EXTENSIVE SEQUENCING APPROACHES TO THE SEARCH FOR LIFE: INITIAL RESULTS FROM THE RIO TINTO. S. S. Johnson[1], C. E. Carr[2], R. Amils[3], M. Zuber[2], and G. Ruvkun[1] [1]Harvard University, Department of Molecular Biology, Simches Research Building, 185 Cambridge Street, Boston, MA 02114, sjohnson@fas.harvard.edu, [2]Massachusetts Institute for Technology, Department of Earth, Atmospheric and Planetary Sciences, 77 Massachusetts Avenue, Cambridge, MA 02139, [3]Centro de Astrobiología, Instituto Nacional de Técnica Aeroespacial, Ctra de Torrejón a Ajalvir, km 4, 28850 Torrejón de Ardoz, Madrid, Spain.

Introduction: While instruments that probe for organic matter and isotopic signatures are critical components of any life detection strategy, a far greater scientific yield may come from the application of new, precision technologies to the search for life, particularly on Mars. One exciting approach is a NASA instrument prototype being developed by the Search for Extraterrestrial Genomes (SETG) Project [1]. SETG, which is designed to search for DNA and RNA molecules, incorporates microfluidic technology and unprecedented sensitivity to detect life in low biomass samples.

If simple organisms adapted to environmental change on Mars and are still present at low levels today, the SETG approach could greatly increase the chances of finding and identifying Martian life. SETG utilizes microfluidic "PCR in a chip" technology that enables hundreds of genetic fragments to be amplified in tiny wells, and subsequent pyrosequencing virtually eliminates false positive results: sequence data from likely contaminants can be immediately identified, whereas any system of life isolated from that on Earth over geologic time will be evident from phylogenetic analysis. Moreover, the applicability of these techniques, which are useful only for RNA/DNA-based life forms, is consistent with an increasingly tenable "shared-ancestry" hypothesis.

Central to SETG is the hypothesis that life on Earth and life on Mars share a common ancestor. This hypothesis is not implausible; indeed, the probability of a common ancestor seems at least as high, if not radically higher, than the alternative of two independent geneses. In the late 1990's, a series of theoretical studies demonstrated that Martian meteorites were transferred to the Earth at shortened time scales and with higher fluxes than previously presumed [2, 3, 4, 5]. In fact, the final destination of 7.5% of all Martian meteorites is believed to be the Earth, delivering over one billion tons of meteoric debris [2, 3]. Within this collection, numerous meteorites would have been delivered with interplanetary transit times of single to thousands of years. Several SNC meteorites of Martian origin have been discovered here on Earth, and magnetic and thermochronological analyses indicate that up to 20 wt% of Martian meteorites have only experienced mild heating (<100ºC, below sterilization temperatures) during ejection and impact [6, 7, 8, 9]. Once life evolved on one of the planets, the rate of material transfer enhances the likelihood that an adjacent planet could "catch" life rather than independently evolving it. Moreover, microbial life has been discovered here on Earth at extremes of temperature and radiation, demonstrating the significant adaptability of microbes and calling in question whether subsequent environmental stress, even if very extreme, could fully sterilize a planet.

Here we consider this new life detection approach vis-à-vis a "training set" of phylotypes detected in the Mars-like chemistry of the Rio Tinto Basin in southwestern Spain, a terrestrial analog for the early Martian environment (see Fig. 1). Much as we might expect for early Mars, the microbial population at Rio Tinto harvests energy from chemical gradients created by the elements iron and sulfur. Here we perform extensive sequencing, collecting more sequence data from prokaryotic organisms than have been collected from any single sampling site at Rio Tinto before, and we discuss the implications of these results for the SETG instrument and its potential use as part of a life detection platform on a future Mars mission.

Sample Collection: Our work focuses on the Northern stretch of the river, where the most acidic conditions exist. Samples were collected in 50 ml Falcon tubes at the confluence of multiple waterways (the Salinas site, coordinates: 37°40'5" N, 6°32'54" W). Sampling conditions and DNA extraction were as described in [10]. Primers 515F, 5'-GTGCCAGCMGCCGCGGTAA -3', and 1391R, 5'-GACGGGCGGTGWGTRCA -3', were chosen to recover maximum prokaryotic diversity [11]. PCR was performed in 20µl reactions. Additives included: 10.75µl H_2O, 2µl Taq buffer (to 1.5mM $MgCl_2$), 2µl DNTPs (to 0.2mM), 2µl forward primer (to 1µM) (Integrated DNA Technologies), 2µl reverse primer (to 1µM) (Integrated DNA Technologies), 0.25µl Taq polymerase (1 U) (Sigma Aldrich).

Thermal cycling was completed under PCR conditions: 2 min initial denaturing at 95°, followed by 30 cycles of 5 s denaturing at 94°, 40 s annealing at 56°, and 1 min extension at 72°, followed by 10 min final extension at 72°. Products from four different PCR amplifications were pooled and then gel purified using the QIAquick Gel Extraction Kit (Qiagen). The resulting 900bp PCR products were TOPO cloned (Invitro-

gen) for ABI sequencing using the Broad Institute's automated sequencing platform.

Fig. 1. The Rio Tinto is located in the Iberian Pyrite Belt, a 250km long geologic structure emplaced by hydrothermal activity in the Late Paleozoic [12].

Results: In sum, 480 clones were sequenced forward and backward, for a total of 960 sequences. Phylotypes include several species of *Acidobacteria*, *Alphaproteobacteria*, *Firmicutes*, and *Gammaproteobacteria*. Several types of microbes detected in the community survey only appeared once or twice within the group of 480 sequences, suggesting they may have been missed if fewer sequences had been returned. Among these were *Legionellales*, *Firmicutes Ruminococcus* and *Alicyclobacillus acidocaldarius*, *Bradyrhizobiales* (an *Alphaproteobacterium*, which can be found in the roots of endemic plants in the Rio Tinto), as well as the *Bacteriodetes Candidatus cardinium*, and *Acidocella* (an *Alphaproteobacterium*). Unclassified *Moraxellaceae* (a family of *Gammaproteobacteria*), were also detected at low levels. Although they have been found deep underground in drilling samples from the Rio Tinto MARTE Project, they have never been detected in any other acid mine site. The detection of *Arcobacteracae* (an *Epsilonproteobacterium*) is also intriguing, as it has never before been identified in a Rio Tinto sampling site.

Others groups, however, were not detected at all. For instance, sulfate-reducing bacteria such as *Desulfosporosinus* are thought to play a role in cycling SO_4^{-2} to S^0/S^{2-} in the Rio Tinto system. Indeed, when three probes specific for sulfate-reducing bacteria were used on Salinas samples from October 1999 and May 2000, positive hybridization signals were detected [13]. Nevertheless, sulfate-reducing bacteria were not seen within our group of sequences, suggesting that more comprehensive look at the ecosystem may be necessary.

Implications: Our analysis of genetic diversity in the Rio Tinto suggests that SETG's life detection strategy should capitalize on the benefits of deep sequencing, incorporate multiple primer pairs and/or random hexamers as allowed by a chip interface, and sample over a range of in situ micro-niches.

Future Work: If present on Mars, the 16S RNA gene may have diverged so much that, even if life on Mars was DNA or RNA-based and ancestrally related to life on Earth, it would not be detectable using rRNA primers. Fortunately, SETG is also being developed for isothermal DNA amplification, utilizing the phage φ29 DNA polymerase and very short random primers (hexamers of all 4^6 possible combinations). Our collaborators have already demonstrated microfluidic isothermal amplification [14, 15], and we plan to show that this approach can be used to amplify and detect nucleic acids in environmental samples with Mars-like chemistry, including the Rio Tinto. SETG will also be able to survey for remnants of the RNA World, as it is plausible that life on Mars diverged from life on Earth before DNA had evolved. To explore such a scenario, the SETG team is developing RT-PCR-based protocols to probe *in situ* for RNA-based life.

Although SETG is being designed in an attempt to detect life on Mars, the remotely sensed, telemetered technology it embraces may eventually be utilized to study biology's adaptability to hostile conditions - as well as the limits of life - in extreme environments here on Earth.

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