

## GOLD FLAKES AND THE ART OF MOLECULAR BIOSCIENCE

### Insights into the molecular methods used to study gold nugget forming microbes— outlook towards a promising future for these organisms in gold ore processing

F. Reith<sup>1</sup> & S.L. Rogers<sup>2</sup>

<sup>1</sup>CRC LEME, Department of Earth and Marine Sciences, Australian National University, ACT, 0200

<sup>2</sup>CRC LEME, CSIRO Land and Water, PMB 2, Glen Osmond, SA, 5064

#### INTRODUCTION

The residential microflora of auriferous soils from the Tomakin Park Gold Mine in temperate south eastern New South Wales and the Hit or Miss Mine in the tropical Palmer River gold fields in north eastern Queensland have been shown to solubilize and precipitate gold (Reith & McPhail, in preparation). To determine if microorganisms also contribute to the authigenic formation of gold nuggets, gold flakes were obtained from soils overlying these deposits and from other prospects in the Palmer River gold fields. Scanning electron microscopy revealed the presence of micrometer-sized 'bubbly' structures, which cover large areas on the surface of these gold flakes. Based on their morphology, these bubbly structures were described as microbial fossils, pseudomorphs or fossilised biofilms of microbial origin. They closely resemble budding microbial cells of the genus *Pedomicrobium* and were detected on gold flakes from various locations in Australia, Alaska and South America (Bischoff *et al.* 1992, Watterson 1992, Bischoff 1997). However, proof of their microbial origin cannot be made based on describing their morphologies alone. Scanning electron microscopy of gold flakes used in this study revealed regions that seemed to be covered by recently active biofilms. This was verified by staining the gold flakes with 4',6'-Diamidino-2-phenylindole (DAPI), a dye that forms fluorescent complexes with natural double-stranded DNA. Using DAPI-staining, combined with confocal laser microscopy, we were able to create 3D-images of these biofilms on the gold flakes.

However, to understand which organisms are involved in the formation of gold flakes and nuggets the DNA of these organisms has to be isolated and studied. We are using denaturing gradient gel electrophoresis (DGGE) of certain variable areas of the 16S rDNA to obtain information relating to the microbial community structure on the gold flakes, such as the organisms present, the number of species and the population diversity. The ribosomal 16S rDNA gene is functionally conserved in all prokaryotic organisms (Amann *et al.* 1995). However, within the 16S rDNA gene are several variable regions where the nucleotide sequence differs between different species, thus 16S rDNA can be used to identify groups and species of organisms (Amann *et al.* 1995). 16S rDNA is today widely used to compare the microbial community structure and diversity of environmental samples (Head *et al.* 1998).

In this paper we aim to explain the methodology we are currently using to obtain and study 16S rDNA from the natural gold grains, describe our first results obtained by using these techniques and give an outlook on how these findings might be used to construct biological accumulation plants for gold processing.

#### MOLECULAR MATERIALS AND METHODS

##### The Polymerase Chain Reaction (PCR)

The PCR amplifies a specific region of DNA as defined by two primer sequences, thus it can be used to examine a particular region of the genome. Starting from a small amount of DNA in a sample a large number of copies of this region of the DNA are created by using PCR-amplification. The PCR-amplification itself is a three-stage process: (i), DNA is denatured (made to a single strand); (ii), the primers bind to their complementary sequence; and (iii), primers are extended by addition of nucleotides complementary to that on the template sequence. This process was repeated 25 times in our experiments. Depending on the focus of the study the primers used can be universal (i.e., used for all bacteria or all fungi), or specific, so as to amplify DNA from one species only. Since the aim of our study was to examine the microbial diversity, we used a universal set of primers (27-GC and 534R), designed to anneal to all 16S rDNA. Because we expected the amount of 16S rDNA on the gold flakes to be minimal, we did not use a conventional DNA extraction method, but used 40 gold flakes directly as templates in our PCR-amplification. The product of this first PCR-amplification was used as a template for a second PCR-amplification.

##### Denaturing gradient gel electrophoresis (DGGE)

The PCR-products, i.e., the amplified 16S rDNA, from the gold flakes were separated by denaturing gradient

gel electrophoresis (DGGE). This technique is used to split up small DNA fragments (200-1000 base pairs—bp) according to their melting behavior. The small fragments of genomic DNA, which are run on a low-to-high denaturing gradient acrylamide gel, initially move according to molecular weight. As they progress into regions of higher denaturing conditions the DNA begins to melt; the point at which this melting occurs depends on its individual nucleotide composition. A shift in mobility of the DNA in the gel occurs after the melting and the DNA gets 'stuck' and forms a characteristic band, which can be stained. In our study we used an 8 % acrylamide gel made up with 40 to 65 urea/formamide denaturing solutions, which was run for 20 h at 60°C and 60 V in 1 % Tris-acetate-EDTA-electrophoresis-buffer (TAE). The gels were stained for band analysis using a silver staining technique (Bio-Rad Laboratories, California, USA). SYBR-gold staining was used on gels used for band excision for sequencing (Invitrogen®, Oregon, USA). SYBR-gold stained gels were digitalized using a UV transluminator system equipped with Diversity database software (Bio-Rad Laboratories, California, USA). Silver stained gels were digitalized using a conventional scanner and scanner software.

### **Cloning and sequencing**

To obtain information on which species are present on these gold flakes individual bands from the SYBR-gold stained gels were excised by inserting a small pipette tip into the gel at each band of interest. The pipette tips were placed into individual microcentrifuge tubes containing 40 µl H<sub>2</sub>O and held at 4°C for 18 h to elute the DNA. The pipette tip was then removed and PCR-amplification was used to multiply the particular bands of 16S rDNA using the 27 GC and 534R primers. The product was run on another DGGE gel, to insure that just single bands had been amplified. These individual bands were excised, amplified by PDR reaction and the PCR-products were run on 2% agarose gels. These were stained for 1 h with ethidium bromide and the 16S rDNA bands were excised using a sterile scalpel and tweezers on a UV-transluminator. These excised bands were gel purified using Eppendorf Perfectprep® Gel Cleanup kit (Eppendorf AG, Hamburg, Germany). Purified fragments were cloned into *E. coli* cells using a Promega pGEM-T SysII (Promega Bioscience, California, USA). Cells were plated out and white colonies selected. Colonies were transferred into LB-broth containing ampicillin and incubated at 37°C overnight. The DNA was extracted using Mobio Ultraclean™ Mini Plasmid Prep kit (Mobio Laboratories Inc., California, USA). Extracted DNA was sequenced using Beckman-Coulter-dye™ Terminator Cycle Sequencing with Quick-Start kit (Beckman-Coulter Inc., California, USA). Samples were run on the Beckman Coulter™ CEQ 8000 capillary sequencer (Beckman-Coulter Inc., California, USA). Base calling was checked by eye.

### **Band and sequence analysis**

To analyze the DGGE gels the Diversity database software is used. The program is designed to identify and group bands and compare band set from different gels. Two reference lanes of *E. coli* 16S rDNA extracts were used as standards in our gels. A dissimilarity matrix will be calculated, based on band presence or absence to compare the community structure of the different gold flakes. These can be analyzed using cluster analysis.

The sequence data was aligned using Bioedit software. The GenBank database was searched for matching sequences using the BLAST software. Furthermore a phylogentic tree will be assembled from the 16S rDNA sequences obtain from the gold flakes to show their relation to other groups of organisms.

## **FIRST RESULTS AND DISCUSSION**

DAPI staining has shown the presence of biofilms on the gold flakes. By successfully extracting 16S rDNA from gold flakes we have now obtained further proof for their existence and are now able to identify their nature. We were able to obtain 16S rDNA from 37 of 40 of gold flakes, indicating that bacteria are commonly associated with gold flakes. Examination of the scanned images of the DGGE gels revealed the presence of 2 to 25 (on average 6 to 8) clearly visible bands in every positive lane, indicating that up to 25 different species of prokaryotes were associated with the gold flakes. A widespread variation within the samples was generally visible. Several bands were only detected on individual flakes or on flakes from one particular site, while some bands occur in almost all samples from one site or in some case can be detected on several samples from both sides. Generally, biofilms work like a well-structured factory where every group of organisms has their specific tasks; while some tasks can be fulfilled by different groups of organisms, others can only be fulfilled by a specific group (Little *et al.* 1997). The 16S rDNA shows that the biofilms consist of a variety of species, but that one or two species seem to occur in most samples, indicating that these organisms might have such a special task, such as the selective precipitation of the gold from solution to form the actual gold flake.

Bands were excised based on the band analysis results of the DGGE gels and to date 8 of these bands have

been sequenced. Six of these sequences are not described in the GenBank database, which could indicate the presence of formerly unknown or undescribed organisms on the gold flakes. Two of our sequences matched a known extremophile. This organism seems to appear on most of the samples from both sampling sites. This organism has been described as a metallophile, i.e., an organism that thrives in the presence of mM concentrations of several heavy metals. It has been shown to harbor a large variety of heavy metal resistances, such as resistance to Zn, Co, Cr, Cd, Ni, Hg on two plasmids. It is commonly found in natural or industrial heavy metal contaminated soils. It has also been shown to accumulate and precipitate heavy metals, such as Cd, and form structures similar to those described on our gold flakes. Commonly it is used in ecotoxicology testing of soils, but has not been tested for its gold resistance or for its use in gold processing.

Organisms such as *Thiobacillus ferrooxidans* are commonly used in mineral processing plants to break down sulfide minerals and liberate the metals bound within (Bosecker 1997). However, to then precipitate the liberated gold the gold-containing solution, the leachate is run over activated carbon, which adsorbs the gold. The adsorbed gold has to then be leached again, usually with concentrated cyanide solutions, before it can be recovered by electro-winning. The process is highly energy consuming and also produces considerable amounts of environmentally dangerous waste. Metal accumulating organisms, especially those showing high metal resistance, could be successfully used to precipitate gold from solution. Apparently, the organisms on our gold flakes selectively adsorb gold from the soil solution and form gold flakes of more than 97% purity. Used in mineral processing, especially in conjunction with a bio-oxidation process using *Thiobacilli*, a highly efficient environmentally friendly gold bio-oxidation/bio-accumulation could be constructed. Further research should be undertaken to secure progress towards an environmentally sustainable and efficient future for the mining- and minerals-processing industries, which make up a large proportion of Australia's GDP.

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## REFERENCES

- AMANN R.I., LUDWIG W. & SCHLEIFER K.H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**, 143-169.
- BISCHOFF G.C.O. 1997. The biological origin of bacterioform gold from Australia. *N. Jb. Geol. Paläont. Abh.* **H6**, 329-338.
- BISCHOFF G.C.O., COENRAADS R.R. & LUSK J. 1992. Microbial accumulation of gold: an example from Venezuela. *N. Jb. Geol. Paläont. Abh.* **194**, 187-209.
- BOSECKER K. 1997. Bioleaching: metal solubilization by microorganisms. *FEMS Microbiol. Rev.* **20**, 591-604.
- HEAD I. SAUNDERS J.R. & PICKUP R.W. 1998. Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbial Ecology* **35**, 1-21.
- LITTLE B.J., WAGNER P.A. & LEWANDROWSKI Z. 1997. Spatial relationships between bacteria and mineral surfaces. In: BANFIELD J.F. & NEALSON K.H. eds. *Geomicrobiology: Interactions Between Microbes and Minerals. Reviews in Mineralogy* **35** Mineralogical Society of America, Washington D.C., USA, 123-155.
- REITH F. & MCPHAIL D.C. in preparation. Effect of the complex natural microflora on the solubilization of gold in soils from the Tomakin Park Gold Mine, New South Wales, Australia. To be submitted to *Geochimica and Cosmochimica Acta*.
- WATTERSON J.R. 1992. Preliminary evidence for the involvement of budding bacteria in the origin of Alaskan placer gold. *Geology* **26**, 1147-1151.