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ORIGINAL ARTICLE

Signaling-mediated cross-talk modulates swarming and biofilm formation in a coral pathogen *Serratia marcescens*

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Interactions within microbial communities associated with marine holobionts contribute importantly to the health of these symbiotic organisms formed by invertebrates, dinoflagellates and bacteria. However, mechanisms that control invertebrate-associated microbiota are not yet fully understood. Hydrophobic compounds that were isolated from surfaces of asymptomatic corals inhibited biofilm formation by the white pox pathogen *Serratia marcescens* PDL100, indicating that signals capable of affecting the associated microbiota are produced *in situ*. However, neither the origin nor structures of these signals are currently known. A functional survey of bacteria recovered from coral mucus and from cultures of the dinoflagellate *Symbiodinium* spp. revealed that they could alter swarming and biofilm formation in *S. marcescens*. As swarming and biofilm formation are inversely regulated, the ability of some native α-proteobacteria to affect both behaviors suggests that the α-proteobacterial signal(s) target a global regulatory switch controlling the behaviors in the pathogen. Isolates of *Marinobacter* sp. inhibited both biofilm formation and swarming in *S. marcescens* PDL100, without affecting growth of the coral pathogen, indicative of the production of multiple inhibitors, likely targeting lower level regulatory genes or functions. A multi-species cocktail containing these strains inhibited progression of a disease caused by *S. marcescens* in a model polyp *Aiptasia pallida*. An α-proteobacterial isolate 44B9 had a similar effect. Even though ~4% of native holobiont-associated bacteria produced compounds capable of triggering responses in well-characterized *N*-acyl homoserine lactone (AHL) biosensors, there was no strong correlation between the production of AHL-like signals and disruption of biofilms or swarming in *S. marcescens*.
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Introduction

Corals and related organisms are intimately co-evolved 'holobionts', complex symbiotic organisms formed by the invertebrate animal, the dinoflagellate, *Symbiodinium* spp. and their associated bacteria. The stability and effectiveness of this symbiosis determine the health of the whole reef ecosystem and its resistance to stresses and diseases. The differences in the microbial species composition of asymptomatic and diseased corals (Gil-Agudelo *et al.*, 2006; Ritchie, 2006; Bourne *et al.*, 2008; Koren and Rosenberg, 2008) imply a role for coral microbiota in coral health, although little is known about the mechanisms which govern the structuring and functions of coral-associated microbiota.

Coral-associated microbial communities are controlled by multiple factors. Nutrients and signals excreted by the coral host with coral mucus contribute to the structuring and functioning of the microbiota. Coral mucus is a polymer made in the specialized cells of the polyp from the photosynthate produced by their dinoflagellate symbionts and then excreted onto the coral surface (Brown and Bythell, 2005). Mucus has at least two roles in the associated microbial communities. Bacterial receptors can bind to mucus (Rosenberg and Falkovitz, 2004; Kvennefors *et al.*, 2008), and this directly controls the composition of the associated microbiota. The mucus polymer itself and small molecular

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weight compounds within it serve as both nutrient sources for the microbes and signals that modulate behavior and gene expression in the associated microbial communities (Vacelet and Thomassin, 1991; Wild *et al.*, 2004; Ritchie, 2006; Sharon and Rosenberg, 2008; Krediet *et al.*, 2009a, 2009b). Although the fate of coral mucus in the reef environment and its role in coral-associated microbial communities are becoming more clear, significantly less is known about the microbe–microbe interactions within the coral surface mucus layer.

Analysis of other host–bacterial systems suggests that microbe–microbe interactions within coral-associated microbial communities may have important functions in coral health and the interactions of native microbiota with pathogens. Models and experimental evidence to support this hypothesis are beginning to emerge (Shnit-Orland and Kushmaro, 2009; Mao-Jones *et al.*, 2010). For example, culturable microbes associated with the elkhorn coral *Acropora palmata* produced an array of anti-bacterials against a broad spectrum of pathogens, including the necrotizing coral pathogen *Serratia marcescens* (Ritchie, 2006). Thus, the *in situ* production of anti-microbials by coral-associated native microbiota may be one of the mechanisms by which interactions within the coral surface mucus layer are controlled. It is reasonable to hypothesize that in addition to the antibiosis, cooperative and antagonistic interactions take place within coral-associated microbial communities (Teplitski and Ritchie, 2009). Mechanisms that govern such interactions are not yet clearly understood.

One of the mechanisms known to be involved in microbe–microbe interactions is the intercellular exchange of small diffusible chemical molecules. The cell-to-cell signaling and the resulting changes in gene expression that link bacterial behaviors to the increases in their population densities within diffusion-limited environments are known as 'quorum sensing' (QS) (Fuqua *et al.*, 2001). The best-characterized QS signals are *N*-acyl homoserine lactones (AHLs) (Eberhard *et al.*, 1981; Fuqua *et al.*, 2001). In many Gram-negative bacteria, including members of the *Serratia* genus, QS controls surface spreading, production of antibiotics and exoenzymes, attachment to surfaces and timing of virulence gene expression (Van Houdt *et al.*, 2007). Production of QS signals has also been reported in cultures of coral-associated vibrios, although the role of QS in coral diseases caused by these microorganisms has not yet been established (Tait *et al.*, 2010). Because QS has important roles in the interactions within microbial communities, various forms of QS manipulation have been documented (Givskov *et al.*, 1996; Pasmore and Costerton, 2003; Skindersoe *et al.*, 2008; Dobretsov *et al.*, 2009). How these interactions carry out in natural habitats is far from being clear. The goal of this study was to test whether bacteria associated with marine invertebrates, and their endosymbiotic dinoflagellates, produce cell-to-cell signals capable of affecting behaviors in opportunistic pathogens by manipulating inversely regulated multicellular behaviors.

Materials and methods

Bacterial strains, media and growth conditions

Bacterial strains used in this study are listed in Table 1. Bacteria were isolated from *Symbiodinium* cultures via serial dilution in liquid f/2 media (Sigma, St Louis, MO, USA) followed by plating onto either Glycerol Artificial Sea Water medium (GASW; 356 mM NaCl, 40 mM $MgSO_4$, 20 mM $MgCl_2 \cdot 6H_2O$, 8 mM KCl, 60 μM K_2HPO_4, 33 μM Tris, and 7 μM $FeSO_4$, with 0.05% peptone, 0.2% yeast extract and 2.0% glycerol, (Ritchie, 2006)) or Marine Broth (Difco-Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Individual bacterial isolates were subcultured to purification on Marine Agar. *Symbiodinium* cultures were provided by T LaJeunesse (Penn State University) and maintained in f/2 medium (Sigma) at 25 °C under a12-h light:dark cycle. Coral-associated bacterial isolates were grown in either GASW or Marine Broth. All purified marine-derived isolates were aliquoted into 96-well plates in 25% glycerol seawater for frozen storage (−80 °C). Reporter strains were cultured in Luria-Bertani (LB) broth (Fisher Scientific, Pittsburgh, PA, USA) with appropriate antibiotics. As needed, ampicillin was added to the final concentration of 100 μg ml^{-1}, tetracycline at 10 μg ml^{-1} and gentamicin at 30 μg ml^{-1}.

The identities of marine bacterial isolates were confirmed by first PCR-amplifying fragments of their 16S rRNA genes with primers 8F (pA) 5′-AGAGTTT GATCCTGGCTCAG-3′ and 1489R 5′-TACCTTGT TACGACTTCA-3′ (Edwards *et al.*, 1989; Bruneel *et al.*, 2006) followed by cloning of the PCR fragments into pCR2.1-TOPO, and sequencing of the resulting constructs and BLAST searching against the NCBI (GenBank) database. Sequencing was carried out at the University of Florida Biotechnology Core Facility. Data are deposited in Genebank (Accession numbers JF263527; JF263528; JF263529; JF263530; JF263531; JF263532; JF346758; JF346759; JF346760; JF346761; JF346762; JF346763; JF3467640).

Field sample collection and handling

Coral mucus samples were collected from apparently asymptomatic *Acropora palmata* colonies at Looe Key Reef, Florida (24° 32.764′ N: 81° 24.304 W) using a needleless syringe in July 2009 and April 2010, as previously described (Ritchie, 2006). Mucus was pre-filtered through glass fiber filter followed by filtration through a 0.22-μm MCE filter, aliquots of mucus were stored frozen at −20 °C.

Table 1 Bacterial strains used in the study

Strain	*Relevant characteristics and source*	*Reference*
Marine isolates		
α-proteobacterium 44B9	Isolated from a culture of *Symbiodiunim* sp clade B1 from Gorgonian (*Pseudopterogorgia bipinnata)*, Caribbean Sea, Jamaica	This study
α-proteobacterium 44F6	Isolated from a culture of *Symbiodiunim* sp clade A1 from jellyfish (*Cassiopeia xamachana*), Caribbean Sea, Florida	This study
α-proteobacterium 45A11	Isolated from a culture of *Symbiodiunim* sp clade D2 from Forams, Red Sea	This study
Marinobacter sp. 47G8	Isolated from a culture of *Symbiodiunim* sp clade D2 from Forams, Red Sea	This study
Caryophanon sp. 52E5	Isolated from surface mucus layer of staghorn coral *Acropora palmata*, Looe Key, Florida	Ritchie, 2006
Marinobacter sp. 46E2	Isolated from a culture of *Symbiodiunim* sp clade C1, crom corallimorph (*Rhodactis* (*Heteractis*) *lucida*), Caribbean Sea, Jamaica	This study
α-proteobacterium 46H6	Isolated from a culture of *Symbiodiunim* sp clade F2, from *Scleractinian* (*Meandrina meandrites*), Caribbean Sea, Jamaica	This study
Marinobacter sp. 47A11	Isolated from a culture of *Symbiodiunim* sp clade A1 from jellyfish (*Cassiopeia xamachana*), Caribbean Sea, Florida	This study
Marinobacter sp. 47E6	Isolated from a culture of *Symbiodiunim* sp clade D1a, from *Acropora* sp., NW Pacific Ocean, Japan, Okinawa	This study
Photobacterium sp. 34E11	Isolated from surface mucus layer of elkhorn coral *Acropora palmata*, Looe Key, Florida,	
Planomicrobium spp. 34D8	Isolated from surface mucus layer of *A. palmata*, Looe Key, Florida	Ritchie, 2006
α-proteobacterium 47H1	Isolated from a culture of *Symbiodiunim* sp clade D2 from Foraminifera, Red Sea	This study
Vibrio spp. 52B8	Isolated from surface mucus layer of *A. palmata*, Looe Key, Florida,	Ritchie, 2006
Serratia marcescens isolates		
S. marcescens MG1	Wild type	Lindum *et al.*, 1998
S. marcescens PDL100	Wild type, isolated from a white pox disease lesion on *Acropora palmata*, Looe Key, Florida	ATCC
S. marcescens MG44	*S. marcescens* MG1 *swrI-*	Givskov *et al.*, 1996; Lindum *et al.*, 1998
QS reporters		
Agrobacterium tumefaciens NTL1 pZLR4	Responds to a wide range of AHLs	Cha *et al.*, 1998
Chromobacterium violaceum CV026	Responds to a wide range of AHLs	McClean *et al.*, 1997
E. coli JM109 pSB1075	AHL reporter based on the LasR receptor from *Pseudomonas aeruginosa*, responds strongly to AHLs with long acyl chains	Winson *et al.*, 1998
E. coli DH5a pTIM2441	Constitutive luminescent reporter, contains Pλ upstream of a promoterless *lux* cassette	Alagely *et al.*, 2011
E. coli JM109 pSB401	AHL reporter based on the LuxR receptor from *Vibrio fischeri*, responds strongly to AHLs with medium acyl chains	Winson *et al.*, 1998
E. coli JM109 pSB536	AHL reporter based on the AhyR receptor from *Aeromonas hydrophila*, responds strongly to AHLs with short acyl chains	Swift *et al.*, 1999

Abbreviation: AHLs, *N*-acyl homoserine lactones.

To collect hydrophobic compounds present within coral mucus surface layer *in situ*, reverse-phase C_{18} Si resin was wetted in ethanol, equilibrated in high-pressure liquid chromatography-grade water and then added into regenerated cellulose dialysis tubing (Fisher Scientific), pre-treated as per manufacturer's instructions. In parallel, cellulose dialysis tubing was filled with Diaion styrenic adsorbent resin HP20SS-1 (Sorbent Technologies, Atlanta, GA, USA), which was pre-treated as per manufacturer's instructions. Tubes with resin were clamped, weighed down on coral surfaces with small dive weights on cotton twines (Supplementary Figure S1). As a control, similarly constructed tubes (with C_{18} reverse-phase Si or HP20SS-1 resin) were deployed on the sand bottom, approximately 10–15 m away from nearest living coral (Supplementary Figure S1). Tubing with resin was deployed at Looe Key Reef, Florida for 3 days in May 2010. Upon completion of the experiment, samples were brought up to the lab, resin was scooped into flash chromatography columns and eluted sequentially with one volume of chloroform, isopropanol, 100% methanol, then 75% and 50% aqueous methanol solutions. Fractions were collected, rotary evaporated to dryness and stored at −20 °C.

Detection of quorum-sensing active compounds
The ability of tested bacteria to affect well-characterized QS reporters was tested using colony overlays and culture filtrate extracts with a suite of biosensors. For colony overlays, collections of bacteria isolated from marine invertebrates or from cultures of their dinoflagellate symbionts were replica plated into microtiter wells containing GASW medium solidified with 1% agar. After 2–3 days incubation at 30 °C, wells were overlaid with the suspension of *Chromobacterium violaceum* CV026 in soft LB agar with C_4-HSL, as described before (McClean *et al.*, 1997). Inhibition of violacein production in this reporter strain was scored visually. Interesting candidates were then re-tested by growing them on glass fiber disks placed onto GASW agar. After 3–5 days, when growth was observed on the surfaces of glass fiber disks, they were lifted from GASW agar and transferred onto the surface of LB agar, which was overlaid with a suspension of the *C. violaceum* CV026 reporter in soft LB agar with C_4-HSL.

In addition to the *Chromobacterium* assay, the presence of compounds affecting AHL reporters was detected using direct assays with *lux*-based bacterial biosensors *E. coli* JM109 pSB401, pSB536 or pSB1075 (Winson *et al.*, 1998). For preliminary screens, 1 ml samples of overnight bacterial cultures of each tested strain were pelleted at 16 000 *g*. Approximately 10 mg of charged Sample Prep C_{18} resin, 35–75U (Alltech, Deerfield, IL, USA) were added to the supernatants and gently mixed. Once resin settled, supernatants were aspirated and discarded. A dilution series of this C_{18} resin slurry was added to the wells of a black polystyrene 96-well plate (Corning Scientific, Corning, NY, USA) and luminescent biosensors were inoculated into the wells and incubated at 37 °C. Biossays were carried out as described previously (Alagely *et al.*, 2011). Luminescence was measured with a multilabel plate reader (PerkinElmer Victor^3, Waltham, MA, USA).

Bioassay-guided characterization of QS activities
A total of 13 bacterial isolates with the ability to promote or inhibit AHL reporters were selected for further characterization using thin-layer chromatography (TLC) aided by the AHL reporter *Agrobacterium tumefaciens* NT1 pZLR4. To prepare extracts, 5 ml aliquots of the overnight starter culture of each isolate were added to 0.5-l shake cultures of GASW and incubated with constant agitation at 200 r.p.m. at 30 °C (we note that AHL production or accumulation in Marine Broth was generally lower). After 48 h (when cultures reached $OD_{600} = 0.9–1.5$), two half volumes of acidified ethyl acetate were gently mixed with each culture, and organic phase was separated using a separatory funnel. The organic phases were frozen at −20 °C to remove residual water, and the solvent was evaporated at 37 °C in Rotavapor R-200 (Büchi Laboratorues, Flawil, Switzerland). The extracts were re-dissolved in a small volume of ethyl acetate, then spotted on C_{18}-reversed phase TLC plates (Whatman, Piscataway, NJ, USA) and developed with 60:40 methanol:water. The TLC plates were dried and overlaid with *A. tumefaciens* NT1 pZLR4 in 0.6% M9 agar supplemented with X-Gal (40 mg l^{-1}) as in Cha *et al.* (1998). The plates were incubated overnight at 30 °C, then air-dried and photographed.

To test whether the QS signals detectable with the *Agrobacterium* bioassay share structural homology with known AHL, the extracts were treated with the AHL-lactonase AiiA. *E. coli* DH5α pDSK*aiiA* and the control strain carrying just the pDSK vector, were grown in 1.5 l of LB with kanamycin (50 μg ml^{-1}) and isopropyl-β-D-thiogalactoside (20 μg ml^{-1}) at 37 °C to $OD_{600} = 1.6$ as previously described (Dong *et al.*, 2000; Gao *et al.*, 2007). The cells were centrifuged and reconstituted in 30-ml LB, then lysed using a French press and centrifuged to remove cellular debris. Assay conditions were optimized using synthetic 3-oxo-C_6-HSL (Sigma). For the treatment of bacterial extracts, 50 μl of the enzymatic preparation were added to 30 μl of dried extracts and incubated at 30 °C for 3 h. After incubation, the contents were extracted twice with 200 μl of ethyl acetate and subjected to the bioassay-aided TLC as described above.

Swarming assays with S. marcescens
AB swarm agar 0.4% (wt/vol) was briefly solidified and then superficial indentations 1/3 along the diameter of the plate were made with a sterile bore (d = 1 cm). Sterile glass filter disks (d = 0.75 cm) with 30 μl of overnight cultures of bacterial isolates (in GASWA) were placed in the recess. A total of 10 μl of *S. marcescens* cultures (subcultured in LB with antibiotics for 2 h) were spotted 2 cm away from the glass filter disk containing another isolate. This method was chosen to promote diffusion of chemical compounds from the marine isolates without direct initial contact with the tested strain.

Negative controls consisted of sterile GASW on the glass filter disk. The plates were incubated at 30 °C for 24–60 h, monitored for the progression of swarming and documented when the control *S. marcescens* swarms covered 50–75% of the plate. Each assay was repeated at least three times.

Swarming assays with *S. marcescens* PDL100 were more consistent if genetic analysis grade agarose (Fisher Scientific) was used instead of granulated molecular genetics bulk agar (Fisher Scientific). The former is typically used for agarose gel electrophoresis, and the latter is often used for laboratory media preparation. Swarm plates must be allowed to set at room temperature for 12 hrs before the assays (excessive dryness or wetness of the plates resulting from longer or shorter set times will confound the experiments). Further, subculturing

the overnight culture for 2 hours with tetracycline and ampicillin before the assays resulted in a consistently observed swarming.

Biofilm assay of co-inoculation of marine isolates and S. marcescens PDL100 in coral mucus

A total of 50 µl of filter-sterilized coral mucus were added to the wells of a 96-well plate. After an hour, 40 µl were aspirated and the remaining mucus was left to dry in a laminar flow hood overnight as in Krediet *et al.*, (2009a). The following day, overnight cultures of *S. marcescens* PDL100 and each of the marine isolates were diluted to OD_{600} of 0.4 and mixed 1:1 (*S. marcescens* PDL100: individual marine isolate) in sterile phosphate buffer solution and added to the wells to a total volume of 100 µl. After 48-h incubation, an aliquot of the supernatant was plated on Marine Agar (1.5% agar wt/vol). Each well was then washed three times with sterile phosphate buffer solution to remove un-attached bacteria; the remaining biofilms were dislodged using a sterile cotton swab, and then streaked to isolation on Marine Agar. The plates were incubated at 30 °C for 48 h and colonies were counted to determine the ratios between the coral pathogen and the marine isolates.

Co-inoculation experiments using marine isolates, S. marcescens *PDL100 and a model polyp* Aiptasia pallida

Clonal lines of *Aiptasia pallida* (from J. Pringle) were maintained in 10-gal saltwater aquaria at ambient temperature (22 °C) under blue actinic (460 nm) and super daylight white −6500 k fluorescent bulbs on a 12 h:12 h light:dark cycle. Polyps were fed weekly with brine shrimp. For the experiments, individual polyps were transferred into wells of six-well plates (Corning Scientific) with 10 ml of artificial seawater (Red Sea Coral Pro Salt, Eilat, Israel) sterilized by passing through a 0.2-µm filter. Polyps were acclimated in the wells for 2 days at room temperature on a shaker at 75 r.p.m. For the inoculations, overnight cultures of commensal strains were inoculated into filter-sterilized artificial seawater at 10^6 cfu ml^{-1}; 10 ml of this suspension were added to the wells with *A. pallida*. After 24 h, the seawater with inocula was aspirated and replaced with filter-sterilized artificial seawater seeded with the overnight culture of *S. marcescens* PDL100 at 5×10^7 cfu ml^{-1}. *A. pallida* sea anemones were photographed daily.

Results and discussion

Small molecular weight compounds present within coral mucus surface layer inhibit biofilm formation in S. marcescens

To survey biologically active substances that are present *in situ* on coral surfaces on the reef tract, we deployed 'chemical traps' consisting of cellulose dialysis tubing filled with reverse-phase Si or HP20SS-1 resin (Supplementary Figure S1). Hydrophobic substances were eluted with organic solvents, concentrated and tested for biological activity in the *S. marcescens* PDL100 biofilm assays. As shown in Figure 1, substances eluted with 75% methanol:25% water inhibited biofilm formation by the coral white pox pathogen *S. marcescens* PDL100. The corresponding fraction collected on the sand bottom did not have a statistically significant effect on the biofilm formation (Figure 1). Substances eluted from HP20SS with 100% methanol and 75% methanol similarly inhibited biofilm formation (data not shown). Other fractions did not inhibit biofilm formation. These observations indicate that the surface mucus layer of asymptomatic corals contains substances capable of inhibiting virulence-related behaviors in opportunistic pathogens.

As most marine invertebrates are 'holobionts' (complex symbiotic organisms formed by the polyp, endosymbiotic dinoflagellates and the associated bacteria (Rosenberg *et al.*, 2007; Rosenberg and Zilber-Rosenberg, 2008)), the bioactive compounds detected with this bioassay could have originated from any of the partners within the holobiont. To begin the characterization of these activities, we focused on testing the hypothesis that commensal bacteria associated with either the surface mucus layer or with the endosymbiotic dinoflagellates contribute to the synthesis of the activities capable of disrupting virulence-related behaviors in a model opportunistic pathogen *Serratia marcescens* PDL100.

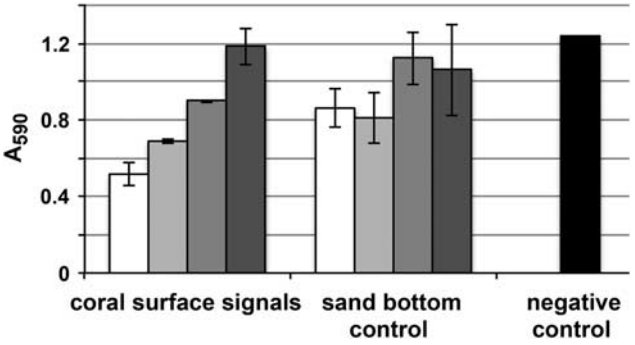

Figure 1 Inhibition of biofilm formation by *Serratia marcescens* PDL100 in the presence of compounds recovered from coral mucus surface layer. Left: Cellulose dialysis pouches containing reverse-phase C_{18} Si or HP20SS resin were deployed on surfaces of the boulder coral *Montastraea faveolata* and the compounds were eluted with a bed volume of chloroform, isopropanol, 100% methanol, 75% methanol and 50% aqueous methanol, evaporated, and reconstituted in 200 µl methanol. A total of 30 µl of serial 10-fold dilutions (left to right) were added to the wells of polystyrene microtiter plates where the pathogen was allowed to settle in colonization factor antigen media. The highest amount corresponds approximately to 37.5 cm^2 of *M. faveolata* coral surface. Activities eluted from the reverse-phase C_{18} Si with 75% methanol:25% water exhibited noticeable inhibitory properties, shown here. Middle: As a control, signals present on the sand bottom 10–15 m from the nearest living coral were similarly tested. Right: Biofilm formation by *S. marcescens* PDL100 in the absence of extracts in colonization factor antigen media. Error bars are standard errors of four technical replications.

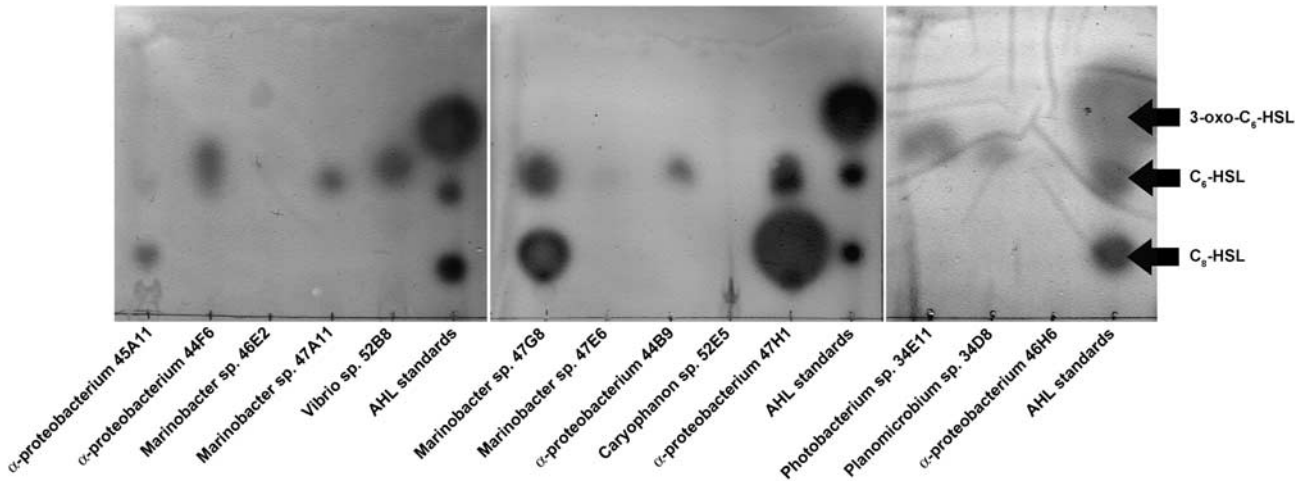

Figure 2 Production of QS signals by bacteria isolated from marine holobionts. Bacterial cultures were extracted twice with half volumes of ethyl acetate acidified with glacial acetic acid (4 ml l^{-1}), and concentrated by rotary evaporation. Samples were spotted on reverse-phase C_{18} Si TLC plates, developed with 60:40 methanol:water and overlaid with a suspension of the *Agrobacterium tumefaciens* NTL1 pZLR4 AHL reporter in soft agar with X-Gal. Synthetic AHL standards (all from Sigma) were similarly subjected to the bioassay-coupled thin-layer chromatography.

Screen and identification of coral-associated bacteria capable of QS manipulation

A library containing over 300 culturable bacterial isolates from mucus of the elkhorn coral *Acropora palmata* (Ritchie, 2006), other marine invertebrates and their dinoflagellate *Symbiodium spp*. symbionts was first screened with the *Chromobacterium violaceum* CV026 reporter. Isolates capable of either stimulating or inhibiting QS-mediated pigment production in the reporter were then subject to direct assays using semi-synthetic luminescent reporters based on the LuxR, AhyR and LasR AHL receptors from *Vibrio fischeri, Aeromonas hydrophila* and *Pseudomonas aeruginosa* (respectively). Approximately 4% of the tested bacteria were capable of affecting at least one QS reporter. Culture filtrates of isolates 44B9, 47H1 and 52E5 (purified with reverse-phase C18 silica resin) stimulated all three luminescent reporters, while similarly prepared culture filtrates from isolates 44F6, 47A11, 47E6 and 52B8 inhibited the LasR reporter, and stimulated the LuxR and AhyR reporters (data not shown). The former observation is consistent with the possibility that the three strains produce AHLs with medium acyl chains that trigger positive responses in the three luminescent reporters (Winson *et al*., 1998). Stimulation of the LuxR and AhyR reporters with the concurrent inhibition of the LasR reporter is less common, and the reverse is usually true (long-chain AHLs that stimulate LasR typically inhibit AhyR (Swift *et al*., 1999)), although some non-AHL QS inhibitors can have diverse effects on bacterial AHL receptors (rev. Teplitski *et al*., 2011). Alternatively, this observation could be explained by the production of multiple signals, capable of either inhibiting or inducing different AHL receptors independently. To test these working hypotheses bioassay-guided thin-layer chromatography was carried out with 13 bacterial isolates.

The thirteen isolates representing different marine invertebrates or their dinoflagellate symbionts were selected for further characterization. Based on partial sequencing of the 16S rRNA gene, the isolates were identified as members of the genera *Photobacterium, Marinobacter, Vibrio, Caryophanon* and *Planomicrobium*; the identity of five α-proteobacteria (Table 1) was difficult to ascertain due to the low homology of their 16S rRNA genes to the sequences deposited and annotated in GenBank. The α- and γ-proteobacteria have been previously reported as over-represented clades in coral-associated microbial communities (Ritchie, 2006; Kooperman *et al*., 2007; Wegley *et al*., 2007); photobacteria and vibrios are commonly found in association with healthy or diseased corals (Ritchie, 2006; Rosenberg *et al*., 2007; Tait *et al*., 2010). However, *Marinobacter* spp. have not been reported in coral-associated microbial communities. *Marinobacter* spp. are most commonly characterized as members of hydrocarbon-degrading consortia. AHL-producing strains of *Marinobacter* have been recovered from 'marine snow' (Gram *et al*., 2002).

Bioassay-guided characterization of QS signals

To better characterize QS-active substances and to separate the inhibitory from the stimulatory QS activities produced by the selected bacteria, organic extracts of their culture filtrates were subjected to the bioassay-coupled reverse-phase C_{18} Si TLC. The biosensor, *Agrobacterium tumefaciens* NT1 pZLR4, produces β-galactidosidase in response to AHL with C_6–C_{18} acyl side chains (Cha *et al*., 1998). Most of the tested strains produced one or two separable activities, which co-migrated with AHLs of medium

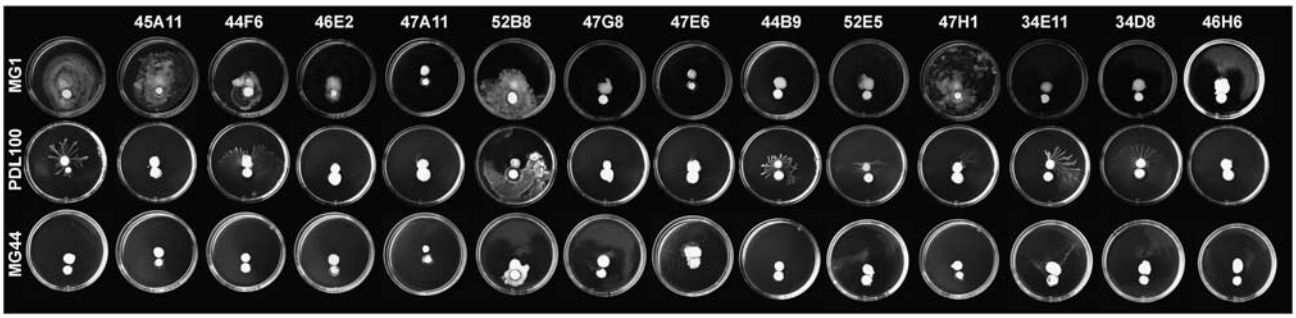

Figure 3 Swarming of *Serratia marcescens* in the presence of marine bacteria. Swarming of the model opportunistic pathogen *S. marcescens* MG1 (top row), coral white pox pathogen *S. marcescens* PDL100 (middle row) and the SwrI AHL synthase mutant *S. marcescens* MG44 (bottom row) was tested. Controls (*Serratia* spotted next to a glass fiber disk without a marine bacterium) are in the far left column. Identities of the tested marine bacteria are listed above the figure.

(C_6–C_8) length (Figure 2). This lack of complexity in QS signal profiles of the tested bacteria is different from the reports, in which α- and γ-proteobacterial associates of sponges and marine dinoflagellates were shown to produce multiple AHLs, including signals with unusually long acyl side chains (Wagner-Dobler *et al.*, 2005; Mohamed *et al.*, 2008).

Treatment with the AHL lactonase AiiA, significantly reduced appearance of the detectable TLC spots produced by the *Marinobacter* sp. 47G8 and α-proteobacteria 45A11 and 47H1 (Supplementary Figure S2). The sensitivity of these signals to the treatment with the AHL lactonase AiiA suggests that these compounds are likely to be AHL. Other signals produced by the marine bacteria and detectable with the *Agrobacterium* bioassay were resistant to the lactonase treatment (Supplementary Figure S2), suggesting that these compounds are not well-characterized AHLs.

As expected, the Gram-positive *Caryophanon* spp. 52E5 did not produce any compounds capable of stimulating the *Agrobacterium* reporter, although it produced yet un-identified substances that stimulated the LuxR- and AhyR-based reporters (pSB401 and pSB536, respectively) 600–200- and 115–138-fold over the background. We note that the production of QS-active substances in α-proteobacterium 46H6 was dependent on culture conditions: QS-active substances were consistently detected in cultures grown in GASW broth, but not in Marine Broth (data not shown).

Effect on swarming in Serratia marcescens

The ability of marine prokaryotes and eukaryotes to excrete compounds that stimulate or inhibit QS reporters is now well documented (Pasmore and Costerton, 2003; Wagner-Dobler *et al.*, 2005; Skindersoe *et al.*, 2008; Teasdale *et al.*, 2009; Kwan *et al.*, 2010); however, ecological roles of these compounds and of the bacteria that produce them are less understood. Therefore, our subsequent experiments focused on testing the behaviors of the isolates in the dual-species microbial consortia consisting of the coral pathogen *S. marcescens* PDL100 and the isolate of interest. We tested the hypothesis that the marine isolates capable of affecting QS reporters will also modulate those behaviors in the coral pathogen *Serratia marcescens*, which are known to be controlled by the AHL-mediated QS in *Serratia* spp. (Van Houdt *et al.*, 2004).

In strains of *S. marcescens*, multicellular surface motility ('swarming') is facilitated by the QS-mediated production of a surfactant (Van Houdt *et al.*, 2007). Wild-type *S. marcescens* MG1 swarms over semi-solid surfaces ((Lindum *et al.*, 1998); Figure 3), while the disruption of the AHL synthase gene *swrI* significantly delays the appearance of the swarm ((Lindum *et al.*, 1998); Figure 3). Despite the fact that 10 of the 13 tested strains produced QS signals detectable with the *A. tumefaciens* reporter, only 2 isolates (*Vibrio* sp. 52B8 and *Marinobacter* sp. 47E6) partially restored swarming in the *swrI* mutant of *S. marcescens* MG44 (Figure 3, bottom row). This suggests that either the QS activities detected with the *Agrobacterium* reporter are not AHLs, or that in addition to the QS signals the tested bacteria produce compounds that specifically disrupt swarming. Similarly to the well-characterized *S. marcescens* MG1, the coral white pox pathogen *S. marcescens* PDL100 was also capable of spreading over semi-solid surfaces, although the appearance of the swarm was distinct (Figure 3).

When inoculated in the vicinity of the wild-type *Serratia* strains, all *Marinobacter* spp. isolates (46E2, 47A11, 47E6 and 47G8) and α-proteobacterial isolate 46H6 inhibited their surface spreading. The α-proteobacterial isolates 44F6 and 44B9, *Photobacterium* sp. 34E11, *Caryophanon* sp. 52E5 and *Planomicrobium* sp. 34D8 inhibited swarming in *S. marcescens* MG1, but not in the coral pathogen *S. marcescens* PDL100 (Figure 3). The α-proteobacterium 47H1 stimulated swarming in *S. marcescens* MG1; however, this phenotype is not likely to be due to the production of AHLs or a surfactant by the strain as evidenced by the lack of swarming restoration in the *swrI* mutant *S. marcescens* MG44 (Figure 3, bottom row). Co-culture of the coral

pathogen *S. marcescens* PDL100 with *Planomicrobium* sp. 34D8 and α-proteobacterial isolate 44F6 stimulated surface spreading in the coral pathogen (Figure 3, middle row). In a co-culture of *S. marcescens* PDL100 with *Vibrio* sp. 52B5, a swarm, likely composed of the two organisms, was observed. These results suggest that in addition to producing compounds that trigger responses in QS reporters, tested strains secrete substances that inhibit swarming in *S. marcescens*, either by interfering with the regulation of the flagellar regulon or by disrupting the synthesis of the surfactant. Both of these functions are required for surface swarming (Lindum *et al*., 1998; Verstraeten *et al*., 2008).

Biofilm formation on coral mucus

In addition to controlling surface spreading, cell-to-cell signaling also has important roles in the formation of sessile microbial communities ('biofilms') and interactions within them. Both swarming and biofilm formation are the behaviors required for surface-associated growth; however, they represent disparate strategies in surface colonization and are inversely regulated in γ-proteobacteria (rev. Verstraeten *et al*., 2008). If the invertebrate-associated bacteria secrete a compound that affects global regulatory systems required for surface-associated growth, then—we hypothesized—in the absence of other interactions, there should be a measurable effect on biofilm formation by *S. marcescens* in the presence of the tested bacterial isolates.

The effects on the biofilm formation by *S. marcescens* PDL100 were tested using dual-species microbial consortia containing bacteria capable of inhibiting swarming. Experiments were carried out in polystyrene microtiter plates coated with mucus of *A. palmata,* as in (Krediet *et al*., 2009a). Consistent with our hypothesis, in dual-species consortia, α-proteobacterium 44B9 stimulated swarming (Figure 3) and also inhibited biofilm formation by the coral pathogen *S. marcescens* PDL100, even though growth of the pathogen in the suspension cultures was not affected (Figure 4). Similar trends (stimulation of swarming and reduced biofilm formation) were observed for α-proteobacterium 47H1, *Photobacterium* sp. 34E11 and *Planomicrobium* sp. 34D8, although statistically significant differences were not observed on all

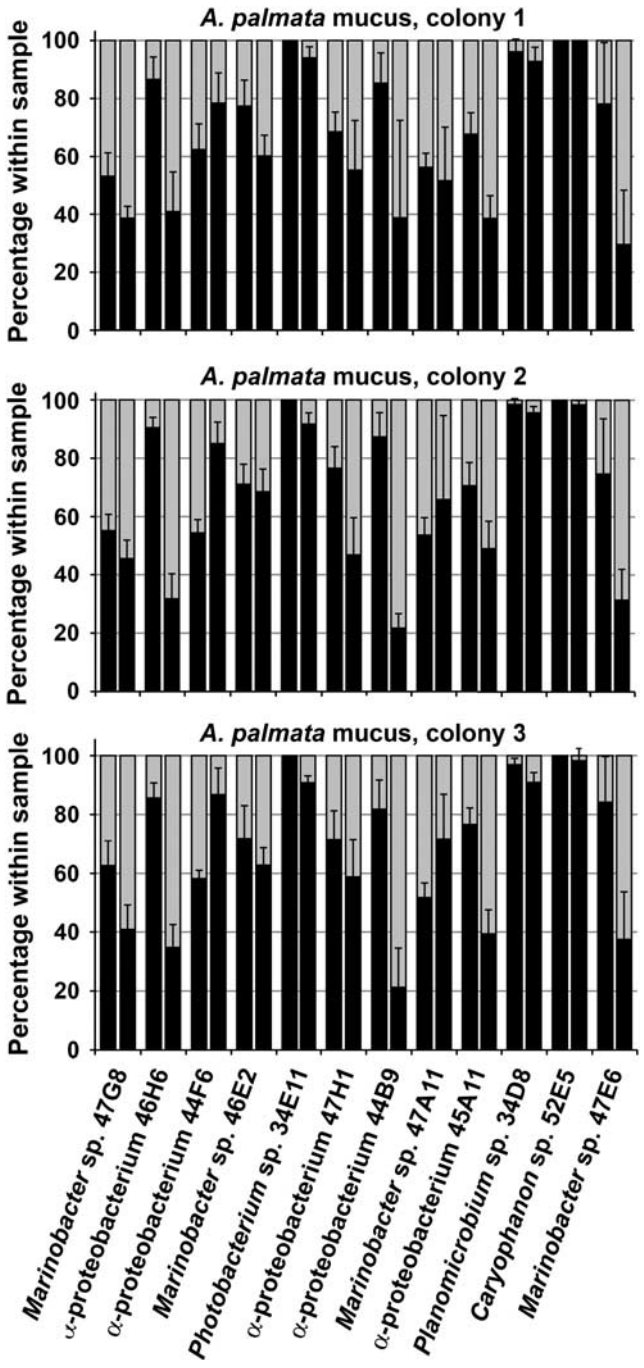

Figure 4 Biofilm formation by the white pox pathogen *S. marcescens* PDL100 on coral mucus in the presence of marine bacteria. Dual-species consortia consisting of the coral pathogen and a marine isolate were set up in microtiter plates, surfaces of which were coated with mucus of *Acropora palmata*. *S. marcescens* PDL100 was inoculated into each well, marine bacteria present within each dual-species consortium are listed at the bottom of the figure. The relative numbers of *S. marcescens* PDL100 and marine isolates were enumerated by dilution plating. *S. marcescens* PDL100 was distinguished from the other marine bacteria based on colony morphology: *S. marcescens* PDL100 forms small cream-color circular convex colonies with an entire edge on Marine Agar (1.5% agar). The black bars indicate the percentage of the coral pathogen and the gray bar is the percentage of the marine isolate in each sample. In each data set, the left bar indicates un-attached bacteria, and the right bar is the biofilm. Inhibition of biofilm formation by the coral pathogen was scored as the decrease of the relative percentage of *S. marcescens* PDL100 within biofilms, compared with the suspension. Data from the dual-species biofilms with *Vibrio spp* 52B8 are not shown because mixed swarms containing the two bacteria formed on agar plates, thus making enumeration of bacteria nearly impossible. Each panel represents data from three independent experiments using mucus harvested from three different colonies of *A. palmata* in April of 2010. Error bars represent standard errors of four biological replications (independent dual-species consortia).

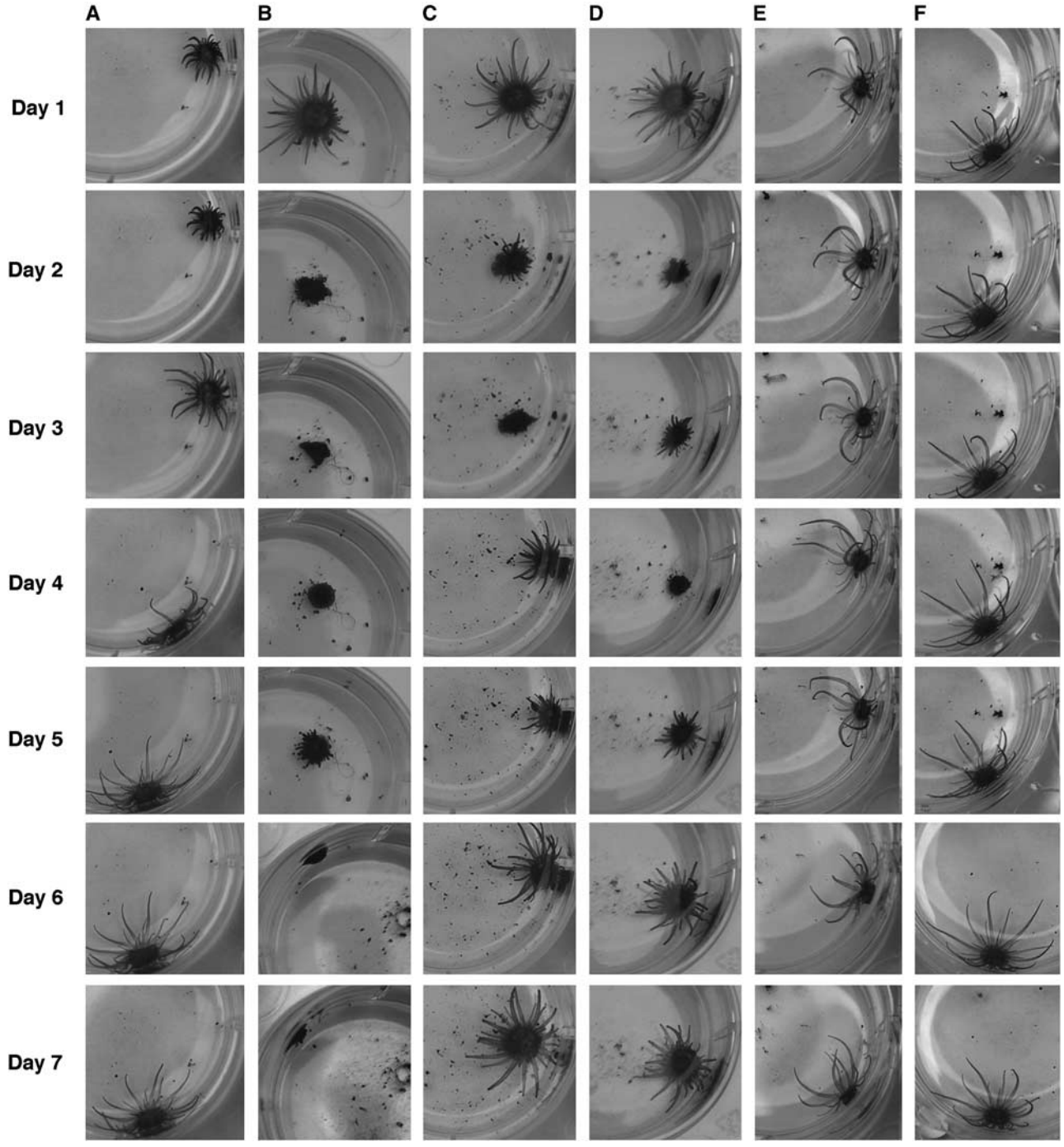

Figure 5 Inhibition of serattiosis in *Aiptasia pallida* by antagonistic holobiont-associated bacteria. Clonal lines of *Aiptasia pallida* were maintained in aquaria. For the experiments, individual polyps were acclimated in six-well plates with 10 ml of sterile artificial seawater for 2 days. Antagonistic marine strains (columns C and E were added as a cocktail containing *Marinobacter* spp. 47E6, 47G8 and 46E2 as well as α-proteobacteria 46H6 and 45A11); polyps shown in columns D and F were treated with α-proteobacterium 44B9 at 10^6 cfu ml^{-1}. Wells in column A contain polyps that were not exposed to *S. marcescens* or marine isolates; polyps in columns B, C and D were infected with *S. marcescens* PDL100 at 5×10^7 cfu ml^{-1} on day 1 of the experiment. Signs of the disease progression (darkened polyp and retracted tentacles) were documented daily for a week. Brightness and color balance of the images were adjusted in Adobe Photoshop CS4 using default settings. Experiments were performed with three biological and six technical replications.

batches of mucus collected from spatially separated *A. palmata* colonies on the same reef (Figure 4). Of the isolates capable of promoting swarming and inhibiting biofilm formation, α-proteobacterium 44B9 both reduced biofilm formation by the pathogen and also dominated the biofilms. Also consistently with the working hypothesis, *Marinobacter* sp. 47A11 inhibited swarming and modestly stimulated biofilm formation by *S. marcescens* PDL100 on some batches of coral mucus (Figure 4). Collectively, these results indicate

that the compound(s) secreted by these organisms may target a global regulatory switch involved in the regulation of swarming and biofilm formation, without affecting growth of another organism.

The phenotypes of seven isolates did not fit the working hypothesis. The α-proteobacterium 44F6 stimulated both swarming and biofilm formation. *Caryophanon* sp. 52E5 promoted swarming of *S. marcescens* PDL100 but had no appreciable effect on biofilm formation on mucus of *A. palmata* (Figure 4). *Marinobacter* spp. 47E6, 46E2, 47G8, α-proteobacteria 45A11 and 46H6 reduced both swarming and biofilm formation (Figure 4). The latter phenotype would be consistent with the production of multiple compounds, each capable of reducing swarming and inhibiting biofilm formation, likely by affecting independent regulatory or functional cascades.

Antagonistic isolates reduce appearance of serratiosis in Aiptasia pallida

To test whether the isolates capable of affecting biofilm formation and swarming in the model opportunistic pathogen *S. marcescens* PDL100, individual polyps were inoculated under laboratory conditions with the pathogen with or without the antagonistic marine isolates. As shown in Figure 5 (column B), *S. marcescens* PDL100 can completely degrade the polyp within 3–5 days. Pre-inoculation of the polyps with either a cocktail of isolates (containing α-proteobacteria 45A11 and 46H6 and *Marinobacter* spp. 47E6, 46E2 and 47G8) or a monoculture of the α-proteobacterium 44B9 reduced the appearance of the disease symptoms in the polyps infected with the white pox pathogen (columns C and D). These results indicate that the native microbiota associated with the invertebrates or their endosymbiotic dinoflagellates are capable of producing activities that reduce susceptibility of marine invertebrates to opportunistic pathogens.

Conclusions

Interactions within the microbiota associated with marine holobionts are undoubtedly complex. Results presented in this manuscript suggest that bacteria, which inhabit the holobiont, can modulate behaviors in the model multi-species opportunistic pathogen *S. marcescens.* In dual-species microbial consortia, swarming of the coral pathogen *S. marcescens* PDL100 was generally inhibited by the bacteria recovered from the associations with the symbiotic dinoflagellate *Symbiodium* spp. (Figure 3). The ability of the antagonistic bacteria to inhibit the progression of a disease caused by *S. marcescens* in a model marine invertebrate holobiont *A. pallida* (Figure 5), is consistent with the fact that swarming is often co-regulated with virulence in γ-proteobacterial pathogens (Verstraeten *et al.*, 2008). Unlike virulence genes that are generally co-regulated with genes involved in swarming, biofilm formation is inversely regulated (Verstraeten *et al.*, 2008). The ability of some strains to inhibit biofilm formation and promote swarming is consistent with the possibility that the native bacteria produce compound(s) that disrupt global regulatory cascades involved in switching from surface motility (swarming) to biofilm formation. Many of the tested native bacteria inhibited both swarming and biofilm formation by *S. marcescens* PDL100 (but not growth of the pathogen), indicative of the ability to produce inhibitory activities capable of disrupting multiple behaviors in the pathogen. Even though bacteria were selected for this study based on their ability to affect well-characterized QS reporters, no strong correlation between their QS activities and the effect on swarming and/or biofilm formation by *S. marcescens* PDL100 was observed.

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