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ENVIRONMENTAL BIOTECHNOLOGY

Novel bacterial sulfur oxygenase reductases from bioreactors treating gold-bearing concentrates

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Abstract The microbial community and sulfur oxygenase reductases of metagenomic DNA from bioreactors treating gold-bearing concentrates were studied by 16S rRNA library, real-time polymerase chain reaction (RT-PCR), conventional cultivation, and molecular cloning. Results indicated that major bacterial species were belonging to the genera *Acidithiobacillus*, *Leptospirillum*, *Sulfobacillus*, and *Sphingomonas*, accounting for 6.3, 66.7, 18.8, and 8.3%, respectively; the sole archaeal species was *Ferroplasma* sp. (100%). Quantitative RT-PCR revealed that the 16S rRNA gene copy numbers (per gram of concentrates) of bacteria and archaea were 4.59×10^9 and 6.68×10^5, respectively. Bacterial strains representing *Acidithiobacillus*, *Leptospirillum*, and *Sulfobacillus* were isolated from the bioreactors. To study sulfur oxidation in the reactors, pairs of new PCR primers were designed for the detection of sulfur oxygenase reductase (SOR) genes. Three *sor*-like genes, namely, sor_{Fx}, sor_{SA}, and sor_{SB} were identified from metagenomic DNAs of the bioreactors. The sor_{Fx} is an inactivated SOR gene and is identical to the *pseudo*-SOR gene of *Ferroplasma acidarmanus*. The sor_{SA} and sor_{SB} showed no significant identity to any genes in GenBank databases. The sor_{SB} was cloned and expressed in *Escherichiacoli*, and SOR activity was determined. Quantitative RT-PCR determination of the gene densities of sor_{SA} and sor_{SB} were 1,000 times higher than archaeal 16S rRNA gene copy numbers, indicating that these genes were mostly impossible from archaea. Furthermore, with primers specific to the sor_{SB} gene, this gene was PCR-amplified from the newly isolated *Acidithiobacillus* sp. strain SM-1. So far as we know, this is the first time to determine SOR activity originating from bacteria and to document SOR gene in bioleaching reactors and *Acidithiobacillus* species.

Keywords Sulfur oxygenase reductase · *Acidithiobacillus* · Sulfur oxidation · Bioleaching · Metagenome

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Introduction

Sulfurous compound-oxidizing bacteria and archaea are widely distributed in soil (Stubner et al. 1998), water (Spring et al. 2000), and in extreme environments including terrestrial hot springs (Yamamoto et al. 1998), solfataras (Kletzin et al. 2004), and oceanic hydrothermal vent (Ruby et al. 1981), and these microbes gain energy from sulfur oxidation processes and play very important roles in the global sulfur cycle (Amend and Shock 2001). Some sulfurous compound-oxidizing bacteria and archaea have been used in bioleaching (Olson et al. 2003; Rawlings 1997, 2002), a process of recovering high valuable metals from the essentially undissolvable metal sulfides, although the same reaction is also well-known for producing acid mine drainages causing serious environmental problem.

A few processes are known to be involved in dissimilatory sulfur oxidation (for review, see Friedrich et al. 2005). In the domain Bacteria, dissimilatory sulfur oxidation is catalyzed by sulfur-oxidizing (Sox) enzyme systems. The α-Proteobacteria harbor the complete *sox* genes

(Friedriech et al. 2001), while β- and γ-Proteobacteria and the *Chlorobiaceae* contain incomplete *sox* gene clusters that lack the genes encoding sulfur dehydroxygenase (Hanson and Tabita 2003). These Sox enzymes have been found in the periplasm of mesophilic and neutrophilic bacteria and characterized. But this system is absent from the acidophilic bacteria and archaea that contribute to bioleaching and acid mine drainage formation. Instead, sulfur oxygenase reductase (SOR) was found in several acidophilic archaea such as *Acidianus ambivalens* (Kletzin 1989) and *Acidianus tengchongensis* (He et al. 2000). The SOR represents the best known example that catalyzes the disproportional reaction of the element sulfur, producing sulfite, thiosulfate, and sulfide (He et al. 2000; Kletzin 1992; Sun et al. 2003). The reaction is dioxygen (O_2)-dependent and it does not require any external cofactors or electron donors (Kletzin 1989). In a high-resolution crystal structure of the *A. ambivalens* SOR, the holoenzyme forms a large hollow sphere enclosing a positively charged nano-compartment, with apolar channels proving access for linear sulfur species (Urich et al. 2006). A cysteine persulfide and a low-potential mononuclear non-heme iron site accorded by a 2-His-1-carboxylate facial triad in a pocket of each subunit constitute the active site accessible from the inside of the sphere where the iron is likely the site of both sulfur oxidation and sulfur reduction (Urich et al. 2004, 2006). The importance of cysteine residues (C^{31}, C^{101}, and C^{104}) and a 2-His-1-carboxylate facial trail (H^{86}, H^{90}, and E^{114}) to the SOR activity has also been demonstrated using recombinant enzymes (Chen et al. 2005; Urich et al. 2005).

Archaeal species of *Acidianus* and *Ferroplasma* have been implicated in biotechnological application of bioleaching processes (Konishi et al. 1999; Golyshina and Timmis 2005). Interestingly, members of both *Acidianus* and *Ferroplasma* encode a SOR. The other microbes carrying a *sor* or *sor*-like gene belonging to *Sulfolobus tokodaii* (Kawarabayasi et al. 2001), *Picrophilus torridus* (Futterer et al. 2004), and *Aquifex aeolicus* (Deckert et al. 1998). Thus, we aimed to determine how widely SOR-encoding microbes were present in natural environments and what roles they played in the bioleaching processes. In this study, we address these issues by the metagenomic method with newly designed polymerase chain reaction (PCR) primers that target *sor* genes.

Materials and methods

Sampling and bioreactors

Samples used in this study were from bioreactors that were used for pre-oxidation of gold-bearing concentrates. The reactors had a total volume of 600 m^3 and were operated at temperature 40–50°C and pH 1.0–1.5. By the sampling time, the bioreactors consumed about 100 tons of low-grade gold-bearing concentrates daily and had been in full operation for more than 2 years. Samples collected from the reactors were transported to the laboratory at room temperature. Upon arrival at laboratory, the samples were used for extraction of metagenomic DNAs and for isolation of bacterial strains.

Bacterial and archaeal strains, isolation, identification, and cultivation

The bacterial and archaeal strains used in this study are listed in Table 1. *Acidianus brierleyi* DSM1651 was cultivated at 70°C in broth containing (per liter) 3 g of $(NH_4)_2SO_4$, 0.5 g of $K_2HPO_4{\cdot}3H_2O$, 0.5 g of $MgSO_4{\cdot}7H_2O$, 0.1 g of KCl, 0.01 g of $Ca(NO_3)_2$, 0.2 g of yeast extract (Oxoid), and 10 g of elemental sulfur. *Acidithiobacillus* and *Sulfobacillus* species were cultivated in media described by Duquesne et al. (2003) and Johnson et al. (1987). All *Escherichia coli* strains were cultivated in Luria–Bertani (LB) broth or on LB agar plates at 30 or 37°C as indicated, and where applicable, antibiotics were added as follows: ampicillin 100 μg/ml, kanamycin 15 μg/ml, tetracycline 12.5 μg/ml, or chloramphenicol 34 μg/ml.

To isolate bacterial strains from bioreactors, samples were inoculated into various broth media corresponding to those used for cultivating *Acidithiobacillus* (Duquesne et al. 2003), *Leptospirillum* (Breed et al. 1999; Pizarro et al. 1996), or *Sulfobacillus* (Norris et al. 1996). Incubation was carried out at 30 and 45°C for 10 days, yielding three enrichment cultures. These enrichments were then diluted and plated onto the same media solidified with 0.7% Gelrite (Sigma) after a series of dilutions. Well-separated colonies were picked up and restreaked onto fresh Gelrite plates. Repeated restreakings were carried out until pure cultures were obtained.

Metagenomic and genomic DNA extraction

Metagenomic DNAs were extracted from 1-g samples collected from the bioreactor slurries according to Martin-Laurent et al. (2001). Genomic DNAs of *Acidithiobacillus*, *Leptospirillum*, and *Sulfobacillus*, *A. brierleyi*, and *E. coli* were extracted according to Marmur (1961). The obtained DNAs were individually dissolved in 30 μl of Tris–EDTA buffer (pH 8.0) and were kept at −73°C until use.

PCR reactions and cloning of PCR fragments

The PCR reactions were carried out as follows: one round of hot-start Taq DNA polymerase activation (10 min at 95°C), 40 cycles of template denaturation (30 s at 94°C), primer

Table 1 Bacterial strains and plasmids used in this study

Strains or plasmids	Usage or characteristics	Source or reference
Strains		
A. brierleyi DSM1651	Used for positive control when SOR gene amplified	Segerer et al. (1986)
E. coli		
Strain XL1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*qZΔ*M15* Tn*10* (Tetr)]	Sambrook and Russell (2001)
Strain HB101	F^-Δ(gpt-proA)62 leuB6 supE44 ara-14 galkK2 lacY1 Δ(mcrC-mrr) rpsL20 (Str^r) xyl-5 mtl-1 recA13(4,5)	Sambrook and Russell (2001)
Strain Rossetta-gamiB(DE3)	Δ*ara-leu 7697* Δ*lacX74* Δ*phoAPvuII phoR araD139 ahpC gale galK rspL* F'[*lac*+(*lacIq*)*pro*] *gor522*::Tn*10* (TcR) *trxB*::kan (DE3) pRARE (CmR)	Novagen
Acidithiobacillus strain SM-1	Utilizing elemental sulfur and other reduced sulfur compounds as energy source for growth	This study
Acidithiobacillus strain SM-2	Utilizing S^o and other reduced sulfur compounds as energy source for growth	This study
Leptospirillum strain LfA-1	Utilizing Fe^{2+}, but not S^o, as energy source for growth	This study
Sulfobacillus strain StA5-4	Utilizing Fe^{2+}, S^o and other reduced sulfur compounds as energy source for growth	This study
Plasmids		
pBluescript SK(-)	Ap^R, cloning vector	Stratagene
pGEM®-T vector	Ap^R, cloning vector	Promega
pBV220	Ap^R, P_LP_R, expression vector	He et al. (2000)
pBV220/sor_{At}	pBV220 harboring *sor* gene from *Acidianus tengchongenses*	He et al. (2000)
pBV220/sor_{SA}	pBV220 harboring sor_{SA}	This study
pBV220/sor_{SA}	pBV220 harboring sor_{SA}	This study
pGEM®-T /sor_{Fx}	pGEM-T-vector harboring sor_{Fx}	This study
pGEM®-T/Ecoli16S	pGEM-T-vector harboring 16S rRNA gene from *E. coli* K12	This study
pGEM®-T/Abr16S	pGEM-T-vector harboring 16S rRNA gene from *Acidianus brierleyi*	This study

annealing (1 min at 31.5°C), and elongation (1 min at 72°C). Genomic DNA of *E. coli* and *A. brierleyi* were used as negative and positive controls, respectively. The obtained PCR products were then purified by gel purification with the Wizard® SV Gel and PCR Clean-Up System by following the instruction of the manufacturer (Promega, Cat# A9281). The purified PCR products were individually cloned onto pGEM®-T vector (Promega, Cat# A3600).

Amplification and construction of 16S rRNA gene library

16S rRNA gene libraries of archaea and bacteria in the bioreactors were individually constructed as follows: two pairs of PCR primers targeting specifically to bacterial 16S rRNA genes, B27F and U1495R (Picard et al. 2000), as well as those annealing to archaeal ones, A21F and A958R (Beja et al. 2002) were utilized to amplify 16S rRNA genes of the microbial population in the samples. The amplified 16S rRNA genes products were individually cloned onto pGEM®-T vector (Promega, Cat# A3600). A 16S rRNA gene library for all bacterial species and another for archaea were created respectively by electroporation of archaeal and bacterial 16S rRNA genes into *E. coli* strain XL1-blue competent cells. Each library was subjected to plasmid extraction and restriction enzyme digestion to evaluate the diversity of the inserts. To further reveal the diversity of microbes in the reactors, 15 clones from the archaeal library and 50 clones from the bacterial library were randomly selected and sequenced.

Amplification of *sor* gene fragments and *sor* whole genes by PCR

Two degenerate primers, sorC1-F and sorH1-R (Table 2), were designed for amplifying *sor* gene fragments from the metagenomic DNAs prepared from the bioreactor samples. PCR reactions and cloning of PCR fragments were conducted as described above. The obtained colonies were screened by plasmid extraction and PCR amplification of the insert DNAs using M13 forward and reverse primers, then subjected to sequencing.

To clone the complete *sor* genes (sor_{SA} and sor_{SB}), the PCR products obtained above were extended using Site-Finding PCR method (Tan et al. 2005). The complete DNA sequences of sor_{SA} and sor_{SB} were obtained by merging the sequences of different DNA fragments that were recovered

Table 2 Primers used in this study for degenerate PCR, SiteFinding PCR, gene cloning and real-time quantitative PCR

Primers	Sequences(5′-3′)	Applications	References
B27F	AGAGTTTGATCCTGGCTCAG	Bacterial 16S rRNA genes cloning	Lane (1991)
U1492R	GGTTACCTTGTTACGACTT	Bacterial 16S rRNA genes cloning	Lane (1991)
U1495R	ACGGCTACCTTGTTACGACT	Bacterial 16S rRNA genes library	Picard et al. (2000)
A25F	CTGGTTGATCCTGCCAG	Archaeal 16S rRNA genes cloning	Robb et al. (1996)
U1525R	AAGGAGGTGATCCAGCC	Archaeal 16S rRNA genes cloning	Robb et al. (1996)
A21F	TTCCGGTTGATCCYGCCRG	Archaeal 16S rRNA genes library	Beja et al. (2002)
A958R	YCCGGCGTTGAMTCCAATT	Archaeal 16S rRNA genes library	Beja et al. (2002)
sorC1-F	GTIGGICCNAARGTNTGY	degenerate PCR	This study
sorH1-R	RTGCATNTCYTCRTGRTC	degenerate PCR	This study
SiteFinder	CACGACACGCTACTCAACACACCACC TCGCACAGCGTCCTCAAGCGGCCGC[a]NNNNNNGCCT	SiteFinding PCR	Tan et al. (2005)
SFP1	CACGACACGCTACTCAACAC	SiteFinding PCR	Tan et al. (2005)
SFP2	ACTCAACACACCACCTCGCACAGC	SiteFinding PCR	Tan et al. (2005)
SA1	CCCACCGCTTCCACATGGTGTAC	SiteFinding PCR	This study
SA2	GAATGCCTAAGGGATTGAGGGTTTGAC	SiteFinding PCR	This study
SA3	CAAATCCGGAATGGTTTGCCGTG	SiteFinding PCR	This study
SA4	GGCAAACCATTCCGGATTTGTAGG	SiteFinding PCR	This study
SA5	CCAAAACCACGTGCAAATCGGG	SiteFinding PCR	This study
SA6	CCGAAATGGACATGCGTCAAACC	SiteFinding PCR	This study
SB1	CACATAGTGTATTGACGAATACCAATGGGG	SiteFinding PCR	This study
SB2	ACGAATACCAATGGGGTTTAGTTCTTCG	SiteFinding PCR	This study
SB3	ATCCGGGGTGATTCGCGGTG	SiteFinding PCR	This study
SB4	CCGCGAATGACCCCGGATTC	SiteFinding PCR	This study
SB5	ACCCCGGATTCGTTGGTTTCCAG	SiteFinding PCR	This study
SB6	GCGCCAAAATGGACATGCACG	SiteFinding PCR	This study
SA-F	CGGAATTC[b]ATGCCAAAACCTTATATTGCGATT	Cloning	This study
SA-R	CGGGATCC[c]GATATTACGAGTGCAAATACTCCCG	Cloning	This study
SB-F	CGGAATTC[b]ATGCCTCGTCCATATATCAC	Cloning	This study
SB-R	CGGGATCC[c]GGATAGAAGAAATAATTACTCGTTG	Cloning	This study
Fx-F	AGTTGGATCC[c]ATAATATGGTGATAAAAAATGCCT	Cloning	This study
Fx-R	TATACTGCAG[d]TTTATGGAGAGTTAAGGTACT	Cloning	This study
BACT1369F	CGGTGAATACGTTCYCGG	Real-time PCR	Suzuki et al. (2000)
PROK1492R	GGWTACCTTGTTACGACTT	Real-time PCR	Suzuki et al. (2000)
A364aF	CGGGGYGCASCAGGCGCGAA	Real-time PCR	Kemnitz et al. (2005)
A934bR	GTGCTCCCCCGCCAATTCCT	Real-time PCR	Kemnitz et al. (2005)
qSA-F	GGTCACGGCAAACCATTCC	Real-time PCR	This study
qSA-R	GTTTGACGCATGTCCATTTCG	Real-time PCR	This study
qSB-F	AGACAACCCACCTAAGAACAAAGAAG	Real-time PCR	This study
qSB-R	AGTTTAAGTCCATCCACTCCATGTG	Real-time PCR	This study
qFx-F	TAAACCTTACGTAGCAATAAACGAAGTG	Real-time PCR	This study
qFx-R	TGGCTGTTACCTGGCATACTTTC	Real-time PCR	This study

Restriction endonuclease sites: a, *Not*I; b, *Eco*RI; c, *Bam*HI; d, *Pst*I
I inosine, *N* = A/T/C/G, *R* = A/G, *W* = A/T, *Y* = T/C

by SiteFinding PCR and with primers of SA1~SA3, SA4~SA6, SB1~SB3, SB4~SB6 (Table 2) and Long Tag DNA polymerase (Tiangen, ET103-01). To reveal any mispairing due to low fidelity of the Long Tag DNA polymerase, the *sor* genes were cloned from metagenomic DNA samples by PCR with Pfu DNA polymerase, an enzyme with higher fidelity (Tiangen, ET10). These PCR fragments were subsequently cloned and characterized by DNA sequencing as described above.

DNA template standards

PCR fragments of the 16S rRNA genes of *A. brierleyi* and *E. coli* and of sor_{SA},sor_{SB} and sor_{Fx} were generated with primer pairs, A25F and U1525R (Robb et al. 1996), B27F and U1492R (Lane 1991), SA-F and SA-R and SC-F and SC-R; these fragments were then cloned into various plasmids. The resulting plasmids, pGEM®-T/Ecoli16S, pGEM®-T/Abr16S, pBV220/sor_{SA}, pBV220/sor_{SB}, and

pGEM®-T/sor_{Fx}, were purified and used as DNA templates and standards for determining the copies of individual genes present in the metagenomic DNAs by quantitative real-time PCR (RT-PCR).

Cloning, expression, and enzymatic assay of the recombinant SOR

Three *sor*-like genes (sor_{Fx}, sor_{SA}, sor_{SB}) were obtained from metagenomic DNAs of the bioreactor with Pfu polymerase. Since the entire sor_{Fx} and sor_{SA} had reading frame shift mutations, only the sor_{SB} genes was cloned into pBV220 (He et al. 2000) and was expressed in *E. coli* HB101 and Rosetta-gamiB (DE3) cells. At cellular optical density ($OD_{600\ nm}$) of 0.6, the synthesis of SOR protein in the culture was induced by shifting its incubation temperature from 30 to 40°C and incubating for 8 h, as described previously (Chen et al. 2005). Cells from culture were harvested and stored at −73°C until use.

Protein concentration was determined using a bicinchoninic acid protein assay kit (Sigma) following the instructions of the manufacturer. The oxygenase activity of SOR was assayed as described previously (Kletzin 1989), except that the assays were conducted at 75°C and pH 7.5, which are the optimal reaction conditions for this enzyme. One unit is defined as the amount of enzyme required for the formation of 1 μmol of sulfite plus thiosulfate per minute. The optimal pH for the enzyme activity was estimated in the pH range of 4.0–10.0 with 0.5 pH unit interval. The following buffer systems were used, all at 20 mM: acetate buffer for pH 4.0–5.5, Bis–Tris–HCl for pH 5.5–7.0, Tris–HCl for pH 7.0–9.0, CHES–HCl for pH 9.0–10.0. The optimal temperature for the enzyme was assayed in the temperature range of 40–90°C with a 5°C interval.

Quantitative RT-PCR

Specific primer pairs qSA-F and qSA-R, qSB-F and qSB-R, and qSC-F and qSC-R (Table 2) were designed to quantify the *sor* genes by using Primer Express 2.0 software. A364aF and A934bR (Kemnitz et al. 2005) as well as BACT1369F and PROK1492R (Suzuki et al. 2000) were used to quantify bacterial and archaeal populations, respectively. All primers were checked for specificity firstly by general PCR and agarose gel electrophoresis and then secondly by dissociation curve during RT-PCR. Absolutely quantitative RT-PCR were performed on an ABI Prism 7000 Sequence Detection System. PCR conditions were set for 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at various temperatures: 66°C for A364aF & A934bR and qSB-F & qSB-R, 60°C for qSA-F & qSA-R and qSC-F & qSC-R, 56°C for BACT1369F & PROK1492R. The reaction volume was 25 μl, containing 1×SYBR® Green PCR Master Mix (ABI, Part No. 4309155), 200 nM of each primer, and 2 μl of different folds diluted DNA preparations. Dissociation curve and cycle thresholds were determined by using the ABI Prism 7000 SDS software.

Phylogenetic analyses of SOR and nucleotide sequence accession numbers

Phylogenetic analyses on these and other SOR or putative SOR sequences were conducted by using MEGA software (version 3.1) (Kumar et al. 2004) with the following parameters: Neighbor-Joining (NJ), bootstrap (1000), random seed, and substitution model (pairwise deletion). SOR and putative SOR sequence identities were evaluated by BioEdit software (Hall 1999). The obtained *sor* sequences were deposited in public databases under the NCBI accession numbers of DQ480731–DQ480734 and those of the 16S rRNA genes of bacterial strains SM-1, SM-2, LfA-1, and StA5-4 are DQ675568, DQ675569, DQ665868, and DQ675570, respectively.

Results

Microbial community in bioreactors

Since some extremophilic microbes are usually resistant to convenient cultivation, we employed microbial molecular ecological method to study the microbial community of microorganisms present in the bioreactors. Bacterial-specific and archaeal-specific 16S rRNA gene libraries were constructed, and 16S rRNA gene sequences of 15 archaeal clones and 50 bacterial ones were determined as described in the "Materials and methods". Whereas the archaeal clones appeared monophyletic, all showing 99–100% sequence identity to the 16S rRNA gene of *F. acidarmanus*, an acidiphilic archaeaon widely present in bioleaching and acidic hot environments (Golyshina and Timmis 2005), the bacterial community was comprised of four distinctive groups, including those closely related to *Leptospirillum* species (32 clones, 16S rRNA gene identity range 98–99.9%), *Sulfobacillus* (9 clones, 16S rRNA gene identity range 98–99.9%), *Acidithiobacillus* (3 clones, 16S rRNA gene identity range 98–99.9%), and *Sphingomonas* (4 clones, 16S rRNA gene identity range 98–99.9%; see Table 3). Members of these genera, except for *Sphingomonas*, have been well-documented for their roles during bioleaching. The *Leptospirillum* species are important players for ferrous ion (Fe^{2+}) oxidation (Breed et al. 1999), and species of *Sulfobacillus* and *Acidithiobacillus* are involved in elemental sulfur and other reduced sulfur compounds oxidation (Valenzuela et al. 2006; Kinnunen et al. 2003).

Next, we were interested in determining the densities of bacterial and archaeal cells in these bioreactors. To avoid

Table 3 Determination of bacterial and archaeal populations by 16S rRNA gene library and copy numbers of *sor*-like genes by real-time PCR

	Amounts (per gram of sample±RSD%)	Ingredients of the microbial community	Percentage (clones/total clones)
Bacterial 16S rRNA gene copy numbers	$4.59\times10^{9}\pm1.24$	*Leptospirillum* species	66.7 (32/48)
		Acidithiobacillus spp.	6.3 (3/48)
		Sulfobacillus species	18.8 (9/48)
		Sphingomonas species	8.3 (4/48)
Archaeal 16S rRNA gene copy numbers	$6.68\times10^{5}\pm4.71$	*Ferroplasma* species	100 (15/15)
Copy number of			
sor_{SA}	$3.18\times10^{8}\pm1.30$		
sor_{Sb}	$2.23\times10^{8}\pm1.83$		
sor_{Fx}	$6.36\times10^{5}\pm3.84$		

The quantifications of archaeal and bacterial populations represented by 16S rRNA genes and the determination of *sor*-like gene densities through real-time PCR were standardized with various DNA templates (Fig. 1). The data are averages of triplicate experiments in parallel and standard deviations are provided.

Average of 16S rRNA gene copy number in bacteria was estimated to be 4.1 (Klappenbach et al. 2001), while there was only one copy of 16S rRNA gene in the *Ferroplasma* genome.

the problem of culturability of bacteria and archaea and given that microbial cells contain fixed numbers of 16S rRNA genes (see the table notes in Table 3), the bacterial and archaeal cell densities were determined by estimatng the copies of 16S rRNA genes in the metagenomic DNA preparations with RT-PCR technology. This analysis revealed that the bacterial and the archaeal populations were 4.59×10^{9} and 6.68×10^{5} copies of 16S rRNA genes per gram sample, respectively, indicating bacteria species dominated microbial population in the bioreactors (Table 3).

Detection and amplification of three *sor*-like genes from the bioreactors

Thus far, six archaeal species and one bacterial species were known to encode an SOR or a homologous sequence (Chen et al. 2005). To reveal if *sor* genes were detectable in bioleaching and natural environments, we amplified *sor* or *sor*-like sequences from the metagenomic DNAs prepared from the bioreactors treating gold-bearing concentrates and from other different environments including sediments from freshwater Tai Lake and sludge from Tengchong hotspring. This was possible since there were two highly conserved motifs within SOR sequences, V^{26}–G–P–K–V–C^{31} and D^{85}–H–E–E/D–M–H^{90} (*A. tengchongensis* SOR number), which were used for designing degenerate primers (sorC1-F and sorH1-R, Table 2). Following the procedure described in the "Materials and methods", we have obtained a PCR product of the predicted size from the examined bioreactors, whereas this product was absent from all other samples (Fig. 1a). Furthermore, sequencing 20 clones from a library generated from the DNA fragments amplified above revealed that they contained three different DNA fragments, which comprised 30, 65, and 5% of total clones. These clones were respectively designated as SA, SB, and SC.

The obtained DNA sequences were then compared with those deposited in public databases. This revealed that the sequence of SC fragment was identical to the corresponding region of the *pseudo-sor* gene (sor_{Fa}) in the genome of *F. acidarmanus* (http://genome.jgi-psf.org/draft_microbes/ferac/ferac.home.html). To obtain the full length of the *sor* gene (sor_{Fx}) containing the SC fragment, we designed the primers that corresponded to sor_{Fa} 5′- and 3′-flanking sequences and used them for PCR amplification. As expected, we cloned a full-length sor_{Fa}-like gene from the metagenomic DNA, which was identical to the one of *F. acidarmanus*. This suggested that the SC fragment was mostly likely originated from a *Ferroplasma* species, of which was detected in the bioreactors, as described above.

Furthermore, the SA and SB sequences did not show any significant identity according to DNA sequences, but their translational products exhibited a significant sequence similarity to the predicted region of the SORs of *A. tengchongensis* (53.2%, AAK58572) and *A. ambivalens* (47.9%, CCA39952) in BLASTX search, suggesting that these sequences might be originated from the genes encoding a sulfur oxygenase reductase. Here, SA and SB were denoted as *sor*-like sequences. To further study environmental *sor* genes, a recently developed technique called the SiteFinding PCR (Tan et al. 2005) was employed to extend sequences from both ends of SA and SB to obtain the complete sequence of the putative *sor* genes (see the "Materials and methods"). Four fragments, designated SA2 (~1.3 kbp), SA5 (~1.4 kbp), SB2 (~800 bp), and SB5 (~2 kbp) were found to extend SA and SB fragments (Fig. 1b). These fragments were then sequenced and compiled to yield draft sequences of *sor*-like genes denoted sor_{SA} and sor_{SB}.

To get an insight into the richness and the microbes that might carry these *sor* homologous genes, we determined the densities of sor_{SA}, sor_{SB}, and sor_{Fx} in the metagenomic

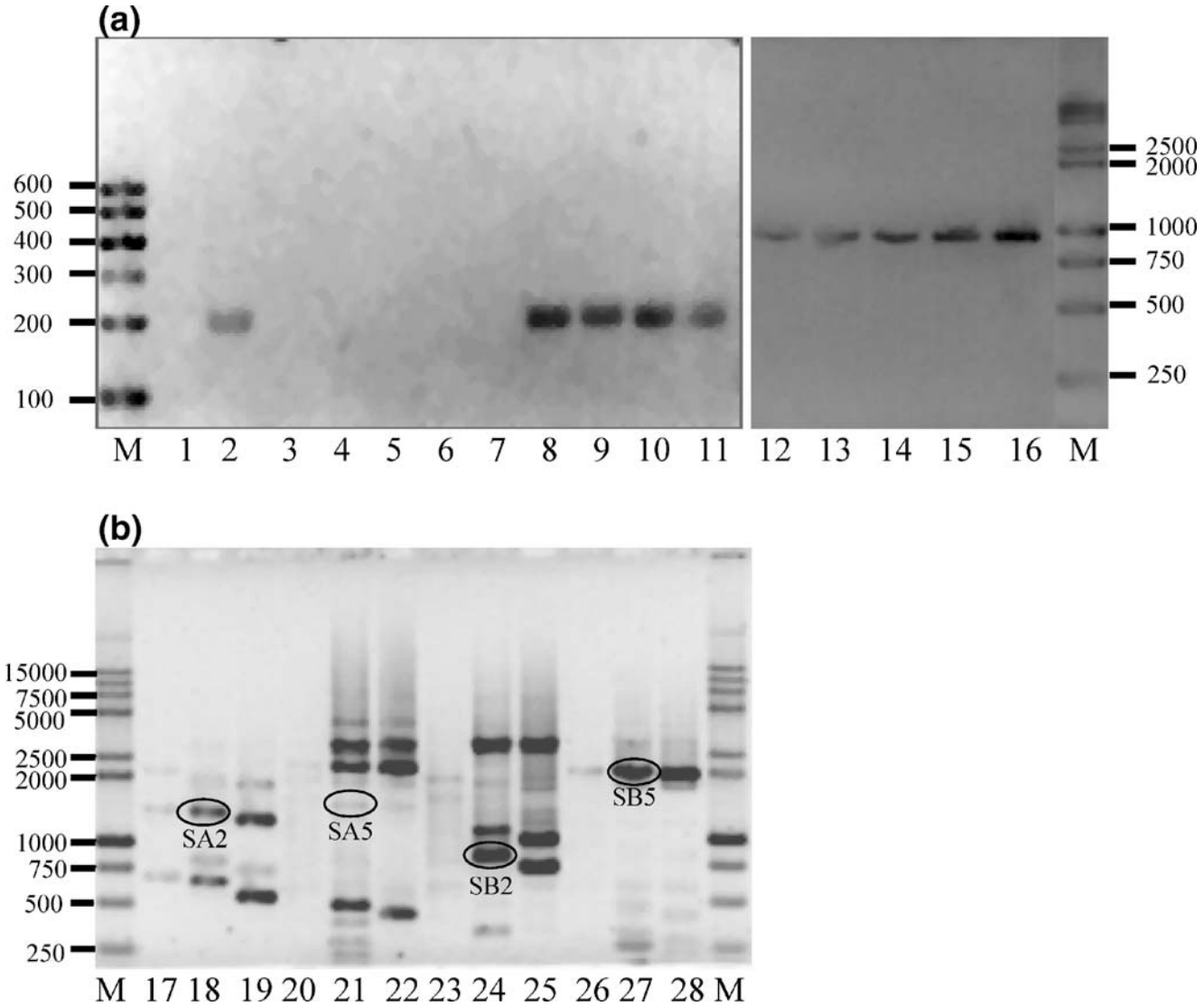

Fig. 1 Detection of *sor*-like gene segments and amplification of sor_{SB} from DNAs extracted from various environmental samples and *Acidithiobacillus* strain SM-1 (**a**) and SiteFinding PCR to obtain the full sequences of the *sor*-like genes sor_{SA} and sor_{SB} (**b**). Lanes: *M* DNA marker (bp), *1* negative control (*E. coli* strain), *2* positive control (*A. brierleyi*), *3* metagenomic DNAs extracted from Tai Lake, *4~7* metagenomic DNAs extracted from Tengchong hotspring samples, *8~11* metagenomic DNAs extracted from bioreactors, *12~15*sor_{SB} amplified from reactors, *16*sor_{SB} amplified from *Acidithiobacillus* strain SM-1. Lanes *17~22* Sitefinding PCR to obtain various fragments of SA2 and SA5 with primers of SA1~SA3, SA4~SA6; *23~28* Sitefinding PCR to obtain various fragments of SB2 and SB5 with primers of SB1~SB3 and SB4~SB6, respectively. PCR products of *SA2*, *SA5*, *SB2*, and *SB5* were selected according to their sizes as expected and were extracted from the gel and subjected sequencing after cloning into pBluescript SK(−)

DNAs using RT-PCR. As shown in Table 3, the three *sor* sequences, sor_{SA}, sor_{SB}, and sor_{Fx}, were estimated to be 3.18×10^8, 2.23×10^8, and 6.36×10^5 copies per gram sample, respectively. These results suggested that (1) the sor_{SA} and sor_{SB} must be originated from bacteria, since copies of sor_{SA} and sor_{SB} genes exceeded the number of the total archaeal population by 1,000 folds; (2) since copies of sor_{Fx} gene and 16S rRNA genes of the *Ferroplasma* species fell into the same range, sor_{Fx} must be originated from *Ferroplasma* species. This revealed, for the first time, that SOR enzymes might occur in bioleaching bacterial communities.

Phylogenetic analysis and functional identification of sor_{SB}

To obtain the authentic *sor* sequences for further analysis, sor_{SA} and sor_{SB} were amplified directly from the metagenomic DNA samples using Pfu polymerase, and the resulting PCR fragments were cloned and sequenced as described in the "Materials and methods". Comparison of

the obtained sequences with the draft version of sor_{SA} and sor_{SB} obtained by SiteFinding PCR revealed several differences. We considered the sequences derived from the PCR fragments of sor_{SA} and sor_{SB} were authentic to the metagenomic DNAs and used them for further studies.

The translated protein sequences of SOR_{SA} and SOR_{SB} showed 75.8% identity to each other. They also exhibited 54.0–55.8% sequence identity to putative SORs of *P. torridus* (SOR_{Pt}) and *F. acidarmanus* (SOR_{Fa}), 50.1–53.2% sequence identity to the SORs of *A. ambivalens* and *A. tengchongensis*, but only 34.9 and 35.7% sequence identity to the putative bacterial SOR_{Aqa}; the last sequence identity was identified in the genome sequence of *A. aeolicus* (Deckert et al. 1998). We then made a phylogenetic analysis of known SORs and their homologous sequences, in which a closely related cluster of *Acidianus–Sulfolobus* SORs appeared (Fig. 2). These SORs, together with putative SOR_{Fa} from *F. acidarmanus* and SOR_{Pt} from *P. torridus*, constituted an archaeal SOR cluster, whereas SOR_{SA} and SOR_{SB} form a cluster that was only distantly related to the former (Fig. 2). These two clusters were joined by a putative bacterial SOR_{Aqa} representing the SOR enzyme family.

To test whether these bacterial *sor*-like genes encode a functional SOR, sor_{SB} was cloned and overexpressed in *E. coli*. Cells of *E. coli* HB101 and Rosetta-gamiB (DE3) were used as hosts for this work. Whereas there was only a poor synthesis of SOR_{SB} protein in the HB101 cells, a significant amount of SOR_{SB} was produced from the Rosetta-gamiB (DE3) cells. Subsequently, recombinant SOR_{SB} protein was purified from the Rosetta-gamiB (DE3) cells and the SOR activity of the obtained enzyme was assayed as previously described (Chen et al. 2005). SOR_{SB} exhibited 3.76 U/mg, the specific oxygenase activity under the optimal reaction condition (75–80°C and pH 7.5). This indicated that SOR_{SB} was a true sulfur oxygenase reductase that catalyzed sulfur disproportional reaction in the same manner as for the known SORs.

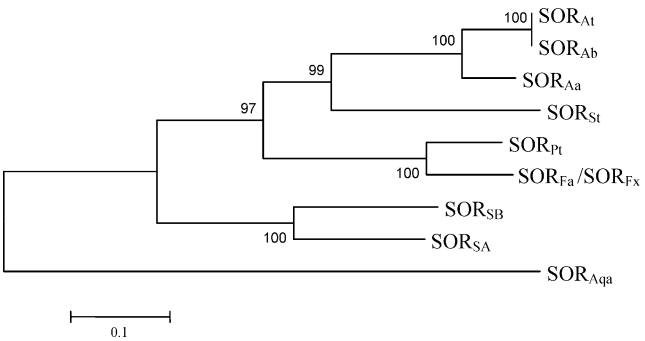

Fig. 2 Phylogenetic tree of nine SOR and putative SOR protein sequences. SOR_{At} SOR from *A. tengchongensis*, SOR_{Ab} SOR from *A. brierleyi*, SOR_{Aa} SOR from *A. ambivalens*, SOR_{St} SOR from *S. tokodaii*, SOR_{Pt} SOR from *P. torridus*, SOR_{Fa} SOR from *F. acidarmanus*, SOR_{Aqa} SOR from *A. aeolicus*.SOR_{SB}, SOR_{SA}, and SOR_{Fx} are SORs from metagenomic DNAs of bioleaching bioreactors, while SOR_{SB} was identified from *Acidithiobacillus* strain SM-1

Isolation and identification of bacterial strains and amplification of sor_{SB} from *Acidithiobacillus* sp. strain SM-1

The presence of high copies of sor_{SA} and sor_{SB} were indicative that they should be related to bacteria rather than to archaea (see above paragraphs). This copy number fell within the range of all major groups of bacteria revealed by 16S RNA gene analyses. These were *Leptospirillum*, *Sulfobacillus*, *Acidithiobacillus*, and *Sphingomonas*. Next, we aimed to enrich these bacterial species by cultivation and to isolate them as pure cultures. Four bacterial strains, LfA1, SM-1, SM-2, and TSB2-6, were obtained, and these were tentatively identified as members representing the genera *Leptospirillum*, *Sulfobacillus*, and *Acidithiobacillus*. Strain LfA1 was Gram-negative, utilized Fe^{2+} for energy source for growth, but not elemental sulfur. Its 16S rRNA gene exhibited 99.9% identity to that of *Leptospirillum ferriphilum*. Strains SM-1 and SM-2 were Gram-negative rods, utilized elemental sulfur, and reduced sulfur as energy sources for growth. The 16S rRNA genes of strain SM-1 and SM-2 showed 99.8 and 99.9% identities to *Acidithiobacillus caldus* and *Acidithiobacillus thiooxidans*, respectively. Strain StA5-4 was Gram-positive rods, utilized both elemental sulfur, and Fe^{2+} as energy source for growth. Its 16S rRNA gene showed 99.6% identity to that of *Sulfobacillus thermosulfidooxidans*.

To identify if anyone of these bacterial isolates harbored the sor_{SB} gene, genomic DNAs were extracted from these strains individually and were used as templates for PCR amplification of the sor_{SA} and sor_{SB} genes. This experiment indicated that the strain SM-1 contained sor_{SB} since it yielded a PCR product with the sor_{SB} specific primers (Fig. 1) and that the sequence of the PCR product was identical to that of SOR_{SB}. None of these strains carried a sor_{SA} gene although it occurred also at high density in the bioreactors.

Discussion

The use of microorganisms to recover metals from low-grade ores and mineral concentrates has developed into a successful and expanding area of biotechnology (Rawlings 2005; Rohwerder et al. 2003). Considering the importance of biomining of metals, it is surprising that only few studies on the microbial community of commercial biomining processes were published (Goebel and Stackebrandt 1994; Okibe et al. 2003). Reports on the composition of microflora in bioleaching reactors revealed that there was

limited biodiversity, and the frequently identified microbial species were *Acidithiobacillus* sp., *Leptospirillum* sp., *Sulfobacillus*, and *Ferroplasma* sp. Our analysis of microbial populations in the commercial bioreactors treating gold-bearing concentrates revealed the same scenario. Four major groups of bacteria have been identified; two-thirds of the sequenced clones were derived from *Leptospirillum*, whereas one-third of the organisms originated from *Sulfobacillus*, *Acidithiobacillus*, or *Sphingomonas*. When the contribution of archaeal species to the bioleaching industry was addressed, we found that *Ferroplasma* species was the sole archaeal species and it accounted for ca. 0.1% of the total microbial population. Okibe et al. (2003) reported that the *Ferroplasma* population increased as mineral oxidation progressed and eventually accounted for >99% (based on plate isolation). In the bioreactors we examined, this archaeon comprised only ca. 0.1% of the microflora, and this may reflect the fact that oxidation of concentrates was at its early stage.

Four archaeal *sor* genes had been functionally identified from *A. tengchongensis* (Chen et al. 2005; He et al. 2000; Sun et al. 2003), *A. brierleyi* (Emmel et al. 1986), *A. ambivalens* (Kletzin 1989), and *S. tokodaii* (unpublished data), respectively. These four SORs formed a tight subcluster of *Acidianus–Sulfolobus* SORs. This *Acidianus–Sulfolobus* subcluster together with two putative SORs (SOR_{Fa} from *F. acidarmanus* and SOR_{Pt} from *P. torridus*) generated the archaeal SOR cluster (Fig. 2). The two newly identified SORs (SOR_{SA} and SOR_{SB}) are distantly related to the archaeal SORs and to the putative bacterial SOR (SOR_{Aqa}), which is represented by a sole sequence from *A. aeolicus*. Although sor_{SA} and sor_{SB} are phylogenetically more related to the putative *sor* gene of *P.torridus* (Fig. 2), which is a theromophilic archaeon of the phylum *Euryarchaeota*, determination of the SOR gene density and prokaryote populations in the reactor suggested that they were from bacterial species rather that from archaeal species. Moreover, this sor_{SB} was PCR-amplified from *Acidithiobacillus* sp. strain SM-1, which was isolated from the reactor. It remains to be tested if the putative bacterial *sor*-like sequence (SOR_{Aqa}) encodes an active enzyme and if so, the enzyme exhibits the archaeal SOR properties. We noticed that SOR_{SB} functioned optimally at 75–80°C, which exhibited a typical thermophilic enzyme nature and this temperature was much higher than the optimal growth temperature for *Acidithiobacillus*. A possible explanation to this might be that the *Acidithiobacillus* strain SM-1 obtained this sor_{SB} gene by horizontal gene transfer from a thermophilic bacterium or a thermophilic archaeon.

Sulfur oxidizing system(s) in thiobacilli has been of interest to microbiologists for a long time. Suzuki (1965) and Sugio et al. (1989) reported that *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* had a glutathione-dependent sulfur oxidizing system. Interestingly, Tano and Imai (1968) detected a SOR-like activity in the cell extracts of a mesophilic bacterium belonging to *A. thiooxidans*, although this has not been confirmed by other independent investigations. Our finding that *Acidithiobacillus* sp. strain SM-1 encodes sor_{SB} suggests that mesophilic or moderately thermophilic bacteria do encode SOR enzymes. Thus, the activity observed by Tano and Imai about 40 years ago might be encoded by a SOR_{SB} homologous enzyme. Furthermore, the other bacterial SOR encoded by sor_{SA} was characterized only by cloning from metagenomic DNA and the organism carrying this gene remains to be isolated. In conclusion, this study revealed, for the first time, that *sor* genes can be present in bioleaching reactors and in *Acidithiobacillus* spp. Apart from these interesting discoveries, this study also raises several questions, such as: how widely are *sor* genes present in *Acidithiobacillus* and mesophilic bacteria? Where did this *sor* gene originate? How does the bacteria carrying a *sor* gene evolve? All these issues remain to be studied to increase our knowledge in SORs and their roles in bioleaching processes and the global sulfur cycle.

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