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Metabolic turnover and catalase activity of biofilms of *Pseudomonas fluorescens* (ATCC 17552) as related to copper corrosion

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ABSTRACT

In this work we report the results of a combined biochemical and electrochemical study aimed to analyze both the growth of biofilms of *Pseudomonas fluorescens* on copper samples and its possible role in the instability of the metal/electrolyte interface.

DNA and RNA were quantified along the time for biofilms grown on copper and glass to estimate both the growth of the bacterial population and its metabolic state (through the RNA/DNA ratio). The expression and specific activity of catalase were also determined to gain insight into their possible role in corrosion acceleration. The electrochemical behavior of the biofilm/copper interface was monitored by Linear Polarization Resistance (Rp) and electrochemical impedance spectroscopy (EIS) along the experiments.

Results showed a longer lag phase for biofilms developing on copper that included a period of high metabolic activity (as measured by the RNA/DNA ratio) without biomass growth. Biological activity introduced a new time constant at intermediate frequencies in EIS spectra whose capacitive behavior increased with the biofilm development. The increment in this biofilm-related signal was accompanied by a strong limitation to charge transfer through a diffusion controlled process probably due to oxygen exhaustion by cells respiration, while the resistance of the interface decreased presumably due to oxide dissolution by local acidification under the colonies. In addition, catalase activity was found to be high in mature copper-tolerant biofilms, which differentially express a catalase isoform not present in biofilms growing on glass.

1. Introduction

The growth of biofilms has been shown to influence the stability of metallic materials used in water distribution systems. In particular, "blue water" corrosion failures of domestic distribution pipes made of copper commonly involve copper-tolerant biofilms (Calle et al., 2007; Lehtola et al., 2004). Although several studies have highlighted the fundamental role of water quality in determining copper dissolution, the worst situation is known to arise when biofilms participate in the process, by imposing local conditions beneath biofilm clusters and interfering with the formation of passivating scales.

Bacterial development in biofilms follows a growth cycle different from that of planktonic cultures (Sauer et al., 2002) and includes structural (O'Toole et al., 2000), functional

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(Sternberg et al., 1999) and temporal heterogeneities (Stewart and Franklin, 2008) that can influence electrochemical corrosion reactions in more than one way. Structural heterogeneity includes clusters, channels and voids that influence liquid flow and mass transport processes at the interface. At the same time functional heterogeneity locally changes the consumption of nutrients or the production of by products (organic acids, reactive oxygen species, enzymes, others), thus leading to the establishment of internal chemical gradients. Indeed, different cells or groups of cells in a biofilm may have a variety of responses to adverse situations such as nutrients limitations or the presence of antibiotics, biocides or heavy metals (Costerton et al., 1995; Elkins et al., 1999; Harrison et al., 2005; Teitzel and Parsek, 2003), thus completing a complex scenario for the study of microbiological influence on corrosion. To gain information about metabolic changes in biofilms developing on a metal surface may help to improve the present understanding of the biological influence on corrosion processes.

In this work we report the results of a combined biochemical and electrochemical study aimed to analyze the growth of biofilms of *Pseudomonas fluorescens* on copper and glass. The general objective is to correlate the biological development and activity with electrochemical information about the copper/electrolyte interface. Of particular interest is the specific response of biofilms to reactive oxygen species (ROS) like hydrogen peroxide (Elkins et al., 1999; Teitzel and Parsek, 2003), in relation to the proposed role of bacterial catalase in corrosion acceleration (Busalmen et al., 1998b, 2002). Hydrogen peroxide is produced during the electrochemical reduction of oxygen in the cathodic counterpart of the corrosion process, so changes in catalase expression or activity might influence the kinetics of this reaction, thus enhancing corrosion.

2. Materials and methods

2.1. Biological material and culture conditions

All the assays were performed with *P. fluorescens* (ATCC 17552), grown in a culture broth composed of 0.1% meat peptone and 0.5% NaCl (pH 6.8).

Biofilms were grown in a cylindrical reactor with a central rotor that holds up to 30 coupons allocated perpendicularly to the rotor axis. It ensured equivalent culture conditions for all the samples, avoiding interferences due to the establishment of nutrient gradients as those found in tubular reactors. Planar samples (4 × 2 × 0.2 cm) of both copper and glass were polished with emery paper to grade 400, washed with distilled water and degreased by immersion in ethanol. The assembled flow system was sterilized in autoclave before use. Biofilm development was performed separately several times on each material as described by (Gilbert and Allison, 1993) with minor modifications. The liquid flow was controlled at 1.5 mL min^{-1} by a peristaltic pump. It ensured a liquid residence time into the reactor lower than the duplication time of the culture, favoring the growth in the biofilm mode. The reactor was inoculated with a bacterial culture grown in batch during 18 h. Copper ions were added to this culture for experiment of biofilm growth on copper (see below). The system was operated without re-circulation during 120 h. The rotation speed of the sample holder was 12 rpm. Samples were collected daily for biological (3 samples) and electrochemical (2 samples) analysis. Control experiments were run with copper samples exposed to sterile culture media following the same procedures.

Batch cultures were grown in 125 mL flask at 30 °C with constant shaking (150 rpm). Copper-adapted batch cultures were performed for comparison with biofilm culture on copper coupons, by adding a stock solution of $CuCl_2$ sterilized by filtration to reach a final concentration of 50 μM. Growth was monitored by measuring the absorbance at 600 nm. Samples were harvested at the exponential and stationary phases of growth by centrifugation at 8000 × g during 10 min, re-suspended in 2 mL of phosphate buffer 0.2 M, pH 7 and frozen for later analysis.

2.2. Biofilm analysis

Biofilms were scrapped from both sides of coupons and collected into 1.5 mL centrifuge tubes. Coupons were washed with 1.5 mL of phosphate buffer 0.2 M, pH7 and the liquid was pooled in the same tube to ensure the exhaustive recovery of the biological material. Biofilms were vortexed (3 pulses of 5 s) to disaggregate cells and samples were utilized for biochemical determinations.

2.2.1. Quantification of nucleic acids and proteins

Aliquots of 700 μL of biofilm were sonicated in 4 pulses of 30 s with a Vibracell VC130 cell processor (Sonics and Materials, USA). Then, 100 μL were used for protein quantification, 500 μL for nucleic acids determinations and the last 100 μL for catalase activity measurement.

Protein content was measured by the colorimetric method described by (Bradford, 1976), using bovine serum albumin for the construction of the standard curve.

For the extraction of nucleic acids, 500 μL of biofilm sample were treated with 500 μL of $HClO_4$ 0.4 N. Samples were centrifuged at 10 000 × g by 10 min at 4 °C and supernatants were discarded. Pellets were processed as described by (Fleck and Munro, 1962) for DNA and RNA hydrolysis and quantification.

2.2.2. Catalase activity determination

Enzyme activity was estimated by the method of (Beers and Sizer, 1952). In the original method the disappearance of H_2O_2 is followed by measuring the absorbance at 240 nm. As copper ions associated to cell debris interfere with this measurement, samples were clarified by centrifugation and the absorbance measurement was performed on the soluble fraction at 220 nm. Specific activity was reported as Units (U), being 1 U = μmol of H_2O_2 consumed by μg of protein in 1 min.

2.2.3. SDS-PAGE and immunodetection of catalase

For the analysis of catalase expression samples containing 25 μg of protein were analyzed by electrophoresis in 12% denaturing polyacrylamide gels containing SDS. Two gels were run in parallel for each experiment. One of them was stained with Coomasie Blue to reveal the total proteins profile, while the other was further analyzed by Western blot to