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Multiple linear regression analysis of bacterial deposition to polyurethane coatings after conditioning film formation in the marine environment

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Many studies have shown relationships of substratum hydrophobicity, charge or roughness with bacterial adhesion, although bacterial adhesion is governed by interplay of different physico-chemical properties and multiple regression analysis would be more suitable to reveal mechanisms of bacterial adhesion. The formation of a conditioning film of organic compounds adsorbed from seawater affects the properties of substratum surfaces prior to bacterial adhesion, which is a complicating factor in studying the mechanism of bacterial adhesion. In this paper, the impact of conditioning films adsorbed from natural seawater to four polyurethane coatings with different hydrophobicity, elasticity and roughness was studied for three different marine bacterial strains in a multiple linear regression analysis. The water contact angle on hydrophobic coatings decreased on average by 8 degrees and increased on average by the same amount on hydrophilic coatings. These changes were accompanied by increased concentrations of oxygen and nitrogen on the surface as determined by X-ray photoelectron spectroscopy, indicative of adsorption of proteinaceous material. Furthermore, the mean surface roughness increased on average by 4 nm after conditioning film formation. Multiple linear regression analysis revealed that changes in deposition due to conditioning film formation of *Marinobacter hydrocarbonoclasticus*, *Psychrobacter* sp. SW5H and *Halomonas pacifica* in a stagnation-point flow chamber could be explained in a model comprising hydrophobicity and the prevalence of nitrogen-rich components on the surface for the most hydrophobic strain. For the two more hydrophilic strains, deposition was governed by a combination of surface roughness and hydrophobicity. Elasticity was not a factor in bacterial adhesion to conditioning films.

INTRODUCTION

Biofilms pose serious problems in many different environments, ranging from the human body to the marine environment. Regardless of the environment, biofilm formation commences with the adsorption of a conditioning film, to which the first-arriving bacteria subsequently adhere. In the marine environment, seasonal changes affect the chemical composition of the conditioning films formed through the adsorption of proteins, lipids, humic acids, nucleic acids, polysaccharides and aromatic amino acids. Marine organisms adhere better to conditioning films formed in the spring than to films formed in the winter, as spring films contain greater amounts of proteins and organic carbon (Bakker *et al.*, 2003b).

Along with changes in the chemical composition of a substratum surface after conditioning film formation (Barth, 1989; Hogt *et al.*, 1985; Oga *et al.*, 1988), physico-chemical properties of the surface such as its hydrophobicity, roughness, charge and elasticity may alter (Bakker *et al.*, 2003b). Although many studies have shown relationships between substratum hydrophobicity, charge or roughness with bacterial adhesion, it is still hard to understand how the multiple changes brought about by the adsorption of a conditioning film affect bacterial adhesion. Conditioning films adsorbed from tear fluid on contact lenses changed the surface properties due to presence of proteinaceous material. Multiple linear regression analysis using the elemental surface composition, presence of proteins, surface

hydrophobicity and surface roughness as input demonstrated that adhesion of *Pseudomonas aeruginosa* could be described by the hydrophobicity, surface roughness, percentage oxygen and nitrogen, O = C/O–C ratio and the presence of proteins on the surface (Bruinsma *et al.*, 2002). The complexity of the changes brought about by the adsorption of a conditioning film and our lack of understanding of its impact on bacterial adhesion has hampered the development of antifouling coatings. This is especially important in the marine environment, as environmental restrictions have impeded the use of bacteriocidal coatings based on tributyltin and cuprous oxide.

The aim of this study was to determine the changes in physico-chemical surface properties of experimental polyurethane antifouling coatings after incubation in natural seawater and to relate these changes to the deposition of three marine bacterial strains in a stagnation-point flow chamber using multiple linear regression analysis.

METHODS

Bacterial strains, culture conditions and harvesting. Three marine bacterial strains with different cell surface hydrophobicities were selected for this study (Bakker *et al.*, 2003a), including the motile rod-shaped bacterium *Marinobacter hydrocarbonoclasticus* ATCC 27132 (water contact angle, θ_w, 85 degrees), the non-motile spherical bacterium *Psychrobacter* sp. strain SW5H (θ_w 54 degrees) and the non-motile spherical bacterium *Halomonas pacifica* ATCC 27122 (θ_w 18 degrees). For each adhesion experiment, bacteria were precultured from marine agar plates (Difco Marine Agar 2216) in batch culture of artificial seawater (Sigma Sea Salts) 38·5 g l^{-1}, supplemented with bacteriological peptone (Oxoid) 5 g l^{-1} and yeast extract (Oxoid) 1 g l^{-1} for 24 h at 30 °C, with vigorous shaking (180 r.p.m.) to avoid aggregate formation. These cultures were used to inoculate second cultures which were grown for 16 h. Bacterial cells were harvested by centrifugation (5 min at 10 000 ***g***) and washed twice with artificial seawater. Subsequently, the OD_{546} was measured with a Spectronic genesys (Spectronic Instruments) and the bacteria were suspended in artificial seawater to a density of 3×10^8 bacteria ml^{-1}.

Substrata, conditioning film formation and surface characterization. Polyurethane coatings with different hydrophobicities (as shown in Table 1) and elastic moduli of 1·9 GPa and 1·5 GPa for hard '650' and soft '670' coatings, respectively, were applied to glass, as described before (Bakker *et al.*, 2003a). The hard '650' coating was obtained by the reaction of the branched, hydroxy-group-containing polyester Desmophen 650 MPA-65 (Bayer) and the aliphatic polyisocyanate, based on hexamethylenediisocyanate (HDI), Desmodur N75. The '670' coating, of lower elastic modulus, was obtained by the reaction of the weakly branched, hydroxy-group-containing polyester Desmophen 670 BA-80 and Desmodur N75. In order to change the hydrophobicity of the coatings, Fluowet EA 600 (Clariant) was added to the Desmodur N75 prior to addition of Desmophen, up to 5·4 mol% and 8·4 mol% in '670F' and '650F', respectively. Conditioning films were allowed to form at room temperature by immersion of the plates in natural seawater, obtained from the Marsdiep near Den Helder, The Netherlands, in September 2002, with gentle shaking (30 r.p.m.) for 60 min. As a control, surfaces were also immersed in artificial seawater. After adsorption, conditioned surfaces were dipped once in demineralized water before further experimentation.

The hydrophobicities of the polyurethane coatings and the conditioned surfaces were determined by the measurement of water contact angles formed by droplets with a volume between 1 and 2 µl. Conditioned surfaces were dried with nitrogen gas until 'plateau' contact angles could be measured. Values for contact angles are the mean of three drops on three surfaces.

X-ray photoelectron spectroscopy (XPS, S-Probe spectrometer, Surface Science Instruments) was performed on the polyurethane coatings prior to and after conditioning film formation to determine the chemical composition of the surfaces. The spectrometer was equipped with an aluminium anode (10 kV, 22 mA) and a quartz monochromator. The angle of the photoelectron collection was 55 degrees with the normal to the sample and the electron flood gun was set at 10 eV. A survey scan was made with a 1000×250 µm spot and a pass energy of 150 eV. Detailed scans of the C_{1s}, O_{1s} and N_{1s} electron-binding energy peaks were obtained using a pass energy of 50 eV. Binding energies were determined by setting the binding energy of the C_{1s} component due to the carbon–carbon bond at 284·8 eV. The experimental peaks were integrated after nonlinear background subtraction and the peaks were decomposed assuming a Gaussian/Lorentzian ratio of 85/15 by using the SSI PC software package. All elemental surface concentrations presented are means of experiments on two polyurethane coatings.

Atomic force microscopy (Nanoscope III, Digital Instruments) was performed on conditioned and unconditioned polyurethane coatings with a silicon nitride cantilever (Veeco) in the contact mode and with a spring-constant of 0·06 N m^{-1} in order to determine the mean surface roughness (R_a). R_a indicates the mean distance of the roughness profile to the centre plane of the profile and was determined on three randomly selected sites per surface. Furthermore, the elastic modulus of the coatings was assessed by measuring their indentation hardness with the atomic force microscope, as described in detail before (Bakker *et al.*, 2003a; Tomasetti *et al.*, 1998).

Flow chamber experiments. A stagnation-point flow chamber was used in this study, as described before in detail (Bakker *et al.*, 2003a; Dabros & Van de Ven, 1983). The flow chamber was incorporated between two communicating vessels placed at different heights and containing the bacterial suspension, to create a pulse-free flow by hydrostatic pressure. Fluid was recirculated with a roller pump between the vessels. In the stagnation-point flow chamber, adhesion in an area 1·1 mm away from the stagnation point was observed with a CCD-LDH camera (Philips) mounted on a dark-field microscope (Leitz) equipped with a 50× objective (Leitz). Live images were acquired with a PC-Vision$^+$ frame grabber (Imaging Technology) and Sharp filtered. Deposited bacteria were discriminated from the background by single grey value thresholding. Experiments were carried out at a flow rate of 0·0088 ml s^{-1}, corresponding to a shear rate at the point of observation equal to 22·5 s^{-1}. The total number of adhering bacteria per unit area of coating was recorded as a function of time by image sequence analysis. All experiments were performed at 20 °C in triplicate, with separately cultured strains.

Data analysis and statistical evaluation. The initial deposition rate, j_0, describing the affinity of a bacterium for a substratum surface, was calculated by linear regression analysis from the initial increase of the numbers of adhering bacteria as a function of time. To analyse the differences in initial deposition rates, a one-way ANOVA was performed with SPSS for Windows (SPSS Inc.), using a significance level of 0·05. Backward multiple linear regression was performed in order to identify the surface properties most predictive for the difference in bacterial deposition with and without a conditioning film. The difference in initial deposition rate was used as a dependent variable, while the differences in water contact angle, mean surface roughness, and the percentage surface composition

(%C, %O, %N), were taken as independent variables. Variables were excluded when equal in both situations or when correlating significantly with other variables, as determined by Pearson's correlation test.

RESULTS

Conditioning film characteristics

Surface characteristics of the substratum surfaces after incubation in natural seawater and artificial seawater as the control are presented in Table 1. After incubation of the polyurethane coatings in natural seawater, the water contact angle for the hydrophilic coatings (650 and 670) increased on average by 8 degrees, whereas for both hydrophobic coatings (650F and 670F) a clear decrease in water contact angles was observed of on average 8 degrees. Longer exposure of the surfaces to seawater might yield a greater convergence in their hydrophobicity. The changes in hydrophobicity were accompanied by changes in surface chemistry of the coatings. All four polyurethane coatings contained carbon as a major constituent, but after incubation in natural seawater a significant decrease in the amount of carbon (on average 5·0 %) was observed for the non-fluoridated coatings (650 and 670), whereas the amount of carbon did not change for the fluoridated polyurethanes (650F and 670F). An increase in the amount of surface nitrogen was observed for all coatings after incubation in natural seawater (on average 0·7 %), indicative of the presence of proteins on the surface. A small increase in silicon was observed after incubation of the surfaces in natural seawater, probably due to small sand particles.

The mean surface roughness of the substratum surfaces, as determined by atomic force microscopy, was on average 2·7 nm for the hard coatings (650 and 650F) and 5·9 nm for the soft coatings (670 and 670F). After conditioning film formation in natural seawater, surface roughness increased to 4·7 nm for the hard coatings (650 and 650F) and to 12·9 nm for the soft coatings (670 and 670F).

Bacterial deposition

Table 2 shows the initial deposition rates for the three strains on substrata after incubation in artificial or natural seawater. In the presence of a conditioning film from natural seawater no significant differences in initial deposition rates between the hydrophobic (650F and 670F) and more hydrophilic (650 and 670) polyurethane coatings were observed in contrast to the bare polyurethane coatings. *Psychrobacter* sp. adhered preferentially to the soft fluoridated coating, whereas in the absence of a conditioning film highest initial deposition rates were observed for the hard non-fluoridated coating. The most hydrophilic strain, *H. pacifica*, showed the highest initial deposition rate to the most hydrophobic coating with a conditioning film of natural seawater.

Multiple linear regression analysis

For multiple linear regression analysis, all data obtained for substrata with a conditioning film were expressed as a difference, relative to the data for the substrata in the absence of a conditioning film. Pearson's correlation test demonstrated a high correlation between %C, %F and %Si, and hence %F and %Si were excluded from the analysis. Furthermore, since the elastic modulus did not change upon incubation, it was also excluded from the analysis.

Table 3 shows that the difference in water contact angle upon conditioning film formation influences the deposition of all three strains, although for *Psychrobacter* sp. (the

Table 1. Water contact angles, percentage elemental surface composition of polyurethane coatings conditioned in artificial seawater and natural seawater (+CF), and mean roughness (R_a) after 1 h incubation in seawater

Mean standard deviations were 2 degrees, 5 % and 0·1 nm for water contact angles, elemental surface compositions and surface roughnesses, respectively.

Substratum	Water contact angle, θ_w (degrees)	Elemental surface analysis					R_a (nm)
		%C	%N	%O	%F	%Si	
650*	70	74·3	5·3	17·3	0·0	3·0	3·0
650 + CF	75	68·7	6·5	19·6	0	5·2	4·7
650F*	108	56·2	5·7	12·0	24·9	1·2	2·3
650F + CF	99	56·0	6·5	13·4	21·7	2·4	4·5
670*	69	73·9	3·6	19·1	0·0	3·6	5·8
670 + CF	80	69·6	4·0	21·1	0·0	5·5	10·5
670F*	112	46·6	5·7	8·2	39·5	0·0	6·0
670F + CF	105	47·4	6·2	9·9	35·8	0·8	12·9

*Data taken from Bakker *et al.* (2003a).

Table 2. Initial deposition rates (j_0) for three marine strains in a stagnation-point flow chamber to polyurethane coatings conditioned in artificial seawater and natural seawater (+CF) with different hydrophobicity and elastic modulus

Substratum	j_0 (cm^{-2} s^{-1})*		
	M. hydrocarbonoclasticus ATCC 27132	*Psychrobacter* sp. SW5H	*H. pacifica* ATCC 27122
650†	1223±82	1827±170	1216±67
650+CF	1562±103	1485±47	736±95
650F†	2075±283	1239±112	778±70
650F+CF	1690±153	1737±86	834±73
670†	798±107	1099±59	657±83
670+CF	1695±190	1224±93	404±55
670F†	1914±188	1098±196	460±90
670F+CF	1699±179	2170±122	1518±103

*All experiments were done in triplicate (± denotes standard deviation over three experiments with separately cultured organisms).
†Data taken from Bakker *et al.* (2003a).

intermediately hydrophobic strain) the influence of the water contact angle difference was not significant. Conditioning film formation also affected the initial deposition rates of *Psychrobacter* sp. and *H. pacifica* through changes of the mean surface roughness, although here too the influence of mean surface roughness on deposition of *Psychrobacter* sp. was not significant. Note that in the case of *M. hydrocarbonoclasticus* ATCC 27132, a small and almost significant influence of the presence of adsorbed nitrogen-rich components on initial deposition was seen.

Fig. 1 shows the influence of water contact angle, mean surface roughness and surface concentration of nitrogen on the changes in initial deposition rates of the strains in a single-parameter model. Fig. 1(a) demonstrates that the deposition of the most hydrophobic strain increases as the substratum becomes more hydrophobic due to conditioning film formation, while the deposition of the more hydrophilic strains decreases. Deposition of *H. pacifica* and *Psychrobacter* sp. increased with increasing surface roughness (see Fig. 1b), whereas increasing surface concentration

Table 3. Standardized coefficients (β) and *P*-values after backward multiple linear regression analysis

In the regression analysis the difference in initial deposition rate was taken as the dependent variable and the difference in %C, %N, %O, the mean surface roughness (R_a) and water contact angle (θ_w) were taken as independent variables.

Variable	*M. hydrocarbonoclasticus* ATCC 27132		*Psychrobacter* sp. SW5H		*H. pacifica* ATCC 27122	
	P	***β***	***P***	***β***	***P***	***β***
Constant	0·04	–	0·34	–	0·03	–
Δ%C	–*	–*	–*	–*	–*	–*
Δ%N	0·07	–0·15	–*	–*	–*	–*
Δ%O	–*	–*	–*	–*	–*	–*
ΔR_a	–*	–*	0·16	0·68	0·02	0·60
$\Delta\theta_w$	0·01	0·98	0·17	−0·64	0·02	−0·74
Sig.	0·02		0·17		0·02	
R^2		99·9		97·2		99·9

*These variables do not play a significant role in determining the difference in initial deposition of the three marine bacterial strains.

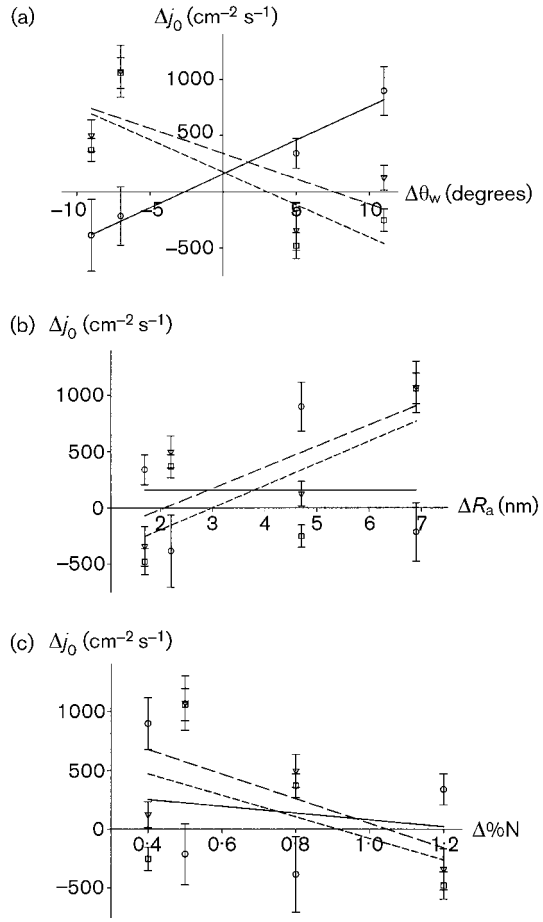

Fig. 1. Changes in initial deposition rates (j_0) to polyurethane coatings in a stagnation-point flow chamber due to the presence of a conditioning film for *M. hydrocarbonoclasticus* (○), *Psychrobacter* sp. (▽) and *H. pacifica* (□) as a function of the change in (a) water contact angle (θ_w), (b) the mean surface roughness (R_a) of the surface, and (c) the percentage nitrogen on the surface (%N). Lines indicate the best fit in a single-parameter linear regression model. Data presented denote values found in the presence of a conditioning film minus those in the absence of a conditioning film. Bars indicate the standard deviations over triplicate runs.

of nitrogen had a negative influence on the deposition rates (see Fig. 1c).

DISCUSSION

In this paper, we have analysed the effects of a conditioning film from natural seawater on polyurethane coatings with different hydrophobicities and elasticities on bacterial adhesion by means of a multiple linear regression analysis. Adsorbed components from the seawater on marine antifouling coatings change the elemental surface composition, hydrophobicity and mean surface roughness. The water contact angles of the submersed coatings tended to converge to an intermediate hydrophobicity, as also shown by others. Incubation of stainless steel and germanium (both fully wettable), polypropylene (water contact angle 100 degrees) and Perspex (water contact angle 73 degrees) in different environmental waters for 1 h resulted in a mean water contact angle for all conditioned surfaces of 61 ± 28 degrees (Schneider & Marshall, 1994). However, also surfaces exposed to other environments, as for example in the oral cavity containing much higher concentrations of dissolved compounds, gave water contact angles between 50 and 70 degrees (Van Dijk *et al.*, 1988). Although surfaces end up with a similar hydrophobicity, heterogeneous dissolved organic carbon compounds in the natural seawater can generate films with different properties with respect to bacterial adhesion. Also, the surface properties of the underlying substratum dictate selective adsorption (Fabrizius-Homan & Cooper, 1991) and conformational changes of adsorbed macromolecules (Elwing *et al.*, 1988; Vermeer & Norde, 2000).

Even though only small amounts of organic carbon (part of it being proteins) are present in the natural seawater used, the results obtained clearly demonstrate that a conditioning film strongly affects the adhesion of bacteria, with a residual influence of the original surface properties. Longer exposure to natural seawaters would possibly have diminished the effects of the underlying surface, as others have reported that the time scale in which the composition of the conditioning film becomes independent of the substratum surface properties ranges from 4 h (Maki *et al.*, 1990) up to 3 days (Little & Zsolnay, 1985).

Multiple linear regression analysis indicated that hydrophobicity was the main determinant for bacterial deposition, as evidenced by the relatively high standardized coefficients and the fact that hydrophobicity was predictive for all strains. Coatings that became more hydrophobic appeared to be more attractive for bacteria that were hydrophobic as well, and vice versa, which is in agreement with the observation on the coatings without a conditioning film (Bakker *et al.*, 2003a) and the general notion that bacteria with hydrophobic surface properties prefer hydrophobic material surfaces and those with hydrophilic surface properties prefer hydrophilic surfaces (Hogt *et al.*, 1985; Satou *et al.*, 1988). Furthermore, hydrophobic bacteria adhere to a greater extent than hydrophilic bacteria (Van Loosdrecht *et al.*, 1987), as evidenced by the higher mean initial deposition rate for *M. hydrocarbonoclasticus* (1582 cm^{-2} s^{-1}) as compared to *H. pacifica* (825 cm^{-2} s^{-1}).

A positive relation was found between mean surface roughness (which ranged from 2·3 nm up to 12·9 nm) and the deposition of micron-sized bacteria. An influence of nanometer-scale roughness on the deposition of *P. aeruginosa* to contact lenses was also observed by Bruinsma *et al.* (2003). This influence of nanometer-scale roughness is surprising and indicates that adhesion of bacteria is mediated by structures much smaller than the bacterium itself, like fimbriae, flagella or polymeric substances excreted by bacteria. Although no significant relation was

shown between elemental surface composition and roughness, adsorbed macromolecules are inevitably involved in the changes of roughness observed.

Summarizing, this study shows the rapid change of the surface properties of surfaces incubated in natural seawater, with subsequent changes in deposition of marine bacteria, which were due to changes in multiple surface properties: hydrophobicity, roughness and the amount of nitrogen on the surface. Attention should be paid to ensure that antifouling coatings based on optimal non-adhesive physicochemical properties maintain their optimal properties after exposure to natural waters, which requires that conditioning film formation should be a first target in the development of antifouling coatings.

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