Bioactive Compounds Produced by *Pseudoalteromonas* Affect Marine Biofilm Formation

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Introduction

Bacterial biofilms, frequently in association with algae, protozoa and fungi, are found on all submerged structures in the marine environment. Biofilms are responsible for a range of surface-associated and diffusible signals, which may moderate the settling behaviour of cells, spores and larvae. Thus, marine microorganisms are a new source of bioactive compounds, which enhance or inhibit the settlement of organisms.

Objectives

In this study, we investigated the effect of the supernatant of a liquid-cultured bacterium strain, *Pseudoalteromonas* 3J6 (SN 3J6), on several marine bacterial strains isolated from coastal waters in Brittany (France). The strain 3J6 was selected for its capacity to inhibit the biofilm formation of other bacteria.

Materials and Methods

Bacteria cultures

The strains were isolated from immersed glass plates during 3h and 6h into the Golf of Morbihan, Brittany, France (*Alteromonas* 1J3, *Pseudoalteromonas* 3J3, *Colwellia* 4J3, *Sulfitobacter* 8J6, *Algibacter* 1M6, *Pseudoalteromonas* 3J6) or from immerse teflon plate during 48h (*Vibrio* D66). All strains were grown in the Vaätanen Nine Salts Solution (VNSS) medium at 20°C and shaking at 150 rpm.

Flow-cell assays {1}

Biofilm flow-cell assays allowed the growth of bacterial biofilm under continuous medium flow condition.
The system contained five components connected via silicon tubbings. After an overnight culture , the bacteria to test were washed and resuspended at an OD_{600} of 0.25 either in artificial sea water (control) or in 3J6 culture supernatant, and 200 µl were introduced into a flow chamber. Bacteria were allowed to attach to the substratum for 2h, and a laminar flow of VNSS medium was then applied at 0.2 mm/s for 48h.

Confocal Laser Scanning Microscopy (CLSM)

Biofilms were 3D structures and required resolution in the direction vertical to the substratum via CLSM [Fig.3]. Cells in biofilms were stained with the Syto 61 dye at 5µM which stained in red all cells regardless of their viability, and with the Sytox dye at 0.5µM which stained dead cells in green. Five observations and mesures were operated for each chamber and the recorded microscopic images were used for quantitative analysis with COMSTAT software. The total biomas was calculated from pixels ocupied by biomass.

Microplate assays {2}

Biofilm microplate assays allowed the biofilm formation in static conditions. We developed a rapid laboratory method for screening potential biofilm inhibiting molecule in 96-well microplates. The Crystal Violet stain was used as an indicator of total attached biomass.

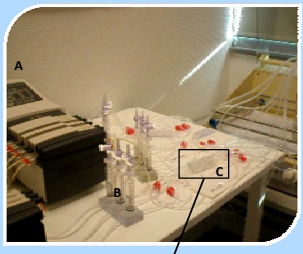
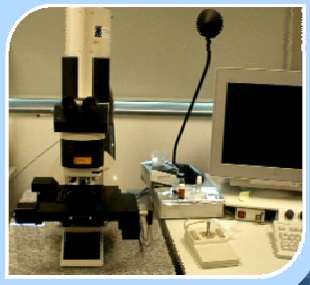


FIG.1. The Biofilm flow-cell system consists of several components connected via silicone tubings: a medium reservoir, a peristaltic pump Watson and Marlow 205S (A), bubble trap (B), flow-cells (C), an effluent reservoir.

FIG.2. Three-chanel flow-cell

FIG.3. Confocal laser Scanning Microscope



Results

Biofilms grown in flow-cells appear to be a useful system to study the antifouling action in biofilms. CLSM observations of 48h-biofilms show an inhibiting activity of the supernatant of *Pseudoalteromonas* 3J6 [Fig.4]. The 3J6 supernatant inhibited strongly the biofilm formation of *Vibrio* D66, *Colwellia* 4J3, *Sulfitobacter* 8J6 and *Algibacter* 1M6. We noticed structural modifications of *Algibacter* 1M6 and *Sulfitobacter* 8J6 biofilms when the adhesion phase occured in the presence of 3J6 supernatant. [Fig.4]

The 3J6 supernatant failed to inhibit the biofilm development of only two of the tested stains, *Pseudoalteromonas* 3J3 and *Alteromonas* 1J3, since the percentages of inhibition were lower than 15% with both flow-cell and microplate assays.

Strain 8J6 is inhibited by the 3J6 supernatant (60%±0.15) in flow-cell, whereas with the microplate protocol this percentage decreased to 40%±0.08. The same phenomenon is observed for D66, 4J3 and 1M6. These bacteria were inhibited up to 80%(±0.02) via the flow-cell system but this value decreased to 53%(±0.03) for D66, 59%(±0.2) for 4J3 and 39%(±0.01) for 1M6.

Tested strains	Flow-cell assays		96-well microplate assays
	Average Thickness (µm)	Total Biomass (µm³/µm²)	OD (590nm)
3J3 (control)	5.75 ± 0.76	3.93 ± 0.90	1.34 ± 0.14
3J3 + SN 3J6	5.71 ± 1.17	3.69 ± 0.64	1.32 ± 0.10
1J3 (control)	5.37 ± 0.67	3.21 ± 0.91	0.35 ± 0.08
1J3 + SN 3J6	4.66 ± 1.44	2.91 ± 0.81	0.34 ± 0.08
8J6 (control)	2.12 ± 0.67	2.04 ± 0.73	0.60 ± 0.02
8J6 + SN 3J6	0.88 ± 0.29	0.66 ± 0.21	0.36 ± 0.03
D66 (control)	38.83 ± 7.60	15.21 ± 3.07	2.00 ± 0.07
D66 + SN 3J6	4.34 ± 1.66	1.87 ± 0.64	0.94 ± 0.08
4J3 (control)	6.53 ± 2.22	5.92 ± 2.47	0.43 ± 0.06
4J3 + SN 3J6	0.35 ± 0.10	0.27 ± 0.10	0.18 ± 0.03
1M6 (control)	18.55 ± 6.62	15.86 ± 5.82	2.81 ± 0.03
1M6 + SN 3J6	0.03 ± 0.01	0.03 ± 0.01	1.71 ± 0.08

FIG.5. Means and standard errors of the mesurements realised for 6 biofilms with or without the 3J6 supernatant (SN 3J6)

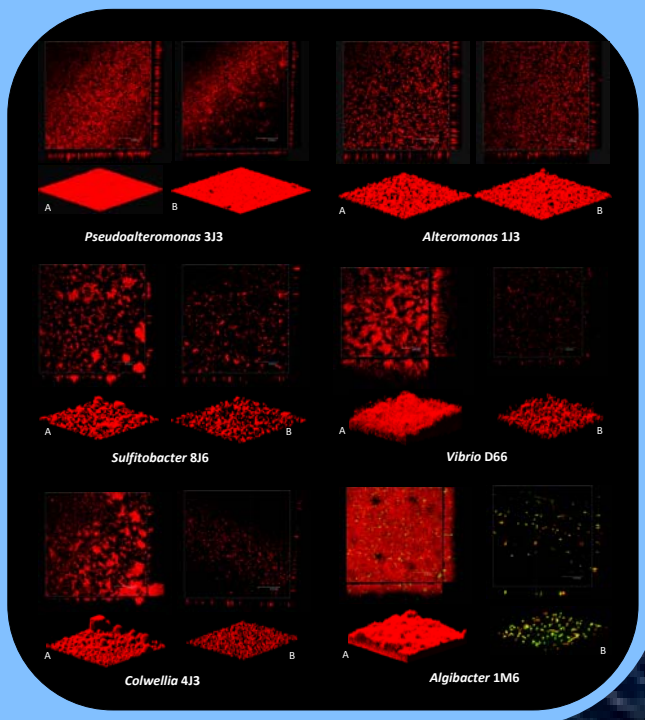


FIG.4. Analysis of a 48h single-specie color-coded biofilm via CLSM. Top views of structures formed are sections and Botom views are 3-dimension projections. Left views (A) are biofilms formed with artificial sea water as adhesion medium as control. Right views (B) are biofilms formed with the 3J6 supernatant as adhesion medium.

If we compare the two assays, we observed that the percentage of inhibition after growth in a microplate (static biofilm) is 25% to 50% lower than growth in a flow-cell system which (dynamic biofilm) [Fig.5].

These differences between the results obtained can be caused by a sedimentation of the bacteria into the well of the microplate which was not involved with the flow-cell process because of a continuous and laminar flow into the chamber and prevented bacteria sedimentation. An other explanation is the process, indeed in the microplate protocole we had several step of well washing that can erode a thick biofilm as the control, thus we underestimate the percentage of inhibition in microplate compared with the flow cell process.

Moreover, a live-dead coloration showed that the 3J6 supernatant did not affect bacterial viability, except for 1M6, the mortality of which was higher than 95% in the presence of 3J6 supernatant [FIG.4].

Conclusions

The *Pseudoalteromonas* 3J6 culture supernatant is able to impair biofilm formation of most of the marine bacteria tested. Further experiments are carrying out to purify and identify the anti-biofilm molecules secreted by Pseudoalteromonas 3J6.

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{1} Pamp *et al.*, Cytometry Part A, 2009, 75A:90-103 {2} Pitts *et al.*, Journal of Microbiological Methods, 2003, 54:269-276