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A member of the *Rhodobacteraceae* promotes initial biofilm formation via the secretion of extracellular factor(s)

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ABSTRACT: The *Rhodobacteraceae* are a relatively dominant bacterial family found in marine biofilms that can rapidly colonize natural and engineered surfaces. However, the role of this group in the development of biofilms is yet to be resolved. This study investigated the contribution of a representative isolate of the *Rhodobacteraceae* in the formation of initial multi-isolate biofilms. Six different bacterial strains, including Rhodo35, the member of the *Rhodobacteraceae*, were isolated from a marine biofilm. These isolates were used as a laboratory model system for studying a biofilm-forming marine community. A significantly higher initial biofilm biomass was observed in the presence of Rhodo35, as revealed by crystal violet staining assay, confocal laser scanning microscopy and quantitative real-time PCR. Moreover, we show that the promotion of the biofilm initiation was mediated by extracellular factor(s) secreted by Rhodo35, which increased the biomass of the biofilms by approx. 50%. In addition, Rhodo35 also increased biofilm formation by a natural marine community by 26%, as measured using crystal violet staining. These findings suggest that *Rhodobacteraceae* may be an important group in marine biofilms, facilitating initial colonization and biofilm formation of other bacterial species through indirect contact.

KEY WORDS: Marine biofilms · Interspecies interactions · *Rhodobacteraceae*

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INTRODUCTION

In marine environments, biofilms are a common mode of life, developing on surfaces such as sediments (Kriwy & Uthicke 2011), ship hulls (Gram et al. 2002), marine macroorganisms (Behrendt et al. 2012), pipes and membranes of seawater desalination facilities (Saeed et al. 2000) and marine snow (Gram et al. 2002). Microbial biofilms are complex multi-cellular communities (O'Toole et al. 2000, Stoodley et al. 2002), developing from specific initial colonizing bacterial groups to mature multi-species communities (Jackson et al. 2001, Rickard et al. 2003, Lyautey et al. 2005). Living in complex multi-species biofilm communities gives bacteria an advantage relative to planktonic life. For example, multi-species biofilms show increased horizontal gene transfer (Molin & Tolker-Nielsen 2003), degradation of organic matter (Peter et al. 2011) and resistance to antibiotics (Elvers et al. 2002, Harriott & Noverr 2010). During biofilm initiation, early surface colonizers may have an impact on biofilm formation and further development of the mature biofilm community by interacting with other bacterial species (Buswell et al. 1997, Leung et al. 1998, Rickard et al. 2003, Yamada et al. 2005, Kuboniwa et al. 2006). However, the role of marine primary colonizers in early developing biofilms, and the mechanisms involved, are not completely established.

Members of the *Rhodobacteraceae* family have been identified in several studies as a dominant bacterial group in marine biofilms which are able to rap-

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idly colonize abiotic (Dang & Lovell 2000, 2002, Dang et al. 2008) as well as biotic (Sharp et al. 2012) surfaces in different parts of the world. More than 40% of the bacteria in 3 and 6 d old biofilms formed by an Atlantic Ocean coastal marine community were affiliated with members of the genus *Rhodobacter*, within the *Rhodobacteraceae* family (Dang & Lovell 2002). Similar results have been obtained from biofilm formed by a Mediterranean Sea coastal marine community in a desalination plant (Elifantz et al. 2013). In this case, *Alphaproteobacteria* (dominated by the *Rhodobacteraceae* family) constituted up to 80% of the initial biofilm biomass (Elifantz et al. 2013).

Because *Rhodobacteraceae* are a common dominant bacterial group in some initial marine biofilms world-wide, we hypothesized that they might contribute to mixed-species biofilm development, interacting with other colonizing bacterial species. Competition for various resources is a common type of negative interaction within biofilms (Zhang et al. 1995, Rao et al. 2005, Tong et al. 2008, Rendueles & Ghigo 2012). Interactions of a cooperative nature also exist within biofilms, such as in the case of biodegradation of complex organic compounds, in which a synergistic cooperation of a consortium of microorganisms is often involved (Cowan et al. 2000, Christensen et al. 2002). Communication by signals, e.g. quorum sensing (QS), can mediate cooperation between different bacterial species within a multispecies biofilm. For example, it has been shown that *Staphylococcus oralis*, when in a mixed-species biofilm with *Actinomyces naeslundii*, produces the autoinducer-2 (AI-2) that promotes mutualistic biofilm formation of both species (Rickard et al. 2006).

In spite of their importance, interspecies interactions during initial biofilm development have not been widely studied, especially in the marine environment. The objective of the present study was to investigate whether members of *Rhodobacteraceae* interact directly or indirectly with other biofilm species during early surface colonization. As mentioned above, several studies have examined interactions between 2 species (Cowan et al. 2000, Rickard et al. 2006), but, as natural biofilms are much more complex in their community structure and interactions, studies of more complex models are required. This work examined the interaction of a member of the *Rhodobacteraceae* family with other community members, both within a model marine community composed of 6 marine biofilm forming isolates, and within the natural complex marine community, in order to find the potential role of *Rhodobacteraceae* in marine biofilm initiation.

MATERIALS AND METHODS

Isolation, identification and growth conditions of biofilm bacteria from Mediterranean Sea water

Isolates were obtained from a flow unit containing a Robbins device (Flemming 1997) that was set in the Palmachim desalination plant, on the coast of Israel (Elifantz et al. 2013). After 4 d of sea water flow, a glass coupon implanted within the device was transferred to the laboratory. Biofilm bacteria were isolated from the coupon by plating on 1/100 strength marine agar medium (0.01 g l^{-1} yeast extract, 0.05 g l^{-1} peptone, 30 g l^{-1} sea salt, 15 g l^{-1} agar) and incubated at 30°C for 48 h. Based on their colony dominance, 16S rRNA sequence and their phylogenetic affiliation to major groups relevant to typical young biofilms, 6 different isolates were chosen for this study: Vib3, Spong12, Pseudoalt17, Halo18, Eryth23 and Rhodo35 (Table 1). Each of the isolates belongs to a different genus.

In order to identify the isolates, DNA was extracted from overnight cultures according to Inbar et al. (2005). Universal bacterial primers (11 forward: GTT TGA TCM TGG CTC AG [Lane 1991] and 1392

Table 1. 16S rRNA based identification of the isolates used to compose the model biofilm community. Identification of phylogenetic affiliation of the isolates was conducted by PCR amplification of 16S rRNA gene fragment of each isolate, sequencing the amplicons and BLAST analysis of the sequences

Closest relative			Isolate	Accession
Max. identity (%)	Class	Species (accession number)	ID	number
99	Gammaproteobacteria	*Vibrio alginolyticus* (JX976307.1)	Vib3	KC691178
99	Gammaproteobacteria	*Spongiibacter marinus* (NR042454.1)	Spong12	KC691179
99	Gammaproteobacteria	*Pseudoalteromonas mariniglutinosa* (AB622472.1)	Pseudoalt17	KC691180
99	Gammaproteobacteria	*Halomonas halodurans* (AB680913.1)	Halo18	KC691181
99	Alphaproteobacteria	*Erythrobacter flavus* (AF500005.1)	Eryth23	KC691182
98	Alphaproteobacteria	*Phaeobacter gallaeciensis* (DQ915619.1)	Rhodo35	KC691183

reverse: ACG GGC GGT GTG TAC [Xu et al. 1998]) were used for PCR amplification of 16S rRNA genes of the isolates. Extracted DNA (1 µl) from each isolate was added to 24 µl of PCR master mix containing 0.125 mg ml^{-1} of BSA (Roche), 0.2 mM of each dNTP (Fermentas), 0.2 µM of each primers (Integrated DNA), 0.025 units $µl^{-1}$ of Taq Polymerase (Fermentas), 2.5 mM of $MgCl_2$ (Fermentas), 2.5 µl of buffer (Fermentas), and ultra-pure water (Fisher). PCR was conducted using the following conditions: initial denaturing for 5 min at 96°C and then 30 cycles of denaturing (95°C; 30 s), annealing (58°C; 30 s) and elongation (70°C; 50 s). A 5 min final extension at 72°C was performed at the end of the PCR program. Fragments of 16S rRNA obtained by PCR of genomic DNA from the isolates were sequenced with the 907 reverse universal primer (CCG TCA ATT CMT TTG AGT TT; Muyzer et al. 1993) using the Sanger method (Macrogen). All sequences were analyzed by BLAST (Altschul et al. 1997) and submitted to NCBI GenBank (for accession numbers, see Table 1).

For preparation of inocula, bacteria were grown in 1/2 strength marine medium (0.5 g l^{-1} yeast extract, 2.5 g l^{-1} peptone, 30 g l^{-1} sea salt) at 30°C (maximum summer temperature in the Levantine basin) with shaking (180 rpm) for 24 h to reach stationary phase. For biofilm experiments, the cultures were incubated in static conditions at 30°C in the dark for 24 h in 1/2 strength marine medium, reaching the stage of biofilm initiation.

Effect of each bacterial isolate on biofilm formation by a mixed culture

The biofilm formed by a 5-isolate culture (excluding each isolate in turn) was measured and compared to a biofilm containing all 6 isolates, in order to determine the impact each isolate has on mixed-isolates biofilm formation. Biofilm biomass, obtained after 24 h, was measured using 3 different methods: crystal violet (CV) staining assay, quantitative real-time PCR (RT-PCR) and confocal laser scanning microscopy (CLSM). The latter 2 methods analyzed the exclusion of only Vib3 and Rhodo35.

In order to form the 6-isolate mixed culture, 6 single-isolate cultures, each grown overnight and at a concentration of approx. 10^7 cells ml^{-1} (optical density [OD] = 0.05), were combined (Table S1 in the Supplement at www.int-res.com/articles/suppl/a075p155_supp.pdf). When using 5-isolate cultures, we compensated for a missing isolate by increasing the relative concentration of each of the 5 isolates so that the total cell concentration was equal to the concentration of a 6-isolate culture at the start of the experiment.

In order to determine whether the presence of a specific isolate also affects the planktonic growth, 6- and 5-isolate mixed cultures, prepared as described above, were incubated at 30°C for 16 h in a Sinergy2 plate reader (BioTek), which measured the absorbance of the cultures at a wavelength of 600 nm every 30 min following 15 s of shaking.

Assessment of the effect of different species on biofilm development using CV assay

For initial biofilm development analysis using CV staining, 200 µl of mixed-isolate cultures were incubated, in 6 replicates, in 96-well microtitre plates for 24 h at 30°C. Biofilm biomass quantification by CV staining was carried out according to Pitts et al. (2003), with some modifications. After 24 h of incubation, the wells were drained and rinsed twice with sterile saline solution (0.9% NaCl). The biofilm forming on well surfaces was then stained with 300 µl of 0.4% (W/V) CV solution (Sigma) for 20 min. The dye of the stained biofilm was removed by gently rinsing with tap water. The stained biofilm was eluted with 300 µl of 95% (v/v) ethanol and the absorbance was measured in a Sinergy2 plate reader (BioTek) at 590 nm.

Assessment of the effect of different species on biofilm development using CLSM analysis

Biofilm analysis using CLSM combined with fluorescent staining (Xu & Liu 2011, Yang et al. 2011) was carried out in order to further examine the effects of isolates Rhodo35 and Vib3 on the structure and the early development of mixed-isolate biofilm. Vib3, an isolate belonging to the genus *Vibrio*, was chosen for further investigation in addition to Rhodo35, a member of the *Rhodobacteraceae* family, based on the results obtained by the CV staining experiments. Five hundred microliters of cultures containing all 6 isolates ($Mix_{(6)}$) and lacking Rhodo35 or Vib3 ($Mix_{(6\text{-}Rhodo35)}$ and $Mix_{(6\text{-}Vib3)}$, respectively) were each placed in triplicate into wells of a 24-well plastic plate, with a round glass coupon (r = 0.5 cm) inserted into each well, and incubated for 24 h at 30°C. Following incubation, the coupons were fixed in 4% paraformaldehyde for 4 h. The coupons were then incubated in phosphate buffer saline (PBS; 0.1 M pH = 7.4; 10.9 g l^{-1} of Na_2HPO_4, 3.2 g l^{-1} of NaH_2PO_4

and 9 g l^{-1} of NaCl) for 20 min and washed 3 times in 1 ml of 0.1 M Tris-HCl (pH = 7.2). Each biofilm was stained with 20 µl of Syto9 (5 µM; cell staining) for 25 min, then washed 3 times with 1 ml of 0.1 M Tris-HCl, followed by staining with 20 µl of ConA (0.2 mg ml^{-1}; extracellular polysaccharide [EPS] staining) for 35 min and additional washing with 0.1 M Tris-HCl. After staining, the coupons were dried at room temperature in the dark and glued to a microscope slide. Slides were covered with Citifluor antifadent mountant solution (Agar Scientific) and examined under an IX81 Olympus FluoView 500 confocal microscope (Olympus). The laser/filter used for Rhodamine and Syto9 were diode/BA560if and argon-ion/BA505-525, respectively (Olympus). Biofilm parameters (thickness, biovolume, coverage, and roughness) were calculated based on the CLSM images using PHLIP-ML software (PHOBIA project; http://phlip.sourceforge.net), which analyses CLSM multichannel data as described elsewhere (Mueller et al. 2006).

Assessment of the effect of different species on biofilm development using quantitative RT-PCR analysis

RT-PCR reactions were performed in order to determine the relative abundance of each of the isolates within the mixed-isolate biofilms (Suzuki et al. 2005). Based on the data obtained from the CV staining assay, 3 combinations of mixed-isolates culture were chosen for further investigation using RT-PCR: $Mix_{(6)}$, $Mix_{(6\text{-}Vib3)}$ and $Mix_{(6\text{-}Rhodo35)}$ (Table S1 in the Supplement). After the preparation of inocula, 200 µl of each culture in 3 replicates were incubated for 24 h at 30°C in 1.5 ml polypropylene plastic tubes (Sarstedt) with four 2 mm glass beads (Marienfeld), for increased surface area. Following incubation, the contents of the tubes, including the glass beads, were rinsed twice with 0.9% saline. DNA was extracted from biofilms formed on surfaces of both the tube and the beads following Inbar et al. (2005).

The primers used for performing RT-PCR reactions were designed based on the 16S rRNA gene sequence of each isolate, using the 'probe design' algorithm in the ARB software package (Ludwig et al. 2004) (Table S2 in the Supplement). Standard curves were prepared with Topo TA plasmids (Invitrogen) containing inserts of the 16S rRNA gene of each isolate in concentrations of 10^1 to 10^9 targets μl^{-1}, diluted according to the quantification of extracted plasmids using a ND1000 spectrophotometer (NanoDrop). The slopes of the standard curves, correlation coefficients and amplification efficiencies of each primer pair were calculated using the MxPro™ QPCR Software analysis tool (Stratagene), based on the standard curves of the primers (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/a075p155_supp.pdf). The specificity of each primer was determined by performing a PCR reaction with isolate-specific primers on plasmids containing each of the other 5 isolates as negative (non-target) control. Additionally, the specificity of the primers was verified using BLAST analysis, which indicated that the primers are specific at least to the family level, and could be used in other studies of taxonomically diverse bacterial classes (data not shown).

RT-PCR assays were conducted in polypropylene 96-well plates in an Mx3000P QPCR System (Stratagene). Each 25 µl contained 1 µl of DNA, 1 µl of each primer (10 µM each), 9.5 µl water and 12.5 µl Absolute Blue SYBR Green ROX mix (Thermo Fisher). PCR conditions were: 15 min at 95°C, followed by 30 cycles (95°C for 30 s, 30 s at the annealing temperature, 30 s at 70°C) and an additional 10 s at 80°C. Annealing temperatures (Table S2 in the Supplement) were optimized for each primer pair in order to maximize the amplification efficiency.

The abundance of each isolate was calculated by dividing the number of targets measured in RT-PCR reaction by the surface area of the solid-liquid interface (calculated from the surface area of the glass beads and the walls of the tube that were in contact with the liquid culture). The calculation of the abundance considered the 16S rRNA gene copy number of each isolate (relative to each other), which was determined by dividing the number of targets of each isolate obtained from RT-PCR analysis using universal bacterial primers (Lane 1991) by the colony forming units (CFU) count of the same sample. Notably, no cell aggregates were detected in the planktonic suspension of the isolates, which enabled us to rely on the CFU counts (data not shown).

Effect of Rhodo35 relative abundances on its ability to promote biofilm formation

An additional experiment examined the effect of different concentrations of Rhodo35 on biofilm formation of the mixed-isolate culture. For this purpose, diluted (OD_{600nm} = 0.05) cultures of the isolates were mixed together in different ratios containing 0, 5, 10, 16.6, 20, 25, 30 and 40% (final concentrations) of Rhodo35. Biofilm formation was measured as described above.

Effect of Rhodo35 on biofilm formation by natural sea water bacterial community

The effect of Rhodo35 on 5-isolate biofilm initiation was studied by including or excluding Rhodo35 from the model mixed culture. However, the exclusion of *Rhodobacteraceae* from the complex natural marine community is impossible. Therefore, in order to study the effect of Rhodo35 within the diverse and complex marine community, Rhodo35 was added to a community of natural sea water bacteria.

Mediterranean Sea water (1 l) was filtered through a 0.2 µm filter using a vacuum pump (Pall). The bacteria on the filter were rinsed and washed in PBS and incubated overnight in 100 ml of 1/2 marine medium at 30°C while shaking (180 rpm). After the incubation, the sea water culture was diluted to OD of 0.05 and mixed with each of the isolates Vib3, Halo18, Eryth23 and Rhodo35 separately, adjusted to OD = 0.05, to obtain cultures with 0, 10, 20 and 40% (v/v) of each isolate. Two-hundred microliters of each culture, in 6 replicates, were tested for biofilm formation in 96-well plate using the CV staining assay described above.

Effect of Rhodo35 extracellular material on biofilm formation by a mixed culture

In order to determine whether Rhodo35 interacts indirectly with other bacteria, affecting their biofilm formation, Rhodo35 was exposed, through semi-permeable membrane, to a mixed culture lacking it. Two hundred microliters of Rhodo35, $Mix_{(6)}$ or $Mix_{(6\text{-}Rhodo35)}$ cultures, in 4 replicates, were placed in cell culture insert chambers with membranes of 0.45 µm pores (pre-tested by plating well content to verify that the membrane blocks Rhodo35 transfer) (BD Bioscience; catalogue number 353095), as described in Fig. S2. These inserts were placed in wells of 24-well plates with 800 µl $Mix_{(6)}$ or $Mix_{(6\text{-}Rhodo35)}$ cultures. The biofilm formed in the wells was measured using CV staining assay, as described above.

Statistical analysis

The results of the experiments on the effect of Rhodo35 or its extracellular material on biofilm formation by the model community were analyzed using an an ANOVA test ($\alpha = 0.05$) performed in SigmaPlot v. 10 (Systat). A Student-Newman-Keuls pairwise comparison was used for the post hoc test. The results of the experiment on the natural complex marine community were analyzed using ANOVA with a post hoc test of Bonferroni comparisons versus controls ($\alpha = 0.05$).

RESULTS

Isolation and identification of Mediterranean Sea biofilm bacteria

Six distinct bacterial isolates were obtained from marine biofilm to constitute model cultures for a biofilm-forming marine community. BLAST analysis of 16S rRNA sequences revealed that the isolates Vib3, Spong12, Pseudoalt17, Halo18, Eryth23 and Rhodo35 were related to the genera *Vibrio*, *Spongiibacter*, *Pseudoalteromonas*, *Halomonas*, *Erythrobacter* and the family *Rhodobacteraceae*, respectively (Table 1).

Effect of the isolates on biofilm formation, analyzed using the CV staining assay

The effect of each isolate on the early development of a mixed-isolate biofilm was tested by comparing 6-isolate biofilm to 5-isolate biofilms by excluding one of the isolates each time, using CV staining assays. In the absence of Rhodo35, a significant decrease of approx. 45% in CV absorbance (as a proxy for biofilm biomass) ($p < 0.05$, $n = 6$, F-test $p < 0.05$) was observed relative to the control containing Rhodo35 (Fig. 1). Notably, Rhodo35 exhibits only a moderate potential for biofilm formation when incubated in a single-isolate culture (biofilm biomass value of 1.3), relative to biofilm formed by a 6-isolate biofilm (biofilm biomass value of 1.8) and to that formed by Eryth23 (biofilm biomass value of 2.5) (Fig. S3 in the Supplement). In contrast, exclusion of isolate Vib3 led to a significant increase of approx. 50% in biofilm formation. Exclusion of any of the other 4 isolates, including Eryth23, from the community had only a relatively small negative effect on biofilm formation. Interestingly, exclusion of Rhodo35 from the mixed culture had no significant effect on planktonic growth of the total mixed culture (Fig. S4 in the Supplement). Based on these results, Vib3 and Rhodo35 were chosen for further investigation.

Effect of the isolates on biofilm formation analyzed using CLSM

CLSM analysis of the biofilms revealed that a mixed culture with all 6 isolates ($Mix_{(6)}$) formed a rel-

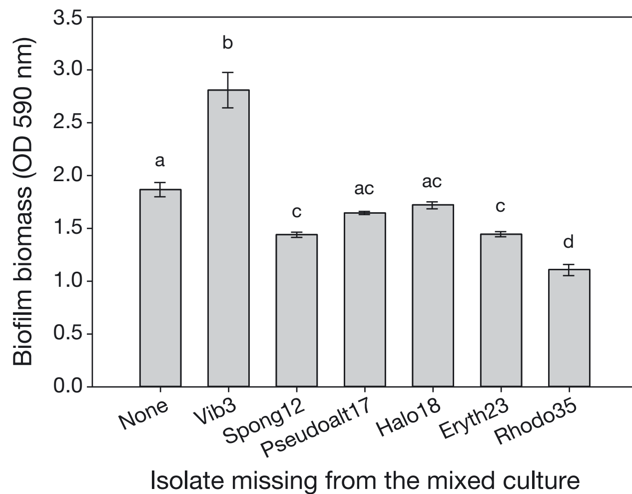

Fig. 1. Effect of absence of specific isolates on biofilm formation by mixed species cultures. Bars represent the biofilm formed by 6- and 5-isolate cultures (see Table S1 in the Supplement for species composition for each culture), missing 1 isolate each. Biofilm formation was measured using the crystal violet (CV) staining assay and the values are the optical density (OD) (wavelength 590 nm) of the CV stain as a proxy for biofilms biomass. Error bars represent SD of 6 replicates and different letters represent significant differences between the means according to ANOVA and Student-Newman-Keuls post hoc tests ($p < 0.05$)

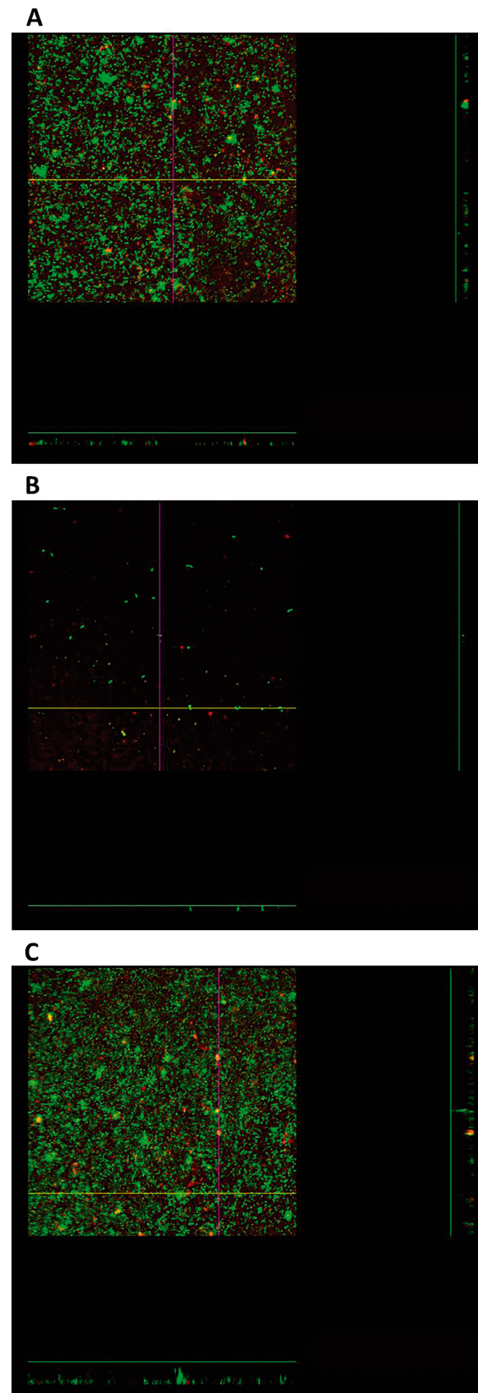

Fig. 2. Effect of Rhodo35 and Vib3 on the development and architecture of mixed-isolate biofilm. Confocal laser scanning microscopy images (500 × 500 µm) of 24 h old mixed-isolate biofilms with or without Rhodo35 or Vib3; green represents cells stained with Syto9 and red represents extracellular polysaccharides (EPS) stained with ConA; yellow represents a combination of Syto9 and ConA. The bottom and the right axes represent XZ and YZ planes, respectively. (A) $Mix_{(6)}$: biofilm formed by culture containing all 6 species (including Rhodo35 and Vib3); (B) $Mix_{(6\text{-Rhodo35})}$: biofilm formed by a 5-isolate culture (excluding Rhodo35); (C) $Mix_{(6\text{-Vib3})}$: biofilm formed by a 5-isolate culture (excluding Vib3)

atively developed early biofilm with visible microcolonies within 24 h of incubation (Fig. 2A). In contrast, biofilm excluding Rhodo35 ($Mix_{(6\text{-Rhodo35})}$) showed only single cells attached to the surface after 24 h of incubation. No microcolonies or any developed structure could be observed in the absence of Rhodo35 (Fig. 2B). While absence of Rhodo35 negatively influenced biofilm initiation, absence of Vib3 ($Mix_{(6\text{-Vib3})}$) led to a more developed biofilm architecture compared to the 6-isolate community ($Mix_{(6)}$) (Fig. 2C). These results were supported by measurement of physical parameters of the biofilms from CLSM images using the PHLIP software. The mean thickness of the biofilms formed by the $Mix_{(6)}$, $Mix_{(6\text{-Rhodo35})}$ and $Mix_{(6\text{-Vib3})}$ cultures were significantly different from each other ($p < 0.05$, $n = 9$, F-test $p > 0.05$), and reached 5.4 ± 1, 2.5 ± 0.8 and 10.4 ± 2.76 µm, respectively. Assuming that the diameter of a bacterial cell is ~1 µm, the biofilm formed by $Mix_{(6)}$ contained 4 to 6 cell layers, while only a monolayer was formed by a culture lacking Rhodo35 and a biofilm of 8 to 10 layers was formed when Vib3 was excluded (Fig. 3A). The biovolume of the biofilm cells significantly ($p < 0.05$, $n = 9$, F-test $p < 0.05$) decreased by almost 2 orders of magnitude due to the absence of Rhodo35 and increased by approx. 2-fold as a result of absence of Vib3 compared to $Mix_{(6)}$ biofilm (Fig. 3B). In

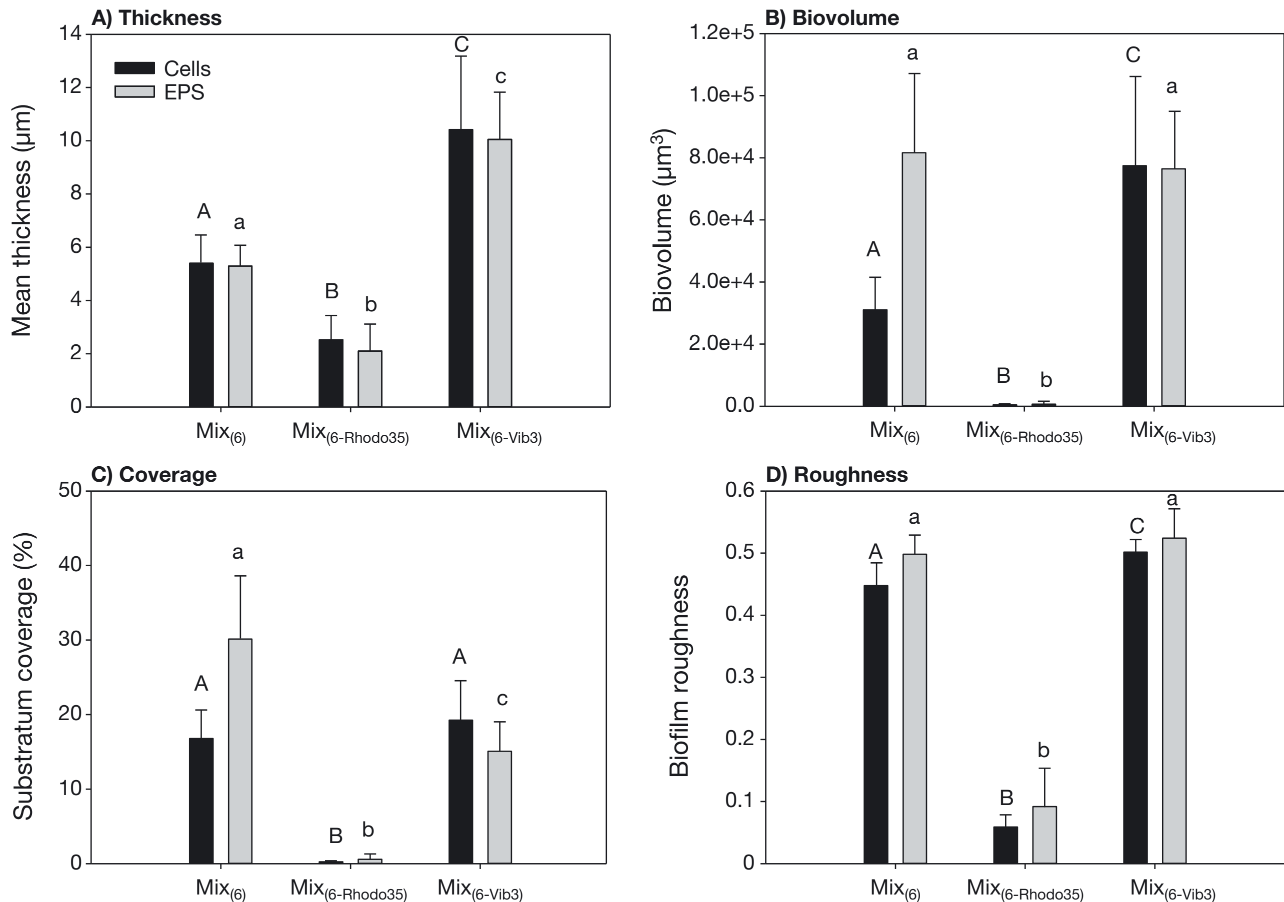

Fig. 3. Effect of Rhodo35 and Vib3 on parameters of biofilm formed by mixed-isolate culture. Bars represent (A) thickness, (B) biovolume, (C) coverage and (D) roughness of 3 different mixed-isolate biofilms. $Mix_{(6)}$: biofilm formed by culture containing all 6 species; $Mix_{(6\text{-Rhodo35})}$: biofilm formed by a 5-isolate culture (excluding Rhodo35); and $Mix_{(6\text{-Vib3})}$: biofilm formed by a 5-isolate culture (excluding Vib3). The parameters were calculated from confocal laser scanning microscopy images analyzed using PHLIP-ML software; EPS: extracellular polysaccharides. Error bars represent SD of 9 replicates. Letters (capital for cells and non-capital for EPS) above bars represent significant differences according to ANOVA and Student-Newman-Keuls post hoc tests ($p < 0.05$)

addition, both roughness and coverage decreased in Rhodo35-lacking culture, while the latter did not change in a Vib3-lacking culture (Fig. 3C,D). The differences in biofilm parameters between cultures including and excluding Rhodo35 (thickness, biovolume, coverage and roughness) were evident both from quantification of Syto9 labeled cells and ConA-labeled EPS (Fig. 3).

Effect of the isolates on biofilm formation analyzed using quantitative RT-PCR analysis

RT-PCR was used to assess the composition of the initial biofilm developed by the model community. The biofilm formed by the 6-isolate culture was found to be composed mainly of 3 dominant isolates: Rhodo35, Vib3 and Pseudoalt17. The 3 other isolates present in the biofilm (Spong12, Halo18 and Eryth23) were found in much lower abundance (Fig. 4). Isolate Vib3, which was one of the dominant bacteria in the mixed-isolate biofilm, had only a low potential for colonization and biofilm formation when grown by itself, giving a value of 0.7 in the CV staining test, while the biofilm biomass in the 6-isolate mixture had a value of 1.8 (Fig. S3). On the other hand, Eryth23, the isolate that had the highest potential for biofilm formation as a single species (with biofilm biomass value of 2.5), was almost completely absent from the mixed-isolate biofilm. In a 5-isolate culture excluding Rhodo35, the 2 remaining dominant species (Vib3 and Pseudoalt17) formed a biofilm in lower levels of isolate abundance compared to a biofilm that included Rhodo35. However, these changes were not statistically significant ($n = 3$). The concentration of Vib3 decreased from 4.0×10^5 to 1.5×10^5 cells mm^{-2} and the concentration of

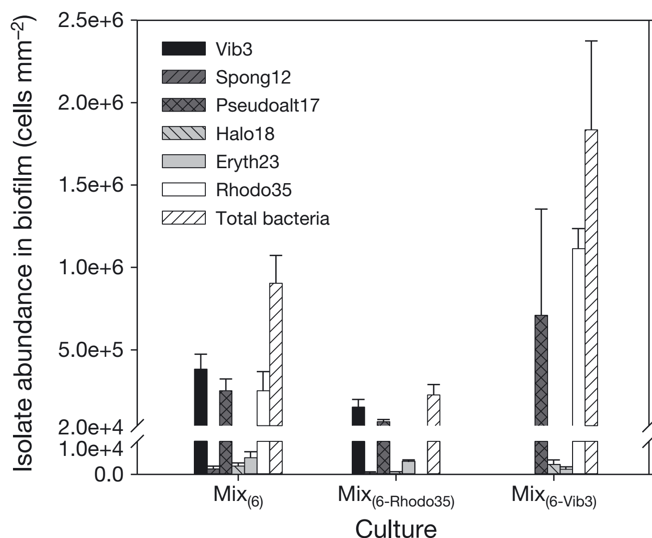

Fig. 4. Effect of Rhodo35 and Vib3 on isolates abundance in mixed species biofilm. Bars represent the abundance of each isolate and of the total bacteria in biofilms formed by 3 different mixed-species cultures. $Mix_{(6)}$: biofilm formed by culture containing all 6 species; $Mix_{(6\text{-Rhodo35})}$: biofilm formed by a 5-isolates culture (excluding Rhodo35); $Mix_{(6\text{-Vib3})}$: biofilm formed by a 5-isolates culture (excluding Vib3). Isolates abundances in the resulting biofilms were measured by quantitative RT-PCR using primers specific for each isolate. Error bars represent SD of 3 replicates

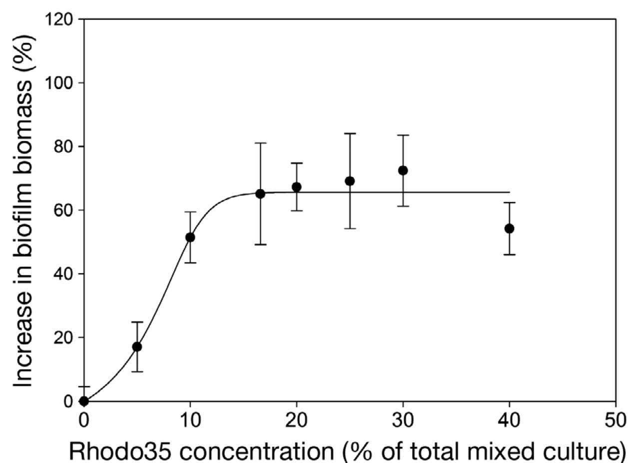

Fig. 5. Effect of relative abundances of Rhodo35 on biofilm formation. Symbols represent the mean increase (%) in biofilm formation of a mixed species biofilm with different Rhodo35 relative abundances (0, 5, 10, 16.6, 20, 25, 30 and 40%). Bars represent SD of 6 replicates

Pseudoalt17 from 2.5×10^5 to 5.0×10^4 cells mm^{-2}, relative to their abundance in biofilm formed by the 6-isolate culture (Fig. 4). Furthermore, the 3 other isolates (Spong12, Eryth23 and Halo18), which were present at low concentrations in the 6-isolate biofilm, were almost completely absent in the biofilm lacking Rhodo35. Based on the quantitative RT-PCR data, total biofilm concentration (sum of the levels of all isolates) decreased from 9.0×10^5 to 2.4×10^5 cells mm^{-2} when Rhodo35 was absent. In contrast, the absence of Vib3 led to an increase, although statistically insignificant, in biofilm formation by the remaining dominant isolates, namely isolates Rhodo35 and Pseudoalt17 (Fig. 4). Vib3 exclusion resulted in Pseudoalt17 concentration increasing from 2.5×10^5 to 7.0×10^5 cells mm^{-2} and Rhodo35 concentration increased from 2.5×10^5 to 1.1×10^6 cells mm^{-2}.

Effect of Rhodo35 relative abundances on its ability to promote biofilm formation

Addition of different concentrations of Rhodo35 to the mixed isolates was conducted in order to evaluate the effect of its relative abundance on biofilm initiation. Higher concentrations of Rhodo35, up to 16.6%, caused an increase in the promotion of biofilm formation. An increase up to 70% in biofilm biomass was observed with added Rhodo35 compared to biofilm biomass formed without added Rhodo35 (Fig. 5). Increasing Rhodo35 to concentrations above 20% did not increase the promotion of biofilm formation any further.

Effect of the isolates on biofilm formation by a complex sea water bacterial community

Inocula of Rhodo35, Vib3, Halo18 and Eryth23 were added separately to a natural marine culture in order to verify that Rhodo35-mediated interaction also takes place in a complex marine microbial community. Addition of 10% of each of the 4 isolates to the sea water culture did not change the biofilm biomass formed relative to the control culture (Fig. 6). However, addition of 20 or 40% of Rhodo35 led to a significant increase of approx. 30% ($p < 0.05$, $n = 6$, *F*-test $p > 0.05$) in biofilm biomass relative to the control treatment, while the addition of Vib3, Halo18 and Eryth23 had no effect (although a negative effect was noted when Halo18 was added at 40%) (Fig. 6).

Effect of Rhodo35 extracellular material on biofilm formation of a mixed culture

In order to determine whether Rhodo35 interacts and promotes biofilm initial formation via direct or indirect contact, mixed cultures were exposed to extracellular material of Rhodo35. The exposure of a

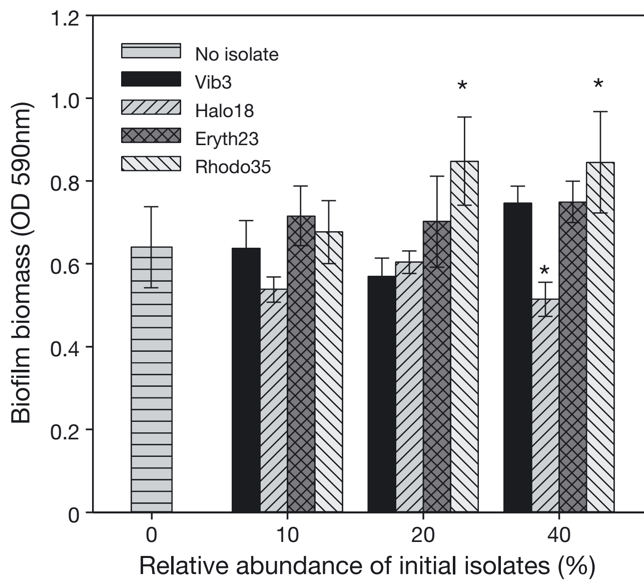

Fig. 6. Effect of the different isolates on biofilm formation by a complex marine microbial community. Bars represent the biofilm biomass formed by concentrated complex seawater community with 0, 10, 20 and 40% of isolates Vib3, Halo18, Eryth23 and Rhodo35. Biofilm biomass was measured by the crystal violet (CV) staining assay and represented by optical density (OD). Error bars represent SD of 6 replicates. Asterisks (*) above the bars represent a significant difference ($p < 0.05$) from the control treatment (0% isolate added) as obtained from Bonferroni post hoc analysis

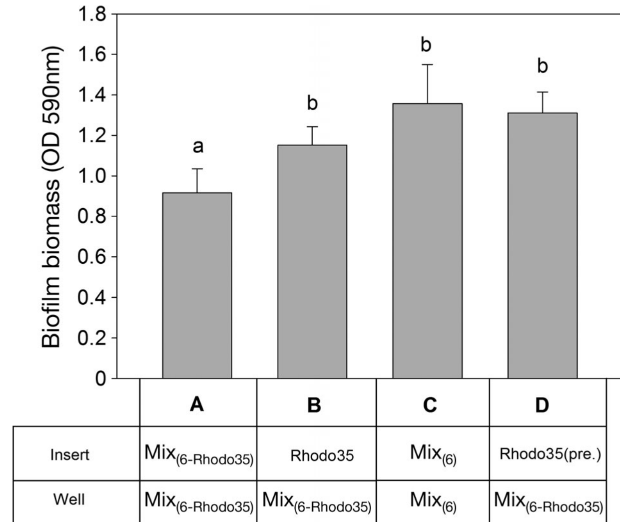

Fig. 7. Effect of Rhodo35 diffusible extracellular material on biofilm formation by a mixed-isolate culture. Bars show biofilm level formed by mixed-isolate culture within the wells exposed to Rhodo35 extracellular material secreted from the insert through a 0.4 µm membrane. Biofilm biomass was measured by the crystal violet (CV) staining assay and represented by optical density (OD). Bar A: control treatment, biofilm formed by culture excluding Rhodo35 ($Mix_{(6\text{-}Rhodo35)}$) that was not exposed to Rhodo35 extracellular material. Bar B: biofilm formed by culture excluding Rhodo35 ($Mix_{(6\text{-}Rhodo35)}$) exposed to Rhodo35 extracellular material. Bar C: biofilm formed by a culture including Rhodo35 ($Mix_{(6)}$) within the well. Bar D: biofilm formed by culture excluding Rhodo35 ($Mix_{(6\text{-}Rhodo35)}$) exposed to exudates of Rhodo35 from the insert (Rhodo35(pre.)) that was inoculated 5 h prior to the well content. Error bars represent SD of 4 replicates. Letters above bars represent significant differences according to ANOVA and Student-Newman-Keuls post hoc tests ($p < 0.05$)

5-isolate culture excluding Rhodo35 ($Mix_{(6\text{-}Rhodo35)}$) to the extracellular material of Rhodo35 through a separating 0.45 µm membrane (Fig. 7, bar B; Fig. S2A) resulted in a 26% increase ($p < 0.05$, $n = 4$, *F*-test $p > 0.05$) in biofilm formation relative to unexposed control cultures (Fig. 7, bar A; Fig. S2B). Exposure of $Mix_{(6\text{-}Rhodo35)}$ culture to extracellular material of Rhodo35, which was inoculated 5 h prior to inoculation of the mixed culture in the well, did not result in an additional increase in biofilm biomass formed by the mixed culture relative to the treatment in which all the isolates were inoculated simultaneously (including Rhodo35) (Fig. 7, bar D). Similar biofilm was formed by mixed culture excluding Rhodo35 that was exposed to Rhodo35 extracellular material (Fig. 7, bar B) and by the mixed culture including Rhodo35 (Fig. 7, bar C). A similar control experiment, carried out with Halo18, resulted in no effect whatsoever on biofilm formation (data not shown).

DISCUSSION

Several studies of diverse marine environments, including the Mediterranean Sea and Atlantic Ocean Coast, have shown *Rhodobacteraceae* to be a dominant bacterial group in initial marine biofilms (Dang & Lovell 2000, 2002, Dang et al. 2008, Elifantz et al. 2013). Despite the high prevalence of *Rhodobacteraceae* in early stage biofilms, their role in multi-species biofilm formation has not yet been elucidated. The present study demonstrates that a randomly isolated member of the *Rhodobacteraceae* promotes initial biofilm formation in marine multi-isolate cultures. It was shown that the biofilm-promoting effect of Rhodo35 is specific to biofilm formation, as Rhodo35 addition did not alter the biomass of the planktonic model mixed-isolate culture.

CLSM analyses revealed that Rhodo35 did not only affect the total biomass of the initial biofilm, but also the level of biofilm development during 24 h of incubation and its overall structure. An early biofilm lacking Rhodo35 was still in its first developmental stage (initial attachment), characterized by single surface-attached cells only, while culture containing Rhodo35

of the same age was at a higher developmental stage, characterized by structured microcolonies (O'Toole et al. 2000), within 24 h of incubation. We therefore hypothesized that Rhodo35 interacts with other bacteria by stimulating their biofilm initiation. This hypothesis was supported and strengthened by findings obtained from RT-PCR analysis. Quantifying separately each isolate in the mixed-isolate biofilm, using specific primers designed in this study for quantitative RT-PCR, enabled us to describe the exact response of each isolate to the presence or absence of the studied *Rhodobacteraceae* member. The abundance of all 5 isolates that constituted the mixed-isolate model biofilm was increased due to the presence of Rhodo35. The results obtained from RT-PCR analyses were based on the calculated relative copy numbers of each of the isolates. This was calculated by dividing the number of targets of each isolate obtained from RT-PCR analysis using universal bacterial primers (Lane 1991) by the CFU count of the same sample. However, we could not eliminate possible biases in this experiment. First, although no cell aggregates were observed by careful microscopic examination in cultures of the isolates, we cannot rule out the possibility that not all of the counted colonies were obtained from single cells. Second, the calculation could be biased by different DNA extraction efficiencies from each of the isolates.

The phenomenon of biofilm initiation was observed in all 3 experimental strategies used in the study (CV staining test, CLSM and quantitative RT-PCR), regardless of the surface on which the biofilm was formed, such as glass or plastic. It is well known that surface properties, such as hydrophobicity and topography, significantly affect biofilm formation and that different species react differentially to these properties (reviewed by Fletcher 1990). However, despite the differences in biofilm initiation levels between the experiments, the same result of biofilm initiation by Rhodo35 was achieved on different surfaces, highlighting the significance of the phenomenon.

The natural marine bacterial community of the Mediterranean Sea is orders of magnitude more diverse and complex than the model community used in this study (Alonso-Sáez et al. 2007). Additionally, several families previously isolated from Mediterranean Sea biofilms, such as *Shewanellaceae* and *Flavobacteraceae* (Brian-Jaisson et al. 2014), were not included in the 6-isolate model community. Thus, it can be assumed that the interactome — the network of interspecies interactions in this natural community (Jenkinson 2011) — is also much more complicated than in the experimentally studied 6-isolate model community. Therefore, in order to assess the significance of Rhodo35 in a complex environment, its role was also studied on a microbial culture obtained from natural sea water. Our findings confirmed that Rhodo35 significantly increased the biofilm formation of the complex community obtained from natural marine water. In contrast, other isolates, including Vib3, which showed strong negative interaction with the strains of the model community, had no detectable impact on the enriched natural marine community.

Inter-species interactions have already been described as promoting biofilm formation, mainly in oral environments, where streptococci and actinomyces coaggregated to form dual-species biofilms (Cisar et al. 1979, Palmer et al. 2003). Analogous results were obtained also in freshwater communities (Simões et al. 2008, Min & Rickard 2009). However, this study suggests that the biofilm-initiating activity of Rhodo35 was mediated by an extracellular factor(s) rather by coaggregation. It was found that Rhodo35 promoted biofilm formation of the model multi-isolate culture without direct contact, presumably by extracellular compounds that diffuse through semi-permeable membranes. The effect of the extracellular material on biofilm formation was at the same level as that of the presence of the bacteria themselves in the mixed culture. Additionally, a pre-inoculation (5 h) of Rhodo35 before the introduction of other species did not result in additional increase of biofilm initiation. This suggests that no accumulation of active product took place during pre-inoculation or that the concentration of the factor or its activity reached saturation within the 5 h. Bacterial species have been shown to communicate with each other within biofilm communities by signal molecules they secrete (Parsek & Greenberg 2005). For example, dental communities have been shown to be controlled by autoinducer-2 (AI-2) mediated interspecies quorum sensing, which promotes multi-species biofilm formation (McNab et al. 2003, Rickard et al. 2006, Cuadra-Saenz et al. 2012). Studies have shown the *Roseobacter* clade (members of the *Rhodobacteraceae*) also to possess QS mechanism (Gram et al. 2002, Martens et al. 2007, Thole et al. 2012), which suggests that the *Rhodobacteraceae* may interact with secondary colonizers using a relatively small diffusible signal factor that induces their colonization. Another possibility is that biofilm induction by Rhodo35 is via extracellular polymeric substance consisting mainly of polysaccharides containing some DNA and proteins, all shown to be crucial components of biofilm matrix (Skillman et al. 1998, Flemming et al. 2007). Future work will be required to address these 2 hypotheses. This proposed research

is based on this present, novel study, which suggests that the common *Rhodobacteraceae* are not only of the most dominant bacterial groups in marine biofilms worldwide, but also have an important role in multispecies marine biofilm initiation.

While it has been shown that some species can cooperate with each other and enhance biofilm initiation, other species may have inhibitive effects on other species (reviewed by Elias & Banin 2012 and Rendueles & Ghigo 2012). Similar results were obtained in this study with a model culture of 6 different isolates. The presence of Vib3 (a member of the genus *Vibrio*), even in a very low relative abundance (5%) within the mixed-isolates model culture, resulted in an inhibition of biofilm formation by the rest of the isolates. Yet, such effect of Vib3, at any concentration, was not found in the natural sea water community. We can hypothesize that the impact of Vib3 in a natural environment could be eliminated due to a different composition of the natural sea water community relative to that of the studied model community (Elifantz et al. 2013), resulting in other interspecies interactions that can inhibit Vib3 or provide resistance to its inhibitive effect. As biofilm inhibition by Vib3 may not be a global mechanism of inhibition, it was not studied further.

CONCLUSIONS

The present study provides an example of interspecies interaction during early biofilm development. The results of the study demonstrated interspecies interaction mediated by primary colonizers during initial biofilm formation, indicating that particular, decipherable traits are involved in succession of the biofilm community. *Rhodobacteraceae* have been shown to include pioneer species in the succession process (Dang et al. 2008, Elifantz et al. 2013). These bacteria are among the first to colonize biotic or abiotic surfaces, probably due to the diverse physiological properties they possess, including rapid response to nutrient input, type I and IV secretion systems and polysaccharide production (Alonso & Pernthaler 2006, Alonso-Sáez & Gasol 2007, Thole et al. 2012). Thus, according to the results of this study, while colonizing the surface, *Rhodobacteraceae* might positively interact with other colonizers, promoting their colonization via extracellular factors, as it did Rhodo35. To summarize, this study uncovers an important part of the interactome that takes place and shapes the initial stages of the succession of biofilm assemblages.

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