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Production of antibacterial compound and biofilm formation in *Roseobacter* species are influenced by culture conditions

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ABSTRACT

Bacterial communities associated with marine algae are often dominated by members of the *Roseobacter* clade, and in the present study we describe *Roseobacter* phenotypes that may provide this group of bacteria with selective advantages when colonizing this niche. Nine of fourteen members of the *Roseobacter* clade, of which half were isolated from cultures of the dinoflagellate *Pfiesteria piscicida*, produced antibacterial compound(s). Many non-*Roseobacter* marine bacteria were inhibited by sterile filtered supernatants of *Silicibacter* sp. TM1040 and *Phaeobacter* (formerly *Roseobacter*) strain 27-4 which had the highest production of antibacterial compound. In contrast, *Roseobacter* strains were only susceptible when exposed to concentrated compound. Production of antibacterial compound was influenced by the growth conditions, as production was most pronounced when bacteria were grown in liquid media under static conditions. Under these conditions, *Silicibacter* sp. TM1040 cells attached to one another forming rosettes, as has previously been reported for *Phaeobacter* 27-4. A spontaneous *Phaeobacter* 27-4 mutant unable to form rosettes was also defective in biofilm formation and production of the antibacterial compound indicating a possible link between these phenotypes. Rosette formation was observed in eight of fourteen *Roseobacter* clade strains examined and was very pronounced under static growth in five of these strains. Attachment to surfaces and biofilm formation at the air-liquid interface by these five strains was greatly facilitated by growth conditions that favored rosette formation, and rosette-forming strains were 13-30 times more efficient in attaching to glass, as compared to conditions where rosette formation was not pronounced. We hypothesize that the ability to produce antibacterial compounds that principally inhibit non-*Roseobacter* species, combined with an enhancement in biofilm formation, may give members of the *Roseobacter* clade a selective advantage, and help to explain the dominance of members of this clade in association with marine algal microbiota.

INTRODUCTION

Bacteria belonging to the α -Proteobacteria are widely distributed in marine waters and are the dominant bacterial group in surface bacterioplankton communities (13,30,40). However the different α-proteobacterial groups are not equally distributed between the different marine niches as members of the *Roseobacter* clade dominate among marine algal associated bacteria (1,9,15,21,31,47), whereas members of the SAR11 (*Pelagibacter ubique*) cluster dominate the total bacterioplankton community (13,30). Understanding how the bacteria interact with other bacteria and with their algal hosts is important, since these interaction can affect the growth and physiology of the algae (1,18), particularly in the production or modification of algal toxins (12). The dominance of the *Roseobacter* clade in bacterial communities associated with algae could indicate that there are specific phenotypic traits that provide these bacteria with a selective advantage in colonizing algae.

Bacteria belonging to the *Roseobacter* clade are rapid colonizer of surfaces (11) and they have been called aggressive colonizers, as a *Phaeobacter gallaeciensis* (Reclassified from *Roseobacter gallaeciensis* (26))invaded and dispersed pre-established biofilm of marine bacteria on the marine plant *Ulva australis* (34). This ability to colonize and form a biofilm may be one of the factors that allow these bacteria to colonize alga. It is not known why bacteria from the *Roseobacter* clade are so efficient in colonizing, but both attachment and biofilm formation have recently been linked to the ability of a *Phaeobacter* strain (27-4) to grow in a multicellular rosette shape (7,8). This rosette shape has previously been reported for members of the *Roseobacter* clade (37,43). The rosette mode of growth has also been described in *Ruegeria atlantica,* a member of the *Roseobacter* clade isolated from the toxic dinoflagellate *Alexandrium catenella* (2) but it is not known if this phenotype facilitates attachment and biofilm formation of that bacterium.

Several studies have found that members of the *Roseobacter* clade inhibit other bacteria (6,8,38) and this may contribute to their dominance among algal associated bacteria. Production of antibacterial compound by *Phaeobacter* 27-4 was influenced by culture conditions and was only detected when the bacteria were grown in liquid nutrient media under static conditions, which also facilitated rosette and biofilm formation (7,8). Indeed particle-associated members of the *Roseobacter* clade are 13 times more likely to produce antimicrobial compounds than free living members (24). Furthermore, a member of the *Roseobacter* clade was able to prevent growth of others bacteria on surfaces, while growing in a biofilm (33,34).

Marine aggregates are characterized by high bacterial densities (10^8-10^9 bacteria/ml) that are 1,000 – 10,000 fold higher than the surrounding water (22,32,39) and comparable to bacterial densities (10^6-10^9 cells/ml) observed in algal cultures (31). At high cell densities, bacteria may use quorum sensing (QS) to regulate production of extracellular compounds (46). QS signals have been detected in *Roseobacter* species (7,16,45) and, since the production of antibacterial compounds in some bacteria is controlled by QS (3,48), it has been suggested as a regulatory mechanisms for *Roseobacter* production of antibacterial compounds (8,44).

One may hypothesize that the dominance of members of the *Roseobacter* clade among algal associated bacteria is influenced by specific phenotypes such as ability to attach and form biofilms or by production of antibacterial compounds. The present study was undertaken to determine if biofilm formation and the production of antibacterial compounds are common phenotypes found in members of the *Roseobacter* clade, especially strains associated with dinoflagellates (e.g., *Pfiesteria piscicida*). In addition, we address if such behaviors occur under specific growth conditions e.g. static or shaken cultures and if they occur when specific morphotypes, such as rosettes are prevalent.

MATERIALS AND METHODS

Bacterial strains and media. Thirteen stains of α-Proteobacteria belonging to the *Roseobacter* clade all isolated from marine environments, most from dinoflagellate cultures of *Pfiesteria piscicida*, were tested in the present study (Table 1). Furthermore the non α-Proteobacteria marine bacteria *Halomonas* sp., *Pseudomonas elongata*, *Spongiobacter* "nickelotolerans", *Shewanella* sp, four environmental strains of *Vibrio cholerae*, two clinical strains of *V. cholerae*, *V. coralliilyticus*, *V. fortis*, *V. mediterranei, V. harveyi, V. shiloi* (Table 3) and *Mycobacterium marinum* were tested for their susceptibility to active compounds produced by *Silicibacter* sp. TM1040 and *Phaeobacter* 27-4. *Phaeobacter* strain 27-4 isolated from turbot larval rearing units was included as a control strain exhibiting inhibitory activity to many γ-Proteobacteria and the ability to form rosettes and biofilms (8,20). This strain was original named *Roseobacter* and clustered close to the *R. gallaciensis* group (6). Recently, this group has been re-classified as *Phaeobacter gallaciensis* (26) and we have used this naming throughout the study. All strains were stored at – 80°C in 30% glycerol. Strains were maintained on Marine Agar (Difco 2216) and liquid broth cultures were prepared in Marine Broth (MB, Difco 2216) incubated at 25ºC under static or shaken (200 rpm) conditions in the dark, as previously described (8).

Attachment assay. Glass cover slips (Corning catalog nr. 2865-22) were dipped into bacterial cultures grown under either static or shaken conditions and left for 5 sec. After removal, non-attached and poorly attached bacteria were removed by placing the glass cover slip on a sterile absorbent paper. The cover slips were incubated at 60ºC for 30 min to fix the bacteria. Bacterial cells attached to the cover slip were stained with 0.1% crystal violet for 15 min at room temperature and unbound dye removed by rinsing the glass cover slip with 1X Phosphate Buffered Saline

(PBS). The cover slips were immersed in 2 ml 33% acetic acid to solubilise the dye bound to the biofilm, which was measured using a Beckman DU640 spectrophotometer at 590 nm.

Cell morphology, motility and biofilm at air-liquid interphase. Bacterial motility, rosette formation, and biofilm formation at the air-liquid interphase in shaken and statically grown cultures was assessed by phase-contrast microscopy using an Olympus BX60 microscope. Images were captured using a Qicam Fast 1394 digital camera (QImaging, Canada) and Volocity version 3.5.1 computer software (Improvision, England). Motility was also measured in both shaken and static grown cultures using marine motility agar (Marine broth (Difco, 2216) supplemented with 3 g Bacto Agar per Liter) (28).

Assessment of antibacterial activity using a well diffusion assay. Sterile filtered (0.22 μm, Millipore) supernatants from outgrown bacterial cultures, as well as ethylacetate extracts of the cultures were tested for antibacterial activity in a well diffusion assay using *V. anguillarum* strain 90-11-287 (serotype O1 strain), as previously described (20). This species of γ-Proteobacteria was chosen as a target organism because of its high sensitivity to the antibacterial compound of *Phaeobacter* 27-4 (Bruhn, Hougaard and Gram personal observation). Sixty μl of each sample were added to a well in an agar cast with *V. anguillarum* 90-11-287. Plates were incubated at 20ºC for 24 h whereupon inhibitory activity was detected as a zone of clearing in the turbid agar around the wells containing antibacterial activity (positive samples). The diameter of the clearing zones was measured to obtain a semi-quantitative determination of the concentration of the antibacterial compound.

To investigate the spectrum of bacterial species inhibited by the antibacterial compounds from *Silicibacter* sp. TM1040 and *Phaeobacter* 27-4, marine bacteria (Table 3) were grown in Marine broth to stationary phase and 100µl of a 10-fold dilution of each culture spread onto the surface of a Petri dish containing Marine Agar. Wells were punched in the agar and supernatants from static grown cultures of *Silicibacter* sp. TM1040 and *Phaeobacter* 27-4 were then added, and the cultures incubated for 1-3 days until a lawn of the target bacteria was visible. As described earlier, a zone of clearing in the bacterial lawn around wells was used to indicate that the target bacteria were sensitive to either the *Silicibacter* sp. TM1040 or *Phaeobacter* 27-4 supernatants.

Screening for acylated homoserine lactones (AHLs). Bacterial strains (Table 1) were grown in MB at 25°C for four days and 20 ml each of the resulting cultures was extracted with 20 ml ethylacetate (containing 0.5% formic acid). The ethylacetate fraction was evaporated under airflow to dryness, reconstituted in 1 ml acidified ethylacetate, and stored at -20ºC. Detection of AHL compounds was done using the two AHL monitor strains: *Agrobacterium tumefaciens* NT1 (pZLR4) (10) and *Chromobacterium violaceum* CV026 (42), using a well diffusion assay (35).

Pigment production. The 14 bacterial species were grown in MB as static or shaken cultures for 4 days at 25°C. The amount of extracellular pigment contained in 0.22 um-filtered supernatants was determined by measuring absorbance at 398 nm (8).

Pigment and antibacterial compound production dependence on bacterial culture conditions. *Roseobacter* 27-4 and *Silicibacter* sp. TM1040 were inoculated in MB at an initial density of 5 x 10^2 cfu/ml and incubated at 25°C under shaken and static conditions. Growth, as measured by CFU/ml, was followed by spread plating on MA (incubated at 25°C for 3 days) and enumeration of

the resulting colonies. Pigment production and antibacterial activity were assessed as described above.

RESULTS

Attachment of *Roseobacter* clade strains during different growth conditions. Fourteen members of the *Roseobacter* clade (Table 1) were tested for their ability to attach to a surface, and the influence of pre-culture conditions (static or shaken) on attachment was determined. Static cultures of *Roseobacter* sp. TM1039, *Silicibacter* sp. TM1040, *Sulfitobacter* EE36, *Sulfitobacter* 1921 and *Phaeobacter* 27-4 attached very efficiently to the surfaces (Fig. 1). Preculture conditions influenced the attachment of these strains, and cells grown under static culture conditions attached 13-30 times more readily than shaken culture counterparts, even though the shaken cultures had approx. 10-fold higher cell density (Table 2). The attachment of the remaining 9 strains was low, and only small differences between shaken and static grown cultures were noted, suggesting that this is a species- or strain-specific trait and not a clade-specific attribute.

Cell morphology, biofilm formation and motility of *Roseobacter* clade strains. It has been suggested that formation of rosettes of cells, which is seen under static growth of *Phaeobacter* strain 27-4, affects the attachment and biofilm formation of the bacterium (7). Several of the strains included in the present study were capable of growing as rosettes, however, the degree to which this occurred differed markedly (Fig. 2 and supplementary data). Rosettes were prominently formed in *Roseobacter* sp. TM1039, *Silicibacter* sp. TM1040, *Sulfitobacter* EE36 and *Sulfitobacter* 1921 in static culture, a condition that also resulted in maximal bacterial attachment (Fig. 1) and biofilm

formation at the air-liquid interphase. Rosettes were also observed in *S. pomeroyi* DSS-3, *Sulfitobacter* SE62 and *R. algicola* ATCC 51442, albeit at a much lower level with a corresponding lower attachment (Table 1, Fig. 2 and supplementary data). Formation of rosettes in cultures of *Silicibacter* sp. TM1040 and *Phaeobacter* 27-4 was very similar and differed somewhat from what was observed in the other strains (Fig. 2 and supplemental data). For example, *R. denitrificans* ATCC 33942 and *R. litoralis* ATCC 49566 produced an aggregate when grown as shaken cultures, but not under static culture conditions, and also had a reduced CFU/ml in the shaken cultures as compared to the static cultures (Table 2). However, these aggregates did not facilitate biofilm formation at the air-liquid interface and did not attach to the glass slide (Fig. 1).

Nine of the 14 roseobacter species were motile, as determined by their outward movement in semi-solid motility agar. Two strains, *R. denitrificans* and *R. algicola*, failed to show overt movement in motility agar, but light microscopy of these cells revealed that they swam (Table 1). Eight of the nine motile strains were capable of forming rosettes, with the exception of *Roseobacter denitrificans* which did not form rosettes yet was motile.

Production of antibacterial compounds. Ethyl acetate extracts from nine of the 14 strains inhibited the growth of *V. anguillarum* (Table 2). Seven of these nine strains only caused inhibition when extracts were prepared from roseobacters grown in static culture conditions. Antibacterial activity was observed from both static and shaken cultures of *Silicibacter* sp. TM1040, however, the inhibition zones from static cultures were much larger than from shaken cultures, despite a higher cell density in the shaken culture. In one strain (*S. pomeroyi* DSS-3), inhibitory activity was seen only in extracts obtained from the shaken culture.

In contrast to the inhibitory activity of ethyl acetate extracts, antibacterial activity, as measured by the zone of clearing in *V. anguillarum* well-diffusion assays, was only detectable in

filtered culture medium supernatants obtained from *Phaeobacter* 27-4 and *Silicibacter* sp. TM1040 (Table 2). This may be due to increase in concentration of the antibacterial compound during ethyl acetate extraction.

Detection of acylated homoserine lactones (AHLs). This set of strains was also tested for the production of QS molecules, under the hypothesis that production of the antibacterial compound could be linked to the production of AHLs. AHLs were detected in five strains, all of which had antibacterial activity, but not in any of the non-inhibitory strains. Ethyl acetate extracts from both shaken and statically-grown cultures of *Roseovarius* sp. TM1035, *Roseovarius* sp. TM1042 and *S. pomeroyi* DSS-3 induced a response from *A. tumefaciens* NT1 (pZLR4) (Table 2), whereas only the static cultures of *Roseovarius* sp. TM1035 and *Roseovarius* sp. TM1042 had detectable antibacterial activity. Only statically grown *Roseovarius* ISM and *Phaeobacter* 27-4 induced the *A. tumefaciens* NT1 (pZLR4), and none of the extracts induced *C. violacerum* CV026.

Pigment production. The amount of a dark brown pigment by *Phaeobacter* 27-4 is correlated with the production of the antibacterial compound, tropodithietic acid (8). Pigment production was therefore measured from all strains grown under both static and shaken conditions. *Phaeobacter* 27-4 and *Silicibacter* sp. TM1040 were the only two strains of the 14 strains that formed visible pigment, although trace amounts of the pigment were detectable in three additional strains (Table 2). These experiments corroborated our earlier work (8) showing that *Phaeobacter* 27-4 only produced pigment when grown under static conditions, and demonstrated that *Silicibacter* sp. TM1040 also produced a brownish/black pigment when grown under static conditions. Pigment was also measurable in *Silicibacter* sp. TM1040 when grown under shaken conditions. However the amount of pigment was much higher (2-10- fold) in the static culture as compared to the shaken

culture despite a 10-fold lower cell density in the former. As observed previously in cultures of *Phaeobacter* 27-4, the production of pigment by *Silicibacter* sp. TM1040 started at the air liquid interface in the static culture.

Bacterial attachment, biofilm formation, and antibacterial activity in spontaneous nonpigmented mutants of *Phaeobacter* 27-4 and *Silicibacter* sp. TM1040. On rare occasions, colorless colonies appeared spontaneously in both *Silicibacter* sp. TM1040 and *Phaeobacter* 27-4. Although the nature of this change is not known, these nonpigmented mutants are stable, as the strains do not regain pigment production upon repeated attempts and prolonged culturing. One colorless mutant from each strain was isolated and tested for ability to attach to surfaces, form rosettes and biofilm, produce pigment and antibacterial activity. A nonpigmented mutant of *Silicibacter* sp. TM1040 lost its ability to produce pigment and antibacterial compounds both under shaken and static culture conditions. Under static condition, these bacteria produced rosettes and attached similarly to the parental strain. On the other hand, a nonpigmented mutant of *Phaeobacter* 27-4 produced trace amounts of pigment (OD_{398} = 0.1 compared to an OD_{398} = 1.4 produced by its parental strain) and also only produced a trace amount of the antibacterial compound when grown under static culture conditions, whereas cells grown in shaken cultures were devoid of antibacterial activity similar to the parental strain. The morphology of the nonpigmented *Phaeobacter* 27-4 cells grown under static conditions differed from the parent strain, as the nonpigmented mutant strain lost its ability to produce rosettes, did not attached to glass slides and did not form biofilm at the air liquid interface.

Correlation between pigment and antibacterial activity in *Phaeobacter* 27-4 and *Silicibacter* sp. TM1040. *Phaeobacter* strain 27-4 and *Silicibacter* sp. TM1040 were grown under static or

shaken culture conditions in MB to further elucidate the co-occurrence of pigment and antibacterial activity. Under shaken conditions the cell density increased for both strains from 1 x 10^3 to 5 x 10^9 CFU/ml within 24 h whereas the static cultures required 36 h to reach a density of 5 x 10^8 CFU/ml (Fig. 3). Pigment was detectable after 24 h in the shaken culture of *Silicibacter* sp. TM1040 and reached a constant level of OD_{398} = 0.2 after 36 h. In the shaken culture of *Phaeobacter* 27-4, pigment was detectable after 36 h at approx. OD_{398} of 0.01 and remained at this level. The amount of pigment was significantly greater in both strains under static culture conditions, reaching a maximum of ca. OD_{398} = 1.5. Pigment was however observed at different times as it was detected after 32 h in static cultures of *Silicibacter* sp. TM1040 but after 48 h in *Phaeobacter* 27-4 cultures, even thought the strains had a similar growth rate. Antibacterial inhibition zones co-appeared at the same time as the pigment was detected for both strains grown under static conditions, reaching a maximum of ca. 25 mm. Inhibition zones from shaken cultures of *Silicibacter* sp TM1040 appeared after 32 h with a zone of ca. 21 mm, thereafter the zone decreased. Shaken cultures of *Phaeobacter* 27-4 did not inhibit *V. anguillarum*. These results indicate a clear correlation between pigment and antibacterial compound production for both *Silicibacter* sp. TM1040 and *Phaeobacter* 27-4 grown under static conditions.

Susceptibility of *Roseobacter* strains to the antibacterial compound produced by *Silicibacter* sp. TM1040 and *Phaeobacter* 27-4. None of the *Roseobacter* clade strains tested (Table 1) were sensitive to filtered culture supernatants containing the antibacterial activity produced by either *Silicibacter* sp. TM1040 or *Phaeobacter* 27-4. This is in contrast to the non-*Roseobacter* marine species tested, many of which were sensitive to the filtered supernatant. *V. anguillarum* 90-11-287, *Pseudomonas elongate, Spongiobacter* "nickelotolerans", environmental and clinical strains of *Vibrio cholerae, V. coralliilyticus*, *V. shiloi,* and *Halomonas* sp. (all members of the γ-

proteobacteria) were sensitive to the compound(s) (Table 3). A strain of *Shewanella, V. harveyi*, *V. fortis,* and *V. mediterranei* were each resistant to the antibacterial activity, showing no zone of inhibition. This pattern of inhibition changed when ethyl acetate extracts were used instead of filtered supernatants. In this case, both *Roseobacter* clade bacteria and non-roseobacters were impacted when ethyl acetate extracts of statically grown cultures of either *Silicibacter* sp. TM1040 and *Phaeobacter* 27-4 were used. Ethyl acetate extracts also inhibited *Mycobacterium marinum*, an acid-fast bacterium and important fish and human pathogen.

DISCUSSION

Bacteria belonging to the α-Proteobacteria, and especially members of the *Roseobacter* clade of the α-Proteobacteria, are widely distributed in marine waters and dominate the microbiota on phytoplankton (1,9,15,21,30,40,47). The reasons for this dominance are not known, but nine of fourteen roseobacters produced antibacterial compound(s) as has previously been reported for several strains belonging to the *Roseobacter* clade (6,8,38), and antagonistic interactions between the bacteria in algal communities may partly explain the dominance of the roseobacters in this niche.

Cell-free culture supernantants from *Phaeobacter inhibens* T5 inhibited marine flavobacteria and actinobacteria, whereas bacteria belonging to the α- and γ-Proteobacteria were mostly unaffected (6). Similarly, we found that none of the roseobacters showed susceptibility to the filtered culture supernatant from either *Silicibacter* sp. TM1040 or *Phaeobacter* 27-4 whereas most non-roseobacter species were inhibited. However concentrated antibacterial compound produced by *Silicibacter* sp. TM1040 or *Phaeobacter* 27-4 inhibited both roseobacters and non-roseobacters, a finding that is correlated with the observation that both α- and γ-Proteobacteria were

inhibited by the pure antibacterial compound of strain T5 (tropodithietic acid) (6). Hence, many members of the *Roseobacter* clade produce active inhibitory compound(s) that are more active against non-roseobacter species than roseobacters themselves.

The antibacterial activity of *Phaeobacter* 27-4 and the closely related *P. inhibens* T5 is due to production of tropodithietic acid, which itself is correlated to the synthesis a brown pigment (6,8). *Silicibacter* sp. TM1040 also produced an antibacterial compound with is correlated to the occurrence of a similar brown pigment. Culture conditions affected the production of both pigment and antibacterial activity in *Phaeobacter* 27-4 and *Silicibacter* sp TM 1040 and both had the highest production under static conditions (Fig 3). Based on these findings, we are currently working on the hypothesis that the antibacterial compound produced by *Silicibacter* sp. TM1040 is tropodithietic acid. Tropodithietic acid contains two sulfur atoms, which is interesting since the *Roseobacter* clade has been linked to the sulfur cycling in the sea specifically by ability of these bacteria to metabolize algal- and dinoflagellate-produced dimethylsulfoniopropionate (DMSP; (29)). *Silicibacter* sp. TM1040 utilizes DMSP through the demethylation pathway (27) and sulfur from DMSP may be used in the synthesis of tropodithietic acid.

The production of antibacterial activity by *Phaeobacter* 27-4 co-occurred with the formation of rosettes, as well as with bacterial attachment and biofilm formation (7,8). We found in the present study, that a spontaneously occurring mutant of *Phaeobacter* 27-4 lacking the dark extracellular pigment simultaneously lost the ability to produce the antibacterial compound and failed to form biofilms suggesting that there is a link between these phenotypes. For members of the *Roseobacter* clade, attached bacteria are 13 times more likely to produce antimicrobial compounds as compared to their free-living counterparts, whereas the frequency of antibacterial strains is similar in particle-attached and free-living bacteria for most non-roseobacter marine species (24). Five of the roseobacters analyzed in the present study displayed proficient attachment capability

when grown under static culture conditions, and four of these showed antibacterial activity at the same time. In contrast, a nonpigmented, antibacterial-activity negative mutant of *Silicibacter* sp. TM1040 retained its biofilm forming ability demonstrating that production of the antibacterial compound is not necessarily linked to biofilm formation in all roseobacters. The molecular mechanisms underlying these differences are under active investigation in our laboratories.

Bacteria of the *Roseobacter* clade are some of the most rapid colonizer of surfaces in the coastal environments (11), and we hypothesize that rosette formation may be an attachment phenotype. Although several strains were capable of growing in a rosette formation, five strains had a more pronounced rosette formation under static growth conditions and this coincided with these five strains being the most efficient in attaching to surfaces and producing a biofilm at the air liquid interface.

The ability to produce an antibacterial compound that is more active against non-roseobacters during the formation of a biofilm may provide the *Roseobacter* clade bacteria a strong adaptive and selective advantage in establishing interactions with their algal and dinoflagellate hosts. Production of antibacterial compound has indeed been demonstrated to have a role in the colonization of *Ulva australis* by *Pseudalteromonas tunicate* (34). Furthermore, antagonistic interaction are common in particle-associated marine bacteria (24,25) and studies have shown that antagonistic attached marine bacteria directly inhibited *V. cholerae* colonization of particles (25). A strain of *Phaeobacter gallaeciensis* was an aggressive colonizer of the alga *Ulva australis,* since it was able to invade and disperse pre-established biofilms of other marine bacteria (34) and the strain was able to prevent the growth of others nonroseobacter bacteria on surfaces, while growing in a biofilm (33). On the other hand, production of antibacterial compounds may not always result in a selective advantage during the colonization of particles, since others have demonstrated that an antibiotic-producing strain had no inhibitory effect on the attachment of two marine bacteria when

compared to mutant unable to produce antibiotic (17). Grossart et al. (17) also found that bacterial growth rate was the most important parameter controlling the long term bacterial population density on agar particles. Taken as a whole, these results suggest that the outcome of interactions, whether antagonistic or mutualistic, between particle-associated bacteria is complex and multifaceted.

V. coralliilyticus and *V. shiloi* are important coral pathogens causing coral bleaching (4,36) and both were inhibited by *Silicibacter* sp. TM1040 and *Phaeobacter* 27-4. The coral polyp is protected by a mucus layer that is populated by α-Proteobacteria group bacteria (5), and bacteria taxonomically related to *Silicibacter* sp. TM1040 are associated with corals (9). One may therefore hypothesize that roseobacters may play a role in preventing coral bleaching, and indeed antibacterial activity has been detected in coral extracts which inhibit members of the Vibrionaceae (19) supporting this hypothesis.

The production of antibacterial compounds is in some bacterial species controlled by AHL in a QS depending manner (3,48). AHL compounds can be isolated from several members of the *Roseobacter* clade (8,16,45), and it has been suggested that AHLs control the production of antibacterial compounds in *Phaeobacter* 27-4 (8). In the present study, five strains induced *A. tumefaciens* NT1 (pZLR4) whereas none induced *C. violacerum* CV026 which could indicate that primarily long chained AHLs were produced in agreement with a recent study (45). AHLs were only detected in strains that produced antibacterial activity; however, there is not a direct correlation between these phenotypes as antibacterial activity was also detected in strains that did not induce the AHL monitor strains. *Silicibacter* sp. TM1040 only produced antibacterial compound at cell densities where QS is expected to occur, however AHL molecules were not detected from this bacterium, and no LuxI homologs are found in the genome of this bacterium (R. Belas, personal observation). The bacteria in which we did not find AHLs may, in principle, produce other QS

molecules that may be detected by other methods, but based on the current data, we find no evidence linking the production of antibacterial compounds to QS in the *Roseobacter* clade.

In conclusion, phenotypes such as production of antibacterial compounds, rosette formation, attachment ability and biofilm formation were detected in several *Roseobacter* strains. Culture conditions influenced both antibacterial activity and bacterial attachment and biofilm formation, as these phenotypes were almost only expressed under static culture conditions. The degree to which the organisms grew as rosettes was linked to attachment and biofilm formation. Collectively, these phenotypes may facilitate the colonization of dinoflagellates, algae and marine particles by *Roseobacter* clade bacteria.

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TABLE 1. Rosette formation, attachment ability and motility of bacteria belonging to the *Roseobacter* clade used in this study.

Bacterial strain	Isolated from	Ability to form rosettes	Attachment ability	Motility	Reference
Sulfitobacter EE36	Coastal seawater	Rosette[a)]	+	+	
Roseovarius ISM	Coastal seawater	Single cells	-	-	(14)
Sulfitobacter 1921	*P. piscicida*	Rosette[a)]	+	+	
Silicibacter pomeroyi DSS-3	Coastal seawater	Rosette	-	+	(14)
Sulfitobacter SE62	Coastal seawater	Rosette	-	+	
Roseobacter litoralis 49566	Seawater	Single cells	-	-	(41)
Roseobacter algicola 51442	*Prorocentrum lima*	Rosette	-	+	(23)
Roseobacter denitrificans 33942	Seawater	Single cells	-	+	(41)
Silicibacter sp. TM1040	*P. piscicida*	Rosette[a)]	+	+	(27)
Roseobacter sp. TM1039	*P. piscicida*	Rosette[a)]	+	+	(27)
Roseovarius sp. TM1042	*P. piscicida*	Single cells	-	-	(27)
Roseovarius sp. TM1035	*P. piscicida*	Single cells	-	-	(27)
Roseobacter sp. TM1038	*P. piscicida*	Single cells	-	-	(27)
Phaeobacter 27-4	Turbot rearing facility	Rosette[a)]	+	+	(20)

[a)] Rosette formation pronounced in static grown cultures

TABLE 2. Detection of antibacterial compound, pigment and acylated homoserine lactones from *Roseobacter* clade strains depending on the growth conditions. Antibacterial activity assessed against *Vibrio anguillarum*.

Bacterial strain	Growth conditions	mm inhibition zone against *V. anguillarum*		Pigment production OD398	AHL induction in *A. tumefaciens* mm zone	Log CFU
		Sterile filtered culture	Ethylacetate extract			
Sulfitobacter EE36	Shaken	ni	ni	0.00	ni	8.78
	Static	ni	13	0.02	ni	8.70
Roseovarius ISM	Shaken	ni	ni	0.00	ni	9.70
	Static	ni	16	0.00	20	9.00
Sulfitobacter 1921	Shaken	ni	ni	0.00	ni	9.95
	Static	ni	ni	0.00	ni	8.60
Silicibacter pomeroyi DSS-3	Shaken	ni	15	0.00	18	7.69
	Static	ni	ni	0.00	25	7.00
Sulfitobacter SE62	Shaken	ni	ni	0.00	ni	9.60
	Static	ni	ni	0.00	ni	8.48
Roseobacter litoralis ATCC 49566	Shaken	ni	ni	0.00	ni	7.30
	Static	ni	ni	0.00	ni	8.78
Roseobacter algicola ATCC 51442	Shaken	ni	ni	0.00	ni	9.00
	Static	ni	ni	0.01	ni	8.30
Roseobacter denitrificans ATCC 33942	Shaken	ni	ni	0.00	ni	8.48
	Static	ni	ni	0.02	ni	9.00
Silicibacter sp. TM1040	Shaken	16	22	0.22	ni	9.30
	Static	27	35	1.81	ni	8.48
Roseobacter sp. TM1039	Shaken	ni	ni	0.00	ni	9.70
	Static	ni	13	0.02	ni	8.90
Roseovarius sp. TM1042	Shaken	ni	ni	0.00	36	9.30
	Static	ni	13	0.00	38	8.60
Roseovarius sp. TM1035	Shaken	ni	ni	0.00	37	9.48
	Static	ni	13	0.00	36	9.00
Roseobacter sp. TM1038	Shaken	ni	ni	0.00	ni	9.70
	Static	ni	16	0.00	ni	8.90
Phaeobacter 27-4	Shaken	ni	ni	0.01	trace	9.90
	Static	27	35	1.46	42	8.48

ni = no inhibition or no induction

Table 3: Inhibition of marine bacteria on marine agar by raw sterile filtered supernatants of *Silicibacter* sp. TM1040 and *Phaeobacter* 27-4

Species	Code	Role	Size of inhibition zone (mm)		Source
			TM1040	27-4	
Vibrio anguillarum	90-11-287	Fish pathogen	17	17	
Pseudomonas elongate		Marine bacterium	11	12	1
Spongiobacter nikelotolerans		Marine bacterium	32	24	1
Vibrio cholerae	C25	Clinical strain	23	21	2
Vibrio cholerae	C42	Clinical strain	36	28	2
Vibrio cholerae	1	Environ. strain	17	19	2
Vibrio cholerae	184	Environ. strain	21	20	2
Vibrio cholerae	4	Environ. strain	27	20	2
Vibrio cholerae	5	Environ. strain	30	23	2
Vibrio coralliilyticus		Coral pathogen	14	14	
Vibrio shiloi		Coral pathogen	14	14	
Halomonas spp.		Marine bacterium	17	18	1
Shewanella spp.		Marine bacterium	ni	ni	1
Vibrio harveyi		Marine bacterium	ni	ni	1
Vibrio fortis		Marine bacterium	ni	ni	1
Vibrio mediterranei		Marine bacterium	ni	ni	1

ni = no inhibition

Souce: The laboratories of Russell T. Hill 1) and Rita R. Colwell 2) Center of Marine Biotechnology, University of Maryland Biotechnology Institute.

Legends to figures

Figure 1: Attachment of *Roseobacter* clade bacteria from static or shaken cultures to glass cover slides measured by crystal violet at OD_{590}. Dotted bars: from static grown cultures. Filled bars: from shaken cultures. The bars represent averages of duplicate determinations and error bars represent standard deviation. Glass cover slides dipped into sterile media served as a control for both static and shaking cultures.

Figure 2: Cell morphology of five *Roseobacter* clade strains grown under static or shaken conditions. Phase contrast light microscopy (400x magnification) was used to observe morphology and biofilm formation at the air-liquid interphase. *Phaeobacter* 27-4, *Silicibacter* sp. TM1040 *Sulfitobacter* 1921 with high attachment capacity from static cultures (Fig. 1), *Sulfitobacter* SE62 forming few rosettes without having high attachment and *Roseovarius* ISM without rosette formation with low attachment capacity from static cultures (Fig.1).

Figure 3. Influence of culture conditions on the growth, production of pigment, and antibacterial activity of *Phaeobacter* 27-4 and *Silicibacter* sp. TM1040 in MB at 25°C. The data represent the mean of duplicate cultures (error bars represent standard deviations). A) Growth measured by plate counts: □ shaken culture of *Phaeobacter* 27-4, ○ static culture of *Phaeobacter* 27-4, ▽ shaken culture of *Silicibacter* sp. TM1040, △ static culture of *Silicibacter* sp. TM1040. B) Pigment formation, as measured by absorbance at OD_{398}. C) Antibacterial activity represented by the zone of inhibition (in mm), as determined using the well diffusion assay with *V. anguillarum.*

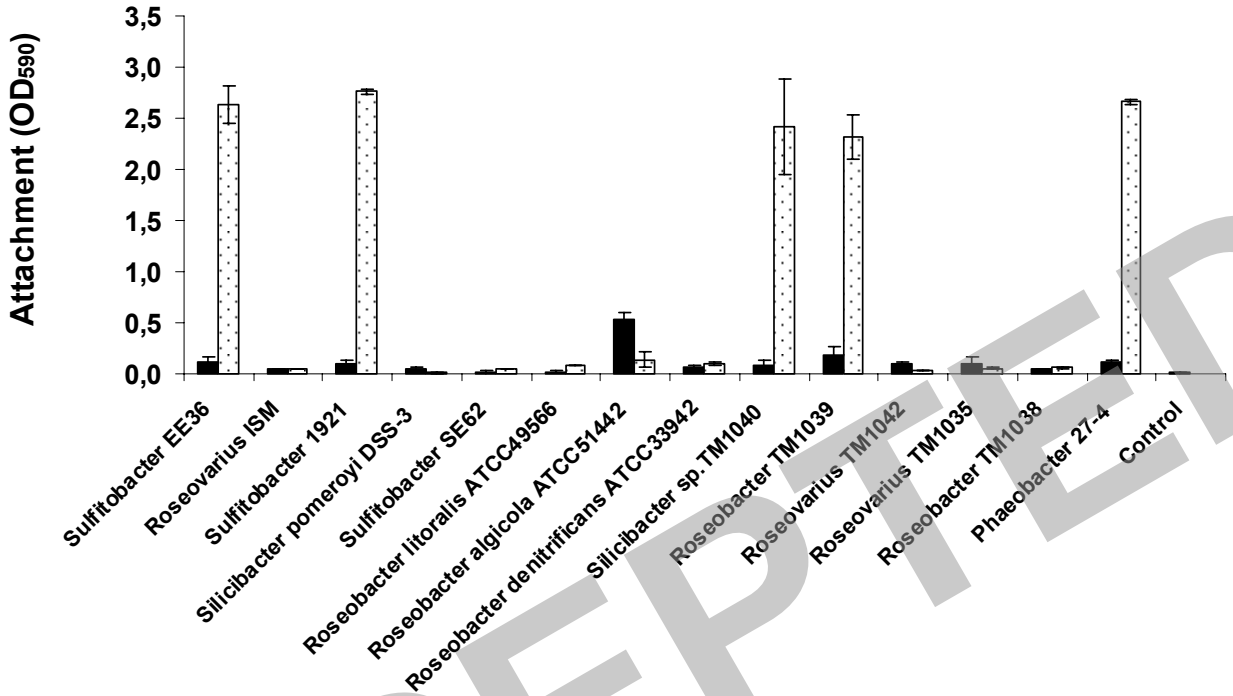


Figure 1

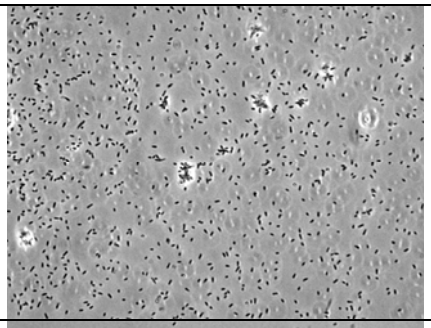
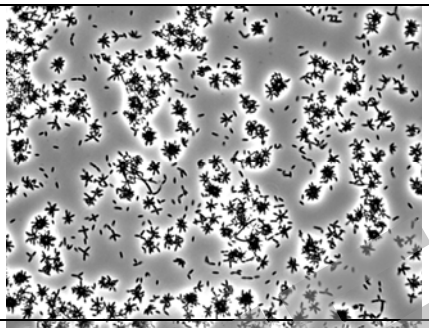
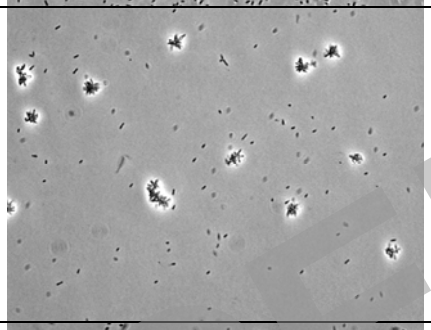
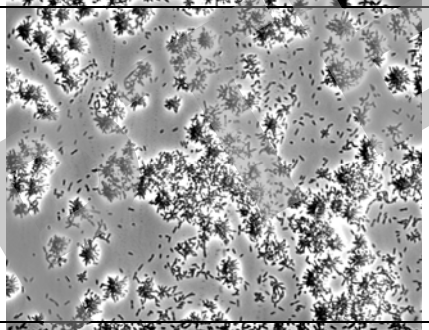
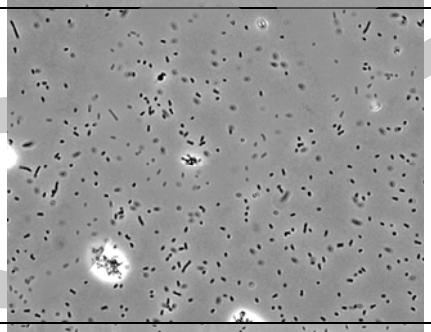
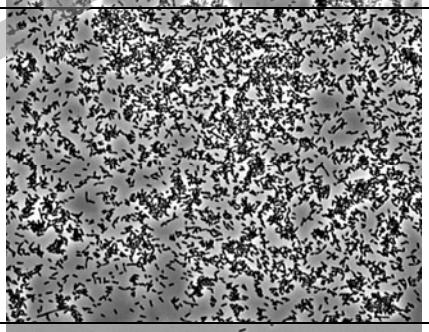
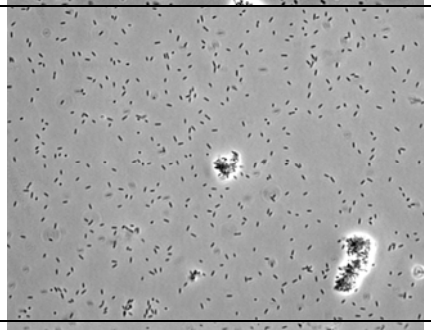
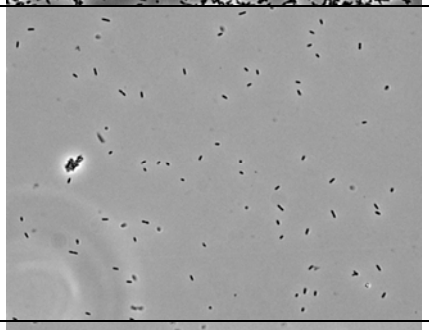
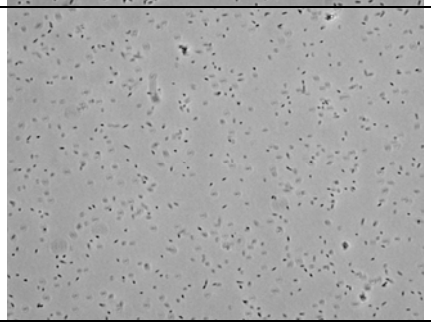
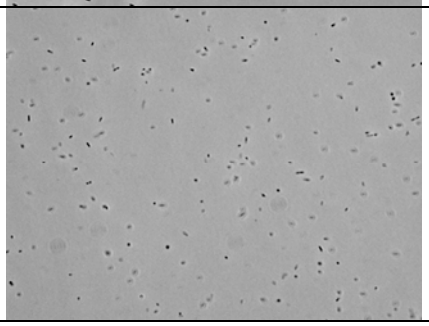
	Shaken	Static
Phaeobacter 27-4		
Silicibacter sp. TM1040		
Sulfitobacter 1921		
Sulfitobacter SE62		
Roseovarius ISM		

Figure 2

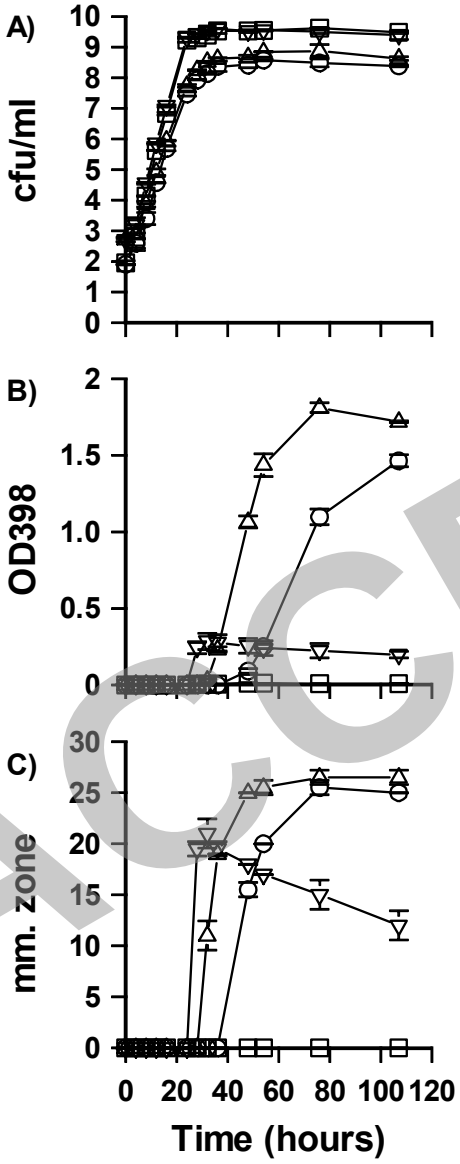


Figure 3

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