

Some Important Developments in Biomining During the Past Thirty Years

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ABSTRACT

A selection of significant discoveries in the general field of biomining over the past thirty years with which the author has been directly or indirectly involved will be reviewed. Early steps in the development of the Biox[®] stirred tank process for the recovery of gold from arsenopyrite ores from laboratory work carried out in the early 1980s up to construction of the first full-scale processes will be described. This will include the development and adaptation of microbial inocula for such processes and that besides selection for arsenic resistance, selection for rapid growth in a continuous-flow system was almost certainly an important, though little recognized, time-consuming adaptation requirement.

How the use of molecular techniques led to the discovery that *Acidithiobacillus ferrooxidans* did not play a major role in continuous-flow biooxidation tanks and why this is so will be discussed. Molecular techniques also clarified our understanding that some of the microorganisms that were initially recognized as being major role players in many bioleaching operations, such as *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans* consisted of more than one species and that the species initially identified were, in many cases, not the main role players. The track record for the development of genetic systems for some of the main microbial role players in bioleaching will be briefly described as well as some of what has been learned during this development. This includes evidence that families of plasmids with similar DNA backbones exist within isolates of certain bacterial species even though the isolates originated from different continents. However, the accessory genes that they carry may be very different. The view that genetically manipulated microorganisms are likely to play a very limited role in tank and heap leaching operations that are open to the environment will be defended.

In conclusion, some of the history of the debate concerning the direct vs indirect mechanisms of bioleaching will be briefly reviewed as well as how the idea of 'contact' vs 'non-contact' leaching has helped to resolve that debate.

Keywords: adaptation of microorganisms; BIOX[®] process; arsenic resistance; genetic systems; plasmids; direct-indirect leaching

1 INTRODUCTION

There is little doubt that one of the major advances in commercial biomining over the past thirty years was the development of the stirred-tank process for the treatment of gold-bearing arsenopyrite concentrates. The two major companies initially involved in this development were Gencor (South Africa) who commercialized the BIOX[®] process that operated at about 40°C and BacTech (Australia) whose BACOX process operated at 50-55°C [1]. Gencor went through a number of alterations and name changes eventually becoming Billiton, which then merged with BHP to form BHP Billiton. Shortly after the acquisition of Billiton, the BIOX[®] process was sold by Gencor to Goldfields, who commercialized the process while the BacTech technology was acquired by MinTek (South Africa). A summary of the commercial BIOX[®] and BACOX/MinTek operations

has been reported by Rawlings et al., [1] and the additional commercial BIOX® operations designed and commissioned by Goldfields up until 2008 by van Niekerk [2]. These stirred tank processes use amongst the largest fermentation tanks in the world and have been highly successful microbially-driven processes by any standards.

The person whose foresight and enthusiasm were crucial for the development of the BIOX® process was the late, Eric Livesey-Goldblatt [3], who was manager of Gencor group laboratories at the time. The researcher who was responsible for much of the early work was Philippa Norman, who wrote up her research as an MSc through the University of Cape Town and for which Professor David Woods and myself were supervisors. This MSc thesis was submitted in March 1986 but was never published in a journal [4].

After batch and continuous culture studies on both run-of-mine ore and arsenopyrite concentrate, it became clear that the possibility existed for the biooxidation of the concentrate to be developed into a commercial scale, continuous-flow process.

2 DEVELOPMENT OF THE BIOX® PROCESS

Initially, the retention time required in a series of three oxidation tanks to sufficiently oxidize the arsenopyrite to allow in excess of 95% gold recovery was 10-12 days. The main reason for this was that the microorganisms were sensitive to the arsenic released from the mineral. If more than 1 g/l As was added (as As₂O₃) to the leaching solution, Fe³⁺ production slowed dramatically. However, by the end of laboratory studies, arsenic resistance had risen to 6 g/l As [4]. After further adaptation of the microorganisms in both pilot plant and the 10 tons of concentrate per day demonstration plant at the Fairview mine (built in 1986), the total retention time required in the series of tanks had fallen to 3.5 days and arsenic resistance had risen to 13 g/l. The retention time was then lengthened to 4 days to provide a margin of safety against cell washout [5]. The microbial culture responsible for arsenopyrite operation quickly established a reputation for being highly robust and much of the further development of the BIOX® process concerned equipment design and process engineering.

2.1 Selection of Efficient Microorganisms for Arsenopyrite Stirred Tanks

Unfortunately, none of the original inoculum was maintained. It was therefore not possible to reconstruct exactly what changes had taken place during adaptation and to what extent the improvement in microbial performance has been due to the recruitment of new microorganisms or the selection of more efficient cells from within the original consortium that was inoculated into the tanks. Irrespective of whether the microorganisms originated from the inoculum or from later arrivals, it is highly likely that a substantial amount of the improvement of an efficient microbial culture would have occurred during growth in the tanks. The changes that are likely to have taken place during adaptation to high levels of arsenic have been extensively researched and will be summarized later. The author believes that what has not been fully appreciated is the extent of changes that take place during adaptation of the natural wild microorganisms to rapid growth in continuous-flow culture. Admittedly, much of this is speculation and difficult to verify without repeating a similar adaptation process, but the theoretical argument for this is as follows.

Iron- and sulfur-oxidizing organisms that are the principle contributors to mineral decomposition are widely distributed in many natural environments. However, these environments would seldom provide the steady state 'near optimal' conditions of a biooxidation tank. Rather, 'wild' microorganisms are likely to experience substantial variations in pH, day-night temperature, the availability of energy, nutrients or water and a number of other possible variables. The fittest 'wild' organisms would be expected to be those that have become adapted to survival in highly variable, feast-famine, cold-warm, wet-dry environmental conditions. Consequently, the gene regulation systems of natural isolates would be expected to have become adapted for survival under frequently adverse conditions. These microorganisms would not have been 'tuned' for rapid growth

in the consistent, near optimal conditions provided by stirred tank bioreactors. This means that potentially there is considerable scope for the selection of mutants with altered gene expression and physiology that permit rapid mineral breakdown under the conditions provided by the bioreactor. This adaptation may take an extended period of time that is difficult to predict, partly due to the random nature of a series of growth rate-improving mutations. For example, a cell with a doubling time of 8 h may acquire a mutation that enables it to divide with a reduced doubling time. This faster-growing mutant would be subject to slightly less washout than the bulk of the cells in a tank and would eventually dominate the population, thereby serving as the starting material for the next growth-rate improving mutation. However, initially there would be only one such mutant cell in a tank outnumbered by, for example, 10^9 cells per ml of the slower-growing, non-mutant cells. Computer simulations (carried out by Prof Johan Rohwer, Biochemistry, Stellenbosch University) indicate that given a residence time of 24 h in a tank of 1 l, it would take a single mutant cell that doubles every 7.8 h about 1100 days to displace a population of non-mutant cells that divides every 8.0 h. Time for displacement is halved to 550 days if the mutant was capable of doubling every 7.6 h. In an alternate simulation, the K_s for a hypothetical rate-limiting metabolic reaction (e.g. CO₂-fixation or iron- or sulfur oxidation) was assumed to be 20 mg/l in a non-mutant cell and reduced to 18 mg/l in a mutant cell. In this case it would take 520 days for the mutant to replace the non-mutant. Although both of these simulations are based on unsubstantiated estimates with the proportional enhancement being of more relevance than the actual values, they suggest that the adaptation of a microbial consortium to rapid growth may take years rather than days or months. This prediction is supported by empirical experience obtained during the adaptation of the microbial consortium to continuous growth in the gold-bearing arsenopyrite concentrate biooxidation tanks at the Fairview mine (Barberton, South Africa) where the rate of growth of the microbial consortium improved over approximately 4 years before stabilizing [5].

2.2 Types of Microorganisms Present in BIOX® tanks

Prior to about 1990, work on *At. ferrooxidans* (then called *Thiobacillus ferrooxidans*) dominated microbiological research on biomining organisms. We too were under the impression that *At. ferrooxidans* dominated the population of biooxidation tanks. One of the reasons for this is that if a sample from a biooxidation tank was grown in a flask on an iron-based medium (such as the well-known 9K medium) and the cells plated and grown on an agarose or phytoigel solidified medium, colonies of *At. ferrooxidans* were readily isolated. However, in the early 1990s, the use of the polymerase chain reaction and 16S rRNA primers began to be applied to the analysis of microbial populations and to microorganisms in bioleaching environments [6]. When these techniques were applied to samples of bacteria isolated directly from biooxidation tanks at the Fairview mine we were stunned [7]. *At. ferrooxidans* was not detected, instead, the population of bacteria was dominated by *Leptospirillum* and the then, recently described, *At. caldus* (at the time called *Thiobacillus caldus* [8] and before that *Thiobacillus thiooxidans* type II).

In retrospect, we should not have been surprised as it was already known that ferrous iron oxidation by *At. ferrooxidans* was inhibited by ferric iron and that *Leptospirillum* was much less inhibited [9]. As the ferric iron concentration in completely mixed continuous-flow tanks operating at steady state was always high relative to ferrous iron, *At. ferrooxidans* cells would not be expected to grow on iron. Furthermore, at an operating temperature of 40°C, *At. caldus* is a far better sulphur-oxidizer than *At. ferrooxidans*. There are two possible reasons why *At. ferrooxidans* was isolated when microorganisms from tank samples were inoculated into shake flasks. Firstly, they were inoculated into a ferrous iron medium in which no inhibitory ferric iron was present and low numbers of *At. ferrooxidans* present in the tank samples would outgrow *Leptospirillum*. A second possibility is that *Leptospirillum* is a lot more fastidious about growing on iron-based plate media. The overlay technique developed by Barrie Johnson and colleagues [10] is required to successfully grow *Leptospirilli*. Since this technique was not being used at the time, *Leptospirillum* would have had difficulty growing on the solid medium even if they had been present in the shake flasks.

The discovery that the arsenopyrite biooxidation tanks were not dominated by *At. ferrooxidans* had a dramatic effect on our research program. We decided not to begin new projects on *At. ferrooxidans* and focussed our attention *Leptospirillum* and *At. caldus*. A review article was published with Geoff Hansford and Helmut Tributsch in which the reasons why *Leptospirilli* dominate the iron-oxidizing population of arsenopyrite biooxidation tanks were explained [11]. Arsenopyrite is an acid-producing reaction and it was subsequently found that if the pH within the BIOX® tanks was not maintained at pH 1.6 but allowed to fall to pH 1.0 or even less, then an archeon of the genus *Ferroplasma* (now identified as *Ferroplasma acidarmus* DR1) [12], became the dominant microorganism (unpublished).

2.3 The identification of *Leptospirillum ferriphilum* in bioleaching processes.

In the days before 16S rDNA sequencing, it was not always easy to distinguish between bacteria that had similar physiologies. This was especially true for autotrophic iron- and sulphur-oxidizing microorganisms. As a result researchers became aware that there were almost certainly more than one species of microorganisms represented by bacteria called *At. ferrooxidans*, *At. thiooxidans* and *Leptospirillum ferrooxidans*. As the grounds for dividing such a species into more than one were not always clear, the approach was to use terminology such as *At. thiooxidans* type II, *Leptospirillum ferrooxidans*, types II and III, while *At. ferrooxidans* had been divided into five groups [13]. Most researchers did not have the technology or comparative strains to know exactly which organism they were working with. It soon became clear that the *Leptospirilli* present in the Fairview mine BIOX® tanks were *Leptospirillum ferrooxidans* type II. As a result of this finding we acquired *Leptospirilli* from several researchers and carried out a genetic and physiological comparison [14]. The result was that all of the isolates could be divided into one of two groups and *Leptospirillum ferrooxidans* type II was named *L. ferriphilum*. Widespread acquisition of the ability to sequence 16S rDNA brought an end to that era of indecisiveness. *At. thiooxidans* type II was reclassified as *At. caldus* [8] and after some effort *L. ferrooxidans* type III was grown in pure culture and named *L. ferrodiazotrophum* [15]. Although *At. ferrooxidans* has been the most studied of all biomining microorganisms, the division of bacteria that go by the name of *At. ferrooxidans* into separate species is still in progress [16].

Once it became possible to distinguish between different types of *Leptospirilli*, it was discovered that *L. ferriphilum* was very widely spread and that many industrial processes contained this species rather than *L. ferrooxidans*. It is uncertain why this species dominated the biooxidation tanks, but we attributed this to its ability to grow comfortably at 45°C whereas *L. ferrooxidans* prefers to grow below 40°C [14]. Although the temperature of the BIOX® process was supposed to be maintained at 40°C, arsenopyrite bioleaching is highly thermophilic and we had assumed any inefficiencies in temperature control would result in an increased temperature favouring *L. ferriphilum*. What cast doubt on this idea was that the BIOX® plant at Tamberaque, Peru, unlike all of the other BIOX® plants built up to that time, was not inoculated with the microbial consortium that originated at Fairview, but with a local consortium adapted from local acid mine drainage. We found that the *Leptospirillum* from those tanks was not *L. ferriphilum* but *L. ferrooxidans* [17]. It would appear that either *L. ferrooxidans*, *L. ferriphilum* or both may be found in bioleaching heaps and tanks. To date *L. ferrodiazotrophum* has been found only in an acid mine drainage sample [15].

2.4 Arsenic resistance of *At. caldus* and *L. ferriphilum* present in BIOX® tanks.

As described earlier, during the period of adaptation to rapid growth in continuous culture, the microorganisms in the biooxidation tanks had become resistant to much higher levels of arsenic than those in the original inoculum. We therefore investigated what changes had taken place to produce such high levels of resistance. This topic has recently been reviewed [18] and so only a brief summary will be presented here.

As none of the original inoculum had been maintained we were unable to directly compare the microorganisms in the inoculum when still sensitive to arsenic with those that had become arsenic

resistant. This prevented us from asking such questions as, did the microorganisms originally present acquire arsenic resistance or were different strains of microorganisms selected that were already resistant to high levels of arsenic? Instead we obtained six isolates of *At. caldus* [19] plus three isolates of *L. ferriphilum* and three of *L. ferrooxidans* from different parts of the world [20]. Only the *At. caldus* and *L. ferriphilum* isolates from the Fairview mine had been known to have been previously exposed to high levels of arsenic. Genomic DNA was extracted from all isolates and examined for the presence of arsenic resistance genes by Southern blot hybridization and by the ability to confer arsenic resistance to arsenic sensitive *Escherichia coli* cells. All isolates of *At. caldus*, *L. ferriphilum* and *L. ferrooxidans* had a set of arsenic resistance genes on their chromosomes. In the case of the *At. caldus* isolates, these encoded for an ArsR (a regulator), ArsB (arsenite export pump) and ArsC (an arsenate reductase) [21]. For *L. ferriphilum* isolates, the genes for the ArsRC proteins appeared to have been fused with a separate gene for ArsB located immediately downstream [18]. *L. ferrooxidans* appeared to have a gene for ArsB only [17]. This *arsB* gene was isolated and sequenced and no equivalent of the *arsR* or *arsC* genes was found immediately upstream or downstream. The *At. caldus* genes conferred low level resistance to an arsenic sensitive *E. coli ars* deletion mutant (0.25 mmol/L arsenite) but the standard *L. ferriphilum* or *L. ferrooxidans* chromosomal genes did not confer any arsenic resistance [20, 21].

The highly arsenic resistant *At. caldus* and *L. ferriphilum* strains that were isolated from the Fairview mine each had a second set of arsenic resistance genes that were clearly different from the standard *ars* genes [20, 22]. For example the amino acid sequences of the ArsB proteins were 60% and 58% identical in the case of *At. caldus* and *L. ferriphilum*, respectively [18]. These additional *ars* genes consisted of homologues of five previously identified *ars* genes, the *arsR*, *arsB* and *arsC* genes already described plus genes coding for an ArsD (arsenite chaperone and secondary regulator) and ArsA (an ATPase associated with arsenite export). In addition, both sets of genes had a gene encoding a CBS-domain protein (CBS domains bind ATP or AMP and regulate the activity of associated enzyme or transport domains) and in the case of the *At. caldus* genes an additional gene for an NADH-oxidase-like protein. Both sets of additional genes were present within a Tn21-like transposon that was transpositionally active when tested in *E. coli* and both sets conferred increased arsenic resistance to an *E. coli ars* deletion mutant [20, 22]. For the *At. caldus* transposon-located *ars* genes, the increase in resistance to arsenite was 20-fold higher (5 mmol/L) than the standard *At. caldus* chromosomal arsenic resistance genes [18].

The two sets of transposon-located *ars* genes, TnAtcArs and TnLfArs, were compared with sequences in data bases in an attempt to discover the possible source of these transposons. TnLfArs was most closely related in gene order and sequence to *ars*-gene containing transposons from the bacteria *Alcaligenes faecalis* and *Methylobacillus flagellatus* [18, 20]. The *arsA*, *arsB*, *arsC*, *arsD* and *arsR* gene products from those two bacteria had between 86.4 and 97.5% amino acid sequence identity to the equivalent genes of TnLfArs [18]. Only the CBS-domain proteins were less related at 30.4 and 88.1% sequence identity for *A. faecalis* and *M. flagellatus* respectively. This level of conservation was a little higher than between the *ars* genes of TnAtcArs and TnLfArs, and no other more closely related sequences were found for TnAtcArs. Differences between the gene sequences of TnAtcArs and TnLfArs, indicated that the *At. caldus* and *L. ferriphilum* isolates from Fairview obtained their arsenic resistance transposons independently of each other, rather than one bacterium having passed the transposon onto the other within the aeration tanks.

In a subsequent study, we investigated whether the *L. ferrooxidans* present in the biooxidation tanks at Tamberaque and that had been adapted to arsenopyrite from acid mine drainage rather than being imported from Fairview, contained an arsenic resistance transposon that was related to the *L. ferriphilum* from Fairview [17]. Using a combination of PCR amplification with primers to several genes, restriction endonuclease mapping and Southern hybridization it was found that *ars* genes that were highly related to the *L. ferriphilum* TnLfArs were present in the *L. ferrooxidans* from Tamberaque. When PCR screening for similar transposon-linked *ars* genes on a number of *Leptospirillum* isolates from Europe and elsewhere, TnLfArs-related genes were found on a *L. ferriphilum* from Rammelsberg (Germany) and a *L. ferrooxidans* from Sao Domingo (Portugal)

[17]. Interestingly, restriction endonuclease mapping suggested that the *ars* transposon of the *L. ferriphilum* from South Africa was more closely related to that of the *L. ferriphilum* from Germany and that the *ars* transposons of the two *L. ferrooxidans* strains (Peru and Portugal) were more closely related to each other than to the transposons of the other *Leptospirillum* species.

An important conclusion from this study is that arsenic resistance transposons appear to be fairly wide-spread amongst Leptospirilli from at least three continents and therefore if high level arsenic resistance is required, it should be possible to isolate suitable strains from the environment.

3 GENETIC MANIPULATION OF BIOMINING ORGANISMS

3.1 Development of genetic systems

In the early 1980s, work began on the development of genetic systems for biomining microorganisms. DNA manipulation was in its early days and it was hoped that genetic engineering of biomining microorganisms would improve their industrial capabilities. Basic genetic systems for some commonly used laboratory bacteria were in place. These included DNA cloning vectors, genes that could be expressed and used as selectable markers, techniques for cutting and joining DNA pieces and methods for inserting DNA into those organisms. However, the physiology of acidophilic, autotrophic, iron- and sulphur-oxidizing biomining bacteria was very different from typical neutrophilic, heterotrophic laboratory bacteria. Researchers were therefore unsure whether DNA cloning vectors, selectable genetic markers or methods for inserting DNA would work in biomining bacteria. Initial work was focussed on attempting to develop a genetic system for *At. ferrooxidans* as, at the time, it was believed to be one of the more important commercial biomining bacterium.

In the early stages of work on a genetic system for *At. ferrooxidans*, it was known that most of the existing bacterial cloning vectors were narrow host-range, there was little chance that they would replicate in *At. ferrooxidans*. Furthermore, it was not known whether the promoter elements required for the expression of genes for selectable antibiotic resistance or metal-ion resistance markers would work in *At. ferrooxidans*, nor how to get any recombinant DNA into the bacterium.

Much of the initial work was on the isolation and study of plasmids from *At. ferrooxidans* as these could serve as sources of DNA that could replicate in *At. ferrooxidans* and also as potential sources of genes that could be expressed in the bacterium. The first two reports of plasmids from biomining bacteria appeared in the publication of papers from the IBS meeting held in Cagliari, Sardinia in 1983 [23, 24]. Since then, many plasmids from many types of biomining microorganisms have been isolated, many of them cloned and sequenced and some found to be broad host-range in that they replicate in very many Gram-negative and Gram-positive bacteria [25, 26]. Two of these broad host-range plasmids have been developed into cloning vectors that have been shown to replicate in several biomining bacteria and may be used also in other bacteria. In addition, many genes have been cloned from *At. ferrooxidans* and related biomining bacteria that have been shown to function in *E. coli* (see [25] for review). It is now known that the Acidithiobacilli are members of the well-studied γ -proteobacteria and therefore many genes from the γ -proteobacteria could be expected to be expressed and their products to function in the Acidithiobacilli. Exceptions are likely include some gene products from neutrophilic bacteria that may have to function in the low pH conditions associated with the cell envelope of the acidophilic bacteria. What has proved to be more difficult is to discover efficient methods for introducing DNA into biomining bacteria like *At. ferrooxidans*. Conjugation and electroporation systems for delivering DNA have been reported [27, 28, 29]. However in the case of conjugation, these systems have proved to be either low frequency and unreliable, or in the case of electroporation, to apply to a restricted number of isolates [28]. Similar conjugation and electroporation systems have been reported for *At. caldus* [30, 31, 32] and some also for *At. thiooxidans* [33], but in general, they suffer from the same problems as *At. ferrooxidans*.

When it comes to other biomining bacteria, such as the Leptospirilli, Sulfobacilli, and Acidimicrobia or the archaea such as *Sulfolobus*, *Metallosphaera* or *Ferroplasma* then genetic systems either do not exist at all or are at best even more primitive.

3.2 Application of genetic systems to biomining microorganisms

An important question is, if one had a workable genetic system for biomining bacteria, what would one do with it to improve commercial bioleaching processes? One obvious answer is attempt to improve metal resistance by insertion of resistance genes to metals that the organisms are currently sensitive to. Another is attempt to reduce certain metabolic bottle-necks by adding, for example, a more effective CO₂-fixing enzyme. However, the use of genetically engineered biomining microorganisms is affected by certain characteristics of the processes in which they are used that are not present in many other processes such as the production of human insulin or growth hormone synthesis in a bacterium like *E. coli*.

Firstly, tank processes are continuous-flow, non-sterile and open to the environment. This means that the organisms able to grow and replicate most efficiently displace the others. If the new gene added does not increase the growth rate of the organism sufficiently for the extra burden it imposes, those organisms that contain the new gene will be eliminated. This is not a problem in sterile, batch processes where one can begin with a pure culture of microorganisms containing the relevant gene each time one begins a new production run. Next, bioleaching processes operate best with a consortium of microorganisms present and if all members of the interdependent consortium have not been modified similarly, then there may be no gain. For example, if only one organism has received a metal resistance gene or only one member of the consortium has had a metabolic bottle-neck reduced, then to operate in the presence of that toxic metal or with a shorter retention time would result in the other members of the consortium being killed or washed out. Furthermore, modifications for more rapid growth are often dependant on other interrelated systems so that once one bottleneck has been removed, the next appears.

In the case of heap leaching operations, these tend to be relatively low-rate processes, with an even greater diversity of microenvironments and microorganisms. Under such circumstances the advantage of genetic modifications is likely to be reduced. Added to the difficulties in both tank and heap processes is the often highly emotional, public resistance to using genetically modified organisms. This is especially the case in non-sterile environments where containment of the modified organisms cannot be guaranteed.

The need for the genetic modification of biomining microorganisms may be reduced by the observation that these microorganisms appear to be naturally highly adaptable [34]. Being open to the environment allows other organisms to continually enter the system. The arrival of new organisms or possibly the genes capable of being transferred horizontal that they contain, may contribute to this adaptability. As described above, resistance to arsenic appears to be acquired quite readily, though whether this takes place in the biooxidation tanks or by selection of microorganisms from the environment that obtained the resistance genes elsewhere, has not been established. Nevertheless, the adaptation to rapid growth and metal resistance may reduce much of the need to genetically manipulate the organisms. Taken together, genetic engineering may have a much smaller role to play in biomining processes than was originally envisaged.

The main value for the development of genetic systems may be in the ability to study the role and functions of different genes and the metabolic processes their products contribute to within the microorganisms. The ability to make defined mutants, that is, the construction of a knockout of a specific gene is a useful technique for studying the function of non-lethal genes. To date only three such specific gene knockouts have been reported. These are a *recA* (DNA recombination and repair) mutant of *At. ferrooxidans* [28] and *arsB* (arsenic resistance) and *tetH* (tetrathionate hydrolase) deletion mutants of *At. caldus* [35]. Defined mutants such as these have been unexpectedly difficult to make, but improvements in DNA delivery techniques should rectify that.

Greatly facilitating these metabolic studies is the rapidly increasing number of biomining microorganisms that have had their entire genomes sequenced and annotated [36, 37, 38]. This information greatly assists in the identification of genes to be studied and also provides the sequence data required for their directed knockout.

3.3 Plasmids of biomining microorganisms

So many plasmids have been isolated from biomining microorganisms that it would be difficult to review these in the confined space of this report. The DNA of many of these has been sequenced. An interesting finding is that there appear to be families of plasmids within a given species of bacterium isolated from different geographic locations on earth, that share a common plasmid backbone although the accessory genes that they carry differ from plasmid to plasmid. There are at least three examples of this to date.

One of these is the pTFI91 group of plasmids from *At. ferrooxidans* [39]. A combination of DNA sequencing and Southern hybridization has indicated that these plasmids share a highly conserved 2.2-kb *SacI-SacI* fragment that includes part of a *repA*-like gene and an origin of replication *oriV*-like site. The adjacent approximately 7.6-kb region is also conserved and in the case of pTF5 contains genes for stability proteins and the transposase of an insertion sequence [40]. Besides pTFI91 (9.8-kb) and pTF5 (19.8-kb), other plasmids with these features include pDSM583 (20.2-kb) and pTFI35 (15-kb). *At. ferrooxidans* strains containing these plasmids have been isolated from environments as varied as a coal mine in Pennsylvania, a chalcopyrite mine in New Mexico, sulphur spring water samples in Italy and a uranium deposit in Japan [40].

In the case of *At. caldus*, three plasmids that share a common 26-kb region were isolated [41]. Two of these, pTcM1 (65.1-kb) and pTcF1 (39.1-kb), were from strains that originated from South African samples whereas the other, pC-SH12 (28.9-kb) originated from Australia. The 26-kb region contained genes for putative replication, partitioning, Pin-like invertase-recombinase and *mobA*-like relaxase proteins as well as small highly conserved proteins of unknown function. There were also several open reading frames that appeared to encode a variety of proteins several of which were related to redox-active proteins plus a putative ABC-transporter system.

The third example was of two plasmids isolated from two *Sulfobacillus thermotolerans* isolates, one from the island of Monserrat (plasmid pL15, 65.9-kb) and the other from Yellowstone National Park (plasmid pY0017, 59.2-kb) [42]. Both plasmids were sequenced and found to be highly related with pL15 having an additional 9.6-kb transposon-associated region. A feature of these plasmids is that they have what appears to be a complete set of conjugation genes which, if functional, would allow these plasmids to transfer themselves by mating to other bacteria.

A disappointing feature of plasmid research so far, is that although many plasmids have been sequenced, no genes have been identified that could be used as selectable markers in studying the genetics of biomining bacteria. An exception is plasmid pTcF1 from *At. caldus* which contains arsenic resistance genes almost identical to those found on Tn*AtcArs*. However, the essential *arsB* gene has been inactivated by the presence of an *ISAtcI*-like insertion sequence [41]. There is, as yet, no clear answer to the question of exactly what are the properties that plasmids from biomining bacteria encode that are of benefit to their hosts. For example, several genes for proteins that appear to redox-active (oxidases or reductases) [41, 43] and others involved in membrane transport [25, 41] have been identified on plasmids from more than one genus of bacterium. However, exactly what the purpose of these putative redox-active proteins is or what substances may be transported is not yet known.

4 DIRECT-INDIRECT MECHANISM OF BIOLEACHING DEBATE

The debate as to whether the mechanism of bioleaching of metals from their ores or concentrates was direct or indirect continued in numerous publications for many years. In essence, this debate revolved around whether metal leaching was mainly chemical with the microorganisms

being responsible for producing the leaching chemicals (ferric iron and protons) or whether microbial attachment resulted in the surface of the cell coming into direct contact with the surface of the mineral and that this resulted in a flow of electrons directly from the mineral surface to the cell. To the author this debate was largely resolved during several talks held on this subject at the IBS99 symposium held in Spain. Several publications from a large number of workers at about that time helped to resolve this debate. In particular those from the laboratories of Wolfgang Sand [44, 45], Helmut Tributsch [46-49], Frank Crundwell [50, 51] and their co-workers, deserve special mention. The major contributions of these and other researchers were carefully reviewed in four pages of a review by the author in 2002 [52] and will not be repeated here.

What made this subject confusing was that if bioleaching was purely chemical, one would expect that leaching should have been as efficient if the chemicals alone were supplied in the absence of microorganisms as when microorganisms were present. However, not only were microorganisms shown to attach with considerable affinity to certain minerals but leaching appeared to be more efficient when cells were attached. Sand, Crundwell and co-workers pointed out that the exopolysaccharide layer that was produced when attached to mineral surfaces, created conditions that were different from the bulk solution and chemical bioleaching of metals took place most efficiently within this layer. Furthermore, that this enhancement of mineral bioleaching within the exopolysaccharide layer did not necessarily imply a direct mechanism but rather was a result of the chemical leaching functioning over short distances and under the improved conditions within the exopolysaccharide layer. The implication of this understanding was that the microorganisms provide not only the chemicals required for bioleaching (a combination of ferric iron and protons depending on the mineral) [44] but also the space where the leaching reactions take place most efficiently, that is, the exopolysaccharide layer. In an attempt to distinguish the direct mechanism taking place within the exopolysaccharide layer from the direct mechanism that might take place in the bulk solution, the term 'contact' leaching was proposed for the former and 'non-contact' for the latter [51]. For acid-soluble minerals like ZnS, 'non-contact' leaching might be as efficient as 'contact' leaching and in such circumstances, the chemical leaching reaction can be separated from the acid generating reaction even if biological.

The recent discovery of electron-conducting nanowires [54, 55] that are produced by certain microorganisms has again raised the possibility of a direct mechanism. Nanowires are modified pilus-like filaments that microorganisms may use to discharge electrons to a non-soluble electron acceptor including certain minerals. As electrons tend to flow both ways this raises the possibility that electrons could be transferred in the other direction as well, from an electron donor such as an iron- or sulphur-containing mineral to a microorganism that has access to an electron acceptor such as oxygen or ferric iron. Since electrons would be drawn directly from the mineral by the microorganism, this mechanism of mineral oxidation would fall within the definition of direct leaching. Such a mechanism is unlikely to be seen in laboratory shake flasks or stirred tanks, but may well be present in a heap where the microorganisms are stationary and would have the time to produce lengthy filaments. Although such a mechanism has not been reported in mineral oxidizing microorganisms as yet, it has probably not been looked for.

5 CONCLUSION

This report has been an attempt by the author to document some of the important findings that have been made during a period of over 30 years of research with bioleaching microorganisms. Work with gold-bearing arsenopyrite biooxidation tanks demonstrated how enormously adaptable microbial bioleaching consortia are and the power of continuous flow tank systems to select for an efficient biooxidation consortium. The performance of the microbial consortium after several years of operation in biooxidation tanks was unrecognisable from the original inoculum. What has probably not been recognised sufficiently is the time that it may take if one begins with environmental samples to adapt to rapid growth in high-performance biooxidation tanks. Besides adaptation to rapid growth, other adaptations, such as the adaptation to arsenic resistance described

here may have to take place. How difficult this may be probably depends on the type and levels of resistance required. Adaptation to arsenic resistance appeared to occur fairly readily and transposon-associated arsenic resistance genes appeared to be widespread. The same widespread occurrence applies to some other resistance genes such as mercury resistance although whether the level of resistance is sufficient for a given mercury-containing mineral has not been reported. Resistance to other toxic substances e.g. fluoride, may be less widespread or even unknown amongst biomining organisms [53].

The lumping of different species of microorganisms under one species, such as happened for *At. ferrooxidans* (and for which the number of species is still being resolved), *At. thiooxidans* and *Leptospirillum* will probably not occur in the future as readily as in the past. This is because widely applied molecular techniques usually provide unequivocal answers to the identity of a microorganism as compared with the criteria that existed for species identification when some of the first iron- and sulphur-oxidizing microorganisms were isolated. This applies also to the surprise received when it was found that *At. ferrooxidans* did not dominate continuous-flow biooxidation tanks. One no longer has to rely on the ability of microorganisms to be isolated in pure culture before identifying them. Instead when examining which microorganisms are present in biooxidation tanks or heaps samples can be taken and analyzed directly without prior cultivation. In this way, so-called 'unculturable' microorganisms, can also be detected.

The development of techniques for the genetic manipulation of biomining microorganisms still requires much work. Although some success has been achieved in the case of the Acidithiobacilli, these techniques remain rudimentary, labour intensive and unreliable. Much of the problem appears to be with the lack of suitable techniques for delivering manipulated DNA at high frequency to cells. There are indications that the more general applicability of electroporation techniques than originally reported might help address this. However, with respect to other important biomining bacteria such as the Leptospirilli and Sulfolobacilli or the archaea like those of the genera *Metallosphaera* and *Sulfolobus*, the work is still in the very early stages. Genetic manipulation is probably going to be more important in the indirect improvement of bioleaching, through an enhanced understanding of the genetics and physiology of the organisms than directly by the addition of genetically manipulated microorganisms to commercial processes.

One can never pronounce any scientific debate as being closed and new evidence is likely to lead to a refinement of our understanding of the bioleaching mechanism. This includes the possibility for elements of what has been understood as the direct mechanism being involved. For example, the direct contact between a cysteine-containing protein with the mineral surface [48] and more recently the possibility of electron-conducting nanowires [54, 55]. However, based on the reaction kinetics, stoichiometry, scanning electron microscope data and several other considerations, a consensus view is that the mechanism of mineral solubilization is indirect (physicochemical), at least under the laboratory conditions so far studied.

One reason for this report is so that a new generation of researchers might learn from experiences of the past. Hopefully, a new generation with an ever increasing number of new techniques at its disposal, will have as much pleasure in gaining new insights into the fascinating world of iron- and sulfur-oxidizing microorganisms and their application to mineral solubilisation in the next three decades as researchers have in the past three.

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