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Metal Resistance and Lithoautotrophy in the Extreme Thermoacidophile *Metallosphaera sedula*

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Archaea such as *Metallosphaera sedula* are thermophilic lithoautotrophs that occupy unusually acidic and metal-rich environments. These traits are thought to underlie their industrial importance for bioleaching of base and precious metals. In this study, a genetic approach was taken to investigate the specific relationship between metal resistance and lithoautotrophy during biotransformation of the primary copper ore, chalcopyrite (CuFeS₂). In this study, a genetic system was developed for *M. sedula* to investigate parameters that limit bioleaching of chalcopyrite. The functional role of the *M. sedula* copRTA operon was demonstrated by cross-species complementation of a copper-sensitive *Sulfolobus solfataricus* copR mutant. Inactivation of the gene encoding the *M. sedula* copper efflux protein, copA, using targeted recombination compromised metal resistance and eliminated chalcopyrite bioleaching. In contrast, a spontaneous *M. sedula* mutant (CuR1) with elevated metal resistance transformed chalcopyrite at an accelerated rate without affecting chemoheterotrophic growth. Proteomic analysis of CuR1 identified pleiotropic changes, including altered abundance of transport proteins having AAA-ATPase motifs. Addition of the insoluble carbonate mineral witherite (BaCO₃) further stimulated chalcopyrite lithotrophy, indicating that carbon was a limiting factor. Since both mineral types were actively colonized, enhanced metal leaching may arise from the cooperative exchange of energy and carbon between surface-adhered populations. Genetic approaches provide a new means of improving the efficiency of metal bioleaching by enhancing the mechanistic understanding of thermophilic lithoautotrophy.

Nearly 80% of current global copper reserves are comprised of low-quality metal ores (10, 25, 44). Consequently, the need for cost-efficient extraction methods has promoted interest in the use of microbe-based processing. Bioleaching is an established approach for the extraction of base and precious metals from sulfidic ores (30, 34, 35, 43). Bioleaching using thermophilic microbes is of particular importance for certain metals. In the case of copper, elevated temperatures produced naturally in heaps overcome recalcitrant extraction due to surface passivation while chemical reaction rates are accelerated (29, 30). A critical disadvantage of bioleaching is the amount of time required for metal solubilization, often spanning years (22, 38). Therefore, improved metal recovery must include factors that accelerate this process, particularly those inspired by biotechnologic approaches (48).

Thermoacidophilic archaea include taxa that are lithoautotrophic and unusually metal resistant (3, 19). These organisms are native to pyritic or sulfur-rich geothermal habitats and are used to recover base and precious metals from low-quality sulfidic minerals through bioleaching processes (31). In the bioleaching process, both direct and indirect leaching mechanisms have been proposed that convert metals to soluble forms (33, 40). Metal release may occur via metabolic oxidation of the sulfur and iron component of these minerals, thereby releasing other complexed metals, or by direct metal oxidation (41). Recovery rates of copper bioleached from chalcopyrite ranged from 10 to 25% (16), and the recently discovered *Metallosphaera cuprina* was shown to mobilize 10.6% of total copper when grown on chalcopyrite (23). Elucidation of the genome sequence of several biomining organisms (5, 24, 45) along with transcriptomic analyses (4, 20) supports investigations into strategies that underlie lithoautotrophic metabolism.

Among archaea, the distribution of putative copper resistance genes is broad. Many archaeal genomes contain *copA* (P-type ATPase) copper efflux transporters, with the most well-characterized example being found in the hyperthermophilic sulfate reducer *Archaeoglobus fulgidus* (1, 13). Multiple mechanisms of copper resistance also have been identified in *Sulfolobus solfataricus*, including a copper efflux system consisting of *copA* and *copB* (P-type ATPase), *copR* (regulator), and *copT* (metallochaperone) (9, 11, 46, 47). An inorganic polyphosphate symport system identified in the related *Sulfolobus metallicus* has also been predicted to play a critical role in copper resistance (36). Additional predictions concerning the identity of archaeal metallochaperones have been made based on the occurrence of a CxCxC motif and extended variants combined with proximity to metal efflux transporters (12).

*Metallosphaera sedula* is a lithoautotrophic archaeon originally isolated from a solfataric field in Italy that grows optimally at 70 to 75°C over a pH range of 1 to 4.5 (18). *M. sedula* differs greatly from its relatives, *S. solfataricus* and *S. acidocaldarius*, because the latter organisms are strict chemoheterotrophs with compatible genetic systems (7, 26). In contrast, genetic-based investigations have not yet been used to study lithoautotrophy. Autotrophy in *M. sedula* uses a recently discovered pathway for carbon fixation (6). Stable carbon isotope fractionation has shown that bicarbonate HCO₃⁻ is used as an inorganic carbon source through the 3-hydroxypropionate/4-hydroxypropionate pathway (6, 32). *M. sedula* is also considerably more resistant to metals than chemoheterotrophic *Sulfolobus* species. For example, cupric ion [Cu(II)] resistance is 20 times greater (18, 46). To gain a
better understanding of the interplay between metal resistance, autotrophy, and lithotrophic metabolism, a genetic system was developed for *M. sedula* and used to evaluate parameters governing the efficiency of copper bioleaching.

**MATERIALS AND METHODS**

Archaeal strains, cultivation, and microscopy. Cell lines used in this study are presented along with their genotypes, origins, and construction details (Table 1). *Metallosphaera sedula* (DSM 5348T) was grown in a basal salts medium (2), adjusted to pH 2.0 at 75°C, in screw-cap flasks with aeration in orbital baths or in glass screw-cap test tubes placed in 150-tube rotary drum agitators mounted in forced-air incubators with external DC motors. Chemoheterotrophic growth used tryptone at 0.05% (wt/vol). A solid complex medium was prepared using basal salts medium mixed with 0.6% (wt/vol) gelrite (Kelco) and supplemented with 0.05% (wt/vol) tryptone. Lithoautotrophic growth used the naturally occurring minerals pyrite (Sargent-Welch) and chalcopyrite (VWR or Alfa Aesar), which had been ground and baked at 200°C for 2 days until sterile. Barium carbonate (witherite) was obtained from Sigma and autoclaved at 120°C for 30 min to sterilize. Planktonic growth was monitored by light adsorption at a wavelength of 540 nm. Epifluorescence microscopy and scanning electron microscopy (SEM) were performed as described previously (5, 37).

**Strain construction.** Mutations in uracil biosynthesis result in uracil auxotrophy and allowed the use of uracil biosynthetic genes as selectable markers for genetic manipulations. To recover such mutants, pyrimidine analogs, including 5-fluoroorotic acid (5-FOA), were tested on a solid medium and found to severely reduce cell-plating efficiencies. Resistance to 5-FOA in yeast and in the related archaea *Sulfolobus acidocaldarius* (17) and *Sulfolobus solfataricus* (28) has been shown to occur in pyrimidine biosynthetic genes, notably orotidine pyrophosphorylase (*pyrE*) and orotidine decarboxylase (*pyrF*). Spontaneous mutation frequencies for *M. sedula* to 5-FOA resistance were evaluated on solid media containing the analog with and without addition of uracil, and 5-FOA resistance was measured at a frequency of 1/10⁶ cells plated. The *pyrEF* genes of one purified 5-FOA-resistant isolate that had been confirmed as a uracil auxotroph were sequenced, and the *pyrE* gene was mutated. *M. sedula* transformation was performed as described for *S. solfataricus* (26, 42). Genetic selections for chromosomal recombination were developed using the *pyrE* analog with and without addition of uracil, and 5-FOA resistance was measured at a frequency of 1/10⁶ cells plated. The *pyrEF* genes of one purified 5-FOA-resistant isolate that had been confirmed as a uracil auxotroph were sequenced, and the *pyrE* gene was mutated. *M. sedula* transformation was performed as described for *S. solfataricus* (26, 42). Genetic selections for chromosomal recombination were developed using the *pyrE*

**TABLE 2 Primers**

<table>
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<tr>
<th>Primer name</th>
<th>Sequence</th>
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<tr>
<td>Mse490F-SphI</td>
<td>5’ AGCTGCATGCAAGGAAAGATGTACTCTT CTGC 3’</td>
</tr>
<tr>
<td>Mse490R-SphI</td>
<td>5’ AGCTGCATGCGATTTCTTGTGAGATACAC GTGC 3’</td>
</tr>
<tr>
<td>MpyrE-NgoMIV</td>
<td>5’ AGCTGCGGCCCAAGGTTGCTGCTAACCCTCGC 3’</td>
</tr>
<tr>
<td>MpyrER-NgoMIV</td>
<td>5’ AGCTGCGGCCCAAGGTTGCTGCTAACCCTCGC 3’</td>
</tr>
<tr>
<td>Mse490RTF</td>
<td>5’ CCACTGAAAGGCACAAAGGAAA 3’</td>
</tr>
<tr>
<td>Mse490NSTR</td>
<td>5’ TATCGGTATCTCAGGCCTTCG 3’</td>
</tr>
</tbody>
</table>

**FIG 1** *M. sedula* copRTA analysis by heterologous complementation. (A) copRTA locus. (B) pJlacS carrying *M. sedula* copRTA. (C) Copper resistance of pJlacS *M. sedula* copRTA in the *S. solfataricus* copR mutant. Strains were the *S. solfataricus* copR mutant strain (PBL2115; closed circles) and the *S. solfataricus* copR mutant complemented with pJlacS *M. sedula* copRTA (PBL2116; open circles). Cultures were treated (arrow) with 0.75 mM Cu(II) (final concentration).
gene. PCR amplicons of \textit{pyrE} and the negative-control \textit{pyrF} were transformed by electroporation into the \textit{pyrE1} \textit{M. sedula} mutant (Table 2). Transformed cells were grown in dilute tryptone medium lacking uracil, thereby allowing selection for prototrophs. Only \textit{pyrE} mutant cells, transformed with the \textit{pyrE} gene, grew normally in the absence of uracil supplementation. Individual transformed cells were isolated and genotyped by colony PCR combined with MsII restriction analysis of the \textit{pyrE} PCR amplicon. The \textit{copA} knockout strain was constructed by successive single crossovers using a plasmid-encoded disruption construct (pBN1260). First, the \textit{copA} open reading frame (ORF) was amplified using primers Mse490F-SphI and Mse490R-SphI and then was cloned into pUC19 at the SphI site. \textit{M. sedula pyrE} then was cloned into pUC19：\textit{copA} (PB1260) at an NgoMIV site, resulting in plasmid pBN1260, which was then transformed into strain PBL4001 with selection for uracil prototrophy. For complementation studies in \textit{S. solfataricus}, the \textit{M. sedula copRTA} operon was cloned into plasmid pBN1078 at the SacII site to make plasmid pBN1126. The \textit{copR}：\textit{lacS} mutation in \textit{S. solfataricus} strain PBL2090 (46) was transferred into strain PBL2091 (termed the \textit{ΔpyrEF} strain) by the allele transfer method (26), and the new strain was used as the parental strain for the transformation of the \textit{M. sedula copRTA} operon located on plasmid pBN1126. A spontaneous copper-resistant strain (CuR1) was isolated by repeated passage of \textit{M. sedula} in media supplemented with increasing concentrations of CuSO$_4$. Passage involved one cycle with 100 mM CuSO$_4$ supplementation and then three cycles with 200 mM CuSO$_4$ supplementation. Cells were transferred in mid-exponential growth phase. The CuR1 isolate was purified by single-colony isolation followed by retesting metal resistance relative to the wild-type (WT) strain.

**qRT-PCR.** Quantitative reverse transcription-PCR (qRT-PCR) using SYBR-I green and a real-time PCR instrument (Eppendorf Mastercycler) as described previously (27). RNA was extracted as described previously (8) and treated to remove DNA by addition of 1 U of DNase I (Fermentas) per μg of total RNA at room temperature for 15 min and then neutralized with 2 μl of 25 mM EDTA and incubated at 70°C for 10 min. cDNA synthesis used 20 pmol of PCR antisense primer, 20 mM deoxynucleoside triphosphate (dNTP) mix (Invitrogen), and 200 U of Moloney-murine leukemia virus RT (Fermentas) for 60 min at 37°C. Synthesized cDNA was subjected to standard PCR and analyzed using 2% (wt/vol) Tris-borate-EDTA agarose gels. Primers used for qRT-PCR were Mse490RTF and Mse490NSTR.

**Proteomics.** Cell suspensions were prepared using intermittent sonication and then analyzed by two-dimensional (2D) SDS-PAGE, and protein identities were determined by tandem mass spectrometry (MS/MS) electrospray analysis using material excised from SDS-polyacrylamide gels (15, 49). Gel slices were infused with trypsin to digest the protein, and the resulting peptides were recovered, separated by capillary electrophoresis prior to MS/MS analysis, and then identified by local BLAST against the \textit{M. sedula} proteome. Fold changes in spot intensity were determined using Phoretix 2D Evolution (Nonlinear Dynamics).

**ICP-MS.** To determine the extracellular concentrations of copper, culture samples were clarified by centrifugation. Samples of the resulting
supernatants were analyzed by inductively coupled plasma-MS (ICP-MS) using an Agilent ICP-MS 7500cx. A certified copper reference standard was used for sample normalization. All values are averages from triplicate samples.

RESULTS

Functional identity of M. sedula copRTA. Metallosphaera sedula is unusually resistant to several metals, including copper (18). Genomic features responsible for this trait include two putative copper-translocating ATP-dependent exporters (copA and copB). A similar situation occurs in the related species Sulfolobus solfataricus, though here only copA has been shown to be copper inducible (11, 46) and the copR transcription factor is transcribed in the opposite direction relative to the situation in M. sedula (Fig. 1A). It has been shown previously that the S. solfataricus copR mutant can be complemented using the S. solfataricus copR gene (46). To verify the functional role and expression of the M. sedula copRTA locus, the region was expressed in an S. solfataricus mutant lacking copper resistance due to a gene disruption of the copR transcription factor gene (46). M. sedula copRTA was cloned into S. solfataricus plasmid pJ1 (pBN1078), a derivative of pJlacS (7) lacking thsAp::lacS. The resulting plasmid (pBN1126) (Fig. 1B) was then transferred into the S. solfataricus copR mutant by transformation followed by selection for uracil prototrophy and the isolation of a clonal population on a solid medium. The copper resistance of the S. solfataricus plasmid transformant (strain PBL2116) was compared to that of the parental strain (PBL2115) in liquid culture under chemoheterotrophic conditions (Fig. 1C). In response to a 0.75 mM cupric ion [Cu(II)] challenge, a concentration shown previously to induce S. solfataricus cop expression (46), heterologous expression of the M. sedula copRTA locus in the S. solfataricus copR mutant complemented copper sensitivity in an inducible fashion. After induction, the complemented strain gained the ability to grow at 10 mM Cu(II), a concentration nearly twice the level observed for growth of wild-type M. sedula (9, 47) and ppx (36). To better understand the importance of copA, it was necessary to develop a genetic system in M. sedula, and this was pursued in part because cross-species gene expression was successful.

Role of the copA gene in copper resistance of M. sedula. While the M. sedula copRTA locus conferred copper resistance when expressed in S. solfataricus, the level produced was more than 8-fold less than the resistance level of wild-type M. sedula (76 mM). This suggested that there are additional factors controlling metal resistance that are native to M. sedula, such as copB (9, 47) and ppx (36). To better understand the importance of copA, it was necessary to develop a genetic system in M. sedula, and this was pursued in part because cross-species gene expression was successful. Mutations in pyrimidine biosynthesis result in uracil auxotrophy and allow the use of pyrimidine biosynthetic genes as selectable markers for genetic manipulations. Spontaneous mutations mapping to the M. sedula pyrE gene were recovered by following the procedures established for other thermoacidophilic archaea (14, 21). One isolate, termed PBL4001, had a −1 deletion mutation in pyrE at nucleotide (nt) 243 relative to the start codon that was called pyrE1 (Fig. 2A). This mutation shifted the reading frame and resulted in a premature stop codon (TAG) at nt 260, producing a C-terminal truncation and shortening the normal protein from 191 to 86 residues. This mutation also resulted in the loss of one of two restriction sites in pyrE for MssI (Fig. 2B and C). Complementation of the pyrE1 mutation used plasmid pJlacS (pBN1090) that carried the S. solfataricus pyrEF genes (7). Recovery of plBN1090 transformants of the M. sedula pyrE1 mutant (PBL4001) resulted from selection for uracil prototrophy, while the continued presence of the plasmid was determined by the expression of the S. solfataricus lacS gene, as indicated by hydrolysis of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) applied to colonies and subsequent formation of a blue color. Having verified that pyrE1 was responsible for the uracil auxotrophic phenotype, repair of this mutation by chromosomal homologous recombination was monitored by changes in pyrE-linked restriction polymorphisms. Purified putative recombinants were characterized by PCR amplification of pyrE followed by restriction analysis (Fig. 2D) to distinguish between mutant and wild-type pyrE alleles. MssI restriction of the wild-type pyrE amplicon produced three fragments of 420, 237, and 244 nt that comigrate, while restriction of the pyrE1 mutant amplicon produced two fragments of 657 and 244 nt. Incompletely digested amplicons are also evident in some lanes as faint large bands. Colony PCR combined with MssI restriction was used as a screen to identify pyrE recombinants. pyrE recombinants exhibited a

![FIG 3 Construction and growth of the M. sedula copA mutant and the supra-normal copper-resistant isolate CuR1. (A) Schematic of the copA disruption construct. (B) PCR genotyping of the copA mutant. Lane 1, recombinant copA allele. Lane 2, wild-type copA allele. Lane 3, copA::pyrE plasmid. Lane 4, no DNA. (C) Heterotrophic growth at 75 mM Cu(II). OD₅₄₀, optical density at 540 nm. (D) Heterotrophic growth at 200 mM Cu(II). Shown are CuR1 (open circles), the wild type (closed squares), and the copA mutant (inverted closed triangles).](image-url)
wild-type pyrE restriction pattern, as indicated by restoration of the second MslI restriction site. Confirmation of homologous recombination at pyrE provided the basis for targeted inactivation of other M. sedula genes. The M. sedula copA gene (Msed_0490) was then disrupted by insertion of the M. sedula wild-type pyrE gene following transformation using a circular nonreplicating (suicide) construct (Fig. 3A). The resulting purified isolate was then geno-
type by restriction analysis and DNA sequencing of the copA locus (Fig. 3B). The level of copper resistance (MIC) of the copA mutant was tested under heterotrophic growth conditions relative to the wild-type strain and was found to have been reduced from 76 to 40 mM Cu(II). While this demonstrated a larger role for copA than in a tryptone medium for 14 days. No carbon source was added to the suspension medium. All three strains exhibited equivalent fitness in the tryptone medium, having cell densities between 10^7 and 10^8 after the incubation period (Fig. 5). However, while there was little apparent growth in the chalcopyrite suspension, the wild-type and CuR1 strains retained viability. In contrast, the copA mutant was unable to retain viability in the chalco-

<table>
<thead>
<tr>
<th>Strain</th>
<th>C_T</th>
<th>Msed_0951 (tbp) C_T</th>
<th>ΔC_T</th>
<th>ΔΔC_T</th>
<th>2^−ΔΔC_T</th>
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<tr>
<td>WT copA</td>
<td>10.08</td>
<td>17.28</td>
<td>−7.20</td>
<td>0.92</td>
<td>0.53</td>
<td>1.88</td>
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<tr>
<td>CuR1 copA</td>
<td>10.18</td>
<td>18.30</td>
<td>−8.12</td>
<td>0</td>
<td>1</td>
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</tr>
<tr>
<td>WT copB</td>
<td>16.32</td>
<td>18.22</td>
<td>−1.90</td>
<td>2.08</td>
<td>0.24</td>
<td>4.17</td>
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<tr>
<td>CuR1 copB</td>
<td>15.33</td>
<td>19.31</td>
<td>−3.98</td>
<td>0</td>
<td>1</td>
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<tr>
<td>WT ppx</td>
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<td>0.81</td>
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<td>18.30</td>
<td>−4.13</td>
<td>0</td>
<td>1</td>
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</tr>
</tbody>
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a C_T, threshold cycle.

b Values were normalized to those of tbp.

c Fold change comparing the WT to CuR1.

Role of copper resistance during lithoautotrophy. The importance of copper resistance during lithoautotrophy was examined using chalcopyrite as the energy source and the wild-type, copA, and CuR1 strains. Growth and retention of viability was examined after incubation in a chalcopyrite suspension relative to that in a tryptone medium for 14 days. No carbon source was added to the suspension medium. All three strains exhibited equivalent fitness in the tryptone medium, having cell densities between 10^7 and 10^8 after the incubation period (Fig. 5). However, while there was little apparent growth in the chalcopyrite suspension, the wild-type and CuR1 strains retained viability. In contrast, the copA mutant was unable to retain viability in the chalco-
pyrite suspension, indicating that a functioning copper pump was essential under these conditions.

One reason for the lack of growth of the wild type and CuR1 strain in the chalcopyrite suspension could be the lack of a carbon source. Therefore, addition of the carbonate mineral witherite (barium carbonate) was tested as a possible and more convenient carbon source than those previously described. Unlike magnesium or calcium carbonate, barium carbonate, with a dissociation constant \(K_d\) of \(2.58 \times 10^{-10}\), is insoluble in hot acid. \(M.\) \textit{sedula} cultured in the presence of witherite colonized the mineral surface, as indicated by epifluorescence microscopy of 4,6-diamidino-2-phenylindole (DAPI)-stained samples (Fig. 6A). Surface colonization and the formation of adherent cells was also evident by SEM when cells were cultured with the addition of both chalcopyrite and witherite (Fig. 6B). The role of metal resistance and carbon supplementation on copper leaching was examined next using the three strains cultivated with chalcopyrite as the energy source and either with or without witherite addition. Lithotrophic-dependent release of copper was monitored by ICP-MS of clarified culture supernatants (Fig. 7). After a 30-day incubation period, it was apparent that both the allelic state of \(\text{copA}\) and carbon supplementation strongly influenced the lithotrophic metabolism of chalcopyrite. Without carbonate addition, the CuR1 strain released 3.3 times more copper than either of the other two strains. However, with carbonate addition, copper release was greatly stimulated in both the CuR1 and wild-type strains, resulting in the mobilization of 41.5% of total copper from chalcopyrite, reaching 0.4 g/liter (6.3 mM). In contrast, release of copper by the \(\text{copA}\) mutant was negligible regardless of carbonate addition and approximated the values observed with uninoculated controls.

**DISCUSSION**

The results presented here demonstrate a direct relationship between the level of copper resistance and the rate of bioleaching of chalcopyrite during lithoautotrophic cultivation of \(M.\) \textit{sedula}. Bioleaching requires the oxidation of iron and/or sulfur and therefore provides an indirect measure of lithotrophic metabolism. Thus, the level of copper resistance influences both lithotrophy and bioleaching of copper. The use of modified cell lines specifically altered in genes associated with copper resistance provided direct evidence that metal resistance influences these processes. The interdependence of these traits is of particular relevance to copper but may be relevant to the recovery of other...
metals where metal toxicity presents a challenge to lithothrophic metabolism.

During lithothrophy, copper is released by iron and sulfur oxidation, becoming available to interact with adherent *M. sedula* cells. The importance of copper resistance most likely stems from the ability of cells to exclude or expel excessive intracellular copper ions. In this study, resistance was dependent on copper translocation as the *copA* mutant lost more than half of wild-type levels of copper resistance along with the ability to retain viability during cultivation on chalcopyrite. Additional support for the importance of this relationship was apparent from the behavior of a mutant that exceeded that of wild-type *M. sedula* copRTA transmembrane transport sites. Proc. Natl. Acad. Sci. U. S. A. 105:1500–1507.


13. Han CJ, Kelly RM. 1998. Biooxidation capacity of the extremely ther-

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### REFERENCES


16. Han CJ, Kelly RM. 1998. Biooxidation capacity of the extremely ther-


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**Bioleaching Genes of Metallosphaera**