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Influence of pH and ionic strength on adhesion of a wild strain of *Pseudomonas* sp. to titanium

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The adherence of an environmental strain of *Pseudomonas* sp. to titanium was evaluated modifying the pH (2 to 8) and ionic strength (0.1 and 0.6 M NaCl) of the electrolyte solution. Results were analyzed considering the participation of the different interfacial forces under the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory. At 0.1 M, maximal bacterial adhesion was at pH 6, in agreement with the point of zero charge of the titanium surface. Similar adhesion values were observed at both sides of this point despite the opposite electrostatic condition of the surface oxide. At 0.6 M an absence of bacterial adhesion was observed throughout the pH range tested. The changes in bacterial adhesion are in agreement with the changes in the number of reinforced H-bond-forming sites on the titanium surface calculated using a simple model for the ionization of OH group adsorbed to the surface. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 303–308.

Keywords: bacterial adhesion; surface oxides; hydrogen bonds; TiO_2

Introduction

Bacterial adherence influences many man-related processes through the formation of biofilms [1,16,27,28]. The growth of biofilm-forming bacteria has been observed in a wide variety of processes from biomaterial-tissue integration [16] to corrosion of heat exchanger tubes [1], and has important economical and public health-associated effects. The progress and structure of biofilms strongly depend on the initial steps of bacterial adhesion [8,31]. Physicochemical features of the bacteria–surface interaction affect this process in terms of the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory of colloidal systems [26]. This theory describes the adhesion as the result of a balance between dispersion and electrostatic interfacial forces. The suitability of this theory to explain experimental results was further probed by other works describing the adherence behavior of bacteria to different materials, including polymers, glass and minerals [12,13,20,28,32].

Due to its excellent response to corrosion, titanium is often selected as a material in heat exchanger tubes exposed to polluted waters of various salinities [1]. However, heat exchange as well as its response to corrosion are seriously diminished by the fouling process [24], which is initiated by primary colonizing bacteria. In addition, titanium and titanium alloys are generally used as implant materials [16]. Bacterial adhesion to and tissue integration with the implant material are competing processes [16] and the determination of the variables that control the former could help to improve the latter [16]. These are reasons why the aim of the present work was to evaluate adhesion of bacteria to titanium.

In the presence of water, most metal and metalloid oxides are covered by hydroxyl groups [30]. Due to the chemisorption of water, the presence of two coexisting types of hydroxyl groups has been proposed for titanium dioxide [5]. On the basis of their coordination with Ti^{4+} ions, hydroxyl groups can be predominantly basic (single coordinated) $\{TiOH\}+[H^+]\leftrightarrow\{TiOH_2\}$ or acidic (double coordinated) $\{Ti_2OH\}\leftrightarrow\{Ti_2O^-\}+[H^+]$ [5]. A consequence of this model is the pH dependence of the net surface charge of the oxide because of the amphoteric nature of the adsorbed ions. There is a pH at which these kinds of surfaces have no net charge; this is the point of zero charge (pzc) of the oxide and corresponds to the pH at which $\{TiOH_2{}^+\}=\{Ti_2O^-\}$ in the absence of other adsorbed ions [30]. The pH of the pzc depends on the value of the proton affinity constant of the adsorbed ions, which in turn depends on the acidity of the metal ions involved and its coordination number with oxygen.

On the other hand, the charge on organic surfaces such as those of bacteria may arise from protolysis of functional amino and carboxyl groups [30]. *Pseudomonas* sp. have an isoelectric point at low pH values, with the bacteria bearing a negative net charge at pH values close to neutrality [9].

Experiments were performed to evaluate the effect of electrolyte pH and ionic strength on irreversible cell adhesion to titanium. Special attention was paid to the electrostatic charge of interacting surfaces and the types of bonds involved in the interaction.

Materials and methods

Biological material

Pure cultures of a strain of *Pseudomonas* sp. isolated from a heat exchanger tube refrigerated with seawater [6] were grown at 32°C with continuous shaking in rich medium containing Lab Lemco (Merck, Darmstadt, Germany) 0.1 g l^{-1}, yeast extract (Sigma) 0.2 g l^{-1}, and peptone (Sigma, St. Louis, MO, USA) 0.5 g l^{-1}, dissolved in 0.1 M NaCl, pH 7. Cells were harvested from cultures at the exponential phase of growth by centrifugation for 10 min at $10{,}000\times g$, suspended in 0.1 M NaCl and divided into four samples of equal volume. After centrifugation cells were suspended in NaCl solutions of the desired ionic strength and pH, at a final cell number of $(2–3)\times10^8$ cells ml^{-1}. Two different ionic strengths (0.6 and

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0.1 M NaCl) were selected for the experiments, to compare natural seawater (0.6 M) with a suitable condition for maximum bacterial adhesion [14,26].

Preparation of metal samples and adherence assay

Pure titanium disks (grade 2, Metal Samples, Munford, USA) were mounted with fast-curing epoxy resin on appropriate PVC holders with an electrical contact on the back. Before each experiment, the electrode surface was smoothed with 600-grit emery paper and then mirror-polished with 0.05-μm alumina powder (Type B, Buehler, Lake Blett, USA). After polishing, samples were aged during 24 h in stagnant conditions in NaCl solution at the different pH values. This treatment allowed the formation of a layer of stable oxides in equilibrium with the solution. The open circuit potential (OCP) of the samples at the end of the aging was recorded using a saturated calomel electrode as a reference. Duplicate measurements were carried out in 0.1 and 0.6 M NaCl solutions.

Aged samples were exposed for 1 h to the corresponding bacterial suspension to evaluate bacterial adhesion on these substrata. Samples were then rinsed gently with the test solution, air-dried, and stained for 1 min with crystal violet. Microscopic cell counts were carried out with an Olympus PMG3 Metallurgical Microscope at 500× magnification. Ten different fields of 0.036 mm^2 on each sample were randomly selected to give statistical significance to the values obtained. Care was taken to select sampling areas distant from the edge of the sample to eliminate distortion of the results. Data are reported as mean number of bacteria by unit area (adhered bacteria per square millimeter).

UV–visible reflectance spectroscopy

UV–visible absorption spectra of metal surface oxides were recorded *in situ* using a Shimadzu UV-160A double beam spectrophotometer, with a differential reflectance dual cell. Baseline correction was made using two identical polished surfaces kept in NaCl solution (pH 2) to avoid growth of oxides. The reference surface was kept in this condition to ensure a common reference line for the surface characterization as described below. No significant changes in the absorbance spectrum of the reference surface were detected during the time of the experiment. To avoid surface oxides changes, the solution in the sample compartment was the same used in the aging period for each sample. Differential absorbance spectra from 200 to 800 nm were recorded.

Electrophoretic mobility

The zeta potential (ζ) of bacterial cells was determined by electrophoretic migration in 0.01 M NaCl at a final cell number of 10^5 cells ml^{-1}. A Rank Bros. Mark II particle microelectrophoresis apparatus (Bottisham, Cambridge, England) was used, and the applied potential was 80 V. The value of ζ was calculated using Smoluchowski's equation as $\zeta = 12.87\mu$, where μ was the measured electrophoretic migration.

Results

Metal surface

After immersion in an electrolyte solution, titanium surfaces accumulate a uniform layer of TiO_2 [17,29]. To verify the presence of this oxide after aging in NaCl solutions at different pH values, the OCP was measured and differential absorbance UV–visible spectra of the oxide were recorded. Mean values of OCP are shown in Table 1. These values are in good agreement with the existence of a TiO_2 layer expected from the Pourbaix diagram [29]. Absorbance spectra of the surface oxide in 0.1 M NaCl at each pH are shown in Figure 1. The main feature is a broad peak of absorption at 300 nm, which was observed at the different pH values tested. These absorption spectra are generally associated with a charge transfer process [23] that can be assigned, in this case, to the transfer of charge from oxygen to Ti^{4+} ions in TiO_2 [23]. The only change observed in the spectra was the height of the peak, which is related to the thickness of the TiO_2 layer. Similar results were obtained in 0.6 M NaCl solutions (data not shown).

Table 1 Influence of pH and ionic strength on adhesion of a wild strain of *Pseudomonas* sp. to titanium

pH	OCP (V/NHE)	
	0.1 M	0.6 M
2	0.17±0.005	0.21±0.001
4	0.17±0.002	0.08±0.000
6	0.17±0.002	0.07±0.000
8	0.32±0.010	0.21±0.002

Open circuit potentials (OCP) measured for titanium electrodes after 24 h in NaCl solutions of various pH and ionic strength. NHE: normal hydrogen electrode. Values are indicated as mean±SD.

Based on protonation of the surface-adsorbed ions cited above, the relation between neutral and charged OH species on the TiO_2 surface was obtained from the expression of the proton affinity constants, pK_{a1} and pK_{a2}, for the basic and acidic sites, respectively [30]. Its dependence on the pH of the solution is:

$$\{\mathrm{TiOH}\} = \frac{10^{-\mathrm{p}K_{\mathrm{a1}}}}{10^{-\mathrm{pH}}}\left\{\mathrm{TiOH}_2^+\right\} \qquad \text{for the basic species}$$

and

$$\{\mathrm{TiOH}\} = \frac{10^{-\mathrm{pH}}}{10^{-\mathrm{p}K_{\mathrm{a2}}}}\left\{\mathrm{TiO}^-\right\} \qquad \text{for the acidic species}$$

where [ ] indicates concentrations of the species at the surface.

The charged site densities (N_{a1} and N_{a2}) can be obtained as:

$$N_{\mathrm{a1}} = \left\{\mathrm{TiOH}_2^+\right\} = N_{\mathrm{s}} - \{\mathrm{TiOH}\}$$

and

$$N_{\mathrm{a2}} = \left\{\mathrm{TiO}^-\right\} = N_{\mathrm{s}} - \{\mathrm{TiOH}\}$$

where N_s is the number of surface sites of every type (either acidic or basic) per square nanometer.

Substituting from Equations 1 and 2 and rearranging:

$$N_{\mathrm{a1}} = \left\{\mathrm{TiOH}_2^+\right\} = \frac{N_{\mathrm{s}}}{1 + \frac{10^{-\mathrm{p}K_{\mathrm{a1}}}}{10^{-\mathrm{pH}}}}$$

and

$$N_{\mathrm{a2}} = \left\{\mathrm{TiO}^-\right\} = \frac{N_{\mathrm{s}}}{1 + \frac{10^{-\mathrm{pH}}}{10^{-\mathrm{p}K_{\mathrm{a2}}}}}$$

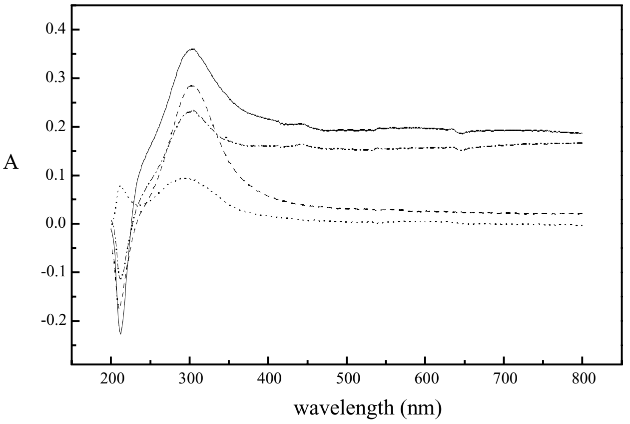

Figure 1 Differential absorbance spectra of the titanium surface aged during 24 h in 0.1 M NaCl at various pH values. (· · ·) pH 2; (– – –) pH 4; (———) pH 6 and (– · – · –) pH 8.

Table 2 Influence of pH and ionic strength on adhesion of a wild strain of *Pseudomonas* sp. to titanium

pH	Zeta potential (ζ) (mV)
2	0.0±0.0
4	−15.0±1.2
6	−28.1±1.8
8	−32.1±1.5

Zeta potential (ζ) of a wild strain of *Pseudomonas* sp. in 0.01 M NaCl at various pH values. Values are indicated as mean±SD.

Calculations were made using Equations 5 and 6 to determine the number of charged sites and the overall charge condition of the surface at every pH value, taking pK_{a1}=4.4, pK_{a2}=7.5 [18], N_s=8 sites nm^{-2} (estimated for rutile on the basis of crystallographic data [22]) and a 1:1 ratio of acidic to basic sites on the TiO_2 surfaces [5]. Results are shown in Figure 2a and b. The pzc is at pH 6.0, where N_{a1}=N_{a2} as described above. This value agrees with previous experimental determinations [2]. It is not possible to obtain the magnitude of the charge at every pH from the results of this simple model. However, a positive charge value at pH values below 6 and a negative one at pH values above 6 can be predicted (Figure 2a). The fraction of charged sites changes from 0.5 to almost 1 at pH 6 indicating that at this pH all the surface sites have a positive or negative charge (Figure 2b).

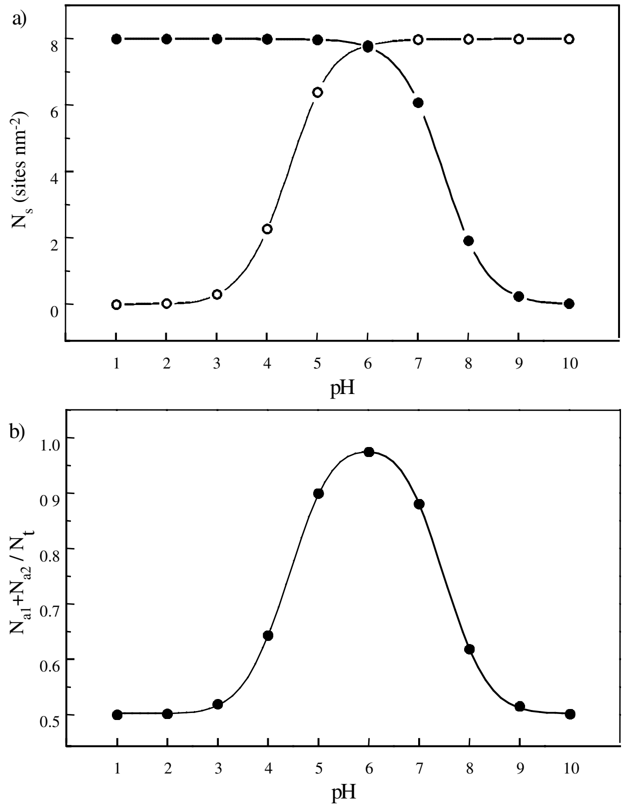

Figure 2 (a) Estimated variation of charged site density (N_s) for (●) basic (Na_1, $TiOH^{2+}$) and (○) acidic (Na_2, TiO^-) OH groups on TiO_2 with the pH of the solution. (b) Fraction of charged sites on the TiO_2 surface. N_t = 16 sites nm^{-2}.

Bacterial cells charge

The ζ values for bacterial cells at the different pH values are listed in Table 2. The isoelectric point occurred at pH 2, with the bacteria bearing a negative charge above this value.

Adherence assays

The influence of pH and ionic strength of the supporting electrolyte on bacterial adhesion was determined. The influence of modifications in pH at a constant ionic strength of 0.1 M NaCl is shown in Figure 3. Bacterial adhesion to the aged titanium surface was minimal at pH 2 and increased with pH until pH 6. At pH 8, the number of adhered bacteria declined to a value close to that at pH 4. At a higher ionic strength (0.6 M NaCl), the adhesion of bacteria was reduced to negligible levels throughout the range of pH tested (Figure 3).

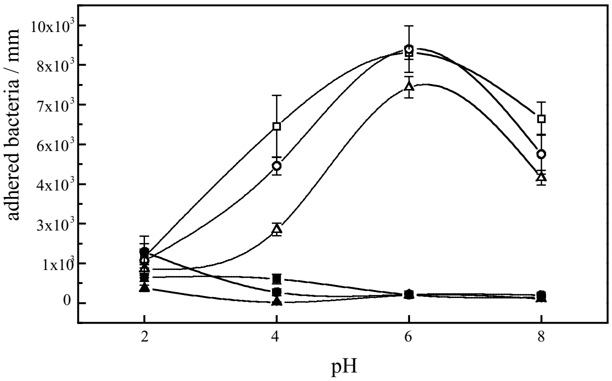

Figure 3 Number of adhered bacteria on titanium electrodes exposed for 1 h to suspensions in (open symbols) 0.1 M and (close symbols) 0.6 M NaCl solutions, at various pH values. Symbols correspond to three replicate experiments. Bars indicate SD of 10 different fields for each sample.

Discussion

Two main variables, pH and ionic strength, play a role on the electrostatic effects in electrolyte solutions due to their influence on the ionization constants of charged groups and the thickness of the double layer, respectively. These parameters give valuable information about the participation of electrostatic forces in bacterial adhesion to charged surfaces. However, these parameters also influence the structure and composition of metal surface oxides [11]. For this reason, although TiO_2 is very stable [29], it was necessary to determine its presence in the different conditions used in the experiments.

Influence of pH

The results shown in Table 1 and the spectra in Figure 1 are in good agreement with the presence of a continuous TiO_2 film on the titanium surface over the range of pH values evaluated [29]. Therefore, it can be assumed that the physicochemical properties of the titanium surface were defined by the properties of the TiO_2 layer. Adopting the DLVO theory, bacterial adhesion depends on the electrostatic interactions between the interacting surfaces [7].

The results in Figures 2 and 3 indicate that the maximum number of adhered bacteria was in agreement with the pzc of titanium oxide at pH 6. Neither attractive nor repulsive electrostatic forces existed in this situation, as the overall charge of the oxide is equal to zero. Bacterial adhesion should then only be the result of van der Waals attractive forces.

The overall charge of the oxide surface changed from positive at $pH<6$ to negative at higher pH values due to variations in the number of charged sites (Figure 2). Consequently, attractive and repulsive forces are expected to act on negatively charged bacteria (Table 2) at pH 4 and 8, respectively. This should lead to differences in bacterial adhesion in these extreme situations, with an increased adhesion at pH 4 and a reduced one at pH 8. In contrast, *similar* degrees of adhesion were observed upon departure from the pzc of the oxide in either direction (Figure 3) indicating that a variable other than electrostatic interactions was involved.

Influence of ionic strength

The presence of surface charge on solids in electrolyte solutions leads to the formation of an electric double layer. In interfacial interactions, this double layer is the closest limit of approach due to electrostatic repulsion for two surfaces charged alike [30]. The position of this limit, i.e., the thickness of the double layer ($1/\kappa$), is dependent of the ionic strength according to [4]:

$$\kappa = \left(\frac{e^2 n^\infty z^2}{\varepsilon kT}\right)^{1/2}$$

where κ is the Debye–Hückel parameter, e the elementary electrical charge, ε the dielectric constant, and z and n^∞ the electrolyte solution valence and concentration, respectively. The influence of the double-layer thickness on bacterial adhesion was proposed by Marshall *et al* [26] in accordance with the predictions of the DLVO theory. As shown by these authors, incremental changes in ionic strength result in a reduction of the double-layer thickness, allowing bacteria to approach closer to the substratum and facilitating interaction and subsequent irreversible adhesion. These results were confirmed by several authors [7,20,32]. Nevertheless, because much of the work done to date involved ionic strength at or below 0.1 M, it is not clear if these concepts are applicable at higher ionic strength, such as that of seawater.

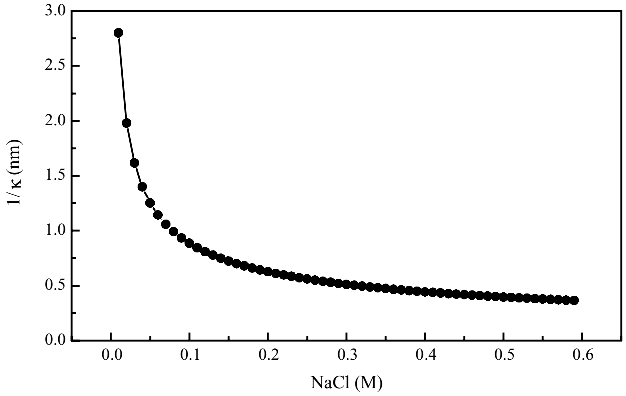

Figure 4 Double-layer thickness as a function of electrolyte ionic strength calculated for NaCl solutions using equation 7 (see text).

Bacterial adhesion to TiO_2 was strongly reduced, instead of increased, in 0.6 M NaCl compared to 0.1 M NaCl (Figure 3). This was in agreement with the results reported by Gordon and Millero [14] that show an inflection point at 0.1 M, with bacterial adherence decreasing exponentially with an increase in ionic strength above this point.

Calculations using Equation 7 show the strong decrease in the thickness of the double layer with an increase in the ionic strength up to 0.1 M NaCl (Figure 4). Additional compression of the double layer above this limit was in the order of 0.5 nm, which, taking into account the bacterial dimensions, seems of minor importance for bacterial adsorption. For this reason, and as pointed out by Gordon and Millero [14], changes in bacterial adherence at ionic strengths higher than 0.1 M cannot explained by a double-layer effect and must be a consequence of some other mechanism.

The effect of changes in pH and ionic strength can not be fully explained by a balance between van der Waals and electrostatic forces alone. As pointed out by Israelachvili [19], there are many aqueous systems where DLVO theory fails to explain the experimental observations. This failure may be due to both the lack of consideration of solvent structuring in relation with surface features [19] or the occurrence of specific interactions that can overcome DLVO barriers [7]. A good example of these systems are oxide or hydroxide surfaces on which hydration and the possibility to form H-bonds are present in this report.

H-bond formation

A hydrogen bond has a partially covalent, partially ionic character and its energy increases when the ionic contribution to the bond increases [15]. Depending on the pH of the solution, hydroxyl ions on the oxide surface can be in the neutral state or have a negative or positive net charge (Equations 1 and 2; Figure 2). Because the strength of the H-bond is related to the electrostatic force of the intervening ion [3], the presence of a net charge on the surface-active groups gives a strong ionic character to its H-bonding with other molecules. This may affect the energy of the bonds, *reinforcing* the interaction with water or other H-bond-forming molecule.

The formation of hydrogen bonds between adsorbed OH groups, water molecules, and lipopolysaccharides (LPS) of bacteria was proposed by Jucker *et al* [21] as a way of bacterial adhesion to oxide surfaces. A two-step adsorption mechanism was described, involving the formation of H-bonds between LPS and the hydration water molecules on the surface in the first step, and displacement of water with the formation of direct H-bonds between adsorbed OH and the LPS in the second step.

Considering the establishment of reinforced H-bonds between charged OH groups on titanium surfaces and the LPS outermost ends, and taking into account variation of the total number of this sites ($N_{a1}+N_{a2}$), it can be observed that at the pzc of the oxide, the surface exposes the maximal number of reinforced bonding sites to interact with bacterial LPS (Figure 2b). This can explain the high number of irreversibly adhered cells at this point (Figure 3).

At either higher or lower pH values, the number of reinforced bonding sites is reduced due to neutralisation of N_{a1} or N_{a2} sites, respectively (Figure 2a). At both pH 4 and 8 the total number of sites is reduced (Figure 2b) in agreement with a proportional decrease in bacterial adhesion at the same pHs (Figure 3). It is assumed in this interpretation of the adhesion results that the possibility to form a bond with the bacterial LPS is the same for acidic as for basic sites on the oxide.

Adsorption of ions

At higher ionic strength, a marked reduction in bacterial adhesion was observed. As the result of the high ionic strength (0.6 M), the specific adsorption of Cl^- and Na^+ ions is expected to occur on both interacting surfaces [4]. Adsorbing cations may adsorb at the same site as protons, or on independent sites (different from those occupied by protons) directly on the surface [22]. In addition, replacement of surface OH ions in oxide films by Cl^- has been described, and its concentration in the film was governed by a chemical equilibrium [25]. Titanium chloride compounds have very low stability [10] and both the adsorption of Cl^- on independent sites and the replacement of adsorbed OH by Cl^- seem to be unlikely, unless a high enough concentration of these ions is present in the solution. At these high concentrations, adsorption of Cl^- on TiO_2 could have result in a reduction of the isoelectric point of the surface, imposing a strong negative charge on the oxide surface and leading to a low adhesion of bacteria. However, as discussed above, the distance between the interacting surfaces may enable short range interactions despite the electrostatic charge. On the other hand, the replacement of OH groups on the surface by Cl^- could reduce the number of H-bonding sites of the substratum leading to the observed decreases in bacterial adhesion (Figure 3).

As a result of their strong dipolar moment, water molecules can be induced to arrange in a molecular network when they are in contact with hydrophilic solid surfaces [19]. Due to the hard polarization induced by Ti^{4+} ions, the H atoms of the adsorbed OH on the TiO_2 are able to form H-bonds with water molecules [3] inducing the structural organization of water layers. The extent of the structural order can vary from one oxide to the other and distances at least as far as four molecular layers were reported on rutile [3].

Bérubé and de Bruyn [3] proposed that Na^+ ions are favored to undergo specific adsorption in the TiO_2-induced water structure especially at high ionic strength, because they fit into the water network distorting but not disrupting the tetrahedral structure. In addition, the occurrence of repulsion forces, as a consequence of cation adsorption at high electrolyte concentrations, was reported for other systems [19], and was attributed to the fact that adsorbed hydrated cations partially retain their hydration water, resulting in a secondary hydration shield.

A similar situation can be proposed for the adsorption of Na^+ on water structured by the TiO_2 surface, which could enlarge the distance from unperturbed bulk water to the surface oxide. The presence of this extra hydration barrier may prevent specific interactions between LPS outer ends and the H-bond-forming sites on the solid substratum. This could contribute to the observed decrease in bacterial adhesion at high ionic strength (Figure 3).

Conclusions

The adhesion of a wild strain of *Pseudomonas* sp. to titanium was strongly influenced by the pH and ionic strength of the electrolyte solution. Under the conditions tested here, adhesion was not determined by the electrostatic charge. The influence of pH was related to the total number of charged OH adsorbed on the oxide surface, which were able to form reinforced H-bonds and determine the irreversible adhesion of bacteria.

However, the complete absence of bacterial adhesion at high ionic strengths can be related to the replacement by Cl^- of H-bond-forming OH on the oxide surface and the development of a hydration repulsion resulting from adsorption of Na^+ ions in the surface-associated water layers.

Although the main conclusion of this work could not at present be extended to other microorganisms or materials, more work is in progress in our laboratory to determine the influence of surface chemistry of different metals and metal oxides on bacterial adhesion.

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