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Antibiofilm activity in the culture supernatant of a marine *Pseudomonas* sp. bacterium

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Abstract

In the marine environment, most solid surfaces are covered by microbial biofilms, mainly composed of bacteria and diatoms. The negative effects of biofilms on materials and equipment are numerous and pose a major problem for industry and human activities. Since marine micro-organisms are an important source of bioactive metabolites, it is possible that they synthesize natural ecofriendly molecules that inhibit the adhesion of organisms. In this work, the antibiofilm potential of marine bacteria was investigated using *Flavobacterium* sp. II2003 as a target. This strain is potentially a pioneer strain of bacteria that was previously selected from marine biofilms for its strong biofilm-forming ability. The culture supernatants of 86 marine heterotrophic bacteria were tested for their ability to inhibit *Flavobacterium* sp. II2003 biofilm formation and the *Pseudomonas* sp. IV2006 strain was identified as producing a strong antibiofilm activity. The *Pseudomonas* sp. IV2006 culture supernatant (SN_{IV2006}) inhibited *Flavobacterium* sp. II2003 adhesion without killing the bacteria or inhibiting its growth. Moreover, SN_{IV2006} had no effect on the *Flavobacterium* sp. II2003 cell surface hydrophilic/hydrophobic and general Lewis acid–base characteristics, but modified the surface properties of glass, making it on the whole more hydrophilic and more alkaline and significantly reducing bacterial cell adhesion. The glass-coating molecules produced by *Pseudomonas* sp. IV2006 were found to probably be polysaccharides, whereas the antibiofilm molecules contained in SN_{IV2006} and acting during the 2 h adhesion step on glass and polystyrene surfaces would be proteinaceous. Finally, SN_{IV2006} exhibited a broad spectrum of antibiofilm activity on other marine bacteria such as *Flavobacterium* species that are pathogenic for fish, and human pathogens in both the medical environment, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and in the food industry, such as *Yersinia enterocolitica*. Thus, a wide range of applications could be envisaged for the SN_{IV2006} compounds, both in aquaculture and human health.

INTRODUCTION

Biofilm is the predominant mode of growth for micro-organisms in most environments. It is defined as dense bacterial communities of cell–cell and cell–surface attachments to a substratum encased in a self-produced extracellular polymeric matrix, which is necessary for the formation and stabilization of biofilm colonies [1–3]. The structural components of the extracellular matrix include proteins, DNA, RNA, lipids and polysaccharides [3]. Compared with the planktonic lifestyle, biofilms improve access to nutrients and protect against stress, predators, antibiotics and the host immune system [1, 2, 4]. Biofilm formation is generally described as a temporal process involving a succession of different stages: first, a reversible attachment of micro-organisms to conditioned surfaces, then an irreversible attachment, and finally biofilm maturation through synergistic and/or competitive interactions between the different microbial communities [4]. Indeed, sessile bacteria may secrete anti-biofilm molecules that regulate biofilm architecture or mediate the release of cells during the dispersal stage of the biofilm life cycle.

Biofilms are known to be responsible for the survival of pathogenic bacteria [5, 6]. They are of great importance for human health because of their role in certain infectious diseases and their development in a wide range of ecosystems in the food industry, medical equipment and natural environments [5, 6]. In the marine environment, biofilms can also alter the properties of colonized metallic structures through the phenomenon of microbiologically influenced corrosion, particularly due to the intense metabolic activity

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of some bacteria [7]. This phenomenon causes great damage to maritime infrastructure, resulting in serious economic losses. The costs related to the fouling of ship hulls also have an important economic impact due to increased fuel consumption and maintenance [8].

Research into the prevention and treatment of biofilms in various industrial and clinical settings is very active and many potential antibiofilm molecules isolated from plants and marine organisms have been identified in the last 10 years [5, 9, 10]. In contrast, despite the major economic challenges, anti-marine biofilm compounds of microbial origin have been relatively neglected [10]. There is a clear need to identify new natural compounds and the large diversity of bacteria in the marine environment constitutes a vast potential reservoir for novel molecules that could be used to control biofilm formation. These compounds should target adhesive properties without affecting bacterial viability in order to avoid the appearance of resistant mutants [11]. Thus, marine actinomycetes were demonstrated to be promising candidates for antifouling [12, 13] or for marine aquaculture because they inhibit the biofilm formation of pathogenic *Vibrio* strains [14]. The genus *Pseudoalteromonas* has also attracted attention because of its members' production of antibiofilm compounds with applications in both the marine environment and the medical field [15–17]. However, most research on antibiofilm compounds produced by marine bacteria has focused on the eradication of bacteria that are pathogenic in humans [18–22].

In a previous study, we built a collection of culturable marine bacteria isolated from different marine biofilms and screened it for the capacity of the bacteria to form biofilms [23]. *Flavobacterium* sp. II2003 was an interesting model because it develops a stable and mushroom-like biofilm. *Flavobacteriales* were identified among the pioneer and sustaining surface colonizers that contribute to the initial formation and development of marine biofilms, particularly on metallic surfaces [7, 24]. Moreover, *Flavobacterium* sp. are known as important pathogens in aquaculture [25] and have been detected in industrial and medical biofilms [26]. It would therefore be particularly interesting to find compounds that inhibit *Flavobacterium* sp. biofilm formation.

In the current study, our objective was to find marine bacteria exhibiting antibiofilm activity against *Flavobacterium* sp. II2003 and to characterize this activity. From our marine bacteria collection, the *Pseudomonas* sp. IV2006 strain was identified as producing a strong antibiofilm activity. The characterization of this activity showed that *Pseudomonas* sp. IV2006 exoproducts were particularly effective at inhibiting bacterial adhesion without killing the bacteria or inhibiting their growth. The effect of the exoproducts on biotic (*Flavobacterium* cells) and abiotic (substratum) surfaces was investigated and the physicochemical characteristics of the active compound(s) were determined.

Table 1. Bacterial strains able to form a biofilm and used for the antibiofilm activity spectrum

Strain	Reference and/or source	Culture conditions
Non-aquatic strains		
Staphylococcus aureus AH478	[66]	Luria–Bertani 37 °C
S. aureus ATCC27217	ATCC	
S. aureus ATCC29247	ATCC	
S. aureus RN4220	[67]	
Pseudomonas aeruginosa PAO1	[68]	
P. aeruginosa PA14	[69]	
Yersinia enterocolitica CIP106.676	CIP	
Bacillus subtilis ND Food	[70]	
B. subtilis 168	[71]	
Bacillus thuringiensis 407	[72]	
Marine strains		
Paracoccus sp. 4M6	[73] / Morbihan Gulf, France	Zobell 22 °C
Micrococcus luteus	LBCM	
Cellulophaga lytica DSM2039	DSMZ	
C. lytica DSM2040	DSMZ	
Tenacibaculum maritimum CIP103528T	CIP	
Vibrio lentus CIP107166T	CIP	
Vibrio anguillarum CIP6336T	CIP	
Roseovarius sp. VA014	[7] / Atlantic harbor, France	
Roseobacter sp. IV 3009	[23] / Intertidal mudflat, France	
Shewanella sp. IV 3014	[23] / Intertidal mudflat, France	
Flavobacterium sp. II2003	[23] / Intertidal mudflat, France	
Freshwater strain		
Flavobacterium psychrophilum CIP103534T	CIP	M36 20 °C

METHODS

Bacterial strains and media

The marine bacteria collection screened for antibiofilm activity is composed of 86 benthic bacteria collected at low tide from the intertidal temperate mudflat biofilm of Marennes-Oléron Bay (45°55′N, 01°06′W, Atlantic coast of France) [23]. The target *Flavobacterium* sp. II2003 strain for antibiofilm assays was isolated from the same location [23]. The bacterial strains able to form a biofilm and used for the antibiofilm activity spectrum are listed in Table 1. Marine isolates were grown in Zobell broth (pastone Bio-Rad, 4 g l^{-1}; yeast extract Bio-Rad, 1 g l^{-1}; sea salts Sigma, 30 g l^{-1}). Luria–Bertani (LB) medium

(Difco) was used for the growth of non-aquatic strains and M36 medium (tryptone Bio-Rad, 5 g l^{-1}; yeast extract Bio-Rad, 0.5 g l^{-1}; beef extract Bio-Rad, 0.2 g l^{-1}; sodium acetate Sigma, 0.2 g l^{-1}) was used for the freshwater strain. Solid media were prepared by adding agar (12 g l^{-1}, Biokar).

Preparation of culture supernatants

For the antibiofilm activity, benthic bacteria from our marine bacteria collection were grown in pools of 8 to 10 strains. Each strain was first individually grown in Zobell broth for 24 h at 22 °C and then diluted in broth to a final optical density of 1 at 600 nm (OD_{600}) before being mixed with the other strains of the pool. Then, 100 ml of 5% Zobell medium were inoculated with the bacterial pool and incubated at 22 °C with shaking for 24 h. The supernatants (SN) of the pool cultures were obtained by centrifuging the cultures (7000 ***g***, 15 min, 4 °C), and they were then passed through a 0.22 µm filter (Millipore PVDF) to sterilize them before storage at −80 °C until use. The SN of individual strain cultures were harvested and stored in the same way. After the screening step, the selected *Pseudomonas* sp. IV2006 strain was grown in Zobell broth supplemented with 30 g l^{-1} of glucose at 22 °C with shaking for 48 h to optimize the production of antibiofilm compounds. For some experiments, 1 vol of the selected active supernatant of *Pseudomonas* sp. IV2006, SN_{IV2006} was frozen at −80 °C, lyophilized at a pressure below 450 mTorr at −80 °C (Cryotec freeze-dryer) and resuspended in 0.1 vol of water to obtain a 10-fold concentrated supernatant (named SN_{IV2006} 10×).

ANTIBIOFILM ASSAYS

Microtitre plate assay (static conditions/ polystyrene surface)

Bacterial biofilms (the marine and non-marine bacteria listed in Table 1) were grown in microtitre plates as previously described by Doghri *et al.* [23]: an overnight bacterial culture was centrifuged for 10 min at 7000 ***g*** and cells were resuspended in artificial seawater for marine strains or saline solution (NaCl 9 g l^{-1}) for non-marine strains to a final OD_{600} of 0.25. One hundred and fifty microlitres of the resulting suspensions were then placed in wells of a 96-well microtitre plate (MICROTEST 96, Falcon). Artificial sea water or saline solution, without bacteria, served as negative controls. After a bacterial adhesion step of 2 h at 22 °C (marine bacteria) or 37 °C (non-marine bacteria), the wells were gently washed three times with artificial seawater or saline solution, respectively, and 150 µl of Zobell or LB medium, respectively, were added to each well. After incubation at 22 °C or 37 °C for 24 h, the microplates were washed three times. Then, the bacterial biofilms were stained with a 0.8% w/v crystal violet solution for 20 min and rinsed with ultra-pure water until the wash liquid was clear. Absorbed crystal violet was then eluted from the attached cells with 96% ethanol (150 µl per well) and quantification was carried out by measuring the OD_{595nm}.

To investigate the effect of the bacterial culture supernatants (grown in pools or as individual strains) on adhesion and biofilm formation, wells were inoculated with biofilm-forming cells resuspended in a solution of 50% v/v supernatant and 50% v/v artificial seawater for marine strains or sterile water for non-marine strains. After a 2 h adhesion step, biofilm formation was performed as described above. Moreover, the effect of the selected supernatant, SN_{IV2006} was further analysed using *Flavobacterium* sp. II2003 in two additional ways. (1) A solution of 50% v/v SN_{IV2006} and 50% v/v artificial seawater without bacterial strains was loaded onto the microplate and left for 2 h at 22 °C to coat the polystyrene surface. Then, the wells were rinsed with artificial seawater, *Flavobacterium* sp. II2003 bacteria were inoculated and biofilms were obtained as described before. (2) A solution of 50% v/v SN_{IV2006} and 50% v/v artificial seawater was loaded onto the wells after the *Flavobacterium* sp. II2003 biofilm maturation step and left for 2 h under static conditions at 22 °C. Biofilms were then quantified by measuring the OD_{595nm} after staining with crystal violet as described above. For each experiment, the culture supernatant was replaced with sterile culture medium in the negative controls. Three independent experiments were performed and for each experiment the test was repeated in at least three wells per microtitre plate.

The activity of SN_{IV2006} was calculated from the minimum concentration of biofilm inhibition. Activity (arbitrary units, a.u.) was determined using the following equation:

(1/last active dilution)×volume of reaction (ml).

Flow cell assay (dynamic conditions/glass surface)

Flavobacterium sp. II2003 biofilm was grown on glass slides in three-channel flow cells (channel dimensions 1×4×40 mm, Technical University of Denmark Systems Biology, Denmark) as previously described [23]: flow cells were inoculated with overnight bacterial cultures diluted in artificial seawater to a final OD_{600} of 0.1. Bacteria were allowed to attach to the substratum (microscope glass coverslip of 24×50 st1, KnittelGlasser) for 2 h at 22 °C without a flow of medium. The channels were then washed to remove non-attached cells by applying a flow of artificial seawater for 15 min at a rate of 2 ml h^{-1}. The biofilm was grown under a constant flow of Zobell medium (2 ml h^{-1}) for 24 h at 22 °C.

To investigate the effect of SN_{IV2006} on the adhesion and biofilm formation of *Flavobacterium* sp. II2003, three protocols were followed, as for the microplates. (1) a solution of 50% v/v SN_{IV2006} and 50 % v/v artificial seawater was injected without bacterial strains into the flow cell channels and left for 2 h at 22 °C without a flow of medium to coat the glass surface. Then, the channels were rinsed with artificial seawater before inoculating *Flavobacterium* sp. II2003 bacteria and growing the biofilms as described before. (2) Flow cells were inoculated with *Flavobacterium* sp. II2003 cultures grown for 24 h and resuspended in a solution of 50% v/v SN_{IV2006} and 50% v/v artificial seawater to a final OD_{600} of 0.1. After the 2 h adhesion step, biofilm formation was performed as initially described. (3) SN_{IV2006} was injected into the channels after the *Flavobacterium* sp. II2003 biofilm maturation step and left for 2 h under static conditions at 22 °C. For each experiment, the culture supernatant was replaced with sterile culture medium in the

negative controls. Microscopic observations were performed by confocal laser scanning microscopy (CLSM) using a TCS-SP2 system (Leica Microsystems). The biofilms were observed by staining cells with 5 µM Syto 61 red for 10 min. The biofilm stacks were analysed with COMSTAT software [developed in MATLAB (Mathworks, Inc., Natick, USA) [27]] to estimate the maximal and average thicknesses (µm) and the biovolume ($\mu m^3\ \mu m^{-2}$) of the biofilm. Each experiment was repeated three times, and three zones of each channel were analysed per experiment.

Coating assay in glass bottom dishes (static conditions/glass surface)

To investigate the coating effect of SN_{IV2006} on the adhesion of *Flavobacterium* sp. II2003, 1 ml of SN_{IV2006} was loaded into chambers of glass bottom dishes (CELLview, Greiner Bio-one) and left for 2 h at 22 °C to coat the glass surface. SN_{IV2006} was replaced with Zobell broth in the negative controls. Then, the chambers were rinsed three times with artificial seawater. Overnight cultures of *Flavobacterium* sp. II2003 were centrifuged for 10 min at 7000 ***g*** and resuspended in artificial seawater to a final OD_{600} of 0.1. Afterward, 1 ml of the resulting suspensions was inoculated into the chambers. After 2 h of incubation at 22 °C, the chambers were washed three times with artificial seawater. Bacteria were stained with DAPI (4µg ml^{-1}). After 20 min in the dark, the glass chambers were rinsed three times with ultra-pure water and dried. The samples were then observed under a Leitz DMR fluorescence microscope (Leica Microsystems). Five fields per chamber were observed. The images were processed with Countpixel software to determine the percentage of the glass surface that was covered by bacteria. Each experiment was repeated three times.

ANTIBACTERIAL ASSAYS

Agar well diffusion assay

The effect of SN_{IV2006} on bacterial growth was assayed by adapting the agar well diffusion assay previously described by Sablé *et al.* [28]. Solid nutrient plates (15 ml) were inoculated with approximately 10^7 target bacteria cells. Sterile glass rings (4 mm inner diameter) were placed on agar medium and filled with 30 µl of previously filter-sterilized supernatant (SN_{IV2006} or 10× SN_{IV2006}). The plates were incubated for 48 h at the optimum growth temperature for each target bacterium to allow bacterial growth and supernatant diffusion. The presence of a halo around the glass cylinder indicates an inhibition of bacterial growth if the halo is clear (without cell growth) or a stimulation of growth if the halo is denser than the remaining plate. SN_{IV2006} and 10× SN_{IV2006} were replaced with Zobell broth and 10× Zobell broth in the respective negative controls.

Liquid antibacterial assay

Target *Flavobacterium* sp. II2003 grown overnight were resuspended in a solution of 50% v/v SN_{IV2006} (or 50% v/v Zobell medium for the control) and 50% v/v artificial seawater at an OD_{600nm} of 0.25. After a 2 h incubation, an aliquot of the cell suspension was serially diluted and 100 µl of each dilution were then plated to count the colony-forming units (c.f.u.). The remaining non-diluted bacterial suspension was centrifuged and resuspended at 20% v/v in fresh medium. Growth was monitored by measuring the absorbance of the cultures at 600 nm.

Each experiment was repeated three times.

Determination of the surface energy properties

The surface energy properties were investigated on the same surface as that used in flow cells, i.e. a glass coverslip (24×50 st1, KnittelGlasser). The glass surface was prepared and sterilized as in a flow cell assay. To eliminate the salt effect, SN_{IV2006} (or Zobell medium for the negative control) was dialysed and then diluted to 50% v/v in ultra-pure water (MilliQ Millipore) instead of seawater. This solution was then used to coat the glass surface for 2 h at 22 °C. After a gentle wash with ultra-pure water and air drying for 40 min, the surface energy properties of the treated glass surfaces were determined from contact angle measurements obtained using a DSA100 Drop Shape Analyzer goniometer. Serial contact angle measurements (around 60 measurements on 3 independent surfaces) were performed for 3 independent pure liquids: ultra-pure water (Milli-Q Millipore), formamide (Sigma-Aldrich) and diiodomethane (Sigma-Aldrich). The three surface tension components of the coated substratum surface energy (γ_S), i.e. the Lifshitz–van der Waals (γs^{LW}), electron-donor (γs^-) and electron-acceptor (γs^+) components, were estimated using the Young–van Oss equation for each liquid [29]: $\cos\theta = -1+2(\gamma_S^{LW}\gamma_L^{LW})^{1/2}/\gamma_L + 2(\gamma_S^+\gamma_L^-)^{1/2}/\gamma_L + 2(\gamma_S^-\gamma_L^+)^{1/2}/\gamma_L$ where γ_L, γ_L^{LW}, γ_L^- and γ_L^+ are the total surface tension, Lifshitz–van der Waals, electron donor and electron acceptor components of the surface tension of a particular liquid, respectively.

The Lewis acid–base component was deduced from:

$$\gamma_s AB = 2\sqrt{\gamma_s^+}\gamma_s$$

and the total surface free energy was defined by:

$$\gamma_s = \gamma_s AB + \gamma_s LW$$

Microbial adhesion to solvent (MATS) assays

The hydrophobic/hydrophilic and Lewis acid–base characteristics of the target *Flavobacterium* sp. II2003 surface were determined using the MATS method described by Bellon-Fontaine *et al.* [30]. This partitioning method is based on the comparison between microbial cell affinity to couples of solvents. In each pair, one solvent is a monopolar solvent and the other is an apolar solvent, and both must have similar Lifshitz–van der Waals surface tension components. The monopolar solvent can be acidic (electron accepting) or alkaline (electron donating). The following couples were used: (i) chloroform (electron accepting)/hexadecane; (ii) ethyl acetate (electron donating)/decane; (iii) dichloromethane (electron accepting)/tetradecane. All solvents were obtained from Sigma-Aldrich and were of the highest purity grade.

Differences between the results obtained with chloroform and hexadecane (or dichloromethane and tetradecane), on the one hand, and between ethyl acetate and decane, on the other hand, indicate the electron donor and electron acceptor character, respectively, of a bacterial surface. The percentage of cells that adhered to hexadecane or tetradecane was used as a measure of cell surface hydrophobicity. Experimentally, a *Flavobacterium* sp. II2003 suspension containing approximately 10^8 cells ml^{-1} ($OD_{400\,nm}$ = 0.8) was prepared in artificial seawater and 1.5 ml of each bacterial suspension was mixed manually for 10 s and vortexed for 120 s with 0.25 ml of the solvent under investigation. The mixture was allowed to stand for 15 min to ensure complete separation of the two phases. The OD of the aqueous phase was measured at 400 nm. The percentage affinity of bacterial cells to each solvent was calculated by % affinity = $(1-A/A_0)\times100$, where A_0 is the $OD_{400\,nm}$ of the bacterial suspension before mixing and A is the $OD_{400\,nm}$ after mixing. Each experiment was repeated three times and five measures were performed per experiment. Percentage affinities are mean values of five measures.

Biosurfactant assay

In order to detect the presence of biosurfactant in SN_{IV2006}, we used the drop collapse test as described by Tugrul and Cansunar [31]. Briefly, drops of SN_{IV2006} were placed in wells of a microtitre plate coated with sunflower oil. Drops containing biosurfactant collapsed whereas non-surfactant-containing drops remained stable. Diluted liquid handwashing cream, distilled water and Zobell broth drops were used as controls.

Preliminary characterization of the active compounds

To elucidate the biochemical nature of the active compounds in SN_{IV2006}, different treatments were performed on SN_{IV2006}. SN_{IV2006} was replaced with Zobell broth in the negative controls. To digest proteins, proteinase K or pronase E was added at a final concentration of 1 mg ml^{-1} and the reaction mixture was incubated for 1 h at 37 °C. To degrade lipids, lipase acrylic resin formulation was used at a final concentration of 2 mg ml^{-1} and the reaction was incubated for 48 h under shaking at 37 °C. To digest the nucleic acids, DNaseI (100 µg ml^{-1}) or RNaseA (25 µg ml^{-1}) was added for 12 h at 37 °C. $NaIO_4$ was used to hydrolyze polysaccharides by cleaving the C–C bonds and oxidizing the carbon of vicinal hydroxyl groups [32]. We improved the standard treatment (20 mM $NaIO_4$, incubation for 2 h at 37 °C) by adding a neutralization step for the excess of $NaIO_4$ with ethylene glycol (1:100) for 2 h at 37 °C followed by a final dialysis step (1000 Da, Spectrum Labs.com) [33]. To evaluate heat sensitivity, SN_{IV2006} samples were incubated for 30 min at 50 °C, 70 and 100 °C.

The activity of all treated SN_{IV2006} samples was then evaluated in two different assays. (1) Treated supernatants were loaded onto chambers of glass bottom dishes and left for 2 h to coat the glass surface before the bacteria were loaded, as described previously. (see coating assay in glass bottom dishes). (2) The microtitre plate assay was employed, in which treated supernatants were mixed with the bacteria during the 2 h adhesion step. The effect of each SN_{IV2006} submitted to a chemical or thermal treatment was then compared with the effect of the native SN_{IV2006}.

Statistical analyses

Standard deviation was calculated using MATLAB software (Mathworks, Inc., Natick, USA). All values presented in the Results section are the averages of data from three independent experiments. For each experiment, at least three technical replicates were performed. In order to analyse differences between a sample and the corresponding control, Student's *t*-tests were performed. Differences were considered significant if P values were <0.05.

RESULTS

Screening of marine bacterial collection for antibiofilm activity against *Flavobacterium* sp. II2003

A wide range of heterotrophic bacteria (86 strains) was previously isolated from mudflat biofilms collected from the Marennes-Oléron Bay (Atlantic coast of France) at low tide. In the present work, this collection was screened for antibiofilm activity against *Flavobacterium* sp. II2003, from the same collection, which had been shown to form a stable biofilm [23]. To rapidly screen the whole collection, 9 pools of 8–10 strains were constituted, and the culture SN of each pool were tested for their potential effects on *Flavobacterium* sp. II2003 biofilm formation by mixing the supernatant with the cells during the 2 h adhesion step in a microplate. Four pool supernatants exhibited a significant inhibitory activity against biofilm formation (data not shown). The $pool_6$ supernatant presented the strongest inhibitory activity (70 %, Fig. 1) against biofilm formation and was chosen for further studies. The culture SN of each bacterial strain of $pool_6$ was then individually tested against the biofilm formation of the *Flavobacterium* sp. II2003 strain (Fig. 1). The strain showing the highest inhibitory activity, almost identical to that of $pool_6$, was *Pseudomonas* sp. IV2006, which was selected to be studied further.

Characterization of the *Pseudomonas* sp. IV2006 antibiofilm activity in microtitre plates

The production of antibiofilm compounds was increased by increasing the *Pseudomonas* sp. IV2006 culture time from 24h to 48h and by adding glucose (30 g l^{-1}) to the culture broth. These new conditions were then used for all experiments. When we examined the effect of increasing SN_{IV2006} concentrations on biofilm formation in 96-well microtitre plate wells, the *Pseudomonas* sp. IV2006 culture supernatant showed a dose-dependent antibiofilm effect (Fig. 2a). A reduction of about 70% of the *Flavobacterium* sp. II2003 biofilm was observed at a 1:2 dilution (50% v/v SN_{IV2006}). In these conditions, the arbitrary activity of SN_{IV2006} was estimated as 27 a.u. ml^{-1}. This concentration was then used in all subsequent experiments. It was shown that SN_{IV2006} affected biofilm formation when it was added to the *Flavobacterium* sp.

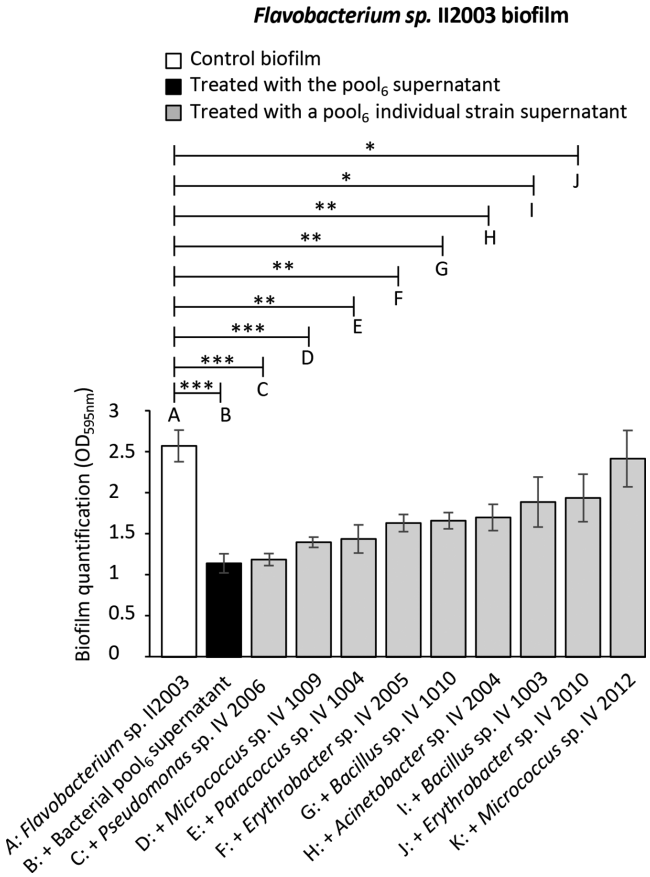

Fig. 1. Effect of the culture supernatants of the pool$_6$ strains, mixed or individual, on *Flavobacterium* sp. II2003 biofilm formation. *Flavobacterium* sp. II2003 was mixed for 2 h with the culture supernatants in 96-well microplates during the adhesion step. The *Flavobacterium* sp. II2003 biofilms were then grown at 22 °C for 24 h. Biofilms were stained with crystal violet and quantified by measuring absorbance at 595 nm. The data represent the mean values±SD of three replicates. Control biofilm: *Flavobacterium* sp. II2003 biofilm treated with culture medium instead of culture supernatants. Each biofilm treated with a culture supernatant was compared with the control biofilm. Significant differences are indicated by * (P<0.05), ** (P<0.01) or *** (P<0.001).

II2003 culture during the adhesion step. To examine whether SN_{IV2006} affected other stages of biofilm formation, its potential substrate coating activity was tested by incubating SN_{IV2006} for 2 h in microplate wells before the addition of *Flavobacterium* sp. II2003 cells. The possibility that SN_{IV2006} was active against the mature *Flavobacterium* sp. II2003 biofilm was also explored. However, these experiments showed that SN_{IV2006} could neither prevent cell adhesion (and consequently biofilm formation) when it was added before adhesion, nor deconstruct the mature biofilm in microtitre plates (Fig. 2b).

Overall characterization of the *Pseudomonas* sp. IV2006 antibiofilm activity in flow cells

SN_{IV2006} activity was studied in flow cells (on a glass surface) for the same three steps as with the microplates (Fig. 3): after coating a surface with SN_{IV2006}, during the 2 h adhesion step and on mature biofilm. The addition of SN_{IV2006} before the *Flavobacterium* sp. II2003 strain (i.e. coating glass with SN_{IV2006}, Fig. 3b) significantly impaired biofilm formation: a reduction of 60% in biofilm biovolume, as well as a reduction of 30% in the average and maximal biofilm thickness, were observed compared with the control. Thus, the biofilm formed on the surface treated with SN_{IV2006} was clearly thinner. Then, when SN_{IV2006} was added during the 2 h adhesion step on the glass surface, the biofilm formation of *Flavobacterium* sp. II2003 was totally inhibited (Fig. 3c). Furthermore, direct microscopic observations of attached cells in the presence of SN_{IV2006}, directly after the 2 h adhesion step, showed that no bacteria adhered during this step (data not shown), indicating that antibiofilm components of SN_{IV2006} influenced cell attachment to the glass surface. Lastly, SN_{IV2006} was not able to significantly modify the properties of the already formed biofilm (Fig. 3d), which demonstrated that SN_{IV2006} lacked components able to destroy the preformed biofilm.

SN_{IV2006} showed no antibacterial activity against free-living *Flavobacterium* sp. II2003 cells

We determined whether SN_{IV2006} contained an antibacterial substance using two distinct methods. With the agar well diffusion assay, no clear halo of *Flavobacterium* sp. II2003 growth inhibition was observed around the wells, indicating that SN_{IV2006} was neither bactericidal nor bacteriostatic for *Flavobacterium* sp. II2003. Similar results were obtained with the liquid antibacterial assay (Fig. 4). The number of c.f.u. ml^{-1} was evaluated after incubation of *Flavobacterium* sp. II2003 cells for 2 h with SN_{IV2006} or with Zobell broth (control), and was similar in both cases ($\approx 4.10^8$ c.f.u. ml^{-1}). Moreover, the number of bacteria was the same for both conditions throughout the experiment (Fig. 4). These results showed that SN_{IV2006} did not reduce cell viability during the 2 h adhesion step and only affected biofilm formation.

SN_{IV2006} modified the glass surface properties

SN_{IV2006} compounds have been shown to inhibit the formation of *Flavobacterium* sp. II2003 biofilm by coating the glass surface (Fig. 3). This activity was then further studied by investigating the overall glass surface energy properties before and after coating with SN_{IV2006}. The contact angles of water, formamide and diiodomethane were measured on both glass treated with Zobell broth (control surface) and glass coated with SN_{IV2006} (Table 2), and the corresponding derived surface energy components were determined (Table 3). Variance analysis of the results indicated a significant effect of SN_{IV2006} on water and formamide contact angles compared with the control surface (P<0.05). Both water and formamide contact angles decreased after coating the glass with SN_{IV2006}. The van der Waals component of the free surface γ^{LW} was 12% higher for the coated surfaces than the control surfaces. The electron donor component of the coated surface was 30% higher and, conversely, the electron acceptor component was 15% lower. The overall Lewis acid–base component and free surface energy of glass were not significantly modified after contact with SN_{IV2006}, but when looking at the individual surface

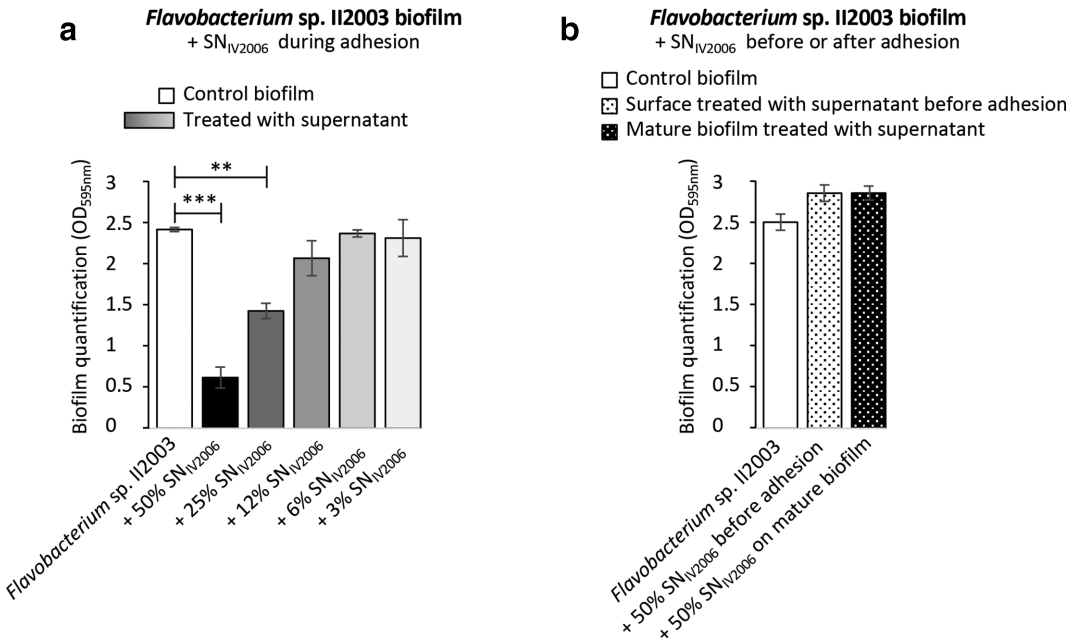

Fig. 2. Effect of SN$_{IV2006}$ on *Flavobacterium* sp. II2003 biofilm formation in a microtitre plate. (a) Effect of decreasing SN$_{IV2006}$ concentration during adhesion. (b) Effect of 50% v/v SN$_{IV2006}$ applied by coating the surface and on mature biofilm. The data represent the mean values±SD of three replicates. Control biofilm: *Flavobacterium* sp. II2003 biofilm treated with Zobell broth instead of SN$_{IV2006}$. Each biofilm treated with a culture supernatant was compared with the control biofilm. Significant differences are indicated by ** (P<0.01) or *** (P<0.001).

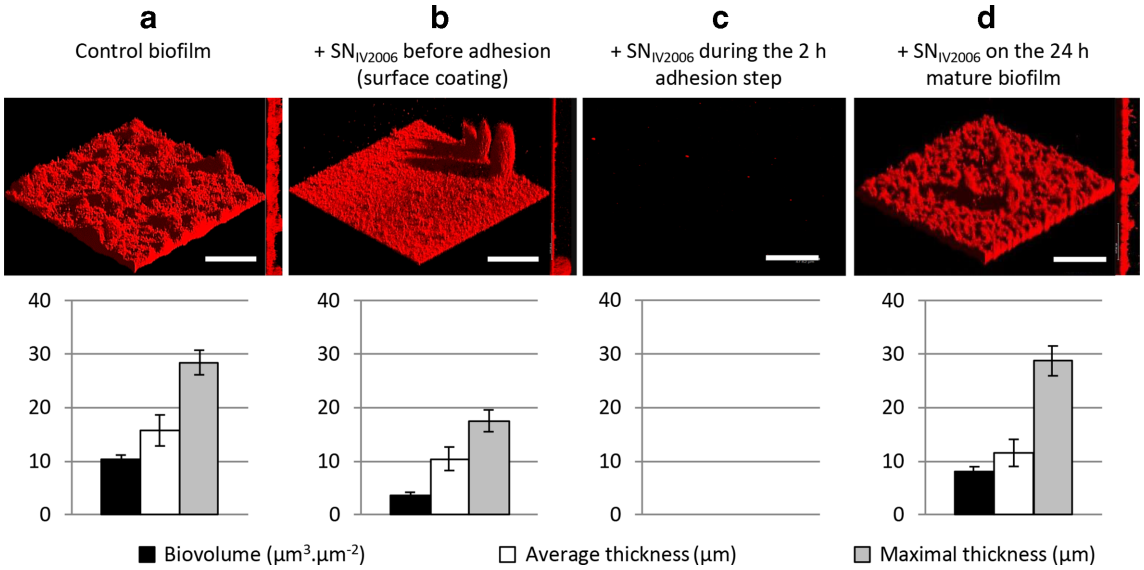

Fig. 3. Effect of SN$_{IV2006}$ on *Flavobacterium* sp. II2003 biofilm formation in flow cell chambers. The *Flavobacterium* sp. II2003 biofilms were grown at 22 °C for 24 h in Zobell broth after the 2 h adhesion step. (a) Control biofilm: SN$_{IV2006}$ was replaced by Zobell broth in assays B, C and D. The biofilms obtained in these three conditions were similar, and a representative biofilm is shown here. (b) SN$_{IV2006}$ was added before the cells to coat the glass surface for 2 h. (c) SN$_{IV2006}$ was added together with the cells during the 2 h adhesion step. (d) SN$_{IV2006}$ was added for 2 h after biofilm maturation. For each experiment, a 3D representation and a side view projection are shown. Average/maximal thickness and biovolume were calculated for each experiment from COMSTAT analyses of 10 image stacks obtained from 2 independent biofilms. The standard deviation was less than 10% of each value. White bars, 67.3 µm.

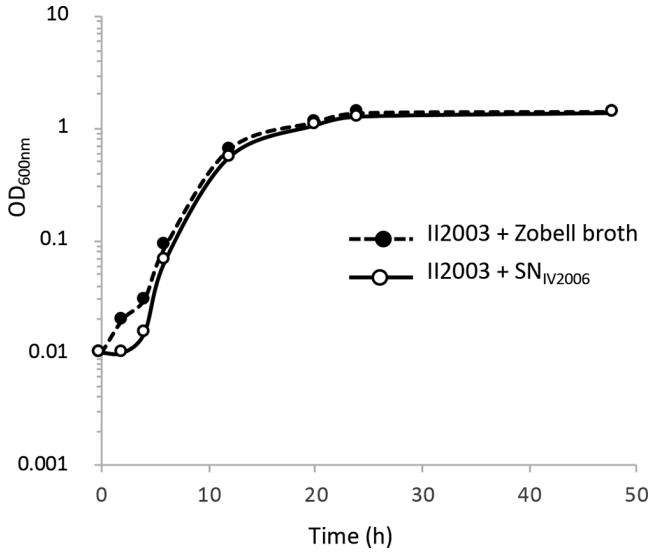

Fig. 4. Growth curves of *Flavobacterium* sp. II2003 after treatment with SN_{IV2006} or with Zobell broth (control) for 2 h**.** The growth was monitored by measuring the OD_{600nm}.

energy components, the results demonstrated that, on the whole, coating the glass with SN_{IV2006} made the glass surface more hydrophilic and more alkaline.

SN_{IV2006} had no effect on the *Flavobacterium* sp. II2003 cell surface hydrophilic/hydrophobic and Lewis acid–base characteristics

The hydrophobic/hydrophilic and Lewis acid–base characteristics of the *Flavobacterium* sp. II2003 surface incubated with SN_{IV2006} (or Zobell broth for the control) for 2 h were determined using the MATS method. This partitioning method is based on the comparison of bacterial cell affinity to a monopolar acidic (electron acceptor) or alkaline (electron donor) solvent and an apolar solvent with similar Lifshitz–van der Waals surface tension components. Hence, the percentage affinity of *Flavobacterium* sp. cells to the following six solvents, classified by pairs, was measured: chloroform (acidic)/hexadecane, dichloromethane (acidic)/tetradecane and ethyl acetate (alkaline)/decane. In all cases, no significant difference in percentage affinity was observed between cells treated with SN_{IV2006} and cells treated with Zobell broth only

Table 2. Contact angle measurements obtained with water, formamide and diiodomethane on a glass surface with SN_{IV2006} or with Zobell broth

	Water	Formamide	Diiodomethane
Glass treated with Zobell broth (control)*	49.05	39.4	55.1
Glass coated with SN_{IV2006}*	37	28.06	50.1

Each value is the mean of approximately 60 droplet measurements performed on 2 independent samples.
*Standard deviation less than 10% of each value.

Table 3. Surface tension components of the uncoated and coated glass surface calculated from the contact angle measurements: Lifshitz–van der Walls (γ^{LW}), electron acceptor (γ^{+}) and electron donor (γ^{-}) components, Lewis acid–base component γ^{AB} and total surface free energy γ_S

	Surface energy ($mJ.m^{-2}$)				
	γ^{LW}	γ_S^{+}	γ_S^{-}	γ^{AB}	γ_S
Glass treated with Zobell broth (control)*	31.6	2.7	29.0	17.5	49.2
Glass coated with SN_{IV2006}*	35.6	1.5	38.9	16.6	51.3

*Standard deviations lower than 10% of each value

(Fig. 5). In both sets of treatment conditions, *Flavobacterium* sp. II2003 cells showed a very high affinity for chloroform and dichloromethane, the acidic solvents, and a relatively high affinity for apolar solvents (hexadecane, decane and tetradecane). These results indicate the broadly alkaline and rather hydrophobic character of the *Flavobacterium* sp. II2003 surface, whether treated with SN_{IV2006} or Zobell broth only.

SN_{IV2006} had no surfactant effect

The drop collapse method was performed in order to detect the presence of biosurfactant in the culture supernatant of *Pseudomonas* sp. IV2006. SN_{IV2006} drops did not collapse

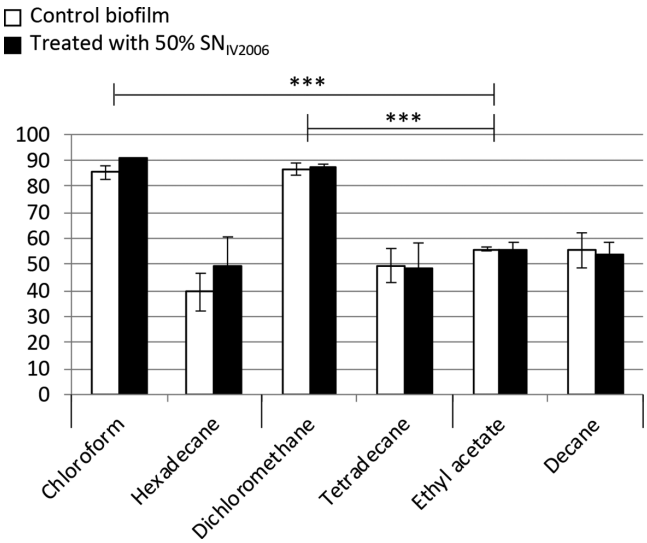

Fig. 5. Percentage affinity of *Flavobacterium* sp. II2003 cells to the six solvents used in the MATS method. *Flavobacterium* sp. II2003 cells were pretreated with SN_{IV2006} or Zobell broth (control). Three couples of solvents were used: chloroform (monopolar acidic)/hexadecane (apolar), dichloromethane (monopolar acidic)/tetradecane (apolar) and ethyl acetate (monopolar alkaline)/decane (apolar). Percentage affinities are mean values (±SD) of five measures obtained from three independent treatments. *Flavobacterium* sp. II2003 cells, treated with SN_{IV2006} or Zobell broth, showed a significantly higher percentage affinity to the acidic solvents than to the alkaline solvent. These differences are indicated by *** (P<0.001).

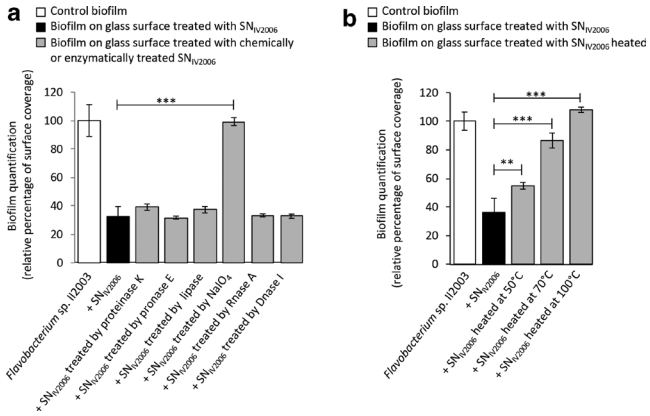

Fig. 6. Effect of various treatments on the coating activity of SN_{IV2006} in glass bottom dishes. (a) Effect of the digestion of proteins (proteinase K, pronase E), lipids (lipase), polysaccharides ($NaIO_4$) and nucleic acids (DNaseI, RNaseA) on the coating activity of SN_{IV2006}. (b) Effect of heat treatment on the coating activity of SN_{IV2006}. Control biofilm: *Flavobacterium* sp. II2003 biofilm obtained when the glass surface was coated with Zobell broth submitted to the same treatments as SN_{IV2006}. Control biofilms obtained in the different conditions were similar. The '100 %' values on the *y*-axis correspond to the control biofilms. The data represent the mean values±SD of at least three replicates. The effect of each SN_{IV2006} submitted to a chemical or thermal treatment was compared with the effect of the native SN_{IV2006}. Significant differences are indicated by ** (P<0.01) and *** (P<0.001).

and remained stable on an oil-coated surface, showing that SN_{IV2006} had no biosurfactant activity.

Preliminary physico-chemical characteristics of SN_{IV2006} antibiofilm compounds

To obtain information about the biochemical nature of the SN_{IV2006} active compounds, the *Pseudomonas* sp. IV2006 culture supernatant was submitted to different treatments. The glass coating effect of the treated supernatants as well as the antibiofilm activity when SN_{IV2006} was added during the 2 h adhesion step were evaluated using the coating assay in glass bottom dishes in static conditions (Fig. 6) and the microtitre plate assay (Fig. 7), respectively.

Concerning the coating activity observed when SN_{IV2006} was added alone before the target bacterium was added, neither protease, lipase nor nuclease treatment significantly affected the activity of SN_{IV2006} (Fig. 6a). Only $NaIO_4$ treatment totally prevented coating activity in *Flavobacterium* sp. II2003 (Fig. 6a). $NaIO_4$ very efficiently hydrolyzes saccharides by oxidizing carbons bearing vicinal hydroxyl groups and cleaving the C–C bonds [20, 32]. The heat sensitivity of the coating compounds of SN_{IV2006} was also investigated (Fig. 6b). The inhibitory effect started to be significantly affected at 50 °C and clearly decreased with increasing temperature. All these preliminary results indicated that the coating molecules would be saccharidic molecules.

For the antibiofilm activity observed when SN_{IV2006} was added during the 2 h adhesion step, neither lipase nor nuclease nor $NaIO_4$ treatment significantly affected the antibiofilm activity of SN_{IV2006} (Fig. 7a). Conversely, treatment with proteinase K and pronase E reduced the biofilm-inhibiting activity in *Flavobacterium* sp. II2003 by 80% (Fig. 7a). This result was confirmed under dynamic conditions in flow cells (data not shown). SN_{IV2006} was also heat-sensitive (Fig. 7b), indicating that the antibiofilm molecules acting during the 2 h adhesion step are likely to be proteinaceous.

SN_{IV2006} exhibited antibiofilm activity against other marine bacteria and human pathogens

The microtitre plate assay was used to determine the spectrum of action of SN_{IV2006} against biofilm-forming bacteria. The supernatant activity was assayed against a large variety of marine and non-marine bacteria (22 strains, Table 1). All of the strains were able to form biofilms in microplates with an OD_{595nm} >0.6 after crystal violet staining. Table 4 presents the percentage of biofilm inhibition of SN_{IV2006} against monospecies biofilms classified in groups according to the effects obtained. The first group represents the most sensitive strains with a percentage biofilm inhibition higher than 50%. This group contains 10 strains including pathogenic marine bacteria such as *Flavobacterium*, *Tenacibaculum maritimum* and *Vibrio lentus*, human pathogenic species such as *Staphylococcus aureus* and *Yersinia enterocolitica*, and the *Bacillus subtilis* ND Food model strain. The second group comprises strains whose biofilm was moderately inhibited (from 32 to 41%). It includes the fish pathogen *Flavobacterium psychrophilum* and the human pathogens *S. aureus* and *Pseudomonas aeruginosa*. Lastly, group 3 contains non-sensitive strains (inhibition <10%). This group is composed of marine bacteria *Micrococcus luteus*, *Shewanella* sp., *Roseobacter* sp., *Roseovarius* sp., *Vibrio anguillarum* and a non-marine bacterium, *P. aeruginosa.* The spectrum of action of the potential antibacterial activity of SN_{IV2006} was also evaluated using the agar well diffusion assay (Table 4). Out of 22 strains tested, SN_{IV2006} prevented the growth of only 3 species, *Y. enterocolitica*, *B. subtilis* and *T. maritimum*. With the exception of the latter three species, our experiment showed that *Pseudomonas* sp. IV2006 produced a specific antibiofilm activity against a wide range of bacterial biofilms (without bactericidal or bacteriostatic activity).

DISCUSSION

Previous reports indicated that secondary metabolites from micro-organisms may constitute one of the most promising sources of environmentally friendly active molecules, such as antifouling compounds, and that bacteria are the organisms of choice to obtain natural biodegradable products for antifouling coatings [34–36]. Indeed, marine bacteria have been shown to produce antibiofilm compounds against Gram-positive and Gram-negative bacteria in their culture supernatants [37, 38]. In the present study, bacterial antibiofilm compounds were looked for, from a collection of 86 marine bacteria, in order

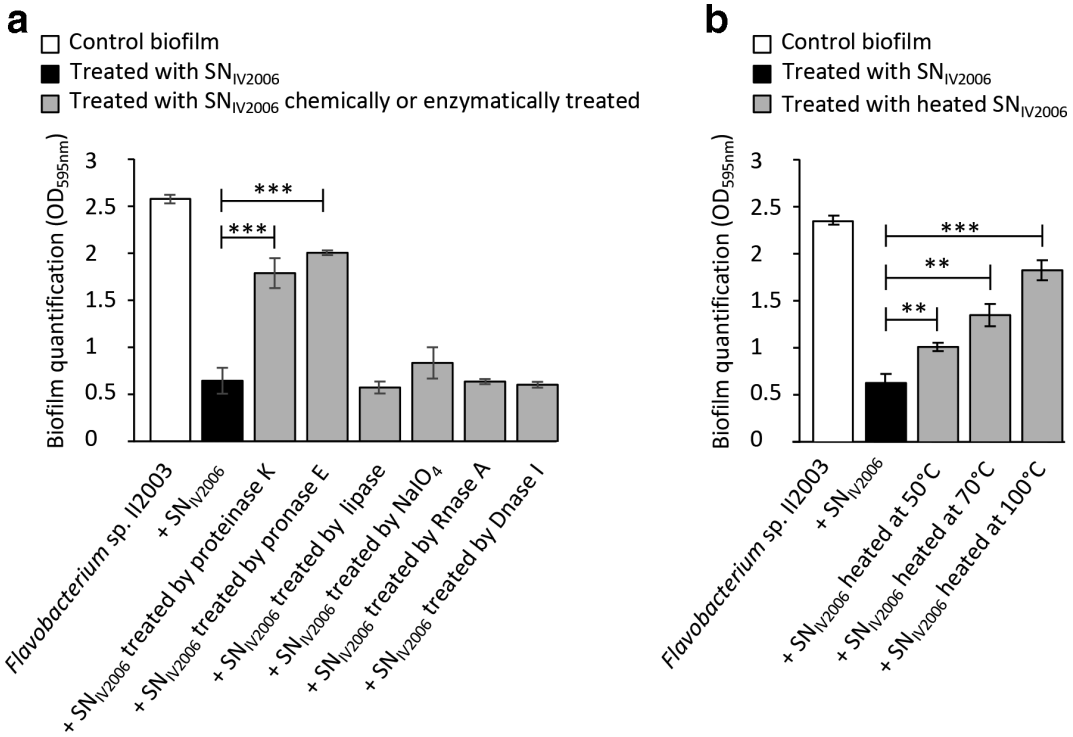

Fig. 7. Effect of various treatments on the antibiofilm activity of SN_{IV2006} added during the 2 h adhesion step in a microtitre plate. (a) Effect of the digestion of proteins (proteinase K, pronase E), lipids (lipase), polysaccharides ($NaIO_4$) and nucleic acids (DNaseI, RNaseA) on the antibiofilm activity of SN_{IV2006}. (b) Effect of heat treatment on the antibiofilm activity of SN_{IV2006}. The data represent the mean values±SD of three replicates. Control biofilm: *Flavobacterium* sp. II2003 biofilm obtained with Zobell broth submitted to the same treatments as SN_{IV2006}. Control biofilms obtained in the different conditions were similar. The effect of each SN_{IV2006} submitted to a chemical or thermal treatment was compared with the effect of the native SN_{IV2006}. Significant differences are indicated by ** (P<0.01) or *** (P<0.001).

to prevent or disrupt the biofilms formed by *Flavobacterium* sp. II2003. This bacterium was selected as a target because it was able to form stable biofilms and originated from the same mudflat as the 86 tested bacteria. Moreover, several bacteria related to *Flavobacterium* sp. are associated with fish disease [26] and cause major economic losses [39]. The natural reservoir of this pathogen, a contagion for farmed fish, is unknown, but its resilience in closed aquaculture systems involves biofilms [39]. Furthermore, many *Flavobacterium* species, including pathogens and opportunistic pathogens, have been identified in biofilm material collected from industrial, domestic and medical environments [26]. In addition, it is known that the biofilm lifestyle enhances bacterial resistance, in particular to antibiotics [40]. The use of antibiofilm compounds, preventing biofilm formation or deconstructing mature biofilms, is thus a promising strategy to increase the antibiotic sensitivity of pathogens [41]. Out of the 86 marine benthic bacteria tested, the culture supernatant of *Pseudomonas* sp. IV2006 (SN_{IV2006}) was selected. It displays a strong antibiofilm activity against *Flavobacterium* sp. II2003 when it is mixed with the cells during the 2 h adhesion step in a microtitre plate. The *Pseudomonas* species (*Gammaproteobacteria*) is one of the ubiquitous groups of metabolically versatile, eukaryote-associated bacteria with effects on host health and survival, and which display a multitude of behaviours [42]. *Pseudomonas* sp. strains may often act as opportunistic pathogens e.g. in plants [43, 44], fish [45, 46] and humans [47, 48]. In contrast, they can also be found in synergistic association with plants [49, 50] and sponges [51], where they play beneficial roles in disease control. Keller-Costa *et al.* [51] pointed out the antibacterial, antiprotozoan and antifungal activities of *Pseudomonas* bacteria isolated from the freshwater sponge *Ephydatia fluviatili*. Moreover, *Pseudomonas fluorescens* was shown to produce several compounds of different classes exhibiting strong antimicrobial activities, such as glycolipids [52], cyclic lipopeptides [53], rhamnolipids [54], phenazines [55] and polyketides [56]. In another study, Farmer *et al.* [57], isolated various *Pseudomonas* sp. strains from soil that inhibited the biofilm formation of *S. aureus* without inhibiting growth.

SN_{IV2006} activity was characterized in microtitre plates (static conditions/polystyrene surface), in glass bottom dishes (static conditions/glass surface) as well as in flow cell chambers (dynamic conditions/glass surface). SN_{IV2006} exhibited a high antibiofilm potential. It was observed that: (i) when the supernatant was added before the *Flavobacterium* sp. II2003 cells (coating step), biofilm formation was significantly inhibited

Table 4. Spectrum of action of SN_{IV2006} determined using a microtitre plate assay for antibiofilm activity and agar well diffusion assay for antibacterial activity

Group* and strain	Antibiofilm assays†	Antibacterial assays‡
1.High inhibition level		
Flavobacterium sp. II2003	70±8.6	–
Staphylococcus aureus AH478	58±3.3	–
S. aureus ATCC27217	51±2.3	–
S. aureus ATCC29247	57±1.1	–
Yersinia enterocolitica CIP106.676	70±4.9	32
Paracoccus sp. 4M6	51±1.2	–
Bacillus subtilis ND Food	55±3.6	18
Cellulophaga lytica DSM2039	54±4.1	–
Tenacibaculum maritimum CIP103528T	56±5.3	9
Vibrio lentus CIP107166T	80±9.2	–
2.Mild inhibition level		
S. aureus RN4220	38±1.0	–
Pseudomonas aeruginosa PAO1	41±3.5	–
Cellulophaga lytica DSM2040	32±1.3	–
Bacillus thuringiensis 407	41±4.9	–
B. subtilis 168	37±4.8	17
Flavobacterium psychrophilum CIP103534T	39±2.1	–
3.Non-sensitive strains		
Micrococcus luteus	0.5±3.1	–
Shewanella sp. IV 3014	1.3±0.8	–
Roseobacter sp. IV 3009	0.8±0.1	–
Roseovarius sp. VA014	2.6±0.4	–
Vibrio anguillarum CIP6336T	0.3±0.1	–
P. aeruginosa PA14	–6±1.1	–

*Groups distinguished on the basis of the percentage of inhibition of biofilm formation in the presence of SN_{IV2006}.
†Percentage of inhibition of biofilm formation in the presence of SN_{IV2006}.
‡Diameters in mm of the inhibition halos on agar plates. –, no inhibition halo.

on the glass surface but not on polystyrene; (ii) when the supernatant was added at the same time as the *Flavobacterium* sp. II2003 cells (adhesion step), the biofilm was totally absent from the glass and was strongly inhibited, but to a lesser extent, on the polystyrene surface; and (iii) when the supernatant was added after *Flavobacterium* sp. II2003 biofilm maturation, the mature biofilm was not disrupted.

We tested different hypotheses to try to explain these effects. We first investigated whether SN_{IV2006} could display a bactericidal or a bacteriostatic activity during the 2 h contact between SN_{IV2006} and the bacterial cells (adhesion step). Interestingly, experiments highlighted the lack of such activity against free-living *Flavobacterium* sp. II2003, suggesting that the SN_{IV2006} activity is specifically directed against biofilms. Few natural molecules have been reported to have antibiofilm activity without also being antibacterial. Such molecules include quorum-sensing inhibitors [58] or biosurfactants [59]. A bacterial exopolysaccharide, whose mode of action is not known, was also shown to inhibit biofilm formation and to disrupt established biofilms of different bacteria without affecting their growth [20].

Since SN_{IV2006} did not reduce cell viability during the adhesion step but strongly affected bacterial attachment to glass surfaces, it was hypothesized that SN_{IV2006} could modify the biotic or abiotic surface properties. The effect of the SN_{IV2006} components on the overall surface properties of *Flavobacterium* sp. II2003 cells was then examined using the MATS method. This showed that the surface of the bacterium was overall relatively hydrophobic and alkaline, and that SN_{IV2006} had no effect on these properties. Another hypothesis was that SN_{IV2006} could create a glass coating that hindered the attachment of bacteria through compounds adsorbing to the glass surface. The goniometric studies showed that the treatment of glass with SN_{IV2006} significantly reduced water and formamide contact angles. Hence, after coating, the glass surface became more hydrophilic and more alkaline. As the MATS method showed that the surface of *Flavobacterium* sp. II2003 cells (treated or not with SN_{IV2006}) exhibited overall a rather hydrophobic and alkaline character, the decrease in adhesion could be explained by a repulsion between the cells and the coated glass surface. It is known that adsorption to a substratum surface of amphipathic molecules such as surfactants alters the hydrophobicity of a surface and thus interferes with microbial adhesion [38, 59, 60]. For instance, pretreatment of glass surfaces with *Escherichia coli* supernatants containing antibiofilm K2 polysaccharides reduced biofilm formation by *E. coli*, *S. aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Klebsiella pneumoniae* and *P. aeruginosa* in microfermentors [61]. Similarly, precoating glass slides with purified *E. coli* Ec300p polysaccharide inhibited *S. aureus* biofilm formation in a flow reactor [38]. As with SN_{IV2006} components, K2 and Ec300p surfactant polysaccharides have been shown to increase the hydrophilicity of a surface [38, 61]. In other studies, biosurfactant lipopeptides from a marine *Bacillus circulans* strain [59] and from a freshwater *P. fluorescens* strain [62] displayed anti-adhesive properties against various bacteria. Moreover, the molecules in SN_{IV2006} that are presumed to be involved in the coating phenomenon were thought to be carbohydrates because only treatments with $NaIO_4$ inhibited the coating activity of SN_{IV2006}. Furthermore, these molecules were eluted with water from Sep-Pak Plus Environmental C_{18} cartridges, demonstrating their relatively hydrophilic character (data not shown). These findings suggest that the reduced bacterial attachment due to

the coating effect of SN_{IV2006} could be due to a carbohydrate molecule. However, we showed that the active molecules in SN_{IV2006} were not surfactants. Other possible modes of action have to be considered to explain the inhibition of adhesion. Antibiofilm compounds might block lectins or sugar-binding proteins present on the surface of bacteria, or block adhesins of fimbriae and pili [63]. They may also act as signalling molecules that modulate the gene expression of recipient bacteria [64].

A preliminary physico-chemical characterization of the compounds responsible for the main SN_{IV2006} inhibitory activity, which is observed on both glass and polystyrene surfaces when SN_{IV2006} is added at the same time as *Flavobacterium* sp. II2003 (e.g. during the 2 h adhesion step), supported the hypothesis of a proteinaceous compound. Indeed, proteases and heat treatment almost totally impaired the capacity of SN_{IV2006} to inhibit biofilm formation. The best-known example of such a proteinaceous compound is the antibacterial/antibiofilm protein AlpP secreted by *Pseudoalteromonas tunicate* D2 [65]. The proteinaceous exoproducts of the marine bacterium *Pseudoalteromonas* sp. 3J6 also inhibit the formation of biofilm by most of the tested marine bacteria without affecting their planktonic growth [15, 16].

To summarize the inhibitory activity of SN_{IV2006} against *Flavobacterium* sp. II2003 biofilm formation, two important effects can be highlighted: (1) a coating effect that significantly inhibits biofilm formation on a glass surface but not on polystyrene, and (2) an antibiofilm effect exerted during the adhesion step on both glass and polystyrene surfaces. SN_{IV2006} could contain two active compounds. One hydrophilic molecule, a carbohydrate, could be active during the coating of glass surfaces only, and the other, a protein, could be active afterward, on both glass and polystyrene surfaces, perhaps because it acts against *Flavobacterium* sp. II2003 and not on the abiotic surface. The two compounds could have a cumulative effect on the glass surface, which would explain the total absence of biofilm when the SN was added during the adhesion step. Understanding the mode of action of SN_{IV2006} antibiofilm compounds will require purification and identification of the molecules responsible.

Finally, the most interesting aspect of the antibiofilm *Pseudomonas* sp. IV2006 exoproducts is their wide spectrum of action. Marine as well as human pathogenic bacteria able to form biofilms were used as target bacteria, and 73% of the strains were sensitive. It is particularly interesting to note that the antibiofilm activity of SN_{IV2006} molecules is effective against several strains of *Flavobacterium* species (both marine and freshwater strains) that are pathogenic in fish, and the related strains *T. maritimum* and *Cellulophaga lytica*. Moreover, the SN_{IV2006} antibiofilm compounds significantly impaired the biofilm formation of human pathogenic bacteria present in both the medical environment, such as *S. aureus* and *P. aeruginosa*, and the food industry, such as *Y. enterocolitica*. Thus, wide-ranging applications of the SN_{IV2006} compounds could be envisaged, both in aquaculture and human health.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study did not include any work on animals or humans of any kind.

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