Fisher alpha log series richness index (15); and Chao1 (8, 9), calculated based on the number of species in a sample that are represented by one or two individuals (16). Rarefaction analysis was conducted on the clone library (25, 28, 67), and a rarefaction curve was produced by using the analytical approximation algorithm of Hurlbert (28). Calculations were performed on a personal computer with the freeware program aRarefact Win (26). Coverage ( $C$ ) values were calculated by the equation  $C = [1 - (n/N)] \times 100$ , where  $n$  is the number of unique clones, and  $N$  is the total number of clones examined (20).

## RESULTS

**SEM analysis.** Analysis of live day 9 PI phyllosomas (1 day premolt) by SEM revealed colonization of the phyllosoma exterior surface by a range of bacteria. Filamentous, rod-shaped, and coccus-shaped bacteria were observed on the anus of the phyllosoma. Often, individual filaments had become entangled among one another as a result of the colonization (Fig. 1a). Colonization of PI phyllosoma mouthparts by filamentous bacteria was low (Fig. 1b); however, extensive colonization of the mouthparts of later-stage PIII phyllosomas (prior to molting) has been recently observed (4; results not shown). Similar extensive colonization of the carapace of later-stage phyllo-

mas by filamentous, rod-shaped, and coccus-shaped bacteria has also been commonly observed (4).

**Clone library analysis.** RFLP analysis of two clone libraries (100 clones each) constructed from live and dead phyllosomas collected on day 10 of a larval rearing trial identified 70 and 36 independent OTUs, respectively (Fig. 2). Representatives of OTUs that contained two or more clones were sequenced, and this consisted of 11 from the live phyllosoma library (LPL) and

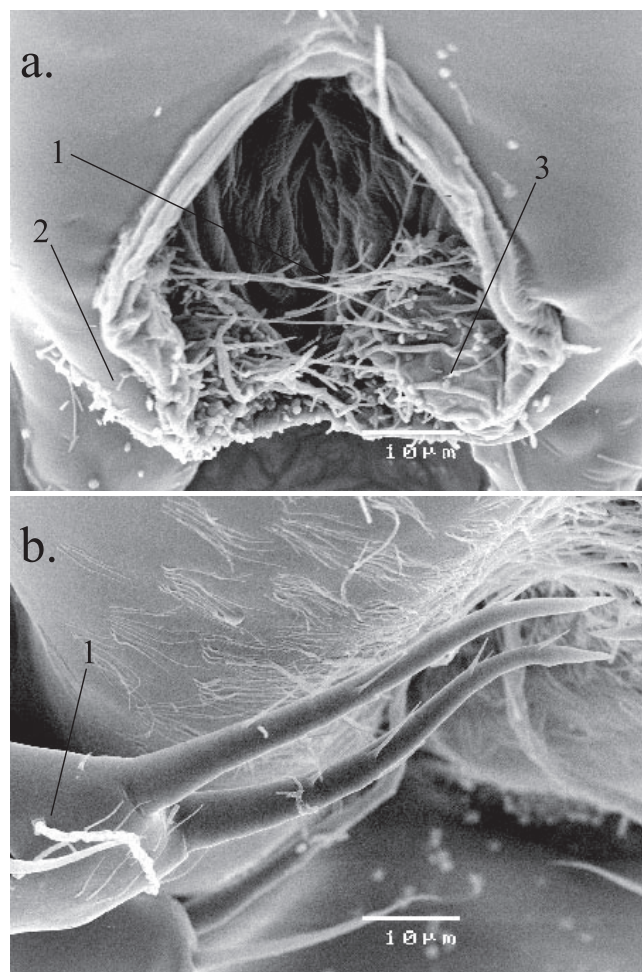


FIG. 1. Colonization of phyllosomas by filamentous, rod-shaped and coccus-shaped bacteria. (a) Scanning electron micrograph of 1 day premolt, PI stage live phyllosoma anus showing colonization by filamentous (1), rod-shaped (2), and coccus-shaped (3) bacteria. (b) Scanning electron micrograph of 1 day premolt, PI stage live phyllosoma mouthparts showing colonization by filamentous bacteria (1).

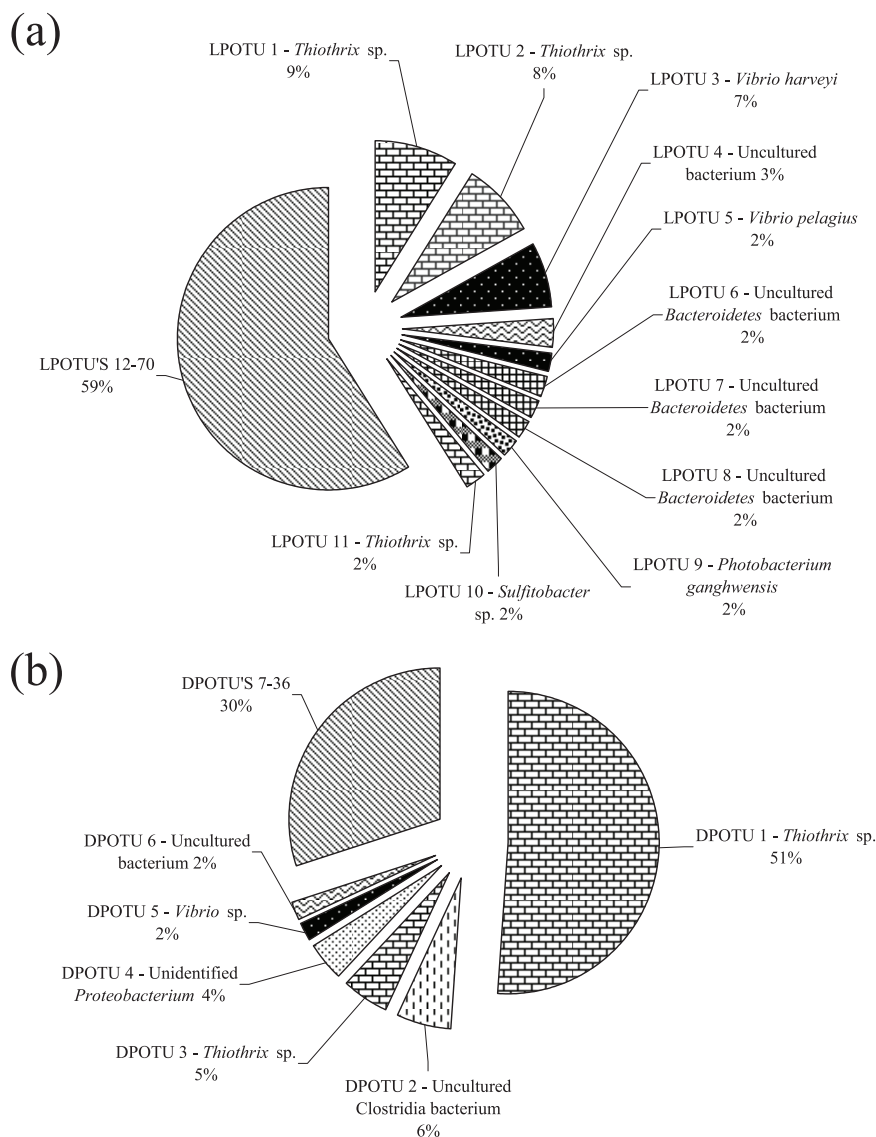


FIG. 2. Frequencies of OTUs detected in 16S rRNA clone libraries derived from live (a) and dead (b) phyllosoma samples. Sequence affiliations are provided for OTUs representative of  $\geq 2\%$  of the total number of clones in the library. Calculations were made based upon the total number of clones associated with an OTU from which one or more representative clones had been sequenced.

6 from the dead phyllosoma library (DPL). The DPL was dominated by a sequence affiliated with a *Thiothrix* sp. that represented 51% of the library. A sequence affiliated with this same *Thiothrix* sp. also represented the largest OTU of the LPL (9%). OTUs 2 and 11 from the LPL and OTU 3 from the DPL were also affiliated with *Thiothrix* species, and each represented 8, 2, and 5% of their respective libraries. In addition, *Vibrio* sp.-affiliated sequences were present in both libraries, with OTU 3 from the LPL being closely affiliated with *Vibrio harveyi* (7% of the library) and OTU 5 affiliated with *Vibrio pelagius* (2% of the library). Within the DPL library, OTU 5, representing 2% of clones, was most closely affiliated with a *Vibrio* sp. Another retrieved sequence of interest, OTU 2, represented 6% of the DPL library and was affiliated with a strict anaerobe marine *Clostridia* bacterium. Within the LPL,

OTU 10 represented 2% of the library and was affiliated with the sulfite-oxidizing bacterium, *Sulfitobacter* sp.

A further 18 clones from the LPL and 7 clones from the DPL, representing individual OTUs, were randomly selected and sequenced to provide additional phylogenetic information on the microbial community associated with live and dead phyllosomas. Within the DPL, three additional clones were affiliated with *Clostridiales* bacteria: *Clostridium halophilum* (OTU 11), *Clostridium litorale* (OTU 18), and *Clostridium collagenovorans* (OTU 43). A *V. harveyi*-affiliated sequence was retrieved from the LPL, along with several *Alphaproteobacteria*-related sequences, including *Roseobacter pelophilus* (OTU 21) and two *Rhodobacteraceae* bacteria (OTUs 23 and 39). *Alphaproteobacteria*-affiliated sequences were also retrieved from the DPL (OTU 23 [*Mesorhizobium* sp.] and OTU 33

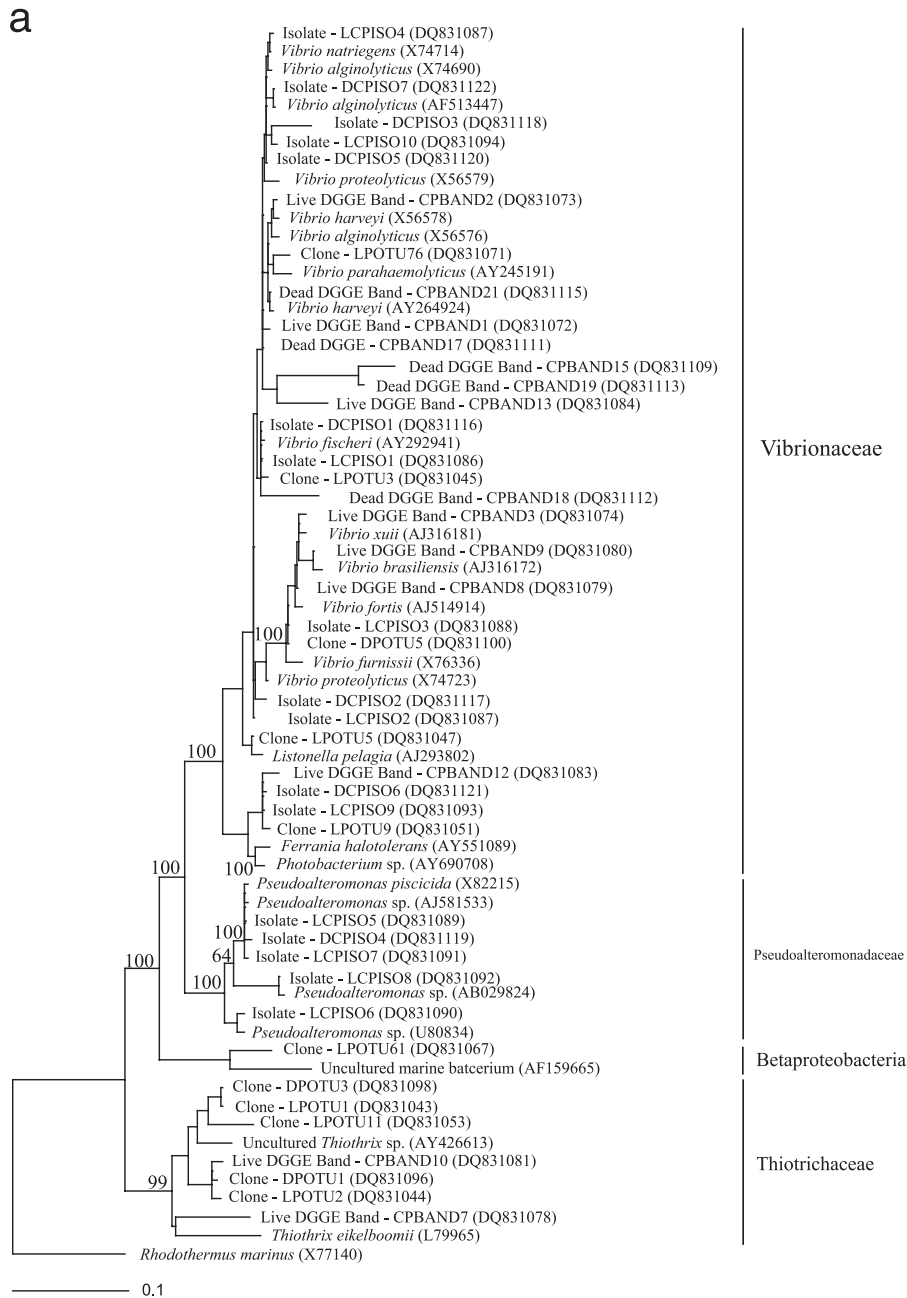


FIG. 3. Phylogenetic tree of 16S rRNA gene sequences recovered from bacterial isolates, clone libraries, and DGGE analysis of live and dead early stage *P. ornatus* phyllosomas taken from the larval rearing system. (a) *Gammaproteobacteria*; (b) sequences within the families *Alphaproteobacteria*, *Betaproteobacteria*, *Epsilonproteobacteria*, and the *Bacteroidetes* group. The scale bar represents 0.1 changes per nucleotide. GenBank accession numbers are provided for all isolate, clone, DGGE, and reference sequences. The proportion of each clone within the clone library is represented in brackets after the clone name. The locations of the DGGE bands are given in Fig. 4. Partial isolate, clone, and DGGE sequences were added to the phylogenetic tree by using the parsimony algorithm tool in the ARB software package (43). *Rhodothermus marinus* was used as an outgroup for the analysis. Bootstrap values of  $\geq 50\%$  are represented at the nodes of branching points.

[*Roseobacter* sp.]). Phylogenetic affiliations for all OTUs sequenced within these libraries are provided in Fig. 3, with most falling in the *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, and *Firmicutes*.

Calculated diversity indices (LPL/DPL [Shannon-Weaver  $H'$ , 3.97/2.32; Fisher alpha, 103.64/20.17; and Chao-1, 283.87/181]), the percentage of library coverage (LPL, 31%; DPL, 64%), and

the absence of a visible asymptote in the LPL rarefaction analysis curve (figure not shown) confirmed higher bacterial diversity within the LPL than within the DPL, supporting the larger number of RFLP patterns detected within this library. All indices for the LPL confirmed that analysis of a larger number of clones would be required to obtain a better representative picture of the bacterial diversity within this sample.

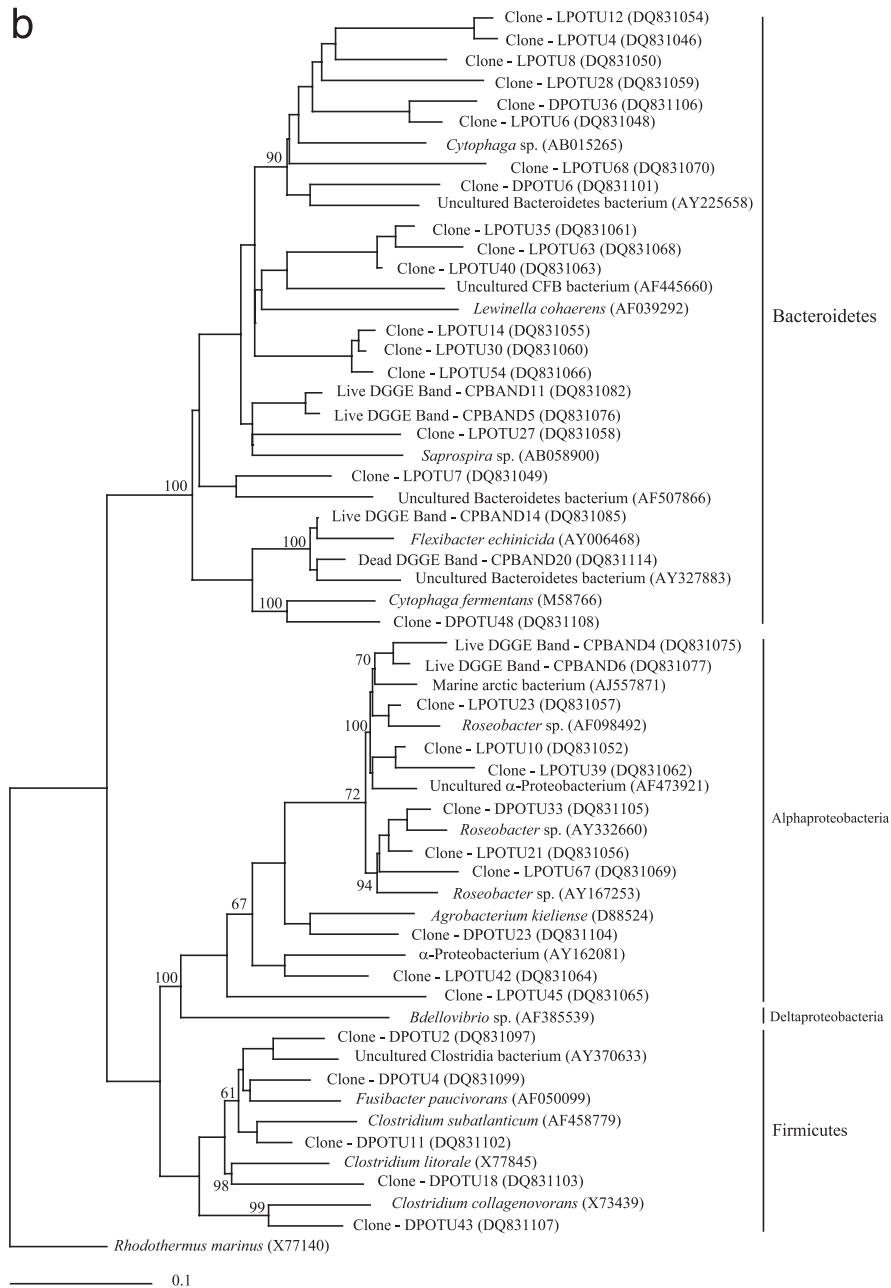


FIG. 3—Continued.

**DGGE analysis.** *Vibrio* sp.-affiliated sequences dominated DGGE profiles of both live phyllosoma (LP) and dead phyllosoma (DP) (LP bands 1 to 3, 8, 9, 12, and 13; DP bands 15 to 18 and 20) (Fig. 4), being present on most days and constituting ca. 54% of retrieved sequences obtained from LP bands and 60% of retrieved sequences obtained from DP bands. Interestingly, a sequence was retrieved from the day 10 LP profile and affiliated with a *Thiothrix* sp. (band 10) (Fig. 4), demonstrating high sequence identity with clone sequences that dominated the LP and DP libraries. Based upon bands of similar mobility for which no sequence information was obtained, its appearance coincided with the day prior to the start of the PI to PII molt (day 6) and was then present for the

remainder of the larval rearing trial. Bands of similar mobility were present in the DP profile, although the sequence information for these bands was not obtained. Further sequences retrieved from the LP profile affiliated with *Methylobacter marinus* (band 7) (Fig. 4) and an alphaproteobacterial sequence related to the *Rhodobacteriaceae* bacterium (band 4) (Fig. 4) and closely affiliated with retrieved sequences from the LP clone library. Other sequence affiliations obtained from the DGGE analysis of LP and DP were related to the *Sphingobacteria*, *Alphaproteobacteria*, and *Flavobacteria* groups (Table 2 and Fig. 3). Interestingly, in both LP and DP DGGE profiles there were no distinct changes in the microbial community observed between days 9 and 10, when high larval mortality



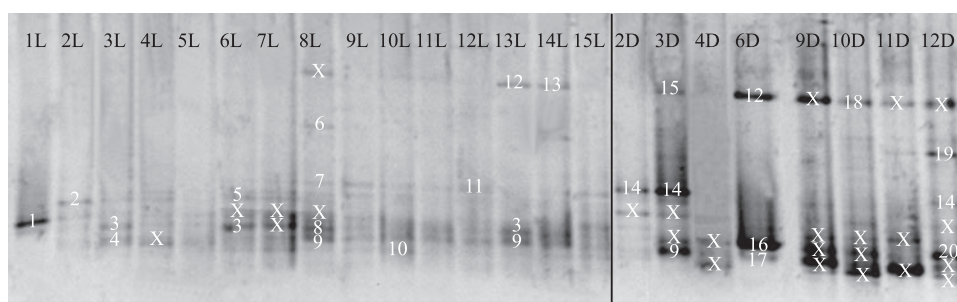


FIG. 4. DGGE profile of 16S rRNA gene fragments of live (L) and dead (D) early-stage *P. ornatus* phyllosomas taken over the duration of a larval rearing trial. The numbers at the top of the figure (in black) represent days. The numbers in the main body of the figure (in white) represent the bands that were cut from the gel and sequenced. These sequences are present in the phylogenetic tree in Fig. 3. Bands marked with the symbol "X" represent bands for which sequence data could not be obtained.

was observed within the larval rearing tank, although a new band appeared on day 8 but was absent from the profile the next day. No sequence information could be obtained for this band.

**FISH analysis.** Probing of whole phyllosomas with the fluorescently labeled domain *Bacteria* probe EUB338 demonstrated extensive colonization by filamentous bacteria. Based upon the large percentage of retrieved clone sequences, a fluorescently labeled oligonucleotide probe targeting *Thiothrix* species (G123T) was used to probe whole animals and confirmed that the extensive colonization by filamentous bacteria on the carapace and appendages (Fig. 5a) of dead phyllosomas sampled on day 10 were *Thiothrix*-related organisms. Surprisingly, analysis of live PII phyllosomas sampled on day 10 did not reveal *Thiothrix* sp. filaments, despite the presence of retrieved sequences in the corresponding clone library. A *Leucothrix mucor* specific probe (LMU) demonstrated the presence of sporadic numbers of *L. mucor* filaments on the carapace and

appendages of dead phyllosomas (Fig. 5b). FISH analysis of live phyllosomas with an LMU probe did not detect the presence of *L. mucor* filaments on live, day 10, PII phyllosomas. Probing of whole animals with a *Vibrionaceae*-specific probe (GV) identified large numbers of *Vibrio* sp. cells externally on both live (Fig. 5c) and dead (Fig. 5d) phyllosomas. Unidentified bacteria, comprised largely of rods and to a lesser extent cocci, were also present on the eyestalks (Fig. 5e), limbs, and carapaces of dead phyllosomas and the appendages and carapaces of live phyllosomas. Analysis of the limbs of some live phyllosomas with a EUB338 probe demonstrated low numbers of filamentous bacteria (Fig. 5f), although since these specific animals were not probed with the *Thiothrix*-specific probe (G123T), it is unknown whether these filaments are indeed *Thiothrix*-related species consistent with sequences retrieved from the clone library analysis.

**Culture-based analysis.** CFU counts on TCBS agar for bacteria associated with phyllosomas detected an increase in *Vibrio*-

TABLE 2. Identification of DNA bands from Fig. 5 based upon a partial 16S rRNA sequence<sup>a</sup>

Band no.	DNA source	Closest relative (database accession no.)	Alignment (bp)	Similarity <sup>b</sup> (%)	Taxonomic description
1	Live phyllosoma	<i>Vibrio corallilyticus</i> , strain LMG 21349 (AJ440004.1)	327/327	100	<i>Gammaproteobacteria</i>
2	Live phyllosoma	<i>Vibrio harveyi</i> , strain LB4 (DQ146935.1)	332/334	99	<i>Gammaproteobacteria</i>
3	Live phyllosoma	<i>Vibrio campbellii</i> , strain 90-69B3 (AY738129.1)	323/325	99	<i>Gammaproteobacteria</i>
4	Live phyllosoma	<i>Rhodobacteraceae</i> bacterium 183, strain 183 (AJ810844.1)	158/163	96	<i>Alphaproteobacteria</i>
5	Live phyllosoma	<i>Saprospira</i> sp., strain SS03-4 (AB191040.1)	203/222	91	<i>Sphingobacteria</i>
6	Live phyllosoma	<i>Roseivivax</i> sp. strain NT N52 (AB166990.1)	165/169	97	<i>Alphaproteobacteria</i>
7	Live phyllosoma	<i>Methylobacter marinus</i> A45 (AF304197.1)	252/265	95	<i>Gammaproteobacteria</i>
8	Live phyllosoma	<i>Vibrio natriegens</i> , strain 01/252 (AJ874353.1)	315/315	100	<i>Gammaproteobacteria</i>
9	Live phyllosoma	<i>Vibrio harveyi</i> , strain S35 (AY750578.1)	321/321	100	<i>Gammaproteobacteria</i>
10	Live phyllosoma	Uncultured <i>Thiothrix</i> sp. clone UP23b (AY426613.1)	308/315	97	<i>Gammaproteobacteria</i>
11	Live phyllosoma	<i>Saprospira</i> sp. SS03-4 (AB191040.1)	246/264	93	<i>Sphingobacteria</i>
12	Live phyllosoma	<i>Vibrio olivaceus</i> (AY827492.1)	71/73	97	<i>Gammaproteobacteria</i>
13	Live phyllosoma	<i>Vibrio hispanicus</i> , strain LMG 13213 (AY254042.2)	256/269	95	<i>Gammaproteobacteria</i>
14	Dead phyllosoma	<i>Flexibacter echinica</i> , strain F11 (AY006470.1)	315/315	100	<i>Sphingobacteria</i>
15	Dead phyllosoma	<i>Vibrio vulnificus</i> (AY264936.1)	184/198	92	<i>Gammaproteobacteria</i>
16	Dead phyllosoma	<i>Vibrio proteolyticus</i> (AF513463.1)	323/323	100	<i>Gammaproteobacteria</i>
17	Live phyllosoma	<i>Vibrio shilonii</i> strain MP-3 (AY911392.1)	285/300	95	<i>Gammaproteobacteria</i>
18	Dead phyllosoma	<i>Vibrio vulnificus</i> , strain 1003(O) (AY676129.1)	193/202	95	<i>Gammaproteobacteria</i>
19	Dead phyllosoma	<i>Psychroserpens mesophilus</i> , strain KOPRI 13650 (DQ001321.1)	301/315	95	<i>Flavobacteria</i>
20	Dead phyllosoma	<i>Vibrio harveyi</i> , strain LB4 (DQ146935.1)	315/315	100	<i>Gammaproteobacteria</i>

<sup>a</sup> Bands marked X in Fig. 5 did not sequence well and, as such, potential identifications were not provided.

<sup>b</sup> Sequences were aligned to the closest relative by using BLAST (3). The similarity was calculated with gaps not taken into account.

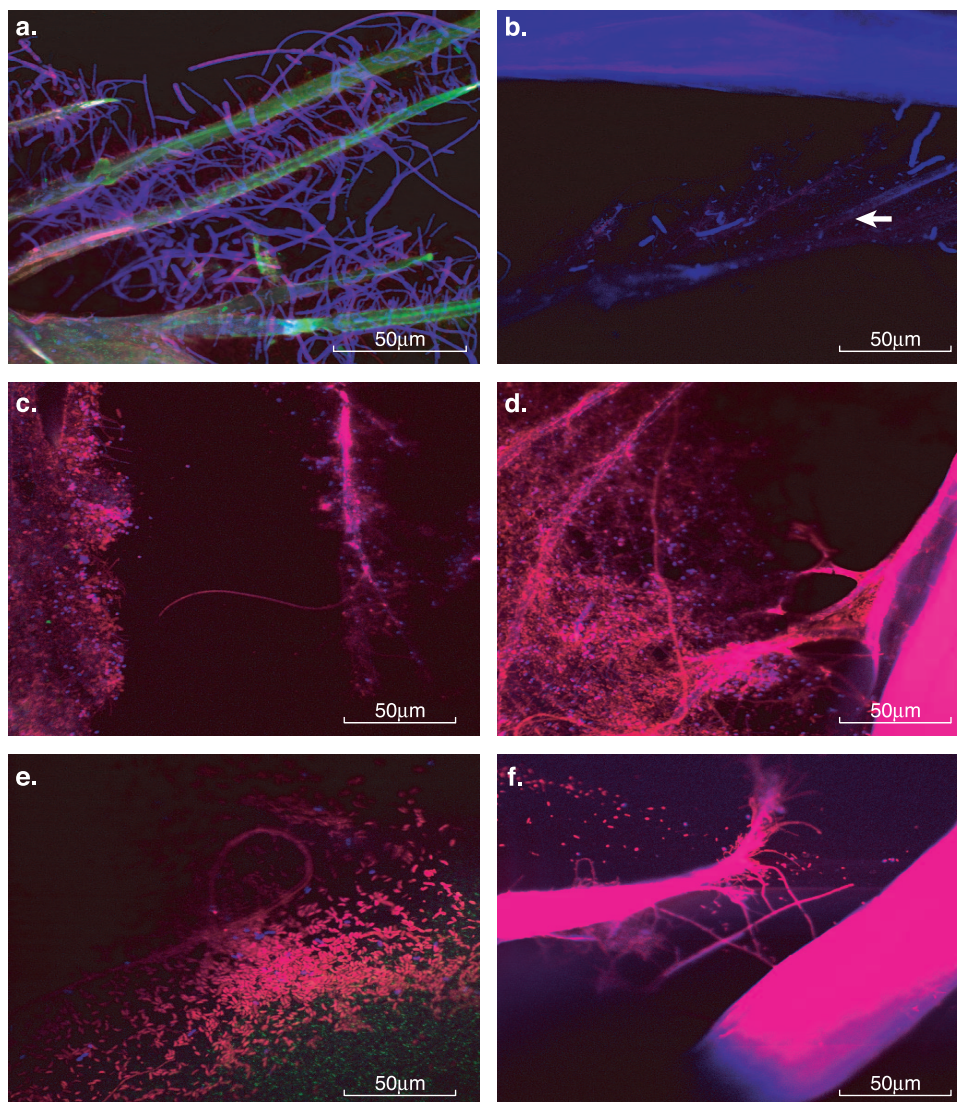


FIG. 5. FISH micrographs of whole animal mounts of *P. ornatus* phyllosomas. (a) FISH image of a dead phyllosoma limb probed with a Cy3-labeled domain *Bacteria*-specific probe (EUB338) and a Cy5-labeled *Thiothrix* sp.-specific probe (G123T). Bacteria on the surface of the animal appear pink; *Thiothrix* sp. bacteria protruding from the appendages of the animal appear blue. (b) FISH image of a dead phyllosoma carapace probed with a Cy5-labeled *Leucothrix mucor*-specific probe. *L. mucor* filaments protruding from the appendages of the animal appear blue. (c) FISH image of a live phyllosoma limb probed with a Cy3-labeled domain *Bacteria*-specific probe (EUB338) and a Cy5-labeled *Vibrio* sp.-specific probe. Bacteria on the surface of the animal appear pink; *Vibrio* sp. appear blue. (d) FISH image of a dead phyllosoma eyestalk probed with a Cy3-labeled domain *Bacteria*-specific probe (EUB338) and a Cy5-labeled *Vibrio* sp.-specific probe. Bacteria on the surface of the animal appear pink; *Vibrio* sp. appear blue. (e) FISH image of a dead phyllosoma eyestalk probed with a Cy3-labeled domain *Bacteria*-specific probe (EUB338) and a Cy5-labeled *Vibrio* sp.-specific probe. Bacteria on the surface of the animal appear pink; *Vibrio* sp. appear blue. (f) FISH image of a live phyllosoma limb probed with a Cy3-labeled domain *Bacteria*-specific probe (EUB338) and a Cy5-labeled *Leucothrix mucor*-specific probe. Bacteria on the surface of the animal appear pink; *Leucothrix mucor* filaments would appear blue.

*naceae* numbers during the early stages of a larval rearing trial. Numbers increased 14% from days 1 to 2 (244 to 284 CFU phyllosoma<sup>-1</sup>), 29% from days 2 to 4 (284 to 398 CFU phyllosoma<sup>-1</sup>), and 39% from days 6 to 7 (388 to 635 CFU phyllosoma<sup>-1</sup>). A large increase of 71% occurred in *Vibrionaceae* CFU counts from days 7 to 8 (635 to 2,220 CFU phyllosoma<sup>-1</sup>), coinciding with the beginning of the PI-PII larval molt. *Vibrionaceae* CFU counts then increased a further 3% from day 8 to day 9 (2,220 to 2,288 CFU phyllosoma<sup>-1</sup>). Statistical analysis of the data demonstrated a significant difference in CFU counts for days 8 and 9 compared to CFU

counts on all other days of the time series (one-way analysis of variance,  $P = 0.00$  and  $P < 0.05$ ). After day 9, swarming bacterial colonies precluded any further statistically valid CFU data from being obtained.

To provide an indication of the microbial diversity and potential pathogen(s) associated with both live and dead phyllosomas on the day of a larval mass mortality event (day 10), morphologically different bacterial strains were isolated on Marine and TCBS agar (Fig. 3a and Table 3). A total of 10 strains were isolated from live phyllosomas, whereas 7 strains were isolated from dead phyllosomas. The dominant genera iso-



TABLE 3. Affiliation of sequences retrieved from bacteria isolated from live and dead cultured phyllosomas based upon a partial (&gt;250-bp) 16S rRNA sequence

Isolate no. <sup>a</sup>	Medium <sup>b</sup>	Closest relative (database accession no.)	Alignment (bp)	Similarity <sup>c</sup> (%)	Taxonomic description
LCPISO1	TCBS	<i>Vibrio harveyi</i> , strain S35 (AY750578.1)	936/938	99	<i>Gammaproteobacteria</i>
LCPISO2	TCBS	<i>Vibrio campbellii</i> , strain 90-69B3 (AY738129.1)	943/944	99	<i>Gammaproteobacteria</i>
LCPISO3	TCBS	<i>Vibrio</i> sp., strain V794 (DQ146993.1)	937/940	99	<i>Gammaproteobacteria</i>
LCPISO4	MA	<i>Photobacterium phosphoreum</i> (AY292916.1)	935/939	99	<i>Gammaproteobacteria</i>
LCPISO5	MA	<i>Pseudoalteromonas piscicida</i> (AF081498.1)	944/945	99	<i>Gammaproteobacteria</i>
LCPISO6	MA	<i>Pseudoalteromonas</i> sp., strain S9 (U80834.1)	906/915	99	<i>Gammaproteobacteria</i>
LCPISO7	MA	<i>Pseudoalteromonas piscicida</i> (AF297959.1)	921/923	99	<i>Gammaproteobacteria</i>
LCPISO8	MA	<i>Pseudoalteromonas</i> sp., strain S511-1 (AB029824.1)	826/826	100	<i>Gammaproteobacteria</i>
LCPISO9	MA	<i>Photobacterium ganghwensis</i> , strain FR1311 (AY960847.1)	834/835	99	<i>Gammaproteobacteria</i>
LCPISO10	MA	<i>Vibrio alginolyticus</i> , strain CIP 70.65 (X74691.1)	878/884	99	<i>Gammaproteobacteria</i>
DCPISO1	TCBS	<i>Vibrio harveyi</i> , strain S35 (AY750578.1)	953/954	99	<i>Gammaproteobacteria</i>
DCPISO2	TCBS	<i>Vibrio campbellii</i> , strain 90-69B3 (AY738129.1)	963/972	99	<i>Gammaproteobacteria</i>
DCPISO3	MA	<i>Vibrio natriegens</i> , strain 01/097 (AJ874352.1)	951/954	99	<i>Gammaproteobacteria</i>
DCPISO4	MA	<i>Pseudoalteromonas piscicida</i> (AB090233.1)	922/928	99	<i>Gammaproteobacteria</i>
DCPISO5	MA	<i>Vibrio natriegens</i> , strain 01/097 (AJ874352.1)	907/908	99	<i>Gammaproteobacteria</i>
DCPISO6	MA	<i>Photobacterium ganghwensis</i> , strain FR1311 (AY960847.1)	781/783	99	<i>Gammaproteobacteria</i>
DCPISO7	MA	<i>Vibrio parahaemolyticus</i> , strain MP-2 (AY911391.1)	831/835	99	<i>Gammaproteobacteria</i>

<sup>a</sup> LCP, live phyllosoma; DCP, dead phyllosoma.

<sup>b</sup> MA, marine agar; TCBS, thiosulfate, bile salt, sucrose agar.

<sup>c</sup> Sequences were aligned to the closest relative by using BLAST (3). The similarity was calculated with gaps not taken into account.

lated were identified as *Vibrio* sp. (nine isolates), with isolates closely affiliated with *V. harveyi* (LCPISO1 and DCPISO1) and *V. campbellii* (LCPISO2 and DCPISO2) being recovered from both live and dead phyllosomas and sharing nearly identical sequence identity. Other isolated *Vibrio* sp. included *V. alginolyticus* (LCPISO11), *V. natriegens* (DCPISO3 and DCPISO5), and *V. parahaemolyticus* (DCPISO7). Isolates affiliated with the genus *Pseudoalteromonas* were also prevalent (five isolates), particularly in live phyllosomas (four of five isolates). Other isolated cultures were affiliated with the genus *Photobacterium* (three isolates).

## DISCUSSION

High mortality during early larval stages, particularly upon initial stocking and around molt periods, is commonly associated with larval rearing of the tropical rock lobster, *Panulirus ornatus* (5). To date, opportunistic bacterial pathogens, particularly *Vibrio* sp., have been implicated as a potential cause of larval mortalities (4, 5, 73). The results from the present study demonstrated colonization by filamentous bacteria as a contributing detrimental factor to phyllosoma survival. SEM identified filamentous bacteria on phyllosomas, and FISH confirmed heavy infestation of dead animals on day 10 by *Thiothrix* sp. bacteria. Clone library analysis supported *Thiothrix* identification with 56% of retrieved sequences from the library of dead phyllosomas sampled on day 10 affiliating with this organism. In addition, 19% of clones from the live phyllosoma library also sampled on day 10 demonstrated sequence identity to *Thiothrix* sp. and a partial 16S rRNA sequence closely affiliated with a *Thiothrix* sp. was retrieved from the DGGE analysis of live phyllosomas on this day. Due to the detection limit of DGGE, it is believed that only predominant species present in a microbial community can be detected (18, 45, 54, 55, 57), suggesting that *Thiothrix* sp. are also a dominant organism associated with live phyllosomas. Recent work by Bourne et al. (4) used SEM to monitor the progressive colonization of fila-

mentous bacteria in later-stage PIII phyllosomas. Extensive colonization and entanglement of mouth parts were observed and believed to render the animal unable to feed adequately. Heavy epibiont growth has also been attributed to increased phyllosoma mortality due to a reduction of respiratory effectiveness as demand for oxygen increases during molt periods (5, 11, 12, 23). In addition, such colonization has compromised animal health in other crustacean species with reports of penaeid prawns affected by the filamentous bacteria, *Leucothrix mucor* (38).

As phyllosomas molt from stage PI to PII they shed the outer shell allowing colonization organisms to also be discarded. Dead phyllosomas sampled on day 10 are stage PI animals that have failed to successfully molt with heavy bacterial colonization likely contributing to mortality. In contrast, live animals sampled on day 10 have successfully shed the outer shell resulting in lower colonization. This contributed to the inability to detect *Thiothrix* sp. during FISH analysis despite the retrieval of sequences in the corresponding clone library. FISH analysis was also limited to a small number of individual animals (~5), whereas clone library analysis was a pooled sample of multiple phyllosomas (~10), allowing a greater representative view of bacterial diversity. Some phyllosomas analyzed with FISH and the EUB338 probe detected filamentous bacteria associated with live phyllosomas, although unfortunately these animals were not probed with the *Thiothrix*-specific probe to confirm phylogenetic identification of these filaments. Many unknown filamentous bacteria which did not hybridize to either *Thiothrix* sp. or *Leucothrix mucor* specific probes were also detected on both live and dead sampled phyllosomas. Although a large number of bacteria have a filamentous morphology it is acknowledged the probes used in the present study do not cover all *Thiothrix* species with a search of Ribosomal Database Project database showing only 80 out of 97 *Thiothrix* sp.-related sequences are specific for the G123T probe. Although molt shedding reduces the microbial

colonization load, it appears to maintain microbial diversity associated with the phyllosomas since indices and rarefaction analysis demonstrated greater microbial diversity associated with live phyllosomas compared to dead animals. Although appearing counterintuitive it is likely that dominant bacteria, in this case, *Thiothrix* sp., proliferate on phyllosoma postmortem. In addition, high bacterial diversity may promote phyllosoma health by preventing the establishment of pathogenic organisms through competition for nutrients and space. Such phenomenon has been previously reported in the gastrointestinal tract of chickens where the mature microbiota confer resistance to infection by *Salmonella enterica* (50, 75).

*Vibrio*-affiliated sequences were commonly retrieved from both live and dead phyllosoma samples with clone library and DGGE analysis, while FISH confirmed the presence of *Vibrio* sp. cells on the external surfaces of phyllosomas on day 10. *Vibrio* sp. dominated isolated bacterial strains from culture-based studies, whereas specific *Vibrio* counts demonstrated a significant increase in *Vibrionaceae*-related organisms from days 7 to 8 correlating with the beginning of the larval molt from PI-PII. This is a time where phyllosomas are particularly susceptible to infection (41) as a result of the phyllosoma's external barriers to pathogen invasion being compromised (73, 74). Previous studies have implicated *Vibrio* sp. as potential pathogens associated with *P. ornatus* and *Jasus verreauxi* phyllosoma mortalities (5, 13, 22, 23, 73). *Vibrio* sp. derived sequences from each analysis method demonstrated a high degree of identity, with one *Vibrio harveyi*-affiliated organism isolated from both live and dead phyllosomas and sequences retrieved from live phyllosoma DGGE analysis and represented as 7% of the live phyllosoma clone library. *V. harveyi* has been implicated as a pathogen in phyllosomas of the New Zealand spiny lobster, *Jasus verreauxi* (13) and also as a pathogen among prawn larvae (24, 37, 39, 40, 51, 53). In addition, sequences affiliated with another known crustacean pathogen, *Vibrio campbellii*, previously associated with disease in the Pacific oyster (66), abalone (44), and in various penaeid prawn species (7, 21, 27, 62, 63), were identified from isolates recovered from both live and dead phyllosomas and in live phyllosoma DGGE analysis. Bands showing similar mobility to the live phyllosoma-*V. campbellii*-affiliated band were also present in dead phyllosoma DGGE analysis.

Numerous clone sequences were retrieved from phyllosomas that affiliated with marine microbial strains involved in nutrient cycling processes, including the methanotrophic bacteria *Methylobacter marinus* (LP DGGE band 7) and the nitrogen-fixing root nodule bacterium *Mesorhizobium* sp. (DP OTU 23). Sulfur cycling appears to be an important process within the aquaculture rearing environment, as indicated by the dominance of retrieved *Thiothrix* sp. sequences, as well as the presence of clostridium-related sequences and a *Sulfitobacter* sp. sequence. *Thiothrix* spp. are sulfur-oxidizing bacteria that have been described in a number of environments, including activated-sludge wastewater treatment plants (31, 56), and as epibionts on a number of aquatic invertebrates (6, 17, 36, 71). *Thiothrix* spp. oxidize sulfide compounds, such as hydrogen sulfide ( $H_2S$ ), to sulfate ( $SO_4$ ). The presence of *Sulfitobacter* sp. indicates the presence of sulfite ( $SO_2$ ), which is a nutrient requirement of this organism, being oxidized to produce  $SO_4$  (32, 69). Environments that contain sulfite may have oxygen-

deficient zones that are likely to be toxic to aquatic species (19). *Clostridium* spp. are strict anaerobes commonly found in soil environments (46) but also isolated from marine systems (2, 49) and able to utilize a range of compounds as energy sources, such as cellulose, sugars, starch, and pectin (46), but of interest to the present study are those that obtain their energy by utilizing amino acids. *Clostridium* spp. ferment individual amino acids or amino acid pairs, which results in the production of a range of foul-smelling substances, including  $H_2S$  (46), potentially acting as a substrate for *Thiothrix* sp. No sequences were obtained that affiliated with sulfate-reducing bacteria (SRB), a group widespread in aquatic and terrestrial environments that become anoxic through microbial decomposition processes (46). SRB are an important group of bacteria in sulfur cycling that reduce the sulfate produced by such organisms as *Thiothrix* sp. and *Sulfitobacter* sp. back to sulfide, allowing the cycle to continue. It is likely that SRB, such as *Desulfovibrio* sp., are present within the larval rearing system, but closer analysis showed that this group of bacteria was poorly represented by the primers used in the present study. Further analysis of the larval rearing system using primers that target the SRB is required to gain an understanding of the genera and species present that represent this group.

Phyllosomas sampled in the present study were defined as "living" as opposed to "healthy" since it is unknown whether the animals are compromised even though they still possess the ability to swim in the water column. Because phyllosomas are small and transparent, high numbers of "dead" phyllosomas could only be sampled from the large 5,000-liter larval rearing tanks by siphoning accumulated dead patches. Although microbial turnover is possible on the dead phyllosomas, dead animals were sampled as soon as possible postmortem (1 to 4 h), and only selected phyllosomas that were not showing obvious signs of decay were incorporated into the analysis. As a result, microbial community changes postmortem are expected to be minimal, and dominant members of the community will be maintained.

One challenge for successful aquaculture of the tropical rock lobster is to establish larval rearing technology on a sufficient scale to make it commercially viable. As a result, larval rearing trials are undertaken in 5,000-liter tanks which, although good for commercial applications, make replication difficult. The results presented in the present study are from one trial, although representative of results seen from other larval rearing runs. This is confirmed by previous published studies that have found both *Thiothrix*- and *Vibrio*-affiliated organisms within the larval rearing environment (4, 5, 73). The present study is unique in that it has provided an in-depth understanding of the community dynamics associated with the phyllosomas, whereas other studies have focused on broader microbial changes within the whole system.

Extensive *Thiothrix* sp. colonization appears to have a negative effect on the health of early-stage phyllosomas by hindering their ability to molt from stage PI to stage PII. Studies of later-stage phyllosomas (PIII to PIV) have recently also confirmed heavy epibiont fouling compromising animal health and supporting the findings of the present study (4). The period around the molt is a time when phyllosomas are particularly susceptible to infection by pathogenic bacteria such as *Vibrio* sp. (41, 73) as a result of the new shell of the phyllosomas being

soft after the shedding of the old carapace (73, 74). This is also a time when *Vibrio* sp. numbers have been reported to dramatically increase within phyllosomas, resulting in subsequent mass larval mortalities (73). In order to control *Thiothrix* sp. proliferation, sulfide compounds within the larval rearing system need to be lowered or removed. Ozonation of all incoming larval rearing water may serve as an effective *Thiothrix* sp. control mechanism by reducing sulfide compounds through the removal of bacteria that produce such compounds. Isolated *V. harveyi* and *V. campbellii* strains will also be tested to determine whether they exhibit a significant pathogenic effect on phyllosomas based upon the satisfaction of Koch's postulates.

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