INL/CON-08-13733
PREPRINT

Monitoring Acidophilic Microbes with Real-Time Polymerase Chain Reaction (PCR) Assays

Hydrometallurgy 2008

F. F. Roberto

August 2008

The INL is a
U.S. Department of Energy
National Laboratory
operated by
Battelle Energy Alliance

MONITORING ACIDOPHILIC MICROBES WITH REAL-TIME POLYMERASE CHAIN REACTION (PCR) ASSAYS

F. F. Roberto, Idaho National Laboratory, Idaho Falls, ID

ABSTRACT

Many techniques that are used to characterize and monitor microbial populations associated with sulfide mineral bioleaching require the cultivation of the organisms on solid or liquid media. Chemolithotrophic species, such as *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, or thermophilic chemolithotrophs, such *as Acidianus brierleyi* and *Sulfolobus solfataricus* can grow quite slowly, requiring weeks to complete efforts to identify and quantify these microbes associated with bioleach samples. Real-time PCR (polymerase chain reaction) assays in which DNA targets are amplified in the presence of fluorescent oligonucleotide primers, allowing the monitoring and quantification of the amplification reactions as they progress, provide a means of rapidly detecting the presence of microbial species of interest, and their relative abundance in a sample. This presentation will describe the design and use of such assays to monitor acidophilic microbes in the environment and in bioleaching operations. These assays provide results within 2-3 hours, and can detect less than 100 individual microbial cells.

INTRODUCTION

Acidophilic and thermoacidophilic microbes associated with natural or engineered dissolution of sulfide minerals, including chemolithotrophic species, such as *Acidithiobacillus ferrooxidans, Leptospirillum ferrooxidans*, and archaea, such as *Acidianus brierleyi, Metallosphaera sedula*, and *Sulfolobus spp.*, are often slow-growing, fastidious organisms. Visual counting of microbial cells in leach solutions can be difficult in the presence of other particulate material, and cells bound to solid surfaces. These organisms may also be reluctant to grow (in some cases impossible to grow) on solid cultivation media, although Johnson (1995) has reported an efficient technique for enumerating acidophilic microbes on selective solid media that is employed in several labs around the world. One of the most common techniques in wide use to quantify cell numbers is the most-probable number (MPN) technique, in which a series of serial dilutions is made in selective liquid media. Cell numbers in the original sample are estimated from the highest (most dilute) dilution in which growth is observed. This approach is also used to recover pure cultures from the environment, and is appropriate for bench studies using monocultures. Unfortunately, given the range of microorganisms that may grow in acidic culture media, such as 9K (Tuovinen and Kelly 1973), it can be difficult to ascribe cell numbers to one species or member of the acidophilic bioleaching community using this approach for field studies.

Thomas Brock, in a seminal paper describing the status of microbial ecology over 20 years ago (Brock 1987) commented “The viable counting procedure, as far as microbial ecology is concerned, was discredited 20 years ago.” In that paper, he discussed the potentially powerful application of molecular biology techniques to the study of microbial environments.

Over the intervening years, molecular techniques have been employed to interrogate natural environments with great success, including a variety of interesting studies focused on mining-related environments. Many of these techniques have relied on the use of phylogenetically informative DNA sequence information, particularly that contained within the 16S (or small subunit; SSU) ribosomal RNA (rRNA) gene. This molecule (or its larger homolog in eukaryotic cells, the 18S rRNA gene) is conserved in all extant life due to its essential role as a structural backbone of the small ribosomal subunit. Only limited changes resulting from mutation have been tolerated over the estimated 3.5 billion years of life on Earth, resulting in what has been termed a “molecular chronometer” (Zuckerkandl and Pauling 1965). The high degree of DNA sequence conservation in this molecule has supported a universally accepted framework in which to taxonomically position microorganisms into what are termed phylogenetic trees. An example of a phylogenetic tree for microbes of interest to the biomining community is shown in Figure 1.

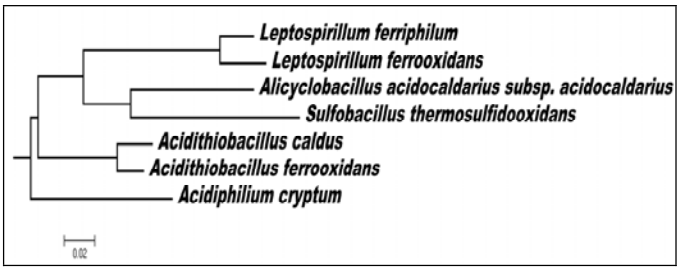

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences for mesophilic bacteria associated with biomining environments. Scale bar represents 0.02 nucleotide substitutions per position.

From rRNA gene sequences, it is possible to design a variety of molecular probes that can be used in techniques such as fluorescence *in situ* hybridization (FISH). The use of FISH probes has been informative in providing a better understanding of in situ distribution of microorganisms, along with estimates of their numbers, without the need of cultivating them from the sample (Amann et al. 1995). Banfield’s group (Schrenk et al. 1998) used this approach to describe the geographic distribution of *Ac. ferrooxidans* and *L. ferrooxidans* in acidic mine drainage at Iron Mountain, CA, and also the seasonal variation in microbial populations (Edwards et al. 1999). This same approach has provided a view of microbial populations associated with a constructed wetlands handling acidic coalmine drainage (Nicomrat et al. 2006). However, FISH can be a challenging technique to apply to environmental samples, due to potentially inhibitory or interfering materials in the samples, and the time and care necessary to achieve good results with a variety of probes and hybridization conditions. The same challenges facing visual enumeration of microbes mentioned before also apply to FISH, although confocal laser microscopy provides a means of optically sectioning through a particulate-containing sample.

The polymerase chain reaction (PCR) has shown promise in detecting the presence of bioleaching microbes. Chilean researchers (Pizarro et al. 1996) used the 16S-23S rRNA intergenic spacer region to determine the distribution of the mesophilic chemolithotrophs, *Acidithiobacillus ferrooxidans, Ac. thiooxidans*, and *Leptospirillum ferrooxidans* in bioleached copper ore samples. A nested PCR approach was used to detect *Acidiphilium cryptum, L. ferrooxidans,*

Sulfobacillus thermosulfidooxidans, Ac. caldus, Ac. ferrooxidans, and *Ac. thiooxidans* in chalcopyrite ore leached in columns (De Wulf-Durand et al. 1997). Goebel and Stackebrandt (1994) first isolated and then sequenced the 16S rRNA genes subsequent to PCR to identify acidophilic microorganisms in natural (acidic runoff from chalcocite overburden) and commercial (1-4 liter) continuous bioleaching environments. Another variant of this approach has characterized isolates recovered using efficient plating techniques with restriction endonuclease digestion of amplified rRNA gene products (amplified ribosomal DNA restriction enzyme analysis; ARDREA) to develop a fingerprint-like method for characterizing known acidophilic iron-oxidizing bacteria (Johnson et al. 2005). Rawlings (2002) noted that PCR had become a significant breakthrough in studying the microbial ecology of acidic environments, but noted that lack of quantitative capability was a limitation of the technique.

Real-time, or quantitative PCR methods have become available in the past few years through the incorporation of fluorescent oligonucleotide primers, allowing monitoring of reaction progress as measured by increased fluorescence released or incorporated into new amplicons (amplification products). Kock and Shippers (2006) were able to estimate total bacterial and archaeal populations associated with acid mine drainage in Sweden and Botswana. Using species-specific primer sets, it was possible to observe population shifts in mesophilic, moderately thermophilic, and extremely thermophilic iron and sulfur-oxidizing bacteria and archaea in bench-scale studies of chalcopyrite bioleaching at 4 increasing temperatures, ranging from 37 to 82°C (Liu et al. 2006). A schematic of the real-time PCR process (TaqMan® variation), depicting how fluorescence is initially quenched by the presence of a fluorescent quencher on the probe during the PCR process, and then observed after the probe is hydrolyzed through the 5'-exonuclease activity of the thermostable DNA polymerase, is shown in Figure 2. Because of the exponential amplification of target sequences through the base PCR reaction (repeated over 25-40 cycles in a typical experiment), single molecule detection is theoretically (and often) possible.

Thus, the use of real-time PCR allows for the detection of a specific organism or group of organisms, as well as provides information about how many of these organisms are present in the sample. It could therefore be employed as a rapid method to monitor shifts in microbial populations during heap leach processes. Understanding how populations shift in terms of numbers present and relative composition with changes in factors such as time, depth, temperature, etc. can provide key information for understanding which microbial populations facilitate increased metal recovery.

This short report describes some of our efforts to develop a panel of real-time PCR probes and primers for use in studying natural and engineered acidic environments of interest to the mining community. Utilizing this approach with acidophilic bacteria and archaea known to be associated with sulfide mineral leaching will lead to a better understanding of population dynamics in extremely acidic, metal-rich environments, and provide new insights into improving the efficiency of industrial-scale bioleaching.

MATERIALS AND METHODS

We have focused initially on development of real-time PCR assays to detect and quantify *Acidithiobacillus ferrooxidans* (and *Ac. thiooxidans*, since these two organisms are virtually indistinguishable when comparing their 16S rRNA genes), *Leptospirillum ferrooxidans*, and the crenarchaeal thermoacidophiles *Acidianus brierleyi*, *Metallosphaera sedula*, *Sulfolobus metallicus*, *S. acidocaldarius*, and *S. solfataricus*. PCR primers were designed to amplify diagnostic regions of the 16S rRNA gene, and hydrolysis (or more commonly, TaqMan®) probes targeting the amplicon have been used to monitor the amplification of the target in real-time.

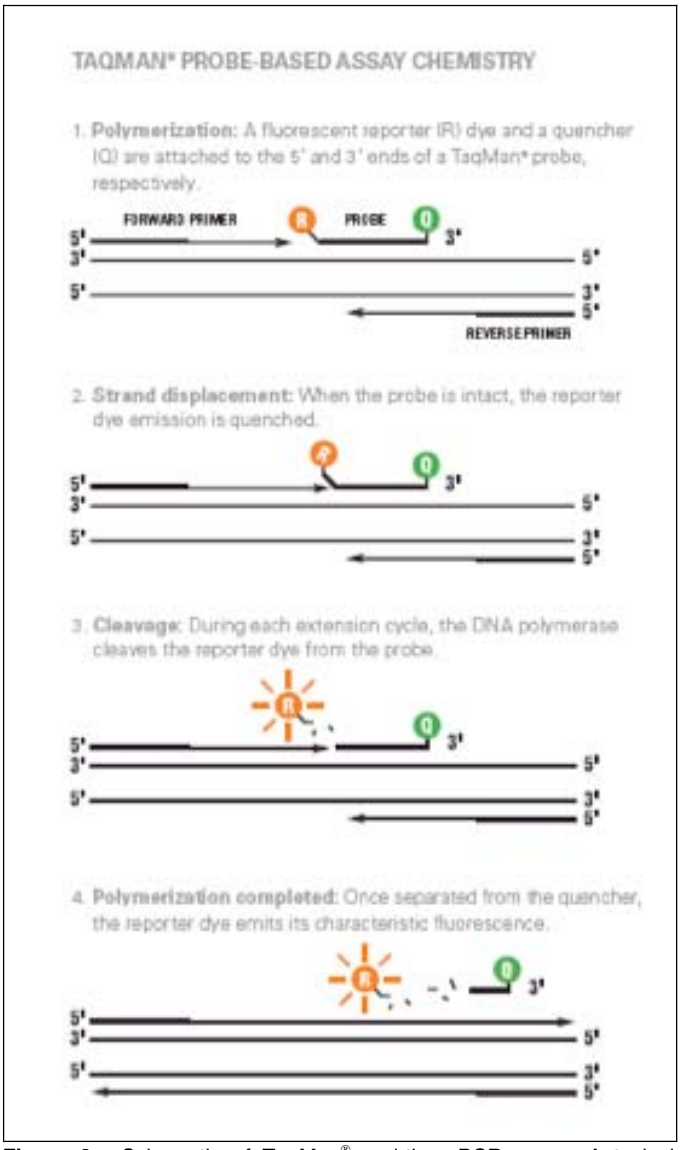

Figure 2. Schematic of TaqMan® real-time PCR assay. A typical assay repeats this process 25-40 times resulting in exponential amplification of the target sequence. (Graphic courtesy M. Luce, Applied Biosystems.)

16S rRNA gene sequences were aligned for probe and primer design using the open-source sequence alignment editor, BioEdit (version 7.0.9, T. Hall, Isis Biosciences, Carlsbad, CA) and the accessory program, ClustalW (Thompson, et al. 1994). The alignment of these sequences is depicted below (Figure 3). Examples of potential forward and reverse primer regions are surrounded by boxes in the figure, and demonstrate that even within conserved regions, differences between kingdoms (Archaea and Bacteria) and phyla (Proteobacteria and Firmicutes) can be observed and used to generate specific primers. The potentially high degree of sequence divergence in unconserved regions of the 16S rRNA gene can also be seen in the figure between the two boxes.

After selection of potential amplicons (the sequence region flanked by the primers), primers and probes were designed using Primer3 online probe design software (http://frodo.wi.mit.edu/primer3/input.htm). Theoretical specificity of selected primers and probes was determined by comparison to the GenBank database using the Basic Local Alignment Search Tool (BLAST). Assays were performed on an Applied Biosystems Prism 7000 Sequence Detection System (Foster City, CA) using 2X TaqMan®

Universal PCR Master Mix (Applied Biosystems, Foster City, CA) in a final volume of 25 μl.

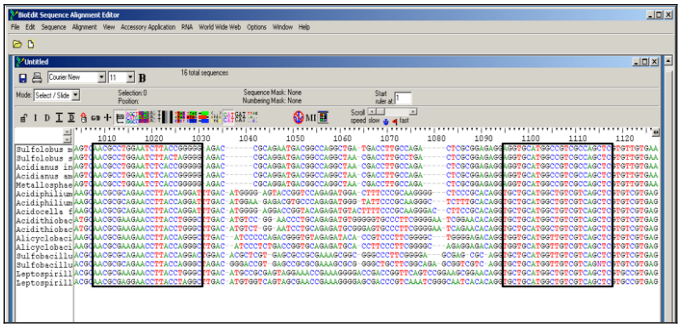

Figure 3. Multiple sequence alignment of 16S rRNA gene sequences from representative acidophilic microorganisms. Boxed regions surround conserved sequence regions that are potential forward and reverse primers for PCR assays.

RESULTS AND CONCLUSIONS

For the purposes of this report, the results of the crenarchaeal real-time PCR assay will be described in detail. The primers and probe were designed to detect Acidianus, Metallosphaera, and Sulfolobus species without further discrimination. Test organisms included *Acidianus brierleyi, A. ambivalens, Sulfolobus acidocaldarius, S. ohwakuensis, S. shibatae, S. solfataricus*, and *Metallosphaera sedula*. The assay was linear over a range of genomic DNA from 50 pg to 5 ng, roughly on the order of 100 cells to 100,000 cells. An example of this dilution series and the corresponding regression analysis producing a standard curve is shown in Figure 4.

Water and sediment samples from hot springs in Yellowstone National Park were also tested directly with this assay to determine if environmental samples could be utilized to detect the microorganisms without performing a DNA isolation step prior to the PCR.

The majority of environmental samples produced a fluorescent signal using the crenarchaeal real-time hybridization probe assay (data not shown). This suggests that field quantification may be achievable with the appropriate positive controls.

The real-time assay provides a means of sensitively detecting thermoacidophilic archaea in DNA extracts and also directly from enrichments without DNA extraction. It may be possible to use the real-time assay to test for the presence of these crenarchaea directly in environmental samples, such as heap leach solutions, in the field. At this time we have not attempted to discriminate between *Acidianus*, *Metallosphaera*, and *Sulfolobus,* but it should be possible to devise assays that do, either independently, or in so-called multiplex assays (where multiple primer sets and probes, labeled with different fluors, are used in the same assay tube). Although we have not described details here, we have similar assays for *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, designed and tested using similar approaches. Through the use of these assays, it should be possible to quantify the relative proportions of different archaea and bacteria present in heap leaches and/or stirred tank reactors.

In addition, it is important to note that the strategy described in this paper has relied on the use of the phylogenetically informative 16S rRNA gene, which allows a common sequence to be compared and used for identification of microorganisms. However, with the advent of high-throughput genomic sequencing, the complete genetic blueprints for microbes important to sulfide mineral dissolution are emerging as sources of gene sequences for use in real-time PCR or other molecular techniques (such as microarrays; Appia-Ayme et al. 2006, Quatrini et al. 2006). Functional genes important to bioleaching (e.g. the rusticyanin gene of *Ac. ferrooxidans*) could be used as targets for real-time PCR assays to directly assess the expression and activity of selected microbes in such operations.

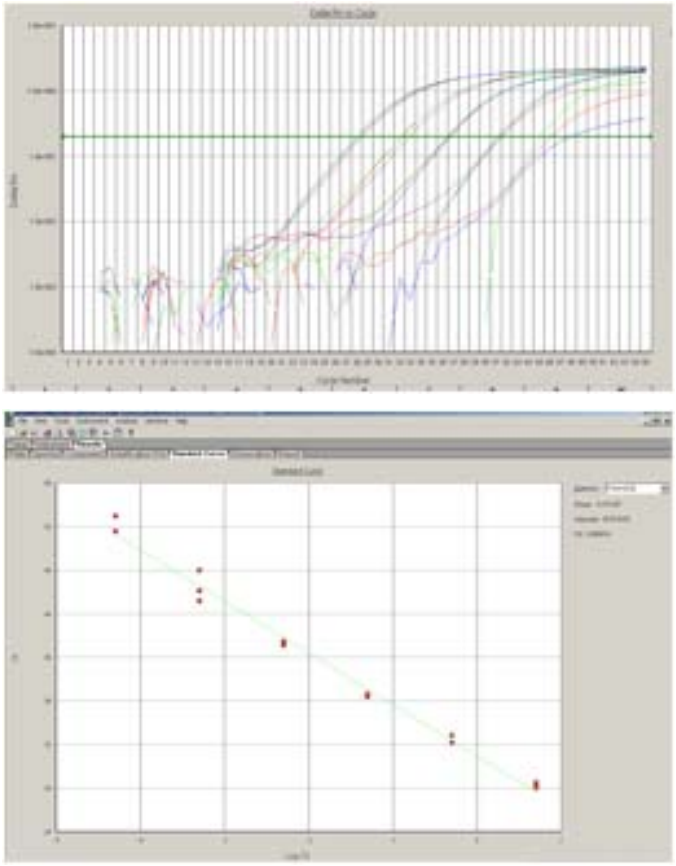

Figure 4. Real-time PCR amplification of crenarchaeal DNA dilution series (*S. ohwakuensis*). Top panel shows amplification with increasing cycle number (left to right) of serial 10-fold dilutions. Bottom panel shows the resulting standard curve plotting increasing cycle threshold (Ct) on the y axis versus log of the DNA concentration (increasing from left to right).

ACKNOWLEDGEMENTS

The author thanks D. Newby for assistance with the development of methods that led to the assays described in this paper. The support of the National Science Foundation Microbial Observatories program for parts of this work is gratefully acknowledged.

REFERENCES

Amann, R. I., W. Ludwig, and K. H. Schleifer (1995), "Phylogenetic identification and in situ detection of individual microbial cells without cultivation", *Microbiology Reviews* 59, 143.

Appia-Ayme, C., R. Quatrini, Y. Denis, F. Denizot, S. Silver, F. Roberto, F. Veloso, J. Valdes, J. P. Cárdenas, M. Esparza, O. Orellana, E. Jedlicki, V. Bonnefoy and D.Holmes (2006), "Insights into the central carbon metabolism of *Acidithiobacillus ferrooxidans* revealed by bioinformatics and microarray analysis", *Hydrometallurgy* 83, 273.

Brock, T.D. (1987), "The study of microorganisms in situ:progress and problems", In M. Fletcher, T. R. G. Gray, and J. G. Jones (ed.), *Symposia of the Society for General Microbiology*, Cambridge University Press 41, 1.

DeWulf-Durand, P., L. J. Bryant, and L.I. Sly (1997), "PCR-mediated detection of acidophilic, bioleaching-associated bacteria", *Applied and Environmental Microbiology* 63(7), 2944.

Edwards, K.J., T. M. Gihring, and J.F. Banfield (1999), "Seasonal variations in microbial populations and environmental conditions

in an extreme acid mine drainage environment", *Applied and Environmental Microbiology* 65(8), 3627.

Goebel, B.M. and E. Stackebrandt (1994), "Cultural and phylogenetic analysis of mixed microbial populations found in natural and commercial bioleaching environments", Applied and Environmental Microbiology 60(5), 1614.

Johnson, D.B. (1995), "Selective solid media for isolating and enumerating acidophilic bacteria", *Journal of Microbiological Methods* 23, 205.

Johnson, D.B., N. Okibe, and K.B. Hallberg (2005), "Differentiation and identification of iron-oxidizing acidophilic bacteria using cultivation techniques and amplified ribosomal DNA restriction enzyme analysis", *Journal of Microbiological Methods* 60, 299.

Kock, D., and A. Shippers (2006), "Geomicrobiological investigation of two different mine waste tailings generating acid mine drainage", *Hydrometallurgy* 83, 167.

Liu, C.-Q., J. Plumb, and P. Hendry (2006), "Rapid specific detection and quantification of bacteria and archaea involved in mineral sulfide bioleaching using real-time PCR", *Biotechnology and Bioengineering* 94(2), 330.

Nicomrat, D., W.A. Dick, and O. H. Tuovinen (2006), "Microbial populations identified by fluorescence in situ hybridization in a constructed wetland treating acid mine drainage", *Journal of Environmental Quality* 35, 1329.

Pizarro, J., E. Jedlicki, O. Orellana, J. Romero, and R.T. Espejo (1996), "Bacterial populations in samples of bioleached copper ore as revealed by analysis of DNA obtained before and after cultivation", *Applied and Environmental Microbiology* 62(4), 1323.

Quatrini, R., C. Appia-Ayme, Y. Denis, J. Ratouchniak, F. Veloso, J. Valdes, C. Lefimil, S. Silver, F. Roberto, O. Orellana, F. Denizot, E. Jedlicki, D. Holmes and V. Bonnefoy (2006), "Global analysis of the ferrous iron and sulphur energetic metabolism of *Acidithiobacillus ferrooxidans* by microarray transcriptome profiling", *Hydrometallurgy* 83, 263.

Rawlings, D.E. (2002), "Heavy metal mining using microbes", *Annual Review of Microbiology* 56, 65.

Shrenk, M.O., K.J. Edwards, R.M. Goodman, R.J. Hamers, and J.F. Banfield (1998), "Distribution *of Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* :implications for generation of acid mine drainage", *Science* 279, 1519.

Thompson, J.D., D.G. Higgins, and T.J. Gibson (1994), "CLUSTALW:Improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position-specific gap penalties, and weight matrix choice", *Nucleic Acids Research* 22, 4673.

Tuovinen, O.H. and D. Kelly (1973), "Studies on the growth of *Thiobacillus ferroooxidans*", *Archives of Microbiology* 88, 285.

Zuckerkandl, E., and L. Pauling (1965), "Molecules as documents of evolutionary history", *Journal of Theoretical Biology* 8, 357.