
Influence of subtilisin on the adhesion of a marine bacterium which produces mainly proteins as extracellular polymers

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Abstract:

Aims: The nature of exopolymers involved in the adhesion of a marine biofilm-forming bacterium *Pseudoalteromonas* sp. D41 was investigated to evaluate and understand the antifouling potential of subtilisin.

Methods and Results: The exopolymers of D41 produced by fermentation were analysed by FTIR and SDS-PAGE showing the presence of polysaccharides, glycoproteins and proteins. A high content of proteins was detected both in soluble and capsular fractions. The microscopic observations of fluorescamine and calcofluor stained adhered D41 indicated mainly the presence of proteins in exopolymers produced during adhesion. Subtilisin, the broad spectrum protease, tested in natural sea water and in polystyrene microplates showed that antifouling activity was higher in the prevention of bacterial adhesion than in the detachment of adhered D41 cells.

Conclusions: Overall, these results demonstrate the involvement of proteins in *Pseudoalteromonas* sp. D41 adhesion and confirm the high antifouling potential of subtilisin.

Significance and Impact of the Study: This study emphasizes the major role of proteins instead of polysaccharides, thus extending our knowledge regarding the nature of extracellular polymers involved in bacterial adhesion. Furthermore, the high antifouling potential of subtilisin evaluated in the very first stages of fouling, bacterial adhesion, could lead to less toxic compounds than organometallic compounds in antifouling paint.

Keywords: biofilm, exopolymer, subtilisin, *Pseudoalteromonas*, antifouling, capsular protein.

1. Introduction

Marine biofilm is described as one of the first steps in fouling formation. It is composed of organic (proteins and osidic fragments) and inorganic compounds like salts (Compère et al., 2001), bacteria and microalgae (Wahl, 1989). Bacteria colonize the surface thanks to appendages and envelope structures such as flagella, pili, peptidoglycan or adhesin (Lejeune, 2003) and consequently build up a biofilm. This latter consists of bacteria embedded in their slime, composed mainly of extracellular polymeric substances (EPS) such as polysaccharides or proteins (Sutherland, 2001; Allison, 2003; Branda et al., 2005).

Exopolysaccharides have been the most studied EPS up to now. They are described as directly involved in the architecture of biofilm and several of them have already been characterised. Surprisingly, extracellular proteins have been less studied and although their presence in biofilm has been demonstrated (Jahn et al., 1999; van der Aa and Dufrene, 2002; Donlan et al., 2004, Kogan et al., 2006), few studies have explored with the nature or primary structure of these proteins and their role in attachment to surfaces has not been elucidated yet. Some proteins could be indirectly involved in biofilm formation such as enzymes which could lead to polysaccharide synthesis as already described in dental biofilm (Welin et al., 2004). Proteins in *Pseudomonas aeruginosa* biofilm can represent 30% of total EPS biomass, with molecular masses averaging 50 to 150 kDa and exhibit a high proteolytic activity (Flemming and Wingender, 2001). Considering cell-wall anchored proteins, some of them can be directly involved in biofilm formation such as adhesins involved in *Escherichia Coli* adhesion (Roux et al., 2005) or like surface proteins Bap, Esp and BapA and have been directly involved in *Staphylococcus aureus*, *Enterococcus faecalis* and *Salmonella enteritidis* biofilms respectively (Curacella et al., 2001; Toledo-Arana et al., 2001; Latasa et al., 2005).

Pseudoalteromonas sp. D41 is a marine biofilm-forming bacteria isolated from a natural 24 h marine biofilm formed in the bay of Brest (France) (Rubio, 2002). A previous study of its chemical composition surface highlighted the presence of proteins in its upmost layer (Pradier et al., 2005). Therefore, we decided to study extracellular polymeric substances (EPS) excreted by *Pseudoalteromonas* sp. D41. In this study, the biochemical analysis of EPS produced by *Pseudoalteromonas* sp. D41 was performed both after fermentation by FTIR and SDS PAGE analysis and upon adhesion by epifluorescence microscopy using DAPI (4'-6-diamidino-2-phenylindole), calcofluor and fluorescamine stains for DNA, β -glucans and protein detection respectively. Results show a high rate of protein content both in EPS excreted during fermentation, especially in capsular fraction, and upon bacterial adhesion.

Fouling on marine underwater surfaces causes critical and economic problems such as extensive material damages and industrial performance reductions. The use of biocides like organometallic compounds causes environmental problems and European regulations regarding antifouling paints are becoming increasingly restrictive, banishing organostannic use on all vessels in 2008 (Directive 98/8/CE). Finding environmentally friendly antifouling compounds has thus reached emergency proportions today. The control of biofilm development by hydrolysing organic compounds involved in the first steps of adhesion using enzymatic treatments is one strategy to prevent fouling. Enzymes compared to organometallic compounds show more biodegradability, weak toxicity and inhibit adhesion instead of killing fouling organisms. Alcalase and Savinase have demonstrated high antifouling potential by inhibiting the adhesion of fouling macroorganisms, such as cypris larvae and the spores of green alga or diatoms (Pettitt et al., 2004), and fouling microorganisms, such as *Pseudoalteromonas* sp. D41 (Leroy et al., in press) respectively. These two commercial enzymatic preparations are based on subtilisin, a broad spectrum protease. Moreover, the effectiveness of enzymatic treatments seems to depend on EPS chemical composition (Chaignon et al., 2007). Therefore, the antifouling properties of subtilisin were studied in solution, in terms of the prevention of bacterial adhesion and of the detachment of adhered bacteria using previous marine bacterial adhesion microplate tests (Leroy et al., 2007).

2. Materials and methods

All the chemicals and reagents were of analytical grade from Sigma-Aldrich.

Biofilm forming bacterium

The D41 strain was isolated from a natural marine biofilm on Teflon coupons after 24 hour immersion in the bay of Brest (France) (Rubio, 2002). For long-term storage, pure culture was maintained on Difco Marine Broth (MB) 2216E (Fischer Scientific Labosi, Elancourt, France) with 15% (v/v) glycerol at -80°C . A comparative 16S rRNA gene sequences analysis indicated that the D41 isolate belonged to the genus *Pseudoalteromonas* sp. (Rubio, 2002).

EPS study

EPS production was performed in a 5 l fermentor as described by Raguenes *et al.* (1997). Detailed procedures relating to extraction/purification were carried out as published by Samaïn *et al.* (1997). Briefly, the two soluble EPS were isolated from the fluid supernatant recovered from the medium by high-speed centrifugation (20 000 g, 2 h, 4°C). EPS2 fraction was obtained from the retentate of an ultrafiltration on 100 kDa membrane. A subsequent ultrafiltration of the filtrate on a 10 kDa membrane gave EPS1 fraction (retentate). Capsular fraction EPS3 was recovered from the cell pellet by dialysis (cut off 6-8 kDa). All fractions were lyophilized.

The amount of neutral carbohydrate was determined using the orcinol-sulfuric method with glucose as the standard as described by Tillmans and Philippi (1929) and modified by Rimington (1931). The protein content was determined by BCA assay with Bovine Serum Albumin (BSA) as the standard (Smith *et al.*, 1985). Inorganic matter content was calculated by thermogravimetric analysis performed with a thermal analysis TGA 92. The weight loss was recorded while the EPS sample was being heated from 20°C to 700°C at a scanning rate of 10°C per minute and maintained at 700°C for 8 hours. The inorganic matter content was evaluated from the residual weight at 700°C (wt%).

EPS fraction composition in carbohydrate and protein was then calculated as the percentage of organic matter (in mg for 100 mg of organic matter).

For infra-red spectroscopy analyses, 2 mg of dried EPS was mixed with dry potassium bromide (KBr) and pelleted. The pellet was analysed on FTIR Bruker Vector 22 and spectra were recorded between 4000 and 400 cm^{-1} region with a resolution of 4 cm^{-1} .

The EPS fractions were analysed under denaturing and reducing conditions on 8% SDS-PAGE according to Laemmli's method (Laemmli, 1970) and using the Mini Protean III Bio-Rad. 0.12 mg of each EPS fraction was loaded per lane and gels were stained with Coomassie blue or Shiff's reagent. For Coomassie blue staining, gels were fixed and colored in 50% ethanol, 10% acetic acid and 0.25% Coomassie blue solution, and discoloured in 30% ethanol, 10% acetic acid. For Shiff's reagent staining, gels were fixed in 25% isopropanol, 10% acetic acid, incubated successively 30 min in 7.5% acetic acid, 1 h in 0.2% periodic acid, 1 h in Shiff's reagent and then discoloured in 7.5% acetic acid.

For microscopic analysis, *Pseudoalteromonas* sp. D41 was grown overnight on Difco Marine Agar (MA) 2216E (Fischer Scientific Labosi, Elancourt, France) at 25°C , scraped and suspended in natural sea water sterilized by filtration through a $0.22\text{ }\mu\text{m}$ to reach an $\text{OD}_{600\text{nm}}$ of 2 ($\sim 2 \times 10^9$ cfu ml $^{-1}$). 900 μl of bacterial suspension were inoculated into Lab-Tek™ wells with a glass surface (Lab-Tek™ NUNC, Fischer Scientific Labosi). Covered Lab-Tek™ were incubated for 24 h at 20°C with orbital shaking (300 r.p.m.). Non adhered bacteria were removed by three successive hand washings: Lab-Tek™ wells were emptied, 900 μl of 36 g l $^{-1}$ sterile NaCl solution was added and Lab-Tek™ were shaken. The adhered bacteria were fixed for 1.5 h at 4°C with 900 μl of sterile 36 g l $^{-1}$ NaCl solution containing 2.5% formaldehyde and then desalted by successive baths with decreasing salinity solutions. For DAPI staining, adhered bacteria were incubated with 900 μl of 4 $\mu\text{g ml}^{-1}$ DAPI for 20 min in the dark. For calcofluor staining, adhered bacteria were incubated with 900 μl of 0.1 mg ml $^{-1}$ calcofluor white M2R for 1 h in the dark. For fluorescamine staining, adhered bacteria were incubated for 5 min in the dark with 900 μl of 0.1 mg ml $^{-1}$ fluorescamine, previously solubilized in acetone at 0.2 mg ml $^{-1}$ and half diluted in buffer phosphate borate 0.4 mol l $^{-1}$ pH 9.7. The excess stain was removed by three hand washings with water or with buffer phosphate borate for the fluorescamine staining protocol. The Lab-Tek™ wells were removed, and the glass surfaces were dried out. The slides were observed under 100 magnification epifluorescence microscopy using an Olympus BH201 under UV filter.

Bacterial adhesion experiments

The subtilisin was purchased from Sigma-Aldrich. Its effect on *Pseudoalteromonas sp.* D41 adhesion in black polystyrene microtiter plates (Microwell F96 FluoroNunc, Bioblock, Illkirch, France), in natural sea water sterilized by filtration through a 0.22 μm , at 20°C was studied as described in Leroy *et al.* (2007).

The enzymes were tested in two ways.

The inhibition of bacterial adhesion (the prevention test),

50 μl of the enzymatic preparation at the desired concentration was placed in the wells one hour before the bacterial suspension (200 μl per well) and incubated for 3 and 24 h at 20°C.

The detachment of adhered bacteria (the detachment test)

The enzymes were added 3 hours after the D41 bacterial suspension and incubated for 1 h at 20°C.

In both tests, three washings in 36 g l⁻¹ NaCl were performed before fixation for 1.5 h at 4°C with 200 μl of 36 g l⁻¹ sterile NaCl containing 2.5% formaldehyde and DAPI staining (4 $\mu\text{g ml}^{-1}$) for 20 min. Three washings were again performed to remove excess DAPI and then, the remaining bound DAPI was solubilized into ethanol 95% for 15 min (200 μl).

Fluorescence was measured at 350 nm excitation and 510 nm emission wavelengths using a Genios Plus microplate fluorescence reader (TECAN, Lyon, France). Each subtilisin solution within a large range of concentrations (from 18 to 0.56 $\times 10^{-3}$ mg ml⁻¹) was tested between twice and five times using two fold serial dilutions. Heat denatured subtilisin was obtained by heating subtilisin at 100°C for 20 min.

A blank with sterile seawater as well as a control with bacterial suspension without enzymes were included in each column of the experimental microplate. The change in bacterial adhesion was calculated as a Percentage Reduction (PR), calculated from the fluorescence of the blank (F_B ; without bacteria), the fluorescence of the control (F_C ; bacteria without enzyme) and the fluorescence of the sample (F_S ; bacteria and enzyme) (Equation (1)).

$$PR = \left\{ \frac{(F_C - F_B) - (F_S - F_B)}{(F_C - F_B)} \right\} \times 100 \quad (1)$$

Subtilisin IC50 values were calculated from a three-parameter logistic equation fitted to all PR values ($p < 0.05$), with their standard deviation, by a non linear regression using Sigmaplot 9.0 software (Systat Software GmbH, Erkrath, Deutschland).

Enzyme assay

Protease activity was assayed with azocasein (Tomarelli *et al.*, 1949). Subtilisin was incubated at different concentrations, from 0.5 to 10 g l⁻¹, in natural sea water (previously filtered through a 0.22 μm membrane), at 25°C with 2% azocasein in phosphate buffer of pH 8.15. Samples were withdrawn at different incubation times from 3 to 12 min. The reaction was stopped by adding 10% trichloroacetic acid. After centrifugation, the absorbance of the supernatant was measured at 450 nm. One unit of protease activity (UP) was defined as the amount of enzyme required to produce an absorbance unit change per minute at 25°C at pH 8.15.

3. Results

EPS characterization

Three EPS fractions were isolated from *Pseudoalteromonas sp.* D41 fermentation: two soluble fractions separated according to their molecular mass during ultrafiltration (EPS1, 10-100 kDa, and EPS2 >100 kDa) and one capsular fraction (EPS3) separated from cell pellets by dialysis and centrifugation. Soluble and capsular EPS fractions were produced at 49.7 and 51.2 mg l⁻¹ of fermentation broth respectively. Biochemical characterization showed that the organic matter in EPS1, EPS2 and EPS3 consisted of 58, 30 and 15% carbohydrates and 38, 69 and 80% proteins respectively. We found a higher amount of carbohydrates in EPS1 while the proteins amount is higher in EPS2 and above all in EPS3. Since glucose was the standard for the orcinol assay and BSA for the protein assay, the obtained values are relative and cannot be compared to each other.

The FTIR analysis of EPS shows high homology between the 3 fractions (Fig. 1). A high absorbance in the region of 1640-1650 and 1530-1550 cm^{-1} respectively considered as amide I and amide II bands and assigned as proteins was observed for the three fractions, with less intensity for EPS1 compared to EPS3. EPS1 shows intense bands at 1100-1000 cm^{-1} usually referred as COC, CO and COH

vibrations indicating a high content of polysaccharides in EPS1 fractions. These results confirm previous colorimetric assays.

Figure 2 shows the SDS PAGE analysis of the three fractions revealed by protein and carbohydrate stains. Unlike proteins, polysaccharide migration depends on their charges as well as on their molecular weight. Thus, the molecular weight of polysaccharides as well as glycoproteins can not be determined with molecular weight markers. A different proteic pattern with molecular weight under 150 kDa was displayed in all fractions. Although the EPS 2 fraction contains molecular weight molecules above 100 kDa, protein bands with molecular masses under 100 kDa were displayed. This may be due to the denaturing and reducing conditions used, suggesting these bands could fit proteic sub-units. The EPS1 fraction contained at least one polysaccharide. It also exhibited two bands which were coloured both by Coomassie blue and Schiff's reagent suggesting that these are two glycoproteins. No carbohydrate was shown in EPS2. However we could not rule out that EPS2 may well contain polysaccharides, as suggested by FTIR and carbohydrate dosages analysis, because even if no carbohydrate containing molecules are retained in the stacking gel, some polysaccharidic compounds might not be detected ; in fact, their size or their charge could prevent them from entering into the gel ; moreover, in the staining method involving oxidation by periodic acid, we expect that only 1,2-diol groups containing carbohydrates would be revealed. A large band between the two molecular weight markers of 100 and 150 kDa was observed both by carbohydrate staining and protein staining suggesting the presence of glycoconjugate molecules such as glycoproteins or proteoglycans in capsular EPS (EPS3 fraction).

Pseudoalteromonas sp. D41 allowed to settle for 24 h on glass slide surfaces was observed by epifluorescence microscopy in order to compare EPS produced in fermented culture and in biofilm structure. Calcofluor, DAPI and fluorescamine were used to stain β -glucans, bacterial DNA and proteins (NH₂ extremity) respectively. Figure 3 shows that adhered bacteria retained more fluorescamine than calcofluor. DAPI staining indicated the total adhered bacteria on the surface. Epifluorescence microscopy showed that adhered *Pseudoalteromonas sp.* D41 produced EPS mainly composed of proteins. These results were correlated with the biochemical characterization of the EPS isolated from D41 fermentation.

Subtilisin effect on bacterial adhesion

Since proteins produced by *Pseudoalteromonas sp.* D41 are the main components of extrapolymeric substances, we investigated the antifouling potential of subtilisin, a protease with a broad substrate spectrum and which is active in sea water conditions (Fig. 4).

A sigmoid curve like dose-response curve was observed when plotting the adhesion inhibitions rate against the subtilisin concentrations in logarithmic scale (Fig. 4, row 2). All data could be fitted to a three-parameter logistic equation leading to IC₅₀ values statistically significant at 99% for the prevention of adhesion and for the detachment of adhered bacteria (Table 1). Subtilisin showed non significant effects up to minimal concentration; above this level the response increased and reached a maximum effect or "plateau". Subtilisin reached a higher percentage reduction at lower proteolytic activity in the prevention than in the detachment test. Moreover, at maximal concentrations tested (0.5 UP l⁻¹), the inhibition rates in the 3 and 24 h prevention tests reached 99.5% but only reached 87% in the detachment of adhered cells. Subtilisin IC₅₀ is 38 times better in the prevention of bacterial adhesion when allowed to set for 24 h than in bacteria detachment ($p = 0.00001$). The effectiveness of subtilisin in preventing bacterial adhesion is 13 times better when bacterial adhesion is allowed for 24 h (IC₅₀ = 0.0252 mg ml⁻¹) than for 3 h (IC₅₀ = 0.333 mg ml⁻¹) ($p = 0.012$). The highest effective values of subtilisin were obtained in the prevention of bacterial adhesion allowed to set for 24 h.

Surprisingly, heat denatured subtilisin showed partial inhibition of bacterial adhesion in the prevention test but not in the detachment test (Fig. 4, row 1).

4. Discussion

In the marine environment, enzymatic preparations can lead to the use of less harmful environmental agents in attempting to fight against the formation of bacterial biofilm. The purpose of our study was to investigate the antifouling potential of subtilisin regarding EPS nature involved in a marine bacterial adhesion model.

EPS studies on bacterial adhesion have mostly focused on exopolysaccharides. Exopolysaccharides are not the sole essential molecules in the development and structure of the biofilm matrix. Proteins in

EPS fractions have been considered as contaminants stemming from the preparation (Nielsen and Jahn, 1999). Latasa *et al.* (2006) reviewed biofilm-associated proteins, in particular, a group of surface proteins promoting adhesion to surfaces; they are called Bap in *Staphylococcus aureus* and *Salmonella enteritidis* or Esp in *Enterococcus faecalis*. They are involved in primary attachment and biofilm formation on abiotic surfaces. Recently, it was shown that *Staphylococcus* biofilm-forming strains produced extracellular acid teichoic and proteins instead of the usual poly-N-acetylglucosamine (Kogan *et al.*, 2006).

Many EPS studies have been performed on EPS isolated from culture media (Wingender *et al.*, 1999) and more recently directly on biofilms (Neu and Lawrence, 1999; Kämpfer *et al.*, 2004). It has been shown that bacteria in biofilm may present a different phenotype from bacteria in suspension, but differences in EPS produced by planktonic or biofilm bacteria could result from environmental adaptation of exopolymers. In this study, we have shown that the D41 EPS seems to have the same biochemical pattern in biofilm as in fermentation. EPS from biofilm and from planktonic bacteria can present differences in conformation, substitution and quantity but not necessarily in composition. Sutherland also thought that EPS from biofilm could have the same composition as EPS in planktonic bacteria (Sutherland, 2001). Beech *et al.* (1999) compared EPS produced by *Pseudomonas sp.* NCIMB 2021 during fermentation and in biofilm. They displayed high homology in IR spectra, an increase in β -sheet conformation of the soluble EPS, and also differences in the level of substitution (O- and N- acetylation). More recently, a great degree of similarity has been shown between protein profiles in the proteomic analysis of *Candida albicans* from planktonic and biofilm growths (Thomas *et al.*, 2006).

In this study, we have shown that the D41 EPS consists mainly of exopolysaccharides, proteins and glycoproteins. Proteins were in great amount in extracellular as well as in capsular fractions from liquid cultures and upon adhesion. These proteins could present, like in the EPS2 fraction, a molecular weight above 100 kDa and could be composed of different sub-units. Moreover, the better effectiveness of subtilisin in the prevention test compared to that in the detachment test suggests that the protease activity is more effective during the very first stage of *Pseudoalteromonas sp.* D41 adhesion. This result stresses the major role of proteins in the initial steps of D41 adhesion.

Moreover, capsular exopolymers are not usually considered as necessary during the initial step of cell adhesion (Sutherland, 2001). We show in this publication that the capsular fraction was the EPS fraction which contains the most proteins. In fact, previous studies have demonstrated the presence of proteins in the chemical composition of *Pseudoalteromonas sp.* D41 surface uppermost layer (Pradier *et al.*, 2005). Since protease activity proved the most effective in reducing D41 adhesion (Leroy *et al.*, in press), *Pseudoalteromonas sp.* D41 may well use a proteic mechanism to adhere to surfaces using extracellular proteins directly involved in bacterial adhesion, and maybe via capsular proteins.

Subtilisin is a serine endopeptidase with a broad substrate range. It has been shown that Alcalase, a commercial enzymatic preparation based on subtilisin, could inhibit the settlement and adhesion of fouling marine organisms such as cypris larvae of the barnacle, spores of green algae and diatoms (Pettitt *et al.*, 2004). Since proteins are present in a great amount in D41 EPS components and Savinase (subtilisin) was the most effective hydrolase in both the prevention of bacterial adhesion and the removal of adhered bacteria (Leroy *et al.*, in press), we have investigated the antifouling potential of subtilisin on D41 adhesion.

Heat denatured subtilisin partially inhibited bacterial adhesion in the prevention test. Since no residual protease activity was detected for heat denatured subtilisin (Leroy, 2006), this result suggests a non specific enzymatic effect, maybe due to the adsorption of enzymatic preparations (denatured enzymes and additives) which could lead to a partial inhibition of bacterial adhesion. This effect could only be observed in the prevention test and not in the detachment test suggesting that the adsorption of additives or denatured subtilisin on the surface, before bacterial adhesion, could modify the chemical properties of the surface and lead to a different bacterial adhesion. However, the effectiveness of subtilisin is significantly better when it is not heat denatured. Moreover, the longer the incubation time the more subtilisin inhibits bacterial adhesion in the prevention test. Furthermore, subtilisin is more effective than Savinase in the same D41 microplate adhesion test (Leroy *et al.*, in press). These results all together suggest that the D41 adhesion inhibition effect is related to the enzymatic role of subtilisin.

As shown in Figure 4 and in Table 1, the effective concentrations of subtilisin for 50% inhibition of D41 adhesion (IC50) are 333, 25.2 and 970 mg l⁻¹ in the 3h prevention test, in the 24h prevention test and in the detachment test respectively. These concentrations are higher than those of other biocides generally used in cooling water (Leroy *et al.*, 2007). But, in general, these products are used in continuous, shock or pulse dosing treatments and for a lower amount of bacteria than in our test

condition. In fact, the number of adhered D41 in the wells is greater than the number of bacteria in a natural seawater biofilm. Under our test conditions, adhered D41 range from 2×10^7 to 2×10^8 bacteria per cm^2 (Leroy et al., 2007) while 2.6×10^3 aerobic bacteria were counted on stainless steel coupons immersed for 60 days in a cooling seawater system (Lutterbach and França, 1996), or, depending on the season and coupon nature, 4×10^5 to 2×10^7 bacteria per cm^2 were enumerated on 15 days immersed coupons in the Atlantic ocean (Brest bay) (Féra, 1985). Moreover, mixing subtilisin with other enzymes could improve the effective concentrations. In fact, a mixture of enzymes may lead to synergistic activity (Leroy, 2006). Moreover, grafting subtilisin onto the surface could also be an alternative in reducing the amount of required subtilisin.

The high amount of proteins produced by the D41 strain either in fermentation broth, particularly in the capsular fraction, or in biofilm together with the high inhibition effect of subtilisin on *Pseudoalteromonas* sp. D41 adhesion suggests that protein hydrolysis is the main mechanism involved in this inhibition process. This emphasizes the major role of proteins in the first step of *Pseudoalteromonas* sp. D41 adhesion.

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Tables

Table 1 Subtilisin IC50 values calculated from a three-parameter logistic equation fitted to experimental values ($p < 0.05$). Data are shown \pm SD (standard deviation).

3 h adhesion prevention	24 h adhesion prevention	3 h adhered bacteria detachment
$0.0092 \pm 0.0015 \text{ UP ml}^{-1}$	$0.0007 \pm 0.0001 \text{ UP ml}^{-1}$	$0.0269 \pm 0.0063 \times 10^{-3} \text{ UP ml}^{-1}$
$0.333 \pm 0.054 \text{ mg ml}^{-1}$	$0.0252 \pm 0.0049 \text{ mg ml}^{-1}$	$0.970 \pm 0.227 \text{ mg ml}^{-1}$

Figures

Figure 1

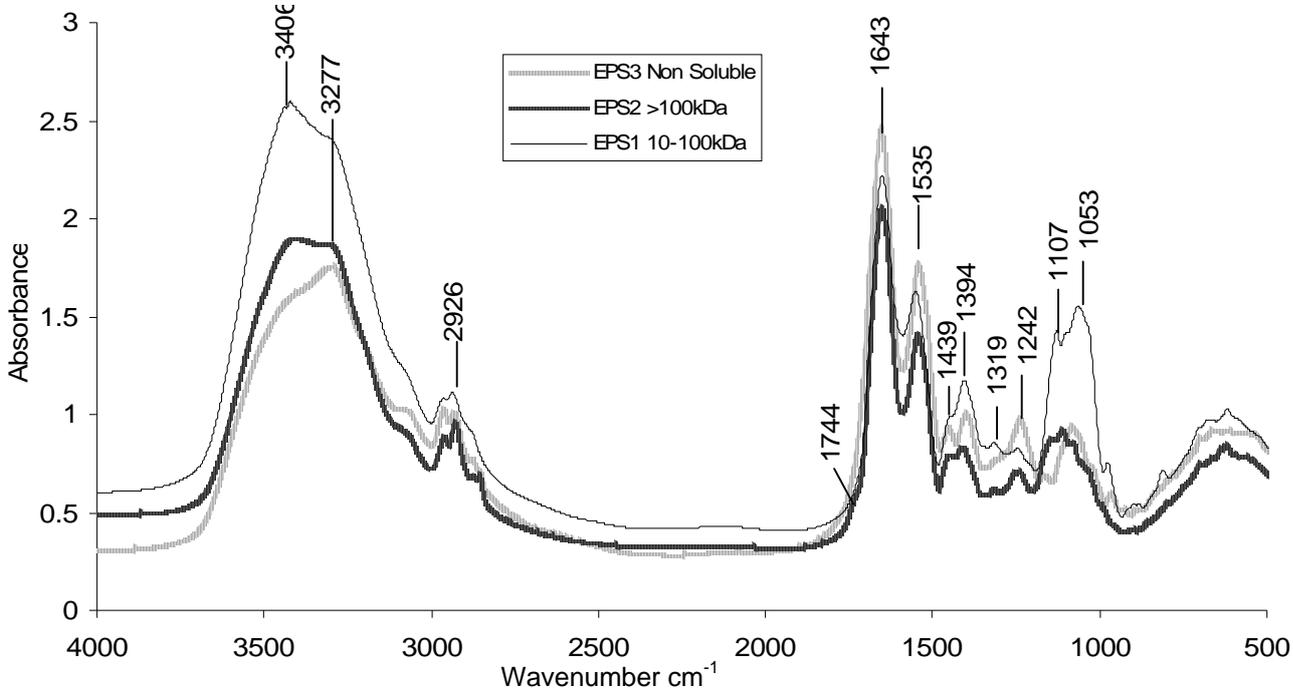


Figure 1 FTIR spectrum of three EPS fractions from *Pseudoalteromonas* sp. D41

Figure 2

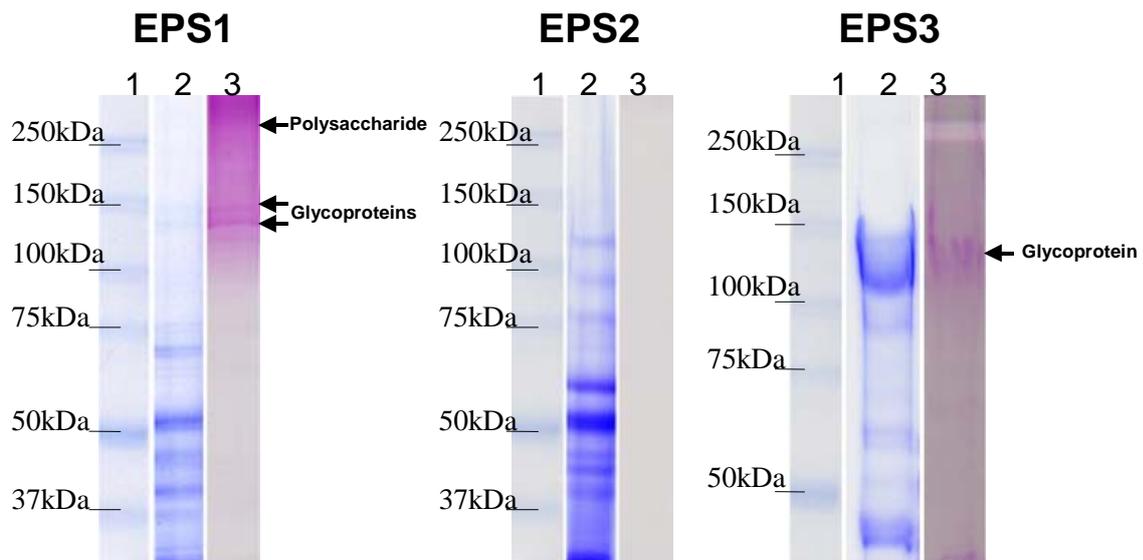


Figure 2 SDS gel (8%) electrophoresis of three EPS fractions from *Pseudoalteromonas* sp. D41 stained with Coomassie (lane 2) and Schiff's reagent (lane 3) to detect proteins and carbohydrates respectively. Molecular weight markers are in lane 1.

Figure 3

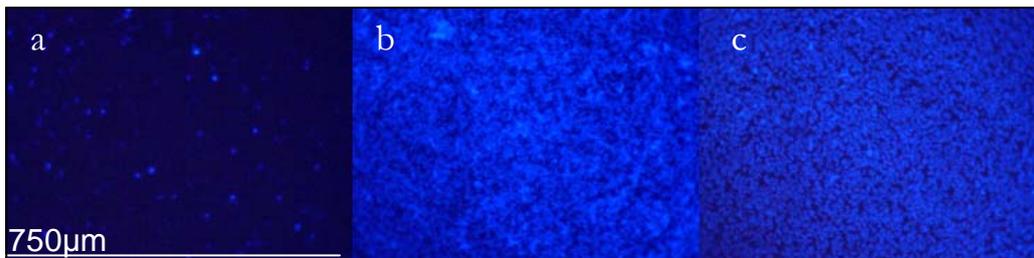


Figure 3 Epifluorescence images of *Pseudoalteromonas* sp. D41 on glass slide after 24 h adhesion and stained with calcofluor (a), DAPI (b) and fluorescamine (c).

Figure 4

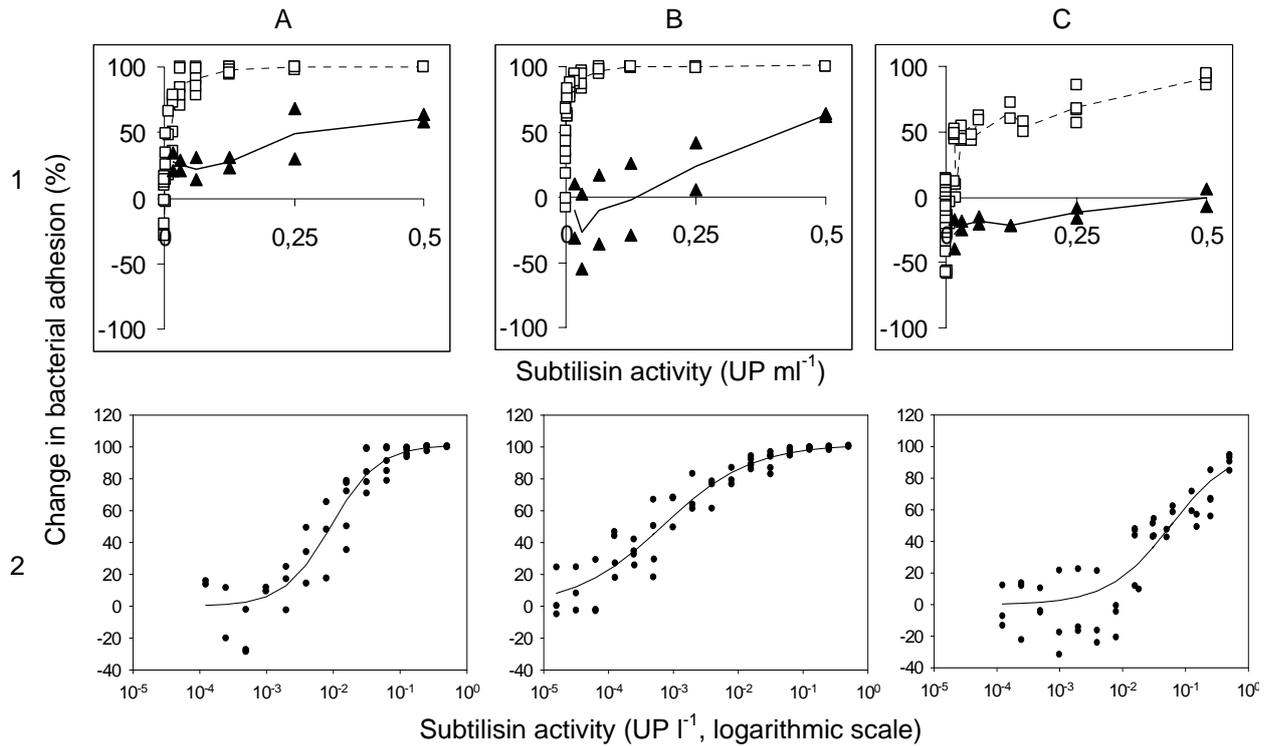


Figure 4 Percentage reduction of bacterial adhesion in microtiter plates against subtilisin concentrations in the well (UP ml⁻¹) with the three protocols tested: prevention of bacterial adhesion measured after 3 h (A), prevention of an adhesion after 24 h (B) and detachment of adhered bacteria after 3 h (C). Row 1, both subtilisin (□) and heat denatured subtilisin (▲) were represented according to subtilisin concentrations in the well (UP ml⁻¹), curves represent average data. Row 2, same data are represented according to logarithmic scale of subtilisin concentrations in the well (UP l⁻¹), curves represent data fitted to a three-parameter logistic equation. All experimental values are shown; four experiments were tested each enzyme concentration and two each heat denatured enzyme. UP is used for the protease activity unit.