Spain’s Rio Tinto, or Red River, an example of an extremely acidic (pH 1.7–2.5) environment with a high metal content, teems with prokaryotic and eukaryotic microbial life. Our recent studies based on small-subunit rRNA genes reveal an unexpectedly high eukaryotic phylogenetic diversity in the river when compared to the relatively low prokaryotic diversity. Protists can therefore thrive in and dominate extremely acidic, heavy-metal-laden environments. Further, because we have discovered protistan acidophiles closely related to neutrophiles, we can hypothesize that the transition from neutral to acidic environments occurs rapidly over geological time scales. How have these organisms adapted to such environments? We are currently exploring the alterations in physiological mechanisms that might allow for growth of eukaryotic microbes at acid extremes. To this end, we are isolating phylogenetically diverse protists in order to characterize and compare ion-transporting ATPases from cultured acidophiles with those from neutrophilic counterparts. We predict that special properties of these ion transporters allow protists to survive in the Rio Tinto.

Earth harbors many extreme environments. Previous investigations of the microbial diversity in these environments have been constrained by preconceived notions about the range of habitability for both eukaryotic and prokaryotic microorganisms. We have been exploring the genetic and physiological diversity of organisms living at pH extremes, both acidic (pH < 3) and alkaline (pH > 10). Our research ranges from environments like the warm (45 °C), acidic (pH 2.7) Nymph Creek in Yellowstone National Park to temperate alkaline lakes in the Sandhills region of western Nebraska. The focus of this report is the acidic, heavy-metal-rich Rio Tinto in southwestern Spain.

The Rio Tinto flows 100 km through the world’s largest pyritic (FeS₂) belt. The river gets its red color from the high levels of iron dissolved in its acidic waters (pH ~2.0). Ferric hydroxide (Fe³⁺/Fe(OH)₃, pKa 2.5) and SO₄²⁻/HSO₄⁻ (pKa 2.0) act as buffers to maintain the pH of the river at about 2. The concentration of iron can be as high as 20 g/l, and the river also contains other heavy metals at concentrations orders of magnitude higher than those in typical freshwater environments.

Much of the past research on the Rio Tinto has focused on the prokaryotes that play an important role in shaping the acidic environment of the Rio Tinto through their metabolism of iron-rich pyrite and chalcopyrite. Recent paleontological research shows that iron-oxidizing bacteria existed in the Rio Tinto river basin 300,000 years ago, long before its 5000-year mining history (Leblanc et al., 2000). Other chemolithotrophs such as sulfur-oxidizing bacteria and archaea also contribute to the river’s probably ancient ecosystem structure (González-Toril et al., 2001).

Some of these prokaryotes, along with fungi, contribute to the formation of biofilms on the surface of rocks. These biofilms, in turn, are the site of metal and mineral precipitation that ultimately forms stromatolites. Biofilms provide a substrate for communities to develop within the river. However, in many parts of this river basin, eukaryotic
microbes (Fig. 1) are the major contributors of biomass (López-Archilla et al., 2001). Eukaryotes not only form the foundations of some of these biofilm communities, but they are also conspicuous inhabitants of them (López-Archilla et al., 1993, 1994, 1995; López-Archilla and Amils, 1999). This makes the Rio Tinto system unique among acidic environments described to date.

Figure 2 shows a phylogenetic tree depicting the breadth of eukaryotic diversity in the river. In our rRNA gene diversity survey of biofilm samples, we obtained sequences representing most of the major eukaryotic lineages, including the fungi, animals, green algae, land plants, stramenopiles, and alveolates (Amaral Zettler, 2002). Many of the sequences from the Rio Tinto represent photosynthetic lineages that were previously identified employing light microscopy (López-Archilla et al., 2001). These included members of the Euglenozoa (euglenids), Chlorophyta (chlamydomonad and chlorella-like relatives), Viridiplantae (Zygnema-relatives), and Bacillariophyta (diatoms). However, our molecular data also unveiled a significant nonphotosynthetic component to the Rio Tinto. Phagotrophic lineages such as the ciliates, cercomonads, and vahlkampfidi amoebae, as well as heterotrophic fungi and possibly myxotrophic lineages—as known to occur in members of the stramenopiles such as Ochromonas (Porter et al., 1985)—also populate the Rio Tinto.

We discovered a diversity of fungi that escaped detection using traditional identification methods. None of our fungal clones showed high similarity to those species already described from the Rio Tinto. Fungi undoubtedly play an important role in community structure in the Tinto since most of them are metal resistant and can sequester specific metals (Durán et al., 1999). Such sequestration of potentially toxic metals could allow less tolerant species to exist where they might not have otherwise, possibly enhancing biodiversity in these areas. Similar studies on metal sequestration have not been conducted on protists living in the Tinto. We hope to apply new tools in genome science to address these questions surrounding the rich microbial diversity of the Rio Tinto.

Our studies also revealed a diversity that we were not able to readily characterize using molecular techniques. Clones such as RT5iin16 and RT5iin14 are most likely examples of novel eukaryotic lineages that at best branch at the base of the animal-fungal-nucleariid radiation. Other clones (RT5iin21 and RT5iin44) branched with the recently sequenced filose amoeba Filamoeba nolandii, whose own taxonomic placement is equivocal. Because our study was

Figure 1. Some representative eukaryotes found in the Rio Tinto. (A) *Euglena cf. mutabilis*; scale bar = 10 μm. Photomicrograph by Linda Amaral Zettler and David Patterson. (B) A heliozoan, most likely belonging to the genus *Actinophrys*; scale bar = 100 μm. Photomicrograph by Linda Amaral Zettler and Erik Zettler.

Figure 2. A minimum evolution phylogeny for small subunit rRNA genes using a likelihood model. Bold letters indicate environmental clones. “RT” indicates the sequence is from the Rio Tinto, and “cul” indicates cultured species. Underlined taxa represent genera that have been identified in the river based on microscopic observation. Sampling sites were as follows: RT1, La Palma; RT3, Berrocal Upper; RT5i, the Origin, black filamentous biofilm; RT5ii, the Origin, green filamentous biofilm; RT7i, Anabel’s Garden green biofilm; RT7ii, Anabel’s Garden yellow biofilm. Bootstrap support values are shown, and the scale bar represents the number of substitutions per site. GenBank accession numbers AY082969-AY083001.
not exhaustive, we surmise that there are still more undiscovered novel lineages in the river.

Despite our growing knowledge of the Tinto’s eukaryotic diversity, we know little about the role eukaryotes play in shaping the varied ecosystems that occur along the river. For example, we do not know if these biofilm communities have microenvironments that enhance survival of their members. Could fungal metal sequestration protect nontolerant species? Furthermore, we know little about how these organisms have evolved adaptations to extreme concentrations of acid and metals.

To explore these questions, we have been isolating organisms from the river for ex situ physiological experiments. We have established monocultures of *Chlamydomonas* sp., *Euglena cf. mutabilis*, *Chlorella* sp., and *Vannella* sp. isolated from enrichments of river water and are currently exploring the physiology of these protists from extreme environments.

We have initiated our physiological studies on an acidophilic species of a chlamydomonad alga isolated from the river—*Chlamydomonas* sp. Our first question about the physiology of the Tinto acidophiles was the nature of the cytosolic pH (pHc). There are published reports of acidophiles from all domains of life with internal pH values that deviate from neutral—these include the archaebacterium *Picrophilus oshimae*, pHc = 4.6 (van de Vossenberg et al., 1998); the eubacterium *Bacillus acidocaldarius*, pHc = 5.6–5.8 (Thomas et al., 1976); and the eukaryotic alga *Euglena mutabilis*, pHc = 5.0–6.4 (Lane and Burris, 1981). Using the fluorescent H+ indicator BCECF, we determined that our acidophilic chlamydomonad isolate maintains an average internal pH of 6.6 at an external pH of 2 (M. A. Messerli, L. A. Amaral Zettler, S.-K. Jung, P. J. S. Smith, and M. L. Sogin, unpubl.). Our other isolates await similar measurements.

Given that there is a 40,000-fold difference in hydrogen ion activity between the inside and the outside of these cells, we propose the existence of active transport mechanisms that help these organisms regulate their internal pH. We hypothesize that novel diversity in H+-ATPases may explain the ability of different protist species to thrive in the low pH, high-metal Rio Tinto environment. There are two major families of H+-ATPases: the V/F/A-ATPases and the P-type-ATPases. The V-type ATPases can occur in the plasma membrane of eukaryotes (but are more commonly associated with vacuolar membranes) and consist of at least 11 subunits and a molecular mass approaching 10^6 Da. In contrast, eukaryotic P-type ATPases consist of either monosubunits (as with H+-ATPases) or a hetero-subunit (alpha and beta, as found in the Na+/K+-ATPases and H+/K+-ATPases); have a molecular weight of about 100 kDa; and form a phosphorylated intermediate during the course of ATP hydrolysis. Indirect evidence of novel ATPases comes from studies of the protozoan parasite *Leishmania dona-

**Literature Cited**

Amaral Zettler, L. A., F. Gómez, E. R. Zettler, B. G. Keenan, R. Amils, and M. L. Sogin. 2002. Eukaryotic diversity in Spain’s River of Fire. *Nature* 417: 137.

Durán, C., I. Marín, and R. Amils. 1999. Specific metal sequestering acidophilic fungi. Pp. 521–530 in *Biohydrometallurgy and the Environment Towards the Mining of the 21st Century*, Proceedings of the International Biohydrometallurgy Symposium, IBS ’99, R. Amils and A. Ballester, eds. Elsevier, Amsterdam.
López-Archilla, A. I., I. Marín, and R. Amils. 1995. Microbial ecology of an acidic river: biotechnological applications. Pp. 63–73 in Biohydrometallurgical Processing, C. A. Jerez, T. Vargas, H. Toledo, and J. W. Wiertz, eds. University of Chile, Santiago.

López-Archilla, A. I., I. Marín, and R. Amils. 2001. Microbial community composition and ecology of an acidic aquatic environment: the Tinto River, Spain. *Microb. Ecol.* 41: 20–35.

Meade, J. C., K. M. Hudson, S. L. Stringer, and J. R. Stringer. 1989. A tandem pair of *Leishmania donovani* cation transporting ATPase genes encode isoforms that are differentially expressed. *Mol. Biochem. Parasitol.* 33: 81–91.

Porter, K. G., E. B. Sherr, B. F. Sherr, M. Pace, and R. W. Sanders. 1985. Protozoa in planktonic food webs. *J. Protozool.* 32: 409–415.

Thomas, J. A., R. E. Cole, and T. A. Langworthy. 1976. Intracellular pH measurements with a spectroscopic probe generated in situ. *Fed. Proc.* 35: 1455.

van de Vossenberg, J. L., A. J. M. Drissen, W. Zillig, and W. N. Konings. 1998. Bioenergetics and cytoplasmic membrane stability of the extremely acidophilic, thermophilic Archaeon *Picrophilus oshimae*. *Extremophiles* 2: 67–74.