

## ORIGINAL ARTICLE

# The onset of microbial associations in the coral *Pocillopora meandrina*

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Associations between healthy adult reef-building corals and bacteria and archaea have been observed in many coral species, but the initiation of their association is not understood. We investigated the onset of association between microorganisms and *Pocillopora meandrina*, a coral that vertically seeds its eggs with symbiotic dinoflagellates before spawning. We compared the bacterial communities associated with prespawmed oocyte bundles, spawned eggs, and week old planulae using multivariate analyses of terminal restriction fragment length polymorphisms of SSU rRNA genes, which revealed that the composition of bacteria differed between these life stages. Additionally, planulae raised in ambient seawater and seawater filtered to reduce the microbial cell density harbored dissimilar bacterial communities, though SSU rRNA gene clone libraries showed that planulae raised in both treatments were primarily associated with different members of the *Roseobacter* clade of *Alphaproteobacteria*. Fluorescent *in situ* hybridization with an oligonucleotide probe suite targeting all bacteria and one oligonucleotide probe targeting members of the *Roseobacter* clade was used to localize the bacterial cells. Only planulae greater than 3 days old were observed to contain internalized bacterial cells, and members of the *Roseobacter* clade were detected in high abundance within planula tissues exposed to the ambient seawater treatment. We conclude that the onset of association between microorganisms and the coral *P. meandrina* appears to occur through horizontal uptake by planulae older than 79 h, and that uptake is preferential to members of the *Roseobacter* clade and potentially sensitive to the ambient seawater microbial community.

The ISME Journal advance online publication, 26 February 2009; doi:10.1038/ismej.2009.3

**Subject Category:** microbe–microbe and microbe–host interactions

**Keywords:** coral; marine bacteria; microorganisms; planula development; *Pocillopora meandrina*; vertically seeded

## Introduction

Corals are best considered as a holobiont organism composed of a dynamic assemblage of anthozoan host polyps, symbiotic dinoflagellates (zooxanthellae), endolithic algae and fungi, bacteria, archaea, and viruses residing within the skeleton, tissues, and mucus layer of adult coral colonies (Rohwer *et al.*, 2002; Knowlton and Rohwer, 2003). The coral–zooxanthellae symbiosis has been well studied, though knowledge of microbial (defined hereafter as bacteria and archaea) communities associated with corals has been greatly advanced in recent years by the emergence of culture independent approaches and molecular biology-based methodology. Studies using these techniques have uncovered high micro-

bial diversity associated with the tissue and mucus layers of adult corals (Rohwer *et al.*, 2001, 2002; Frias-Lopez *et al.*, 2002; Kellogg, 2004; Wegley *et al.*, 2004), showed stable associations between geographically separated corals and certain bacterial taxa (Rohwer *et al.*, 2002), and revealed the presence of microbial functional genes that may play a role in coral health (Beman *et al.*, 2007; Wegley *et al.*, 2007). Microorganisms are also becoming an increasingly important component of studies investigating the variables leading to coral bleaching and disease (Beleneva *et al.*, 2005; Bourne, 2005; Guppy and Bythell, 2006; Ainsworth *et al.*, 2007; Bourne *et al.*, 2007) but, in general, these studies have been hampered by the many unknowns that still remain regarding microbial associations in healthy corals.

Fundamental to the study of coral–microbial associations is an understanding of when and how the relationships are established, and the specificity of the established associations. The onset of association has not been studied in any coral species but could provide important insight into the nature of

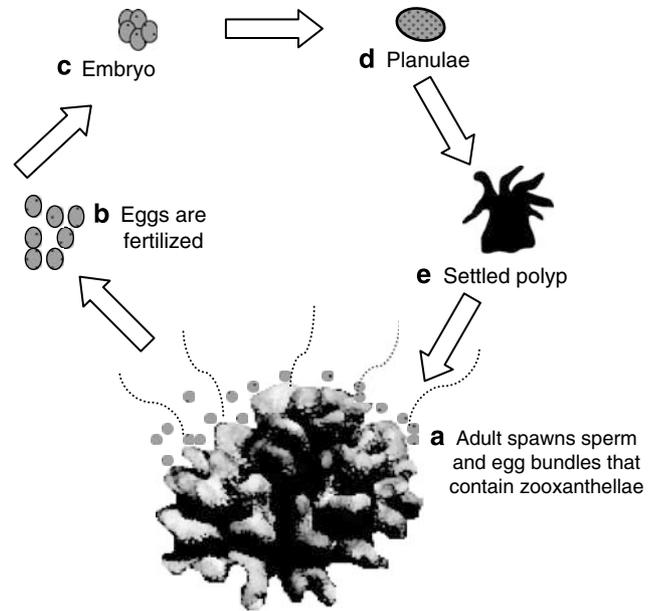
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Received 17 September 2008; revised 12 December 2008; accepted 21 December 2008

coral–microbial relationships. In some invertebrates, vertical transmission of microbial symbionts is common, and ensures the longevity of the partnership (Smith and Douglas, 1987). Vertical transmission has been shown in such marine invertebrates as tropical sponges (Sharp *et al.*, 2007), bivalves (Krueger *et al.*, 1996) and bryozoa (Haygood and Davidson, 1997), and is well studied in terrestrial aphids, where there is evidence that vertical transmission has permitted opportunities for co-evolution and co-diversification of the hosts and their symbionts (Munson *et al.*, 1991). Acquisition of microbial symbionts through horizontal transmission also occurs in many invertebrate species through a variety of acquisition mechanisms (Moran and Baumann, 2000; Nussbaumer *et al.*, 2006; Kikuchi *et al.*, 2007). Many of these associations are also species specific, but the onset of the associations are affected by the availability of the symbionts in their environment, and the host requires a specific mechanism for acquisition (Moran and Baumann, 2000).

The onset of the coral–dinoflagellate symbiosis in broadcast spawning coral species occurs either before spawning, with the maternal colony seeding the eggs, or, more commonly, by the acquisition of dinoflagellate cells from the environment by the coral (Trench, 1983). In more rare cases, fertilization takes place within the maternal polyp (brooding), and these species release fully developed planula larvae that typically contain zooxanthellae (Harrison and Wallace, 1990; Richmond and Hunter, 1990). The goal of this study was to examine the onset of microbial associations in colonies of *Pocillopora meandrina*, a globally distributed reef-building coral that acquires zooxanthellae vertically from the maternal colony. *P. meandrina* has a known spawning history in Hawaii and a well-studied developmental period (Hirose *et al.*, 2001; Marlow and Martindale, 2007). *P. meandrina* colonies are simultaneous hermaphrodites in which each polyp seasonally spawns sperm and eggs that contain zooxanthellae (Figure 1a). During spawning, sperm and individual eggs are released and the eggs become fertilized by sperm (potentially only from a different colony) (Figure 1b), and each egg develops into an embryo (Figure 1c) and subsequently into a motile planula (Figure 1d). The planula finally settles onto a benthic substrate and develops into a single polyp (Figure 1e), which then grows into a colony of calcifying polyps.

In this study we investigate the acquisition of microorganisms at different developmental stages (prespawmed oocyte bundles, spawned eggs, and week old planulae) of the coral *P. meandrina* using a cultivation independent, ribosomal RNA gene-based approach in conjunction with fluorescence microscopy. Our results show that, although microbes are associated with the oocyte bundle and egg life stages, they are not localized internally within *P. meandrina* tissues until the planulae are 79 h old.



**Figure 1** Developmental stages of the coral *P. meandrina*. (a) Adult colonies simultaneously spawn both sperm and eggs that contain zooxanthellae. (b) The eggs are fertilized by another colony's sperm, and individually develop into (c) embryo and subsequently (d) motile planula stages. (e) Planula settle onto a benthic substrate and metamorphose into a single adult polyp, which is then capable of self-replicating to form an adult colony. Illustrations are not to scale.

## Materials and methods

**Summary of spawning events and sample collections**  
Our goal was to collect prespawmed oocyte bundles, freshly spawned eggs, and rear planulae to 1 week from material obtained from the same colony. In April 2007, we identified 26 colonies from Kaneohe Bay, Oahu (21° 27.345' N, 157° 46.961' W) (hereafter labeled A–Z), which were located at the same sampling site but separated by a distance of at least 5 m from each other to limit the probability that colonies represented genetic clones. Spawning was not observed in April but was observed following the full moon in May and June 2007, with a large spawning event occurring in May and a smaller event in June. Oocyte bundles were collected in April and May 2007, whereas freshly spawned eggs were collected and planulae were reared up to 1 week from the May and June 2007 spawning events.

Eggs from spawning colonies were incubated at room temperature with a mixture of sperm from the different colonies for 20 min, rinsed three times in sterile seawater, and incubated in replicate petri dishes at 29 °C under a 12/12 light/dark cycle in an ambient microbial seawater (AMSW) or low microbial seawater (LMSW) treatment. For AMSW, seawater was filtered through a GF/A filter (Whatman International Ltd., Kent, United Kingdom) to remove zooplankton and other large organisms. LMSW was prepared by filtering seawater through a 0.2- $\mu$ m pore-sized filter (Supor-200; Pall Corp., East Hills,

NY, USA) to remove most cells. Cell densities were microscopically determined to be  $6.6 \times 10^5$  cells ml<sup>-1</sup> in AMSW and  $5.8 \times 10^4$  cells ml<sup>-1</sup> in LMSW (Porter and Feig, 1980). The live planulae were transferred daily into fresh AMSW or LMSW seawater and a new petri dish, and they were preserved at densities of 20–50 planula/sample when they reached 1 week old (168 h).

Before preservation, all samples were rinsed three times in sterile seawater to remove loosely associated microbial cells. Subsamples of oocyte bundles, eggs and planulae were preserved for DNA analysis in 250 µl of lysis buffer [20 mM Tris/HCl (pH 8.0), 2 mM EDTA (pH 8.0), 1.2% (vol/vol) Triton X100, and 20 µg/ml lysozyme] and frozen to -80 °C. Samples were also preserved for microscopic analysis by fixation with 3.7% paraformaldehyde in sterile seawater for 6 h at 4 °C, followed by five washes in PTW buffer (phosphate buffered saline (PBS) and 1% tween) for 5 min at room temperature, and finally frozen to -20 °C in methanol. Additional details about the samples collected from each colony and life stage are outlined in Supplementary online material, and the type of analysis performed on each colony and life stage is presented in Table 1.

Water samples were collected from the microcosm aquaria in which the *P. meandrina* fragments were held before and during spawning. One hour before the spawning event, 1 l of seawater was collected from the aquaria. A 1-l subsample of the AMSW media water was also collected before placing the eggs in the treatment. For each water sample, 900 ml to 1 l was filtered sequentially through a GF/A glass microfiber (Whatman International Ltd.) and 0.2 µm pore-sized polyethersulfone membrane filters (Supor-200; Pall Corp.) and frozen for DNA analysis in 250–500 µl of lysis buffer.

Archived samples obtained from *P. meandrina* spawning events in 2005 and 2006 were also used in this study. During May 2005, *P. meandrina* eggs were obtained from 20 colonies, mixed together, reared, and sampled at various developmental stages (detailed in Marlow and Martindale, 2007). A subset of these samples was analyzed using fluorescence microscopy with fluorescence *in situ* hybridization (FISH) to target microbial communities. During May 2006, *P. meandrina* eggs were collected from five individual colonies. These samples were used to develop the DNA preservation methods and polymerase chain reaction (PCR) reaction conditions used in this study. From these preliminary surveys, data are presented below from two of these colonies (colonies 1 and 5) that were examined for archaeal diversity by constructing small clone libraries of archaeal SSU rRNA genes.

#### T-RFLP of bacterial SSU rRNA genes

DNA was extracted from the oocyte bundle, egg, and planula samples using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA) with modifications (Becker *et al.*, 2007), and quantified using the PicoGreen fluorescent assay (Invitrogen Corp., Carlsbad, CA, USA) on a SpectraMax M2 plate reader (Molecular Devices Corp., Sunnyvale, CA, USA). For terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu *et al.*, 1997), bacterial SSU rRNA genes (SSU rDNA) were first amplified through the PCR using oligonucleotide primers 27F-B-FAM (5'-AGRGTTYGATYMTGGCTCAG-3') and 519R-VIC (5'-GWATTACCGCGGCKGCTG-3'), with 'FAM' and 'VIC' indicating 5' end-labeling with FAM or VIC fluorochromes. Each 50 µl PCR reaction contained 2 U of Sahara enzyme (Biolone USA Inc., Taunton, MA, USA), 1 × Sahara reaction buffer,

**Table 1** Summary of analyses performed on *P. meandrina* samples from different life stages of colonies sampled during April–June, 2007

Colony	Pre-spawned oocyte bundles (April, May)			Spawned eggs (May)			AMSW planulae (May)			Spawned eggs (June)			AMSW planulae (June)			LMSW planulae (June)		
	T	Nu	Ba	T	Nu	Ba	T	Ba	R	T	Nu	T	Ba	R	T	Ba	R	
C										✓		✓			✓		✓	
E				✓	✓					✓								
F	✓	✓	✓	✓	✓		✓	✓										
G				✓	✓													
I				✓	✓	✓	✓	✓		✓		✓	✓	✓	✓	✓	✓	
S	✓	✓	✓		✓		✓											
U				✓	✓		✓			✓		✓						
X				✓			✓	✓	✓									
Y				✓	✓													
Z				✓	✓	✓												
Mixed										✓	✓	✓	✓	✓	✓	✓	✓	

T, T-RFLP; Nu, nucleic acid staining for microscopy; Ba, FISH with bacterial probe suite; R, FISH with *Roseobacter* clade probe. Boxes indicate samples used to construct SSU rRNA gene clone libraries.

2 mM Sahara MgCl<sub>2</sub>, 200 μM of each dNTPs, 200 nM of each primer, and 100 ng of genomic template (concentrated up to 1 μg for samples that did not amplify at lower concentrations). After an initial denaturation step at 95 °C for 5 min, the reaction conditions were: 29 cycles of 95 °C denaturation for 30 s, 55 °C annealing for 1 min, and 72 °C extension for 2 min, concluding with an extension at 72 °C for 20 min. The reactions were carried out in a MyCycler Personal Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Amplification products were purified using the QIAquick PCR Purification Kit (Qiagen Inc.), and subsequently restricted in a 10-μl reaction containing 100 ng of purified amplification product, 2 μg of bovine serum albumin, 1 × enzymatic reaction buffer, and 5 units of HaeIII restriction endonuclease (10 units μl<sup>-1</sup>, Promega, Madison, WI, USA) for 7 h at 37 °C. Restriction digests were purified using the QIAquick Nucleotide Removal Kit (Qiagen Inc.), and 30 ng/ml of each product was subsequently electrophoresed on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Operational taxonomic units were identified as peaks from both the forward and reverse labeled primer for fragments between 33 and 550 bp in length. To account for small differences in the amount of DNA loaded on the ABI 3100, data were normalized by excluding peaks that contributed <0.05% of the total peak area for each sample. An average of 0.81% of the peaks was removed from samples, with a minimum of no peaks and a maximum of 3.2% of peaks removed. The normalized matrix was analyzed for species richness,  $S$  ( $S$  = number of T-RF's resulting from combined forward and reverse labeled primers), Shannon's diversity index,  $H$  [ $H = -\sum [p_i \times \ln(p_i)]$ ] where  $p_i$  is the percentage of each species], and evenness,  $E$  [ $E = H/\ln(S)$ ] using PC-ORD software (MjM Software Design, Gleneden Beach, OR, USA). The T-RF matrix was additionally analyzed using cluster analysis with the Sorensen (Bray-Curtis) distance measure and flexible beta linkage method ( $\beta = -0.25$ ) with PC-ORD software, and parameters were based on recommendations for nonparametric comparisons from McCune and Grace (2002). To test for significant differences between the bacterial communities associated with the different life stages, multiresponse permutation procedure was also conducted on the matrix by comparing the different life stages using the Sorensen (Bray-Curtis) distance measure (rank transformed) with the  $n/\text{sum}(n)$  weighting option using PC-ORD software program (parameters recommend by McCune and Grace, 2002).

#### Bacterial SSU rDNA clone libraries

SSU rRNA gene cloning and sequencing was used to identify the bacterial communities associated with oocyte bundles (20–30 bundles), spawned eggs (ca. 100 eggs) and 168-h-old AMSW planulae (ca.

40 planula) collected from a single colony (colony F), and 168-h-old LMSW planulae (ca. 40 planula) from colony I. Because of limited genomic DNA, purified unrestricted PCR product amplified with fluorescently labeled primers for T-RFLP analysis were used for cloning and sequencing. The labeled products were re-amplified to reduce the concentration of fluorescent labels in a 20-μl reaction volume containing 1 U of Sahara enzyme, 1 × Sahara buffer, 2 mM Sahara MgCl<sub>2</sub> (Bioline USA Inc.), 200 μM of each dNTPs, 200 nM each of unlabeled 27F-B and 519R primers and 40 ng of amplified sample. The reaction consisted of 25 cycles of the same PCR conditions described earlier. To reduce the formation of heteroduplexes, a reconditioning PCR step was preformed (Thompson *et al.*, 2002) that consisted of 2 U of Sahara enzyme, 1 × Sahara buffer, 2 mM Sahara MgCl<sub>2</sub> (Bioline USA Inc.), 200 μM of each dNTPs, 200 nM of each 27F-B and 519R primer, and 500 ng of amplified template in duplicate 50 μl reactions. Reaction conditions consisting of an initial denaturation step at 95 °C for 5 min followed by two cycles of 95 °C denaturation for 30 s, 55 °C annealing for 1 min, and 72 °C extension for 2 min, and concluding with a cycle containing an extension step of 20 min, were used. The duplicate reactions were combined and purified as above. Amplification products were cloned using the pGem-T Easy system (Promega), and sequenced on an ABI 3730XL capillary-based DNA sequencer (Applied Biosystems). Sequence alignments and phylogenetic analyses were carried out using the ARB software package (Ludwig *et al.*, 2004) and the 'All-Species Living Tree' project SSU rRNA gene database (Yarza *et al.*, 2008). Clone sequences were identified to the family level and closest recognized species or lineage.

A phylogenetic tree was constructed using sequences belonging to the *Roseobacter* clade of the Alphaproteobacteria obtained in this study, reference sequences of recognized species from the *Rhodobacteraceae* lineage, and previously published environmental gene clones of high similarity to the clone sequences obtained in this study. Because of the short clone sequences (approximately 500 bp), the tree was constructed in two parts. First, the base of the tree was constructed using the RAxML maximum likelihood method (Stamatakis, 2006) with nearly full-length reference sequences. The clone sequences obtained in this study were subsequently added to the tree without changing the topology by use of the ARB parsimony interactive method. Bootstrap values were obtained in ARB using upper bootstrap limits dependent on nearest-neighbor interchange.

#### Archaeal SSU rDNA clone libraries

Archaeal SSU rRNA genes were amplified with the oligonucleotide primers Arch21F (DeLong, 1992) and Arch915R (Stahl and Amann, 1991) from egg

samples collected from two different *P. meandrina* colonies (colonies 1 and 5, about 200 eggs per sample) during the May 2006 spawning event. The 20- $\mu$ l PCR reactions contained 0.5 U of PicoMaxx enzyme, 1  $\times$  PicoMaxx buffer (Stratagene, La Jolla, CA, USA), 200  $\mu$ M of each dNTPs, 200 nM of each primer, and 80 ng of genomic template. The reaction was conducted for 40 cycles of a touchdown protocol of an initial denaturation step at 95 °C for 3 min followed by 40 cycles of 95 °C denaturation for 30 s, 65 °C annealing for 1 min (decreasing by 0.5 °C every cycle until 50 °C), and 72 °C extension for 2 min, and concluding with a cycle containing an extension step of 20 min. Cloning, sequencing, and phylogenetic analyses were carried out as described earlier.

All sequences generated in this study have been deposited in GenBank under accession numbers FJ497059–FJ497209.

#### *Nucleic acid and membrane staining for microscopy*

The DNA of oocyte bundles, spawned eggs, and planulae was visualized with propidium iodide (1 mg ml<sup>-1</sup>) and RNaseA (1  $\mu$ g ml<sup>-1</sup>), or DAPI (5  $\mu$ g ml<sup>-1</sup>). The cell membranes of the oocyte bundles were visualized with alexa-fluor 488-conjugated phalloidin (Molecular Probes, Eugene, OR, USA), at a concentration of 2 U ml<sup>-1</sup> (66 nM). Propidium iodide and phalloidin staining was carried out at room temperature in PTW buffer for 1 h, followed by 3  $\times$  5 min washes in PBS. Whole samples were subsequently dehydrated through an isopropanol series (30%, 50%, 80% isopropanol in PBS) followed by 100% isopropanol, and mounted in one part benzyl alcohol to two parts benzyl benzoate. DAPI staining was carried out in 15% formamide solution [0.9 M NaCl, 20 mM Tris/HCl (pH 7.4), 15% formamide, 0.01% SDS] for 10 min at room temperature, followed by 3  $\times$  5 min washes in 15% formamide solution. Samples were diluted into PBS and mounted.

#### *Fluorescence in situ hybridization*

Oocyte bundle, egg, embryo, and planula samples from 2005 and 2007 were examined using FISH with general bacterial and taxon-specific oligonucleotide probes. To prepare samples for hybridization, methanol-preserved samples were re-hydrated in PTW followed by 5  $\times$  5 min washes in PTW. All manipulations and washes were carried out at room temperature. Tissues were then subjected to a prehybridization procedure that included a 10-min enzymatic digestion with proteinase K (2.5  $\mu$ l/5 ml PTW buffer), 2  $\times$  5 min glycine (2 mg ml<sup>-1</sup>) washes, one 5-min wash in 1% triethanolamine, one 5-min wash in 6  $\mu$ l ml<sup>-1</sup> acetic anhydride in 1% triethanolamine, one 5-min wash in 12  $\mu$ l ml<sup>-1</sup> acetic anhydride in 1% triethanolamine, and 2  $\times$  5 min washes in PTW. The tissues were secondarily fixed with

3.7% formaldehyde in PTW for 30 min, followed by 5  $\times$  5 min washes in PTW. For hybridization reactions using the general bacteria probe suite, the samples were subsequently washed for 10 min at room temperature in 15% formamide hybridization solution [0.9 M NaCl, 20 mM Tris/HCl (pH 7.4), 15% formamide, 0.01% SDS], followed by incubation in fresh solution for 30 min at 37 °C. A suite of five Cy3 labeled bacterial probes (EUB-27R, EUB-338Rpl, EUB-700R, EUB-700Ral, and EUB-1522R) (Morris *et al.*, 2002) were each added at 2 ng  $\mu$ l<sup>-1</sup> in 15% formamide hybridization solution and hybridized at 37 °C for 14 h in the dark. Negative control samples were incubated with nonsense oligonucleotide 338F-Cy3 (Morris *et al.*, 2002), added at 2 ng  $\mu$ l<sup>-1</sup>, as well as a no probe control, under the same conditions. At the end of the hybridization period, the samples were washed 2  $\times$  10 min at 50 °C using 0.15 M NaCl hybridization wash [150 mM NaCl, 20 mM Tris/HCl (pH 7.4), 6 mM EDTA, 0.01% SDS], dehydrated through an isopropanol series (30%, 50%, 80% isopropanol in PBS), and mounted in one part benzyl alcohol to two parts benzyl benzoate.

A subset of samples were examined for the presence of members of the *Roseobacter* clade using the Cy3-labeled oligonucleotide probe Roseo536R (Brinkmeyer *et al.*, 2000) and the same hybridization protocol used for the bacteria probe suite described earlier, with the following exceptions: the pre-washes and hybridization were carried out in a 35% formamide hybridization solution [0.9 M NaCl, 20 mM Tris/HCl (pH 7.4), 35% formamide, 0.01% SDS], and the posthybridization washes were carried out at 52 °C and used 0.07 M NaCl hybridization wash [70 mM NaCl, 20 mM Tris/HCl (pH 7.4), 5 mM EDTA, 0.01% SDS].

#### *Microscopy*

Samples prepared with DAPI were visualized using a Leica DM5000B phase contrast/fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with 63  $\times$  or 100  $\times$  objectives. All other samples were visualized using a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss Inc., Jena, Germany) and a 63  $\times$  or 40  $\times$  objective. From each sample, 5–15 specimens were examined and compared with 5–10 specimens of the control samples (no stain, no probe, or nonsense probe). Images (optical slices or stacks) were obtained from 2 to 3 representative specimens of each sample and were processed using Zeiss LSM Image Browser version 4.2.0.121 (Carl Zeiss Inc.). Postprocessing of confocal stack images was carried out using Volocity software (Improvision, Coventry, United Kingdom) by applying an intensity rendering. Pixel brightness and contrast was altered uniformly for each image using Photoshop version 7.01 (Adobe Systems Inc., San Jose, CA, USA) to enhance the visualization of probe signals.

**Table 2** Richness and diversity indices for bacteria T-RFs associated with different *P. meandrina* life stages

Life stage	Richness ( <i>S</i> )	Shannon's diversity index ( <i>H</i> )	Evenness ( <i>E</i> )
Oocyte bundles ( <i>n</i> = 3)	72.00 ± 13.11	3.14 ± 0.18	0.74 ± 0.04
Spawned eggs ( <i>n</i> = 18)	105.83 ± 44.55	3.28 ± 0.71	0.71 ± 0.08
AMSW planulae ( <i>n</i> = 9)	28.22 ± 21.30	2.12 ± 0.52	0.66 ± 0.12
LMSW planulae ( <i>n</i> = 3)	21.67 ± 8.39	1.53 ± 0.56	0.51 ± 0.07
Seawater controls ( <i>n</i> = 4)	83.75 ± 21.32	3.28 ± 0.29	0.74 ± 0.02

*n*, number of T-RFLP samples. Values are mean ± standard deviation.

## Results

### *T-RFLP analysis of bacterial communities associated with different coral life stages*

T-RFLP fingerprinting of bacterial communities associated with *P. meandrina* revealed differences in the richness of T-RFs identified per life stage. T-RF richness was highest for the spawned eggs, seawater controls, and oocyte bundles, and much lower for the planulae reared in the ambient and LMSW treatments (Table 2). The Shannon's diversity index (*H*) similarly showed lower bacterial diversity for the planulae samples. Bacteria associated with the planulae also exhibited a less even community compared with the oocyte bundle, egg, and seawater control associated communities (Table 2).

Multidimensional cluster analysis of the T-RFs in each sample resulted in a dendrogram that revealed clustering, or similarity, between the bacterial communities associated with the oocyte bundles, LMSW planulae, AMSW planulae, and the seawater controls (Figure 2). Distance is an objective function based on the linkage method used during the analysis; lower distances indicate higher similarity between samples. From samples obtained solely from colony F (denoted by ^ in Figure 2), the oocyte bundles, eggs, AMSW reared planulae, and AMSW media that the planulae were reared in were all associated with separate clusters, or distinct communities of bacteria. The raw T-RFLP electropherograms similarly illustrated that unique T-RFs were present in each life stage (Supplementary Figures S1 and S2).

Cluster analysis also revealed that samples from the same life stage but from different spawning months formed distinct clusters (Figure 2). For example, egg samples obtained in May and June clustered by month, with limited overlap between the two groups. In addition, the eggs from one colony (colony E) were sampled during both spawning months (May and June) and these samples did not cluster together. A similar trend of clustering by month was shown for the AMSW reared planulae (Figure 2). The raw T-RFLP electropherograms from May and June AMSW reared planulae similarly revealed two distinct bacterial communities (Supplementary Figure S3). Finally, planulae

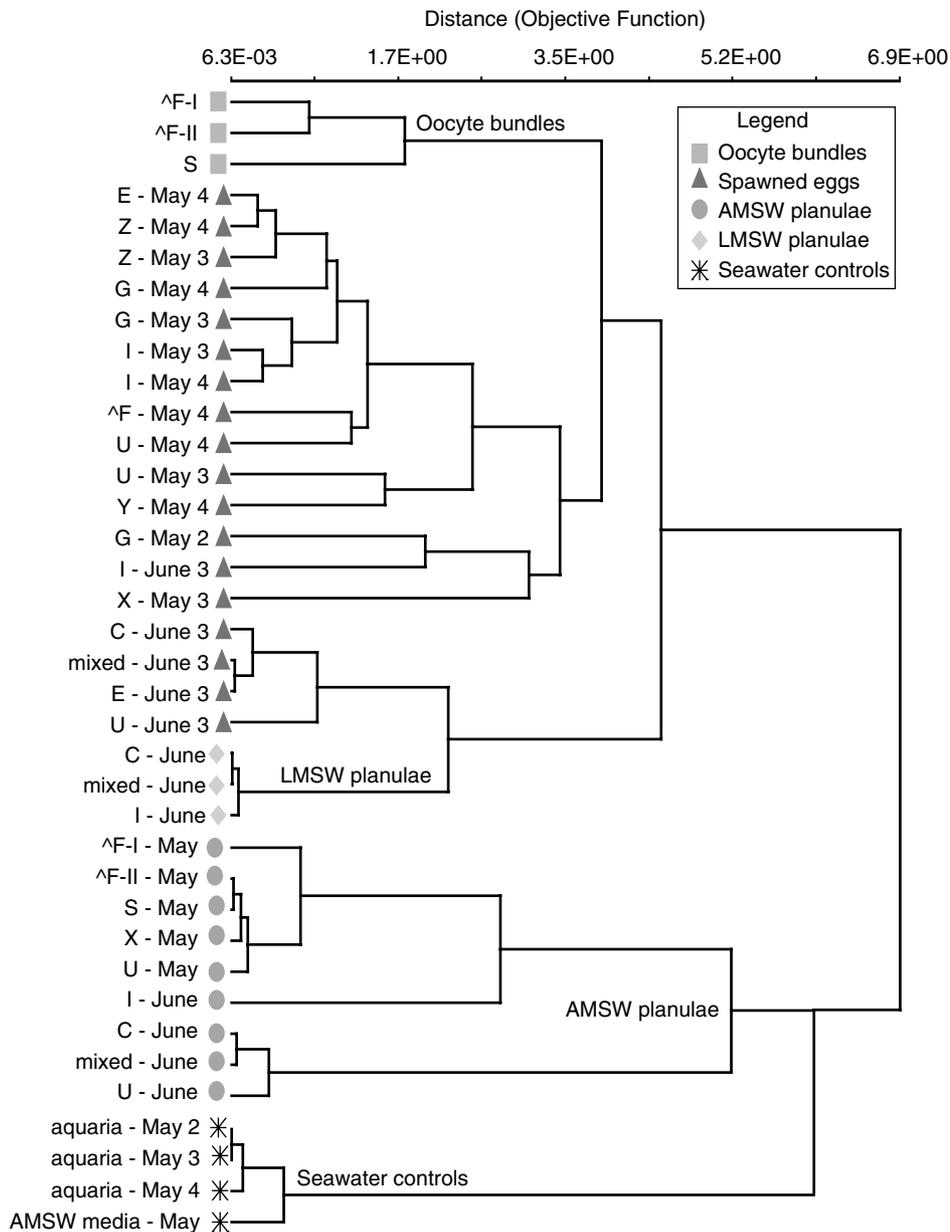
reared in AMSW clustered separately from those reared in LMSW (Figure 2; Supplementary Figure S4), even for planulae obtained from the same colony.

A multiresponse permutation procedure (MRPP) test found significant differences in bacterial community composition between the predefined sample groups. The MRPP analysis provides statistical A-values, with values closer to zero reflecting heterogeneity and values closer to one reflecting homogeneity between the bacterial communities associated with the compared samples. All predefined life stage groupings exhibited A-values less than 0.42, and all life stages were significantly different at a 5% significance level (Table 3). In combination, the cluster and MRPP analyses showed that each life stage of *P. meandrina* examined in this study harbored a distinct community of bacteria, and that the community of bacteria associated with each developmental stage was different from the community found in the surrounding seawater.

### *Clone library analysis of bacterial and archaeal communities associated with different coral life stages*

Phylogenetic analyses of bacterial SSU rRNA clone libraries from developmental stages obtained from the same colony (F) revealed that the oocyte bundles and spawned eggs were associated with diverse members of the bacterial phyla *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Planctomycetes*, *Proteobacteria*, and *Firmicutes*, with limited overlap between the two libraries (Supplementary Tables S1 and S2). A group of 18 clones related to the genus *Streptococcus* were recovered from the egg bundle sample (Supplementary Table S1) and were found to be nearly identical to bacteria associated with humans (data not shown). These may be the result of contamination introduced when these samples were manually dissected from the adult tissues. The remaining spawned egg and oocyte bundle associated gene clones were closely related to bacteria typically found in marine environments.

Nearly 70% of the SSU rDNA sequences obtained from the AMSW planulae clone library (48/70 total clones) were identified as members of the *Roseobacter* clade within the *Alphaproteobacteria*



**Figure 2** Comparison of bacterial community T-RFLP profiles associated with different *P. meandrina* developmental stages and seawater controls through cluster analysis. Letters denote the different colonies, with the mixed sample containing samples from multiple colonies as described in the Materials and methods section. Replicate oocyte bundle and AMSW reared planula samples were obtained from colony F and are denoted as I or II. For the egg samples and aquaria controls, the date indicates when the collections were made and, for the planulae and AMSW media, the month corresponds to the spawning month.

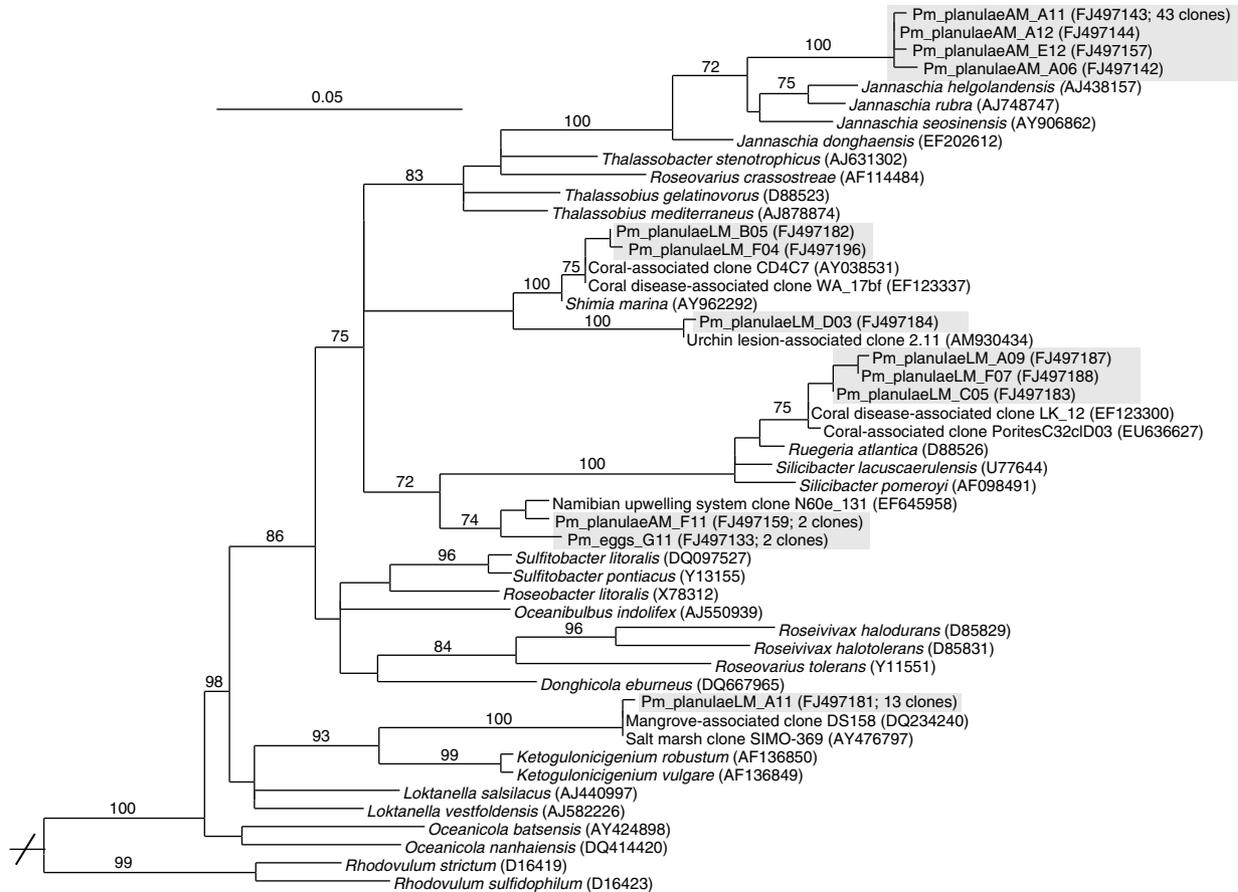
**Table 3** MRPP test for significant differences between predefined groups determined by T-RFLP profiles

Sample	Spawned eggs	AMSW planulae	LMSW planulae	Seawater controls
Egg bundles	0.15 ( $P < 0.002$ )	0.20 ( $P < 0.02$ )	0.42 ( $P < 0.02$ )	0.40 ( $P < 0.01$ )
Spawned eggs		0.28 ( $P < 10^{-5}$ )	0.19 ( $P = 0.001$ )	0.30 ( $P < 10^{-5}$ )
AMSW planulae			0.27 ( $P = 0.003$ )	0.35 ( $P < 10^{-4}$ )
LMSW planulae				0.44 ( $P < 0.01$ )

*P*-values are given in parentheses.

(Supplementary Table S3). Of the *Roseobacter* clade sequences, 96% (46/48 clones) formed a novel lineage within the genus *Jannaschia* (Figure 3).

Forty-three of the *Jannaschia*-related clones were identical. Two clones from each of the colony F egg and planulae clone libraries were also identified as



**Figure 3** Phylogenetic relationships between bacterial SSU rRNA gene clones obtained from *P. meandrina* spawned eggs (Pm\_eggs), planulae reared in AMSW (Pm\_planulaeAM), and planulae reared in LMSW (Pm\_planulaeLM) and representatives of the *Roseobacter* clade of the Alphaproteobacteria. Clones sequenced in this study are highlighted in gray. The scale bar corresponds to 0.05 substitutions per nucleotide position. Only bootstrap values >70% are listed. The Alphaproteobacteria *Bradyrhizobium japonicum* (U69638), *Caulobacter vibrioides* (AJ009957), and *Rhizobium leguminosarum* (U29386) were used as outgroups.

members of the *Roseobacter* clade but were not affiliated with *Jannaschia* (Figure 3). The remainder of bacterial clones recovered in the library from AMSW reared planulae were associated with the phyla *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, OP11, and *Gamma*-subphylum of the *Proteobacteria* (Supplementary Table S3).

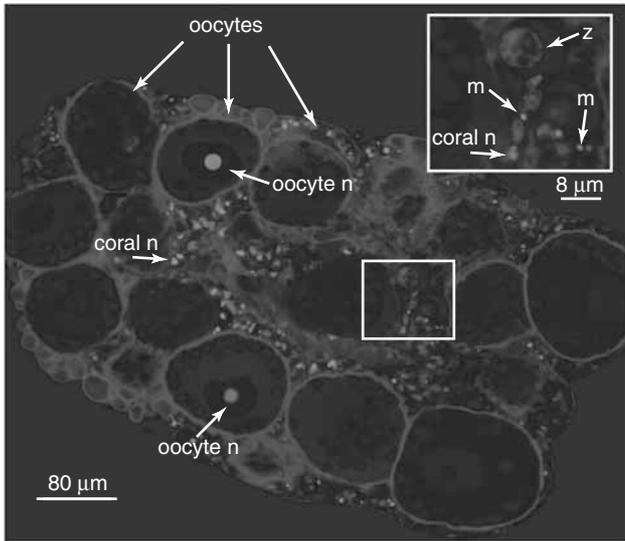
Similar to the bacterial community associated with planulae reared in AMSW, phylogenetic analyses of the LMSW reared planulae revealed a high proportion of bacteria belonging to the *Roseobacter* clade (34%, 20/58 total clones; Supplementary Table S4). These clones were closely related to sequences obtained from healthy and diseased corals but were not associated with the *Jannaschia* lineage (Figure 3). A diverse assemblage of bacteria belonging to the phyla *Bacteroidetes*, *Cyanobacteria*, and *Firmicutes*, and the *Gamma*-subphylum of the *Proteobacteria* were also represented in this clone library (Supplementary Table S4).

The presence of archaea was examined in *P. meandrina* egg bundles, spawned eggs and planulae sampled during 2007, as well as eggs

sampled in 2006, using archaeal specific SSU rRNA gene primers and the PCR. Only the spawned egg life stage samples from 2006 yielded putatively archaeal PCR amplicons, and modest clone libraries were constructed from two samples to confirm that the products were indeed archaea. All of the clones sequenced from these two samples were identified as marine Crenarchaea (Supplementary Table S5).

#### *Microscopic analysis of microorganisms associated with different coral life stages*

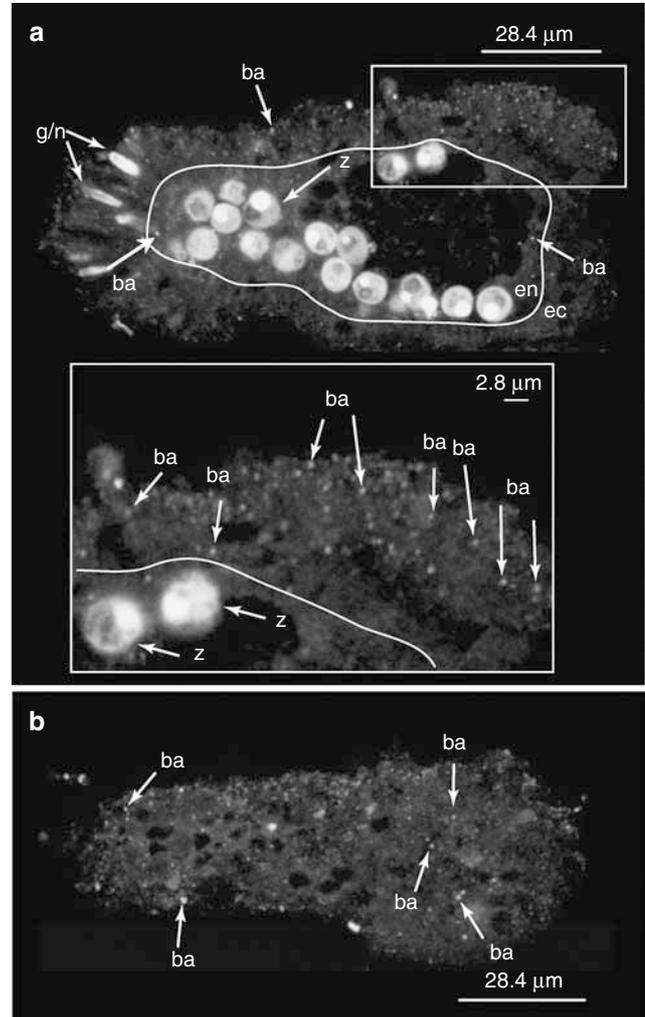
Although the coral and zooxanthellae both exhibited visible autofluorescence, no microbial or zooxanthellae cells were detected within individual oocytes of the bundles from colonies stained with propidium iodide (Figure 4). Zooxanthellae cells, coral mesentery nuclei, and putative microbial cells (which were smaller sized than coral mesentery nuclei) were instead located in the tissue external to the oocytes (Figure 4, inset; Supplementary Figure S5). Spawned eggs examined from eight colonies (Table 1) using either propidium iodide or DAPI staining were found to contain zooxanthellae



**Figure 4** Examination of a single *P. meandrina* oocyte bundle stained with propidium iodide and viewed by confocal microscopy. The prespawned oocyte bundle contains individual oocytes (propidium iodide stained the nuclear material and phalloidin stained f-actin, a component of cell membranes). Coral oocyte nuclei (oocyte n) are visible inside the individual oocytes, whereas zooxanthellae (z) and putative microorganisms (m) are visible inside the bundle, but not within individual oocytes (magnified inset). Coral mesenterial nuclei (coral n) are larger than the putative microbial cells. A colored version of this figure is available as Supplementary Figure S5.

internally, but, again, no microbial cells were present inside the eggs or on the external surface of the eggs. In the propidium iodide stained egg samples, no fluorescence signal outside of that associated with zooxanthellae cells was detected (Supplementary Figure S6a); the unstained control sample (Supplementary Figure S6b) revealed that zooxanthellae cell autofluorescence was visible in both samples. Microorganisms associated with fully developed, 168 h planulae could not be visualized by propidium iodide or DAPI staining due to strong fluorescence from the coral nuclei, which are also stained by these methods.

Using FISH in conjunction with a probe suite targeting the domain bacteria and confocal microscopy, bacterial cells were observed both internally (Figure 5a) and externally (Figure 5b) associated with 168-h-old planulae raised in AMSW. These cells were concentrated internally in the ectoderm and a few were also present in endoderm (Figure 5a) and were not observed in identical preparations containing no oligonucleotide probes (no probe controls, not shown) or a Cy3-labeled nonsense oligonucleotide probe control (Supplementary Figure S7). The location of bacterial cells associated with planulae was consistent for samples prepared from multiple colonies of *P. meandrina* reared in AMSW. Planulae samples from two colonies reared in LMSW were examined with identical methods and found to also harbor bacteria within the ectodermal and endodermal tissues, but at reduced



**Figure 5** Fluorescent *in situ* hybridization of bacterial cells internally and externally associated with 168-h *P. meandrina* planulae. (a) Internal (with magnified inset) and (b) external confocal stacks (4–5 slices) displaying a planula hybridized with Cy3-labeled oligonucleotide probes specific to the domain bacteria (ba = bacterial cells). Planulae in all images are oriented with the oral end on the right. Coral tissues and zooxanthellae (z, representative cells designated with arrows) are illuminated due to autofluorescence in all images. Gland cells and/or nematocysts (g/n) are prominent in the probe and nonsense control sample. The ectodermal (ec) and endodermal (en) differentiation is drawn for reference in the internal stack.

densities compared with AMSW reared planulae (data not shown).

A series of developmental stages spanning the time interval between freshly spawned eggs and 168-h-old planulae that were hybridized with the bacteria-specific probe suite revealed that no bacterial cells were detected in 5 h (18/32 cell division stage; Supplementary Figure S8a) and 7 h (32/64 cell division stage) embryonic stages, or 26 h (Supplementary Figure S8b) and 51 h planulae (Table 4). Bacteria were detected in low densities within the outer ectoderm of 79 h planulae (Figure 6a, inset) and were more concentrated within the ectodermal

**Table 4** Summary of FISH identifications of internally associated bacteria and *Roseobacter* clade cells identified in different *P. meandrina* developmental stages

Developmental stage	Bacteria		Roseobacter clade	
	No. of colonies examined	Internally associated	No. of colonies examined	Internally associated
Egg bundles (2007)	2	0	0	—
<1 h(2007)	2	0	0	—
5 h(2005)	Mixed	0	0	—
7 h (2005)	Mixed	0	0	—
27 h (2005)	Mixed	0	0	—
51 h (2005)	Mixed	0	0	—
79 h (2005)	Mixed	+	0	—
131 h (2005)	Mixed	+++	Mixed	+++
168 h, AMSW (2007)	5	+++	1	+++
168 h, LMSW (2007)	2	+	1	+

0 indicates no cells observed; dashes indicate no data; + indicates qualitative indication of the density of probe identified in the samples.

and outer endodermal tissues of 131 h planulae, which had developed a mouth, gut, and differentiated cells such as gland cells and nematocysts (Figure 6b, inset).

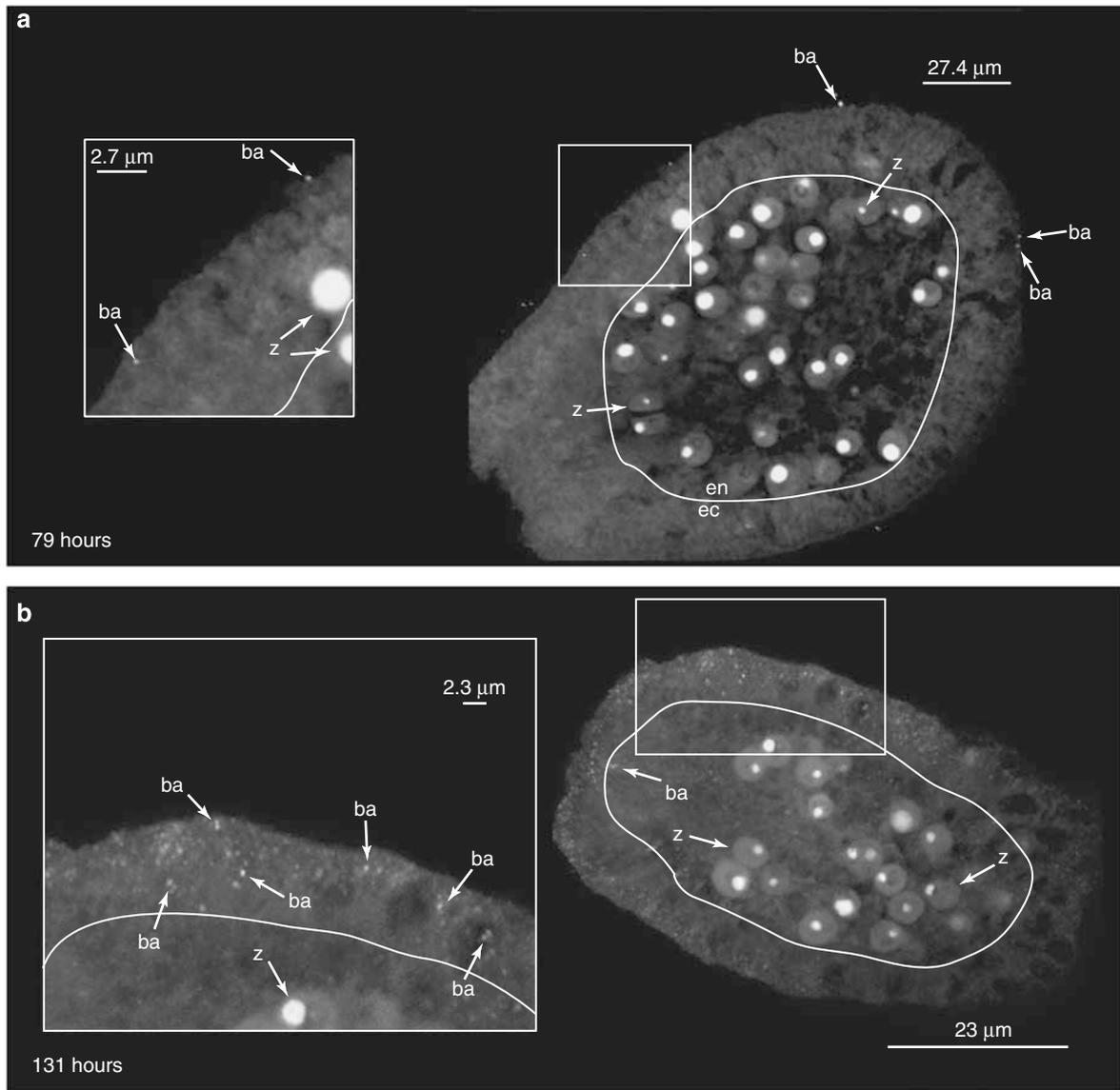
FISH experiments with a fluorescently labeled oligonucleotide probe specific to the *Roseobacter* clade revealed that 131-h-old planulae were associated with *Roseobacter* cells. The *Roseobacter* cells were densely associated with the ectoderm and frequently concentrated on the aboral end of the planulae (Figure 7a). Microbial-sized cells were not observed within a control, no probe sample (Supplementary Figure S9a). A similar pattern of distribution in the ectoderm, and an increased number of cells in the endoderm, was observed in 168-h planulae from 2007 reared in AMSW (Figure 7b, inset). In agreement with observations made with the general bacterial probe suite, fewer *Roseobacter* clade cells were observed in the planulae from 2007 reared in LMSW (Supplementary Figure S9b, inset). Table 4 summarizes the results of these observations.

## Discussion

This study is the first to examine the onset of microbial associations in a coral that obtains symbiotic dinoflagellates vertically from the maternal colony. The SSU rDNA data presented here shows that distinct communities of bacteria are associated with the prespawning oocyte bundles, spawned eggs, and planulae developmental stages of *P. meandrina*. However, microscopic analyses revealed that bacterial cells are not internally present until the planulae are fully developed. Bacteria belonging to the *Roseobacter* clade were identified as important members of the community within planulae reared in both ambient as well as reduced microbial cell density seawater from different colonies over two spawning years.

A diverse community of bacteria associated with *P. meandrina* oocyte bundles was detected, and this community was found to be distinct from those associated with the egg and planulae life stages. The oocyte bundle bacteria were localized within the bundle rather than within individual oocytes. A diverse community of bacteria was also found to be associated with the freshly spawned eggs but was microscopically determined not to be localized within the eggs themselves. A large number of clones recovered from the egg-associated library consisted of groups of marine bacteria previously identified in Kaneohe Bay seawater, including the SAR11 cluster of the *Alphaproteobacteria*, the SAR86 cluster of the *Gammaproteobacteria*, and the *Cyanobacteria* genus *Synechococcus* (M Rappé, unpublished data). Bacteria belonging to these groups likely originated from the associated seawater, though a number of other diverse microbial groups were also identified. It is possible that some of these bacteria may be externally associated with the eggs but were removed from the mucus surface during rinsing of the samples and were therefore not observed by microscopy. Archaea were not found to be ubiquitously associated with developing *P. meandrina*. Despite screening oocyte bundle, egg and planulae life stages, only spawned eggs of *P. meandrina* were associated with archaea, and were all found to belong to the marine Crenarchaeota group.

Our microscopy observations revealed an established coral–microbial relationship in late stage developing corals. The bacterial cells were primarily concentrated on the external surface and within the ectodermal tissue layer, though some cells were also found associated with the endoderm. The primary acquisition method may be phagocytosis by the ectoderm, which is in closest contact with the seawater microbial community. Acquisition through exterior tissue layers is a mechanism used by the Hawaiian bobtail squid (Foster and McFall-Ngai, 1998) and hydrothermal vent tube worms (Nussbaumer *et al.*, 2006) to acquire and concentrate micro-



**Figure 6** Localization of bacteria in developing *P. meandrina* through fluorescent *in situ* hybridization with a suite of Cy3-labeled oligonucleotide probes specific to the domain bacteria. Bacteria (ba, representative cells designated with arrows) were observed in the external ectoderm (ec) of 79 h planulae (a) and were heavily concentrated within 131-h-old ectodermal (ec) tissues (b) with fewer cells located in the endodermal (en) tissues. Autofluorescent coral tissue and zooxanthellae (z, representative cells designated with arrows) were present in all life stages. All images are projections of confocal stacks containing 7–8, 0.1  $\mu\text{m}$ -thick image slices. The ectodermal and endodermal boundary is drawn for reference.

bial symbionts, albeit through different strategies. In corals, the most common mode for acquiring zooxanthellae symbionts is horizontally through the mouth and into the gastrodermal cavity, where they avoid digestion and are incorporated into the animal's tissues (Kinzie, 1974; Marlow and Martindale, 2007). In *P. meandrina*, we observed bacteria in 79-h-old planula, which already have a gut and mouth. Although ingestion through the mouth and subsequent transfer to the ectoderm is a possible acquisition method, we did not observe bacterial cells concentrated within the gastrodermal cavity. Rather, bacterial cells were mostly concentrated in ectodermal cells at the aboral pole of the planulae. The incorporation of microorganisms during the

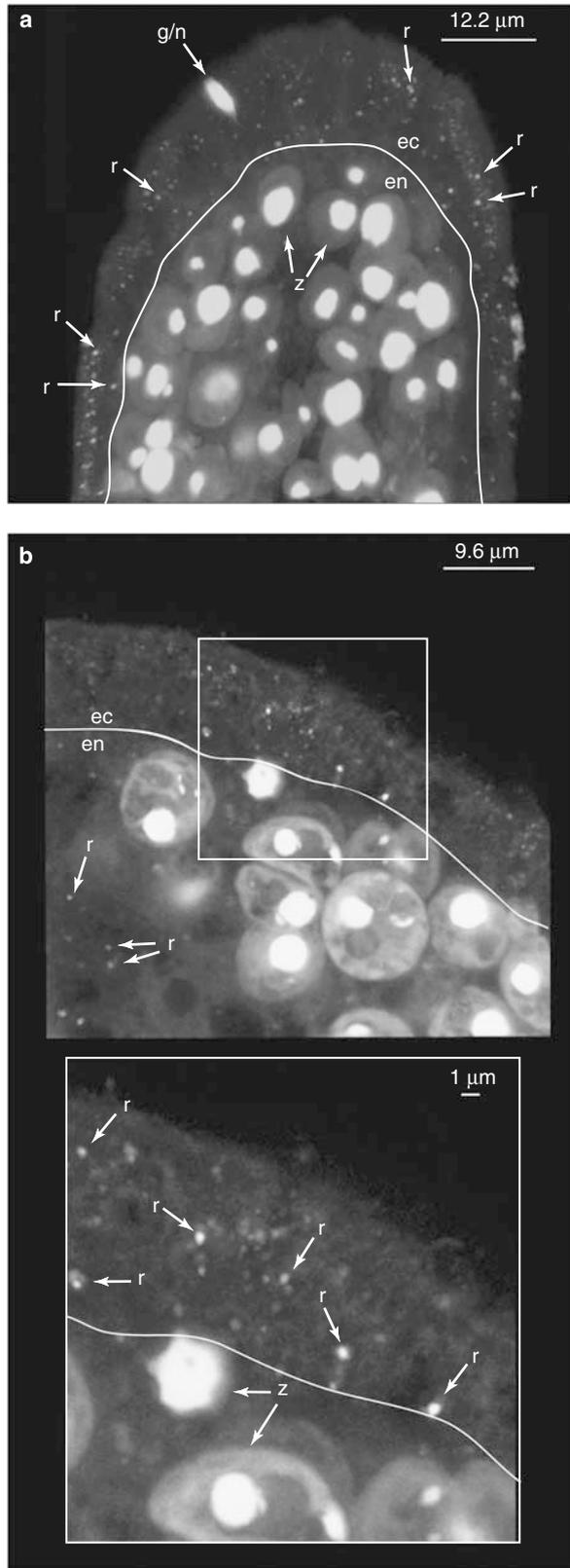
later stages of planulae development indicates that they may play a role in processes specific to activities during this life stage, such as settlement onto a benthic substrate. Fully developed coral planulae generally settle at about 168 h postfertilization (Babcock and Heyward, 1986), and it is possible that bacteria play an important role in providing cues regarding suitable substrates (Negri *et al.*, 2001; Webster *et al.*, 2004).

The prominence of microorganisms of the *Roseobacter* clade in the AMSW reared planulae that we preliminarily identified as related to the genus *Jannaschia*, and confirmation of the same *Jannaschia*-related T-RF from multiple coral colonies, indicates a potentially important association for

*P. meandrina* planulae. On the basis of T-RFLP analyses, the *Jannaschia* lineage made up 0.40% of the SSU rRNA genes in the AMSW seawater media and was detectable at an average abundance of

0.68% in southern Kaneohe Bay (A Apprill and M Rappé, unpublished data). Because of the presence of members of the *Jannaschia* lineage in the AMSW media coupled with the high SSU rRNA gene similarity of *Jannaschia*-related gene clone sequences associated with the planulae, we hypothesize that this lineage proliferated in *P. meandrina* after being horizontally incorporated into the larval tissues. Although difficult to enumerate accurately, we have conservatively calculated a doubling time of 10–16 h for internally associated microbial cells, based on micrographs taken from samples hybridized with the *Roseobacter* clade-specific probe. This doubling time is consistent with other organism-associated marine bacteria (Ruby and Asato, 1993). However, it is also plausible that the microbial cells are being actively acquired from the seawater. The planulae reared in seawater containing less concentrated microorganisms (LMSW) harbored substantially fewer associated bacterial cells, though they were also disproportionately high in members of the *Roseobacter* clade. These findings suggest that the planulae preferentially take up certain groups of bacteria when they are available, such as microorganisms of the *Jannaschia* lineage, and may take up other members of the *Roseobacter* clade when *Jannaschia* are unavailable.

It is possible that the dominance of our clone libraries by SSU rRNA genes related to *Jannaschia* may be due to methodological issues. In particular, a high number of PCR amplification cycles and semi-nested reactions were required, which have the potential to bias the composition of the amplicons used to construct the libraries. However, several lines of evidence support our findings. First, we observed the same prominent *Jannaschia*-related T-RF in planulae samples originating from different coral colonies. These T-RFs resulted from single, un-nested 30-cycle PCR reactions. Second, we observed a high prevalence of cells of the *Roseobacter* clade internally associated with *P. meandrina* planulae through FISH. Finally, a clone library constructed from a planula sample in an un-nested, single 30-cycle PCR amplification reaction that used general bacterial primers that targeted nearly full length SSU rRNA genes was also found to be dominated by *Jannaschia*-related bacterial clones in preliminary analyses (data not shown). The congruence between



**Figure 7** Localization of cells of the *Roseobacter* clade in *P. meandrina* planulae. (a) The 131-h-old planula from 2005 reared in nonfiltered seawater, hybridized with a Cy3-labeled oligonucleotide probe specific to the *Roseobacter* clade. (b) The 168-h-old planulae from 2007 reared in AMSW (full panel with magnified section). *Roseobacter* (r, designated with arrows) were abundant in the external ectoderm (ec) of (a) and (b) were also located in the endoderm (en) of (b). All images contain autofluorescent coral and zooxanthellae (z) tissues (representative zooxanthellae are designated with arrows). All images are projections of confocal stacks containing 7–8, 0.1- $\mu\text{m}$  thick image slices, and the ectodermal and endodermal differentiation is drawn for reference.

different analyses gives us confidence that the relationship between *Jannaschia*-related microorganisms and *P. meandrina* planulae is not an artifact of our methodology.

Members of the *Roseobacter* clade are known to form relationships with phytoplankton, invertebrates and vertebrates (Hjelm *et al.*, 2004). They are prevalent, and often numerically dominant, in laboratory cultures of dinoflagellates, and may even be intracellular within the dinoflagellates (Alavi *et al.*, 2001). The relationship between members of this phylogenetic clade and dinoflagellate cultures, dinoflagellate blooms, and zooxanthellae corals is potentially linked to the metabolism of dimethylsulfoniopropionate (DMSP), which is produced in high concentrations by dinoflagellates, including many species of zooxanthellae corals (Hill *et al.*, 1995; Broadbent *et al.*, 2002; Van Alstyne *et al.*, 2006). The genome of *Jannaschia* sp. strain CCS1 includes *dmdA*, a demethylase enzyme-coding gene that mediates the first step in DMSP degradation (Moran *et al.*, 2007). This strain also contains multiple chemotaxis receptor genes and may exhibit an attraction toward DMSP or other algal osmolytes (Moran *et al.*, 2007). It is plausible that the prevalence of cells of the *Roseobacter* clade within developed planulae may be in response to the availability of DMSP produced by the zooxanthellae. For corals that acquire zooxanthellae symbionts horizontally, other modes of chemical attraction may be used to acquire bacteria. It is also possible that the free-living zooxanthellae cells may contain intra- or extracellular bacteria, which are incorporated along with the zooxanthellae. It is interesting to note that, although other members of the *Roseobacter* clade frequently associate with adult scleractinian corals (Rohwer *et al.*, 2001; Cooney *et al.*, 2002; Sekar *et al.*, 2006), members of the genus *Jannaschia* have not been detected previously.

Coral planulae reside in dynamic reef environments and, similar to observations made with zooxanthellae (Little *et al.*, 2004), altered environmental conditions undoubtedly impact their infection by or incorporation of other microbial partners. We observed that the composition of the bacterial community incorporated into planulae tissues was impacted by the density of bacteria in the incubation media. This finding indicates that the planulae may be affected by the composition of the surrounding bacterioplankton community. In reef environments, the composition of pelagic microbial communities quickly changes in response to nutrient alterations from storms (Cox *et al.*, 2006) and potentially other anthropogenic interactions (Dinsdale *et al.*, 2008), and nutrient loading events have already been correlated with reduced coral development (Loya *et al.*, 2004). Continued research on the necessity of specific microbial associations in developing corals is essential to predict the response of corals and their recruitment success in the wake of globally changing environmental conditions.

In summary, we found that the coral *P. meandrina* acquires an internal microbial flora early in its development cycle. Conservatively, we detected internally associated microorganisms within planulae as early as 79 h after fertilization but did not detect them within eggs or younger embryos. Although *P. meandrina* seeds its eggs with zooxanthellae endosymbionts, it does not seed the eggs with microbial cells. Acquisition of microbial cells through the planular ectoderm is a plausible entry route due to the concentration of the microorganisms within the ectodermal tissues, and the phagocytosis of bacteria may depend on characteristics of the surrounding planktonic microbial community, such as cell density or community structure. Members of the *Roseobacter* clade and, in particular, the *Jannaschia* lineage, appear to be an important component of the microbial community associated with *P. meandrina*, though additional work is needed to comprehensively assess the specificity of microbial uptake by the planulae, the mechanism of uptake and acquisition of microbial cells, and the stability of this association throughout settlement and adulthood. Investigating these interactions in other coral species will advance our understanding of the fundamental interactions between microorganisms and the developing coral holobiont, and aid in our assessment of the onset, longevity, and potential symbiotic nature of coral–microbial relationships.

## Acknowledgements

We thank M van Oppen and E Rottinger for assistance with coral collections, P Jokiel and K Rodgers for use of aquaria, and M Miller for assistance with egg collections. Coral collections were conducted under the State of Hawaii's Department of Land and Natural Resources special activity permit # 2007-02. This research was supported by funding from a research partnership between the Northwestern Hawaiian Island Coral Reef Ecosystem Reserve and the Hawaii Institute of Marine Biology (NMSP MOA 2005-008/66882) to MSR, and a NSF predoctoral research fellowship to AA. This is SOEST contribution 7555 and HIMB contribution 1337.

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