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# Metamorphosis of broadcast spawning corals in response to bacteria isolated from crustose algae

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ABSTRACT: External chemical signals provide a mechanism for broadcast-spawning scleractinian corals to recognise suitable substrata for larval settlement and metamorphosis. These morphogens can be extracted from crustose coralline algae (CCA) and the skeletons of some coral species, however the precise origin of the chemical inducers has not yet been conclusively demonstrated. Microorganisms have been reported to induce metamorphosis in various species of echinoderms, molluscs, polychaetes and cnidarians. We report that Strain A3, a species of *Pseudoalteromonas* isolated from the CCA *Hydrolithon onkodes* (Heydrich), was able to induce significant levels (up to 51.5% ± 5.8 SE) of metamorphosis of *Acropora willisae* Veron & Wallace, 1984 and *A. millepora* (Ehrenberg, 1834) larvae in laboratory assays. This experiment was repeated daily over 4 d, and the spat developed normally into juvenile polyps in flow-through aquaria. Approximately the same number of larvae underwent partial metamorphosis, forming flattened discs that were not attached to the substrata. Larvae underwent full settlement, attachment and metamorphosis only in the presence of *Pseudoalteromonas* A3 plus inert chips of the coral skeleton *Porites* sp., indicating that the calcareous matrix may play a role in the synthesis of inducers from *Pseudoalteromonas* Strain A3. This discovery provides evidence for a widening range of morphogenic sources and demonstrates the role that microorganisms may play in fine-scale coral recruitment. In addition, the synthesis of chemical inducers by *Pseudoalteromonas* Strain A3 may have biotechnological applications for reef re-seeding.

KEY WORDS: Coral · Metamorphosis · Settlement · Bacteria



## INTRODUCTION

### Reproduction and recruitment

Corals exhibit a range of reproductive strategies, which include both sexual and asexual propagation. Many species of brooding coral exhibit internal fertilisation and expel well-developed larvae at various times of the year. However, most species of coral reproduce during annual spawning events whereby gametes are ejected into the water column synchronously and fertilisation occurs outside the polyp (Harrison & Wallace 1990). Both strategies result in the development of free-swimming planula larvae that seek appropriate substrata upon which to settle, attach and undergo metamorphosis. The larvae of broadcast-spawning scleractinian corals typically become competent to metamorphose into juvenile polyps within a week of the spawning and fertilisation event (e.g. Babcock & Heyward 1986). At this stage, larvae have developed cilia for motility and are able to use sensory and secretory cells to actively 'taste' and subsequently adhere to appropriate surfaces (Heyward 1987). Larvae at this stage of development have been reported to probe or crawl over available surfaces and are thought to actively select suitable substrata upon which to attach and metamorphose (Fadlallah 1983, Harrison & Wallace 1990).

The terms settlement and metamorphosis continue to be used interchangeably in the literature. In this

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paper we use the term 'settle' to describe the point where pelagic larvae have become pear-shaped and casually attached to the substrata at the aboral end. This is a behavioural description. Metamorphosis occurs when larvae undergo physiological and morphological changes that are largely irreversible. Early metamorphosis in acroporid species often occurs within 12 h of settlement, when larvae have developed into firmly attached disc-shaped structures with pronounced flattening of the oral-aboral axis and typically obvious septal mesenteries radiating from the central mouth region (Harrison & Wallace 1990, Heyward & Negri 1999). Subsequent calcification proceeds rapidly and the development of tentacles usually occurs within a week. As corals are sessile animals, the choice of an appropriate substrata and habitat for settlement of planula larvae is crucial to survival. In field studies, larvae have been reported to prefer undersurfaces and, in general, favour locations of low light intensity and irregular or rough substrata (see Harrison & Wallace 1990, Mundy & Babcock 1998).

### Chemical signals for metamorphosis

In addition to physical influences, increasing evidence indicates that many corals, such as the brooding agariciids and the broadcast-spawning acroporid and favid species, require an external chemical signal to trigger settlement and metamorphosis (Morse et al. 1988, 1994, 1996, Heyward & Negri 1999). The best-known source of chemical morphogens for coral larvae are various species of non-geniculate calcareous red algae (CCA). Larvae of agariciid (Morse et al. 1988) and acroporid corals (Morse et al. 1996, Heyward & Negri 1999) do not require continuous contact with the water-insoluble cue(s) throughout metamorphosis, but are able to proceed to other substrata for permanent attachment. In addition, such coral species may have evolved chemosensory receptors in order to recognise the clean, high-current habitats often colonised by CCA and most suitable for successful survival of the adult colonies. The identity of these CCA-associated chemical cues has been reported as cell-wall-bound, high molecular mass polysaccharides that can only be released following decalcification (Morse et al. 1994, 1996). Chemical inducers for the metamorphosis of *Acropora millepora* larvae were later found to be readily extractable without decalcification from various species of CCA using methanol (Heyward & Negri 1999). Inducers for metamorphosis were also discovered in the skeleton of the massive coral *Goneastrea* sp. and coral rubble (Heyward and Negri 1999). These observations indicate that either multiple inducers for metamorphosis exist or specific inducers may originate from a variety of sources.

### Microbial sources of morphogens

The role of microorganisms in the induction of settlement and metamorphosis in marine invertebrates across a wide array of phyla is becoming increasingly apparent (see Johnson & Sutton 1994, Leitz 1997, Bryan & Qian 1998, Unabia & Hadfield 1999). Most studies have examined the role of microbial biofilms in preference to specific activity of particular bacterial isolates. Microalgal and bacterial biofilms of indeterminate species composition induced metamorphosis in up to 16% of individual agariciid larvae (Morse et al. 1988). In the same experiments, CCA induced almost 90% metamorphosis. Larvae of 2 additional brooding corals have been shown to settle and metamorphose in response to biofilms. The soft coral *Heteroxenia fuscenscens* underwent 60 and 40% metamorphosis in the presence of 2 strains of unknown bacteria isolated from a coral skeleton (Henning et al. 1991), while the miniature scleractinian coral *Stylaraea punctata* responded to natural microbial biofilms in preference to other substrata (Golbuu et al. 1995). This raises the possibility that microbial biofilms, in addition to CCA, may function to influence metamorphosis in some coral species. Microorganisms associated with CCA are thought to play a role in triggering metamorphosis of other marine invertebrates. For instance, bacterial films associated with CCA induce metamorphosis in the crown-of-thorns starfish (Johnson & Sutton 1994). In fact, the larvae of many cnidarian species, including hydrozoans and scyphozoans, undergo settlement and metamorphosis in response to bacteria isolated from natural substrata including CCA (see comprehensive review by Leitz 1997).

Electron microscopy techniques have been used to visualise high densities of bacteria on the surface of various CCA species (Galbary & Veltkamp 1980, Garland et al. 1985, Lewis et al. 1985, Johnson et al. 1991a,b). It is possible that bacteria associated with CCA may be involved in the synthesis of inducers for metamorphosis of scleractinian corals. In this study, we isolated various species of heterotrophic bacteria from the surface of CCA and examined their potential to induce the settlement and metamorphosis of larvae from 2 broadcast-spawning scleractinian corals from the Great Barrier Reef.

## MATERIALS AND METHODS

Experiments were conducted with widespread plateforming acroporid species from 2 mass spawnings, the first at Lizard Island on the Great Barrier Reef (14° 40′ S, 145° 26′ E) on November 18, 1997, and the second at Nelly Bay, Magnetic Island (19° 10′ S, 146° 51′ E), on November 27, 1999.

**Spawning, gamete collection and larval cultivation.** Expt 1 was conducted at the Lizard Island Research Station. Eight live colonies of the reef-building coral *Acropora millepora* (Ehrenberg, 1834), each measuring approximately 30 × 30 cm, were collected by hand from fringing reef crests and maintained in outdoor, flow-through seawater tanks for 2 d before spawning. Prior to sunset on the night of November 18, 1997, the individual colony pieces were isolated in 50 l plastic containers. Synchronous spawning occurred between 21:30 and 22:00 h. The released gametes, in the form of buoyant egg-sperm bundles 1 to 1.5 mm in diameter, were collected in 250 ml plastic containers from the water surface by gentle suction. Gametes from all colonies were cross-fertilised for 1 h in a single 200 l plastic tank. The eggs, which formed a monolayer on the water surface, were then transferred to a 3000 l plastic tank for primary rearing. A 75% water change was performed after 2 h. The embryos were then left undisturbed until the following morning, when gentle aeration was introduced. Fresh seawater changes were performed twice daily as the larvae developed cilia and became motile. Tank water temperature fluctuated daily between 27 and 31°C and was monitored by an *in situ* data logger.

The same procedures were adopted in 1999 for Expt 2, which was conducted using similar-sized colonies of *Acropora willisae* Veron & Wallace (1984) collected from fringing reefs around Magnetic Island. Sixteen colonies were transported to the aquarium system at Reef HQ Aquarium of the Great Barrier Reef Marine Park Authority in Townsville, and spawning occurred between 21:00 and 22:00 h. Ciliated planula were transported to the aquarium systems at the Australian Institute of Marine Science (AIMS) 3 d post-fertilisation and maintained in 100 l flow-through tanks with 1 µm-filtered seawater and aeration at 26 to 28°C.

**Preparation of substrata.** ***Expt 1a:*** Small chips (5 × 5 × 2 mm) of CCA can be used in laboratory experiments to induce a high proportion of coral larval metamorphosis (Heyward & Negri 1999). Chips of a single continuous colony of CCA (*Hydrolithon onkodes* Heydrich) collected from Lizard Island in 1997 were prepared as positive control substrata. Two inert substrata (negative controls) were prepared by cutting similar-sized chips from autoclaved terracotta tiles and autoclaved skeleton from the massive coral *Porites* sp. (Heyward & Negri 1999).

Johnson & Sutton (1994) reduced the level of bacteria from CCA using antibiotic treatment. We treated CCA chips with the antibiotics tetracycline and streptomycin (30 mg $l^{-1}$ each) in a similar attempt. Separate sterilisation treatments were performed by oxidation of CCA chips with 2 g $l^{-1}$ sodium hypochlorite or by autoclaving at 121°C and 1.5 atm for 30 min.

***Expt 2:*** Similar substrata were prepared in 1999 using the CCA *Lithophyllum sp.* collected from Magnetic Island. The negative controls used in this experiment were also chips produced from fragments of terracotta tiles and skeleton of *Porites* sp.

**Bacterial isolation and cultivation.** ***Expt 1b:*** All procedures in Expt 1b in 1997 were performed aseptically and used sterile artificial seawater (ASW). Live cells from the outer 0.5 mm tissue were scraped from 1 $cm^2$ sections of a live continuous colony of *Hydrolithon onkodes* using a sterile scalpel, and were ground with a mortar and pestle. This material was suspended in 9 ml ASW and mixed by vortexing for 10 min. Isolates were labelled A,B,C to signify the 3 replicate sections from the same algal colony. Ten-fold serial dilutions of each suspension were prepared to $10^{-4}$, and 100 µl of each dilution was spread-plated in triplicate on Bacto Marine Agar 2216 (2MA) (Difco Laboratories, Detroit, USA). This medium is designed for isolation and enumeration of heterotrophic marine bacteria. All plates were incubated at 27°C for 72 h, and representatives of each colony morphotype were serially streak-plated on Marine Agar 2216 in order to obtain pure cultures. Bacterial films of pure cultures were prepared on drops of Marine Agar 2216 and glass coverslips. Bacterial suspensions were prepared by placing a small swab of culture from individual colonies into 5 ml of Marine Broth 2216. Biofilms were established by adding a single piece of sterile substrate (agar drop or glass coverslip) to each well. The biofilms were subsequently incubated for 24 h at 27°C prior to removal from marine broth and placement in assay wells containing ASW.

***Expt 2:*** The same procedures were used in 1999 to isolate bacteria from the surface of *Lithophyllum* sp. collected from Magnetic Island. All procedures in Expt 2 were performed aseptically and used 0.2 µm filter-sterilised seawater (FSW). Bacterial films were prepared on *Porites* sp. and terracotta chips by incubating each substratum in 5 ml of each bacterial culture for 24 h. The Strain A3 isolated from Expt 1 in 1997 was re-cultured from cryopreserved samples and also prepared as a biofilm in these experiments. After incubation of the biofilm for 24 h, the substrata were transferred to assay wells containing 10 ml FSW and coral larvae. In separate experiments, assay wells containing sterile terracotta or *Porites* sp. chips and coral larvae were inoculated directly with 2 µl of Strain A3 suspended in Marine Broth at $10^8$ CFU $ml^{-1}$ (final concentration approximately $2 \times 10^4$ cells $ml^{-1}$).

**Bacterial identification by 16S rRNA gene-sequence analysis.** The heterotrophic bacterial isolate Strain A3 was identified by 16S ribosomal RNA (rRNA) gene sequence analysis. Strain A3 was grown overnight in 100 ml Marine Broth 2216 and a DNA extract was pre-

pared using a method based on that of Ausubel et al. (1987). Bacterial-specific oligonucleotide primers (forward primer 8-27: 5′-GAGTTTGATCCTGGCTCAG-3′ [Weisburg et al. 1991] and reverse primer 1492: 5′-GGTTACCTTGTTACGACTT-3′ [Reysenbach et al. 1992]) were used to amplify 16S rRNA gene fragments from Strain A3. PCR products were purified by electrophoresis in a 1% (w/v) agarose gel, excised and recovered using a gel extraction kit (Qiagen, Inc., Chatsworth, CA), and sequenced on an ABI 310 automated sequencer using the PRISM Ready Reaction Kit (PE Applied BioSystems). Sequence data was analysed by comparison to 16S rRNA genes in the Ribosomal Data Base Project (Maidak et al. 1999) and the Genbank database. All sequences were manually aligned to *E. coli* using Phydit software (Chun 1995). The nearest relatives of each organism were obtained by BLAST searches (Altschul et al. 1990). Phylogenetic trees were then inferred by comparing homologous nucleotides using the neighbour-joining (Saitou & Nei 1987), Fitch-Margoliash (Fitch & Margoliash 1967) and maximum parsimony (Kluge & Farris 1969) algorithms in the PHYLIP package (Felsenstein 1993). Evolutionary distance matrices for the neighbour-joining and Fitch-Margoliash methods were generated as described by Jukes & Cantor (1969). Tree topologies were evaluated after 1000 bootstrap re-samplings of the neighbour-joining data.

**Extraction of chemical inducers.** Methanol extracts of CCA were performed as described in Heyward & Negri (1999). Bacterial extracts were prepared by fermenting bacteria in 1000 ml of Marine Broth 2216 overnight at 27°C and 180 rpm in a Bioline orbital shaker (Edwards Instrument Company, Australia). Cells were concentrated by centrifugation (3000 × *g*) and the pellets lyophilised. The dry pellets were extracted twice with HPLC-grade methanol (BDH Laboratory Supplies, Poole, Dorset, UK) and the combined methanol extracts were pooled and the solvent removed under vacuum. The extract was introduced to assay wells as a suspension in 0.2 µm FSW (at concentrations between 0.05 and 10% v/v).

**Metamorphosis assays.** Larval metamorphosis assays were performed in sterile 6-well cell culture plates (Iwaki, Japan) maintained at a constant temperature of 27 to 28°C. Coral larvae (n = 10 to 20) of various ages (depending on the experiment) were introduced to each well containing the substrata and bacteria to be tested and ASW or FSW (depending on the experiment) to a final volume of 10 ml. For each bacterium, 6 or 12 replicate wells were used to provide sufficient levels of replication, rapid throughput and assessment of many treatments while maintaining high levels of water quality. Control wells contained either chips of CCA (5 × 5 × 2 mm), which typically induces high levels of metamorphosis (Heyward & Negri 1999), or FSW with chips of *Porites* sp. or terracotta as negative controls. The endpoint chosen for scoring early-stage metamorphosis was 24 h (Heyward & Negri 1999). At this time, planula larvae had permanently attached and primary polyps were developing. Metamorphosis was scored by direct counting of all larvae and newly metamorphosed polyps in each well using a dissecting microscope.

The artificial inducer tumour-promoting phorbol ester (TPA) has been shown to induce metamorphosis in planula of numerous cnidarians, including a broadcast-spawning octocoral, 4 brooding octocorals, and a single brooding scleractinian from the Red Sea (Henning et al. 1996). We repeated this experiment with the broadcast-spawning scleractinian *Acropora millepora* by exposing larvae to $10^{-5}$ to $10^{-12}$ M TPA (Sigma) in FSW.

**Data treatment.** Mean and standard error (SE) values were calculated and plotted (Sigmaplot 4, SPSS Science, Chicago, IL). For bar graphs only data +SE are presented. Differences in numbers of settled larvae between treatments required that a comparative measure of metamorphosis be obtained using the log-transformed data according to Eq. (1) below (McCullagh & Nelder 1989). Comparisons were made on all treatment groups within an experiment, and degrees of freedom and *F* statistics were calculated accordingly. An analysis of variance (ANOVA) using the Tukey honest-significant-difference test was performed on each transformed data set (Statistica for Windows, v. 5.1, StatSoft Inc., Tulsa, OK).

$$lp_i = \log_{10}(a + 1)$$

where $lp_i$ = the log-transformed probability of metamorphosis, and $a$ = the proportion of larvae metamorphosed (%).

## RESULTS

### Expt 1

A high proportion of *Acropora millepora* larvae (81.2% ± 6.8 SE) metamorphosed in the wells containing CCA chips in FSW (Fig. 1). Larvae settled and metamorphosed predominantly on the floor and edges of the polystyrene wells and not directly on the CCA (see Heyward & Negri 1999). The sterile CCA chips also induced a relatively high proportion of metamorphosis, not significantly different from CCA (Fig. 1, $p > 0.05$, $F_{6,33} = 54.2$). No metamorphosis was observed in the negative control wells in response to chips of terracotta tiles, *Porites* sp. skeleton or FSW alone. The phorbol ester TPA, did not induce metamorphosis across the concentration range $10^{-5}$ to $10^{-12}$ M (results not shown).

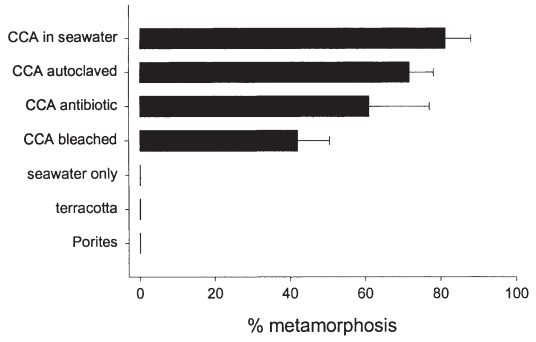


Fig. 1. *Acropora millepora.* Proportion (+SE) of metamorphosis of 7 d-old larvae exposed to live and sterilised chips of the crustose coralline alga (CCA) *Hydrolithon onkodes* and control sterile and terracotta chips *Porites* sp. (Expt 1a). Six replicate wells containing filter-sterilised seawater (FSW) were used for each treatment

Artificial seawater was used to encourage establishment of microbial biofilms and to test for subsequent larval metamorphosis. Under these conditions, however, less than 10% settlement and metamorphosis was observed in the presence of CCA and sterile agar or coverslips (Fig. 2). This reduction in metamorphosis was probably due to the use of ASW, which was subsequently replaced by FSW in Expt 2. Approximately 10% of larvae underwent partial metamorphosis, flattening into discs and displaying obvious septal mesenteries radiating from the central mouth region. This indicated that a significant development event had occurred, but none of these larvae had permanently attached to a surface and instead floated freely in the ASW.

Of the 20 heterotrophic bacteria isolated from *Hydrolithon onkodes*, 1 isolate, designated Strain A3, induced partial metamorphosis in 76 ± 15 and 46 ± 21% of larvae when presented as a biofilm on coverslips or agar drops respectively (Fig. 2). All of these partially metamorphosed larvae remained unattached. The experiment could not be repeated in 1997 using FSW due to the time required to produce new biofilms. This isolate was therefore cryopreserved for the later experiments in 1999.

## Expt 2

Chips of *Lithophyllum* sp. induced 60 ± 12% metamorphosis of 7 d-old *Acropora willisae* larvae (Fig. 3). Larvae were most competent to metamorphose in response to CCA 8 d after spawning (86.4 ± 3.4%) (Fig. 4A). At Day 8, a small number of larvae (2.2 ± 1.1%) also underwent partial metamorphosis as described for *A. millepora* in Expt 1 and were not attached to the substrata. While the number of fully metamorphosed larvae peaked, the number of larvae undergoing partial metamorphosis in response to CCA increased over time to a maximum of 15.0 ± 5.3% after 10 d. No settlement and metamorphosis was observed in response to either the control terracotta chips or *Porites* sp. or FSW alone (Fig. 3).

The biofilms of all 47 Gram-negative bacteria and 13 Gram-positive filamentous bacteria isolated from *Lithophyllum* sp. were tested for morphogenic activity with 7 d-old larvae. None of these microorganisms induced over 10% metamorphosis (data not shown). In contrast, Strain A3 isolated from *Hydrolithon onkodes* in 1997 induced high levels of metamorphosis of *Acropora willisae* larvae (Fig. 3). The biofilms grown on terracotta and *Porites* sp. chips induced over 90% metamorphosis, but most of these polyps were unattached in the presence of terracotta. In contrast, biofilms of Strain A3 grown on *Porites* sp. induced 45.5 ± 9.7% settlement and metamorphosis in *A. willisae* larvae ($p < 0.001$, $F_{9,49.2} = 121$), with 53 ± 10% of larvae remaining partially metamorphosed in the water column. Almost identical results were observed when Strain A3 was

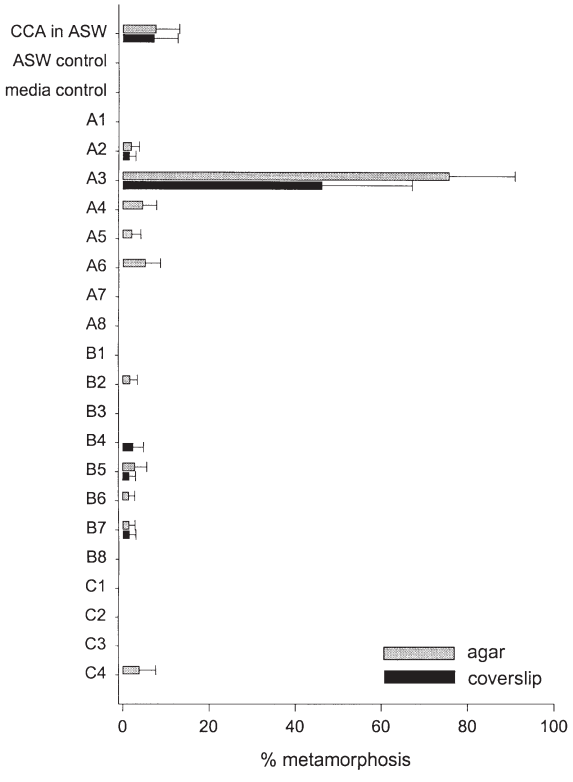


Fig. 2. *Acropora millepora.* Proportion (+SE) of partial metamorphosis (i.e. no attachment) of 7 d-old larvae induced by biofilms of bacteria (A1 to C4) isolated from *Hydrolithon onkodes* (Expt 1b). Biofilms were prepared on both agar drops and glass coverslips; 6 replicate wells containing artificial seawater (ASW) were used for each treatment

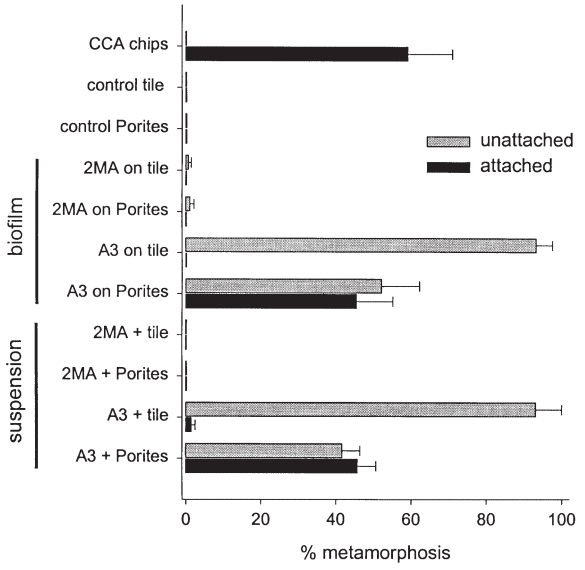


Fig. 3. *Acropora willisae.* Proportion (+SE) of metamorphosis of 7 d-old larvae induced by biofilms of Strain A3 on terracotta chips and *Porites* sp. and inoculations with suspended cells of Strain A3 in the presence of the same substrata (Expt 2). Control treatments contained identical films and inoculations with the growth media 2MA: 12 replicate wells containing filtered seawater (FSW) were used for each treatment. The positive control for metamorphosis consisted of chips of CCA (*Lithophyllum* sp.)

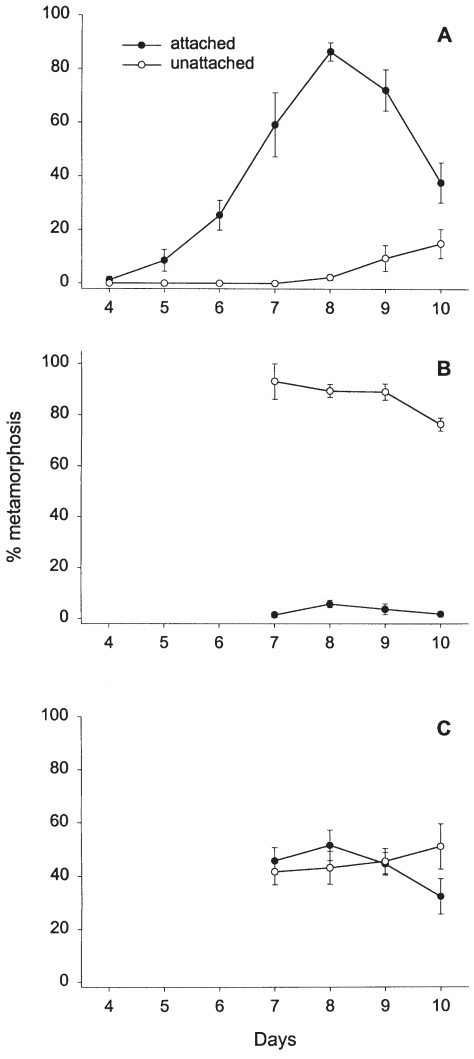


Fig. 4. *Acropora willisae.* Proportion (±SE) of metamorphosis of larvae induced by chips of the CCA *Lithophyllum* sp. (A), inoculation with Strain A3 in the presence of terracotta (B), and inoculation with Strain A3 in the presence of *Porites* sp. (C) as a function of larval age (Expt 2); 6 replicate wells containing FSW were used for each treatment

added as a suspension of cells with an approximate concentration of $2 \times 10^4$ colony-forming units $ml^{-1}$ FSW (Fig. 3). These induction experiments with suspended A3 cells were repeated with 8, 9 and 10 d-old larvae using both terracotta and *Porites* sp. chips as substrata (Figs 4B & 5). Settlement and metamorphosis was significantly higher for 7 to 10 d-old larvae in the presence of *Porites* sp. than terracotta chips (Fig. 4B,C, $p < 0.001$, $F_{7, 34.9} = 39.2$). The overall level of metamorphosis and the proportions of attached and unattached polyps remained relatively constant over this period. The control treatments, containing terracotta chips and *Porites* sp. incubated in Marine Agar 2216 (media used to culture A3) resulted in less than 5% metamorphosis.

Several of the assay plates with newly settled and metamorphosed larvae were transferred to outdoor, unfiltered flow-through seawater tanks at AIMS. Polyps were observed to develop normally, with calcification, the development of tentacles and colouration, due to the uptake of zooxanthellae observed within a week of settlement (Fig. 5).

### Chemical extracts

Methanolic extraction of *Lithophyllum* sp. induced 76.1 ± 2.3% metamorphosis of *Acropora willisae* larvae. Extracts of *Porites* sp. and Marine Agar 2216 were inactive. Extracts of Strain A3 cultured in Marine Agar 2216 and Marine Agar 2216 + 0.1% w/v ground *Porites* sp.-induced highly variable levels of partial metamorphosis ranging from 0 to 68% over several experiments (data not shown). However, neither extract type induced settlement, attachment and metamorphosis.

### Phylogenetic analysis

Phylogenetic analysis of Strain A3 using 16S RNA gene-sequencing showed it to be a member of the

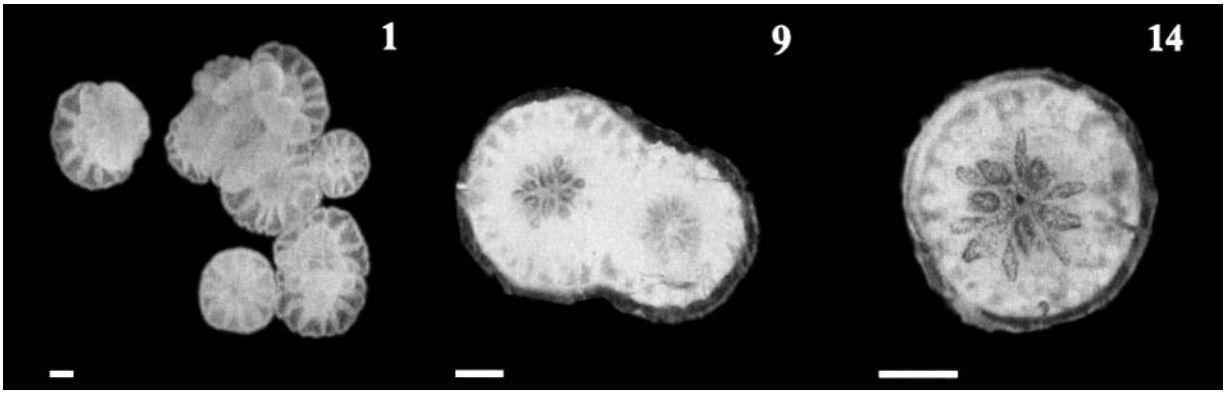


Fig. 5. *Acropora willisae.* Larvae that have undergone settlement, attachment and metamorphosis in response to Strain A3 biofilms grown upon *Porites* sp. skeleton (Expt 2). Photographs were taken at 1, 9 and 14 d post-fertilisation (scale bars = 200 µm)

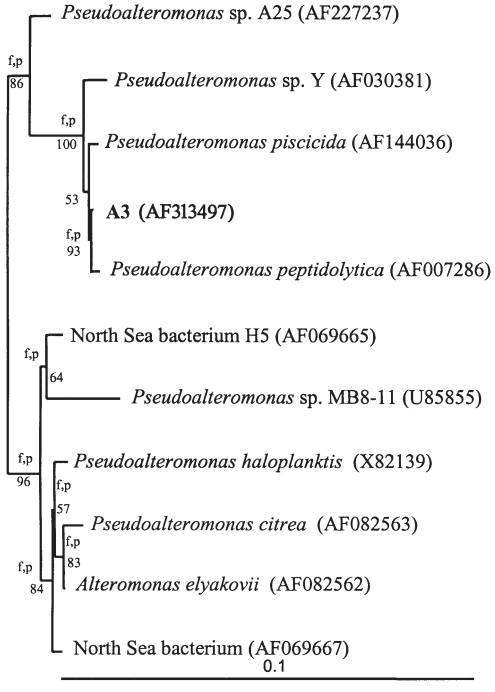


Fig. 6. Neighbour-joining phylogenetic genus tree generated from analysis of 1366 bp of 16S rRNA gene sequence from Strain A3, revealing it to be a member of the *Pseudoalteromonas*. f, p: branches also found using the Fitch-Margoliash or maximum parsimony methods, respectively; numbers on the nodes: percentages indicating levels of bootstrap support, based on a neighbour-joining analysis of 1000 re-sampled data sets; only values > 50% are shown. Scale bar represents 0.1 substitutions per nucleotide position. *E. coli* was used as an outgroup

*Pseudoalteromonas* genus (Fig. 6) most closely phylogenetically allied with a bacterium capable of mussel thread degradation (Accession No. AF007286: Venkateswaran & Dohmoto 2000). Other close relatives include a bacterium isolated from an Australian sponge species (AF144036) and an algicidal strain (AF030381) with activity against toxic dinoflagellate species (Lovejoy et al. 1998).

## DISCUSSION

### Larval response to live and sterilised CCA

Larvae of both acroporid species (*Acropora millepora* and *Acropora willisae*) tested underwent over 80% metamorphosis in response to chips of CCA, as previously described for this genus in laboratory experiments (Morse et al. 1996, Heyward & Negri 1999). Neither treatment of CCA with antibiotics nor autoclaving significantly reduced the morphogenic activity of CCA (Fig. 1). This suggests that either bacteria associated with CCA do not directly influence metamorphosis, or active compounds synthesised by bacteria are not affected by autoclaving. Surprisingly, treatment with 2% (v/v) hypochlorite solution also failed to eliminate morphogenic activity of *Hydrolithon onkodes*, indicating that not only are the morphogens resistant to high temperatures, but they are also chemically stable under highly oxidative conditions. It is also possible that bacterial-derived compounds are able to be incorporated in the CCA skeleton and are in this way protected from degradation. Morse et al. (1988) showed that the activity of inducers associated with natural bacterial and algal biofilms on coral skeleton reduced coral larval metamorphosis from 7.5 to 1.4% following immersion of the substrata in boiling water. Johnson & Sutton (1994) were able to eliminate the morphogenic activity of CCA towards crown-of-thorns starfish by treatment with antibiotics and were hence able to perform isolation and re-inoculation experiments of individual bacteria with CCA. This was not possible in the

current study, as the morphogenic activity of the CCA remained after antibiotic treatment. For this reason, inert *Porites* sp. skeleton was chosen as the substratum for biofilm growth.

### Partial metamorphosis

In Expt 1, poor levels of larval metamorphosis were observed in the presence of CCA (Fig. 2) due to the use of artificial seawater. This result indicates that coral larvae may have a specific requirement for natural seawater, although other ASW preparations may also be satisfactory. It is possible that the ASW used in this experiment is limiting in key essential amino acids and minerals required for metamorphosis to proceed. In all subsequent experiments, ASW was replaced by FSW. When using ASW, it was observed that some larvae had undergone partial metamorphosis into floating flattened discs with septal mesenteries that were not attached to the substrata or to the assay wells as expected. Sammarco (1982) described polyp 'bail out', whereby polyps abandon adult colonies of the brooder *Seriatopora hystrix* under environmental stress and are later able to re-settle. Another type of unattached polyp or 'secondary larvae' has been described for *Pocillopora damicornis* (Richmond 1985). In this case 'demetamorphosis' occurred when water quality declined and newly settled and metamorphosed polyps absorbed their tentacles and retracted from the exoskeleton, forming 'secondary larvae' that were able to later settle and attach under more favourable conditions. These are true planktonic polyps with the ability to feed and contain symbiotic zooxanthellae. Our observations that the larvae sometimes undergo partial metamorphosis in the water column may be more closely related to observations by Henning et al. (1996), who noted that some octocoral larvae, which had undergone metamorphosis in response to artificial cues, formed typical polyps with normal basal discs but that these were unattached. Partial metamorphosis has also been observed for brooding scleractinian corals deprived of natural settlement substrata. In fact, some corals have been described as undergoing complete metamorphosis including calcification without attachment (see Harrison & Wallace 1990).

### Larval response to biofilms

Previous studies on the induction of metamorphosis of corals have reported both low (Morse et al. 1988) and high (Golbuu 1996) metamorphosis in response to microbial biofilms. Both of these studies utilised brooding corals and unidentified microbial films. In our experiments, a single bacterium isolated from *Hydrolithon onkodes* was able to induce significant settlement, attachment and metamorphosis in acroporid larvae in the presence of inactive *Porites* sp. skeleton chips. The procedure used to isolate Strain A3 ensured that this bacterium originated from the surface of the algae where the interaction between larvae and microorganisms occurs. The discovery of a bacterium that induces significant levels of metamorphosis supports earlier research indicating that the source of metamorphic inducers for scleractinian corals extends to other organisms besides CCA (Heyward & Negri 1999). Complete metamorphosis was achieved only in FSW, and the new coral spat that were observed for over 20 d exhibited normal polyp development, including the uptake of zooxanthellae from aquarium seawater.

A high number of larvae underwent partial metamorphosis (unattached) in the presence of Strain A3 with sterile terracotta chips, suggesting a requirement for a calcium carbonate source (or another component of coral skeleton) during the metamorphosis process. It is unlikely that the larvae require both a chemical cue from the bacteria and a suitable physical structure like the coral chip in order to attach. Previous studies have shown that coral larvae readily settle directly on polystyrene surfaces in the absence of biological substrata when induced by a chemical extract of crustose algae (Morse et al. 1994, Heyward & Negri 1999). A purified sulfated glycosaminoglycan isolated from CCA was shown to induce coral metamorphosis (Morse et al. 1994), further reducing the possibility that calcareous substrata or chemistry is required as a direct co-factor for metamorphosis. It is more likely that the presence of *Porites* sp. skeleton activates or enhances the biosynthetic pathway for production of a chemical inducer by the bacterium. Alternatively, bacteria may synthesise fully active inducer molecules only when they are also attached to a suitable substratum such as the coral skeleton. Similar observations were made by Johnson & Sutton (1994), in whose study bacteria isolated from crustose coralline algae induced metamorphosis in crown-of-thorns starfish larva only when crustose algae was also present. These authors proposed that the inducer chemical may be produced by bacteria from substrates derived from the algae host. Bacteria which induce metamorphosis in hydrozoans are more potent when attached to filters (Leitz 1997).

The proportion of metamorphosis of *Acropora willisae* in response to Strain A3 was not affected by larval age up to 10 d (Fig. 4B,C). In contrast, the response to CCA peaked dramatically with 8 d-old larvae (Fig. 4A), indicating possible differences in physiological response or perhaps inducer concentration. The majority of microbial biofilms were tested with 7 d-old

larvae, which were almost 30% less competent to metamorphose than those after Day 8. This raises the possibility that some strains exhibiting low metamorphic activity may have been overlooked. Future studies with coral larvae should take this into consideration when examining rates of metamorphosis.

Larval metamorphosis was apparently unaffected by the mode of introduction of A3 cells to the test wells (biofilm or cell suspension), suggesting that the morphogenic compound produced by Strain A3 may be water-soluble. Previous research has shown that inducers associated with CCA are not water-soluble or available to larvae in the water column (Morse et al. 1988). It is possible that in the artificial environment of an assay well contact between suspended cells of Strain A3 and the chemoreceptors on the larvae may initiate metamorphosis, although this is not a likely event in the marine environment. The planula of the scyphozoan *Cassiopea andromeda* also underwent metamorphosis in response to suspensions of *Vibrio* sp. cells as well as chemical extracts of this bacterium (Neumann 1979).

### Chemical extracts

Chemical cues for coral larval metamorphosis have been extracted from both CCA and skeleton of the coral *Goneastrea* sp. (Morse et al. 1988, 1994, 1996, Heyward & Negri 1999). Methanolic extracts were performed on cells of Strain A3, cultured both in the presence and absence of 0.1% (w/v) *Porites* sp. skeleton, in an attempt to isolate the morphogen associated with Strain A3. Interestingly, only partial metamorphosis was achieved at a range of concentrations (0.05 to 10% v/v), and the levels of metamorphosis were highly variable between treatments. It remains unclear why full metamorphosis in the presence of these extracts and *Porites* sp. chips did not occur. Methanol, which readily extracts morphogens from CCA (Heyward & Negri 1999), may be unsuitable for the extraction of morphogens from bacteria, as these compounds are not necessarily related to their CCA-derived counterparts.

*Acropora millepora* larvae in these experiments did not undergo metamorphosis in response to the artificial inducer TPA, in contrast to several Red Sea corals (Henning et al. 1996). This primary or external chemical signal is thought to induce a response in secondary or internal transduction mechanisms, which in some corals can be activated by TPA (Leitz 1997). It is possible that the Red Sea octocorals and brooding scleractinian share a common primary receptor type with broadcast spawners to recognise CCA, but the secondary transduction pathways leading to the onset of metamorphosis may differ between some coral taxa.

### Phylogenetic affiliations

Strain A3, a member of the *Pseudoalteromonas* genus, was phylogenetically most closely allied with *P. peptidolytica*, which has been implicated in the production and secretion of powerful proteases that cleave complex proteins in the foot of the mussel *Mytilus edulis* (Venkateswaran & Dohmoto 2000). Other close relatives include *P. piscicida* and *Pseudoalteromonas* sp. Y. Interestingly, *Pseudoalteromonas* sp. Y exhibited potent algicidal activity against the toxic dinoflagellate *Gymnodinium catenatum* and also against numerous species of raphidophytes and armoured dinoflagellates (Lovejoy et al. 1998). Antifouling (Holmstrom et al. 1998) and antimicrobial activity against marine Gram-negative bacteria (Yoshikawa et al. 1997) has also been reported for *P. tunicata* and *Pseudoalteromonas* sp. F-420 respectively. Members of the *Pseudoalteromonas* genus were also reported to form complex biofilms on the surface of the bivalve *Montacuta ferruginosa*, and were postulated to play a role in mineral formation within the biofilm (Gillan et al. 1998). Hydrozoans are another class of cnidarian that are known to undergo metamorphosis in response to bacteria including several *Alteromonas* and *Oceanospirillum* species (Leitz 1997).

### Ecological significance

The ability of *Pseudoalteromonas* Strain A3 to induce settlement and metamorphosis in the planula of *Acropora willisae* has been clearly demonstrated. However, the ecological significance of the role Strain A3 plays in coral metamorphosis requires further research. In particular, the natural distribution of this strain needs to be determined, and its ability to induce metamorphosis at ecologically relevant concentrations must also be tested. The inability to obtain inert CCA for re-infection experiments poses problems for unequivocally assigning a role for this bacterium on its natural substratum. It may be possible, however, to trigger or enhance morphogen production in this species by establishing mixed-culture biofilms. In addition, there is a need to elucidate the role of environmental co-factors, such as the presence of calcareous substrate like the inactive *Porites* sp. skeleton used here, in the production of morphogens.

The large number of bacterial morphotypes isolated from the surfaces of *Hydrolithon onkodes* and *Lithophyllum* sp. indicates that these crustose algae species maintain diverse microbial biofilms on their surface cells. It is possible that some of these species are common environmental strains that may originate from the water column. Several studies have used electron

microscopy to demonstrate close affiliations between bacteria and the surface of CCA (Galbary & Veltkamp 1980, Garland et al. 1985, Lewis et al. 1985, Johnson et al. 1991a). The contribution of microbial symbionts to the production of active metabolites in marine invertebrates is becoming increasingly apparent (Leitz 1997, Schmidt et al. 2000). Despite the high diversity of bacteria isolated from CCA, it is highly likely that this represents a tiny fraction of the total microbial community. Generally, less than 1% of marine bacteria are able to be cultivated using standard techniques (Hugenholtz et al. 1998). Our preliminary examinations indicate that, despite describing a single species which exhibits interesting bioactivity towards coral larvae, a great potential for further discovery remains. An examination of the total microbial community associated with the surface of CCA is required before we can fully elucidate the role of microorganisms in the metamorphosis process.

## Conclusions

Despite the obvious potential for CCA to influence microhabitat selection by coral larvae, evidence is mounting that CCA is not the sole source or repository of chemical inducers in reef environments. The porous coral skeleton of *Goneastrea* sp. actively induces metamorphosis (Heyward & Negri 1999), and now the morphogenic activity of *Pseudoalteromonas* Strain A3 has been described. The discovery that marine bacteria from CCA can induce metamorphosis in some corals may have significant practical applications for coral reef conservation and reef re-seeding activities. Only by further exploring the relationships between CCA and its associated microbial community will we understand the ecological relevance of microorganisms in coral metamorphosis.

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