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The marine bacteria *Shewanella frigidimarina* NCIMB400 upregulates the type VI secretion system during early biofilm formation

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Summary

Shewanella sp. are facultative anaerobic Gram-negative bacteria, extensively studied for their electron transfer ability. *Shewanella frigidimarina* has been detected and isolated from marine environments, and in particular, from biofilms. However, its ability to adhere to surfaces and form a biofilm is poorly understood. In this study, we show that the ability to adhere and to form a biofilm of *S. frigidimarina* NCIMB400 is significantly higher than that of *Shewanella oneidensis* in our conditions. We also show that this strain forms a biofilm in artificial seawater, whereas in Luria–Bertani, this capacity is reduced. To identify proteins involved in early biofilm formation, a proteomic analysis of sessile versus planktonic membrane-enriched fractions allowed the identification of several components of the same type VI secretion system gene cluster: putative Hcp1 and ImpB proteins as well as a forkhead-associated domain-containing protein. The upregulation of Hcp1 a marker of active translocation has been confirmed using quantitative reverse transcription polymerase chain reaction. Our data demonstrated the presence of a single and complete type VI secretion system in *S. frigidimarina* NCIMB400 genome, upregulated in sessile compared with planktonic conditions. The fact that three proteins including the secreted protein Hcp1 have been identified may suggest that this type VI secretion system is functional.

Introduction

The capacity of bacterial species to adhere and form a biofilm *in situ* and *in vitro* has been shown to vary depending on several factors such as gene expression at the time of adhesion, environmental conditions and types of surface involved. In the marine sector, ecological and economic consequences of biofilm and subsequent biofouling formation on immersed artificial surfaces are numerous, such as ship drag reduction or species relocation (Yebra *et al.*, 2004). Several anti-biofilm strategies are being studied in virtually all industrial or medical sectors. The main difficulty in the marine environment is to find ecofriendly alternatives to limit biofilm development that would be non-toxic for other organisms. One of the potential straightforward strategy focuses on the inhibition of adhesion and the early step of biofilm formation on an inert surface. This could represent in combination with other strategies integrating for instance surface properties and structuration as well as other anti-biofilm molecules (targeting quorum sensing or c-di GMP regulatory networks), a way to limit biofilm and biofouling development on ship hull paints. Therefore, understanding the mechanism of adhesion and early biofilm formation of marine bacteria developing on inert surfaces is the first step toward the discovery of potential adhesion inhibitors that could be effective in marine conditions.

Shewanella sp. are facultative anaerobic Gram-negative, motile, rod-like bacteria frequently found and isolated in marine environments as are *Pseudoalteromonas* sp. or *Vibrio* sp. (Patel *et al.*, 2003; Stin *et al.*, 2003; Huggett *et al.*, 2006; Hau and Gralnick, 2007; Fredrickson *et al.*, 2008; Brian-Jaisson *et al.*, 2014; Doghri *et al.*, 2015). The *Shewanella frigidimarina* NCIMB400 strain, a marine isolate, was originally referred to as *Pseudomonas*-like marine bacteria then as *Shewanella putrefaciens* prior to re-classification. *Shewanellae* in particular *Shewanella oneidensis* MR-1, a fresh water isolate have been extensively studied for their ability of electron transfer, with the utilization of a broad array of final electron acceptors in anaerobic conditions (Lee *et al.*, 1977; Reyes-Ramirez *et al.*, 2003; Paquete *et al.*, 2010). Because of their diverse metabolic abilities such as carbon cycling, remediation, microbial fuel cells, *Shewanella* sp. are able to survive in diverse

environments and they have been isolated from habitats characterized by diverse salt concentrations, temperatures and barometric pressures (reviewed in Hau and Gralnick, 2007). Since its first isolation in grease ice and the ice-free zone in Antarctica (Bowman *et al.*, 1997), detection by microarray analysis and isolation of *S. frigidimarina* strains have been performed from marine biofilm samples (Patel *et al.*, 2003; Stin *et al.*, 2003; Brian-Jaisson *et al.*, 2014; Doghri *et al.*, 2015). Overall, little data are available on *S. frigidimarina* ability to adhere and form biofilm on surfaces, in contrast with its freshwater counterpart *S. oneidensis* MR-1 (Vanrobaeys *et al.*, 2003; Thormann *et al.*, 2004; De Vriendt *et al.*, 2005; Learman *et al.*, 2009; Kouzuma *et al.*, 2010; Zhang *et al.*, 2010).

Bacteria are capable of expressing a whole set of diverse adhesins on their surface, such as fimbrial adhesins (i.e. Type IV pili), curli and non-fimbrial adhesins (i.e. integral outer membrane adhesins) and secreted adhesins. Expression of these proteins, which can play a role in adhesion and biofilm formation, can differ in function of species or strains, environmental conditions and surfaces (reviewed in Gerlach and Hensel, 2007). In this study, we have examined adhesion and biofilm formation of *S. frigidimarina* NCIMB400 in comparison with that of *S. oneidensis* MR-1. A two-dimensional (2D) proteomic approach was used to compare the membrane-enriched proteins fractions from sessile versus planktonic *S. frigidimarina* with the objective of identifying proteins of *S. frigidimarina* that play a role in adhesion and the early step of biofilm formation.

Results and discussion

Comparative analysis of adhesion kinetics of S. frigidimarina and S. oneidensis

Because adhesion is a key step of biofilm formation, the adhesion ability of *S. frigidimarina* NCIMB400 was evaluated using a bacterial nucleic acid staining assay adapted from previous studies (Leroy *et al.*, 2007; Camps *et al.*, 2011). PBS and ASW (artificial seawater), two buffered saline (organic free) solutions are often used for adhesion assays (Zampini *et al.*, 2005; Stauder *et al.*, 2010; Camps *et al.*, 2011) to avoid potential bacterial growth over the time of the experiment. Both *Shewanella* species were unable to grow in ASW and PBS and gave similar results in this adhesion assay (data not shown). The number of adhered bacteria increased with time for both *S. frigidimarina* NCIMB400 and *S. oneidensis* MR-1 in ASW (Fig. 1A and B). A saturation of *S. frigidimarina* adhesion can be observed at OD₆₀₀ of 0.5 following 5 h incubation, with no further increase in the number of bacteria adhered to the bottom of the microplates. The most

important progression was observed at OD₆₀₀ 0.1 and 0.3 for both *Shewanella* species. At OD₆₀₀ 0.3, the number of *S. frigidimarina* NCIMB400 adhered to the surface was significantly higher than that of *S. oneidensis* MR-1 at 5, 12, 24 h ($P < 0.05$, $P < 0.001$ and $P < 0.001$ respectively) but not at 48 h, whereas at OD₆₀₀ 0.1, the difference was significant only at 48 h ($P < 0.05$) (Fig. 1C). As expected, *Pseudomonas aeruginosa* PAO1 used as a test control adhered more efficiently at 24 h than the *Shewanella* strains (Fig. 1D). Taken together, these results show that *S. frigidimarina* is able to adhere *in vitro* to microplates in a similar manner than *S. oneidensis*, with a significantly higher number of adhered bacteria for *S. frigidimarina* NCIMB400 at all time points.

Evaluation of biofilm formation of S. frigidimarina and S. oneidensis using the Biofilm Ring Test®

The biofilm formation of *S. frigidimarina* NCIMB400 and *S. oneidensis* MR-1 was evaluated at different time points, either in Luria–Bertani (LB) broth (rich medium), ASW or PBS (both organic free) solutions for each of the strains using the Biofilm Ring Test® (Fig. 2A–E). The Biofilm Ring Test®, an innovative tool allowing the evaluation of biofilm formation based on the motility of inert paramagnetic microbeads, has the advantage of bringing a different approach to study biofilms, focusing rather on density and extracellular networks (Chavant *et al.*, 2007). At most time points (Fig. 2D and E), significantly highest Δ BFI (BFI for Biofilm Formation Index) for *S. frigidimarina* NCIMB400 and *S. oneidensis* MR-1 were obtained when bacteria were suspended in PBS or ASW but not in LB ($P < 0.001$), suggesting that ASW promotes the formation of biofilm of *S. frigidimarina* NCIMB400 and *S. oneidensis* MR-1, whereas LB represses this ability. The results also show that in ASW, *S. frigidimarina* biofilm reaches a plateau at 12 h, whereas the progression of biofilm formation is slower for *S. oneidensis* (Fig. 2B,D and E). The Δ BFI of the biofilm formed in ASW at 12 and 24 h are significantly higher ($P < 0.001$) for *S. frigidimarina* compared with *S. oneidensis*, demonstrating that *S. frigidimarina* biofilm formation is faster than that of *S. oneidensis* in ASW. *Pseudomonas aeruginosa* PAO1, as previously observed (Nagant *et al.*, 2010), show very high Δ BFI values at 0 and 5 h (Fig. 2A–C and data not shown), probably due to a high initial number of adhered bacteria for this strain. *Shewanella oneidensis* presents the same pattern in LB only, while the Δ BFI of *S. frigidimarina* is only positive after 5 h. Taken together, these data indicate that in ASW, *S. frigidimarina* NCIMB400 is able to form a biofilm faster than *S. oneidensis* MR-1.

In this experiment, *S. frigidimarina* biofilm formation was triggered in the ASW and PBS solutions, but not in

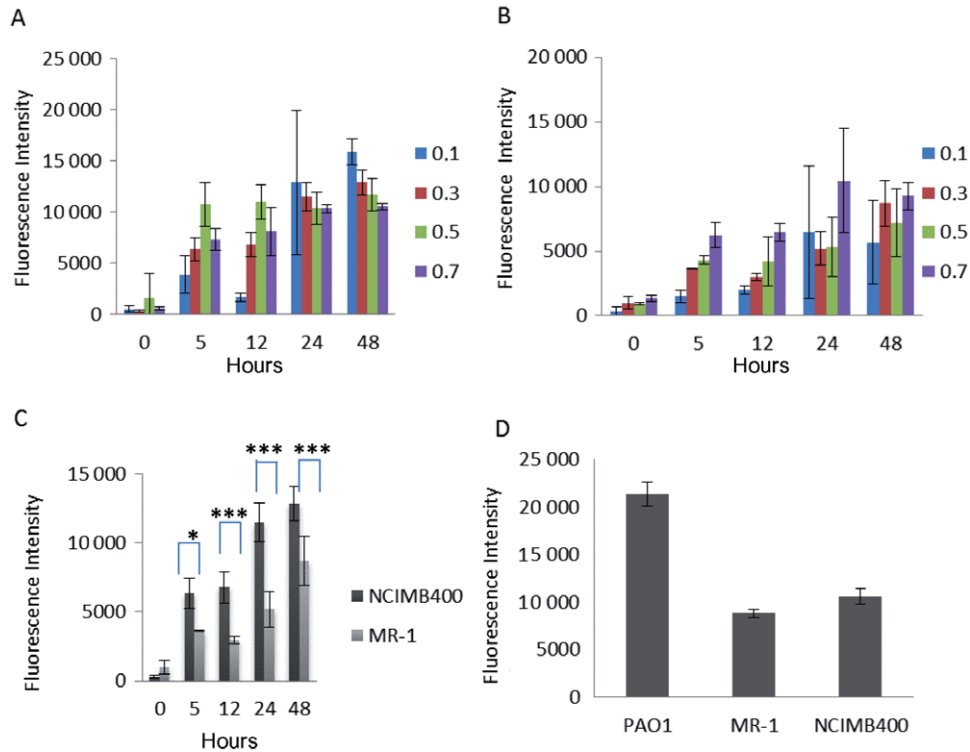


Fig. 1. Adhesion assays of *S. frigidimarina* NCIMB400. Post-exponential strains of *S. frigidimarina* NCIMB400, *S. oneidensis* MR-1 and *P. aeruginosa* PAO1 were inoculated in ASW solution and stained with Syto61. The fluorescence intensity of bacteria adhered at the bottom of the plates was evaluated at 0, 5, 12, 24 and 48 h. Adhesion of (A) *S. frigidimarina* and (B) *S. oneidensis*, inoculated at different concentrations ($OD_{600nm} = 0.1, 0.3, 0.5$ and 0.7); Comparison of *S. frigidimarina* and *S. oneidensis* adhesions (C) when inoculated at an OD_{600nm} of 0.3 ; adhesion of *S. frigidimarina* compared with that of *S. oneidensis* and of *P. aeruginosa* (D) at 24 h with an initial OD_{600nm} of 0.3 . The error bars indicate the standard errors of the means. Statistical significance was accepted at $p < 0.05$. * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

LB. LB is a nutrient-rich medium, in which *S. frigidimarina* and *S. oneidensis* are frequently propagated (as they have no real salt requirement) (Bowman *et al.*, 1997; Bozal *et al.*, 2002). PBS, which has no carbon source and ASW, a sea salt solution, resembling the composition of the salts dissolved in seawater, with bicarbonate as sole carbon source, were used in this assay as nutrient-poor medium. Tween 20 was added to these solutions for the Biofilm Ring Test[®], at the concentration 0.004% w/v, below the inhibiting concentration for growth or biofilm formation (Slijkhuis *et al.*, 1984; Toutain-Kidd *et al.*, 2009; Brian-Jaisson *et al.*, 2014). It is important to note that no bacterial growth was observed at this concentration of Tween in ASW and PBS (data not shown). It has been shown that bicarbonate can be assimilated by numerous heterotrophic or mixotrophic bacteria (DeLorenzo *et al.*, 2012), and Tween 20 can be hydrolysed by both *Shewanella* strains (Bowman *et al.*, 1997; Bozal *et al.*, 2002). The fact that in PBS (no carbon source), the biofilm formation of *S. frigidimarina* was more important than in LB, suggests that carbonate does not play a role in the biofilm development in this assay for this strain, but it remains to check if Tween does. An earlier study, under nutrient-limiting conditions, also resulted in the formation

of a more robust biofilm by *S. oneidensis* (Thormann *et al.*, 2004). It is known that environmental conditions influence bacterial biofilm formation in different ways. For some bacteria, such as *E. coli* O157:H7 and *Salmonella enterica* serovar *typhimurium*, nutrient limitation triggers the development of biofilm through the stationary-phase sigma factor RpoS activation of biofilm-related genes. Furthermore, under starvation or stress conditions, the extracellular polysaccharides (EPS) composition and biofilm structure have also been shown to be modified (Parsek and Tolker-Nielsen, 2008; Myszka and Czacyk, 2009). However, in other bacteria, such as *Vibrio cholerae*, biofilm formation is stimulated by nutrient-rich environments, which are also regulated by RpoS (Dewanti and Wong, 1995; Karatan and Watnick, 2009). Some osmolytes, including NaCl and/or sucrose, that produce a high osmolarity environment can also inhibit the biofilm formation of *S. typhimurium* (White *et al.*, 2010). *Vibrio cholerae* can form a biofilm under high-salt solutions, a phenomenon controlled by the regulator OscR (osmolarity controlled regulator), which can modulate gene transcription involved in biofilm matrix production (Karatan and Watnick, 2009; Shikuma and Yildiz, 2009). Therefore, the variation of the response in

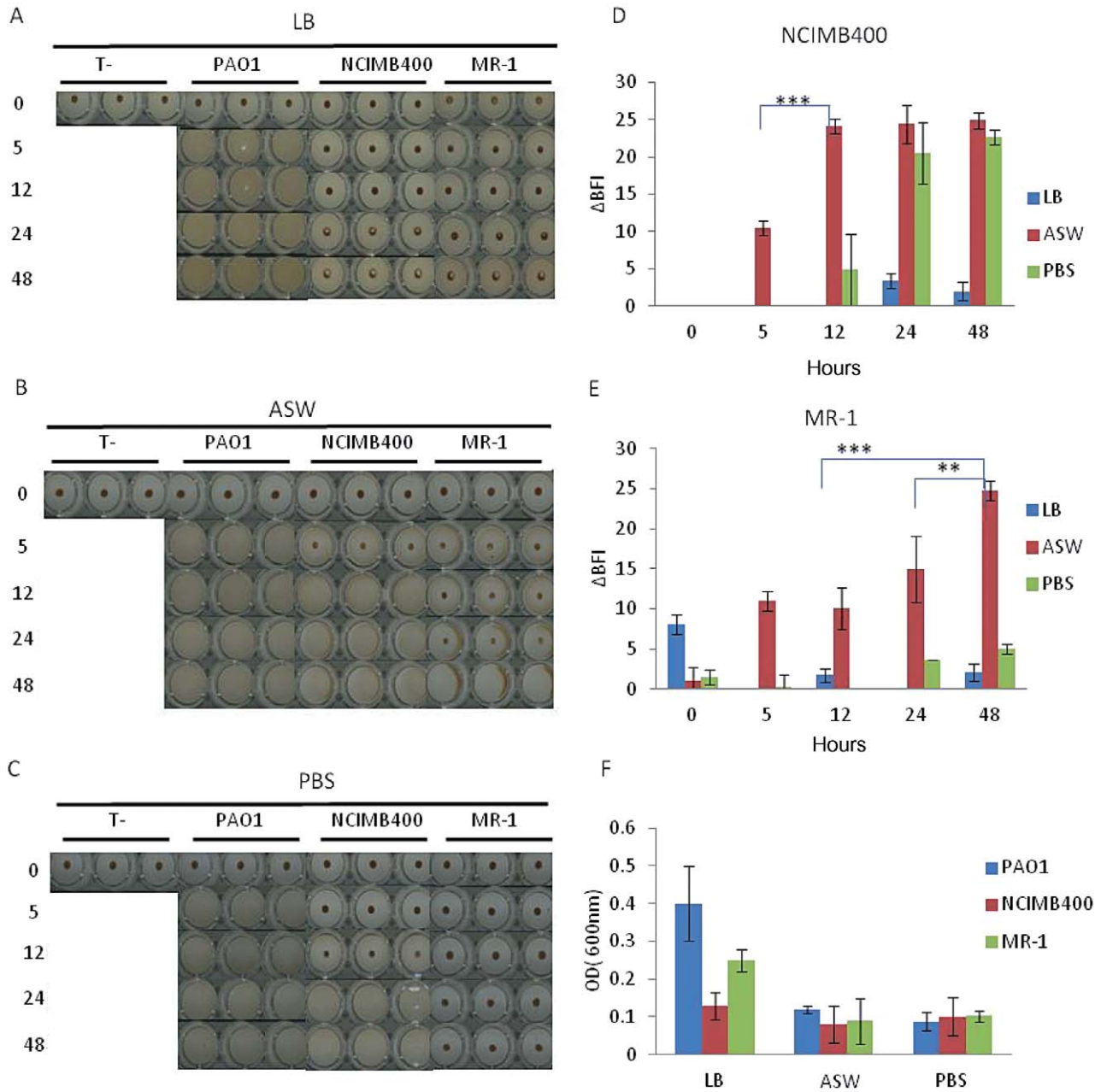


Fig. 2. Biofilm formation of *S. frigidimarina* NCIMB400. Post-exponential cultures of *S. frigidimarina* NCIMB400, *S. oneidensis* MR-1 and *P. aeruginosa* PAO1 were incubated in microplates for 0, 5, 12, 24 or 48 h in LB, ASW or PBS as shown in the photographs in A, B, C in presence of paramagnetic microbead. After incubation and magnetization of the microplates, mobility of the beads was evaluated using Biofilm Control plate reader. Kinetics of Δ BFI (Biofilm Formation Index) are presented in D and E for NCIMB400 and MR-1 respectively. Bacterial concentrations after biofilm formation have been evaluated at 24 h (F). After elimination of supernatants, cells were washed, scraped and re-suspended in sterile LB, ASW or PBS before OD₆₀₀ was measurement. The error bars indicate the standard errors of the means. Statistical significance was accepted at $p < 0.05$. * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

adhesion and biofilm formation in different nutrient-limiting and osmolarity environments may explain the results found for *Shewanella* sp. using the Biofilm Ring Test®. We believe that it is rather exudates (types and density) of the biofilm matrix that are produced in ASW than an increase in bacterial number, which is responsible for the high Δ BFI

level observed for *S. frigidimarina*. Studying more precisely which components of the media are responsible for biofilm formation by *S. frigidimarina* would be of interest, in particular what is triggering biofilm formation in ASW and/or what is inhibiting biofilm formation in rich media such as LB.

To determine if the immobilization of the magnetic beads in the previous assay by *S. frigidimarina* NCIMB400 and *S. oneidensis* MR-1 in ASW or PBS solutions was due to a higher number of bacteria bound to the surface of the wells or by the biofilm matrix itself, a strip well inoculated in the same conditions was kept to evaluate bacterial concentration for each condition at 24 h. The results in Fig. 2F show that except for *P. aeruginosa* PAO1, the concentrations of resuspended bacteria present in the wells at 24 h are very close to each other whether LB, ASW or LB was used, using the spectrophotometer and the CFUs numeration (data not shown). No statistical difference was found for *S. frigidimarina*, when comparing these media. This suggests that at 24 h, for *S. frigidimarina* despite significantly different Δ BFI in LB, ASW and PBS, the microbeads were trapped in the actual biofilm matrix of *S. frigidimarina* rather than by an increased number of bacteria in the wells. The study of the structural composition of the biofilm matrix formed in ASW should confirm that *S. frigidimarina* produces more (and/or different) extracellular components in ASW than in LB, as suggested by the results shown in Fig. 2F.

Proteomic analysis of sessile versus planktonic S. frigidimarina

To compare and identify bacterial membrane proteins involved in adhesion and early biofilm formation, 2D proteomic analysis of adhered versus planktonic *S. frigidimarina* have been performed in LB broth, its growth medium (Dobbin *et al.*, 1999; Gordon *et al.*, 2000; Reyes-Ramirez *et al.*, 2003). The reasons why we selected LB for this approach are as following: (i) planktonic bacteria cannot be produced in ASW as *S. frigidimarina* NCIMB400 cannot grow in this solution; (ii) the use of LB limits the upregulation of stress-related genes/proteins and phenotypical modifications due to the change of medium that would be required if we grow first the bacteria in LB and then switch to ASW; (iii) we are rather looking for bacterial surface components involved in adhesion and early steps of biofilm formation than extracellular exudates of the biofilm matrix; (iv) LB provides a similar number of adhered bacteria than in ASW and PBS (Fig. 2F), which means that bacteria develop similar initial strategies to adhere onto a polystyrene surface.

Post-exponential *S. frigidimarina* NCIMB400 were inoculated in LB medium, either in Petri dishes or in an Erlenmeyer flasks under agitated conditions for 24 h, to constitute respectively the sessile and the planktonic samples to be analysed. It is noteworthy to precise that for the sessile samples, bacteria are inoculated directly into multiples Petri dishes (without agar) in order for the bacte-

ria to adhere directly onto the polystyrene surface of the dishes (similar to adhesion and biofilm assays described above). A membrane enrichment step was performed on the pellet following the harvest, and proteins were extracted, quantified and subjected to a 2D electrophoresis approach using pH 3–10 or pH 4–7 IPG (immobilized pH gradient) Strips (Table S1). A total number of 105 and 267 spots were detected with the PDQuest 2D – Analysis software for the non-enriched (pH 3–10 linear Ready Strip only) fractions from both planktonic and sessile bacteria respectively. For the membrane-enriched fractions, 167 and 185 spots with the pH 3–10 IPG Strips versus 564 and 416 spots using the 4–7 IPG Strips were found from both planktonic and sessile bacteria respectively (Table S1). The total number of matched spots and the total number of upregulated spots detected in each condition show a higher number of spots in both planktonic and sessile conditions when pH 4–7 IPG Strips was used (Table S1). The pH 4–7 IPS Strips re-centred the spot window in the 2D gels, increasing both resolution and separation of detectable spots (Fig. S1). As a result, using the gels pH 4–7, 22 spots in the membrane-enriched fraction from the sessile sample were selected for protein sequencing.

The result of sequencing provided a list of proteins for each spot sequenced (Table 1) corresponding to a specific annotated gene tag in the *S. frigidimarina* NCIMB400 sequenced genome (<http://genome.jgi.doe.gov/>), including those for uncharacterized proteins. For all these proteins, we checked using the UniProtKB (<http://www.uniprot.org/>) and NCBI websites that the gene tag corresponded to each protein-coding name. Information given by the gene related to the identified peptides on the annotated sequences genome proved to be very useful to finalize the analysis including for the putative uncharacterized proteins (Table 1). The predicted subcellular localization tool was provided by PSIPREP (<http://www.imtech.res.in/raghava/pslpred/submit.html>) and PSORTB (<http://www.psort.org/psortb/>). Among the 22 spots listed in Table 1, at least 12 potential surface or membrane-associated protein candidates were identified along with 10 presumptive cytosolic proteins.

The most striking results of this 2D proteomic analysis concern the proteins Q080U3, Q080V9 and Q080V1, which according to the genome annotation of *S. frigidimarina* NCIMB400 (<http://img.jgi.doe.gov/>), belong to a gene cluster related to the type VI secretion system (T6SS). The BLAST of nucleic sequences of all putative components of this T6SS suggests that this T6SS is complete, including all basal core components as found in other bacteria (Filloux *et al.*, 2008; Pukatzki *et al.*, 2009; Barret *et al.*, 2011), and all of these 27 sequences are unique ones in the NCIMB400 genome (Fig. 3). Up to 6 T6SS loci, which are localized in genomic islands acquired by HGT (horizontal gene transfer), have been

Table 1. Identification of the protein spots upregulated in *S. frigidimarina* NCIMB400 in sessile conditions.

Spot	Name	Gene ID	MW	log(E value)	Coverage (%)	Spectra	Uniques	PAI	Predicted subcellular localization ^a
1	Q084W3_ABC transporter related	Sfri_1349	58,7	-85,4	41	28	20	1,6	Inner-membrane
2	Q080U3_FHA domain-containing protein	Sfri_2377	50,9	-71,4	50	29	17	1,6	Periplasmic
3	Q07YN4_Band 7 protein	Sfri_3042	32,5	-74,2	76	29	22	1,7	Inner-membrane
4	Q080V9_Putative uncharacterized protein	Sfri_2361	17,5	-21,6	45	14	9	2,0	Surface associated, Extracellular
5	Q07ZX4_Peptidyl-prolyl cis-trans isomerase	Sfri_2600	17,8	-25,3	57	14	7	2,5	Cytoplasmic
6	Q084W3_ABC transporter related	Sfri_1349	58,7	-146,0	63	89	34	5,0	Redundant with n°1 ^b
7	Q088S7_Shikimate kinase	aroK	19,1	-33,8	55	12	8	1,5	cytoplasmic
8	Q088X8_GTP-binding protein TypA	Sfri_0324	66,2	-151,5	57	77	46	4,4	Cytoplasmic
9	Q086D0_ABC transporter related	Sfri_1032	62	-136,5	67	56	34	2,4	Inner-membrane
10	Q07Z26_OmpA/MotB domain protein	Sfri_2899	41,2	-86,9	59	43	24	3,0	Outer-membrane
11	Q080V1_Uncharacterized conserved protein	Sfri_2369	18,8	-28,6	61	11	8	2,2	Cytoplasmic
12	Q07Z26_OmpA/MotB domain protein	Sfri_2899	41,2	-70,9	62	26	21	1,9	Redundant with n°10
13	Q07Z30_Peptidase M28	Sfri_2895	51,2	-116,9	51	51	26	3,1	Extracellular
14	IQ08A48_DNA gyrase subunit B	Sfri_0004	89,7	-95,0	39	37	28	1,5	Cytoplasmic
15	Q086D0_ABC transporter related	Sfri_1032	62	-153,8	73	90	39	3,8	Redundant with n°9
16	Q07Z90_HAD-superfamily phosphatase, subfamily IIIC	Sfri_2835	68,3	-100,2	57	45	29	1,6	Cytoplasmic
17	Q07YL8_MiIA-interacting MipA family protein	Sfri_3058	27,8	-32,1	49	11	8	2,8	Outer-membrane
18	Q084C6_Chaperone protein htpG	htpG	71,4	-103,3	48	34	26	1,2	Cytoplasmic
19	Q084W6_DNA polymerase I	Sfri_3943	101,6	-93,4	39	44	29	1,2	Cytoplasmic
20	Q086K8_NADH:ubiquinone oxidoreductase, subunit F	Sfri_0954	45,7	-104,1	62	67	27	3,7	Cytoplasmic
21	Q07Z82_Polysaccharide export protein	Sfri_2843	99,7	-158,0	61	67	44	2,5	Periplasmic
22	Q07W99_Rod shape-determining protein MreB	Sfri_3738	37,2	-73,2	52	38	18	2,7	Cytoplasmic

a. Predicted subcellular localization using PSIPREP and PSORTB.

b. Redundant spots; putative isoforms.

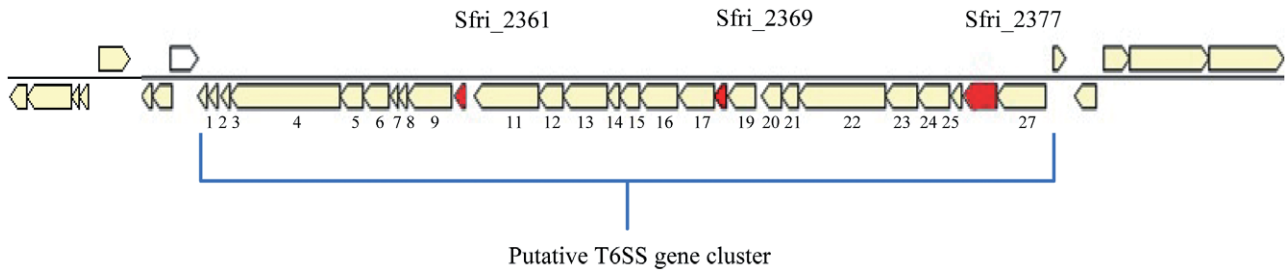


Fig. 3. T6SS putative gene cluster of *S. frigidimarina* NCIMB400. The genes Sfri_2361 (n°10), Sri_2369 (n°18) and Sfr_2377 (n°26) are located within a putative T6SS gene cluster in the genome of *S. frigidimarina* NCIMB400. Annotations in the JGI genome website (<http://img.jgi.doe.gov/>) include the following information:

- 1 (Sfri_2352)-3(Sfri_2354): Hypothetical proteins
- 4 (Sfri_2355): RHS-repeat associated core domain-containing protein
- 5 (Sfri_2356)-6 (Sfri_2357): Hypothetical proteins
- 7 (Sfri_2358): Zn-binding-PAAR domain-containing protein (involved in T6SS)
- 8 (Sfri_2359): Hypothetical protein
- 9 (Sfri_2360): T6SS secreted protein VgrG
- 10 (Sfri_2361): T6SS secreted protein HCP
- 11 (Sfri_2362): T6SS protein VasG (ATPase, ClpV family protein)
- 12 (Sfri_2363): T6SS protein ImpH
- 13 (Sfri_2364): T6SS protein ImpG
- 14 (Sfri_2365): T6SS protein ImpF
- 15 (Sfri_2366): T6SS protein ImpE
- 16 (Sfri_2367): T6SS protein ImpF
- 17 (Sfri_2368): T6SS protein ImpE
- 18 (Sfri_2369): T6SS protein ImpD
- 19 (Sfri_2370): T6SS protein ImpA
- 20 (Sfri_2371): Protein serine/threonine phosphatase
- 21 (Sfri_2372): T6SS protein ImpM
- 22 (Sfri_2373): T6SS protein ImpL(lcmF domain protein)
- 23 (Sfri_2374): T6SS protein ImpK (OmpA/MotB domain)
- 24 (Sfri_2375): T6SS protein ImpJ
- 25 (Sfri_2376): T6SS protein VasD
- 26 (Sfri_2377): FHA domain-containing protein
- 27 (Sfri_2378): Serine/threonine protein kinase

found within the same bacterial strains (Barret *et al.*, 2011; De Maayer *et al.*, 2011; Mulder *et al.*, 2012). This recently discovered secretion system has been shown in many Gram-negative bacteria to be involved in bacterial pathogenicity, in inter-bacterial communication, intra- and inter-species competition, biofilm formation and environmental stress response (Sheng *et al.*, 2013; Zhang *et al.*, 2014; Alcoforado Diniz and Coulthurst, 2015; Aragon *et al.*, 2015). Almost no references are available on its link with biofilm formation of marine bacteria (Vaysse *et al.*, 2009; Sheng *et al.*, 2013).

The Sfri_2361 gene (protein Q080V9) is related to the T6SS effector Hcp1 (haemolysin-coregulated protein), as suggested by its annotation (Joint Genome Institute website) and its sequence alignments (NCBI, UniProtKB websites). One key characteristic of T6SS activity is the expression of phage-like proteins Hcp and Vgr (valine-glycine-repeat protein) G/S, part of the contractile sheath and tailspike of the secretion apparatus. They are secreted structural T6SS components (De Maayer *et al.*, 2011; Records, 2011) whose expression can be dependent on experimental conditions in some bacteria (Mulder *et al.*,

2012). The Hcp protein is secreted by all bacteria with a functional T6SS and has become a reliable indicator of T6SS function, even if its gene is not always found in T6SS loci (Pukatzki *et al.*, 2009). Therefore, it is most likely that the T6SS of *S. frigidimarina* NCIMB400 is functional.

The Sfri_2369 gene corresponds to an uncharacterized protein (Q080V1) orthologous to T6SS *impB* of the plant symbiont *Rhizobium leguminosarum*. The locus *imp* (for impaired in nodulation), comprising the first described genes of T6SS, is essential for nodulation and subsequent stages of symbiosis. The ImpB protein appears to be part of the secretion apparatus across the bacterial membranes, but its exact localization has to be confirmed (Filloux *et al.*, 2008). Analysis of its peptidic sequence using PSLPRED and PSORTB suggests, however, a cytoplasmic localization.

The gene Sfri_2377, which belongs to the same T6SS gene cluster, corresponds to a FHA (forkhead-associated) domain-containing protein (Q080V3), specifically recognizing phosphothreonine residues (Mahajan *et al.*, 2008; Spivey *et al.*, 2011). Some FHA domain-containing proteins have been shown to be present in ABC transporters

or T6SS components (Bernard *et al.*, 2010). For instance, the *fhA1* gene of *P. aeruginosa* (PA0081) containing an amino-terminal FHA domain, is part of the Hcp secretion island-I-encoded type VI secretion system (H-T6SS) loci (Boyer *et al.*, 2009). The active form of Fha1 protein is required for secretion of the T6SS substrate Hcp1 (Mougous *et al.*, 2007; Hsu *et al.*, 2009). The activation of the T6SS involves a post-translational regulatory cascade requiring a serine-threonine kinase and a phosphatase for the regulation of the FHA inner membrane protein (Bernard *et al.*, 2010). Interestingly, analysis of the surrounding genes within the T6SS gene cluster of *S. frigidimarina* NCIMB400 showed the presence of a serine/threonine kinase (Sfr_2378) and a serine/threonine phosphatase (Sfri_2371), upstream and downstream, respectively, of the FHA domain-containing protein (Fig. 3). Although *S. frigidimarina* T6SS includes approximately 20–27 proteins (Fig. 3), as previously shown in other bacterial T6SS (De Maayer *et al.*, 2011; Mulder *et al.*, 2012), whether this T6SS is functional in the *S. frigidimarina* NCIMB400 strain and is involved in adhesion and biofilm formation remain to be further investigated. Overall, these results suggest that these three proteins (Q080U3, Q080V9, Q080V1), upregulated in sessile conditions, are part of a same putative T6SS gene cluster in *S. frigidimarina* NCIMB400.

Among the other interesting candidates potentially involved in adhesion and early biofilm formation, the polysaccharide export protein Q07Z82 is a putative membrane-associated protein that is potentially involved in the export of EPS or capsular polysaccharides that form a layer associated to the cell surface (Cuthbertson *et al.*, 2009). Several *Shewanella* sp. lipopolysaccharides (LPSs) have been studied, although little is known about the EPS and their export/biogenesis in this genus (Korenevsky *et al.*, 2002). *Shewanella oneidensis*, *Shewanella algae*, *S. frigidimarina*, and *Shewanella* sp. strain MR-4 possess either O-side chains or capsular fringes, albeit are technically difficult to distinguish. The composition and length of surface polysaccharide polymers could play a role in its ability to adhere to surfaces (Korenevsky *et al.*, 2002; Vinogradov *et al.*, 2005; Kouzuma *et al.*, 2010).

The proteomic analysis of the sessile membrane fraction of *S. frigidimarina* NCIMB400 reveals two ABC transporter-related proteins, Q084W3 and Q086D0 (Table 1). Transporters with ATP-binding cassette (ABC) are part of a superfamily of ubiquitous proteins located in the periplasm and the inner membrane (as found using PSLPRED and PSORTB) (Davidson and Maloney, 2007; Davidson *et al.*, 2008). It has been reported for instance that transition from reversible to irreversible attachment prior to biofilm formation of *P. fluorescens* involves an ABC transporter LapEBC (Hinsa *et al.*, 2003). The *lap*

mutants are unable to attach in an irreversible way and to form a biofilm including inert surfaces (Hinsa *et al.*, 2003).

The OmpA/MotB domain protein identified in this study (Q07Z26) is usually located in the C-terminal region, and this structure is found in outer membrane proteins of many Gram-negative bacteria, such as the porin-like integral membrane proteins (like OmpA), the small lipid-anchored proteins (such as Pal) and MotB, a component of a transmembrane proton channel required for anchoring the stator complex of the flagellar motor to the peptidoglycan of the bacterial cell wall (Van Way *et al.*, 2000; Dumetz *et al.*, 2007; Goulter *et al.*, 2009). The N-terminal half is variable, although some of the proteins in this group have the OmpA-like transmembrane domain at the N terminus, which is also the case of Q07Z26. The role of Omp proteins such as *E. coli* OmpA, in various phenotypes including adhesion and biofilm formation, has been extensively studied. OmpA influences bacterial attachment by modulating Type 1 fimbrial expression (reviewed in Smith *et al.*, 2007, and in Krishnan and Prasadarao, 2012).

qRT-PCR of sessile versus planktonic S. frigidimarina in LB and ASW

To verify the results found using the 2D proteomic analysis on some of the candidates, qRT-PCR was used to analyse gene transcriptional expression of *hcp1* (Q080V9; Sfri_2361) and *impB* (Q080V1; Sfri_2369) both part of the same T6SS gene cluster and the *pep* gene (Q07Z82; Sfri_2843) corresponding to the polysaccharide export protein. The comparison of their transcription in ASW and in LB (Fig. 4) shows that at least the *hcp1* gene was significantly upregulated in the LB sessile fraction confirming the result found in the proteomic analysis. The result with the *impB* gene showed the same profile but with no significant difference. The *pep* gene was also upregulated in LB planktonic and more importantly sessile conditions. As flagella genes and proteins are upregulated in many cases in planktonic compared with sessile conditions (Guttenplan and Kearns, 2013), the *flgE* gene from the flagella apparatus was used to verify if we would obtain a different pattern. This gene involved in flagella motility was only upregulated in the LB and planktonic fractions. Taken together, these results corroborate the results found with the 2D proteomic analysis regarding the upregulation of the T6SS functional marker *hcp1* as well as of the polysaccharide export protein in LB and sessile conditions. The reason why the *hcp1* or *pep* genes are not upregulated in ASW is not known. However, as said above, the medium and the presence of some osmolytes could modulate the expression of these genes. It is possible that the T6SS (through its involvement in competition) is more relevant for *S. frigidimarina* in rich media and

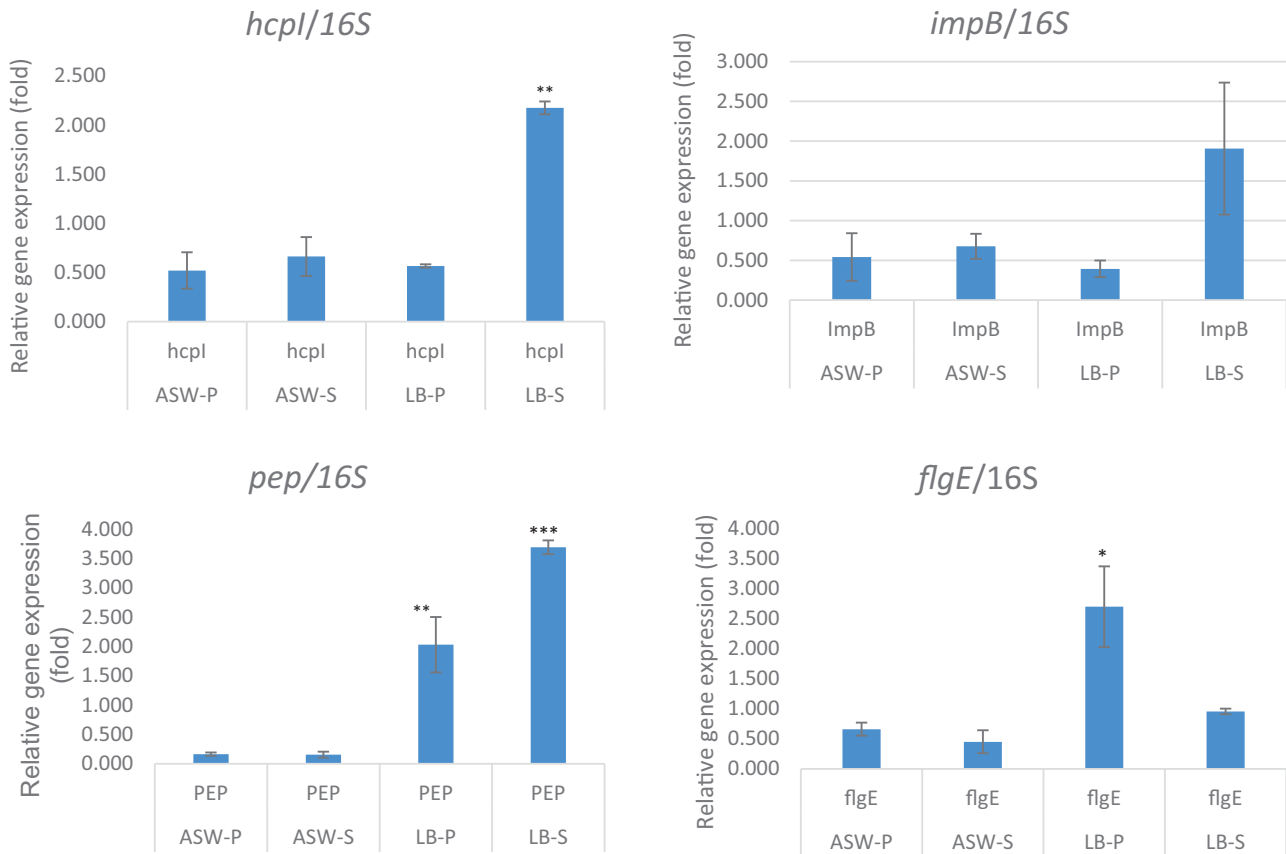


Fig. 4. qRT-PCR analysis of *hcpI*, *impB*, *pep* and *flgE* transcript levels in *S. fridigimarina* in the sessile (S) versus planktonic (P) fractions. The samples have been prepared in LB or ASW media. The relative difference has been calculated using the control gene 16S rRNA. Statistical significance was accepted at $p < 0.05$. * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

that the *pep* gene synthesizes a type of polysaccharide that is only produced in LB. Other polysaccharides may be produced in ASW. *Pseudomonas aeruginosa* is known to produce different types of polysaccharides such as Pel (pellicle) and Psl (*polysaccharide synthesis locus*). Both are regulated by high level of intracellular c-di-GMP as well as by the stationary-phase σ -factor RpoS, which induces *psl* transcription in response to changes in global physiology (Mann and Wozniak, 2012). The regulation of the T6SS and secretion of polysaccharides are tied to several and complex regulatory networks that remain to be investigated (Bernard *et al.*, 2010). The verification of the actual secretion of Hcp1-like protein by *S. fridigimarina* NCIMB400 and of *pep* gene-associated polysaccharides in these media would fully demonstrate the functionality and the regulation of these genes in different situations.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. 2D polyacrylamide gels of planktonic and sessile membrane protein enriched fractions from *S. frigidimarina* NCIMB400, performed using pH 3-10 (A and B) or pH 4–7 IPG strips (C and D). Upregulated spots obtained under sessile conditions (B and D) are indicated by arrows. Spot numbers in D correspond to those displayed in Table 1. The pI and molecular weight (MW) scales are indicated for the horizontal and vertical axis, respectively.

Table S1. Number of protein spots of non-soluble and membrane-enriched fractions, separated by 2D electrophoresis comparing planktonic and sessile conditions.