NOUBA Project
Novel Natural Molecules Inhibiting the Development of Marine Bacteria in Biofilms

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Marine organisms represent a rather unexplored source of new activity and biological functions of molecules for biotechnologies. The research in chemical ecology of the marine environment leads to looking at metabolites of recognition or defence produced by these **models. 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. The marine *Pseudoalteromonas* sp. 3J6 and D41 were selected for their capacity to inhibit the biofilm formation of other bacteria.** The study of antibiofilm metabolites synthesised by marine **bacteria** 3J6 and D41 biofilms can lead to the development of new anti-fouling compounds or applications in surface hygiene. The main originality of these compounds is to be specifically antibiofilm, they have no activity against planctonic bacteria.

A major objective of this project is to screen the activity spectrum and to caracterise and identify these original compounds produced by the bacteria 3J6 and D41.

Investigating the antibiofilm activity spectrum

Two different approaches were experimentally used in biofilm studies:

- one method that quantifies the total biomass, the thickness, the architecture and the viability of a biofilm involving growth under flow conditions (Flowcell system, Fig.1), staining by Syto 61, Sytox and with observations in Confocal Laser Scanning Mocroscopy (Fig.2 and 3).

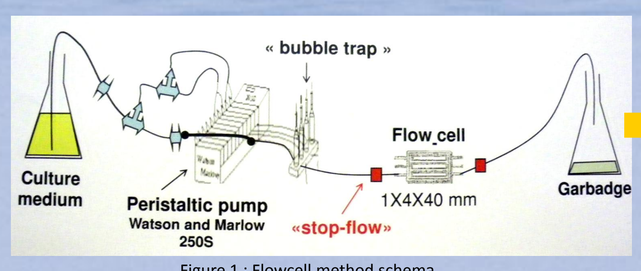


Figure 1 : Flowcell method schema

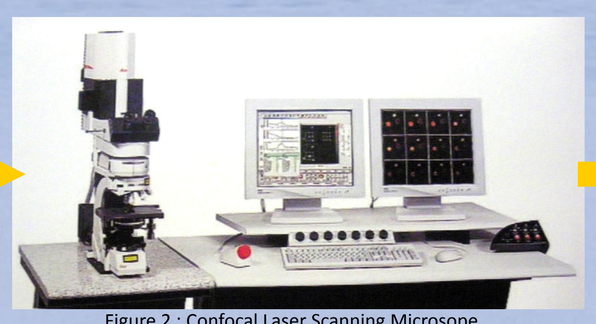
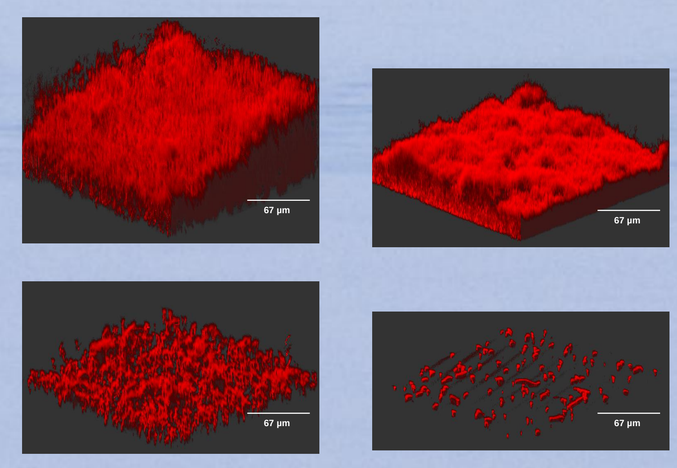


Figure 2 : Confocal Laser Scanning Microsope

Figure 3 : Effect of molecules produced by the bacteria 3J6 on bacterial adhesion at 20°C. *Vibrio* sp. D66 (left panel) and *Algibacter* sp. 1M6 (right panel) were incubated for 2 h under static condition in flow cell channels in the presence (bottom images) or absence (top images) of molecules produced by the bacteria 3J6. Biofilms were grown on glass surfaces at 20°C for 48 h under a flow of VNSS medium.



- another that allows the determination of the total biomass involving staining by crystal violet in a 96-wells microplate in static conditions (Fig.4).

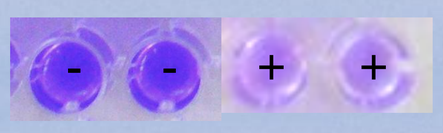


Figure 4 : Biofilm formation in microplate well by *Algibacter* sp. 1M6 in the presence ('+'-wells) and absence ('-'-wells) of the molecule produced by the bacteria D41.

The molecules produced by the bacteria 3J6 and D41 had a wide spectrum of activity since it affected all the Gram negative marine strains tested except other *Pseudoalteromonas* strains (Fig. 5). Biofilm biovolumes of the sensitive strains were reduced 3 to 530 fold and the percentages of non viable cells were increased 3 to 225 fold.

Interestingly, molecules produced by the bacteria 3J6 also impaired biofilm formation by three strains belonging to the human pathogenic species *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Escherichia coli*.

Tested strains	Flow-cell assays		96-well microplate assays
	Average Thickness (µm)	Total Biomass ($\mu m^3/\mu m^2$)	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

Figure 5 : Activity spectrum of the bacteria 3J6

Perspective : Identify the antibiofilm compounds

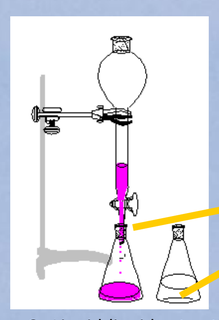


Figure 6 : Liquid-liquid extraction

Figure 7 : Separation with C18 column

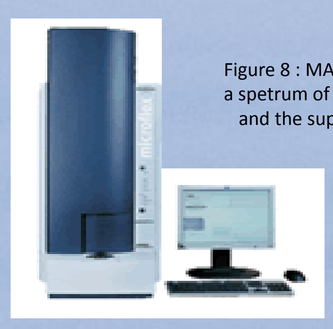
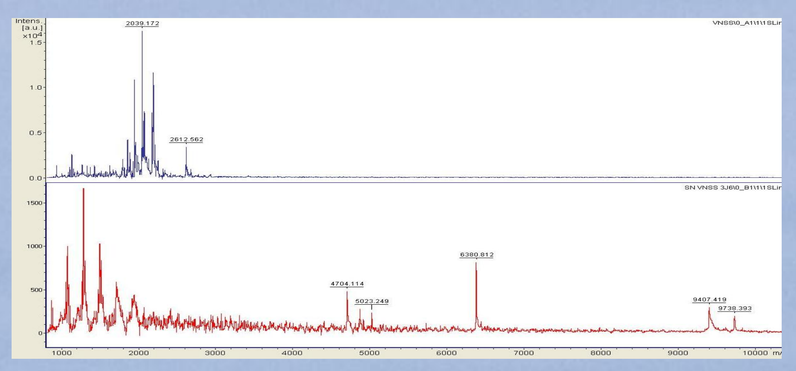


Figure 8 : MALDI-TOF Mass Spectrometer and a spetrum of the culture medium VNSS (Blue) and the supernatant of the bacteria 3J6 in VNSS(Red).



The development of techniques and methods for molecules purification will be an essential step. The purification scheme choosen involve differents extractions (Fig. 6) and chromatography techniques (Fig. 7) with differents selectivities (hydrophobocity, size exclusion and ion exchange chromatography). All separation methods will be coupled with a MALDI-TOF-Mass Spectrometer (Fig. 8).

Conclusion

The anti-biofilm molecule produced by *Pseudoalteromonas* sp. 3J6 and D41 presents the originality of differing from other classical anti-bacterial compounds by its biofilm specific activity. Further experiments has been undertaken to purify and identify the anti-biofilm molecules secreted by these bacteria.

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