African Journal of Biotechnology Vol. 10(20), pp. 4121-4126, 16 May, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2406
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Isolation and characterization of a novel sulfur-oxidizing chemolithoautotroph *Halothiobacillus* from Pb polluted paddy soil

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Accepted 7 April, 2011

A new mesophilic and chemolithoautotrophic sulfur oxidizing bacterium (SOB) strain HT1 was isolated from a rice rhizosphere soil polluted by Pb using thiosulfate as electron donor at pH 7.0. The 16S rRNA gene sequence showed that the new isolate was a sulfur oxidizing obligate chemolithotroph belonging to Gammaproteobacteria, *Halothiobacillus* and utilizing different reduced sulfur compounds (sulfide, elemental sulfur, thiosulfate and sulfite) as chemolithotrophic substrates. Strain HT1 was able to use CO_2 as a carbon source responsible for the reduction of nitrate to nitrite, which represented a halophilic SOB capable of growth within a broad salinity range of 0 to 3 M NaCl and a heavy-metals-tolerant SOB. HT1 was Gram negative, motile and was proposed as the type strain of a novel species of sulfur oxidizing bacteria. The *soxB* gene could not be detected in strain HT1 during thiosulfate oxidation. The metabolism pathway of HT1 was 'S4 intermediate' (S4I) pathway. Sulfur globules accumulated in HT1 were mainly S_8.

Keywords: *Halothiobacillus,* heavy-metals-tolerant, *soxB* gene, 16S rRNA gene, sulfur globule.

INTRODUCTION

Sulfur oxidizing bacteria play an important role in mineral cycling in environments. The species of the genus *Thiobacillus* include a wide diversity of Gram negative, rod sulfur oxidizing bacteria which obtain energy from oxidation of reduced sulfur. They fall into α, β and γ subclasses of the *Proteobacteria* and could be classified as *Acidithiobacillus*, *Halothiobacillus* and *Thermithiobacillus* (Kelly and Wood, 2000). Biological reduced sulfur species such as aqueous (hydro) sulfide as well as insoluble metal sulfides, polysulfides, elemental sulfur, sulfite, thiosulfate and polythionates are finally oxidized to sulfate, or more precisely to sulfuric acid. During these processes, protons are produced. The oxidation of reduced sulfur to sulfuric acid is of great importance for biohydrometallurgical technologies. Contrary to its significant role in the global sulfur cycle and its biotechnological importance, the microbial fundamentals of sulfur oxidation are incompletely understood.

Sulfur oxidizing bacterium (SOB) exhibits a wide range of metabolic flexibility, particularly with respect to processes involving respiration and energy transduction. Previous studies concluded that there were at least two metabolic pathways of SOB during the thiosulfate oxidation (Friedrich et al., 2001). Some SOB was involved in the toxicity of heavy metal ion and possesses unique metabolic and ecophysiological features with extraordinary properties. Novel SOB with extraordinary properties was reported (Sorokin et al., 2006; Ghosh and Roy, 2007). Wood and Kelly (1991) found the first halophilic SOB capable of growing at very high salt concentrations (4 M NaCl). Some SOB that is capable of

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anaerobic growth with sulfur compounds and nitrogen oxides as electron acceptors play an important role in mineral cycle.

Herein, we isolated a novel SOB from a Pb polluted paddy soil. The nearly complete 16 s rRNA gene sequences and physiological characteristics of the new isolate were analyzed. Functional *soxB* gene was detected in order to investigate its metabolic pathway. Sulfur K-edge x-ray absorption near edge structure spectroscopy (XANES) was used to identify the speciation of sulfur in the cells of HT1.

MATERIALS AND METHODS

Rhizosphere soil of rice was used as the inoculums for enrichment cultures. Selected physical and chemical properties of the soil used are: organic matter, 2.26%; pH, 5.63; Pb concentration, 680.09 $mgkg^{-1}$; Zn concentration, 68.96 $mgkg^{-1}$; Cu concentration, 21.10 $mgkg^{-1}$; S concentration, 247 $mgkg^{-1}$. Mineral medium used for enrichment, isolation and cultivation of SOB contained (per liter of deionised water, added 15 agar for solid medium): Na_2HPO_4 1.2 g, KH_2PO_4 1.8 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 0.1 g, $CaCl_2$ 0.03 g, $FeCl_3$ 0.02 g and $MnSO_4$ 0.02 g. Ten grams of $Na_2S_2O_3$ was added as model sulfur source (Graff and Stubner, 2003). Enrichment of SOB was conducted with liquid medium on a rotary shaker at 200 rpm at 28°C. For isolation and purification, solid medium was prepared. 0.2 ml of aliquots of the enrichment cultures were transferred onto the solid medium. The plates were incubated in an incubator at 28°C. Colonies formed on agar plates of the mineral medium were transferred at least three times to be pure.

DNA of the new isolate -was obtained for amplification and sequencing of the 16S rRNA gene. The 16S rRNA gene was amplified with primers BSF8/20: 5'-AGAGT TTGAT CCTGG CTCAG-3' and BSR1541/20: 5'-AAGGA GGTGA TCCAG CCGCA-3'. PCR was run using 50 µl reaction volumes and reactant concentrations in each 50 µl reaction which were 10 to 15 ng of DNA template, 25 pM of each primer, 2.5 mM deoxynucleotide triphosphates (dNTPs, Proega, USA), 10×PCR buffer (applied Biosystems, USA), 0.1 mM $MgCl_2$ solution (Sigma) and 1 U of Taq polymerase (Applied Biosystems, USA) in nuclease-free water. The reaction was carried out as follows: 4 min initial denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 50 s, annealing at 57°C for 50 s and DNA extension at 72°C for 40 s. Cycling was completed by a final elongation step of 72°C for 10 min. The PCR product were purified and sequenced by the Invitrogen Corporation (USA) in China. The nearly complete 16S rRNA gene sequences of the new isolate were compared using the BLAST program. A phylogenetic tree was constructed with sequences aligned with the CLUSTAL X 1.83 and the Paup v.4.0b.8.a program.

Physiological characteristics were analyzed as described by Dong and Cai (2001). Cells were suspended in sterile double distilled water and adhered on copper mesh. Then, the cells were dyed with 1% uranygl acetate for about 15 s. Transmission electron microscope (H-7650, HITACHI) was used to observe the morphology. Different reduced sulfur compounds (thiosulfate, thiocyanate, elemental sulfur, sulfide, sulfite and tetrathionate) were used to determine the utilization of substrates as energy sources. The sulfite solution was prepared in 50 mM EDTA to prevent autooxidation (Sievert et al., 2000). Nitrogen (nitrate, nitrite) was used to test as an electron acceptor in the absence of oxygen with KNO_3 and KNO_2. Effects of salinity (NaCl) and heavy metal tolerance (Pb, Zn and Cu) on bacterial growth were examined in Luria-Bertani medium containing varying concentrations.

PCR amplifications of *soxB* gene fragments with extracted DNA were performed as a two-step PCR using the primer sets and protocols as described by Petri et al., (2001). Sulfur species in the cell were analyzed by XANES as described by Prange et al., (1999) at Beijing synchrotron radiation facility, institute of high energy physics of China. Spectra were recorded at 4B7A beam line and scanned in the region between 2420 and 2520 eV.

RESULTS AND DISCUSSION

A new isolate using thiosulfate as sulfur was obtained and named as strain HT1. Colonies grown on mineral medium with thiosulfate as sulfur source were smooth and white-light yellow (1 to 3 mm). The cells of strain HT1 appeared singly or in pairs and motile with flagellums (Figure 1).

The 16S rRNA genes were amplified using general bacterial primers. A phylogenetic tree was obtained as shown in Figure 2. Comparison of the nearly complete 16S rRNA genes showed that strain HT1 was closely related to *Halothiobacillus* and belonged to the γ subclass of the Proteobacteria. Its lineage is Bacteria, Proteobacteria, Gammaproteobacteria, Chromatiales, Halothiobacillaceae and *Halothiobacillus.* The GenBank accession number for the nearly complete 16S rRNA gene sequence of *Halothiobacillus* HT1 is GU013549.

Table 1 shows the physiological characteristics of HT1 when compared with known *Halothiobacillus*. Reduced inorganic sulfur compounds sulfide, polysulfides, elemental sulfur, sulfite and thiosulfate were oxidized as electron donors for energy by HT1. No growth was observed on a mineral medium with thiocyanate as energy source.

Under aerobic conditions, the bacterium was able to grow with nitrate as electron acceptor. However, no growth was observed with nitrite. It was concluded that strain HT1 is a nitrate to nitrite reducer and can serve as a nitrite provider. The isolate grew autotrophically on thiosulfate and can use carbon dioxide as carbon source. HT1 was capable of adapting to high salt (3 M) and could use CO_2 as a carbon source, suggesting that it possessed carboxysomes that contain enzymes involved in carbon fixation (Tsai et al., 2007).

HT1 could grow in 621 $mg.kg^{-1}$ of Pb^{2+}, 192 $mgkg^{-1}$ of Cu^{2+} and 325 $mgkg^{-1}$ of Zn^{2+}, respectively, showing relatively high tolerance of heavy metals. Possible toxicity and mechanisms of resistance to Cu have been studied in *Sulfolobus solfataricus* and *Sulfolobus metallicus* (Ettema et al., 2006; Remonsellez et al., 2006). Microbial sulfur oxidation is of great importance in influencing the transformation of heavy metals. This useful application requires heavy-metals-tolerant SOB which can grow in heavy metals polluted environment. They may possess genetic determinants for metal resistance encoding bacterial metallothioneins and heavy metal-transporting ATPases to exist in toxic heavy metal ions and contribute to sulfur oxidation (Auernik et al., 2008).

As a result of the phylogenetic and physiological diversity of sulfur oxidizing bacteria, several different enzymatic

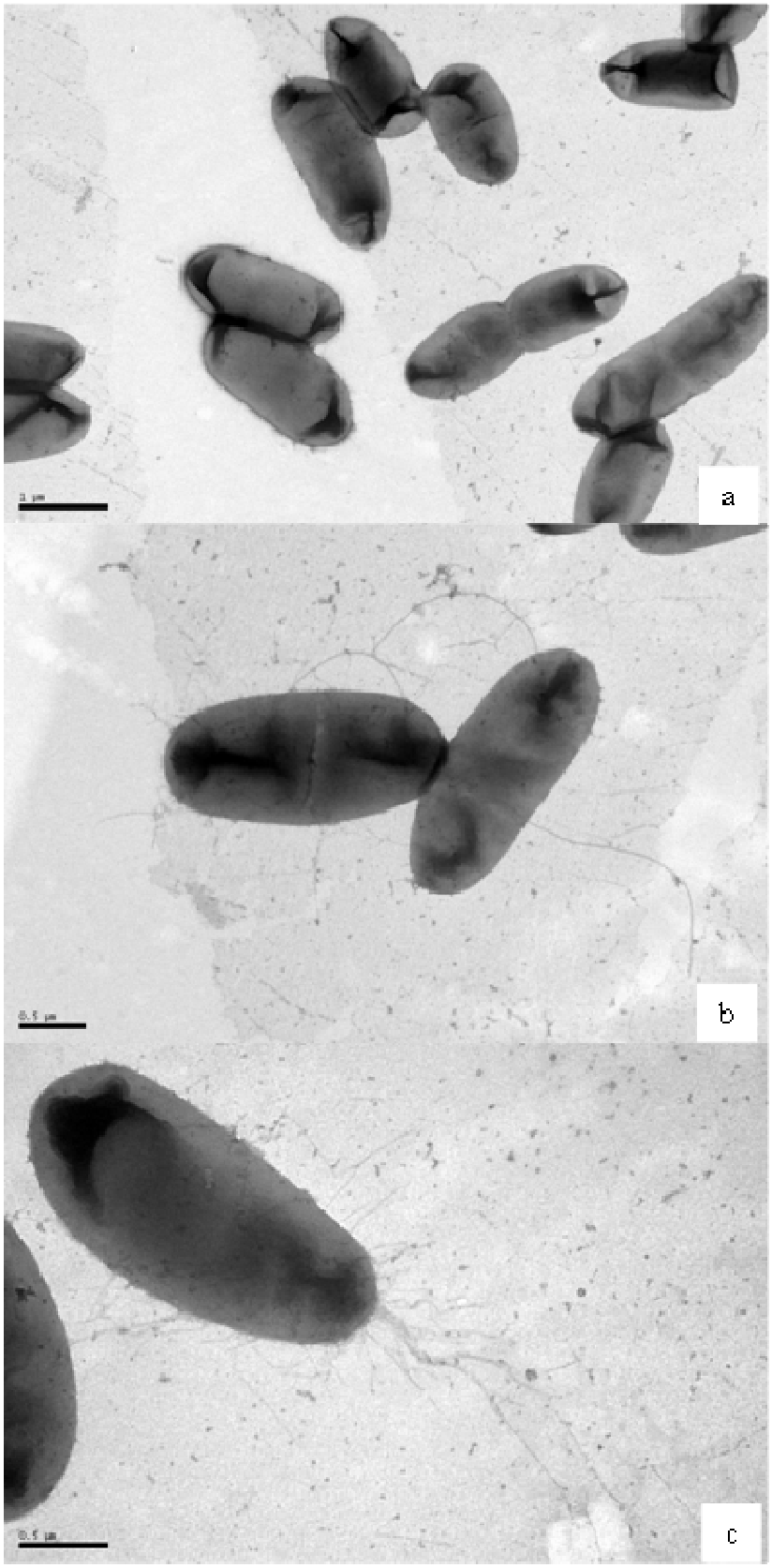

Figure 1. Morphology of cells of strain HT1 grown in LB medium. (a) In pair; (b) in pair with flagellums; (c) single.

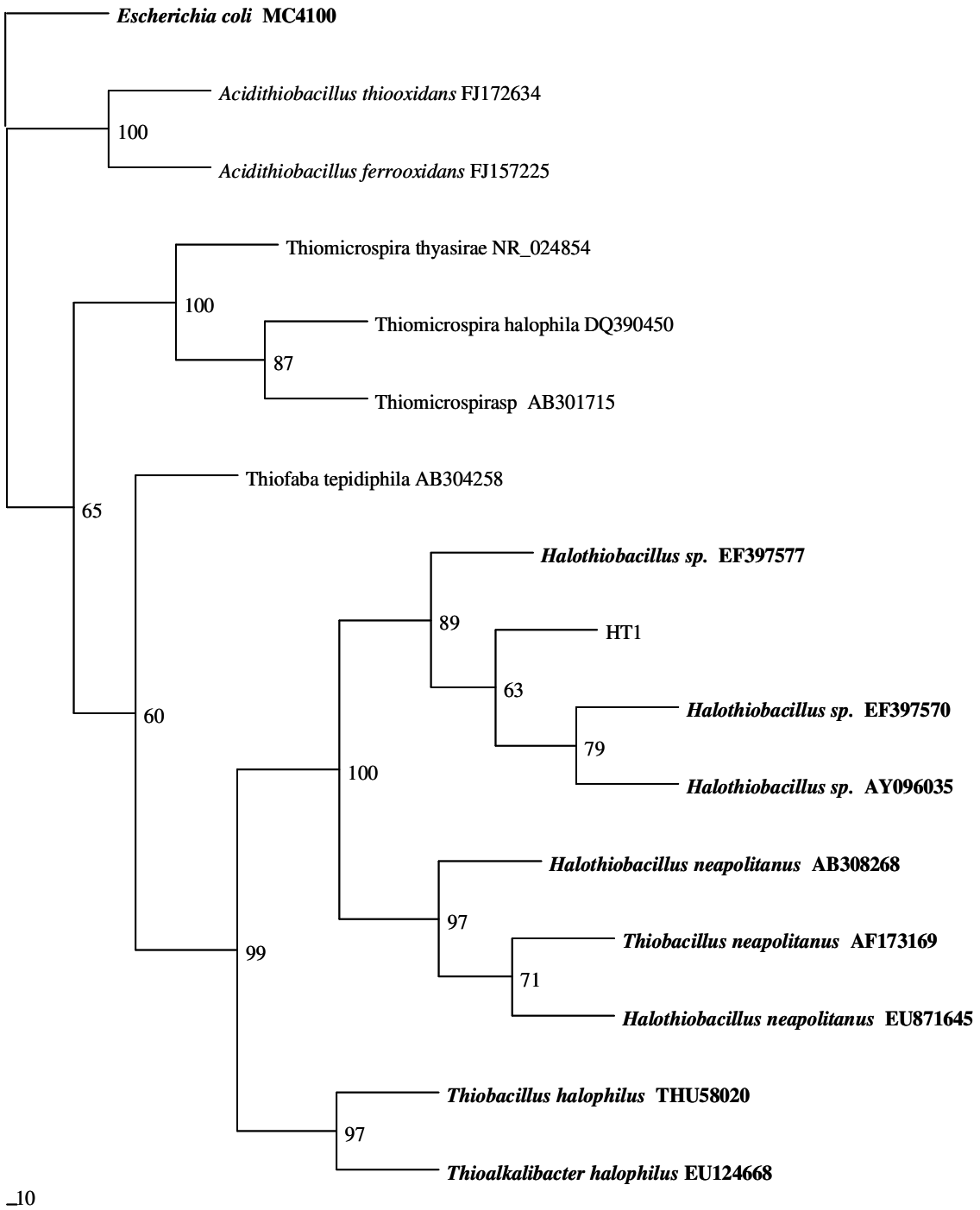

Figure 2. Phylogenetic tree derived from 16S rDNA sequence data of strain selected and other related species. The tree was rooted with *Escherichia coli* as an out group.

systems and pathways are involved in the dissimilatory oxidation of thiosulfate. At least two major pathways have been proposed for different SOB: (1) the sulfur oxidation pathway (PSO) and (2) the S4 intermediate pathway involving polythionates (S4I) (Friedrich et al., 2001). The S4I pathway includes the formation and oxidation of polythionate or S and sulfite from thiosulfate. In the PSO pathway, thiosulfate is oxidized directly to sulfate and does not accumulate intermediate products such as polythionate, S and sulfite. Thiosulfate oxidation is carried out by a thiosulfate-oxidizing multi-enzyme system in which one enzyme is coded by *soxB* (Anandham et al., 2008). *SoxB* contains a prosthetic manganese cluster in the reaction center and is essential for thiosulfate

Table 1. Comparative characteristics of strain HT1 and typical *Halothiobacillus.*

Characteristic		HT1	*Halothiobacillus**
Gram stain test		-	-
Oxidase		/	/
Catalase		-	/
Indole		+	/
Methyl red test		-	/
Amylohydrolysis		-	-
Oxidative fermentation of glucose		Ferment	/
Nitrate reduction		+	+
Nitrite reduction		-	-
Denitrification		-	-
Gelatin liquefaction		-	/
H_2S		+	/
Cu tolerance		621 $mgkg^{-1}$	/
Pb tolerance		192 $mgkg^{-1}$	/
Zn tolerance		325 $mgkg^{-1}$	/
NaCl tolerance		175.5 gkg^{-1}	0-234 $g.kg^{-1}$
Sulfur sources	sulfide	+	+
	S	+	+
	sulfite	+	+
	thiosulfate	+	+
	tetrathionate	+	+
	thiocyanate	-	-

"/" Denotes this index does not test or exist; "*" denotes reference control

oxidation. HT1 is a sulfur oxidizing obligate chemolithotroph capable of using thiosulfate as substrate. In order to detect the pathway of HT1, different primers were conducted on it to detect the existence of *soxB.* PCR-based analysis showed that no specific bands were obtained in HT1. Our results were consistent with *soxB* gene analysis with *Halothiobacillus hydrothermalis* conducted by Petri et al., (2001), suggesting that HT1 oxidize thosulfate via the S4I path way which is enzymatically different from the PSO path-way.

It was reported that many SOB took up and stored sulfur globules in the reduced forms (usually as elemental sulfur). Different bacterial groups stored various forms of globules (Prange et al., 1999). Sulfur in the globules of *Beggiatoa alba* and *Thiomargarita namibiensis* consisted of S_8, whereas *Acidithiobacillus ferrrooxidans* stored polythionates as globules. Purple and green sulfur bacteria also consisted mainly of polymeric sulfur chains while the sulfur chains were found in the globules of *Thermoanaerobacter sulfurigignens* and *Thermoanaerobacterium thermosulfurigenes*. As an essential macronutrient for microorganisms, plants and animals, sulfur (S) exists in soils in a great variety of organic and inorganic species with oxidation states ranging from -2 to +6. Reference compounds were used to explore different sulfur oxidation states and chemical structures. For analysis, the fitting and plotting package WinXAS version 3.1 was used. The spectra were normalized by fitting first and second order polynomial functions to the pre- and post-edge regions respectively. Sulfur K-edge XANES analysis showed that sulfur globules in HT1 fit well with cyclooctasulfur (S_8) which was consistent with the results of Engel et al., (2007) (Figure 3). This could be either a biosignature of bacteria that preferentially take up and oxidize cyclooctasulfur (S_8) or a sign that the polymeric form of sulfur is preferred, thus leaving cyclooctasulfur to accumulate.

HT1 was a heavy-metals-tolerant SOB which might play an important role in biogeochemical cycle. Microorganisms are considered to be important contributors to the biogeochemical cycle of trace elements through several microbe mediated processes. Most sulfur transformations are fundamentally controlled by biosphere processes, especially by the specialized metabolisms of microorganisms. Biological oxidation was the principal pathway for mineralization of organic S. Sulfur oxidation by metal resistant SOB contributed to the formation of SO_4^{2-}, leading to the transformation of sulfur.

ACKNOWLEDGEMENTS

This work wassupported by Zhejiang Provincial Natural Science Foundation of China (R5110031), the National

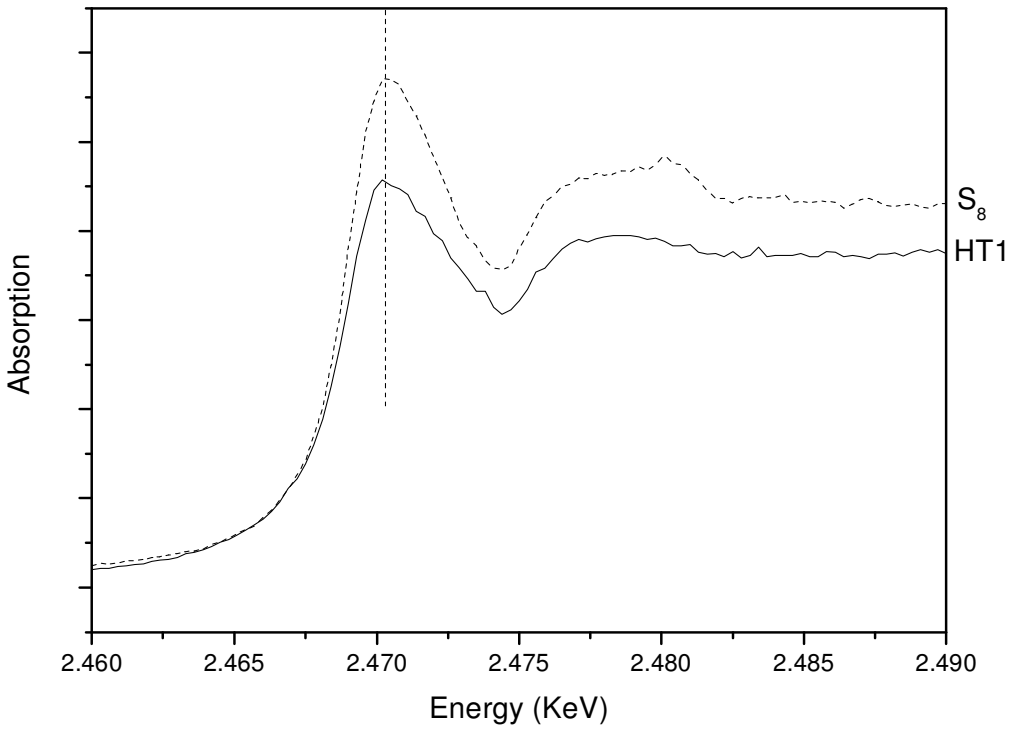

Figure 3. Sulfur K-edge XANES spectra of HT1 cells.

Natural Science Foundation of China (40601086, 20777066), and the Fundamental Research Funds for the Central Universities (2010QNA6025).

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