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Microbe–surface interactions in biofouling and biocorrosion processes

Summary. The presence of microorganisms on material surfaces can have a profound effect on materials performance. Surface-associated microbial growth, i.e. a biofilm, is known to instigate biofouling. The presence of biofilms may promote interfacial physico-chemical reactions that are not favored under abiotic conditions. In the case of metallic materials, undesirable changes in material properties due to a biofilm (or a biofouling layer) are referred to as biocorrosion or microbially influenced corrosion (MIC). Biofouling and biocorrosion occur in aquatic and terrestrial habitats varying in nutrient content, temperature, pressure and pH. Interfacial chemistry in such systems reflects a wide variety of physiological activities carried out by diverse microbial populations thriving within biofilms. Biocorrosion can be viewed as a consequence of coupled biological and abiotic electron-transfer reactions, i.e. redox reactions of metals, enabled by microbial ecology. Microbially produced extracellular polymeric substances (EPS), which comprise different macromolecules, mediate initial cell adhesion to the material surface and constitute a biofilm matrix. Despite their unquestionable importance in biofilm development, the extent to which EPS contribute to biocorrosion is not well-understood. This review offers a current perspective on material/microbe interactions pertinent to biocorrosion and biofouling, with EPS as a focal point, while emphasizing the role atomic force spectroscopy and mass spectrometry techniques can play in elucidating such interactions. [**Int Microbiol** 2005; 8(3):157-168]

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Introduction

The study of bacterial interactions with metallic materials led recently to the formulation of a unifying electron-transfer hypothesis of biocorrosion, using microbially influenced corrosion (MIC) of ferrous metals as a model system [23]. According to this hypothesis, biocorrosion is a process in which metabolic activities of microorganisms supply insoluble products that can accept electrons from the base metal. This sequence of biotic and abiotic reactions produces a kinetically favored pathway of electron flow from the metal anode to the universal electron acceptor, oxygen. Although convincing and based on sound scientific evidence, this theory does not take into account the involvement of ultimate electron acceptors other than oxygen. Indeed, the theory has recently been challenged based on a study of marine biocorrosion of carbon steel under anoxic conditions [28]. The role that the organic component, i.e. the biofilm matrix, plays in the electron transfer processes has not been considered in the unified electron transfer hypothesis, despite evidence that enzymes active within the biofilm matrix, and metal ions bound by extracellular polymeric substances (EPS) can catalyze cathodic reactions [8]. The importance of EPS to bio-

corrosion has rarely been addressed, and data available on corrosion rates due to EPS are scarce, in spite of the evidence that EPS alone can accelerate corrosion reactions [9,13].

The production of extracellular polymeric substances by microorganisms is unequivocally accepted as a key mechanism facilitating irreversible cell attachment to inanimate surfaces in aqueous environments, thus promoting the development of a biofilm. A recent review on bacterial biofilms is provided by Hall-Stoodly et al. [22]. It is generally acknowledged that microbial EPS are a complex mixture of macromolecules, such as proteins, polysaccharides, lipids and nucleic acids, and that their composition changes with microbial species, physiological status of the cells, and a wide range of environmental factors [48 and references therein]. In bacteria, EPS are associated with cells as capsules or sheaths; they are an integral part of the biofilm matrix, and are released into the bulk phase of surrounding liquid as planktonic or "free" EPS. Furthermore, evidence that the chemical composition of these different types of EPS is dissimilar has been presented [6]. EPS produced by some bacteria facilitate cell adhesion to hydrophilic surfaces while exopolymers of other bacteria may show a preference for hydrophobic substrata. Moreover, certain bacteria, such as *Vibrio proteolytica*, have separate adhesion mechanisms, i.e. different macromolecules are involved in EPS–surface interaction, depending on the wettability (or surface energy) of the colonized material [39].

Apart from bacteria, unicellular microalgae, such as diatoms, are ubiquitous members of fouling communities and significant contributors to biofilms. Adhesive exopolymers associated with benthic—attached to a sediment surface—diatoms are known to comprise polysaccharides, proteins, and glycoproteins [17]. The characterization of the EPS produced by different genera of marine and freshwater diatoms, with respect to morphology, serological analysis, and lectin interaction, has led to a broad classification of EPS materials into different types, i.e. frustule EPS; outer capsular EPS; motility EPS; and matrix EPS [47]. However, reports related to diatom EPS are infrequent, and knowledge about both the strength and the nature of forces between diatoms and the different surfaces that they colonize remains limited.

Polysaccharides, proteins, and lipids present in microbial EPS have all been implicated in microbial cell adhesion to hydrophobic (low surface energy) substrates, whereas acidic and neutral polysaccharides have been proposed to facilitate attachment to hydrophilic (high surface energy) materials [35]. Exopolymeric material composed of macromolecules with varying hydrophilicity, or of macromolecules that have both hydrophilic and hydrophobic regions (e.g. hydrophobic polypeptides and hydrophilic saccharides on glycoproteins) can adhere to a wide range of surfaces.

This review presents the current understanding of microbe/surface interactions from the perspective of gaining insight into mechanisms of biofouling and biocorrosion. The following topics are emphasized: (i) the use of atomic force spectroscopy (AFS) techniques in elucidating cell adhesion to solid substrata; (ii) the chemical properties of EPS matrix pertinent to biocorrosion processes; and (iii) current and future applications of modern mass spectrometry in characterizing biofilm populations and biofilm matrix.

Techniques to study biofouling and biocorrosion processes

One of the most promising techniques for the characterization of microbial cell adhesion and cell-surface interactions is AFS. This method uses atomic force microscopy (AFM) operating in force mode, which offers both imaging capabilities and quantitative measurements of forces between the AFM tip and the sample. Several reviews have been published on the fundamentals and applications of AFM/AFS relevant to biofilm systems [20 and references therein]. A recently reported investigation combined AFS with confocal force microscopy to demonstrate, in situ, interaction between a living bacterium, *Escherichia coli*, and the silicon nitride surface of the AFM tip [33]. The study revealed that an outer-membrane protein of *E. coli* extending outside the cell wall was responsible for cell adhesion to the tip surface.

Apart from facilitating bacterial attachment to substrata, EPS form the dynamic biofilm matrix [48]. The majority of studies on EPS have focused on an analysis of the structure and function of polysaccharides, often ignoring the presence and possible significance of other types of macromolecules. In most cases, carbohydrates, including neutral sugars, acidic polysaccharides, and aminosugars, are indeed the most abundant chemical species and, therefore, are proposed to act as structural elements responsible for the mechanical stability of biofilms. However, one must be cautious when claiming the importance of a certain type of polysaccharides in biofilm development. An investigation has demonstrated that the exopolysaccharide alginate, produced by mucoidal strains of *Pseudomonas aeruginosa*, is, in fact, not required to form the biofilms of two predominant environmental phenotypes of non-mucoidal strains of *P. aeruginosa* [49]. These two strains, PA14 and PAO1, have traditionally been used to study biofilms, and in several reports alginate has been assumed to be a key EPS component mediating cell adhesion of the above strains. Evidence that a polysaccharide different than alginate facilitates attachment of *P. aeruginosa* PA14 and PAO1 strains to surfaces would probably have consider-

able implications in developing strategies for preventing biofilm development by these bacteria. In contrast to the abundant literature on polysaccharides, reports on EPS proteins are scarce. Yet, extracellular enzyme activities are readily observed in biofilms, and it has been proposed that exoenzymes ought to be considered an integral part of the EPS matrix [48 and references therein].

The most powerful tool for the characterization of proteins is mass spectrometry (MS). In the past 10–15 years, MS has revolutionized bioanalytical chemistry. Analytical MS has undergone rapid development with respect to ionization methods, instrumentation, and the ability to detect large molecules and deal with complex mixtures. Undisputedly, MS has the potential to expand our ability to monitor microbiological systems and to elucidate the molecular mechanisms and interrelationships in such systems. Reaching a deeper understanding of the interfacial chemistry governing biofouling and biocorrosion processes is likely to require the extensive use of modern MS. The remarkable progress in MS is due primarily to two ionization methods developed in the late 1970s, namely, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). In ESI, a liquid solution containing a sample is sprayed from a narrow capillary with a high electric potential applied to the capillary tip. Electrically charged droplets are formed, and analyte ions develop as a result of droplet disintegration. In laser spray ionization (LSI), the electrospray tip is irradiated with a high-energy infrared laser [26]. We have found that this ionization method has unique advantages for the MS characterization of complex biological mixtures, such as EPS (Beech, unpublished data).

In MALDI, analytes are co-crystallized with a UV-absorbing matrix compound. Analyte ions are then desorbed from the matrix by a short UV laser pulse. In the most common use of the technique, the ions are desorbed inside a vacuum chamber and mass analyzed in a time-of-flight (TOF) mass spectrometer. In parallel with the development of MALDI and ESI ionization methods, mass spectrometric instruments have undergone an extremely rapid evolution. The capabilities of modern instruments, such as TOF, ion trap, and Fourier transform mass spectrometers (FTMS) were unknown only a few years ago. Tandem MS (MS/MS), which consists of two mass analyzers in series, are powerful instrument for the analysis of complex materials and, for example, quadrupole/TOF (Q-TOF) have been successfully applied to the analysis of biological materials. In addition to characterizing proteins, MALDI and ESI enable analysis of nucleic acids, lipids, and phospholipids. Several applications of MALDI and ESI of interest to environmental microbiology have been demonstrated and/or are under active development. These include bacterial identification, monitoring bacterial protein expression and post-translational modifications in response to environmental or metabolic stimuli, and measuring bacterial cell growth. However, the application of modern MS to biofilms research, including biofouling and biocorrosion, remains virtually unexplored.

To prove that MS can help to elucidate the composition of complex biological materials with minimum sample preparation and with high sensitivity, we will present a few examples from our own unpublished studies on the characterization of organic and inorganic components using EPS, as well as mixtures of model compounds, using ESI and LSI MS. The EPS used in these studies were recovered from cultures of a recently described, new strain of a sulfate-reducing bacterium *Desulfovibrio alaskensis* [21].

Apart from AFM/AFS and MS, other techniques of surface science and numerous forms of light and electron microscopy are valuable tools to understand the nature of microbially influenced interfacial processes and are extensively used for that purpose [4,10,37,45] (see Table 1 for a list of techniques currently used to study biofouling and biocorrosion).

Table 1. Techniques used in the study of biofouling and biocorrosion [4,10,37,45]

Technique	Abbreviation
Atomic force spectroscopy	AFS
Atomic force microscopy	AFM
Biological force microscopy	BFM
Biological force spectroscopy	BFS
Confocal force microscopy	CFM
Mass spectrometry	MS
Tandem MS	MS/MS
Fourier transform mass spectrometry	FTMS
Electrospray ionization	ESI
Matrix-assisted laser desorption ionization	MALDI
Laser spray ionization	LSI
Time-of-flight (ToF) mass spectrometry	ToFMS
Quadruple/ToF	Q-TOF
X-Ray diffraction	XRD
X-Ray photoelectron spectrometry	XPS
Near-edge X-ray absorption fine structure spectroscopy	NEXAFS
Energy dispersive X-ray analysis	EDX
Scanning transmission X-ray microscopy	STXM
Secondary ion mass spectrometry	SIMS
Surface-enhanced laser desorption ionization	SELDI

Atomic force spectroscopy studies of microbial adhesion to solid surfaces

In addition to AFM imaging of single microbial cells and biofilms [7], AFS has helped to determine the physical properties of EPS either associated with surfaces of microbial cells or occupying cell-free areas on the material surface. This has been achieved by measuring forces between silicon nitride AFM tips, either as received or chemically modified, and EPS macromolecules. Both, cell-free EPS (Beech, unpublished) (Fig. 1) and EPS associated with surfaces of living or dead microbial cells immobilized on different substrata have been characterized [1,14,40,46]. Some investigators have followed the latter approach to demonstrate the complexity of species-dependent macromolecular composition and heterogeneous spatial distribution of different types of EPS on the surface of living marine diatoms [25]. A few studies have reported the use of AFM tips functionalized with microbial cells, termed bioprobes (Fig. 2), for determining cell–material surface interactions [32,40]. This latter AFM technique, known as biological force microscopy (BFM), offers a great advantage for quantitative measurements of the adhesion characteristics of microorganisms on surfaces with varied physicochemical properties. A recent example is the use of BFM to study living marine diatoms of *Navicula* genus as bioprobe. The adhesive properties of the EPS associated with individual diatom cells to two physicochemically different materials, mica and a fouling-release silicone elastomer, Intersleek, were quantitatively characterized [2]. Using different diatoms, as well as the same diatom in different growth stages, it was found that the work of detachment of a single diatom from either type of surface strongly depended on the individual *Navicula* cell but not on its

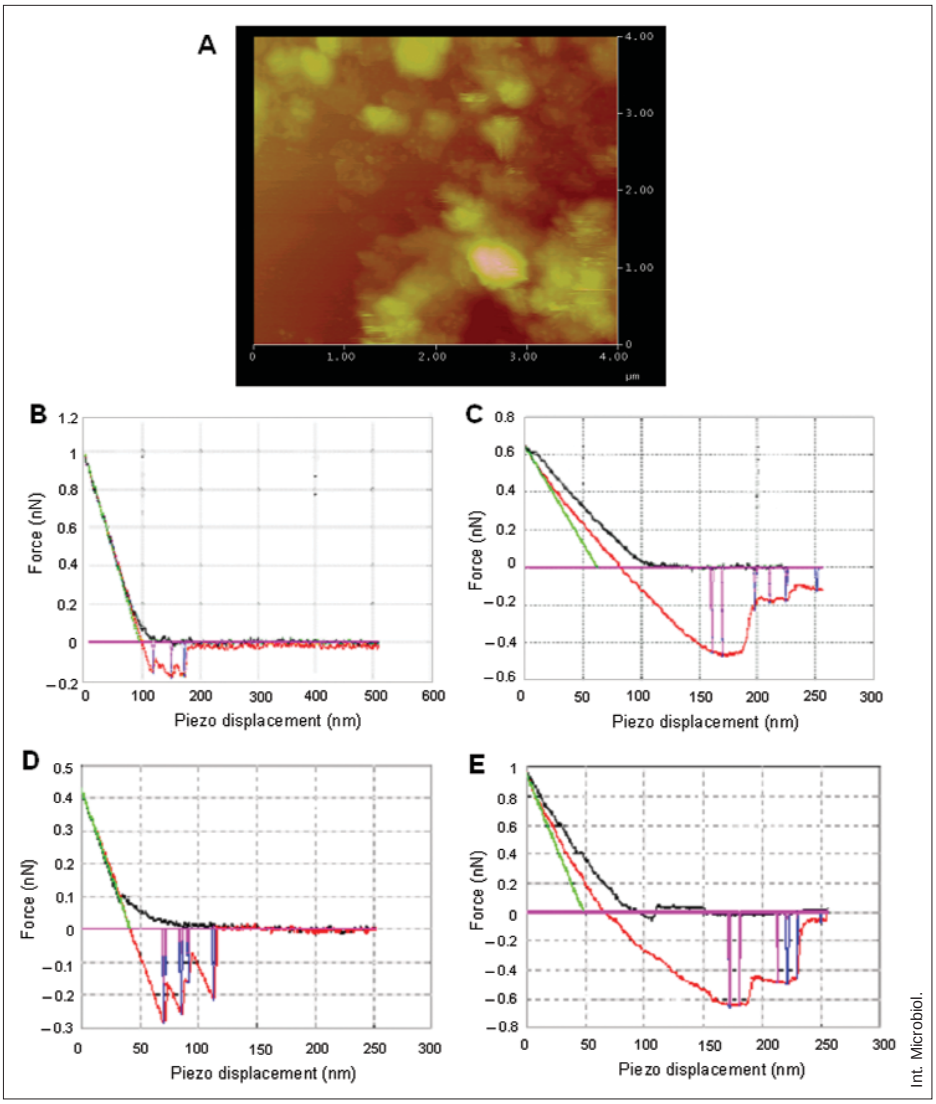

Fig. 1. (**A**) Atomic force microscopy (AFM) image in water of *Desulfovibrio alaskensis* extracellular polymeric substances (EPS) on the surface of mica. (**B**, **C**) Representative force curves (FC) from two main groups of FC obtained when characterizing the strength of adhesion of *D. alaskensis* EPS depicted in A to AFM silicon nitride tip. (**D**, **E**) FC obtained when characterizing the adhesion in water of protein cytochrome *c* and polysaccharide dextran, deposited on mica, to the AFM tip. The profile of the EPS force curve depicted in B is similar to that of cytochrome *c* shown in D, while the FC profile of EPS in C closely resembles the FC of dextran presented in E.

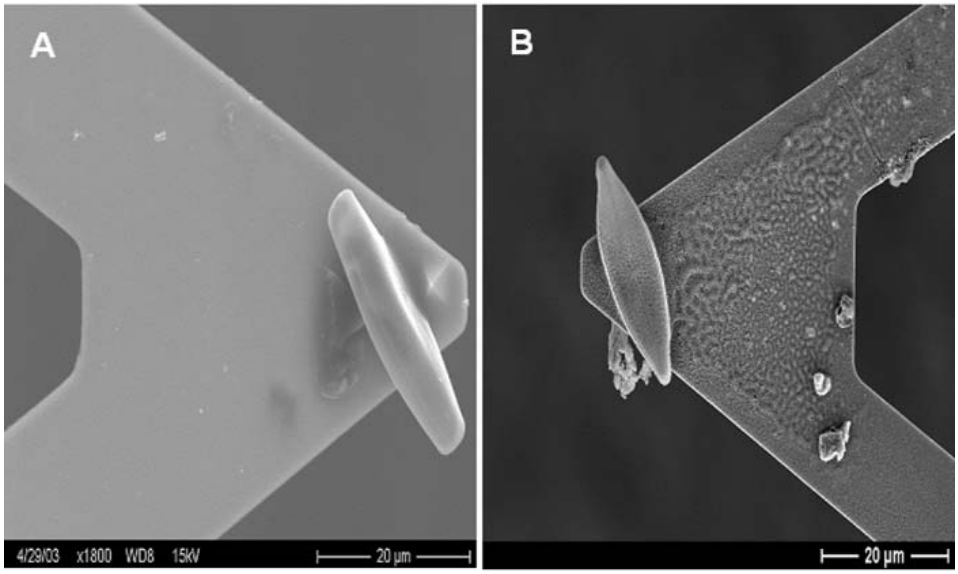

Fig. 2. Scanning electron microscopy (SEM) micrographs of single marine diatoms of *Navicula* genus immobilized (**A**) sideways on an AFM cantilever manufactured with the silicon nitride tip and (**B**) on a tipless AFM cantilever with the slit (raphe) of the diatom facing down. Living diatoms immobilized to tipless cantilevers were used as bioprobes for the study of single-cell adhesion to hydrophobic and hydrophilic surfaces in a simulated marine environment. The micrographs were obtained at Montana State University Imaging and Chemical Analysis Laboratory, Bozeman, USA.

growth stage. Generally, the adhesion forces to the hydrophobic Intersleek surface and the hydrophilic mica surface were of similar strength. The study concluded that the adhesion of *Navicula* to surfaces with different physicochemical properties is governed by the macromolecular specificity of diatom EPS. However, the EPS macromolecules that mediate diatom cell adhesion have not been identified, and curve profiles strongly suggested that both proteins and polysaccharides are the likely bioadhesives.

Colloid probe AFM, which is another version of AFS, has recently been used to demonstrate differences in the adhesive properties of three *E. coli* strains to the surface of glass [29]. The study employed a gradient force analysis method. The authors proposed that the values of adhesion (sticking) coefficients between bacterial cells and glass were correlated solely with the length of the exopolymers on surfaces of *E. coli*. As in the diatom study by Arce et al. [2], the type of EPS macromolecules governing adhesion process were not determined. Undisputedly, AFS and in particular biological force spectroscopy (BFS) can considerably improve our understanding of microbial adhesion to surfaces in aqueous environments, both at the single cell and the single macromolecule level.

The capability of AFS to generate unique force signatures characteristic of specific macromolecules is of great advantage when elucidating fundamental aspects of microbial cell/solid surface interactions. Force profiles of polysaccharides differ from those of proteins or nucleic acids [1 and references therein]. Furthermore, each individual macromolecule has its own force signature. It is anticipated that, ultimately, AFS will aid in the in situ characterization of complex mixtures of surface-associated macromolecules. Obvious applications of AFS techniques to biofouling and biocorrosion are in the area of testing novel materials for their antifouling properties and in elucidating the chemistry of microbial adhesives.

Enzymes and biocorrosion

Bacteria are generally regarded as the primary colonizers of surfaces submerged in water; hence numerous investigations have focused on the chemical characterization of bacterial exopolymers [42]. Bacteria produce a wide range of enzymes, e.g. hydrolytic and proteolytic enzymes, as well as lyases, which are able to react with substrates beyond the cell wall. Such enzymes can be broadly categorized as ectoenzymes (associated with the cell, but expressed outside the cytoplasmic membrane), and extracellular enzymes, i.e. present as free forms. The latter include polysaccharidases, proteases, lipases, esterases, peptidases, glycosidases, phosphatases, and oxidoreductases.

The release of enzymes by microorganisms into their external environment provides the basis for the interaction between cells and substrates. In order for such an interaction to be energy-efficient, the enzymes, substrates, and hydrolysis products should remain in close proximity (within no more than 500 nm) of the cells. The biofilm environment facilitates the latter relationship. The chemical properties of the biofilm matrix, such as the presence of different types of binding sites within macromolecules forming the matrix, are likely to promote close association between EPS

enzymes and exogenous substrates, thus enabling enzymatic reactions.

Despite evidence of the spatially independent distribution of EPS and cells obtained using different forms of microscopy [7], most reports on monitoring enzyme expression in biofilms focus on cell-associated activities and rarely address cell-free areas of the biofilm matrix. Enzymes, such as the hydrogenases of anaerobic sulfate-reducing bacteria (SRB), are known to stay active within the biofilm matrix, irrespective of the absence of living cells, and can play a significant role in the biocorrosion of iron and ferrous alloys [3]. In addition to hydrogenases, the activity of enzymes, such as catalases, phosphatases, lipases and esterases, can be readily detected in aqueous oxygenated solutions of freeze-dried SRB exopolymer [5]. The impact of these enzymes on steel corrosion is not fully understood. However, progress in microbial genomics allowed a comparative analysis of two available SRB genomes, those of *Desulfovibrio vulgaris* and *Desulfitobacterium hafniense*. While only a plasmid-encoded catalase gene is present in gram-negative *D. vulgaris* [24], gram-positive *D. hafniense* encodes three catalases, of which at least one is secretable, i.e. extracellular (Galperin and Beech, unpublished). These catalases are HPI (CatA_ECOLI), HPII (CatE_ECOLI), and Mn-containing catalase PMID: 8939876, gi|1752756. Interestingly, an abundant EPS producer, *P. aeruginosa*, encodes two catalases, CatE and an Mn-dependent catalase, but CatE is present in three copies. Whether catalases encoded in some, but not all, SRB species are active in biofilms under oxygen stress and can contribute to oxygen reduction reactions, thus influencing corrosion processes, requires further studies.

The ecology of microbial communities and, therefore, their metabolic output are of great importance when investigating the effect of biofilms on the corrosion behavior of metallic materials. Many studies have been carried out in the areas of bacterial genomics and molecular environmental ecology related to biocorrosion, but work in this field is outside the scope of this review. Briefly, emerging data indicate the enormous physiological versatility of different bacterial species and suggest that species specificity could certainly explain why, despite identical environmental conditions, biofilms consisting of different bacterial strains belonging to the same genus differ in their ability to deteriorate colonized materials [18 and references therein]. The possibility to monitor microbial processes on surfaces at the single-cell level would certainly help to document such specificity.

Of considerable research interest is the process of direct electron transfer between the metallic surface and enzymes, whether present in the outer membrane of a cell or absorbed to metallic surfaces [5]. Numerous microorganisms are known to promote the corrosion of iron and its alloys through dissimilatory reduction reactions [36]. The consequence of these reactions, in which enzymes play a key role, is the dissolution of protective oxide/hydroxide films on the metal surface. Thus, passive layers on steel surfaces can be lost or replaced by less stable, reduced metal films that allow further corrosion to occur. One of the best examples of a microorganism causing biocorrosion due to dissimilatory iron reduction is *Shewanella oneidensis*, an extensively studied, gram-negative, facultatively anaerobic bacterium, formerly classified as *S. putrefaciens*. This bacterium oxidizes various carbon substrates by reductively dissolving Fe(III)-containing minerals, such as ferrihydrite, goethite, and hematite. The biocorrosion of steel in the presence of *S. oneidensis* has been documented [27]. The corrosion rate was first measured by Little et al. [30], who also showed that the rate depended on the type of oxide film under attack. The authors emphasized the importance of the proximity of cells to the surface of an iron-oxide layer, but did not otherwise propose a mechanism.

However, using biological force spectrometry, i.e. employing living bacterial cells as AFM probes, it was determined that, out of several type of iron-reductases expressed in *S. oneidensis* and mobilized within the outer membrane of this bacterium, a 150-kDa protein was the most likely candidate responsible for the transfer of electrons directly to Fe(III) in the crystal structure of goethite [32]. This electron transfer weakened the iron–oxygen bond and caused the reductive dissolution of the mineral layer. The minimum cell-surface distance required to observe the bioprobe–iron-oxide interaction was in the range of several hundred nanometers, which is enough for an enzyme to act on its substrate. In another study, AFM and X-ray photoelectron spectroscopy (XPS) combined with molecular modeling provided a better understanding of the role of surface structure in iron-oxide reduction by *S. oneidensis* [34]. Three single crystal faces, hematite (001), hematite (111), and magnetite (100), were used as model iron oxides. The theoretical calculations indicated that electron transfer rates to either of the two hematite surfaces are slower than that to magnetite by up to two orders of magnitude. The study concluded that the structure of the iron oxide has a clear influence on the rate of electron transfer. The theoretical calculations were supported by laboratory experiments, which revealed that hematite accumulated a higher density of cells and showed a greater accumulation of Fe(II) than did magnetite.

Investigation of parameters governing cell interactions with mineral surfaces is of obvious importance to biocorrosion, since passive layers composed of oxides, hydroxides, and abiotically produced corrosion products are prime examples of such minerals on surfaces of metallic materials.

Metal binding by the biofilm matrix

The metal-binding/sorption capacity of the biofilm matrix is important to MIC, and sorption is both bacteria and metal species-specific. Metal ions, trapped within the biofilm matrix, are expected to be coordinated by a variety of ligands, and the resulting complexes will have a range of redox potentials. Such complexes may participate in the electron-transfer processes that drive corrosion reactions [8]. The importance of this mechanism in the overall corrosion process strongly depends on the chemistry of the material surface [13].

Current reports and models of EPS–metal ions interactions emphasize the role of anionic polysaccharides in metal binding but rarely consider the participation of protein, nucleic acid, or lipid. Using IR techniques, it was recently found that nucleic acid in EPS secreted by *Bacillus subtilis* and *P. aeruginosa* form monodentate complexes with Fe centers on goethite [37]. Microbial EPS contain many functional groups that are either negatively or positively charged at near neutral pH values.

Polysaccharides owe their negative charge either to carboxyl groups of uronic acids or to non-carbohydrate substituents, such as phosphate, sulfate, glycerate, pyruvate, or succinate [42]. Proteins rich in acidic amino acids, including aspartic and glutamic acid, contain carboxyl groups that also contribute to the anionic properties of EPS. Nucleic acids are polyanionic due to the phosphate residues in the nucleotide moiety. As negatively charged components of EPS, uronic acids, acidic amino acids, and phosphate-containing nucleotides are expected to be involved in electrostatic interactions with multivalent cations (e.g. Ca^{2+}, Cu^{2+}, Mg^{2+}, Fe^{3+}).

A recent study of iron-hydroxide-encrusted biofilms collected from a subterranean location revealed that bacterial exopolymers, most likely acidic polysaccharides, act as a template for the assembly of akaganeite (β-FeOOH) pseudo-single crystals [15]. Mineralization was shown to result from contact between the EPS and oxidized iron, through the binding of ferric iron with carboxylic groups on the polymers. The authors pointed out that oxidation of ferrous ions and subsequent precipitation of iron oxyhydroxide on the biofilm exopolymers releases protons, leading to a decrease in the pH outside the cell membrane. They proposed that the purpose of exopolymer production is to ensure that iron oxyhydroxide mineral precipitation is localized immediately outside the cell, as this increases metabolic energy generation of the cell through enhancement of the proton-motive force.

Apart from demonstrating that mineral precipitation within a biofilm matrix can have a major influence on the activity levels of biofilm organisms, the process has implications also for biocorrosion. The oxidation of ferrous ions adsorbed on the surfaces of iron oxyhydroxide assembled on biofilm polymers would provide an additional contribution to the cathodic reaction. Such biominerals may be thought of as electron-conducting fibers dispersed within the biofilms matrix. A close spatial relationship of, and interaction between, inorganic and organic components within the biofilms matrix is further supported by a number of recent studies using near-edge X-ray absorption fine structure spectroscopy (NEXAFS) and scanning transmission X-ray microscopy (STXM) [10,45].

Biochemical mass spectrometry

Proteomics. MS is now the core technology in proteomics and has allowed the enormous progress made in that field during the last few years [50]. Current practice in proteomics constitutes a modern example of the separation/MS analysis approach. Proteins from biological material are commonly separated on two-dimensional gels, followed by digestion, mass analysis, and possibly partial sequencing of the digested peptides. In an alternative approach, a complex mixture of proteins is digested, and the resulting peptide mixture separated prior to (partial) sequencing of the peptides by MS/MS. MS has been extensively used to characterize bacterial proteomes and to identify proteins that have been up-or down-regulated by genetic mutations, virus infections, and environmental stress factors [11].

Usually, the proteomes characterized are those of pure cell strains, but the methods should also be applicable to EPS and bacterial consortia in natural environments. However, the effort required to characterize a proteome is presently very substantial; thus, to study the variation of a proteome as a function of several environmental variables, although pursued at the fundamental research level, is not yet practical for routine monitoring. The characterization of proteins and peptides in EPS of model biofilms using proteomics would seem to be long overdue and very worthwhile undertaking.

At present, proteomics methods are unable to deal with the additional level of proteome complexity introduced by protein–protein or protein–carbohydrate interactions [19]. Clearly, characterizing such complexes will be critical to understanding molecular mechanisms of a biofilm matrix, in which macromolecules provide a unique 3-D architecture. There has been significant progress in detecting non-covalent (protein) complexes using ESI [31], and the strength of such complexes is advantageously determined employing LSI [43]. It should be noted that MS characterization of non-

covalent complexes has generally been restricted to highly purified samples, containing only the culprit macromolecules. The MS detection of non-covalent complexes directly from biological material, for example EPS, remains a challenge for the future.

Analysis of polysaccharides and complex mixtures. Glycosylation of proteins modulate protein functions, such as folding and non-covalent interaction patterns. Of particular importance are cell-surface carbohydrates, which control interactions between the cell and its environment. As already stated, polysaccharides are often a key constituent of EPS, and they play important roles in cell adhesion to solid surfaces, as well as providing structural stability and metal-binding sites within the biofilms matrix. Analysis of carbohydrates is thus important for the characterization of biofilm communities.

The MS study of both free carbohydrates and protein-bound glycans is complicated by the fact that they are expressed as extensive variations on a core structure. Thus, the characterization of oligosaccharides represents a greater challenge than the characterization of proteins. Still, MS does offer the potential for precise determination of carbohydrate sequence, linkage, and branching. Considerable progress has been made in this field, both in instrumentation and in gaining a fundamental understanding of ion-formation processes [38]. Applications to life sciences and medicine are rapidly expanding, and there is no doubt that these methods will become powerful tools in environmental microbiology.

While separation followed by MS analysis is the most powerful approach towards a complete molecular-level characterization of complex biological materials, sample handling and separation procedures are typically both costly and time-consuming. Therefore, methods for direct analysis of a complex mixture or with fast and simple "sample" cleanup will continue to be of great interest. The critical analytical challenge is a "classic" one, i.e. to obtain relevant molecular information with a minimum of effort put into expensive sample-handling procedures. Recognized analysis applications with no or minimal separation include monitoring, fingerprinting, detection of biomarkers, and analysis of various targeted analytes. Such limited approaches may well dominate MS applications to environmental microbiology for the foreseeable future because of the complexity of the systems and the multitude of environmental variables.

Our unpublished study demonstrated that the use of LSI followed by MS/MS, both organic and inorganic components can be detected in exopolymers produced in anaerobic cultures of *Desulfovibrio alaskensis* (Fig. 3). We have also showed that, for example, LSI is a very useful technique for detecting, at a femtomole level, both polysaccharides and proteins in a model mixture comprising aqueous solution of acidic polysaccharide alginate and a cytochrome (unpublished). LSI spectra revealing characteristic signatures of cytochrome and alginate are depicted in Fig. 4. Furthermore, using LSI, the detection of proteins in predominantly polysaccharide-containing native EPS of *D. alaskensis* [21] has also been achieved (Fig. 5).

Surface-enhanced laser desorption ionization. SELDI is a general method for targeted biomolecular MS analysis. In this approach, the laser desorption substrate is modified to selectively extract targeted analytes [44]. Common variants of this technique use antibodies immobilized on dextran to selectively capture targeted proteins and metal affinity probes to selectively capture metal-binding peptides and proteins. Immobilized lectins have been used to capture microbes via exposed glycosylation sites [12]. Protein biochips that enable facile creation of bio-specific probes are commercially available and have found extensive use in clinical proteomics studies. The use of SELDI and related methods would seem to have a great potential as a rapid technique for monitoring and characterization of microbial biofilms.

Biomarkers—identification and characterization of bacteria. The application of MS to bacterial taxonomy has been pursued for several decades using a variety

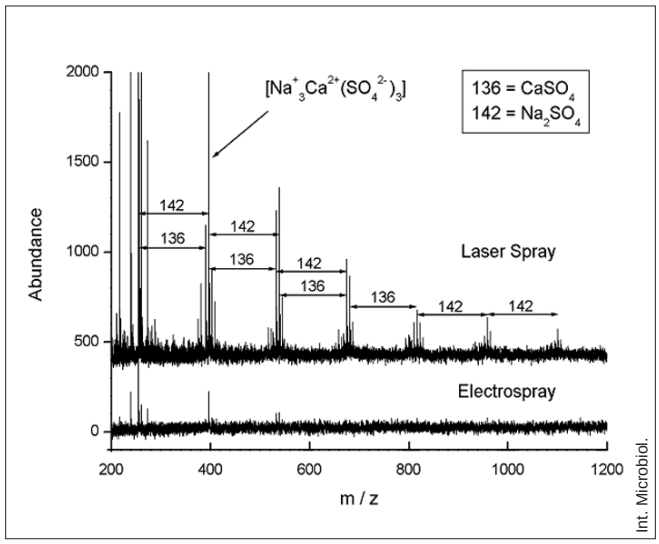

Fig. 3. Laser spray ionization (LSI) and electrospray ionization (ESI) negative-mode mass spectra from aqueous solution of *D. alaskensis* EPS. The peaks in LSI are due to clusters of Na^+, Ca^{2+}, and SO_4^{2-}. The spectrum illustrates the ability of mass spectrometry (MS) to obtain information about inorganic compounds in a complex biological material, such as EPS. The upper spectral trace was obtained with LSI and the lower trace using ESI under identical experimental conditions.

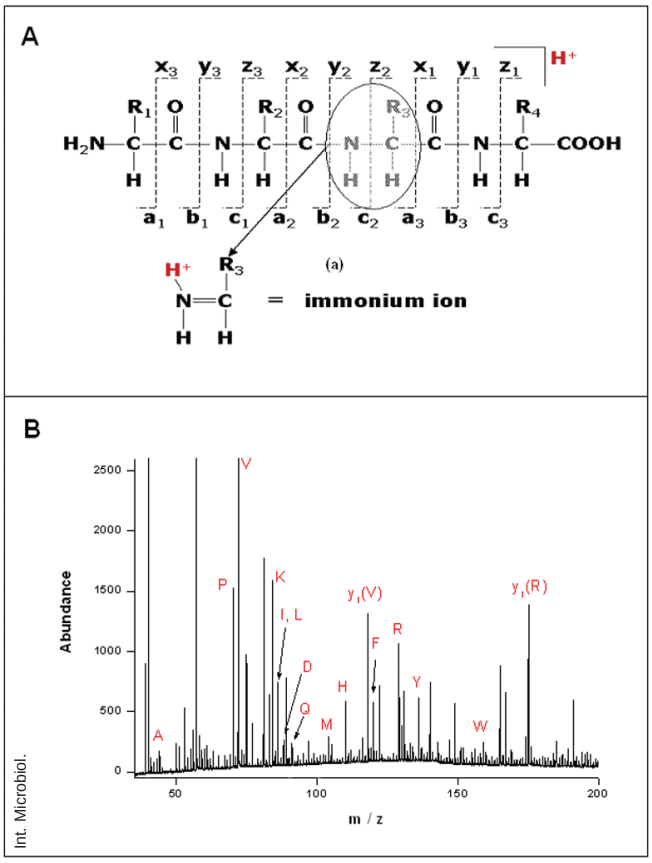

Fig. 4. (**A**) Common types of MS fragment ions from a protonated peptide. The immonium ions are formed by breaking two bonds in the backbone of the peptide, accompanied by proton transfer. (**B**) Low-mass region of LSI mass spectra from an aqueous solution of *D. alaskensis* EPS, with tentative peak assignments to immonium ions of different amino acids. The amino acids that are known to generate intense immonium ions (proline, P; valine, V; isoleucine or leucine, I/L; and histidine, H) are all identified in the *D. alaskensis* EPS mass spectrum.

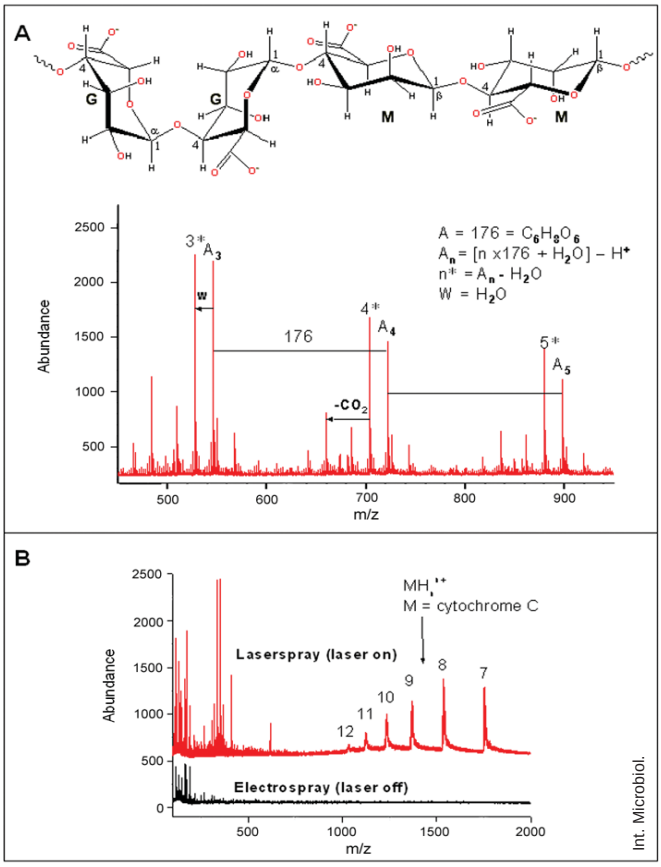

Fig. 5. (**A**): The upper part of the figure depicts the structure of linear, unbranched polymer alginate produced by brown algae *Mycrocystis pyrifera* (kelp), containing 61% D-mannuronic acid (M) and 39% L-glucuronic acid (G). The lower part of the figure presents a LSI mass spectrum of 10^{-6} M aqueous alginate solution obtained in negative ion mode. The spectrum shows ion fragments characteristic of the 240-kDa polysaccharide. The mass spectral pattern is repeated every 176 Da, which is the mass A of the $C_6H_8O_6$ residue of alginate. The mass of the alginate monomers ($C_6H_{10}O_7$) is 194 Da. (**B**): LSI and ESI positive ion mass spectra of an aqueous mixture of *D. alaskensis* EPS and 10^{-5} M, 12,370 Da bovine cytochrome *c*. The cytochrome *c* peaks, labeled 7–12, and the clusters of peaks from both cytochrome and EPS in the 300 to 700 Da mass range appear only in LSI and not in ESI. Peak labels represent numbers of protons added to the protein in the electrospray process. Masses of protein peaks are calculated according to the formula m/z = (M+n)/n, where M is the mass of protein and n is the number of protons. Cytochrome c peaks were also detected in positive ion mode LSI mass spectra of the cytochrome *c* and alginate mixture.

of techniques. Recently, promising results for MALDI/MS-based methods for the identification of bacterial strains have been presented [41]. Generally, the identification is based on the presence in the mass spectra of species- and strain-specific "biomarkers" at certain mass values. While apparently not yet demonstrated, a logical extension of MS bacterial identification would be to also determine the metabolic and life cycle status of cells, using "metabolic biomarkers". There is a growing awareness that the "metabolome", i.e. the collection of cellular metabolites, represents an important complement to the genome and proteome. The metabolome consists of thousands of compounds of highly varying structure, but typically with molecular masses of less than 1000 Da. MS is the central analytical technique in this emerging field, and the dominating methods are GC/MS, liquid chromatography MS (LC/MS), and capillary electrophoreses MS (CE/MS). The metabolome represents a signature of the physiological state of a cell or cell population and reflects changes in specific biochemical processes that may be induced by environmental stress or opportunities and interactions with other species.

The ability to rapidly detect, identify, and determine the metabolic status of bacteria in situ would clearly be of great interest to environmental and clinical microbiology. Indeed, it would likely revolutionize microbiology, especially if such

information were to be obtained from single cells. Current developments in imaging MS hold such promise.

Molecular imaging. While many methods are used for imaging, MS has a unique potential to characterize complex molecular systems. Imaging by secondary ion mass spectrometry (SIMS) is well-established and yields images with a high sensitivity and a lateral resolution of 100 nm or better. This technique is used primarily for metal and inorganic materials. For organic and bioorganic analysis, SIMS has severe limitations. It is a very "hard" ionization method that results in extensive ion fragmentation, and it is typically impossible to detect and identify large molecules. Moreover, SIMS is destructive to any organic surface layer and, after a dose of about 10^{13} primary ions per cm^2, the secondary ion mass spectra are totally degraded.

The need for a non-destructive, soft-ionization method to obtain mass spectra of intermediate and large molecules, with a high sensitivity and sub-micrometer lateral spatial resolution, from organic and biological surfaces is apparent. Several of the most exciting, recent developments in MS, one of which is imaging using MALDI [16], represent progress in achieving this aim. There are also promising SIMS-related developments that, at least partially, have overcome the limitations of SIMS. Greatly improved high mass capability has been achieved in matrix-assisted SIMS, which uses a MALDI-type of matrix in SIMS experiments. Other approaches for detection and imaging of large molecules involve the use of polyatomic ions, such as SiF_5^+, Au_n^+, and C_{60}^+, or massive clusters, such as primary ions. The advantages include less surface damage and dramatically higher yields for higher-molecular-mass species.

While imaging MS technology is still in its infancy, the ability to obtain high-resolution 3-D molecular maps of, for example, biofilms is only a matter of time.

Conclusions

The presence of microorganisms on materials surfaces can modify their chemistry and morphology, often promoting the establishment and/or maintenance of physico-chemical reactions not normally favored in the absence of the microbes and harmful to the performance and integrity of the material. Microorganisms such as bacteria, fungi and algae, interact with solid surfaces by releasing EPS, which facilitate cell adhesion and biofilm development. Understanding, at the molecular level, interactions of microbial cells and their metabolic products, including EPS, with materials surfaces is, therefore, of paramount importance to the ability to prevent biofouling and biocorrosion. Recent developments in microscopy imaging and surface-analytical techniques are changing our perception of the impact of microorganisms on materials in both natural environments and man-made systems. In particular, BFS, NEXAFS, and STXM are techniques that allow quantitative in situ investigation of cell/surface interactions at submicron scale, providing information on the strength of microbial cell attachment to solid substrata and the properties of macromolecules involved in this process. Exciting developments in biochemical MS have opened up new analytical approaches to the study of complex mixtures of macromolecules and offer the not too distant possibility of in situ chemical imaging at high resolution. Gaining deeper insight into the fundamental mechanisms of biofilm-mediated deleterious interfacial processes will, undoubtedly, result in the development of practices that will aid in their control.

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Revisión de las interacciones entre microorganismos y superficies en los procesos de bioensuciamiento y de biocorrosión

Resumen. La presencia de microorganismos en las superficies de materiales puede tener un efecto profundo en el funcionamiento de dichos materiales. El crecimiento microbiano asociado a superficies, por ejemplo un biofilm, se sabe que estimula el desarrollo del bioensuciamiento (*biofouling*). La presencia de biofilms puede promover en las interfaces reacciones fisicoquímicas no favorecidas en condiciones abióticas. En el caso de materiales metálicos, los cambios no deseados en las características del material y debidos a un biofilm (o a una capa de bioensuciamiento) se denominan biocorrosión o corrosión microbiana (MIC, microbially influenced corrosion). El bioensuciamiento y la biocorrosión se producen en hábitat acuáticos y terrestres con diferentes contenidos de nutrientes, temperatura, presión y pH. En dichos sistemas, la química de las interfaces refleja una gran variedad de actividades fisiológicas realizadas por poblaciones microbianas diversas que crecen muy bien en los biofilms. La biocorrosión puede verse como la consecuencia de un conjunto de reacciones biológicas y abióticas de transferencia de electrones de los metales, por ejemplo reacciones redox, favorecidas por la ecología microbiana. Las sustancias poliméricas extracelulares (EPS) producidas por microorganismos, que comprenden diferentes macromoléculas, median la adherencia inicial de la célula a la superficie del material y constituyen la matriz del biofilm. A pesar de su importancia indiscutible en el desarrollo del biofilm, no se sabe muy bien hasta qué punto contribuyen las EPS a la biocorrosión. Esta revisión describe la percepción actual de las interacciones material/microorganismo relativas a la biocorrosión y al bioensuciamiento, centrándose en las EPS, y destacando el papel que las técnicas de espectroscopia de fuerza atómica y de espectrometría de masas pueden desempeñar en la aclaración de tales interacciones. [**Int Microbiol** 2005; 8(3):157-168]

Palabras clave: sustancias poliméricas extracelulares · bioensuciamiento (*biofouling*) · biocorrosión · biofilm · espectrometría de masas · espectroscopia de fuerza atómica

Revisão das interações entre microorganismos e superfícies nos processos de bioensuciamiento e de biocorrosão

Resumo. A presença de microorganismos nas superfícies de materiais pode ter um efeito profundo no funcionamento de ditos materiais. O crescimento microbiano associado a superfícies, por exemplo um biofilm, se sabe que estimula o desenvolvimento do bioincrustação (*biofouling*). A presença de biofilms pode promover nas interfaces reações físico-químicas não favorecidas em condições abióticas. No caso de materiais metálicos, as mudanças não desejadas nas características do material e devidas a um biofilme (ou a uma capa de bioincrustação) se denominam biocorrosão ou corrosão microbiana (MIC, microbially influenced corrosion). A bioincrustação e a biocorrosão se produzem em hábitats aquáticos e terrestres com diferentes conteúdos de nutrientes, temperatura, pressão e pH. Em ditos sistemas, a química das interfaces reflete uma grande variedade de atividades fisiológicas realizadas por povoações microbianas diversas que crescem muito bem nos biofilms. A biocorrosão pode ver-se como a conseqüência de um conjunto de reações biológicas e abióticas de transferência de eléctrons dos metais, por exemplo reações redox, favorecidas pela ecologia microbiana. As substâncias poliméricas extracelulares (EPS) produzidas por microoorganismos, que compreendem diferentes macromoléculas, intermedeiam a aderência inicial da célula à superfície do material e constituem a matriz do biofilm. Apesar de sua importância indiscutível no desenvolvimento do biofilm, não se sabe muito bem até que ponto contribuem as EPS à biocorrosão. Esta revisão descreve a percepção atual das interações material/microorganismo relativas à biocorrosão e à bioincrustação, centrando-se nas EPS, e destacando o papel que as técnicas de espectroscopia de força atômica e de espectrometria de volumes podem desempenhar no esclarecimento de tais interações. [**Int Microbiol** 2005; 8(3):157-168]

Palavras chave: substâncias poliméricas extracelulares · bioensuciamiento (*biofouling*) · biocorrosão · biofilm · espectrometria de massas · espectroscopia de força atômica