Microbial Populations in Surface Spoil at the Abandoned Mynydd Parys Copper Mines

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Abstract

A study was undertaken to examine whether old and highly weathered mine spoil found at Mynydd Parys, Anglesey U.K., possessed the microbial mechanism in the form of mineral-oxidising bacteria necessary to generate acid mine drainage (AMD), i.e. does it therefore pose a threat to the local environment independent of that posed by the underground workings. Geochemical data indicated that the spoil still contained significant amounts of readily mobilised metals and maintained a low pH. While microbes were isolated which are capable of mineral oxidation, molecular community analysis indicated the site was dominated by hitherto uncultured and unknown actinomycetes.

1 Introduction

Mynydd Parys, located in the northern part of the Isle of Anglesey, North Wales is an extremely old mine site with evidence of Bronze Age and Roman workings. However, large-scale operations did not begin until the discovery of a huge sulfidic copper ore body in the 1760's, which turned it into one of the most important copper mines in Europe (Southwood and Bevins 1995). This ore body was depleted by the 1790's and while more lodes were discovered northwards, by the 1890's most large-scale operations had ceased. As a result of these activities, there are extensive dumps

of highly pyritic mine spoil totalling several million tonnes (Jenkins et al. 2000).

Mynydd Parys, like many other decommissioned mines, presents two key environmental problems. Firstly, where previously exposed underground workings have flooded when pumping stopped, microbially oxidised sulfidic minerals on the rock face have been mobilised. This has led to the generation of acid mine drainage (AMD) within the mine. This metal-rich, highly acidic water presents itself as chronic point source pollution where it drains the mine along natural fissures and man-made adits. Secondly, a more diffuse and insidious generation of AMD occurs at this site where mine spoil has been left on the surface exposed to the elements. The effects of this are potentially more long-term than that of AMD from inside the mine, since where water levels are stable, little pyrite oxidation occurs below the water line and there is limited mobilisation of metals from above (Banks et al. 1997).

While microbes associated with AMD itself are well documented (e.g. Johnson and Hallberg 2003), little is known about the microflora of weathered mine spoil dumps or how this changes over time. The microbial community of a site is implicit to its overall environmental impact. If mineral-oxidising microbes are not present then there will be very little mobilisation of exposed sulfide minerals (Gleisner and Herbert Jr. 2002).

Organisms responsible for mineral oxidation, and thus AMD generation, are usually acidophilic (optimal growth pH<3) iron and/or sulfur oxidisers. Well-known examples of these include *Leptospirillum ferrooxidans* (an iron oxidiser), *Acidithiobacillus ferrooxidans* (iron and sulfur) and *Acidithiobacillus thiooxidans* (sulfur only) (Johnson and Hallberg 2003). Both *L. ferrooxidans* and *At. ferrooxidans* have been previously detected in the Afon Goch (red river) south, which until recently received much of the AMD draining Mynydd Parys (Walton and Johnson 1992). Such bacteria are considered the most central to mineral oxidation, but not necessarily the most numerically dominant (Jenkins et al. 2000). Heterotrophic bacteria which do not oxidise iron or sulfur such as *Acidiphilium* spp. and *Acidocella* spp. are almost always found (e.g. deWulf-Durrand et al. 1997; Berthelot et al. 1997; Johnson and Roberto 1997). These heterotrophs are not thought to contribute to mineral dissolution directly, but exist synergistically – stimulating the activity of the mineral oxidisers.

While such organisms may be readily found in AMD itself, it is unclear as to whether they would dominate mine spoils. Brofft et al. (2002) studied a forest wetland that had been receiving acidic, metal rich water (effectively AMD) from a poorly contained reject coal pile for over 22 years. They found the site was dominated by *Acidobacterium* spp. and uncultured actinomycetes. Bacteria that belong to the same phylogenetic group as

Acidobacterium spp. are extremely ubiquitous, having been detected in many different types of environment (Barns et al. 1999). There are no reports of the presence of actinomycetes in mine spoils, nor in AMD.

Somewhat more research has been carried out on active dumps, where the spoil is managed in order to accelerate the process of mineral oxidation. Heap leaching (or bioleaching) is employed to extract metals (most usually copper or gold) from low-grade ores and tailings. Many studies have detected the presence of *At. ferrooxidans, At. thiooxidans, At. caldus* and *L. ferrooxidans* (Rawlings et al. 2003; Pizarro et al. 1996) while the occurrence of *Acidiphilium* spp. and *Sulfobacillus* spp. has also been reported (Goebel and Stackebrandt 1994; deWulf-Durrand et al. 1997).

Mynydd Parys presents an interesting opportunity to study the microflora of an unmanaged, mineralogically active spoil. Of importance is what are the main microbes there and are they capable of mineral oxidation. An absence of such microbes may suggest that in the hundreds of years since it was first deposited, the ability of Mynydd Parys spoil to generated AMD has been attenuated.

2 Materials and Methods

Spoil material was taken from within the Great Opencast, a massive void in the west of the site. The material was collected in a sterile bag and transported back to the laboratory where it was processed immediately.

To measure spoil pH, 1 g spoil was added to 2.5 ml Reverse Osmosis water and left for 1 h. The pH of the liquor then measured using an Accumet pH meter 50 with a BDH pHase Rapid Renew D/J Epoxy electrode.

Extractable metals were determined by shaking 5 g spoil in 100 ml 0.1 M H_2SO_4 for 1 h. The liquor from this was filtered through a nitrocellulose filter (0.2 μm pore size) and metal concentration assayed by atomic absorbance spectroscopy (AAS) using a PYE Unicam SD9 Atomic Absorbance Spectrophotometer.

DNA was extracted from 1 g of spoil using the UltraClean™ Soil DNA Kit (MO BIO Laboratories, Inc., USA) in accordance with the manufacturer's instructions.

To detach microorganisms from the spoil matrix, 1 g spoil was added to 10 ml basal salts solution [(g l^{-1}) $Na_2SO_4 \cdot 10H_2O$ (0.15); $(NH_4)_2SO_4$ (0.45); KCl (0.05); $MgSO_4 \cdot 7H_2O$ (0.5); KH_2PO_4 (0.05); $Ca(NO_3) \cdot 4H_2O$ (0.015)], adjusted to pH 2.5 with H_2SO_4 and shaken for 30 min. Once the solid phase had settled, the resultant liquor was serially diluted and plated onto Iron (Fe<u>o</u>), Iron:Tetrathionate (FeS<u>o</u>) and Iron:Thiosulfate (FeT<u>o</u>) overlay

plates, Yeast Extract pH 3 and 4 (YE_3O, YE_4O) overlay plates (Johnson, 1995; Hallberg and Johnson, 2003) and R2A plates (Reasoner and Geldreich 1985). Solid media were incubated for four to six weeks at 20°C.

Colonies were differentiated based on their appearance. Iron-oxidising colonies appeared orange-dark brown, sulfur-oxidising colonies appeared bright white, and heterotrophic, non-iron-sulfur-oxidising colonies appeared as a variety of colours from clear to off white, to yellow to pink. Colonies were sub-cultured onto appropriate media and further identification carried out on unique isolates by analysis of their 16S rRNA gene.

Amplification of 16S rRNA genes from extracted DNA by the polymerase chain reaction was as described by Okibe (2003). A clone library of the 16S rRNA genes from the spoil was constructed by ligating PCR products to the pGEM®-T Easy Vector (Promega) according to the manufacturer's instructions. Inserts were amplified using the vector specific primers SP6 and T7, and were screened for uniqueness by restriction enzyme digestion using *Eco*R I and *Cfo* I.

Unique clones and individual isolates were sequenced on the CEQ8000 genetic analysis system with the DTCS QuickStart kit (Beckman Coulter). The sequences obtained were compared to gene sequences in public databases by BLAST searching (Altschul 1997).

3 Results

Basic geochemical data are summarised in Table 1. Extractable metal is a measure of how readily mobilised a metal is, given in mg g^{-1} dry spoil. The spoil is acidic and relatively metal rich with iron exhibiting the greatest potential for mobilisation.

Table 1. Basic geochemical data from Mynydd Parys spoil.

Spoil pH	extractable Fe [mg g^{-1}]	extractable Cu [mg g^{-1}]	extractable Zn [mg g^{-1}]	extractable Mn [mg g^{-1}]	extractable Al [mg g^{-1}]
2.8	0.740	0.030	0.004	0.002	0.058

A variety of organisms were isolated from the mine spoil, all of which are extremely or moderately acidophilic bacteria (pH optima for growth of <3 and around 4, respectively); no neutrophilic organisms were isolated. Table 2 summarises the colony morphology of each isolate, the medium it was isolated on and the nearest known organism or organisms based on partial 16S rRNA gene DNA homology. Isolate Pa9 is related to the obligate iron-oxidising autotroph *Leptospirillum ferrooxidans*. Isolates Pa11

and Pa17 are closely related to an iron- (and possibly sulfur) oxidising γ-proteobacterium, WJ2, isolated from a constructed wetland receiving AMD from an abandoned tin mine (Hallberg and Johnson 2003). Pa17 initially oxidised sulfur preferentially, but after subsequent re-plating began to exclusively oxidise iron. Isolate Pa13 is closely related to the obligate

Table 2. Colony morphologies and nearest known organisms based on 16S rRNA gene homology.

Isolate:	Description:	Nearest organism(s) based on partial 16S rRNA gene DNA sequence homology:	Accession ID:	Homology:
Pa9	Iron oxidising colony from Feo plate	*Leptospirillum ferrooxidans* CF12	AF356834	96.90%
Pa10	Large pink colony from Feo plate	Uncultured bacterium clone RCP2-4, and	AF523897	97.60%
		Acidobacterium sp. WJ7	AY096034	94.90%
Pa11	Small, off-white colony from Feo plate	γ-proteobacterium WJ2	AY096032	98.60%
Pa13	Large white colony from FeSo plate	*Acidithiobacillus thiooxidans* ATCC19377	Y11596	98.00%
Pa17	Concentric circular colony from FeTo plate	γ-proteobacterium WJ2	AY096032	97.80%
Pa18	Ruby-red colony from YE_4o	*Acidobacterium* sp. WJ7	AY096034	98.40%
Pa19	Yellow cone-shaped colony from YE_4o	Gram-positive iron-oxidizing acidophile SLC66	AY040739	97.70%
Pa20	Yellow colony from YE_4o	*Acidiphilium acidophilum*	D30769	97.30%
Pa22	Tiny pale colony from YE_4o plate	Uncultured bacterium clone RCP2-2, and	AF523916	96.60%
		"*Actinomyces* sp." TM213	X92705	91.70%

Table 3. Clones from Mynydd Parys and nearest known organisms based on 16S rRNA gene homology.

Clone:	Nearest organism(s) based on partial 16S rRNA gene DNA sequence homology:	Accession ID:	Homology:
pCBPa1b1	*Ralstonia pickettii*	AY268176	99.4%
pCBPa1b2	Uncultured bacterium clone RCP1-37, and	AF523912	94.5%
	"*Actinomyces* sp." TM226	X92708	93.0%
pCBPa1b3	Uncultured bacterium - DGGE band C4	AJ517308	97.0%
pCBPa1b4	"*Actinomyces* sp." TM56	X92695	94.1%
pCBPa1b5	"*Actinomyces* sp." TM208	X92703	97.9%
pCBPa1b6	Uncultured bacterium - DGGE band C4	AJ517308	97.4%
pCBPa1b7	Uncultured bacterium clone RCP1-34, and	AF523911	95.8%
	"*Actinomyces* sp." TM208	X92703	95.6%
pCBPa1b10	Uncultured bacterium - DGGE band C4	AJ517308	97.2%
pCBPa1b11	Uncultured bacterium clone RCP1-37, and	AF523912	94.4%
	"*Actinomyces* sp." TM226	X92708	93.8%
pCBPa1b12	"*Actinomyces* sp." TM210	X92704	94.0%
pCBPa1b13	Uncultured bacterium - DGGE band C4	AJ517308	97.4%
pCBPa1b15	Uncultured bacterium clone RCP2-68	AF523918	99.5%
pCBPa1b16	Uncultured bacterium clone RCP2-4, and	AF523897	96.8%
	Acidobacterium sp. WJ7	AY096034	93.1%
pCBPa1b17	Uncultured bacterium clone RCP2-68	AF523918	99.0%

sulfur-oxidising autotroph *Acidithiobacillus thiooxidans*. Isolate Pa19 is related to an iron-oxidising, Gram-positive facultative heterotroph SLC66 described by Johnson et al. (2001). However, this isolate seems to be unable to oxidise iron and would not grow on the Feo or FeSo solid media. Isolates Pa10 and Pa18 are related to the obligate heterotroph *Acidobacterium*. Pa10 is most closely related to an uncultured clone, RCP2-4, from a wetland receiving acidic drainage from a reject coal pile (Brofft et al. 2002) while Pa18 is more closely related to an *Acidobacterium* sp., WJ7, isolated by Hallberg and Johnson (2003). Isolate Pa20 is closely related to the obligate heterotroph *Acidiphilium acidophilum*. Isolate Pa22 is related

to an uncultured actinomycete clone, RCP2-2, found at the same site as RCP2-4. It has no close described relatives and is the first of this group of extremely acidophilic actinomycetes to be cultured in the laboratory.

Plate-count data (not shown) show isolates Pa11 and Pa17 accounted for nearly half of the colony forming units (CFU) between them. Pa22 accounts for a fifth, while the other isolates make up the remainder.

Of twenty clones picked from the 16S rRNA gene clone library, RFLP analysis suggested there were fourteen major individual species. The closest relatives of these 14, as determined by gene homology, are summarised in Table 3.

The clone library was found to be dominated (13 out of 20) by clones related to a group of actinomycete-like clones found in the study on a wetland receiving drainage from a reject coal pile noted above. Many were also related to clones found during a study on acidic peat bogs, described as "*Actinomyces* sp." (Rheims et al. 1996). However, the closest known cultured relative of this group is isolate Pa22 above.

Four of the 20 clones are related to an organism found during DGGE analysis of the Tinto river, Spain (Gonzalez-Toril et al. 2003). These clones represent an organism that is quite unique phylogenetically, and has no known relatives at all, although it may be more closely related to the actinobacteria than to any other group.

Only two other organisms were represented in the library, an organism very closely related to *Ralstonia pickettii* (1/20) and an organism related to an *Acidobacterium* sp. (2/20). The latter clone is most closely related to isolate Pa10, of the two *Acidobacterium*-like isolates found in this study.

4 Conclusions and Discussion

The Mynydd Parys spoil presents a specialised environment, which is highly acidic and metal rich due to the dissolution of the mineral sulfides contained therein. The fact that only acidophilic organisms were isolated supports this. The range of organisms isolated includes a variety of autotrophs and heterotrophs often associated with AMD and mineral rich dumps.

However, whereas AMD and active dumps are often found to be dominated by strict autotrophs such as *Acidithiobacillus* spp. and *Leptospirillum* spp., the most numerically dominant organism isolated here, in terms of plate-counts, was an iron and sulfur-oxidising heterotroph. It may be possible that this site, being less extreme, lends itself more to organisms with more adaptable metabolic capabilities; there may not be a high enough

concentration of available minerals to sustain a large population of mineral-oxidising autotrophs. This notion is exemplified by the isolation of what appears to be a non-mineral-oxidising relative (Pa19) of a mineral-oxidising heterotroph (SLC66).

The fact that molecular analysis revealed a group of organisms, which represented only a fifth of the total isolates, dominated the population is unsurprising. It is testament to the caution which needs to be taken when applying culture-based quantitative data to an entire population. Use of the DNA-staining dye DAPI demonstrated (data not shown) that only around 10% of the total population could be cultured on the media used, and that while many mineral oxidisers were found, they do not appear to numerically dominate the spoil microbial community.

That this group of hitherto uncultured actinomycetes apparently dominate this site is most intriguing. The diversity of organisms found at Mynydd Parys echoes the findings of Brofft et al. (2002) in their study. Both these sites seem initially quite different, one is essentially generating AMD the other is essentially receiving it. The major common factor between them may be time. Both the Mynydd Parys spoil and the forest wetland may represent how the microflora of sites change following many years of exposure.

The question, however, that still remains is whether the biological mechanism for mineral oxidation and mobilisation at this site exists. The geochemical data suggest it must. The site maintains a low pH and high soluble mineral concentration, but is this due to the presumably small population of mineral oxidisers isolated? It would be easy to say that it must as there are no known examples of mineral oxidising actinomycetes. However, in a subsequent sample from the site, another actinomycete was isolated and initial studies have shown that this isolate appears to be capable of oxidising ferrous iron. It may therefore turn out to be the case that mineral oxidising actinomycetes are of great importance in weathered sites.

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