PROTEOMIC APPROACHES APPLIED TO ADHESION FACTORS IN MARINE BIOFILM-FORMING BACTERIA

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Introduction

Biofouling is ubiquitous in marine environment, and bacteria are among the first organisms to foul surfaces. They form biofilms which serve as focus for the attachment and growth of other organisms, such as invertebrates, sessile plants, and animals (Davis et al., 1989). Mature marine biofouling communities are complex, highly dynamic ecosystems (Fig.1) and once established are extremely difficult to eradicate (Holmstrom et al., 2002). For this reason the understanding of the mechanisms leading to marine bacterial attachment and its subsequent biofilm development are of great biological importance with obvious potential industrial outcomes. This development is conditioned by complex processes involving bacterial attachment to surfaces, growth, cell-to-cell communication, mobility and production of exoproducts constituting the biofilm matrix. Concerning attachment, the molecular strategies used by bacteria are diverse. They can employ pili, fimbria and a plethora of proteins regrouped under the term "adhesins" that recognize many different elements of the target (living or inert) surfaces (Pizarro-Cerda and Cossart, 2006; Fronzes et al., 2008). Although there is consistent data on human pathogenic bacteria attachment mechanisms, scarce information is available for marine bacteria. For this reason this project focused in the marine biofilm-forming bacterium *Pseudoalteromonas sp.* D41 (P. D41). This organism displays strong and competitive adhesion onto a wide variety of substrates, promoting subsequent biofilm development (Fig2.). Previous physicochemical studies in this strain related the high outer-shell protein content to its adhesion properties (Pradier et al., 2005; Leroy et al., 2008). For this reason, we attempted to unravel the molecular mechanisms responsible for these adhesive and competitive properties though a proteomic strategy, with particular attention to the outer membrane (OM) fraction.

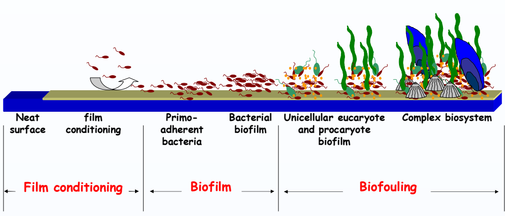
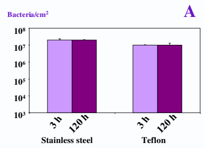


Fig 1. Steps of marine biofouling formation.



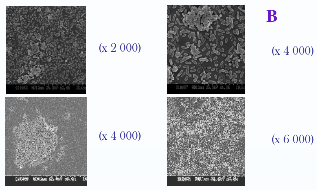
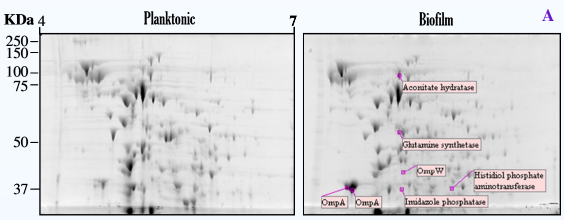


Fig 2. Characterization of *Pseudoalteromonas sp.* D41 adhesion and biofiom formation features. (A) Biomass quantification of P. D41 biofilms developed after 3 and 120 hours on teflon and stainless steel surfaces. (B) Electron microscopy images of P. D41 entrapped in their own exopolymers after 3 hours of attachment on stainless steel surfaces.

Results

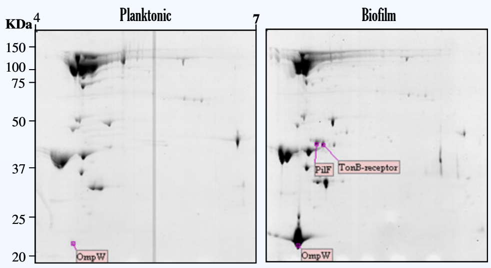
In order to screen for proteins regulated during biofilm formation in P. D41, we carried out 2D-PAGE proteome profiling of the total soluble and the OM proteome fractions (Fig4). The differential expression of 10 biofilm-related proteins was detected and they were subsequently identified by MS/MS *de novo* sequencing. These proteins were identified as involved in primary cellular metabolic functions, membrane transport, stress resistance and cell adhesion.Of particular interest, we detected four strongly induced OM proteins presenting high homologies to, a TonB-dependent receptor, an OmpW-like porin, an OmpA-like porin, and a type IV pilus biogenesis protein (PilF). The gene transcription levels of these proteins were followed by RT-qPCR in biofilms cultivated either on hydrophobic (silicone) or hydrophilic (glass) surfaces at early and late biofilm developmental stages. The expression of all the four corresponding genes was upregulated on both surfaces suggesting their importance in biofilm formation(Fig 5). Unfortunately, we were unable to further address this question by inactivating the corresponding genes in P. D41 due to the lack of appropriate genetic tool. We therefore examined if the homologous genes are involved in biofilm development of *Pseudomonas aeruginosa* PAO1, which is a widely used bacterial model for biofilm studies. The inactivated *P. aeruginosa* genes were *oprF, oprG, oprC* (homologous to ompA, ompW, and the TonB-dependent receptor, respectively), and *pilF.* The biofilm development of the wildtype strain PAO1 and of the four mutants were followed by confocal laser scanning microscopy. All of mutants but *oprC* showed a significant reduction of the biomass and an altered architecture of the biofilm structure (Fig 6).

1. Proteomics



B

	Protein Name	Accession	Peptides	Score%	Coverage%	MW theo	MW obs	Fold-change
Total proteome fraction	histidinol phosphate aminotransferase [*Pseudoalteromonas haloplanktis* TAC125]	gi|77362401	5	98	8	40	37	-11.3
	aconitate hydratase [*Pseudoalteromonas haloplanktis* TAC125]	gi|77359131	11	99	5	100	100	5.0
	glutamine synthetase [*Alteromonadales bacterium* TW-7]	gi|119468251	5	99	8	52	55	>1000
	OmpA-like hypothetical protein PSHAa0183 [*Pseudoalteromonas haloplanktis* TAC125]	gi|77359154	8	99	9	38	38	19.1
	OmpA-like hypothetical protein PSHAa0183 [*Pseudoalteromonas haloplanktis* TAC125]	gi|77359154	41	99	15	38	38	3.4
	imidazole glycerol-phosphate dehydratase [*Pseudoalteromonas haloplanktis* TAC125]	gi|77362400	7	99	12	39	37	-4.3
	putative protein W (OmpW) family [*Alteromonadales bacterium* TW-7	gi|119470014	10	29	23	23	40	8.2



D

	Protein Name	Accession	Peptides	Score%	Coverage%	MW theo	MW obs	Fold-change
Outer membrane proteome fraction	type IV pili biogenesis protein PilF [*Pseudomonas entomophila* L48]	gi|104780238	2	62	9	28	30	>1000
	TonB-dependent receptor [*Shewanella sp.* MR-7]	gi|114046006	3	91	1	78	30	>1000
	protein W (OmpW) [*Pseudoalteromonas haloplanktis* TAC125]	gi|77361078	37	31	33	23	20	81.7

Fig 4. Proteomic profiling of the P. D41 biofilm development on hydrophobic surface (silicone). Representative 2-DE gels of the total soluble (A) and OM (C) proteomes fractions extracted from planktonic P. D41 (stationary phase) and from P. D41 grown in biofilm on a silicone surface for 10 days. Proteins with amounts significantly changed ($p<0.05$) in biofilm are highlighted on the gels. Proteins shown on panels A-C are listed in tables B-D respectively. Differentially expressed spots were detected using Image Master 5.0 (G.E. healthcare) and identified by MS/MS *de novo* sequencing analysis using PEAKS (Bioinformatic Solutions).

2. Transcript accumulation

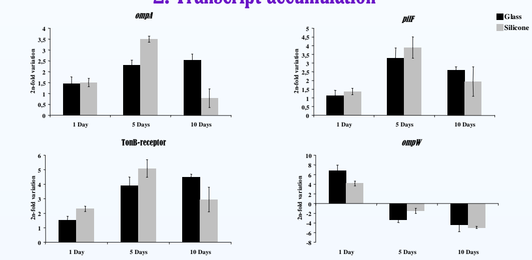
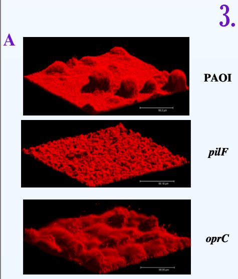


Fig 5. Changes in outer-membrane gene expression in P. D41monitored by RT-qPCR. Expression profiles of ompA, pilF,TonB-receptor and ompW genes was followed on silicone (black) and glass (grey) biofilm cultures for 1;5 and 5 days. The rpoB, rho and rplB genes were used as internal controls. The relative variations in gene expression were calculated as x-fold changes to the appropriate planktonic control conditions and presented as 2n-fold variation. Results are means ± SE from three independent biological samples.

3. Functional studies



B

	Mean biomass ($\mu m^3.\mu m^{-2}$)	Mean thickness (μm)	Maximal thickness (μm)
PAOI	2.27 ± 0.39	8.23 ± 3.28	25.89 ± 4.88
pilF	0.52 ± 0.20	1.08 ±0.53	30.76 ± 3.25
oprC	6.32 ± 0.59	21.31 ± 2.44	29.19 ± 2.07
oprF	1.18 ± 0.16	6.32 ± 2.12	28.89 ± 4.45
oprG	1.14 ± 0.50	3.61 ±1.62	42.17 ± 6.46

Fig 6. Biofilm formation by *Pseudomonas aeruginosa,* strain PAOI and the outer membrane mutants *pilF , oprG, oprF* and *oprC.* Stains were grown at 20 ℃ for 48 h in LB broth. Biofilms were stained with 5 µM of Syto 61 red and observed by confocal laser scanning microscopy. (A) 3D-shadow representations are shown for PAOI, *pilF* and *oprC.* Mean biomass (biovolume), mean and maximal thicknesses were calculated from COMSTAT analyses.

Conclusion and perspectives

Bacterial adhesion and biofilm development implies profound physiological changes and particularly with relation to cell surface structure. Our studies report for global changes in physiology during biofilm-forming stages of a marine bacterium, *Pseudoalteromonas sp.* D41. Interestingly P. D41 up-regulates a type IV fimbrial biogenesis protein PilF. Type IV pili were already shown as essential components for cell attachment to biotic and abiotic surfaces in pathogenic bacteria (Pizarro-Cerda and Cossart, 2006; Fronzes et al., 2008). Therefore, it is likely type IV pili structures play similar roles in the of P. D41adhesion.

The porin channel proteins OmpA and OmpW were also induced in P.D41 under biofilm conditions. In *E. coli*, OmpA is a key adhesin for the colonization of brain microvascular endothelial cells, through the direct interaction between OmpA and a glycoprotein (Prasadarao et al., 1996). Besides, OmpA exerts its influence on bacterial binding by modulating type I fimbriae expression (Teng et al., 2006). Based on these results, it is likely that OmpA could also represent an adhesion factor in P. D41. Much less is known about the role of OmpW in other bacteria. This protein might be an important stress resistance factor, which could therefore be in relation to the establishment of biofilm resistance mechanisms (Asakura et al., 2008). In agreement to our results, Calcium-induced biofilm formation is related to the expression of OmpW in *Pseudoalteromonas sp.* 1398 (Patrauchan, 2005). P. D41 upregulates a TonB dependent receptor which is involved in other organisms to the uptake of large molecules such as iron-siderophores or vitamin B12 (Nikaido, 2003). These receptors are uncommonly related to biofilms in non-marine bacteria, and hence represent a feature specific to the marine environments. Iron is a major limiting factor in open oceans , concentrations are typically as low as 0.2 nM to 1 nM and can vary depending on salinity and oxygenation (Wu & Luther III, 1996). As an adaptation to this limitation, iron binding siderophores are produced by many marine bacteria (Rue & Bruland, 1995). Previous studies in *Pseudoalteromonas tunicata* showed that a biofilm-associated transcriptional regulator ToxR-like induced the transcription of a TonB dependent receptor (Stelzer et al., 2006). These results together with ours indicate that iron regulation may play an important role for *Pseudoalteromonas* biofilm development. According to the interesting phenotypes of corresponding mutants, actual efforts are now being developed to complement *P. aeruginosa* mutants with the corresponding P. D41 homologues genes.

Taken together, our results indicate that the marine fouling bacterium *Pseudoalteromonas sp.* D41 utilizes mechanisms that are common to human pathogenic bacteria, and other that seem to be more specific to the marine environment. Our results open promising research perspectives with potential industrial applications for targeted anti-biofouling strategies. This work was founded by the Axis 1 (Genomics and Blue Chemistry) of the GIS Europôle Mer.

References

Asakura H, Kawamoto K, Haishima Y, Igimi S, Yamamoto S, Makino S (2008) Research in Microbiology **159:** 709-717
Davis AR, Targett NM, McConnell OJ, Young CM (1989) Bioorganic Marine Chemistry **3:** 85-114
Fronzes R, Remaut H, Waksman G (2008) Embo Journal **27:** 2271-2280
Holmstrom C, Egan S, Franks A, McCloy S, Kjelleberg S (2002) Fems Microbiology Ecology **41:** 47-58
Leroy C, Delbarre-Ladrat C, Ghillebaert F, Compere C, Combes D (2008) Journal of Applied Microbiology **105:** 791-799
Nikaido H (2003) Microbiology and Molecular Biology Reviews **67:** 593-+
Patrauchan MA, Sarkisova S, Sauer K, Franklin MJ (2005) Microbiology **151**:2885-2897
Pizarro-Cerda J, Cossart P (2006) Cell **124:** 715-727
Pradier CM, Rubio C, Poleunis C, Bertrand P, Marcus P, Compere C (2005) Journal of Physical Chemistry B **109:** 9540-9549
Rue, E. L. & Bruland, K. W. (1995). Marine Chemistry **50**, 117-138
Prasadarao NV, Wass CA, Weiser JN, Stins MF, Huang SH, Kim KS (1996). Infection and Immunity **64:** 146-153
Stelzer S, Egan S, Larsen MR, Bartlett DH, Kjelleberg S (2006) Microbiology-Sgm **152:** 1385-1394
Teng CH, Xie Y, Shin S, Di Cello F, Paul-Satyaseela M, Cai M, Kim KS (2006) Infection and Immunity **74:** 5609-5616
Wu, J. & Luther III, G. W. (1996). Geochim Cosmochim Acta **60**: 2729-2741