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Antibiofilm Activity of the Marine Bacterium *Pseudoalteromonas* sp. Strain 3J6

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Abstract:

Biofilm formation results in medical threats or economic losses and is therefore a major concern in a variety of domains. In two-species biofilms of marine bacteria grown under dynamic conditions, *Pseudoalteromonas* sp. strain 3J6 formed mixed biofilms with *Bacillus* sp. strain 4J6 but was largely predominant over *Paracoccus* sp. strain 4M6 and *Vibrio* sp. strain D01. The supernatant of *Pseudoalteromonas* sp. 3J6 liquid culture (SN_{3J6}) was devoid of antibacterial activity against free-living *Paracoccus* sp. 4M6 and *Vibrio* sp. D01 cells, but it impaired their ability to grow as single-species biofilms and led to higher percentages of nonviable cells in 48-h biofilms. Antibiofilm molecules of SN_{3J6} were able to coat the glass surfaces used to grow biofilms and reduced bacterial attachment about 2-fold, which might partly explain the biofilm formation defect but not the loss of cell viability. SN_{3J6} had a wide spectrum of activity since it affected all Gram-negative marine strains tested except other *Pseudoalteromonas* strains. Biofilm biovolumes of the sensitive strains were reduced 3- to 530-fold, and the percentages of nonviable cells were increased 3- to 225-fold. Interestingly, SN_{3J6} also impaired biofilm formation by three strains belonging to the human-pathogenic species *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Escherichia coli*. Such an antibiofilm activity is original and opens up a variety of applications for *Pseudoalteromonas* sp. 3J6 and/or its active exoproducts in biofilm prevention strategies.

INTRODUCTION

Biofilms are defined as microbial communities of cells that are irreversibly attached to a substratum or to an interface or to each other, and are embedded into a matrix of extracellular polymeric substances that they have produced (8). It is now considered that most (if not all) bacteria are capable of forming biofilms and that this is their predominant bacterial lifestyle. Biofilm formation is a complex biological phenomenon and was generally described as a temporal process involving a succession of distinct stages: reversible then irreversible attachment of planktonic bacteria onto a surface, formation of microcolonies either by clonal growth of attached cells or by active translocation of cells across the surface, coalescence of growing microcolonies to form a macrocolony, and cell dispersal. It should however be noted that this developmental model still requires further experimental validation, especially concerning the possibility of a hierarchical order of genetic pathways (26). Furthermore, Karatan and Watnick (17) pointed out that there are as many different types of biofilms as there are bacteria, and that a single bacterium may even make several different types of biofilms under different environmental conditions. Biofilm formation is associated with the virulence of pathogenic bacteria, and cells included within a biofilm are generally more resistant (up to 1000-fold) to antibiotics and disinfectants than free-living bacteria (8, 26). Biofilms are therefore a major concern in medicine and in medical environments, but also in all domains where their growth constitutes a source of contamination for humans or animals (food industry, cooling towers, water pipes, ...) or leads to economical losses (biofouling of boats and immersed structures, material biocorrosion, ...). The development of anti-biofilm strategies is therefore of major interest and currently constitutes an important field of investigation, in which environmentally friendly anti-biofilm molecules or organisms are highly valuable (5, 7, 9).

Marine bacteria belonging to the *Pseudoalteromonas* genus of the *Gammaproteobacteria* class are often found in association with marine eukaryotes, and their ability to produce a variety of biological activities attracted a particular attention (2, 11, 13, 15, 28). We previously isolated marine bacteria attached to solid surfaces (glass in most of the cases) immersed for 3 or 6 h in the Morbihan gulf or in the bay of Brest, France (10, 20, 21, 27). Out of the three *Pseudoalteromonas* strains isolated, we were able to tag the 3J6 strain with a green fluorescent protein (GFP)-encoding plasmid. This allowed us to investigate whether the *Pseudoalteromonas* sp. 3J6 strain affected the biofilm growth of other marine bacterial isolates. Here, we report that the 3J6 strain predominated in two-species biofilms over *Paracoccus* sp. 4M6 and *Vibrio* sp. D01. Although devoid of anti-bacterial activity towards planktonic cells, *Pseudoalteromonas* sp. 3J6 exoproducts impaired biofilm formation by *Paracoccus* sp. 4M6 and *Vibrio* sp. D01. We characterized the effects of these exoproducts on the latter strains and on other bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this work are listed in Table 1. The strain 3J6 was affiliated to the *Pseudoalteromonas* genus (10). Its 16S rDNA sequence (GenBank entry FJ966949) is the most closely related (95.5% identity) to that of *Pseudoalteromonas* sp. SM9913 strain, which would belong to a new species (28). *Pseudoalteromonas* sp. 3J6 and most of the strains from marine origin that we used were isolated from the same location in the Morbihan gulf within a two-month period (10), and therefore shared the same natural habitat. Marine bacteria were grown in Vaatanen nine-salt solution (VNSS) (24) at 20°C with shaking (120 rpm). *Pseudoalteromonas* sp. 3J6

was selected when required with 200 µg ml^{-1} streptomycin, and *Pseudoalteromonas* sp. 3J6(pCJS10) was grown with 125 µg ml^{-1} chloramphenicol. *Escherichia coli* DH5α cells containing pCJS10 or pRK2013 were grown at 37°C with shaking (180 rpm) in LB broth containing 50 µg ml^{-1} chloramphenicol or kanamycin, respectively. *Pseudomonas aeruginosa* $PAO1_{YFP}$, *E. coli* $CC118_{DsRed}$, and *Salmonella enterica* $MB1409_{HcRed}$ were grown with shaking (180 rpm) at 37°C in Luria-Bertani (LB) medium (34) supplemented with 50 µg ml^{-1} gentamycin, 15 µg ml^{-1} gentamycin, and a mix of 50 µg ml^{-1} kanamycin and 10 µg ml^{-1} chloramphenicol, respectively.

***Pseudoalteromonas* sp. 3J6 tagging.** The chloramphenicol-resistant plasmid pCJS10 (30) carrying the *gfp*mut3 gene which encodes a green fluorescent protein (GFP) (3) was introduced into the *Pseudoalteromonas* sp. 3J6 strain (streptomycin resistant) by conjugation using *E. coli* DH5α(pCJS10) and *E. coli* DH5α(pRK2013) as donor and helper strains, respectively. Triparental conjugation was carried out as described by Rao et al. (30) and *Pseudoalteromonas* sp. 3J6 transconjugants were isolated on VNSS-agar plates containing chloramphenicol and streptomycin.

Biofilm formation. Biofilms were grown in continuous-culture three-channel flow cells (channel dimensions, 1 by 4 by 40 mm). The system was assembled and prepared as previously described (36). The substratum was a microscope coverslip st1 (VWR International, Fontenay sous Bois, France). Channels were inoculated with overnight bacterial cultures which were diluted to a final OD_{600} of 0.25 after three washes in Filtered Marine Water (FMW) for marine bacteria or in NaCl 0.9% for the other strains. To obtain FMW, sea water from Kernevel habor (Larmor Plage, France) was sterilized first by filtration through a membrane with pores of 0.22 µm diameter and then by autoclaving at 121°C for 20 min.

Bacteria were allowed to attach to the glass surface during 2 h at 20°C under static condition. Biofilm growth was then performed under a constant flow of VNSS or LB medium (0.2 mm s^{-1}) for 48 h at 20°C.

In the case of two-species biofilms, channels were inoculated with a mixture of *Pseudoalteromonas* sp. 3J6(pCJS10) and one of the other strains in a ratio leading to similar attachment of each strain to the glass surface (ie similar percentages of glass surface covered by each strain) after the 2 h adhesion step. This ratio was 1:1 for all strain couples except for *Pseudoalteromonas* sp. 3J6(pCJS10) - *Bacillus* sp. 4J6, which needed a 1:4 ratio.

To investigate the effects of *Pseudoalteromonas* sp. 3J6 exoproducts on adhesion and biofilm formation by other bacteria, these bacteria were diluted to an OD_{600} of 0.25 in SN_{3J6}. The latter is a supernatant of a liquid overnight culture of *Pseudoalteromonas* sp. 3J6 sterilized by filtration through a membrane with pores of 0.22 µm diameter. Bacteria diluted in SN_{3J6} were then inoculated in flow cell channels, and the 2 h adhesion step under static condition followed by the biofilm growth under a flow of fresh VNSS medium were performed as described above. For a better understanding of the SN_{3J6} action, we modified our procedure as follows: i) bacteria at an OD_{600} of 0.25 were incubated with SN_{3J6} outside of flow cell channels for 2 h at 20°C with shaking at 120 rpm, were washed twice in FMW and were then inoculated into channels; or ii) SN_{3J6} was injected without any bacterium into flow cell channels, was left for 2 hours at 20°C to coat the glass surface, and the channels were then rinsed with FMW before inoculating bacteria.

Confocal laser scanning microscopy and image analyses. Microscopic observations were performed on a TCS-SP2 system (Leica Microsystems, Germany) using the oil immersion ×63 objective. To study the attachment of each strain on the glass surface after the 2 h-adhesion step, a VNSS flow (0.2 mm s^{-1}) was applied for 15 min to remove planktonic

144 cells and the attached bacteria were observed. The glass surfaces covered by bacteria were
145 evaluated using the Image Tool software (University of Texas Health Science Center at San
146 Antonio, USA). Biofilms were observed by monitoring the GFP fluorescence of
147 *Pseudoalteromonas* sp. 3J6(pCJS10) (λ excitation, 488 nm ; λ emission, 510 nm) or after a 10
148 min-incubation with 5 µM Syto 61 Red nucleic acid stain (λ excitation and emission: 633 and
149 645 nm, respectively). For cell viability assays, bacteria were stained with 5 µM Syto 61 Red
150 and 0.5 µM Sytox Green (λ excitation and emission: 488 and 510 nm, respectively).
151 Fluorescence of *P. aeruginosa* $PAO1_{YFP}$, *E. coli* $CC118_{DsRed}$, and *S. enterica* $MB1409_{HcRed}$
152 were detected at 535, 590, and 647 nm, respectively. Images were obtained using the Leica
153 Confocal Software and biofilm stacks were analyzed with the COMSTAT software (12). The
154 calculated parameters were the maximal and average thicknesses of the biofilms, and the
155 biovolume which is the volume of bacteria (in μm^3) per μm^2 of glass surface. The results are
156 representative of at least two independent experiments from which a total of 12 to 18 image
157 stacks were obtained.
158
159 **Determination of strain proportions in two-species biofilms by quantitative PCR.**
160 Two-species biofilms were grown as described above, except that we used BST FC 81 Flow-
161 cells (BioSurface Technologies, Montana, USA) with channel dimensions of 50.8 x 12.7 x
162 2.54 mm. These flow-cells were chosen because they were easily disassembled, allowing total
163 bacterial DNA extraction from biofilms using the DNeasy tissue kit (Qiagen, Germany). The
164 same kit was used to extract bacterial DNA from planktonic cells for control experiments.
165 Quantitative PCR (qPCR) was performed using primers targeting the 16S rDNA sequences
166 (Table 2). The PCR products were detected with the SYBR Green stain or with TaqMan
167 probes (Table 2). Primers and Taqman probes were custom-synthesized by Eurogentec
168 (Belgium) and Applied Biosystems (Forster City, California, USA), respectively. The 25-µl

reactions were performed in triplicate with the 7300 Real-Time PCR System apparatus (Applied Biosystems). The reaction mixture contained between 0.001 to 10 ng of DNA, 300 nM of each primer, and either 12.5 µl of SYBR Green PCR Master Mix (including AmpliTaq Gold DNA Polymerase) (Applied Biosystems) or 200 nM of TaqMan probes and 12.5 µl of TaqMan Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems). The PCR conditions were 50°C for 2 min, 95°C for 10 min for polymerase activation, followed by 40 cycles at 95 and 60°C for 15 and 60 s, respectively. ROX dye was used as passive reference to normalize the non-PCR related fluorescence variations. Each qPCR reaction was performed in triplicate and the standard deviations were lower than 0.15 C_T. Each primer pair was validated by verifying that the PCR efficiency was above 0.95, and that a single PCR product with the expected Tm was obtained. The DNA of a given strain in the sample was quantified by reporting the C_T on a standard curve (C_T = f[log DNA amount]) obtained with serial dilutions of total DNA extracted from a pure culture of the corresponding strain (22). For each analysis, total DNAs were extracted from two different biofilms and the two samples were analyzed independently.

Liquid co-cultures of bacteria and population analyses. *Pseudoalteromas* sp. 3J6(pCJS10) and *Vibrio* sp. D01 were each inoculated into VNSS medium at an OD_{600} of 0.25 and bacteria were grown for 24 h at 20°C with shaking. Dilutions were then plated onto VNSS agar without antibiotic (total cell count) and onto VNSS agar containing both streptomycin and chloramphenicol to select *Pseudoalteromas* sp. 3J6(pCJS10). The numbers of total cfu and of *Pseudoalteromas* sp. 3J6(pCJS10) cfu per ml allowed to calculate the percentage of each of the two strains in a co-culture. Experiments were performed three times.

193 **Concentration of the anti-biofilm activity.** 200 ml of SN_{3J6} were passed through a
194 Sep-Pak® Plus C18 cartridge (Waters Corporation, Milford, Massachusetts, USA). The
195 cartridge was rinsed three fold with water, and the anti-biofilm molecules were eluted with 50
196 ml of 50% acetonitrile. The solvent was evaporated using a rotary evaporator under vacuum
197 at 35°C. The residues were dissolved in 2 ml of phosphate buffered saline (NaCl 8.0 g l^{-1},
198 KCl 0.2 g l^{-1}, Na_2HPO_4 1.44 g l^{-1}, KH_2PO_4 0.24 g l^{-1}, pH 7.4) and sterilized by filtration
199 through a membrane with 0.22 µm pores. A 10^{-3} dilution of this preparation prevented
200 *Paracoccus* sp. 4M6 biofilm formation as efficiently as undiluted SN_{3J6}, whereas a 10^{-1}
201 dilution of SN_{3J6} displayed a significantly reduced anti-biofilm activity. This showed that the
202 anti-biofilm activity was concentrated at least 100 fold after elution from the C18 cartridge.
203 No anti-biofilm activity was observed with a preparation resulting from the passage of VNSS
204 culture medium on a C18 cartridge.
205
206 **Bacteriocin-like activity tests.** We used the culture supernatant diffusion method (33),
207 in which SN_{3J6} was loaded into wells of VNSS agar plates seeded with *Vibrio* sp. D01 or
208 *Paracoccus* sp. 4M6. The plates were incubated at 20°C for 24 to 48 h to allow diffusion of
209 SN_{3J6} molecules from the wells to the agar medium and growth of *Vibrio* sp. D01 or
210 *Paracoccus* sp. 4M6. The presence of a bacteriocin-like molecule in SN_{3J6} would lead to clear
211 halos of bacterial growth inhibition around the wells. Alternatively, disks soaked with SN_{3J6}
212 were placed onto the seeded agar medium, instead of using wells. This disk assay was also
213 used to test the antimicrobial activity of anti-biofilm molecules which had been concentrated
214 as described in the above paragraph.
215
216 **RESULTS**
217

Single-species biofilms of *Pseudoalteromonas* sp. 3J6 and three other marine bacteria. We first introduced the GFP-encoding plasmid pCJS10 (Table 1) into *Pseudoalteromonas* sp. 3J6 as described in Materials and Methods. This fluorescent tagging allowed the observation of *Pseudoalteromonas* sp. 3J6 biofilms by confocal laser scanning microscopy (CLSM) without further staining the cells. Since bacteria in a biofilm are generally more antibiotic-resistant than planktonic cells, *Pseudoalteromonas* sp. 3J6(pCJS10) biofilms were grown without antibiotic (liquid precultures were performed in the presence of chloramphenicol). This required that pCJS10 was stably maintained into the strain without selective pressure. Biofilms grown in a flow cell under dynamic condition for 48 h were observed using CLSM by detecting the green fluorescence of GFP (Fig. 1A), and after staining bacteria with Syto 61 Red and detecting the red fluorescence. When overlaying the resulting images, GFP-producing cells were yellow (overlay of green and red) or green, whereas GFP-free bacteria were red. Fig. 1B shows very few red-stain patches of cells, indicating that the vast majority of bacteria were GFP producers and had thus retained pCJS10. This plasmid is therefore suitable for our study. *Pseudoalteromonas* sp. 3J6(pCJS10) biofilms were heterogeneous with the presence of protruding mushroom-like microcolonies which were up to 21 μm high, whereas the average thickness of the biofilms was 4.8 μm (Fig. 1A and B; Table 3).

We failed to introduce pCJS10 and other plasmids encoding fluorescent proteins in our model strains *Paracoccus* sp. 4M6, *Vibrio* sp. D01, and *Bacillus* sp. 4J6. We nevertheless grew single-species biofilms of these strains in the same conditions as above, and stained them with Syto 61 Red prior to CLSM observation (Fig. 1C, E, and G). Each of these three strains led to thicker biofilms than *Pseudoalteromonas* sp. 3J6(pCJS10) (Table 3). *Paracoccus* sp. 4M6 biofilms were heterogeneous and contained cell aggregates but no mushroom-like structure (Fig. 1C). They were in average 1.7 fold thicker than

Pseudoalteromonas sp. 3J6(pCJS10) biofilms, while their biovolumes were identical (Table 3), indicating that cell density is lower in *Paracoccus* sp. 4M6 biofilms. Although not flat, *Vibrio* sp. D01 biofilms were less heterogeneous: they were devoid of mushroom-like structure and cell aggregates were less obvious (Fig. 1E). Their average thickness and biovolume were 1.8 and 2 fold higher, respectively, than those of *Pseudoalteromonas* sp. 3J6(pCJS10) biofilms (Table 3). The *Bacillus* sp. 4J6 biofilms were the thickest, which led to the highest biovolume, and they displayed a hairy surface although they contained homogeneously distributed cells (Fig. 1G, Table 3). In our conditions, each of the four strains was therefore able to develop a single-species biofilm with a specific structure.

***Pseudoalteromonas* sp. 3J6 inhibits the development of *Paracoccus* sp. 4M6 and *Vibrio* sp. D01 in two-species biofilms.** The GFP-tagging of *Pseudoalteromonas* sp. 3J6 allowed growing two-species biofilms with any unlabeled strain, and distinguishing between the two strains by staining biofilms with Syto 61 Red and overlaying green and red fluorescences. Co-inoculating *Pseudoalteromonas* sp. 3J6(pCJS10) and *Bacillus* sp. 4J6 led to biofilms in which each strain is abundant: the biofilms consisted in *Pseudoalteromonas* sp. 3J6(pCJS10) aggregates (green-yellow) surrounded by *Bacillus* sp. 4J6 cells (red) (Fig. 1H). Furthermore, the biofilm thickness and biovolume were closer to *Bacillus* sp. 4J6 single-species biofilms than to *Pseudoalteromonas* sp. 3J6(pCJS10) biofilms (Table 3). By contrast, when *Pseudoalteromonas* sp. 3J6(pCJS10) was co-inoculated with either *Paracoccus* sp. 4M6 or *Vibrio* sp. D01, the resulting biofilms contained only few red cells and were similar to single-species *Pseudoalteromonas* sp. 3J6(pCJS10) biofilms, with in particular the presence of mushroom-like structures (Fig. 1, compare D and F to B). This suggested that *Pseudoalteromonas* sp. 3J6(pCJS10) was the predominant strain in these biofilms. This visual impression was confirmed by determining the biovolume of each strain within the two-species

biofilms using the COMSTAT software: *Pseudoalteromonas* sp. 3J6(pCJS10) was estimated to account for 76 and 84 % of the total biovolumes in mixed biofilms including *Vibrio* sp. D01 and *Paracoccus* sp. 4M6, respectively (Table 4). On the opposite, *Pseudoalteromonas* sp. 3J6(pCJS10) accounted for only 35 % of the total biovolume in *Pseudoalteromonas* sp. 3J6(pCJS10) - *Bacillus* sp. 4J6 biofilms (Table 4).

To ascertain the proportion of each strain in two-species biofilms, we set up qPCR assays using primers targeting the 16S rDNA sequences of the four strains (Table 2). To validate the assays, total DNAs independently extracted from pure liquid cultures of the four strains were mixed in known ratios (1:1 and 9:1) and values closed to these ratios had to be obtained by qPCR analyses. When studying a mix of *Pseudoalteromonas* sp. 3J6(pCJS10) and *Paracoccus* sp. 4M6 DNAs, the corresponding primers were specific enough of each strain to be detected using the SYBR green dye. Analyses of mixes including *Pseudoalteromonas* sp. 3J6(pCJS10) DNA and either *Vibrio* sp. D01 or *Bacillus* sp. 4J6 DNAs required the use of TaqMan probes (Table 2) to detect more specifically the amplicons resulting from each strain. Two-species biofilms were then grown, total DNAs were extracted from each biofilm, and the proportion of each DNA was determined by qPCR. These assays strengthened the above conclusions, confirming i) the co-development of *Pseudoalteromonas* sp. 3J6(pCJS10) and *Bacillus* sp. 4J6 in mixed biofilms, since *Bacillus* DNA represented almost 40 % of total DNA; and ii) that *Pseudoalteromonas* sp. 3J6(pCJS10) cells were largely prevailing (89 and 95 % of total DNA) over *Paracoccus* sp. 4M6 and *Vibrio* sp. D01 in two-species biofilms (Table 4).

Pseudoalteromonas sp. 3J6(pCJS10) therefore inhibited the growth of *Vibrio* sp. D01 and *Paracoccus* sp. 4M6, but not of *Bacillus* sp. 4J6, in two-species biofilms. This could not be simply correlated to the growth parameters of the strains: *Pseudoalteromonas* sp. 3J6(pCJS10) displayed a doubling time of 53 min in liquid VNSS culture at 20°C, whereas

doubling times of *Vibrio* sp. D01, *Paracoccus* sp. 4M6, and *Bacillus* sp. 4J6 were 55, 160, and 80 min, respectively. In our conditions, *Pseudoalteromonas* sp. 3J6(pCJS10) was therefore unlikely to outcompete *Vibrio* sp. D01 because of a faster growth. This was confirmed by liquid co-cultures of *Pseudoalteromonas* sp. 3J6(pCJS10) and *Vibrio* sp. D01: after 24h of growth, *Pseudoalteromonas* sp. 3J6(pCJS10) accounted for about 50% of the total cultivable cell population. This furthermore indicated that the negative effect of *Pseudoalteromonas* sp. 3J6 on *Vibrio* sp. D01 growth occurred specifically in biofilms.

***Pseudoalteromonas* sp. 3J6 exoproducts inhibit the formation of *Paracoccus* sp. 4M6 and *Vibrio* sp. D01 biofilms.** We examined whether the supernatants of liquid *Pseudoalteromonas* sp. 3J6(pCJS10) cultures (SN_{3J6}) affected *Vibrio* sp. D01 and *Paracoccus* sp. 4M6 biofilm formation. These two strains were independently resuspended either in Filtered Marine Water (FMW) as positive controls or in SN_{3J6}. The bacterial suspensions were introduced into flow-cell chambers and incubated for 2h at 20°C to allow bacterial attachment onto the glass surface (a control experiment in which FMW was replaced by fresh VNSS medium showed that the VNSS components of SN_{3J6} did not affect bacterial attachment). Flows of VNSS medium free of *Pseudoalteromonas* sp. 3J6 exoproducts were then applied for 48 h to allow biofilm formation. Incubating *Vibrio* sp. D01 or *Paracoccus* sp. 4M6 cells with SN_{3J6} strongly impaired the ability of *Paracoccus* sp. 4M6 and *Vibrio* sp. D01 to develop single-species biofilms (Fig. 2, compare C and D to A and B): the residual biovolumes were about 5 and 14%, respectively, of the SN_{3J6}-free positive control biovolumes (Table 5). In the subsequent experiments, we attempted to identify at which level(s) is acting SN_{3J6}.

SN_{3J6} moderately affects bacterial attachment. The glass surfaces covered by bacteria were determined after a 2 h step of adhesion in the presence or not of SN_{3J6}. These surfaces

were about 2 fold lower in the presence of SN_{3J6} (Fig. 3). This effect seems however too mild to fully explain the subsequent inability of the attached bacteria to develop biofilms. We furthermore observed that the presence of SN_{3J6} during the attachment step did not favor the detachment of bacteria after applying the medium flow (data not shown).

SN_{3J6} is devoid of bactericidal activity against free-living cells. We examined if SN_{3J6} contained a bacteriocin-like inhibitory substance, using two classical methods (diffusion into agar medium from wells or disks) as described in Materials and Methods. We failed to observe any clear halo of inhibition around the wells or the disks, indicating that SN_{3J6} was neither bactericidal nor bacteriostatic towards *Vibrio* sp. D01 and *Paracoccus* sp. 4M6. Consistently, adding 4.5 ml of SN_{3J6} to 0.5 ml of fresh liquid 10x VNSS medium before inoculating *Vibrio* sp. D01 or *Paracoccus* sp. 4M6 did not prevent growth of these two strains. After 24 h of growth, the numbers of *Vibrio* sp. D01 and *Paracoccus* sp. 4M6 cfu were 88 and 94%, respectively, compared to control cultures in SN_{3J6}-free VNSS. Furthermore, we used a double staining with Syto 61 Red (staining all bacteria) and Sytox Green (staining in green only bacteria with damaged membranes, which are therefore considered as non viable bacteria) on bacteria attached to the glass surface after a 2 h adhesion step. This showed that the presence of SN_{3J6} during this step did not decrease the percentage of viable bacteria (about 90% and 100% of viable *Vibrio* sp. D01 and *Paracoccus* sp. 4M6, respectively, with or without SN_{3J6}). This set of experiments indicated that the anti-biofilm action of SN_{3J6} did not result from a bactericidal activity exerted during the 2 h contact between SN_{3J6} and the bacteria (adhesion step). To ascertain this conclusion, *Vibrio* sp. D01 or *Paracoccus* sp. 4M6 cells were incubated in SN_{3J6} for 2 h at 20°C, then washed and resuspended in FMW, and the cell suspensions were injected in flow-cell chambers. As in previous experiments, a 2 h adhesion step was then performed and biofilms were allowed to

grow under a VNSS flow for 48 h. This led to full biofilm formation (Fig. 2E and F), showing that the incubation of bacteria with SN_{3J6} outside of the flow-cell chamber had no effect on the ability of the bacteria to subsequently form a biofilm. Finally, we used a C18 cartridge to concentrate at least 100 fold the anti-biofilm activity from SN_{3J6}. This preparation also failed to display a bactericidal activity using the disk assay, invalidating the possibility that SN_{3J6} would contain a sub-inhibitory concentration of a bacteriocin-like molecule which would nevertheless be responsible for the anti-biofilm activity.

Glass coating ability of anti-biofilm components of SN_{3J6}. Since SN_{3J6} did not reduce cell viability during the adhesion step and only mildly affected cell attachment, it was likely to act during biofilm formation, *ie* after applying the flow of VNSS medium although this flow is removing SN_{3J6} as well as unattached cells. This suggested that anti-biofilm components of SN_{3J6} could coat the glass surface during the adhesion step. This hypothesis was tested by incubating for 2 h SN_{3J6} in flow-cell chambers without bacteria before extensively rinsing the chambers with FMW and inoculating them with *Vibrio* sp. D01 or *Paracoccus* sp. 4M6 cells in the absence of SN_{3J6}. This impaired the biofilm formation by the two strains almost as efficiently as the presence of SN_{3J6} with the bacteria during the adhesion step (Fig. 2, compare G and H to C and D). The coating of the glass surface by anti-biofilm components of SN_{3J6} explains that these components can act during biofilm growth, even though SN_{3J6} was removed from the flow-cell chambers after the adhesion step by the medium flow.

SN_{3J6}-dependent reduction of cell viability in biofilms. As shown above, various approaches led to the conclusion that SN_{3J6} was not bactericidal toward *Vibrio* sp. D01 and *Paracoccus* sp. 4M6, and that its anti-biofilm action was not resulting from a bactericidal

activity exerted during the adhesion step. We therefore expected that SN_{3J6} would not affect the viability of cells within the subsequently obtained biofilms. We nevertheless assessed the bacterial viability in 48 h biofilms by using a Syto 61 Red/Sytox Green double staining. Whereas control biofilms of *Paracoccus* sp. 4M6 contained only 1.2% of non viable cells, we were surprised to observe that none of the cells were viable in biofilms obtained after incubation with SN_{3J6} (Table 5). Albeit not so pronounced, this effect was also observed with *Vibrio* sp. D01: control biofilms contained 13% of non viable cells, while this value reached 78% when cells had been incubated with SN_{3J6} (Table 5). SN_{3J6} therefore led to loss of cell viability occurring in a biofilm-specific manner.

SN_{3J6} anti-biofilm activity against other marine bacterial isolates. We examined if SN_{3J6} acts on a larger variety of marine bacteria. Five groups of bacteria could be distinguished (Table 5).

Group 1 contains only the most sensitive strain of the whole study, *Algibacter* sp. 1M6: SN_{3J6} abolished its ability to develop a biofilm covering the glass surface, and the biovolume of the dispersed cell clusters attached to the glass surface was 530 fold lower than the biovolume of control biofilms. Furthermore, the SN_{3J6} treatment led to a dramatic drop of cell viability after 48 h of biofilm growth.

Group 2 includes strains strongly impaired by SN_{3J6} in their ability to form biofilms (biovolumes 6.9 to 22 fold lower) and in the viability of biofilm bacteria. This group contains *Colwellia* sp. 4J3 in addition to *Paracoccus* sp. 4M6 and *Vibrio* sp. D01.

Group 3 consists in *Vibrio* sp. D66 and *Sulfitobacter* sp. 8J6, the biofilm formation of which was strongly or mildly inhibited by SN_{3J6} (biovolumes 3 to 8 fold lower), while SN_{3J6} did not lead to higher percentages of non viable cells in biofilms (the percentage of non viable

392 *Sulfitobacter* sp. 8J6 cells was even 3.2 fold lower in biofilms obtained after incubation with
393 SN_{3J6}).

394 Group 4 is defined by *Alteromonas* sp. 1J3, which was unaffected in its ability to form
395 biofilms, although the incubation with SN_{3J6} resulted in a 9.4 fold higher percentage of non
396 viable cells within the biofilms.

397 Group 5 is composed of fully non-sensitive isolates, *ie* the two *Pseudoalteromonas* sp.
398 strains 3J3 and D41: they were essentially unaffected by SN_{3J6} in their ability to develop
399 biofilms, and SN_{3J6} either failed to increase the percentage of non viable cells in biofilms
400 (3J3) or increased it to only 12% (D41).

401 Altogether, most of the tested isolates were affected: SN_{3J6} impaired the biofilm
402 formation of six strains (groups 1, 2, and 3) out of nine, and led to increase the percentages of
403 non viable cells from the 0.4-29% range to 78-100% in biofilms of five strains (groups 1, 2,
404 and 4). Groups 3 and 4 showed that the anti-biofilm action of SN_{3J6} and the loss of cell
405 viability in biofilms can occur independently from each other. Interestingly, the two non-
406 sensitive isolates (group 5) belong to the same genus as the 3J6 strain, *Pseudoalteromonas*.
407 Although the number of tested strains remains small, these data suggest that
408 *Pseudoalteromonas* sp. 3J6 allows the growth of *Pseudoalteromonas* in marine biofilms while
409 impairing the biofilm development of a variety of other marine bacteria. The sensitive strains
410 indeed displayed various taxonomic relationships with the *Pseudoalteromonas* genus: they
411 belong to the same class and order (*Gammaproteobacteria*, *Alteromonadales* in the
412 *Alteromonas* sp. 1J3 and *Colwellia* sp. 4J3 cases), to a different order (Vibrionales for *Vibrio*
413 sp. D01 and D66) of the same class, to a different class (*Alphaproteobacteria* for *Paracoccus*
414 sp. 4M6 and *Sulfitobacter* sp. 8J6), or even to a different superphylum
415 (*Bacteroidetes*/*Chlorobi* group for *Algibacter* 1M6).

416

SN_{3J6} anti-biofilm activity against human pathogens. We furthermore examined the effect of SN_{3J6} on bacteria belonging to the human pathogen species *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Escherichia coli*. These bacteria belong to the same class as *Pseudoalteromonas* (*Gammaproteobacteria*), but to different orders (*Pseudomonadales* for *Pseudomonas*; *Enterobacteriales* for *Salmonella* and *Escherichia*). We used strains tagged by genes encoding a yellow (eYFP) or red (HcRed or DsRed) fluorescent protein in order to visualize biofilms without the addition of fluorescent dye. Consistently with its wide spectrum of action observed above, SN_{3J6} impaired the biofilm formation of all three strains: the biovolumes of *P. aeruginosa* $PAO1_{YFP}$, *S. enterica* $MB1409_{HcRed}$, and *E. coli* $CC118_{DsRed}$ biofilms were 3, 5.3, and 2.7 fold lower, respectively, when the adhesion step was performed in the presence of SN_{3J6} (Fig. 4). CLSM images showed that these biovolume reductions resulted from peculiar effects on *P. aeruginosa* and *E. coli*. *P. aeruginosa* $PAO1_{YFP}$ control biofilms (about 50 μm thick) were not homogeneous with respect to the cell density: it was high in the base of the biofilms, from which it decreased progressively in the upper part (Fig. 4A). This suggested that cells were embedded into increasing amounts of extracellular matrix from the base to the top of the biofilms. By contrast, the biofilm resulting from incubation with SN_{3J6} was thinner (about 5 fold) but homogeneous with a high cell density, which led to a relatively high biovolume (Fig. 4D). The *P. aeruginosa* $PAO1_{YFP}$ biofilm formation was therefore more impaired by SN_{3J6} than indicated by the sole biovolume comparison. On the opposite, SN_{3J6}-resulting biofilms of *E. coli* $CC118_{DsRed}$ differed from control biofilms by their biovolumes, whereas the biofilm structures and maximal thicknesses were similar (Fig. 4C and F). The biovolume reduction in *E. coli* biofilms obtained after SN_{3J6} incubation therefore resulted from a lower cell density. In this study, this is the only example of a biovolume reduction which occurred without a lowering of biofilm maximal thickness.

DISCUSSION

We report here an anti-biofilm activity exerted by *Pseudoalteromonas* sp. 3J6 exoproducts on a wide range of Gram negative bacteria, but not on the other tested strains of the same genus. This activity leads *Pseudoalteromonas* sp. 3J6 to predominate over other bacterial isolates such as *Vibrio* sp. D01 and *Paracoccus* sp. 4M6 in two-species biofilms. Marine *Pseudoalteromonas* bacteria are known as producers of a variety of biologically active extracellular compounds, including anti-bacterial agents which can lead to anti-fouling effects (2, 11, 13, 15). The best known example is the anti-bacterial protein AlpP secreted by *Pseudoalteromonas tunicata* D2 (16). AlpP provides to the latter a competitive advantage for biofilm formation in the marine environment and for the colonization of the surface of the green alga *Ulva australis* (30, 31). Consistently, AlpP producer strains seem rather widespread in Northern Europe since *P. tunica* D2 was isolated from the surface of the tunicate *Ciona intestinalis* collected from waters off the Swedish west coast (14), and the *alpP* gene was PCR-amplified from marine samples collected on *Ciona intestinalis* and on the green algae *Ulva lactuca* and *Ulvaria fusca* in the waters around Aarhus, Denmark (35). AlpP furthermore mediates cell death in mature biofilms of its own producer strain, leading to the dispersal of surviving cells within the marine environment (23). *Pseudoalteromonas* sp. 3J6 is unlikely to produce AlpP since we failed to PCR-amplify any *alpP* gene fragment from *Pseudoalteromonas* sp. 3J6 DNA with four different primer couples (data not shown). Furthermore, the anti-biofilm molecule produced by *Pseudoalteromonas* sp. 3J6 presents the originality of differing from AlpP and other classical anti-bacterial compounds by its biofilm-specific activity: SN_{3J6} displayed no anti-bacterial activity against *Paracoccus* sp. 4M6 and *Vibrio* sp. D01 in liquid, on agar plates or during the adhesion step, whereas SN_{3J6} impaired biofilm formation by these two strains and, more generally, by a variety of Gram negative

467 bacteria. Interestingly, this anti-biofilm effect was accompanied in most of the cases by a loss
468 of cell viability in biofilms. We propose the two following hypotheses to explain this
469 observation. SN_{3J6} would display a direct bactericidal activity against biofilm-included cells,
470 but not against free-living cells of the same strains. This could occur if the target of the anti-
471 bacterial compound is produced (or accessible) only in biofilm cells. The anti-bacterial agent
472 is unlikely to be a lytic phage released by *Pseudoalteromonas* sp. 3J6 that targets a biofilm
473 specific receptor, since we did not observe any phage-like particle in SN_{3J6} by scanning
474 electron microscopy (data not shown). In an alternative hypothesis, SN_{3J6} would act indirectly
475 on biofilm cells, perhaps by somehow stimulating the regulated cell death which takes place
476 during biofilm maturation and is thought to be caused by the self-destruction (suicide) of
477 individual cells (1). Cell death contributes to normal biofilm development by leading to
478 release of genomic DNA, which can be a component of the biofilm extracellular matrix, and
479 to the dispersal of surviving cells (1, 38). Although it is obvious that the loss of cell viability
480 could contribute to the anti-biofilm action of SN_{3J6}, these two phenomena occurred
481 independently from each other in three examples (Table 5, strains of groups 3 and 4), which
482 suggests that none of them is the consequence of the other one.

483 To our knowledge, very few anti-biofilm molecules secreted by bacteria and devoid of
484 anti-bacterial activity against free-living cells were previously reported. *E. coli* strains
485 producing group II capsules release in their environment a soluble polysaccharide preventing
486 biofilm formation by a wide range of Gram positive and negative bacteria (37). This
487 polysaccharide acts both on the initial adhesion by weakening cell-surface contacts, and on
488 the subsequent biofilm formation by reducing cell-cell interactions. In another study, a
489 lipopeptide biosurfactant from a *Bacillus circulans* strain of marine origin displayed anti-
490 adhesive properties against various bacteria but its effect on biofilm formation was not
491 investigated (4). This suggests that the reduced bacterial attachment due to SN_{3J6} could result

492 from a polysaccharidic molecule or a biosurfactant. However this adhesion impairment might
493 not fully account for the subsequent biofilm formation defect, and does not explain the
494 decrease in cell viability within the biofilms. Quorum sensing (QS) contributes to the control
495 of biofilm formation either negatively in the cases of *Vibrio cholerae* and *Staphylococcus*
496 *aureus*, or positively in the case of *Pseudomonas aeruginosa* (17). QS inhibitors are
497 considered as anti-pathogenic drugs, which can be used to reduce virulence and to increase
498 biofilm susceptibility to anti-microbial compounds and human immunity cells (32), and as
499 species-specific anti-biofilm molecules (9). QS signal molecules of the homoserine lactone
500 type were shown to accumulate in some marine biofilms (6) and the production of QS
501 inhibitor(s) by *Pseudoalteromonas* sp. 3J6 is a possibility. It would not however fully explain
502 the anti-biofilm activity of SN_{3J6}, since all SN_{3J6}-sensitive strains do not share similar QS
503 systems: *P. aeruginosa* produces several homoserine lactones whereas *E. coli* does not use
504 such signaling molecules. Alternatively, the anti-biofilm effects of *Pseudoalteromonas* sp.
505 3J6 exoproducts could be due to a novel molecule or on the complementary actions of several
506 molecules. We are currently undertaking the purification and identification of the active
507 molecule(s), which is required to understand its (their) mode(s) of action and to more
508 precisely define which of the potential applications can be drawn. The latter could extend
509 from the use of *Pseudoalteromonas* sp. 3J6 to prevent biofilm formation of undesirable
510 bacteria in aquaculture or fish farming to the use of its exoproducts in anti-biofilm strategies.
511 From this point of view, several features of *Pseudoalteromonas* sp. 3J6 exoproducts are
512 particularly attractive: i) the fact that, in addition to impair biofilm growth, they lead to
513 biofilms containing a majority of non viable cells; and ii) their wide spectrum of action, which
514 does not limit their use to the marine environment. Medical applications could be imagined
515 since this spectrum includes human pathogens such as *P. aeruginosa*, the biofilms of which

516 are particularly difficult to reduce during chronic infection of the lung of cystic fibrosis
517 patients (29).
518
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530

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638

FIGURE LEGENDS

FIG. 1. Confocal laser scanning microscopy (CLSM) images of single- and two-species biofilms. Biofilms were grown on glass surfaces at 20°C for 48 h under a flow of VNSS medium. Single-species biofilms of *Pseudoalteromonas* sp. 3J6(pCJS10) (A, B), *Paracoccus* sp. 4M6 (C), *Vibrio* sp. D01 (E) and *Bacillus* sp. 4J6 (G) are shown. *Pseudoalteromonas* sp. 3J6(pCJS10) biofilm was visualized by detecting only the GFP fluorescence (A) and by staining the cells with Syto 61 Red and overlaying the GFP and the Syto 61 Red fluorescences (B). The other single-species biofilms were stained with Syto 61 Red (C, E, G). Two-species biofilms resulted from co-inoculations of *Pseudoalteromonas* sp. 3J6(pCJS10) and *Paracoccus* sp. 4M6 (D), or *Vibrio* sp. D01 (F), or *Bacillus* sp. 4J6 (H). Overlays of GFP fluorescence from *Pseudoalteromonas* sp. 3J6(pCJS10) and of Syto 61 Red fluorescence from all cells of the biofilms are displayed (D, F, H). Maximal projections in the *xy* plane (top of each panel) and 3D-shadow projections (bottom of each panel) are presented. Bar, 30 µm.

FIG. 2. Activity of *Pseudoalteromonas* sp. 3J6 culture supernatant (SN_{3J6}) on biofilm formation by *Paracoccus* sp. 4M6 and *Vibrio* sp. D01. Biofilms of *Paracoccus* sp. 4M6 (left panels) and *Vibrio* sp. D01 (right panels) were grown in flow cells (FC) for 48 h after a 2 h adhesion step in FC without SN_{3J6} (A, B) or in the presence of SN_{3J6} (C, D). Alternatively, bacteria were incubated with SN_{3J6} outside of the FC and washed prior to inoculation (E, F), or the FC glass coverslip was coated with SN_{3J6} and washed before inoculating SN_{3J6}-free bacteria (G, H). Biofilms were stained with Syto 61 Red. A 3D-shadow projection is shown for each biofilm. Bar, 30 µm.

663 FIG. 3. Effect of SN_{3J6} on bacterial adhesion at 20°C. *Vibrio* sp. D01 and *Paracoccus* sp. 4M6
664 were incubated for 2 h under static condition in flow cell channels in the presence or absence
665 of SN_{3J6}. CLSM images of attached bacteria were recorded, and the glass surfaces covered by
666 attached bacteria were calculated using the Image Tool software and normalized relatively to
667 the values obtained in the absence of SN_{3J6}, which were set to 100%. Each experiment was
668 performed twice, and the bacterial attachment was determined on 8 different sites of each
669 glass slide. The bars indicate the standard deviations, which were too small to be visible in the
670 absence of SN_{3J6}.
671
672 FIG. 4. Effect of SN_{3J6} on biofilm formation by *Pseudomonas aeruginosa*, *Salmonella*
673 *enterica*, and *Escherichia coli*. Biofilms of *P. aeruginosa* $PAO1_{YFP}$ (A, D), *Salmonella*
674 *enterica* $MB1409_{HcRed}$ (B, E), and *Escherichia coli* $CC118_{DsRed}$ (C, F) were grown at 20°C for
675 48 h in LB broth after a 2 h adhesion step without SN_{3J6} (A, B, C) or in the presence of SN_{3J6}
676 (D, E, F). The yellow (eYFP) or red (HcRed or DsRed) fluorescent protein produced by each
677 strain was detected. A 3D-shadow representation and a side view projection are shown on the
678 top and bottom, respectively, of each panel. For each experiment, maximal thicknesses and
679 biovolumes were calculated from COMSTAT analyses of 12 to 18 images stacks obtained
680 from at least two independent biofilms. Standard deviations were lower than 10% of the
681 values. Bar, 30 µm.

682 TABLE 1. Strains and plasmid used in this study

Strain or plasmid	Origin or construction	Characteristics[d]	Reference or source
Strain			
Pseudoalteromonas sp. 3J6	Morbihan gulf, France[a]	Wild type strain, Sm^R	10
Paracoccus sp. 4M6	Morbihan gulf [a]	Wild type strain	10
Bacillus sp. 4J6	Morbihan gulf [a]	Wild type strain	10
Vibrio sp. D01	Bay of Brest, France[b]	Wild type strain	27
Algibacter sp. 1M6	Morbihan gulf [a]	Wild type strain, initially affiliated to the *Cytophaga* genus	10
Colwellia sp. 4J3	Morbihan gulf [a]	Wild type strain	10
Sulfitobacter sp. 8J6	Morbihan gulf [a]	Wild type strain	10
Vibrio sp. D66	Bay of Brest[b]	Wild type strain	IFREMER collection
Alteromonas sp. 1J3	Morbihan gulf [a]	Wild type strain	LBCM collection
Pseudoalteromonas sp. 3J3	Morbihan gulf [a]	Wild type strain	10
Pseudoalteromonas sp. D41	Bay of Brest[c]	Wild type strain	20, 21, 27
Escherichia coli DH5α		*sup*E44, Δ*lac*U169(Φ*lac*ZΔM15), *rec*A1, *end*A1, *hsd*R17, *thi*-1, *gyr*A96, *rel*A1	Biomedal S.L., Spain

Pseudomonas aeruginosa $PAO1_{YFP}$	Wild type PAO1 strain tagged with eYFP in a mini-Tn*7* construct	miniTn*7*(Gm)$P_{A1/04/03}$-*eyfp*-a, Gm^R	18
Escherichia coli $CC118_{DsRed}$	CC118(λpir) strain containing pUT-miniTn*5*(Gm)$P_{rrnB\ P1}$ *DsRed*	DsRed, Gm^R	T. Tolker-Nielsen
Salmonella enterica $MB1409_{HcRed}$	Wild type MB1409 strain containing pBK-miniTn*7*(Km, Sm)$P_{A1/04/03}$-*HcRed*-a	HcRed, Kan^R, Cm^R	19, 25, G. Legendre
Plasmid			
pCJS10	*gfp*mut3 gene cloned into plasmid RSF1010	GFP, Cm^R	30
pRK2013	Helper plasmid for pCJS10 transfer	oriColE1, RK2-Mob^+, RK2-Tra^+, Kan^R	Biomedal S.L., Spain

[a] Isolated from a glass slide immersed for 3 or 6 h in the Morbihan gulf, France

[b] Isolated from a glass slide immersed for 6 h in the bay of Brest, France

[c] Isolated from a Teflon coupon immersed for 24 h in the bay of Brest, France

[d] Cm^R, chloramphenicol resistance; Kan^R, kanamycin resistance; Sm^R, streptomycin resistance; Gm^R, gentamycin resistance.

690 TABLE 2. Quantitative PCR primers and TaqMan probes used in this study

Primer or probe	Sequence (5'-3')[a]	Target
Primer		
3J6 F	CGAACTGGCAAACTAGAGTGTGAG	*Pseudoalteromonas* sp. 3J6
3J6 R	CCGAGGCTCCGAGCTTCTA	
D01 F	TGAAACTGGTGAACTAGAGTGCTGT	*Vibrio* sp. D01
D01 R	CTCAAGGCCACAACCTCCA	
4J6 F	CAACCGTGGAGGGTCATTG	*Bacillus* sp. 4J6
4J6 R	GCGGAAACCCTCTAACACCTT	
4M6 F	TGGAACTGCCTTTGAAACTATCAG	*Paracoccus* sp. 4M6
4M6 R	CATGCTTGCCGACGTCTG	
TaqMan probe		
AD3J6	VIC-CACTGACGCTCATGTAC-MGB-NFQ	*Pseudoalteromonas* sp. 3J6
ADD01	6-FAM-CTGACACTCAGATGCGA-MGB-NFQ	*Vibrio* sp. D01
AD4J6	6-FAM-TTCGCGCCTCAGTGTCA-MGB-NFQ	*Bacillus* sp. 4J6

691

692 [a] VIC and 6-FAM (6-carboxyfluorescein), fluorescent dyes; MGB, minor groove binder;
693 NFQ, non-fluorescent quencher.

694

695

696 TABLE 3. COMSTAT analyses of single- and two-species biofilms after 48 h of growth.

Biofilm	Maximal thickness (μm)[a]	Average thickness (μm)[a]	Biovolume ($\mu m^3/\mu m^2$)[a]
Single-species			
3J6(pCJS10)	21	4.8	2.6
4M6	68	8.2	2.6
D01	34	8.7	5.4
4J6	76	19.0	10.4
Two-species			
3J6(pCJS10) + 4M6	24	2.5	2.5
3J6(pCJS10) + D01	44	5.8	4.6
3J6(pCJS10) + 4J6	92	12.0	17.6

697

698 [a] Averages of data from three independent experiments, with standard deviations lower than

699 10 % of each value.

700

701 TABLE 4. Quantification of each strain in two-species biofilms.

Strain	Proportion of each strain (%)	
	Biofilm biovolume[a]	qPCR analyses[b]
3J6(pCJS10)-4M6 biofilms		
3J6(pCJS10)	84	89
4M6	16	11
3J6(pCJS10)-D01 biofilms		
3J6(pCJS10)	76	95
D01	24	5
3J6(pCJS10)-4J6 biofilms		
3J6(pCJS10)	35	62
4J6	65	38

702

703 [a] Biovolumes were obtained by COMSTAT analyses performed on three independent
704 experiments, with standard deviations lower than 10 % of each value.

705 [b] For each biofilm analysis, total DNAs were extracted from two different biofilms and the
706 two samples were analyzed independently, with standard deviations lower than 10 % of each
707 value.

708

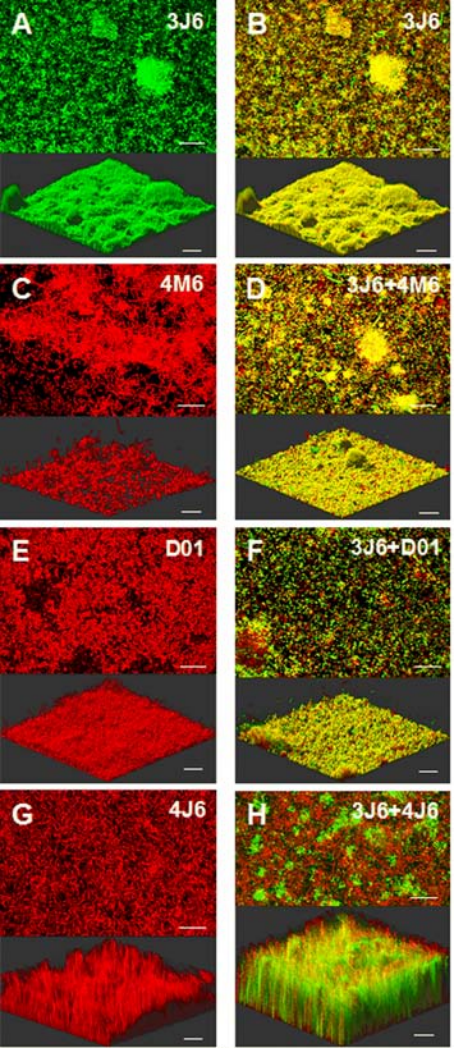
709 TABLE 5. Effect of SN_{3J6} on marine bacteria: characteristics of single-species biofilms
710 obtained without SN_{3J6} or in the presence of SN_{3J6} during the adhesion step.

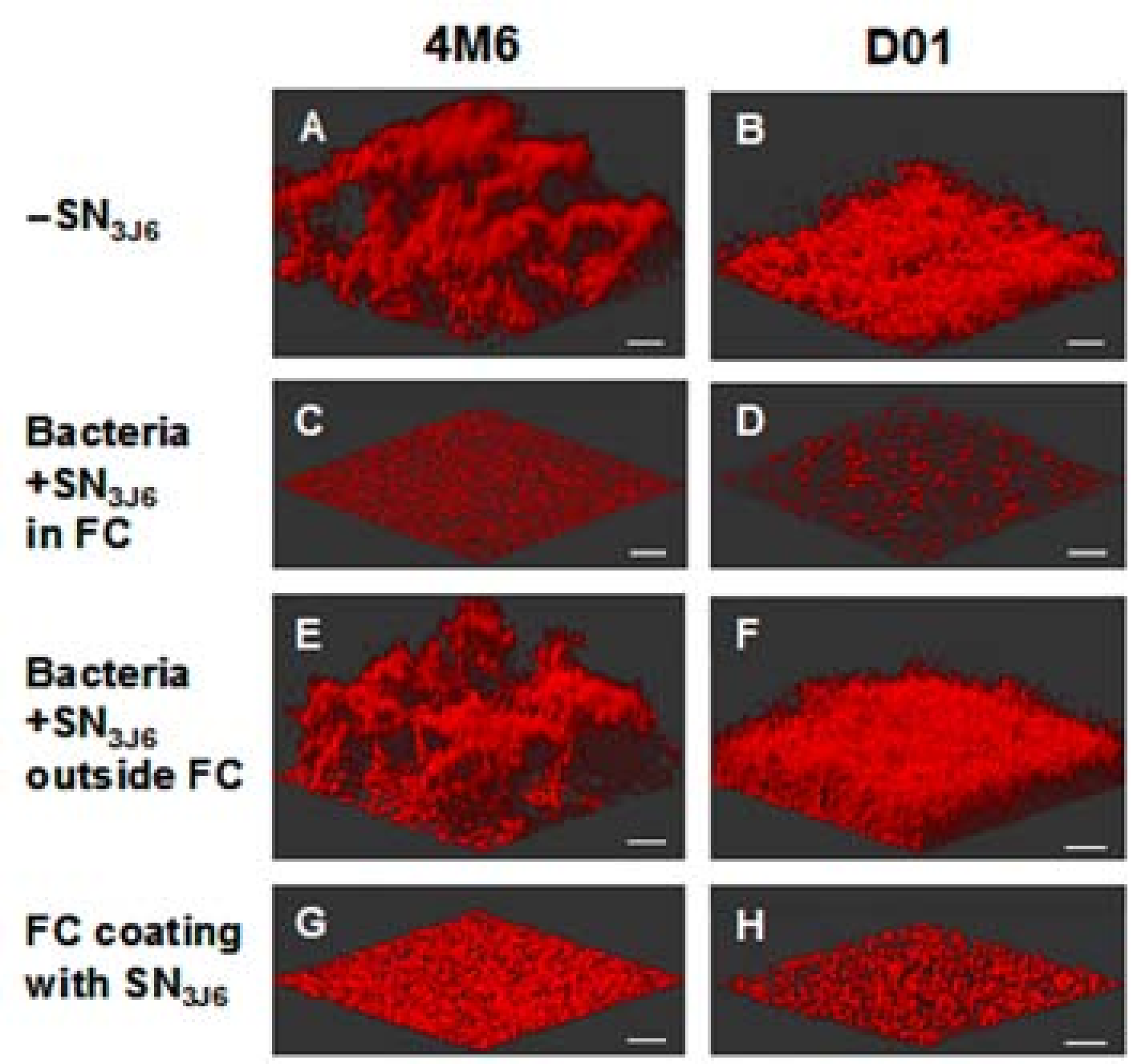
Strain[a]	Maximal thickness (μm)[b]		Biovolume ($\mu m^3/\mu m^2$)[b]		Non viable cells (%)[b]	
	− SN_{3J6}	+ SN_{3J6}	− SN_{3J6}	+ SN_{3J6}	− SN_{3J6}	+ SN_{3J6}
Group 1						
Algibacter sp. 1M6	44.6	7.4	15.9	0.03	0.4	89
Group 2						
Paracoccus sp. 4M6	95.0	13.2	6.1	0.88	1.2	100
Vibrio sp. D01	45.2	16	14.2	0.68	13	78
Colwellia sp. 4J3	28.7	11.3	5.9	0.27	25	97
Group 3						
Sulfitobacter sp. 8J6	14.4	9.0	2.0	0.66	29	8.9
Vibrio sp. D66	122	46.8	15.2	1.9	3.7	2.5
Group 4						
Alteromonas sp. 1J3	14.5	16.9	3.2	2.9	8.5	80
Group 5						
Pseudoalteromonas sp. 3J3	10.4	14.5	3.9	3.7	46	36
Pseudoalteromonas sp. D41	11.8	10.2	2.1	1.9	0.6	12.2

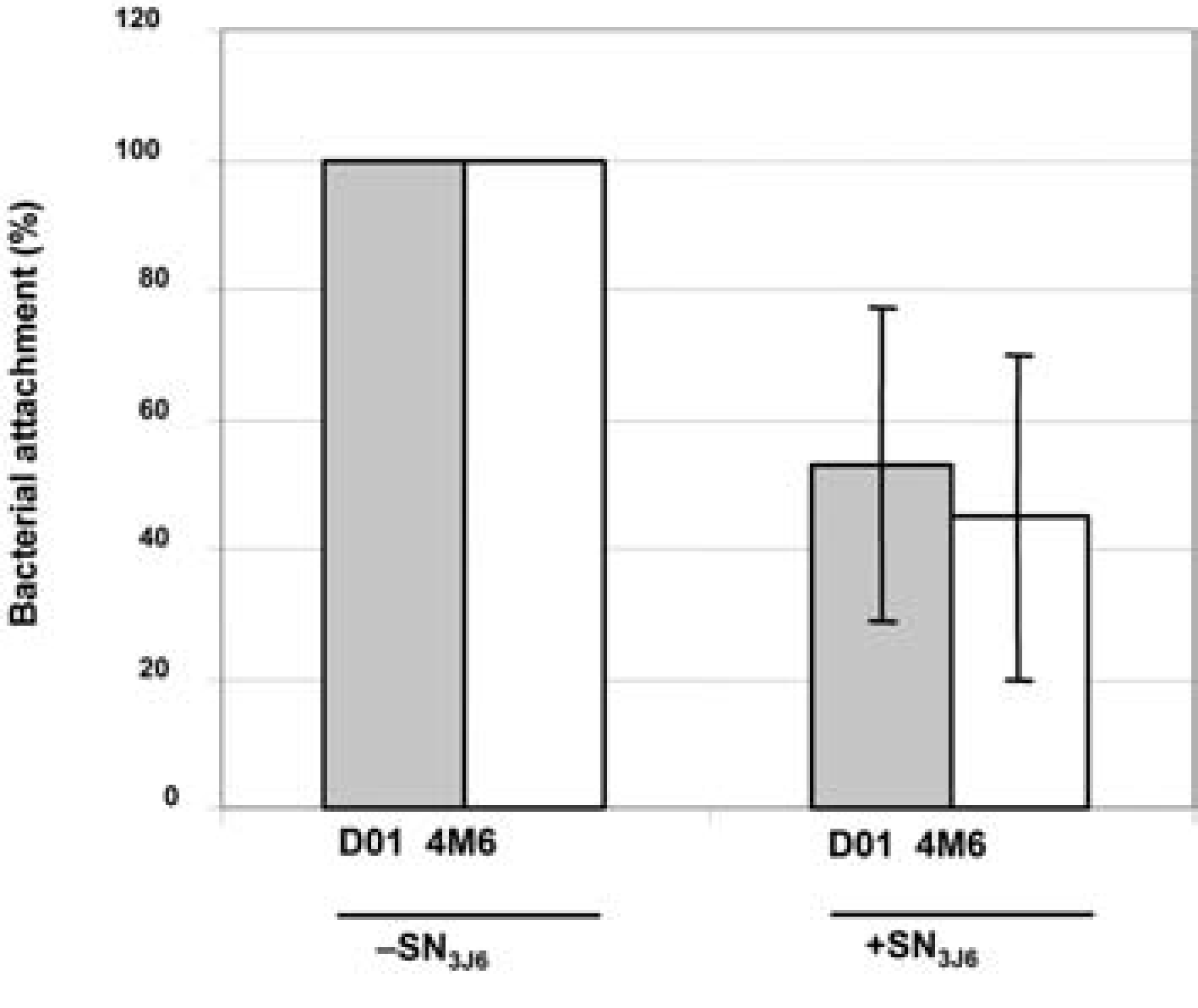
711

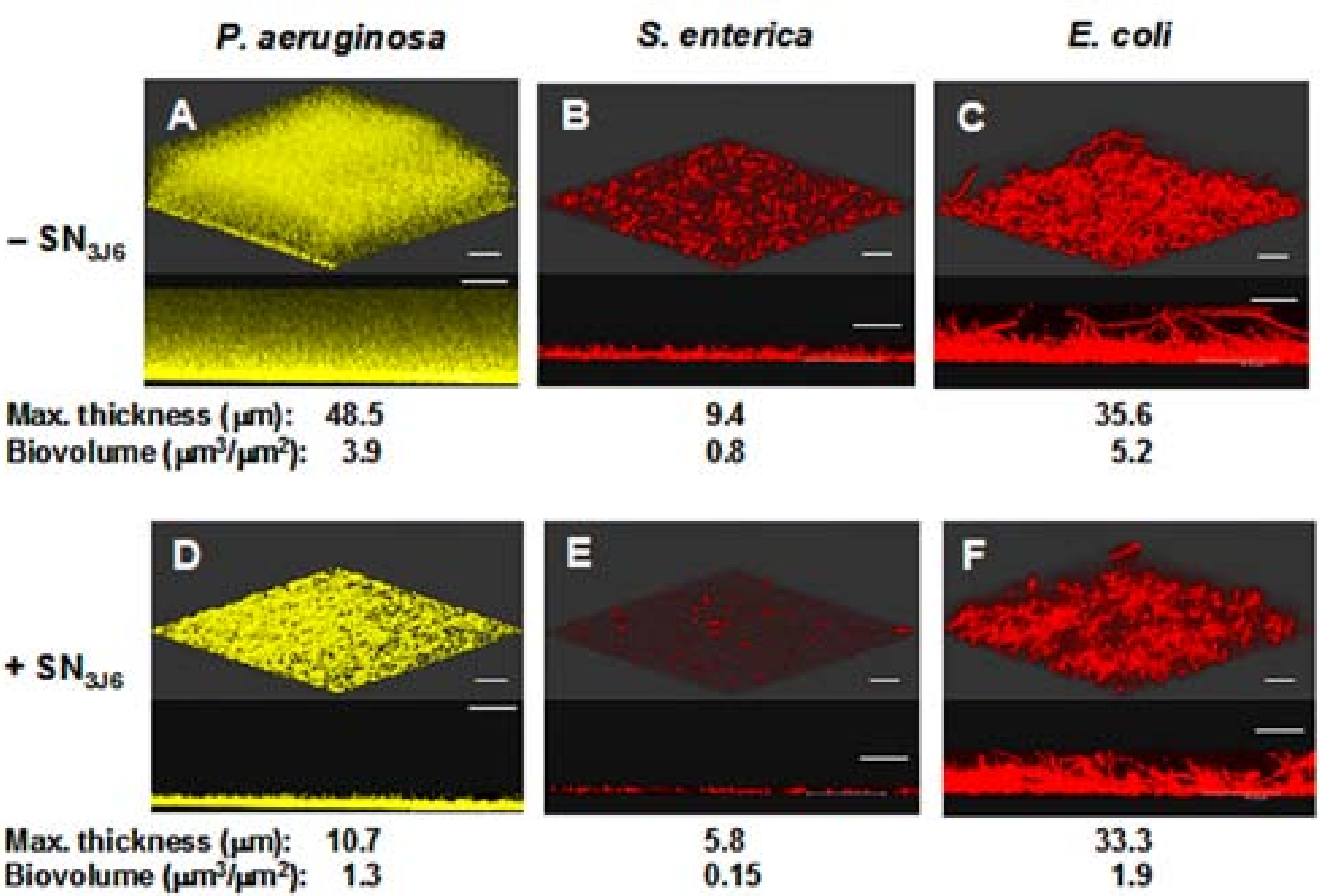
712 [a] Groups were defined according to the sensitivity of each strain to SN_{3J6} (see text for details).
713 [b] COMSTAT analyses were performed on three independent experiments, and standards
714 deviations were lower than 10% of each value.

715


A
3J6
B
3J6
C
4M6
D
3J6+4M6
E
D01
F
3J6+D01
G
4J6
H
3J6+4J6


4M6
D01
A
B
C
D
E
F
G
H
–SN3J6
Bacteria +SN3J6 in FC
Bacteria +SN3J6 outside FC
FC coating with SN3J6


120
100
80
60
40
20
0
Bacterial attachment (%)
D01 4M6
D01 4M6
–SN3J6
+SN3J6


P. aeruginosa
S. enterica
E. coli
A
B
C
– SN3J6
Max. thickness (µm): 48.5 9.4 35.6
Biovolume (µm³/µm²): 3.9 0.8 5.2
D
E
F
+ SN3J6
Max. thickness (µm): 10.7 5.8 33.3
Biovolume (µm³/µm²): 1.3 0.15 1.9