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## Induction of Larval Settlement in the Reef Coral *Porites astreoides* by a Cultivated Marine *Roseobacter* Strain

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**Abstract.** Successful larval settlement and recruitment by corals is critical for the survival of coral reef ecosystems. Several closely related strains of  $\gamma$ -proteobacteria have been identified as cues for coral larval settlement, but the inductive properties of other bacterial taxa naturally occurring in reef ecosystems have not yet been explored. In this study, we assayed bacterial strains representing taxonomic groups consistently detected in corals for their ability to influence larval settlement in the coral *Porites astreoides*. We identified one  $\alpha$ -proteobacterial strain, *Roseivivax* sp. 46E8, which significantly increased larval settlement in *P. astreoides*. Logarithmic growth phase (log phase) cell cultures of *Roseivivax* sp. 46E8 and filtrates (0.22 $\mu$ m) from log phase *Roseivivax* sp. 46E8 cultures significantly increased settlement, suggesting that an extracellular settlement factor is produced during active growth phase. Filtrates from log phase cultures of two other bacterial isolates, *Marinobacter* sp. 46E3, and *Cytophaga* sp. 46B6, also significantly increased settlement, but the cell cultures themselves did not. Monospecific biofilms of the three strains did not result in significant increases in larval settlement. Organic and aqueous/methanol extracts of *Roseivivax* sp. 46E8 cultures did not affect larval settlement. Examination of filtrates from cell cultures showed that *Roseivivax* sp. 46E8 spontaneously generated virus-like particles in log and stationary phase growth. Though the mechanism of settlement enhancement by *Roseivivax* sp. 46E8 is not yet elucidated, our findings point to a new aspect of coral-*Roseobacter* interactions that should be further investigated, especially in

naturally occurring, complex microbial biofilms on reef surfaces.

### Introduction

The settlement, survival, and growth of coral larvae play a critical role in the resilience and repopulation of coral reef ecosystems (Hughes *et al.*, 2003). The ultimate settlement and post-settlement survival of coral larvae is regulated by several biotic and abiotic factors, including availability of suitable substrate for settlement. Various surfaces in the reef environment have differential effects on larval settlement. Larval recruitment rates are lower in areas with abundant macroalgae (Edmunds and Carpenter, 2001) due to allelopathic compounds produced by macroalgae (Kuffner and Paul, 2004; Birrell *et al.*, 2005, 2008; Kuffner *et al.*, 2006; Ritson-Williams *et al.*, 2009, 2010; Paul *et al.*, 2011). Recent evidence also suggests that macroalgae can shape the bacterial community composition in the benthos (Barott *et al.*, 2011; Morrow *et al.*, 2012; Vega Thurber *et al.*, 2012). In contrast, other algal species, including particular species of crustose coralline algae (CCA) (Ritson-Williams *et al.*, 2010, 2014) and compounds derived from CCA (Kitamura *et al.*, 2007, 2009) facilitate larval settlement of scleractinian corals.

The positive influence of specific surfaces in the marine environment on larval settlement is likely due to a combination of microbe-microbe interactions within surface biofilms, physical factors of the benthic environment on a microscopic scale, and the production of chemical cues. Bacteria in multi-species biofilms on surfaces in benthic ecosystems influence settlement in diverse marine invertebrate phyla (reviewed in Hadfield, 2011). Successful settlement of invertebrate larvae (attachment and metamorphosis)

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is strongly governed by the density, taxonomic composition, and activity of bacteria in reef environments (Huang and Hadfield, 2003). Marhaver *et al.* (2013) showed that manipulation of microbes in reef seawater influences coral larval settlement and survival and, ultimately, the large-scale landscape of coral reefs. A specific strain from the CCA *Neogoniolithon fosliei*, produces the small molecule tetrabromopyrrole (TBP) that induces metamorphosis but not attachment in the Pacific coral *Acropora millepora* (Tebben *et al.*, 2011; Siboni *et al.*, 2012, 2014). However, a closely related strain of *Pseudoalteromonas* sp., cultured from the CCA *Paragoniolithon solubile*, produces TBP and induces both attachment and metamorphosis in multiple species of Caribbean corals (Sneed *et al.*, 2014). In the Pacific coral *Pocillopora damicornis*, strains of *Pseudoalteromonas* and *Thalassomonas* isolated from marine surfaces also induce both metamorphosis and attachment (Tran and Hadfield, 2011). Inductive capabilities were found in bacteria from both genera, and in isolates from a range of benthic surfaces, including macroalgae, corals, and bio-filmed glass slides (Tran and Hadfield, 2011), suggesting that the inductive properties are not exclusive to one genus of bacterium nor to bacteria from particular surfaces (Tran and Hadfield, 2011). An additional mechanism of bacterial induction of larval settlement has been recently identified. A strain of *Pseudoalteromonas luteoviolacea*, isolated from a marine biofilm, produces arrays of contractile phage tail-like structures that induce larval settlement of the serpulid worm *Hydroides elegans* (Shikuma *et al.*, 2014). This finding demonstrates that phage-associated structures may play a role in induction of settlement although the exact mechanism of induction is yet unknown.

Fluorescence *in situ* hybridization (FISH) with sequence-specific probes suggests that the most abundant groups of bacteria on biofilms inductive to the coral *Acropora microphthalma* are  $\gamma$ -proteobacteria,  $\alpha$ -proteobacteria, and *Cytophaga* (Webster *et al.*, 2004). However, of that described diversity, to date, all of the cultured bacterial isolates shown to influence coral larval metamorphosis or settlement belong to the  $\gamma$ -proteobacteria. Other representatives of this diverse bacterial assemblage, such as the group of  $\alpha$ -proteobacteria known as the *Roseobacter*-affiliated bacteria, have been shown to be abundant in the seawater during spawning of multiple coral species (Apprill and Rappe, 2011; Ceh *et al.*, 2012). *Roseobacter*-affiliated sequences have been consistently recovered from larvae and post-settlement juvenile stages of multiple coral species from the Pacific and Caribbean (Apprill *et al.*, 2009, 2012; Sharp *et al.*, 2011). Though their contributions to the coral host are not yet well understood, there is mounting evidence that this clade of bacteria may fix nitrogen and translocate organic nitrogenous compounds to *Symbiodinium* spp. (Ceh *et al.*, 2013); produce antibacterial compounds for host defense

(Nissimov *et al.*, 2009); or metabolize dimethylsulfoniopropionate (DMSP) (Raina *et al.*, 2009, 2010; Bourne *et al.*, 2013). *Roseobacter* bacteria are among the main taxonomic groups consistently associated with cnidarian-associated *Symbiodinium* spp. In addition, bacteria from the genera *Marinobacter* and *Cytophaga* are consistently associated with *Symbiodinium* (Ritchie, 2011).

In this study, we explored the ability of three bacterial strains, *Roseivivax* sp. 46E8, *Marinobacter* sp. 46E3, and *Cytophaga* sp. 46B6, isolated from a collection of *Symbiodinium* spp. cultures, to influence larval settlement in the coral *Porites astreoides* Lamarck, 1816. We used *P. astreoides* because it is common throughout the Caribbean and the Atlantic basin; it is robust to laboratory manipulation; and it predictably releases large numbers of competent larvae on a seasonal basis. We exposed *P. astreoides* larvae to liquid cultures—both logarithmic and stationary growth phase—and monospecific biofilms of the three bacterial strains, and we assayed them for induction of larval settlement. We also tested cell-free filtrates and organic and aqueous/methanol extracts of the cultures in larval settlement assays. We imaged filtrates from inductive liquid cultures in order to test for the presence of potential inductive particles not identified with traditional methods of extracting bioactive compounds.

## Materials and Methods

### Colony collection and larval rearing

Collection efforts were timed around the new moon in the months of April, May, and June in 2012. *Porites astreoides* colonies were collected at least 24 h before the new moon from Wonderland Reef, located in the Lower Florida Keys (24°33.69'N, 081°30.08'W). Colonies were transported in coolers to Mote Tropical Research Laboratory in Summerland Key, Florida, and maintained in a flow-through seawater system. Larvae were released each night for 3–5 nights after collection. Throughout the larval release period, larvae were collected and maintained in tanks as previously described (Kuffner *et al.*, 2006).

### Isolation of bacterial strains

*Symbiodinium* sp. cultures 151/Clade C1 and 147/Clade B1 (originally isolated by R. Trench from the corallimorph *Rhodactis* [*Heteractis*] *lucida*, and the soft coral *Pseudopterogorgia bipinnata* from Jamaica, respectively) were serially diluted in sterile F/2 medium (Sigma), plated onto marine agar (Sigma), and grown at 25 °C for 3–5 days. Bacteria exhibiting unique colony or cellular morphology compared to other colonies on a single plate were subcultured to purification under the same set of growth conditions. *Roseivivax* sp. 46E8 and *Marinobacter* sp. 46E3 were isolated from a *Symbiodinium* sp. culture derived from the

cnidarian host corallimorph *Rhodactis* [*Heteractis*] *lucida*, and *Cytophaga* sp. 46B6 was isolated from a *Symbiodinium* sp. culture derived from the gorgonian *Pseudopterogorgia bipinnata*. Bacteria were identified using 16S rRNA gene sequence analysis as previously described (Ritchie, 2006).

#### Preparation of filtrates from bacterial cultures

Each strain was grown in marine broth (MB) 2216 (Difco Laboratories, Sparks, MD). Growth of the cultures was monitored by absorbance at 595 nm with a spectrophotometer. Cell-free filtrate of the culture, once it reached the appropriate growth phase (log or stationary), was prepared from the liquid cell culture by spinning the liquid culture for 10 min at maximum speed ( $16,000 \times g$ , VWR Galaxy) and filtering the supernatant through a  $0.22\text{-}\mu\text{m}$  syringe SUPOR disc filter (Acrodisc, Pall Life Sciences, Inc).

#### Preparation of extracts of bacterial cultures

Organic extracts were prepared from plated cells, cells from liquid culture, and cell-free filtered media of liquid cultures. Extracts of plated cells were prepared by spreading  $100\ \mu\text{l}$  of a 24-h liquid culture onto each of 10 MB agar plates and grown for 48 h to create thick bacterial lawns. Bacteria were scraped from the surface of the agar using a sterile razor blade and lyophilized. Cells from liquid cultures were harvested by centrifuging a 48-h liquid culture at  $4700 \times g$  (Beckman Model TJ-6) for 15 min, removing the supernatant, and lyophilizing the pelleted cells. Dried bacteria were extracted overnight in an excess of methanol/ethyl acetate (1:1). Solvents were removed by SpeedVac. The supernatant from the liquid cultures was filtered through a  $0.2\text{-}\mu\text{m}$  filtration unit (Millepore Express PLUS) to remove any remaining cells, and the organic compounds were separated by liquid-liquid partitioning with ethyl acetate and butanol, sequentially. Aqueous methanol extracts of bacterial cells were prepared in the same way, but dried cells were extracted in an excess of methanol/water (1:1). Salts precipitated out and were removed by centrifuging extracts at maximum speed ( $16,100 \times g$  (Eppendorf 5415D) for 2 min and filtering the supernatant through a  $0.45\text{-}\mu\text{m}$  syringe filter (Target2 PTFE). Solvents were evaporated from aqueous methanol extracts under a constant flow of air. All extracts were stored at  $-20\ ^\circ\text{C}$ .

#### Settlement assays

We tested liquid cultures, filtrates from liquid cultures, and monospecific biofilms from each tested strain. For the assays in which liquid cultures or filtrates from liquid cultures were added to larvae, the settlement assay dishes were made using deep polystyrene petri dishes ( $100\ \text{mm} \times 25\ \text{mm}$ ) with 40 ml of filter-sterilized ( $0.22\ \mu\text{m}$ ) seawater (FSW) and a single autoclaved ceramic coral propagation disk (OceansWonders, Inc., Decorah, IA). Larvae were col-

lected each morning into bowls of FSW, counted into aliquots of 20 larvae in 10 ml of FSW, and then added to the settlement assay dishes. After larvae were added,  $300\ \mu\text{l}$  of treatment (liquid culture or filtrate) was immediately added to each disk.

Monospecific biofilms were prepared on ceramic frag disks (Bulk Reef Supply, Minneapolis, MN). Disks were autoclaved for sterilization and then incubated in 5 ml sterile Difco MB in plastic 6-well plates (one disk per well). For the biofilm treatments, wells were inoculated with  $100\ \mu\text{l}$  of log phase liquid culture and allowed to grow for 24 h. In the MB control, the disks were incubated for 24 h in sterile MB only.

Crude extracts of the cultures were also assayed. Larvae were prepared similarly, and 10 larvae were added to plastic 6-well plates in a total volume of 10 ml of FSW. Crude extracts were dissolved in dimethyl sulfoxide (DMSO) and were added at two concentrations ( $1\ \mu\text{g ml}^{-1}$  and  $2\ \mu\text{g ml}^{-1}$ ) in a volume of  $10\ \mu\text{l}$ . In positive control treatments, 10 larvae were added to wells with FSW and a small piece of *Hydrolithon boergesenii*, a species of CCA shown to be inductive to larval settlement in multiple species of corals (Ritson-Williams *et al.*, 2010, 2014).

In all assays, 24 h after addition of the treatments, settlement was scored as the number of larvae that had attached and metamorphosed anywhere in the well, including on the ceramic disks and on the plastic well itself. The number of settled larvae did not include larvae that had metamorphosed but were still free-floating in the water column (or on the water surface). Larvae were considered attached if they could not be dislodged by light agitation of the water with a glass Pasteur pipet during the scoring.

#### Statistical analysis

In the liquid culture treatments and biofilm treatments, each of the 20 larvae within an individual dish was classified into one of two categories: settled (success) or not settled (failure). Because there were only two mutually exclusive outcomes where the probability of success ( $p$ ) and failure ( $q$ ) is  $p = 1 - q$ , this was a binomial distribution. All data were therefore analyzed using a binomial exact test that compares the observed distribution to an expected probability distribution when there are only two categorical response variables. Using an ANOVA or other statistical analysis that requires a continuous response variable was not appropriate.

We calculated the expected distribution for the binomial exact test based on the probability of larval settlement in the control dishes (MB controls). In both the liquid culture and biofilm experiments, settlement in MB controls was very low, with just three larvae settling out of the 200 total larvae



tested across all replicates. We therefore calculated a conservative probability of settlement in control conditions of 0.015 ( $3 \div 200$ ).

We used the binomial exact test to calculate the probability that the observed number of settled larvae occurred by chance given the expected settlement probability (0.015) and tested the null hypothesis that the number of larvae that settled in each experimental dish of 20 larvae for a treatment was not significantly different from settlement occurring in the control. For each of the 10 dishes of 20 larvae per treatment ( $n = 10$ ; 20 larvae per dish), we calculated the probability that the settlement observed occurred by chance. We used the median value of these probabilities to accept or reject the null hypothesis for each treatment.

There was no settlement in response to any of the added bacterial extracts (see Appendix, Figs. A1 and A2), and therefore no statistical analyses were performed on these samples.

### Imaging analysis of culture filtrates

Triplicate sterile flasks of nutrient MB (equivalent to Difco MB 2216) were inoculated with *Roseivivax* 46E8 and grown at 26 °C with shaking. Growth of the cultures was monitored by spectrophotometer at wavelength 595 nm. Once the culture reached the appropriate growth phase (log or stationary), subsamples were removed, and bacterial cells were pelleted *via* centrifugation ( $9500 \times g$  for 5 min at room temperature). Supernatant was filtered through a 0.22- $\mu\text{m}$  pore size syringe filter (Millipore) to remove any remaining cells and debris. Filtrates were diluted with sterile filtered artificial seawater and immediately stained with SYBR Gold according to standard protocols (Patel *et al.*, 2007). Subsamples of each filtrate were subjected to RQ1 DNase ( $2.5 \text{ U ml}^{-1}$ ) incubation, according to the manufacturer's instructions (Promega). Additional subsamples were mixed with an equal volume of molecular-biology grade chloroform and centrifuged ( $12,000 \times g$  for 5 min at room temperature) in a phase-lock tube (5Prime, Gaithersburg, MD), for chloroform removal. The DNase- and chloroform-treated filtrates were stained with SYBR Gold as above. Slides were examined on an Olympus BX60 epifluorescence microscope. Particles were detected in these treatments and were enumerated in triplicate for each replicate of log or stationary phase growth cultures ( $n = 9$  for each time point). Images were acquired on an Olympus IX71 inverted epifluorescence microscope equipped with an Olympus DP70 digital camera.

## Results

Three bacterial strains, *Roseivivax* sp. 46E8, *Marinobacter* sp. 46E3, and *Cytophaga* sp. 46B6, showed varying degrees of influence over larval settlement in the coral *Porites astreoides*. Of the three strains, only the *Roseivivax*

sp. 46E8 culture significantly increased larval settlement. However, cell-free filtrates of all three cultures caused significant increases in larval settlement.

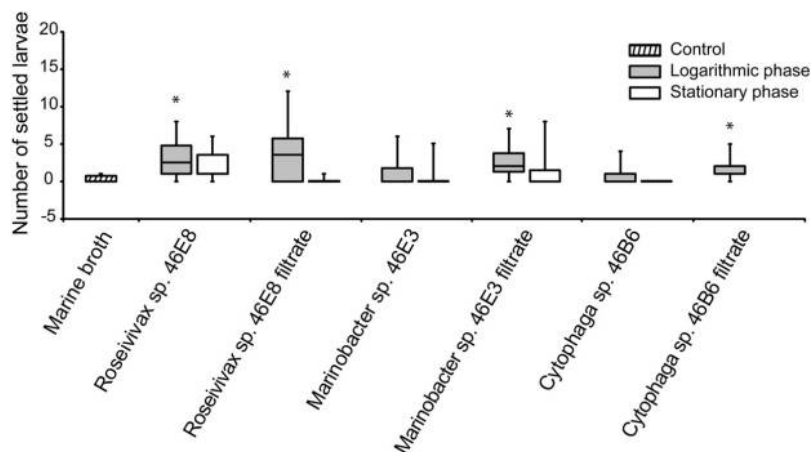
When exposed to log phase liquid cultures of *Roseivivax* sp. 46E8, settlement rates of *P. astreoides* planula larvae significantly increased, relative to uninoculated culture medium (MB) controls ( $P = 0.018$ , Fig. 1). Larval settlement did not significantly change in response to *Marinobacter* sp. 46E3 or *Cytophaga* sp. 46B6 log phase liquid cultures ( $P = 0.739$  and  $0.225$ , respectively). Stationary phase cultures of the three strains did not elicit a significant increase in settlement (Fig. 1). Exposure to cell-free filtrates of log phase cultures of *Roseivivax* sp. 46E8, *Marinobacter* sp. 46E3, and *Cytophaga* sp. 46B6 resulted in significantly increased settlement ( $P = 0.016$ ,  $0.033$ , and  $0.033$ , respectively, Fig. 1), but filtrates of the stationary liquid cultures did not (Fig. 1). Exposure of the larvae to ceramic disks coated in monospecific biofilms of each of the three test strains did not result in a significant increase in larval settlement, relative to disks incubated in uninoculated MB (Fig. 2).

None of the tested crude organic extracts prepared from *Roseivivax* sp. 46E8 cultures resulted in larval settlement over 1% (Appendix Fig. A1). These extracts, prepared from liquid culture, from liquid culture filtrate, and from plated culture, were tested at  $1 \mu\text{g ml}^{-1}$  and  $2 \mu\text{g ml}^{-1}$  concentrations, and no settlement was observed at either concentration. Similarly, aqueous/methanol extracts from the culture in various growth phases did not affect larval settlement (Appendix Fig. A2). In both assays, positive controls (pieces of the crustose coralline alga *Hydrolithon boergeseni*) demonstrated high settlement rates (as high as 85%), indicating that the larvae were competent and capable of settlement.

Epifluorescence imaging of SYBR Gold-stained *Roseivivax* sp. 46E8 filtrates from both log phase growth ( $A_{595}$  range 0.537–0.655) and stationary phase growth ( $A_{595}$  range 1.152–1.460) revealed small ( $<0.2 \mu\text{m}$ ) DNA-containing particles (Fig. 3). Logarithmic phase cell-free filtrate contained an average of  $7.4 \times 10^7$  particles  $\text{ml}^{-1}$  and the stationary phase cell-free filtrate contained  $2.09 \times 10^8$  particles  $\text{ml}^{-1}$ . The particles were resistant to both DNase and chloroform treatments.

## Discussion

Our study revealed that a strain of bacteria (*Roseivivax* sp. 46E8), representing the *Roseobacter* clade of  $\alpha$ -proteobacteria, induces larval settlement in the coral *Porites astreoides*. This finding adds to the accumulating evidence that *Roseobacter*-affiliated bacteria play an important role in the larval ecology and survival of early life stages in corals. Bacteria from the *Roseobacter* clade are among the most



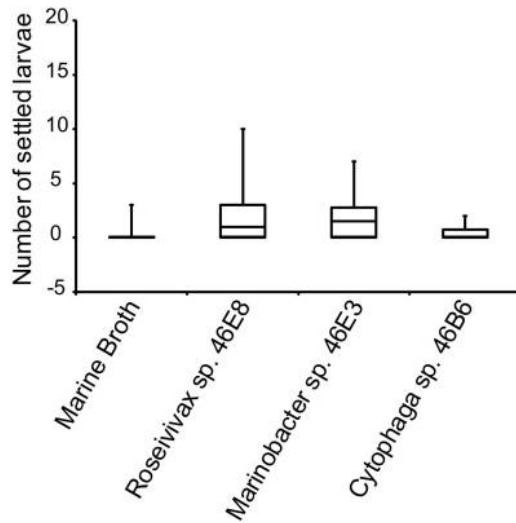
**Figure 1.** Settlement of *Porites astreoides* larvae exposed to liquid bacterial cultures and to filtrates from the liquid cultures. Boxplots representing the median number of larvae settled in dishes with ceramic settlement disks after 24 h in response to exposure to *Roseivivax* sp. 46E8, *Marinobacter* sp. 46E3, and *Cytophaga* sp. 46B6 liquid cultures or filtrates (0.22  $\mu$ m) from the cultures. Error bars represent the range (maximum and minimum number of settled larvae) out of 20 larvae per dish. Data are shown for stationary phase cultures and logarithmic phase cultures, in comparison to the negative control sterile marine broth (MB) ( $n = 10$ ; 20 larvae per dish). Filtrate from stationary phase of *Cytophaga* sp. 46B6 was not tested. Symbol (\*) above bars represents significant differences in settlement ( $P < 0.05$ , median of 10 replicates with 20 larvae per replicate, binomial exact test), relative to the MB control.

abundant bacterial groups in the oceans, and they are important in global biogeochemical cycling (Buchan *et al.*, 2003). *Roseobacter* clade bacteria have consistently been detected as abundant members of seawater-associated bacterial communities during reproduction of both brooding and spawning coral colonies (Aprill and Rappe, 2011; Ceh *et al.*, 2012), and they are prevalent in larvae, juveniles, and adults of diverse corals (Aprill *et al.*, 2009, 2012; Sharp *et al.*, 2011; Ceh *et al.*, 2013). The consistent detection of these taxa in early life stages of diverse corals suggests that they engage in long-term symbioses with corals and may therefore have important functional roles in their coral hosts. Bacteria from the *Roseobacter* clade have been proposed to defend coral larvae from pathogenic bacteria (Nissimov *et al.*, 2009) and provide fixed organic nitrogen to the partner *Symbiodinium* spp. (Ceh *et al.*, 2013).

In this study, larvae settled in response to cell-free filtrates of *Roseivivax* sp. 46E8 cultures, indicating that these bacteria produce some type of extracellular cue that signals coral morphogenesis. The results of the assays in this study suggest that the mechanism of action is unlike many that have been previously described to induce larval metamorphosis or settlement in marine invertebrates *via* small, bioactive organic compounds (Hadfield and Paul, 2001; Sneed *et al.*, 2014). In this study, crude organic and aqueous extracts of *Roseivivax* sp. 46E8 (prepared as in Sneed *et al.*, 2014) did not elicit an increase in *P. astreoides* settlement, suggesting that the observed induction is not due to a small molecule like TBP or other compound that would be extracted into organic solvents.

Analysis of *Roseivivax* sp. 46E8 filtrates by microscopy revealed nucleic acid-containing particles in both logarithmic and stationary phase growth. The nature of the particles has yet to be determined, but their size relative to the bacteria and their resistance to DNase and chloroform treatments are consistent with protein-encapsidated viruses. One possibility is that the *Roseivivax* isolate is lysogenic, meaning it may contain a latent viral infection and spontaneously produce low levels of viral particles. Bacterial isolates representing a wide range of environments are lysogenic, and production of lytic viruses occurs at a low level. This production can be induced by genetic damage to the host bacterium, including damage by UV light or toxic chemicals (Wommack and Colwell, 2000). It is estimated that approximately 50% of marine bacterial isolates contain inducible prophages (Paul, 2008). However, the magnitude of spontaneous viral production by true lysogens is generally thought to be much lower (Ackermann and DuBow, 1987). Another possible explanation is that *Roseivivax* sp. 46E8 is a pseudolysogen, a bacterial strain with an unstable latent viral infection that produces high levels of viral particles in conjunction with rapid growth of the bacterial host (Williamson *et al.*, 2001).

Alternatively, the particles could be gene transfer agents (GTAs), spontaneously produced virus-like particles that function as vectors of gene transfer and can transform the metabolic activity of surrounding microorganisms (Lang *et al.*, 2012). It has only recently been recognized that the virus-like GTAs can be active and functional in marine



**Figure 2.** Settlement of *Porites astreoides* larvae exposed to monospecific bacterial biofilms grown on ceramic discs. Boxplots representing median number of larvae settled after 24 h in response to exposure to *Roseivivax* sp. 46E8, *Marinobacter* sp. 46E3, or *Cytophaga* sp. 46B6 biofilms on ceramic coral fragmentation disks. Error bars represent the range (maximum and minimum number of settled larvae) out of 20 larvae per dish. Data are shown in comparison to a negative control, a dish containing a ceramic disk incubated in sterile marine broth (MB) ( $n = 10$ ; 20 larvae per dish). There was no significant effect on settlement from treatment relative to the MB control ( $P < 0.05$ , median of 10 replicates with 20 larvae per replicate, binomial exact test).

systems and can catalyze high rates of gene transfer between diverse taxa (McDaniel *et al.*, 2010). Though it is not yet clear how or if the particles detected in this study facilitate *P. astreoides* larval settlement, our data are consistent with mounting evidence that marine bacteria produce viral particles or phase-associated structures that influence marine invertebrate larval settlement (Shikuma *et al.*, 2014).

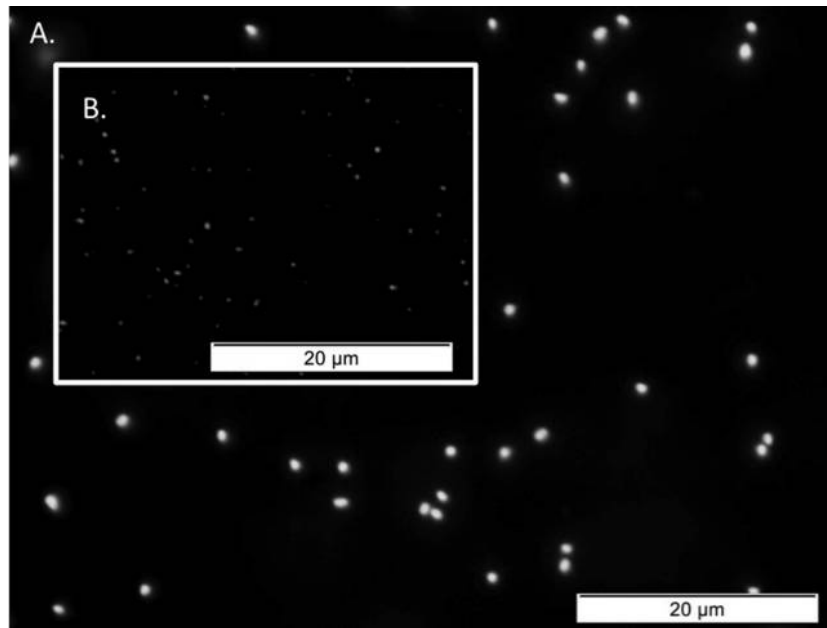
*Roseivivax* sp. 46E8 cultures and cell-free filtrates from the cultures elicited increased settlement in *P. astreoides* larvae during logarithmic, but not stationary, phase. These data support the hypothesis that *Roseivivax* sp. 46E8 produces settlement cues exclusively during log growth. Further investigation will be required to identify particles produced by the bacteria, and whether or not particles are involved in settlement induction. In addition, the identity of the particles and whether the effect is dependent on number of particles or bacterial growth phase are yet to be determined. Nevertheless, these results point to an aspect of bacterial-eukaryote interactions that should be further explored. Cell-free filtrates of log phase *Marinobacter* sp. 46E3 and *Cytophaga* sp. 46B6 also increased settlement, but cell cultures did not. Further investigation of filtrates from *Marinobacter* sp. 46E3 and *Cytophaga* sp. 46B6 will aim to clarify their role in the observed larval settlement.

Exposure of *P. astreoides* larvae to a monospecific biofilm of *Roseivivax* sp. 46E8 did not significantly increase

settlement. However, the tested biofilms were 24 h old; the biofilms may have to mature for longer periods (as in Tran and Hadfield, 2011) to exhibit induction in the larval settlement assays. Alternatively the observed effect may be due to the production of a cue in liquid log phase growth that is not produced in biofilm growth phases.

There are limitations to culture-dependent approaches, including the inability to capture wild diversity and strain-level genomic variation. Cultured bacteria often do not reflect naturally occurring taxonomic and functional diversity (Barrott and Rowher, 2012; Montalvo *et al.*, 2014). In this study, our aim was to identify specific strains and the inductive bioactive compounds that they produce in order to identify ecologically important members of wild bacterial assemblages in reef surfaces. Our study tested only cultured, single-species biofilms, rather than wild, mixed biofilms. Therefore, uncultured members of the biofilm, naturally occurring microbe-microbe interactions, and spatial organization of the microbial assemblages were not replicated in the assays. Further research on the genetic basis for induction—and the environmental regulation of those genes—will reveal more about the ecological relevance and mechanism of action of *Roseivivax* sp. 46E8. In addition, identification of coral biosynthetic and metabolic pathways affected by the presence of *Roseivivax* sp. 46E8 should be compared with those previously identified in the coral *Acropora millepora* (Siboni *et al.*, 2012, 2014). Transcription-level expression profiles of coral larvae that have undergone complete settlement (attachment and metamorphosis) in response to *Roseivivax* sp. 46E8 should be compared to larvae exposed to natural cues and to larvae exposed to other bacterial strains shown to induce only metamorphosis (as in Siboni *et al.*, 2012, 2014). This continued analysis will offer deeper characterization of the genetic basis for regulation of attachment and metamorphosis.

*Roseivivax* sp. 46E8 induces larval settlement in laboratory assays, but a single strain is probably not the only settlement factor in the natural environment. Rather, it is likely that uncultivated, multi-domain microbial interactions, including *Symbiodinium*, produce the most effective settlement cues on reef surfaces. Although *Roseivivax* sp. 46E8 caused a significant increase in larval settlement compared to sterile seawater controls, the amount of settlement was less than that in our other experimental treatments with naturally occurring crustose coralline algal biofilms (Appendix Figs. A1 and A2). The “invisible majority”—bacteria, viruses, and organic matter—are important drivers of coral reef health and resilience (reviewed in Barrott and Rohwer, 2012). Our data, together with findings from recent studies on bacterial influence on larval settlement, highlight the importance of characterizing interactions among diverse microorganisms on benthic surfaces. In natural conditions, induction of settlement by one bacterial strain may be compounded by activity of other microbes, production of



**Figure 3.** Epifluorescence micrograph of (A) *Roseivivax* sp. 46E8 cell culture and (B) 0.22- $\mu\text{m}$  filtrate of *Roseivivax* sp. 46E8 cell culture, stained with SYBRGold I. (A) *Roseivivax* sp. 46E8 cells stained with SYBR. Images were acquired on an Olympus IX71 inverted epifluorescence microscope equipped with an Olympus DP70 digital camera. Bar = 20  $\mu\text{m}$ .

bioactive compounds by other key organisms, and interactions among these different organisms, including possible interactions with viruses. In fact, tetrabromopyrrole produced by bacteria living on surfaces of crustose coralline algae has been shown to exhibit antibacterial properties against other bacteria (Tebben *et al.*, 2014). Here, larval settlement is induced by the presence of a *Roseobacter*, a group commonly detected in coral larvae and in seawater during coral spawning. This additional role in coral morphogenesis and settlement, a key component of coral reef recovery and resilience, illustrates a need to understand the *Roseobacter* clade and its potential for impacting coral reef ecosystem health and resilience.

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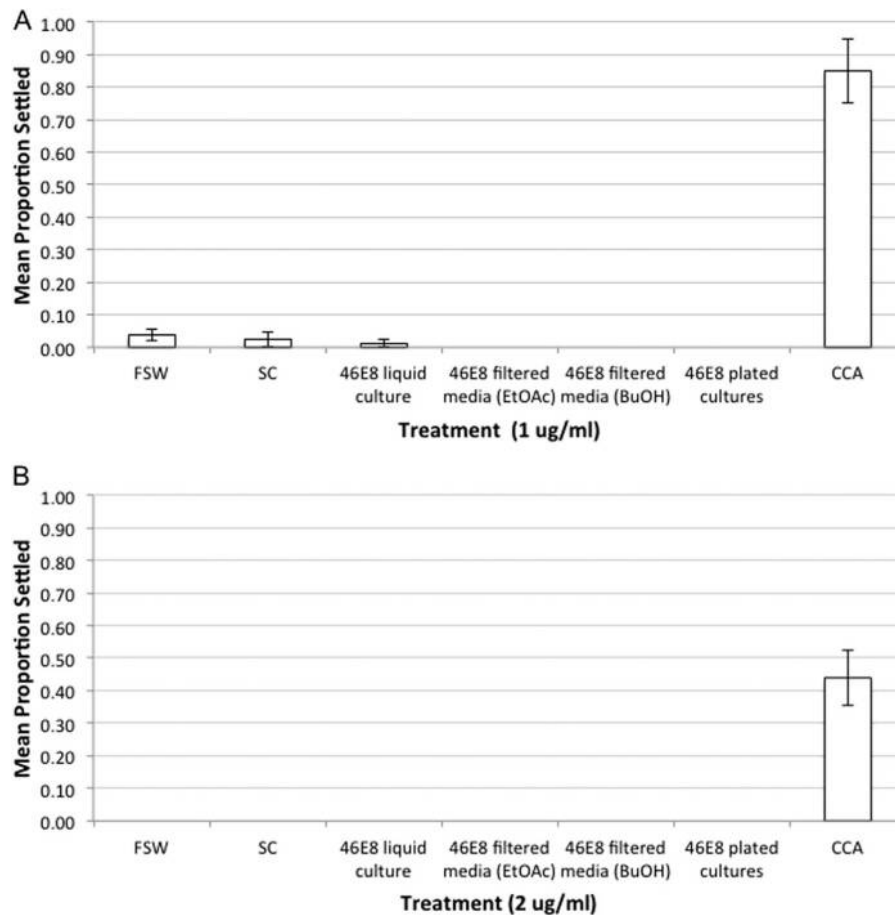
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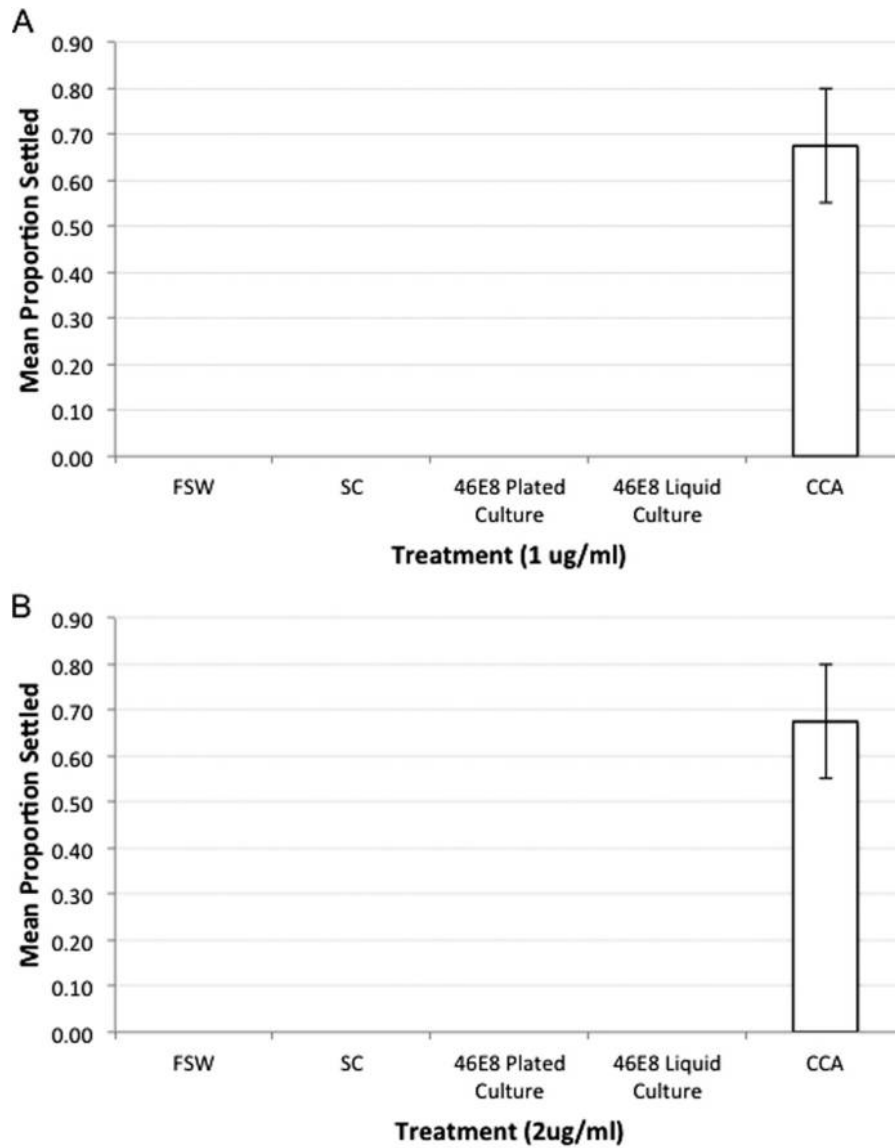
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## Appendix

### Settlement in *Porites* larvae



**Figure A1.** Mean proportion of settlement in *Porites astreoides* larvae exposed to crude organic extracts of *Roseivivax* sp. 46E8 cultures. Bars represent total settlement (attachment and metamorphosis) after 24-h exposure to extracts (mean  $\pm$  SE). Organic extracts (EtOAc:MeOH 1:1) were prepared from cells from liquid culture in marine broth in log phase and cells from plated cultures, and ethyl acetate and butanol partitions were prepared from filtrate (0.22  $\mu$ m) from log phase liquid culture. Crude extracts were measured at 1  $\mu$ g/ml (A) and 2  $\mu$ g/ml (B), in comparison to the negative controls sterile filtered seawater (FSW) and DMSO solvent control (SC) and the positive control crustose coralline alga piece (*Hydrolithon boergesenii*) (CCA).  $n = 10$ ; 10 larvae per treatment.



**Figure A2.** Mean proportion of settlement in *Porites astreoides* larvae exposed to crude aqueous methanol extracts of *Roseivivax* sp. 46E8 cultures. Bars represent mean total settlement (attachment and metamorphosis) after 24-h exposure to extracts (mean  $\pm$  SE). Aqueous methanol extracts were prepared from log phase liquid culture in marine broth and plated cultures at 1  $\mu\text{g}/\text{ml}$  (A) and 2  $\mu\text{g}/\text{ml}$  (B), in comparison to the negative controls sterile filtered seawater (FSW) and DMSO solvent control (SC) and the positive control crustose coralline alga piece (CCA) (*Hydrolithon boergesenii*).  $n = 10$ ; 10 larvae per treatment.