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SHORT COMMUNICATION

Metals tolerance in moderately thermophilic isolates from a spent copper sulfide heap, closely related to *Acidithiobacillus caldus*, *Acidimicrobium ferrooxidans* and *Sulfobacillus thermosulfidooxidans*

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Abstract Selective enrichments enabled the recovery of moderately thermophilic isolates with copper bioleaching ability from a spent copper sulfide heap. Phylogenetic and physiological characterization revealed that the isolates were closely related to *Sulfobacillus thermosulfidooxidans*, *Acidithiobacillus caldus* and *Acidimicrobium ferrooxidans*. While isolates exhibited similar physiological characteristics to their corresponding type strains, in general they displayed similar or greater tolerance of high copper, zinc, nickel and cobalt concentrations. Considerable variation was found between species and between several strains related to *S. thermosulfidooxidans*. It is concluded that adaptation to metals present in the bioleaching heap from which they were isolated contributed to but did not entirely explain high metals tolerances. Higher metals tolerance did not confer stronger bioleaching performance, suggesting that a physical, mineralogical or chemical process is rate limiting for a specific ore or concentrate.

Keywords *Sulfobacillus thermosulfidooxidans* · *Acidithiobacillus caldus* · *Acidimicrobium ferrooxidans* · Bioleaching · Heavy metal tolerance

Introduction

The microbial contribution to mineral sulfide oxidation was discovered more than 50 years ago [2]. Since then, researchers have been investigating the possibilities of exploiting bacterially enhanced oxidation reactions for the bioleaching of sulfide minerals at industrial scale [22]. However, relatively few acidophiles have been identified that can tolerate the metals concentrations typically experienced in acid mine drainage environments and bioleaching reactors [6, 22].

Elemental concentrations in acidic drainage from mines or other sulfide rich deposits vary enormously depending on the mineral composition of the deposit and the acidity of the water [4, 13]. Bioleaching operations, particularly managed sulfide heaps, constitute extreme examples of 'acid drainage'. Typically, elemental concentrations in heap or dump leachates are 2–6 g/L Cu, 2–5 g/L Ni, <0.1 g/L Co and up to 23 g/L Zn; iron concentrations may exceed 20 g/L. Agitated tank reactors for the processing of sulfide concentrates constitute more extreme environments: up to 19 g/L Cu, 23 g/L Ni, 65 g/L Zn, 3 g/L Co, 14 g/L As and 40 g/L Fe [7, 10, 14].

Acidophilic microorganisms that thrive in such extreme conditions encounter selective pressure to develop resistance mechanisms to the heavy metals that will provide them with a competitive selective advantage [3]. This will result in a tolerance of much higher concentrations of metallic ions than seen in neutrophiles. Adaptation through

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exposure to a metal is likely to be responsible for the considerable strain variation that has been reported [9, 19, 20].

It has been shown that the oxidation of iron(II) or reduced sulfur species by acidophilic microorganisms is inhibited by the presence of heavy metals [1, 3, 9, 17]. Heavy metals have been shown to block vital enzymes, inhibit transport systems and disrupt cell membrane integrity [12]. The exact impact of heavy metals on cellular processes appears to vary between microbial genera. Both copper and zinc have been shown to inhibit the growth and iron(II) oxidation by *Sulfobacillus thermosulfooxidans* via competitive inhibition [21], whereas a non-competitive effect for a range of heavy metals on iron(II) oxidation by *Acidithiobacillus ferrooxidans* has been demonstrated [1].

In this paper we identify ten isolates enriched from a spent chalcocite heap [15], which exhibited strong ability to enhance copper extraction from a chalcopyrite concentrate in controlled bioleaching tests. Differences in their physiological characteristics and heavy metals tolerances were examined using screening tests and these were compared with the characteristics of their most closely related type strains (determined under similarly controlled conditions).

Materials and methods

Microbial enrichment, physiological characterization and metals tolerance

The mixed chalcocite–chalcopyrite test heap [15] was systematically sampled at the end of its life cycle and portions of the ore from selected locations were used to enrich bioleaching microorganisms [8]. The resulting isolates were screened for growth on a variety of substrates, in a media of varying compositions and pH, in the temperature range 30–60°C [8]. Screening for metals tolerance was undertaken using a mixed iron(II)–tetrathionate medium with yeast extract. The enhancement of metals extraction was examined for the mineral sulfides, chalcopyrite, sphalerite and pentlandite, in medium containing 0.1 g/L yeast extract, as described previously [24]. Two spore-forming bacillus isolates in addition to those sequenced were included in the bioleaching tests. Subsequent to isolate identification, the type strains *S. thermosulfidooxidans* DSM 9293^T, *At. caldus* DSM 8584^T and *Am. ferrooxidans* DSM 10331^T were subjected to the same test protocols to generate directly comparable physiological and bioleaching data.

Phylogenetic characterization

The selected isolates were grown in basal salts medium [8] at either 45 or 30°C (for N39-30-02 and N39-30-03 only) and DNA was extracted [25]. The 16S rRNA gene was amplified as previously described [11] and purified using QIAquick® PCR Purification Kit protocol (QIAGEN Pty Ltd) according to the manufacturer's instructions. The PCR product was sequenced using the ABI protocol for sequencing using a Big Dye Terminator v3.0 Cycle Sequencing Kit in accordance with the manufacturer's directions. Sequence data were assembled using the Gene Tool (Lite Version 1.0) program and aligned with sequences of selected type strains obtained from GenBank using CLUSTALW version 1.8 [18]. A phylogenetic tree was constructed by the neighbor-joining method [16] using PHYLIP Version 3.6a3 [5].

These sequence data have been submitted to the GenBank database and assigned accession numbers EF199628, EF199986 to EF199991 and EU499918 to EU499920.

Results

Iron(II) and sulfur oxidizing bacteria with bacillus morphology were recovered from enrichments at 30, 45 and 50°C. No isolates were recovered from enrichments at 60°C. Comparative sequence analysis of the 16S rRNA gene in selected bacterial isolates indicated high levels of similarity with *S. thermosulfidooxidans* (seven isolates), *At. caldus* (two isolates), and *Am. ferrooxidans* (one isolate). A phylogenetic tree showing the relationship between the ten isolates and associated type strains is shown in Fig. 1. Physiological screening of the selected indigenous isolates revealed a variety of profiles but all showed similar physiological traits to their corresponding type strains (data not shown). When compared for their ability to enhance the extraction of zinc, copper or nickel from sphalerite, chalcopyrite and pentlandite concentrates, the *Sulfobacillus* and *Acidithiobacillus*-like isolates performed similarly to their type strains. The *Acidimicrobium*-like isolate grew very poorly in sulfide bioleaching tests without pH control, but performed better when the pH was controlled at pH 1.8.

The greatest variability was observed for metals tolerances both between species and between isolates of the same species. In general the isolates grew well in the presence of 5–50 g/L copper (Table 1). The isolates also grew well in the presence of 5–50 g/L zinc. Of the metals tested, nickel and cobalt were the least tolerated by the isolates.

The seven *S. thermosulfidooxidans*-like isolates were as tolerant as the type strain if not more tolerant of copper, zinc and cobalt. N39-45-02 and *At. caldus* showed similar tolerances except for copper, where the indigenous isolate could grow in the presence of up to 10 g/L copper. Both were extremely tolerant of zinc (>50 g/L). The *At. caldus*-like isolate N39-30-02 was, in addition, tolerant of both nickel and cobalt. The distinguishing feature of the *Am. ferrooxidans*-like isolate N39-30-03 was its strong adaptation to high metal concentrations, as evidenced by tolerance of

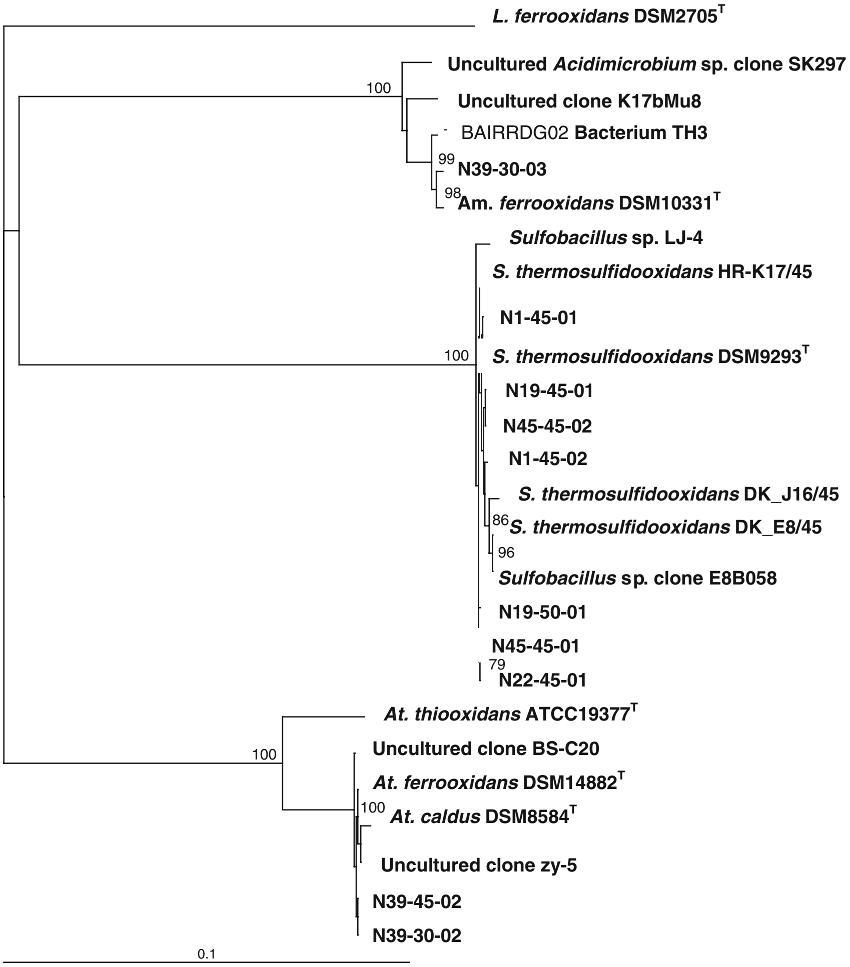

Fig. 1 Rooted neighbor joining tree based on 16S rDNA sequences showing the relationship of isolates obtained from the Nifty copper mine and their associated type stains. Bootstrap values after 1,000 iterations shown above 70% only are given at branching points. *Scale bar* represents the number of inferred nucleotide substitutions per site. Sequences of the following species and strains were used to construct the tree: *At. caldus* DSM 8584^T (Z29975), *At. ferrooxidans* DSM 14882^T (AJ278718), *At. thiooxidans* ATCC 19377^T (Y11596), *Am. ferrooxidans* DSM 10331^T (U75647), *Leptospirillum ferrooxidans* DSM 2705^T (X86776), *S. thermosulfidooxidans* DSM 9293^T (AB089844), *S. thermosulfidooxidans* strain HR-K17/45 (EU419199), *S. thermosulfidooxidans* strain DK_E8/45 (EU419198), *S. thermosulfidooxidans* strain DK_J16/45 (EU419197), *Sulfobacillus* sp. LJ-4 (DQ673613), *Sulfobacillus* sp. clone E8B058 (DQ455581), uncultured bacterium clone BS-C20 (DQ661642), uncultured bacterium clone zy-5 (EF672753), BAIRRDG02 Bacterium TH3 (M79434), uncultured bacterium clone K17bMu8 (EU419133) and uncultured *Acidimicrobium* sp. clone SK297 (AY882846)

up to 50 g/L copper or zinc, 40 g/L nickel or 45 g/L cobalt compared with *Am. ferrooxidans* DSM 10331^T (Table 1). In terms of metals tolerances, N39-30-03 out-performed all other isolates and the three type strains.

Iron tolerance was estimated indirectly from ferrous ion oxidation screening tests undertaken as part of the physiological characterization of the isolates. Tolerance to extreme iron concentrations was not investigated. During these tests, three isolates grew in medium which initially contained 4.4 g/L Fe as ferrous ion and were still active after 2 weeks. Iron concentrations up to 3.5 g/L were experienced by the isolates in 4-week bioleaching tests.

The isolates were tested and compared for their ability to enhance the extraction of zinc, copper or nickel from sphalerite, chalcopyrite and pentlandite concentrates, respectively. No correlation was observed between metals tolerance and the enhanced ability to leach the metal sulfides (Fig. 2). Excepting iron (see above), maximum metal concentrations in bioleach test liquors were lower than the maximum tolerated concentrations determined in screening tests.

Discussion

The isolates obtained from the spent-heap enrichments show a high degree of similarity to the known, moderately thermophilic bioleaching bacteria *At. caldus*, *S. thermosulfidooxidans* and *Am. ferrooxidans*. Data obtained on metals tolerances for the indigenous isolates and their

Table 1 Highest metal concentration in solution in which bacterial growth was observed in metals tolerance screening tests

Isolate	Metal tolerance (g/L)				
	Fe	Cu	Zn	Ni	Co
N1-45-01	2.5[a]	15	35	5	5
N1-45-02	3.2[a]	35	13	5	5
N19-45-01	≥4.4	50	50	5	<5
N19-50-01	2.5[a]	50	35	10	<5
N22-45-01	4.4	≥50	≥50	<5	<5
N45-45-01	2.8[a]	20	10	5	5
N45-45-02	2.5[a]	35	10	5	10
S. thermosulfidooxidans DSM 9293^T	4.4	19	13	9	1.5
N39-45-02	2.0[b]	10	≥50	<5	5
N39-30-02	2.4[b]	5	50	50	50
At. caldus DSM 8584^T	1.9[b]	1.5	65	2.3	5.9
N39-30-03	≥4.4	≥50	≥50	40	45
Am. ferrooxidans DSM 10331^T	4.4	0.6	3.3	1.5	0.6

[a] Maximum concentration in bioleach test solutions

[b] N39-45-02, N39-30-02 and *At. caldus* growth on reduced sulfur species in the presence of soluble iron

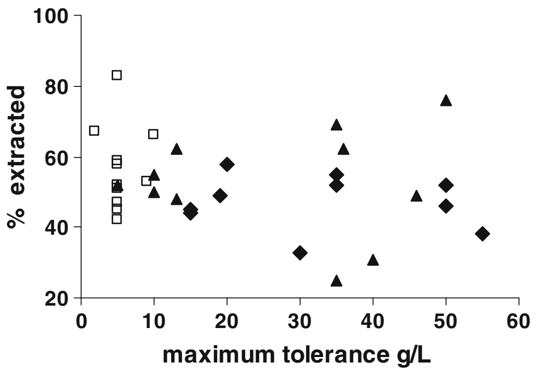

Fig. 2 The percent metals extractions at 30–35 days bioleaching as a function of the tolerance of the *S. thermosulfidooxidans*-like isolates to the particular metals. *Filled diamond* Cu, *open square* Ni, *filled triangle* Zn

respective type strains (Table 1) therefore supplement the few published data for moderately thermophilic bacteria.

Resistance to high copper concentrations by the isolates was anticipated due to their adaptation to the copper sulfide ore (1.2% Cu) from which they were isolated. In general, the isolates grew well in the presence of 5–50 g/L copper, many being more tolerant than their type strains, a result that is consistent with adaptation. Surprisingly, the isolates also grew well in the presence of zinc. Analysis of the ore from which the isolates were enriched showed that it contained 0.01% Zn as sulfide. However, it is not clear whether the tolerance to zinc is a natural attribute of these species or acquired through adaptation.

While high tolerance to copper and zinc in the indigenous strains may be attributed to the extreme environment from which they were sourced, this particular ore did not contain significant nickel or cobalt. It is not surprising, therefore, that nickel is the least well tolerated by most of the isolates. Only two isolates, N39-30-03 and N39-30-02 exhibit high tolerance (40 and 50 g/L, respectively). Their corresponding type strains, *At. caldus* DSM 8584^T and *Am. ferrooxidans* DSM 10331^T do not grow well in the presence of nickel (1.5 and 2.3 g/L, respectively), suggesting an adaptive response in the isolates. The *Sulfobacillus*-like strains and the type strain *S. thermosulfidooxidans* DSM 9293^T tolerate low nickel concentrations.

The *Sulfobacillus* -like isolates show considerable strain variation in their heavy metals tolerances (Table 1). It has previously been established that copper and zinc inhibits both growth and iron(II) oxidation by a strain of *S. thermosulfidooxidans* [21] and considerable variation in metals tolerances between the four recognized *Sulfobacillus* species has been shown [24]. However, there have been no previous reports of strain variation in metals tolerance in the moderately thermophilic bioleaching bacteria tested in this study.

The most likely methods of processing low-grade base metal sulfide ores are heap or dump leaching [22]. All of the isolates have been shown to tolerate copper, nickel and cobalt at concentrations which may be experienced in heap leaching environments. However, the low tolerance to iron by all isolates and their type strains seems to preclude their activity in heap leach operations. Clearly, adaptation and/or physical protective devices such as selection of less exposed sites for attachment must play roles because the three species have been isolated from heaps of low-grade sulfide ores and also agitated tank reactors for the processing of sulfide concentrates.

Further, superior tolerance to metals fails to result in greater metals extraction efficiency from mineral sulfides (Fig. 2). None of the metals show a trend toward higher extraction when strains with high tolerance are used in the standardized tests. This result suggests that other factors control the rates and extent of metals extraction from sulfide minerals and that bacterial activity is not rate limiting except if the bacteria are inactive (not present or not growing).

Based on the results obtained for the moderately thermophilic isolates and their type strains *S. thermosulfidooxidans* DSM 9293^T, *At. caldus* DSM 8584^T and *Am. ferrooxidans* DSM 10331^T, it is concluded that tolerance to high metals concentrations confers the advantage of survival in extreme environments without a corresponding enhanced capability in metals extraction from mineral sulfide concentrates. The results indicate that metals tolerances can vary significantly between species and between strains of the same species as has previously been reported ([23] and references therein).

Given the differences between exhibited metals tolerances and metals concentrations in heap or agitated-tank leachates, an adaptive response by these bacteria is necessitated to enable them to grow in extreme, bioleaching environments.

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