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Extracellular matrix assembly in extreme acidic eukaryotic biofilms and their possible implications in heavy metal adsorption

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ABSTRACT

To evaluate the importance of the extracellular matrix in relation to heavy metal binding capacity in extreme acidic environments, the extracellular polymeric substances (EPS) composition of 12 biofilms isolated from Río Tinto (SW, Spain) was analyzed. Each biofilm was composed mainly by one or two species of eukaryotes, although other microorganisms were present. EPS ranged from 130 to 439 mg g^{-1} biofilm dry weight, representing between 15% and the 40% of the total biofilm dry weight (DW). Statistically significant differences ($p < 0.05$) were found in the amount of total EPS extracted from biofilms dominated by the same organism at different sampling points. The amount of EPS varied among different biofilms collected from the same sampling location. Colloidal EPS ranged from 42 to 313 mg g^{-1} dry weight; 10% to 30% of the total biofilm dry weight. Capsular EPS ranged from 50 to 318 mg g^{-1} dry weight; 5% to 30% of the total biofilm dry weight. Seven of the 12 biofilms showed higher amounts of capsular than colloidal EPS ($p < 0.05$). Total amount of EPS decreased when total cell numbers and pH increased. There was a positive correlation between EPS concentration and heavy metal concentration in the water. Observations by low temperature scanning electron microscopy (LTSEM) revealed the mineral adsorption in the matrix of EPS and onto the cell walls. EPS in all biofilms were primarily composed of carbohydrates, heavy metals and humic acid, plus small quantities of proteins and DNA. After carbohydrates, heavy metals were the second main constituents of the extracellular matrix. Their total concentrations ranged from 3 to 32 mg g^{-1} biofilm dry weight, reaching up to 16% of the total composition. In general, the heavy metal composition of the EPS extracted from the biofilms closely resembled the metal composition of the water from which the biofilms were collected.

1. Introduction

With a length of ca. 100 km, Río Tinto (SW, Spain) provides one of the largest extreme acidic habitats for a broad range of organisms, including different species of bacteria, fungi, algae and protozoa (López-Archilla et al., 2001; Amaral et al., 2002; González-Toril et al., 2003; Gadanho et al., 2005; Aguilera et al., 2006a; Gadanho and Sampaio, 2006). These organisms can persist at the extremes of known physiological tolerance of organisms to low pH and high heavy metal concentrations, yet their survival mechanisms are not well understood.

Río Tinto flows through the Iberian Pyritic Belt, one of the richest metal sulfide ore deposits on Earth (Bourter, 1996), and the extreme conditions of its water are the product of the metabolic activity of chemolithotrophic microorganisms, mostly iron- and sulfur-oxidizing bacteria, that can be found in high concentrations in its waters. The iron-oxidizing metabolism is responsible for the solubilization of sulfidic minerals (mainly FeS_2) and the correspondent high concentration of ferric iron, sulfate and protons in the water column (Fernández-Remolar et al., 2003; González-Toril et al., 2003). The result is a strong acidic solution of ferric iron which dissolves other cationic metals into solution.

Besides the extreme physicochemical characteristics of water, what makes Río Tinto a unique extreme environment is that eukaryotic organisms are the principal contributors of biomass in the river, over 65% of the total biomass is due to the remarkable degree of eukaryotic diversity found in its waters (Amaral et al., 2002; Liu and Fang, 2002). However, despite extensive efforts devoted to studying the biodiversity and geochemistry of this system (López-Archilla et al., 2001; Amaral et al., 2002; Fernández-Remolar et al., 2003; González-Toril et al., 2003; Aguilera et al., 2006a), little is known regarding their ecophysiology.

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Most of the eukaryotic microbial communities found in the river are distributed in extensive biofilms along the riverbed. The macroscopic shape and species composition of the biofilms vary greatly throughout the river. Some of them adopt filamentous morphologies in flowing water while others form thick colourful patches firmly attached to the mineral substrates. Microbial biofilms at Río Tinto are three-dimensional structures that show a spectrum of structurally heterogeneous forms determined by the dominating organisms (Aguilera et al., 2007). In addition, the distribution of these communities seems to be more influenced by the presence of heavy metals than by the pH (Aguilera et al., 2006b). The analysis of similarities among different sampling sites showed three areas that indicated a gradient of pH and heavy metals along the river. Although the development of biofilms in aquatic and terrestrial extreme environments has been documented (Ferris et al., 1989; Hughes and Poole, 1989; García-Meza et al., 2005), the mechanisms of adaptation are not well understood. Previous studies have shown that the extracellular matrix could be partly responsible for the increased tolerance of biofilms, particularly to heavy metals (Flemming, 1993; Barranguet et al., 2000).

The biofilm matrix is a dynamic environment that organizes microbial cells (Wingender et al., 1999; Sutherland, 2001). Their major components, besides water, are extracellular polymeric substances (EPS). Microorganisms in natural environments produce EPS, which in turn determine the structural and functional integrity of the biofilms and are considered the key component responsible for their physicochemical and biological properties (Christensen and Characklis, 1990). The EPS consists of a complex mixture of proteins, carbohydrates, acid polysaccharides, lipids, DNA and humic acid substances, although the wide range of environments in which biofilms are found makes it extremely difficult to generalize about their structure and physiological activities (Jenkinson and Lappin-Scott, 2001). Polysaccharides are the most abundant component, generally representing 40–95% of the EPS (Flemming and Wingender, 2001).

The adsorption of heavy metals by EPS is attributed to their large number of negatively charged functional groups such as carboxyl, phosphate and sulfate at neutral pH (Bitton and Friehofer, 1978; Kaplan et al., 1987). However, the heavy metal complexing properties of the EPS may be altered with pH, since pH determines metal solubility and also the organic ligand adsorption capacities (Stone, 1997). In general, at acidic pH most heavy metals are in free cationic form, and more available to microorganisms, whereas at higher pH they tend to precipitate as insoluble compounds (Förstener and Prosi, 1979; Rai et al., 1981). In the same regard, low pH modifies the ionic status of the different EPS functional groups, changing their electrochemical properties (Ferris et al., 1989). Thus, at low pH the availability of negatively charged sites such as carboxylates and phosphates is greatly reduced so fewer metal cations are absorbed. The reduction of surface charge density with decreasing pH levels also serves to weaken electrostatic free energy contributions to metallic ion adsorption (Jefree and Read, 1991).

We reported here a qualitative description of the extracellular matrix assembly and the evaluation of the importance of EPS of several eukaryotic biofilms collected from an extreme acidic environment in relation to their heavy metal binding capacity. For this purpose the biofilms were selected according to their dominant species composition and from locations along the river characterized by different physicochemical characteristics. Results may lead to a better understanding of the role of the extracellular matrix assembly and the importance of EPS in acidic environments in relation to their heavy metal adsorption capacities.

2. Materials and methods

2.1. Field sites and biofilm sampling

Six sites along Río Tinto were selected for *in situ* measurements, water sampling and biofilm collection (Fig. 1). The sampling sites were selected taking into account previous studies carried out in Río Tinto regarding their eukaryotic biodiversity and water physicochemical characteristics (Aguilera et al., 2006a,b, 2007). Samples were taken for all sites in May 2006 during the day (between 9.00 am and 11.00 am).

In situ measurements of water conductivity, temperature, redox potential and pH, were carried out as described previously (Fernández-Remolar et al., 2003). Water samples were filtered through 0.45 μm Millipore membranes. The total concentrations of nine recoverable metals were measured for each water sample (Zn, Cu, Fe, Co, Ni, As, Cd, Cr and Pb) using X-ray fluorescence reflection (TXRF) and inductively coupled plasma-mass spectrometry (ICP-MS).

Twelve eukaryotic biofilms were taken from the riverbed surface (maximum water depth <5 cm) using a sterile plastic spatula and then placed in 1.5 mL cryotubes. The samples were immediately frozen in dry ice and kept in the laboratory at −20 °C until the experiments were carried out. Besides the samples taken for the EPS analysis, a subsample was also taken for the microscopy identification of the eukaryotic species. Identification of algae and heterotrophic protists was carried out by direct microscopic observation down to the lowest possible taxonomic level using different phenotypic features based on previous studies of the eukaryotic

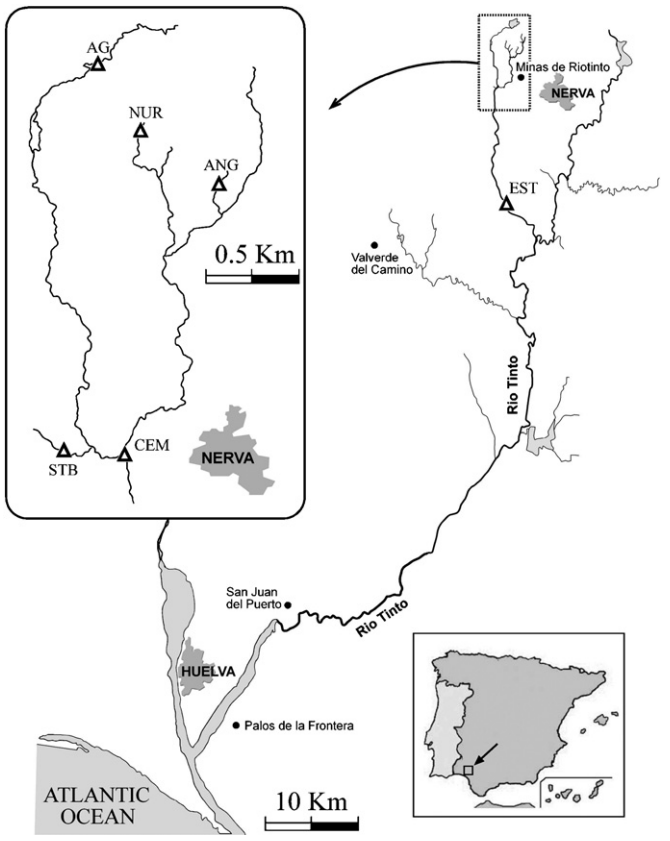

Fig. 1. Schematic map of the Río Tinto from the source near the town of Nerva to the ocean near the town of Huelva. The relative location of each sampling site is shown. Inset in lower right shows general location of the river in Spain, and at upper left is a detailed map of the headwaters.