A BIOFILM-FORMING MARINE BACTERIUM PRODUCING PROTEINS AS EXTRACELLULAR POLYMERIC SUBSTANCES

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INTRODUCTION

Any surface immersed in an aqueous environment is rapidly colonized by microorganisms, building up a biofilm. Biofilm consists on bacteria embedded into their slime composed essentially of extracellular polymeric substances (EPS) such as polysaccharides or proteins. Searching on marine biofilm formation will be helpful in fighting against marine biofouling.

AIMS : EPS biochemical characterization and observation by epifluorescence microscopy.
Development of a marine biofilm formation model onto a polystyrene microtiter plate using the "O' Toole and Kolter method" [1] adapted with the well known fluorescent DNA staining DAPI.

EPS Biochemical characterization

Bacterial Model: *Pseudoalteromonas sp.*D41 marine bacteria was isolated from a natural biofilm on Teflon immersed for 24 hours in the Atlantic Ocean at Brest.

EPS Production: The EPS production was performed in fermentor as described by Raguénès et al. [2]. Two soluble EPS were isolated from the fluid supernatant recovered from the medium by high-speed centrifugation (20 000g, 2h, 4°C). EPS2 fraction was obtained by ultrafiltration of the supernatant onto 100kDa membrane and EPS1 fraction was further obtained by ultrafiltration of the EPS2 onto a 10kDa membrane. Non soluble EPS3 fraction was recovered from the cell pellet by dialysis.

Tab. 1. Biochemical characteristics of the 3 EPS fractions (mg.g-1 dry weight)

EPS Fraction	10 - 100kDa EPS1	> 100kDa EPS2	Non Soluble EPS3
Proteins	303	410	592
Total carbohydrates	430	153	82
Total Uronic acids	32	24	26
Ratio Proteins/Carbohydrates	0.7	2.68	7.22

Proteins, total carbohydrates and total uronic acids contents were determined by BCA, orcinol-sulfuric method and meta-hydroxydiphenyl method respectively [2].

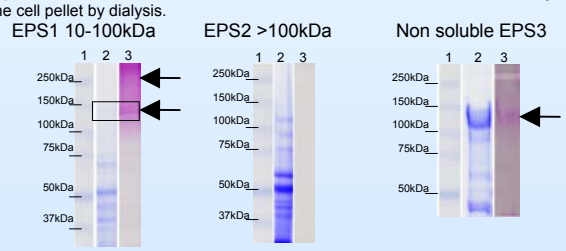

Fig. 1. SDS gel (8%) electrophoreses of EPS fractions stained with Coomassie (lane 2) and Schiff's reagent (lane 3) to detect proteins and carbohydrates respectively. Molecular weight markers were in lane 1.

❖*Pseudoalteromonas sp.* D41 fermentation lead to the production of 3 EPS fractions: two soluble fractions separated according to their molecular weight and another cell bound fraction, non soluble in a salt solution (36g/l). None of them is pure. EPS1 consists mainly in a majority of carbohydrates while the two others consist mainly in a majority of proteins.

❖Gel electrophoresis analysis showed different EPS proteins composition with molecular weight lower than 150kDa. EPS1 fraction contains a polysaccharide. It also exhibits 2 bands both coloured by Coomassie and Schiff, maybe corresponding to 2 glycoproteins. EPS2 fraction is not coloured by schiff reagent. A main band between 150 and 100kDa could be observed both in carbohydrate and protein stained gel for EPS3 fraction.

❖Most of studies on EPS are performed on EPS isolated from culture media and little on EPS isolated from biofilm. But we can hypothesize that these biochemical features are similar to EPS which are excreted in a biofilm structure.

Model of marine biofilm formation in a microtiter plate

Microtiter plate biofilm formation: *Pseudoalteromonas sp.* D41 was inoculated at approximately 2.10^9 cfu/ml in sterile natural sea water in a sterile black polystyrene microtiter plate and incubated from 45 min. to 24 h. at 20°C under horizontally shaking. After washings, wells were stained with 4µg/ml DAPI for 20 min. and washed again. The absorbed stain was then solubilized in 95% ethanol for 15min. and fluorescence was measured.

Biofilm quantification: *Pseudoalteromonas sp.* D41 in natural sea water was stained with DAPI. A part of the liquid sample was filtered on 0,22µm black polycarbonate filter, and counted by epifluorescence microscopy. Another part of DAPI stained bacteria was washed by centrifugation and absorbed DAPI was solubilized in 95% ethanol for 15min. under shaking. Fluorescence intensity was measured at 350nm ex. /510nm em. in black microtiter plate.

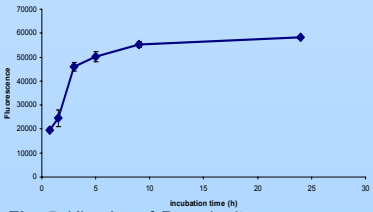

Fig. 5. Kinetics of *Pseudoalteromona sp.* D41 attachement in microtiter plate.

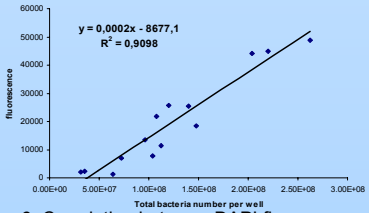

Fig. 6. Correlation between DAPI fluorescence measurement and DAPI stained bacteria counted by epifluorescence microscopy.

❖The number of attached bacteria to polystyrene surface seems to increase very quickly at the beginning of the biofilm formation. At about 3h incubation, the number of attached bacteria is stabilised. In this assay, a 3h. incubation time is enough to get a biofilm.

❖Correlation between fluorescence measurement and microscopy counting is good. About 10^7 attached cells in the well or one bacteria for $5\mu m^2$ is the detection limit. This assay is about five times more sensitive than crystal violet staining [1].

EPS epifluorescence microscopy observation

Calcofluor and fluorescamine were used to stain β-glucans and proteins (NH_2) respectively in a 24hours biofilm adhered onto a glass slide.

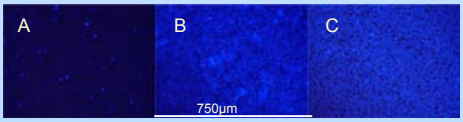

Fig. 7. Epifluorescence microscopy images of a 24h bacteria adhered onto glass surface and stained with A) Calcofluor B)DAPI and C)Fluorescamine

❖*Pseudoalteromonas sp.* D41 seems to produce EPS composed mainly of proteins. β-glucans represent a small part of the EPS. These results are correlated with the biochemical characterisation of the EPS isolated from D41 fermentation.

References

[1] G.A. O'Toole *et al.* (1999) Methods in Enzymology. 310: 91-109

[2] G. Raguénès et al. (1997) J. Appl. Microbiol. 82: 422-430

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Conclusion

❖*Pseudoalteromonas sp.* D41 can be used as a model of marine biofilm forming bacteria, in order to study marine biofilm formation or EPS excretion and either in microscopy and in microplate. The perspective in microplate will be the screening of new anti-fouling molecules in order to fight marine fouling.

❖This bacterial strain produces in fermentor EPS mainly composed of protein. Their characterization will be helpfull for the knowledge of adhesion mechanism and they could be a target to fight against the biofilm formation. We have shown the presence of glycoproteins in excreted EPS fraction.

❖The presence of protein has also been shown in a 24 hours marine biofilm, confirming the biochemical analysis of EPS produced in fermentor.