Review Growth in sulfidic mineral environments: metal resistance mechanisms in acidophilic micro-organisms

Mark Dopson, Craig Baker-Austin, P. Ram Koppineedi and Philip L. Bond

School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK

Acidophilic micro-organisms inhabit some of the most metal-rich environments known, including both natural and man-made ecosystems, and as such are ideal model systems for study of microbial metal resistance. Although metal resistance systems have been studied in neutrophilic micro-organisms, it is only in recent years that attention has been placed on metal resistance in acidophiles. The five metal resistance mechanisms identified in neutrophiles are also present in acidophiles, in some cases utilizing homologous proteins, but in many cases the degree of resistance is greater in acidophiles. This review summarizes the knowledge of acidophile metal resistance and presents preliminary in silico studies on a few known metal resistance systems in the sequenced acidophile genomes.

Overview
Environments containing high levels of dissolved metals include active and disused mines, where the production of acid mine drainage (AMD) and acid rock drainage (ARD) is catalysed by the action of micro-organisms (reviewed by Ledin & Pedersen, 1996). Catalysis occurs via the regeneration of Fe(III), which oxidizes the metal sulfide bond, releasing Fe(II) and reduced inorganic sulfur compounds (RISCs). The RISCs are subsequently oxidized to sulfuric acid, the source of acid in these environments, and the Fe(II) is reoxidized to Fe(III). The generation of mine wastes causes a huge environmental problem where drainage from tailings (generated whilst processing the ore) and waste rock (produced when uncovering the ore) releases high levels of acid and metals. If these metal-rich acidic solutions enter natural water systems they can cause devastating effects, altering river ecologies, destroying commercial and recreational fishing industries and contaminating drinking water.

Acid-leaching solutions are characterized by high metal concentrations that are toxic to most life and have historically been considered ‘sterile’. Solutions that contain the highest recorded ‘natural’ levels of soluble metals occur at the Iron Mountain site in California, USA. In those solutions, iron has been measured at 1-99 M (111 g l⁻¹) and concentrations of copper, arsenic, cadmium and zinc have all been recorded in the tenths of grams to grams per litre range (Nordstrom & Alpers, 1999). It is well known that AMD solutions are far from ‘sterile’ and that acidophilic micro-organisms not only tolerate, but thrive in these acidic metal-rich solutions (Hallberg & Johnson, 2001).

The aim of this communication is to review the knowledge of metal resistance in acidophilic micro-organisms. Considerable insight into general, neutrophilic, microbial resistance mechanisms is currently available (see Nies, 1999). However, micro-organisms surviving in acid-leaching environments should possess the most advanced metal resistance mechanisms, making them ideal systems to study and improve understanding of metal resistance. Here we summarize the current knowledge of this topic, highlighting where gaps and questions are revealed, as well as suggesting some future directions.

Some environmental and economic aspects of acid leaching
It is known that acidophilic micro-organisms can enhance AMD production and there is strong interest to better understand the geochemical and biochemical influences on acid leaching as remediation of contaminated water is expensive. Other types of acidic metal-rich environments are created during the biotechnological process termed bioleaching or bioremediation of waste waters. Bioleaching exploits the action of acidophilic micro-organisms for the extraction of metals and is extensively employed around the world, such as the extraction of mineral from low-grade ores that would not be economically viable by any other method, for example copper extraction by heap leaching.

Knowledge of metal toxicity and how acidophiles survive in acidic metal-rich environments may provide insights into bioremediation of AMD and ARD sites, the optimization of existing techniques and the development of novel biotechnological processes. A possible example for the optimization of an existing technique is the transfer of metal resistance
gene operons between acidophilic micro-organisms, creating multi-metal resistant strains. This would be highly advantageous for bioleaching of metal sulfides and metal sequestration systems for bioremediation of metal-contaminated sites. For instance, bioleaching of arsenic-containing minerals is accelerated at higher temperatures due to increased chemical reaction rates. Unfortunately, typical acidophilic thermophiles (usually archaea) used for bioleaching are not as resistant as moderately thermophilic bacteria to arsenic. Therefore, the ability to transfer arsenic resistance genes to thermophilic archaea could significantly increase the bioleaching efficiency.

**Ecology of acid-leaching environments**

Acidophiles grow in environments of low pH (<3) and include bacteria, archaea and eukaryotes that are capable of growing chemolithoautotrophically, chemomixotrophically or chemoheterotrophically (reviewed by Hallberg & Johnson, 2001). Autotrophic and mixotrophic micro-organisms are able to utilize Fe(II) and/or RISCs solubilized during Fe(III) oxidation of the sulfidic mineral. The end product of RISC oxidation is sulfuric acid, the source of the acid in acid mine environments. Due to its ease of culturing on plates, it was originally thought that the micro-organism population at AMD, ARD and bioleaching sites was dominated by the bacterium *Acidithiobacillus ferrooxidans* (formerly *Thiobacillus ferrooxidans*). Since then, molecular phylogenetic techniques such as fluorescent in situ hybridization and PCR amplification of 16S rRNA genes have shown that other species are more important in natural and commercial bioleaching sites. Molecular phylogenetic techniques have been used to identify the microbiological populations in AMD sites (Bond et al., 2000a, b), biooxidation plants (De Wulf-Durand et al., 1997; Espejo & Romero, 1997; Goebel & Stackebrandt, 1994) and acidic geothermal sites (Burton & Norris, 2000).

Micro-organisms inhabiting AMD, ARD and bioleaching environments encounter considerable selective pressure to develop resistance mechanisms to metal ions, providing them with a competitive selective advantage. As a result, the effectiveness of different heavy metal resistance mechanisms would play a significant role in affecting the functional and

**Table 1. Upper level concentrations of some common metals in a variety of acidophiles where metabolic activity has been recorded**

Values of metal toxicity are often defined differently. Therefore, for this table the term ‘metal concentration whereby metabolic activity occurs’ has been defined as the maximum metal concentration where either growth or a defined enzyme activity [e.g. Fe(II) oxidation] still occurs. The *E. coli* MIC has been included as a neutrophilic example for comparison of the degree of metal resistance between these types of micro-organisms. ND, Not determined.

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Metal concentration whereby metabolic activity occurs (mM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>As(III)</td>
</tr>
<tr>
<td>Neutrophilic bacterium</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>4b</td>
</tr>
<tr>
<td>Acidophilic bacteria</td>
<td></td>
</tr>
<tr>
<td><em>Acidiphilum cryptum</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>Acidiphilum multivorum</em></td>
<td>30f</td>
</tr>
<tr>
<td>‘<em>Acidiphilum symbioticum</em>’ KM2</td>
<td>ND</td>
</tr>
<tr>
<td>‘<em>Acidiphilum symbioticum</em>’ H8</td>
<td>ND</td>
</tr>
<tr>
<td><em>Acidiphilum angustum</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>Acidiphilum strain GS18h</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>Acidocella aminolytica</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>Acidocella facilis</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>Acidocella strain GS19h</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>Acidithiobacillus ferrooxidans</em></td>
<td>84f</td>
</tr>
<tr>
<td><em>Sulfobacillus thermosulfidooxidans</em></td>
<td>ND</td>
</tr>
</tbody>
</table>

*Acidophilic archaea*  

| ‘*Ferroplasma acidarmanus*’ | 13l     | 16f    | ND     | 9f     | ND     |
| *Metallosphaera sedula*    | 1m      | 16m    | 150m   | 1m     | ND     |
| *Sulfolobus acidocaldarius* | ND     | 1''   | 10''   | 10''   | 1''    |
| *Sulfolobus sulfataricus*  | ND      | 1''   | 10''   | 10''   | 0-1''  |

*Data have been taken from the following references: a, Carlin et al., (1995); b, Nies (1999); c, Mahapatra & Banerjee (1996); d, Suzuki et al. (1997); e, Ghosh et al. (1997); f, Harvey & Grundwell (1996); g, Kondratyeva et al. (1995); h, Baillet et al. (1997); i, Dew et al. (1999); j, Vartanyan et al. (1990); k, Gihring et al. (2003); l, unpublished results; m, Huber et al. (1989); n, Miller et al. (1992).
structural characteristics of microbial communities in acidic environments. Another area of considerable interest is prokaryote metal resistance in the context of surface-attached/biofilm modes of growth. Many micro-organisms grow naturally within biofilms and this form of growth exhibits significant resistance to physio-chemical conditions compared to planktonic modes of growth (LaPlagia & Hartzell, 1997). It is possible that there is a link between acidophile biofilm formation and metal ion concentration, as well as higher metal resistance as compared to planktonic cells. Some examples of the degree of metal resistance in acidophilic micro-organisms are listed in Table 1; for ease of comparison all concentrations have been converted to molarity (in the text, for those cases where a conversion has been carried out the original values are given in parentheses).

Metal toxicity and general mechanisms of microbial resistance

Micro-organisms require the presence of a number of metals that play essential biochemical roles such as catalysts, enzyme co-factors, activity in redox processes and stabilizing protein structures (reviewed by Bruins et al., 2000). Metals may accumulate above normal physiological concentrations by the action of unspécific, constitutively expressed transport systems, whereby they become toxic. Intracellular metals can exert a toxic effect by forming coordinate bonds with anions blocking functional groups of enzymes, inhibiting transport systems, displacing essential metals from their native binding sites and disrupting cellular membrane integrity (reviewed by Nies, 1999). There are five basic mechanisms (Fig. 1) that convey an increased level of cellular resistance to metals: (1) efflux of the toxic metal out of the cell; (2) enzymic conversion; (3) intra- or extracellular sequestration; (4) exclusion by a permeability barrier; and (5) reduction in sensitivity of cellular targets. In the following sections of the review, we describe the current knowledge of metal resistance mechanisms in acidophilic micro-organisms.

Acidophile metal resistance mechanisms

Arsenate and arsenite

Arsenic is toxic to most micro-organisms and exists as either arsenate [As(V)] or arsenite [As(III)]. It is a common metalloid in acidic environments due to the breakdown of arsenic-containing minerals (e.g. arsenical pyrite), and in most cases acidophilic species are more tolerant than Escherichia coli to As(III) (Table 1). The toxicity of As(III) has been documented in Acidiphilium multivorum, At. ferrooxidans, Ferroplasma acidarmanus and Metallosphaera sedula (Table 1). As(III) is the more toxic of the species and is shown to inhibit At. ferrooxidans growth in a redox-controlled reactor that maintains a constant As(III) concentration (Harvey & Crundwell, 1996). As(III) also causes the formation of extracellular sulfur in growing cultures of Acidithiobacillus caldus (formerly Thiobacillus caldus). The sulfur production was not due to inhibition of RISC metabolism, but rather due to a general toxicity effect (Hallberg et al., 1996). The toxicity of As(V) to micro-organisms is due to replacement of phosphate in cellular processes, inhibiting a plethora of biological reactions. In metal-leaching biooxidation vessels, As(V) inhibition can be alleviated by increasing phosphate concentrations (M. Dopson & E. Lindström, unpublished), likely countering the toxic action of As(V).

Neutrophiles are resistant to arsenic via energy-dependent efflux encoded by the ars operon, containing the genes arsRBC. This constitutes an As(V) reductase (ArsC) that reduces As(V) to As(III) prior to efflux via a membrane potential driven pump (ArsB) controlled by a trans-acting repressor (ArsR). In some instances, an ATPase (ArsA) is attached to the ArsB membrane pump that confines a higher level of resistance to arsenic, and a second regulator (ArsD) is sometimes present that controls the upper level of expression (Fig. 2; reviewed by Xu et al., 1998). Acidophilic arsenic resistance operons have been cloned from At. ferrooxidans, At. caldus and A. multivorum (Fig. 2) and all three operons conferred As(III) resistance in E. coli (Butcher et al., 2000; de Groot et al., 2001; Suzuki et al., 1998). The At. ferrooxidans ArsR protein has been found to be atypical in that it is not conserved in regions formerly shown to be important for As(III) binding and that the arsRC genes were induced irrespective of the form of arsenic added (Butcher &
The presence of the \textit{arsB} gene (efflux pump) has been identified by Southern hybridization in various acidophilic micro-organisms; these include \textit{At. caldus}, \textit{Acidithiobacillus thiooxidans} (formerly \textit{Thiobacillus thiooxidans}), \textit{At. ferrooxidans}, \textit{Acidiphilium acidophilum} (formerly \textit{Thiobacillus acidophilus}), \textit{Thiomonas caprina} and \textit{Acidocella facilis} (Dopson et al., 2001). Neutrophiles have an inside negative membrane potential, whereas acidophiles have a reversed membrane potential (inside positive). Despite the physiological differences and the reversed membrane potential, the \textit{arsR} genes in an unusual configuration (Fig. 2; Butcher et al., 2000). By Southern hybridization two sets of arsenic resistance operons have been identified from \textit{At. caldus} strains and it has been postulated that all \textit{At. caldus} strains have a basic set of chromosomal arsenic resistance genes (putatively\textit{arsRBC}; de Groot et al., 2001). In addition, three \textit{At. caldus} strains isolated from a South African biooxidation plant operating for the oxidation of arsopyrite have been found to contain an additional transposon located set of\textit{ars} genes (\textit{At. caldus} #6; Fig. 2). One of these genes is homologous to the \textit{E. coli} \textit{arsA} which when cloned into \textit{E. coli} confers resistance to \textit{As}(III) but not to \textit{As}(V) (de Groot et al., 2001), although the \textit{arsA} has not been shown to be functional biochemically. By inference from PFGE of \textit{At. ferrooxidans} strains Kondratyeva et al. (1995) suggested that amplification of certain chromosomal fragments in resistant strains, resulting in increased copy number of putative chromosomal resistance genes, was responsible for arsenic resistance. However, no direct evidence that these fragments contained the resistance genes was presented in that study. Although the presence of an \textit{arsB}-like gene has been identified by Southern hybridization in all of the micro-organisms tested (Dopson et al., 2001), chromosomally encoded \textit{As}(V) reduction to \textit{As}(III) (\textit{arsC} activity) followed by energy-dependent efflux of \textit{As}(III) has only been demonstrated experimentally in a single acidophile, \textit{At. caldus} KU (Dopson et al., 2001). \textit{At. caldus} KU was shown to be resistant to higher concentrations of \textit{As}(III) and \textit{As}(V) than \textit{E. coli} with MICs of 13 and 310 mM, respectively. Although \textit{At. caldus} KU is resistant to \textit{As}(V) and \textit{As}(III), inclusion of 100 and 5 mM, respectively, reduced the growth rate (Hallberg, 1995).

So far the investigations of archaea have added further to the enigma of arsenic resistance in acidophilic micro-organisms. \textit{Sulfolobus metallicus} strain BC (formerly \textit{Sulfolobus} strain BC or \textit{Sulfolobus acidocaldarius} strain BC) contains an enzymic, membrane-bound \textit{As}(III) oxidase that was induced eightfold by growth in a subtoxic concentration of \textit{As}(III) (Sehlin & Lindström, 1992). The \textit{As}(III) oxidase may be involved in resistance as \textit{As}(V) is less toxic than \textit{As}(III), or act as an electron acceptor in a respiration mechanism. No difference in growth rates between the induced and non-induced cultures was observed. \textit{S. metallicus} strain BC also contains a proteinase-K-insensitive tetrahionate-dependent \textit{As}(V)-reducing activity that has a 20-fold greater rate than the arsenite oxidase activity. During growth it is possible that the chemical reduction of \textit{As}(V) could mask the possible resistance to \textit{As}(III) conferred by the arsenite oxidase. If the cells were grown in the absence of tetrahionate (e.g. on Fe(II)), induction of the \textit{As}(III)-oxidizing activity may confer resistance to \textit{As}(III).

\textit{In silico} studies indicate the presence of an \textit{arsB} homologue on a number of genomes from acidophilic micro-organisms (Fig. 3), including the archaeon ‘\textit{Fp. acidarmanus}’. In ‘\textit{Fp. acidarmanus}’, a single operon containing genes homologous to \textit{arsRB} was found, as well as a separate gene encoding a protein similar to \textit{ArsA} (Gühring et al., 2003). Unusually, no genes homologous to \textit{arsC} were identified in ‘\textit{Fp. acidarmanus}’. The \textit{in silico} data are supported by biochemical results whereby ‘\textit{Fp. acidarmanus}’ is resistant to \textit{>13-3 mM (>-1000 p.p.m.) As(V) and As(III)}, although no \textit{As(V)} to \textit{As(III)} reduction is observed, suggesting that ‘\textit{Fp. acidarmanus}’ may have a novel arsenic resistance...
system. A similar arrangement of \textit{arsRB} and a partial \textit{arsA} gene is observed in a related archaeon, \textit{Thermoplasma acidophilum} (Ruepp et al., 2000).

We have also carried out some initial \textit{in silico} studies using motif-based searches for identification of genes involved in As(III) resistance (unpublished results). This was performed by aligning genes from acidophilic and neutrophilic microorganisms, constructing motif-based hidden Markov models using Meta-MEME (Grundy et al., 1997) and subsequently searching genomes of acidophilic microorganisms to identify putative proteins involved in As(III) resistance. The \textit{in silico} data confirm the previously identified \textit{arsB} homologues in \textit{A. ferrooxidans} and \textit{'Fp. acidarmanus'}, and suggest the presence of \textit{arsB} homologues in \textit{T. acidophilum} and \textit{Tp. acidophilum} and \textit{Thermoplasma volcanium}, but no significant homologies were found for \textit{Sulfolobus solfataricus} and \textit{Sulfolobus tokodaii} (Fig. 3). This is supported in \textit{S. metallicus} strain BC, which appeared to lack a typical arsenic resistance mechanism, even though it was putatively resistant to As(III) (Sehlin & Lindström, 1992). Therefore, these species could also be resistant to arsenic via an As(III) oxidase activity rather than the ArsB As(III) efflux system observed in many other microorganisms. Alternatively, these archaea may have an undetected As(V) reductase that does not have significant sequence homology to other ArsC proteins or, finally, completely novel resistance mechanisms may be in operation. Much of the understanding of acidophilic arsenic resistance has been extrapolated from knowledge of neutrophilic resistance mechanisms. However, it is apparent there are differences in the mechanisms that are yet to be resolved.

**Copper**

Copper is used in cytochrome-c oxidase and other related terminal oxygen acceptors and is therefore required in many organisms. Copper is released at AMD, ARD and bioleaching sites and \textit{A. ferrooxidans} strains adapted to increased levels of Cu(II) have been found to be tolerant to 800 mM (Dew et al., 1999). Other acidophiles shown to be resistant to copper include \textit{Leptospirillum ferrooxidans}, which shows growth in 5 mM Cu(II) (Johnson et al., 1992), and those listed in Table 1. Although \textit{A. ferrooxidans} was shown to be the most resistant to Cu(II), virtually all of the acidophiles were found to be more resistant than \textit{E. coli} (Das et al., 1997) found that \textit{A. ferrooxidans} copper resistance was inducible and that the resistant strain extracts Cu(II) more rapidly compared to an unadapted strain. Cu(II) inhibition of growth and Fe(II) oxidation has also been demonstrated in \textit{Sulfobacillus thermosulfidooxidans} subsp. \textit{asporogenes} via competitive inhibition of Fe(II) oxidation (Vartanyan et al., 1990).

Two copper resistance systems have been characterized in neutrophiles. The first system consists of P-type ATPases in Gram-positive bacteria that efflux copper out of the cell and the second is the \textit{Pseudomonas} copper sequestration system that binds copper in the periplasm or close to the outer

![Fig. 3. Amino acid sequence of the \textit{arsB} gene motif model aligned with hits from the sequenced genomes of acidophilic micro-organisms. The amino acid sequence motif models were constructed with the Meta-MEME motif-based modelling program and aligned using ClustalW. The numbers of amino acids quoted are for the residues for each of the sequences that precede or follow each of the motifs.](http://mic.sgmjournals.org)
membrane (reviewed by Nies, 1999). Most investigations of copper resistance in acidophilic micro-organisms have been restricted to *At. ferrooxidans*. A strain of *At. ferrooxidans* that took up 700 mg Cu(II) (g dry weight)$^{-1}$ was adapted to growth in the presence of 600 mM Cu(II) and subsequently Cu(II) uptake decreased to 90 mg Cu(II) (g dry weight)$^{-1}$ (Boyer et al., 1998), suggesting that the Cu(II) was excluded from the cell possibly via an inducible efflux system. The copy number of plasmids in *At. ferrooxidans* decreased with exposure to Cu(II), and increased with subsequent growth in its absence (Pramila et al., 1996). The change in copy number may simply be a stress response, as *At. ferrooxidans* plasmid fragments transformed into *E. coli* did not confer increased Cu(II) resistance (Chisholm et al., 1998). This suggests that *At. ferrooxidans* Cu(II) resistance is chromosomal and not plasmid-based. *At. ferrooxidans* exposure to Cu(II) results in formation of a proteinase-K-sensitive cell-surface component, which upon loss results in decreased Cu(II) adsorption and loss of tolerance, suggesting that some cell-surface components are involved in *At. ferrooxidans* Cu(II) resistance (Das et al., 1998). Differential protein expression induced by exposure to Cu(II) in *At. ferrooxidans* resulted in induction of three proteins (Novo et al., 2000). Also, RNA arbitrarily primed PCR identified 17 genes induced by Cu(II) in *At. ferrooxidans* (Paulino et al., 2002). However, on those occasions no genes with homologies to Cu(II) resistance genes were identified. No reports into archaeal Cu(II) resistance have been published, but Cu(II) resistance has been shown to be inducible in 'Fp. acidarmanus' (unpublished results) and *M. sedula* is also tolerant to 16 mM Cu(II) (Huber et al., 1989).

Zinc

Zinc occurs as the divalent cation Zn(II) and although it cannot undergo redox reactions under biological conditions, it is present in a number of enzymes. Zn(II) toxicity is based upon complexation with various cellular components, and in *Su. thermosulfidooxidans* it has been demonstrated to be due to competitive inhibition of Fe(II) oxidation (Vartanyan et al., 1990). The toxicity of Zn(II) to *At. ferrooxidans* depends on the growth substrate. One strain resistant to 153 mM (10 g l$^{-1}$) Zn(II) whilst growing on Fe(II) is sensitive to 92 μM (0.6 mg l$^{-1}$) when growing on thiosulfate (Trevors et al., 1985). It was found that *At. ferrooxidans* strain TFZ was adapted to growth in 1·071 M (70 g l$^{-1}$) Zn(II) (Kondratyeva et al., 1995; Table 1). Other acidophiles resistant to Zn(II) are listed in Table 1.

The mechanism of zinc resistance in neutrophiles is efflux by P-type ATPases, cation-diffusion facilitator transporters or high-efficiency efflux proteins such as Czc (reviewed by Nies, 1999). Few investigations have gone towards resolving the genetics of acidophile zinc resistance, but from Table 1 it is clear that acidophiles are more resistant than neutrophiles. As was the case for *At. ferrooxidans* arsenic resistance, based on PFGE Kondratyeva et al. (1995) suggested zinc resistance to be chromosomally encoded. Adaptation of the strain to increased levels of Zn(II) resulted in an increase in genome fragment size, suggesting increased copy numbers of the operon encoding the putative Zn(II) resistance genes. However, once again no direct evidence that these chromosomal fragments contained the zinc resistance genes was presented. Plasmids isolated from *Acidocella* strain GS19h have been transformed into sensitive strains of *A. multivorum* and *E. coli*. These events conferred increased Zn(II) resistance to the host organisms, suggesting that it is plasmid-based in strain GS19h (Ghosh et al., 1997). Curing *Acidocella* strain GS19h of the plasmid resulted in loss of resistance and the MIC fell from 1 M to 5 mM (Ghosh et al., 2000). Also, *Acidiphilium symbioticum* KM2 has been shown to harbour three plasmids which, when a mini-plasmid library was created and transformed into *E. coli*, conferred resistance to Zn(II) and Cd(II). This suggests that Zn(II) and Cd(II) resistance is plasmid-based in *A. symbioticum* KM2. The genes encoded by the recombinant KM2 plasmid had no sequence similarity to reported metal resistance genes. Therefore, it is likely that a new Zn(II) and Cd(II) resistance mechanism may be in operation (Mahapatra et al., 2002).

Cadmium

A plethora of studies have demonstrated the toxicity of cadmium to micro-organisms, however, specific mechanisms have yet to be defined. In some micro-organisms, cadmium is taken up via the magnesium or manganese uptake systems (reviewed by Nies, 1999), but the mechanisms in acidophiles have not been elucidated. A possible commercial biotechnological application of acidophiles adapted to high levels of cadmium is for the solubilization of nickel and cadmium from batteries by *At. ferrooxidans* (Cerruti et al., 1998). Cd(II) is toxic to micro-organisms through a variety of mechanisms, including binding to thiol groups, protein denaturation, and interaction with calcium and zinc metabolism. The toxicity of Cd(II) to *At. ferrooxidans* was found to be low in comparison to Hg(II), inhibiting Fe(II) oxidation by 2% at 8·90 μM (10 mg l$^{-1}$) whilst similar levels of Hg(II) caused 63% reduction (De et al., 1997). However, Cd(II) toxicity is greater than that of zinc and copper to *At. ferrooxidans* (see previous sections). Cd(II) uptake in *At. ferrooxidans* has also been reported, and a greater uptake was observed in tolerant strains (Baillet et al., 1997). Other acidophiles resistant to Cd(II) include a number of *Acidiphilum* spp. and an *Acidocella* strain resistant to 700 mM Cd(II), whilst the maximum resistance in archaea was 10 mM in *Sulfobolus acidocaldarius* and *S. solfataricus* (Table 1).

Cadmium ion efflux systems are present in a number of micro-organisms; for example, the Czc system and a P-type ATPase pump (Cada) in Gram-negative and Gram-positive bacteria, respectively (reviewed by Nies, 1999). Ghosh et al. (2000) found that *Acidocella* strain GS19h contained three
plasmids, one of which when transferred to *A. multivorum* and *E. coli* increased the MIC. Also, when GS19h was cured of the plasmids, the MIC dropped, confirming that the plasmids contained genes encoding Cd(II) resistance. The genes contained in these plasmids have not been characterized. As described for Zn(II), the ‘*A. symbioticum*’ KM2 Cd(II) resistance mechanism has been cloned into *E. coli*, conferring Cd(II) resistance (Mahapatra et al., 2002).

In the manner used to search for acidophile As(III) resistance genes, we used the *in silico* approach to detect genes involved in acidophile Cd(II) resistance. A number of cadmium resistance operons are present in acidophiles (unpublished data). This is indicated by the presence of gene sequences with homology to the P-type export ATPase gene cadA on many of the sequenced genomes from acidophilic micro-organisms (Fig. 4). The species with the highest homology to the cadA motif was *At. ferrooxidans* and then the *Thermoplasma* spp. These high similarities suggest that Cd(II) export may be a common resistance mechanism among acidophiles. However, some care must be exercised with interpretation of this Meta-MEME result, as motif matches may occur with other members of the P-type ATPase family.

**Nickel**

Nickel [commonly Ni(II)] is used in a few biologically important reactions, for example to form complexes with polypeptide chains. There have been few investigations with regard to acidophile nickel tolerance. *At. ferrooxidans* has been used to dissolve Ni(II) from spent batteries (Cerruti et al., 1998) and Sulfolobus sp. has been used for bioleaching of Ni(II)/Co(II) minerals (Miller et al., 1999). Of 15 *At. ferrooxidans* isolates from a Cu(II)/Ni(II) tailings environment, 10 were inhibited by >320 mM Ni(II) (Chisholm et al., 1998) and adapted strains have been isolated that can tolerate up to 1 M Ni(II) (Dew et al., 1999). Other acidophiles resistant to Ni(II) include *A. multivorum*, *Acidocella aminolytica* and *Acidocella* strain GS19 (Table 1). The oxidation of Fe(II) by an unadapted mixed culture of mesophilic acidophiles was unaffected by Ni(II) concentrations below 150 mM, and metal release from pyrite by the same mixed culture was almost unaffected by 40 mM Ni(II) (Sampson & Phillips, 2001). In contrast, sulfur and sulfite oxidation and CO2 incorporation were strongly inhibited by 320 mM Ni(II) in *At. thiooxidans*. It was shown that Ni(II) binds to the cell surface, where it inhibits the RISC enzymes sulfur dioxygenase and sulfite oxidase, and ultimately growth (Maeda et al., 1996; Nogami et al., 1997). Ni(II) has been shown to repress the synthesis of proteins via two-dimensional polyacrylamide gel analysis (Novo et al., 2000), but no resistance mechanisms have been characterized.

**Mercury**

Mercury is found in cinnabar (HgS) and its oxidation results in the production of Hg(II) and RISCs. *M. sedula* has an MIC of 5 μM (Huber et al., 1989) and certain strains of *At. ferrooxidans* and *S. metallicus* strain BC have been shown to be sensitive to mercury (Imai et al., 1975; Mier et al., 1996) via uncompetitive inhibition of Fe(II) oxidation. This suggests that Hg(II) binds to the Fe(II)-oxidizing enzyme (De et al., 1997).

A mercury resistance system, analogous to the neutrophilic mechanism, based on an Hg(II)-reducing flavoprotein producing Hg(0) which volatilizes out of the cell, has been found in *At. ferrooxidans* strains (Booth & Williams, 1984; Olson et al., 1982). Several different mercury resistance operons have been characterized from strains of *At. ferrooxidans* and are listed below. The mercury resistance operon from *Thiobacillus* T3.2 and *At. ferrooxidans* Tn5037 (Kalyaeva et al., 2001; Velasco et al., 1999) consists of: *merR*, which encodes a unique positive regulatory protein that twists the operator DNA to allow mRNA formation; *merT*, encoding a mercury uptake protein that may act in association with MerP [a periplasmic Hg(II)-binding protein]; and the final gene, *merA*, encoding the Hg(II)-reducing flavoprotein (reviewed by Osborn et al., 1997). The **Metal resistance in acidophiles**

---

**Fig. 4.** Alignments of amino acid sequences from the sequenced genomes from acidophilic micro-organisms compared to sequence motif models detected in the cadmium efflux ATPase, CadA.
mercury resistance operon from \textit{At. ferrooxidans} E15 has also been cloned and differs from that of most other Gram-negative bacteria in that it does not start with a \textit{merR} gene. Instead it consists of a \textit{merC} gene [Hg(II) transporter into the cell] and a \textit{merA} gene. In addition, a separate gene cluster occurs containing two functional \textit{merR} genes, a \textit{merC} and two non-functional \textit{merA} genes. The presence of a \textit{tn}7 \textit{tnsA} homologue next to the \textit{mer} genes suggests that transposition may be responsible for the gene arrangement (Inoue et al., 1991).

A second type of mercury resistance, not found in neutrophiles, has been identified in \textit{At. ferrooxidans} SUG 2-2. This strain does not have a Hg(II)-reducing flavo-protein analogous to MerA, but nonetheless catalyses the reduction of Hg(II) to Hg(0) in the presence of Fe(II). The reduction is thought to be catalysed by the cytochrome-c oxidase (Sugio et al., 2001). There is interest in making use of this strain’s Hg(II)-reducing capabilities and it has been studied for use in bioremediation of mercury-polluted acidic sites (Takeuchi et al., 2001). The difference between mercury-sensitive and -resistant strains of \textit{At. ferrooxidans} has been characterized. It is apparent that purified cytochrome-c oxidase from resistant strains is more tolerant to increased levels Hg(II), which, due to its importance in Fe(II)-oxidizing activity, likely contributes to the resistant strain’s increased growth rate (Takeuchi et al., 1999).

**Silver**

Silver is released during the oxidation of refractory sulfide ores, where it is trapped as fine particles within the mineral matrix. Silver has been shown to be toxic to \textit{M. sedula}, \textit{L. ferrooxidans} and \textit{Acidiphilium cryptum} at concentrations of 900, 2 and 0.5 \textmu M, respectively (Huber et al., 1989; Johnson et al., 1992). The toxicity of Ag(I) to \textit{At. ferrooxidans} growth and oxidation of Fe(II) has also been recorded (Imai et al., 1975), and Guay et al. (1989) found that Ag(I) was the most toxic of the metals and ion studied (silver, cadmium, cobalt, copper, zinc, uranyl and As(III)). Silver is toxic to \textit{At. ferrooxidans} Fe(II) oxidation at a concentration of 0.93 \textmu M (0.10 mg l^{-1}) (De et al., 1996 and references therein), and at 927 \textmu M (100 mg l^{-1}) pyrite oxidation is severely reduced (Norris & Kelly, 1978). In contrast, after previous silver exposure \textit{At. ferrooxidans} has been observed to be tolerant to 1 mM (108 mg l^{-1}) Ag(I) (Ehrlich, 1984). The Ag(I) mode of inhibition of Fe(II) oxidation has been attributed to a mixed mechanism whereby Ag(I) replaces Fe(II) in the active site of the oxidizing enzyme (De et al., 1996). Inhibition of \textit{S. metallicus} strain BC (adapted strains were less sensitive) and ‘Sulfolobus rivetincti’ by Ag(I) has also been reported (Gomez et al., 1999; Mier et al., 1996). Silver efflux has been demonstrated in neutrophiles, but the mechanism of Ag(I) resistance in acidophilic microorganisms has not been elucidated. Bioaccumulation was observed in \textit{At. ferrooxidans} and \textit{At. thiooxidans} during biooxidation of sulfide ores (Pooley, 1982), although it is not known if this conferred higher levels of resistance.

**Ferric iron**

Iron is the most biologically important metal and is therefore an essential requirement for growth. In most aerated environments, the bioavailability of iron is very low due to chemical oxidation of soluble Fe(II) to Fe(III) in the presence of oxygen and the low solubility of Fe(III) above pH 1.6. However, in acidic environments the solubility of Fe(III) is much greater, and in many cases can reach the g l^{-1} range. A tolerance to high levels of Fe(III) is required in sulfidic ore leaching environments and one ecological example of this is the competition between \textit{At. ferrooxidans} and \textit{Leptospirillum}-like micro-organisms in biooxidation tanks. Both these acidophiles can grow via the oxidation of Fe(II), causing accumulation of Fe(III); however, selection of the \textit{Leptospirillum}-like microorganisms can occur and is thought to be due to their greater tolerance to Fe(III) (Rawlings et al., 1999). Another example is the greater tolerance of \textit{Sulfobacillus acidophilus} than \textit{Acidimicrobium ferrooxidans} to Fe(III), resulting in dominance of the former in mixed-culture Fe(II)-oxidizing environments (Clark & Norris, 1996). Fe(III) toxicity has been detected in batch and chemostat studies of Fe(II) oxidation by \textit{At. ferrooxidans} and chemostat studies of \textit{L. ferrooxidans}. These show that Fe(III) competitively inhibits Fe(II) oxidation (Boon et al., 1999a, b; van Scherpenzel et al., 1988). The presence of 358 mM (20 g l^{-1}) Fe(III) has been shown to inhibit Fe(II) oxidation and cause cell lysis in \textit{At. ferrooxidans} (Shrihari & Gandhi, 1990) and its growth is inhibited by \geq 36 mM (2 g l^{-1}) Fe(III) (Curutchet et al., 1992). In another study of suspended cells of \textit{At. ferrooxidans}, Fe(III) inhibition of Fe(II) oxidation occurred above 107 mM (6 g l^{-1}). However, when growing as a biofilm the cells were unaffected by 251 mM (14 g l^{-1}) Fe(III) (Karamanev & Nikolov, 1988). Also, \textit{L. ferrooxidans} and \textit{A. cryptum} have been shown to be resistant to 500 and 300 mM Fe(III), respectively (Johnson et al., 1992). While acidophiles often tolerate elevated concentrations of iron, no information is available regarding iron resistance mechanisms.

**Other metals**

Many other metals are found in acidic environments, including uranium, molybdenum and chromium. A number of different acidophiles have been isolated from locations containing these metals, and the following species have been isolated from environments containing uranium: \textit{At. ferrooxidans}, \textit{L. ferrooxidans}, \textit{Tn. cuprina}, cells resembling \textit{Sulfobolus/Acididans} spp., \textit{Acidiphilium} spp. and other heterotrophic iron-oxidizing acidophiles (reviewed by Tuovinen & Bhatti, 1999). In many cases, toxicity studies have been carried out with these metals on acidophiles. Examples are U(VI) toxicity via inhibition of Fe(II) oxidation and CO_{2} fixation (Dispiroto et al., 1983) and \textit{At. ferrooxidans} growth inhibition by >15 mM Cr(III) (Wong et al., 1982).
A number of mechanisms of resistance to these metals have been identified in acidophiles, including the mode of toxicity to and putative Mo(V) resistance mechanism in *At. ferrooxidans* strain Funis 2-1 (Yong et al., 1997). Mo(VI) is chemically reduced by Fe(II), and the Mo(V) formed binds to the plasma membrane, probably to the cytochrome-c oxidase (lowering its activity), inhibiting Fe(II) oxidation and consequently growth. Resistance is based on a combination of a cytochrome-c oxidase that is tolerant to higher concentrations of Mo(V) and on an Mo(V)-oxidizing activity sixfold greater than that detected in the sensitive *At. ferrooxidans* strain AP19-3. A chromium resistance mechanism has been identified in *At. ferrooxidans* and *Acidiphilium rubrum* whereby they take up and subsequently precipitate a Cr(VI)-rich compound on the surface of the cell, increasing chromium tolerance (Baillet et al., 1998; Itoh et al., 1998).

**Future directions**

To date, virtually all of the research into acidophile metal resistance has focused on toxicity studies and adaptation/induction of laboratory and natural cultures to increasing concentrations of metals. It is clear that there is a lack of completed investigations into acidophile metal resistance mechanisms, thus there are many unanswered questions. However, it is apparent that these organisms cope with the highest levels of toxic soluble metals and much could be learned about general biological resistance mechanisms. Much of the effort has been focused on *At. ferrooxidans*; however, more recent molecular ecological investigations indicate that other bacteria and archaea are numerically dominant in acid-leaching environments (Bond et al., 2000b). Compilation of such molecular surveys of acid-leaching environments would indicate relevant microorganisms on which to focus investigations.

At this time, the genomes of the following six acidophiles have been sequenced: *At. ferrooxidans*, 'Fp. acidarmanus', *Tp. acidophilum*, *Tp. volcanium*, *S. sulfataricus* and *S. tokodaii*. This opens the door to a whole new area of research that can be carried out on acidophiles. As can be seen from the unpublished results presented in this review, it is now possible to putatively identify genes associated with metal resistance by *in silico* studies of the genomes, by searching for analogues of known metal resistance genes. A further strategy that may be employed to elucidate metal resistance systems in acidophilic micro-organisms is experimentally based proteome and transcriptome investigations. These investigations could examine differential expression for detection of 'metal resistance' genes expressed as a response to the addition of metals. Such techniques are increasingly relevant as microbial genome sequence data accumulate.

Presently uncultured acidophiles are detected as important constituents of bioleaching microbial communities (Bond et al., 2000b). Investigation of the metal resistance of these uncultured microbes remains a major challenge. In comparison to other natural microbial communities, acidic environments have a reduced biodiversity as indicated by molecular phylotypes (Bond et al., 2000b). Such microbial systems perhaps lend themselves to investigation by a mixed-community genome analysis. Another approach is to screen for gene function directly from environmental DNA. This approach has been termed metagenomics and has enormous potential (Rondon et al., 2000).

**Concluding remarks**

Acidophiles must be resistant to heavy metals due to the selective pressures that metal-rich acidic environments pose. The lack of understanding within this area is surprising given the importance of obligate acidophiles in the partitioning of metal species between aqueous and terrestrial environments and the role that these organisms play as intermediates in biogeochemical cycling. Some of these gaps are highlighted from the examples drawn from the data and sequenced genomes quoted in this review. Many of the experimental investigations have been conducted on a very limited number of acidophiles. Clearly investigations of a broader range of acidophiles are required to gain a fuller understanding. The prospect of using the genomic information and expression studies is a fruitful avenue of investigation and much could be discovered using this approach. A large portion of the microbial diversity in these acidic metal-rich environments is yet to be cultured and a further challenge will then be to discover the secrets of metal resistance of uncultured microorganisms, possibly employing metagenomics. Another possibility is to perform similar genomic and expression studies directly on mixed-culture environmental samples. Many answers to acidophile metal resistance should be forthcoming by application of these technologies.

**References**


