Oxidation of Elemental Sulfur by *Fusarium solani* Strain THIF01 Harboring Endobacterium *Bradyrhizobium* sp.

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**Abstract** Nineteen fungal strains having an ability to oxidize elemental sulfur in mineral salts medium were isolated from deteriorated sandstones of Angkor monuments. These fungi formed clearing zone on agar medium supplemented with powder sulfur due to the dissolution of sulfur. Representative of the isolates, strain THIF01, was identified as *Fusarium solani* on the basis of morphological characteristics and phylogenetic analyses. PCR amplification targeting 16S rRNA gene and analyses of full 16S rRNA gene sequence indicated strain THIF01 harbors an endobacterium *Bradyrhizobium* sp.; however, involvement of the bacterium in the sulfur oxidation is still unclear. Strain THIF01 oxidized elemental sulfur to thiosulfate and then sulfate. Germination of the spores of strain THIF01 was observed in a liquid medium containing mineral salts supplemented with elemental sulfur (rate of germinated spores against total spores was 60.2%), and the culture pH decreased from pH 4.8 to 4.0. On the contrary, neither germination (rate of germinated spores against total spores was 1.0%) nor pH decrease was observed without the supplement of elemental sulfur. Strain THIF01 could also degrade 30 ppmv and ambient level (approximate 500 pptv) of carbonyl sulfide.

**Introduction**

Biodeterioration of building materials and bas-relief by microorganisms is serious problems for conservation and protection of sandstone-made cultural heritage, especially in Angkor Wat, and Bayon temple of Angkor monuments in Cambodia. Microorganisms are capable of forming biofilms on surfaces of inorganic materials with different communities in succession over time, and the surface colonizers may damage the substratum through physical, chemical, and biological processes at the interface. They change the moisture contents in stone and cause difference in expansion between the sun facing and shading sides, and they also release organic and inorganic acids through their biochemical metabolisms of the colonizers [42]. End products of chemolithotrophic metabolism such as sulfuric acid and sulfate are produced by sulfur-oxidizing bacteria, and these products can cause damage of stone materials by corroding constituting elements and formation of crystals in the material matrices, resulting in expansion and subsequently physical damage. However, the importance of the chemolithotrophs in the biodeterioration process is still largely unknown because sulfur-oxidizing bacteria have
been found as a minor population in the area of deterioration [33]. Concerning the degradation of concrete, sulfur-oxidizing chemolithoautotrophic bacteria of such Thiobacillus curvatus (renamed as Thiobacillus thiooxidans) and Thiobacillus neapolitanus (renamed as Halothiobacillus neapolitanus) have been isolated from corroded concrete by Parker and co-workers [26, 27]. On the other hand, Gu et al. suggested that Fusarium cause concrete surfaces to produce organic acids [13]. Fungi have usually been considered as chemosynthetic microorganisms that utilize organic compounds as the major carbon and energy source. However, ability of conversion of inorganic sulfur compounds has been reported in various groups of fungi, for example Acremonium, Aurobasidium, Penicillium, and others [12, 41] including yeasts of Debaryomyces,Saccharomyces, and Rhodotorula [18, 40]. These experiments were carried out by using microtrophic medium supplemented with both reduced sulfur compound and organic compound such as sucrose [41]. Since nutrients are limited on the surface of sandstone, utilization of sulfur by fungi should be most likely carried out under oligotrophic or autotrophic condition.

In the Angkor Wat, deterioration of pillars of the first floor especially located at the inner wall was remarkable in the lower part. The floor level, little or no visible bioturbation was observed, however deterioration and exfoliation of the stone surfaces were found, suggesting elution of minerals and lower elevation of inner-pressure by crystal formation, e.g., CaSO₄. Since we have encountered sulfur-oxidizing microorganisms colonizing the deteriorating sandstones in Angkor Wat, Bayon temple, and Phnom Krom temple to evaluate contribution of the microorganisms to the biodeterioration processes [20]. By using mineral salts medium supplemented with elemental sulfur, cultivable sulfur-oxidizing microorganisms ranging between 10⁴ and 10⁸ most probable number (MPN) per gram of the deteriorated stone sample were obtained during period of the experiment (1 year). Since fungal-like microorganisms were often observed in positive MPN cultures exhibiting a decrease of the culture pH, we tried to isolate fungi that can grow with sulfur in chemolithoautotrophic condition. However, attaching microorganisms that produce acidish metabolite as a metabolite. Because endosymbiotic bacteria in fungi have been reported for arbuscular mycorrhizal fungi [5, 6], ectomycorrhizal fungi [4], basidiomycetes [21], and plant pathogenic fungi [25], we also examined the presence of bacteria associated with fungi by PCR amplification of 18S rRNA gene to know a possible involvement of bacteria on the elemental sulfur oxidation by a fungal isolate. This paper deals with characteristics of an elemental sulfur-oxidizing fungal strain that was isolated from deteriorated sandstone of Angkor Wat. Although the participation of fungal phenotype is unclear, the harboring endobacterium in the fungus was investigated by PCR amplification of 16S rRNA gene.

Methods

Isolation of Sulfur-Oxidizing Fungi

Deteriorated sandstone samples were collected at Angkor site, Cambodia in August 2003 and September 2008. Details on sampling site have been reported previously [20]. Fungal strains were isolated from positive culture of MPN to enumerate sulfur-oxidizing microorganisms in deteriorated stones or directly from deteriorated sandstone samples collected in September 2008 as described below. The Northern Library where sample for the isolation of strain THF01 was collected is located inside the outer enclosure of the temple, and the side chamber and porch roofs of the library have been reconstructed by Japanese Government Team for Safeguarding Angkor, however pillars inside the building were untouched during the restoration activities. Medium used for MPN enumeration of sulfur-oxidizing microorganisms was W5 containing KH₂PO₄, 3 g/L MgSO₄.7H₂O, 1 g/L FeCl₃.6H₂O, 0.01 g/L CaCl₂.2H₂O, 0.25 g/L elemental sulfur (granular), 10 g dissolved in 1,000 mL of deionized water, pH 5.0. Oxidation of sulfur was measured by monitoring the decrease of medium pH due to the production of sulfuric acid with pH meter (Twin pH, Type B-212, Horiba Ltd, Kyoto, Japan). Sulfur-oxidizing fungi were isolated on W5 agar plates which contained powder sulfur (0.2 g per 1,000 mL as final concentration) instead of granular sulfur and 30 g per 1,000 mL of Bacto agar (Becton, Dickinson and Co., USA). W5 agar medium was prepared according to the procedure described by Wieringa [45].

Morphological characteristics of THF01, from well developed mycelium on Corn Meal Agar (CMA, Becton, Dickinson and Co., USA) and W5 agar media, were examined by using a differential interference contrast microscope BX50 (OLYMPUS, Tokyo, Japan). Morphological identification of Fusarium species was done based on the manual described by Nelson et al. [25].

Enumeration of Sulfur-Oxidizing Fungi in Deteriorating Stones

Sulfur-oxidizing fungi in deterioration of sandstone collected in Aug 2006, Aug 2007, and Sept 2008 were enumerated based on CFU method using W5S agar medium. Samples were diluted by ten times in sterilized 0.85% NaCl solution and then vortexed for 10 s. The resultant suspension (0.1 mL) was spread onto the agar medium and the incubation was conducted at 30°C. Sulfur-oxidizing fungi were distinguished from other fungi based on the ability to produce clear zone around the colony due to a solubilization of powder sulfur [45]. From the agar plates inoculated with samples of AW, A2W, BY2-3, BY2-3, BY2-3, BY2-3, and PK3, 19 fungal strains were obtained after repeated isolation. The isolates were maintained on potato dextrose agar plate (DAIAGO, NIHON SEIKAKU Co., Tokyo, Japan) medium supplemented with 50 mg per 1L of chloramphenicol.

Polymerase Chain Reactions and Cycle Sequence

DNA was extracted from mycelium of the isolated fungal grown on PDA medium by using an ISOLPLAN DNA extraction kit (NIPPON GENE, Toyama, Japan). Unless otherwise noted, all polymerase chain reactions (PCR) were performed in 100 μL reaction volumes with 10 μL of 10×PCR Buffer (HotStarTaq Plus, QIAGEN), 200 μM of dNTPs, 0.5 μM primers (upstream and downstream), 2.5 units of HotStarTaq Plus DNA polymerase, DNA template, and MiLiQ water to total reaction volume. The PCR products were purified using the UltraClean 15 DNA Purification Kit (QIAGEN) Laboratory, Santa Clarita, CA, USA). Cloned PCR products were sequenced by BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) in the reaction: 0.5 μL of Ready Reaction Premix, 2 μL of BigDye Sequencing Buffer, 0.32 μL primer, cleaned PCR template and MiLiQ water to 10 μL total reaction volume. Cycle sequencing was achieved with an initial denaturation step of 1 min at 96°C, 25 cycles of 10 s at 96°C, 1 s at 50°C, and 4 min at 60°C. Sequencing reactions were cleaned by ethanol precipitation and sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems, CA, USA). PCR amplification parameters for each gene region are provided below. The 18S rRNA gene was amplified with primers NS1 and NS8, and sequenced with NS1, NS2, NS3, NS4, NS5, and NS6 [44]. Amplification was achieved with an initial denaturation step of 5 min at 95°C, 32 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C; and a final extension step of 7 min at 72°C.

The internal-transcribed spacer (ITS) between 18S rRNA and 28S rRNA genes was amplified by PCR with ITS1 and ITS4 [44]. Amplification was carried out under the same program as for the 18S rRNA gene, except for the annealing step at 55°C, and final extension was 5 min at 72°C.

The 16S rRNA gene was amplified with primers 27F and 1523R, and sequenced with 27F, 1523R, 785F, and 907R [2, 19]. Amplification was carried out under the same program as for the 18S rRNA gene, except the annealing step was carried out 1 min at 55°C, and final extension was 10 min at 72°C.

Phylogenetic Analysis

All sequences obtained were compared with those in the GenBank database using BLAST [1], and analyzed by Clustal W program [37] using Kimura's distance [17]. To evaluate phylogenetic position of strain THF01 and the endobacterium, analysis were performed on 18S rRNA gene and ITS region, and 16S rRNA gene, respectively. To reveal the phylogenetic distribution of sulfur-oxidizing fungi other than THF01, analysis on ITS region was performed.

A multiple sequence alignment was performed and the Infernal software [36] was utilized to produce a neighboring tree. Statistical support for inferred groups was estimated by bootstrap analysis [9] using 1,000 replications and was performed using the Bootstrap NJ-tree option. The final tree is displayed with NJ plot [31].

Nucleotide Sequence Accession Numbers

The nucleotide sequence of strain THF01 have been deposited in the DNA Data Bank of Japan (DDBJ) under the following accession numbers: AB473809 for ITS gene; AB473810 for 18S rRNA gene; AB474797 for endobacterial 16S rRNA gene. The ITS sequence of strain THF01, which was isolated from the 16S rRNA gene of the isolates in the DDBJ under the following accession numbers: AB558272 for AW1-1111, AB558273 for PK2-M223, AB558274 for AW1-1121, AB558275 for BY2-2124, AB558276 for BY2-2121, AB558277 for BY2-2310, AB558278 for BY2-2211, AB558279 for AW2-2311, AB558280 for BY2-2311, AB558281 for BY2-2311, AB558282 for BY2-2311, AB558283 for PK2-1131, AB558284 for PK2-1131, AB558285 for BY2-2111, AB558286 for BY2-1231, AB558287 for AW2-1231, AB558288 for AW2-1131, AB558289 for AW2-1130.

Oxidation of Elemental Sulfur by Strain THF01

Oxidation of elemental sulfur was examined by measuring concentration of thiosulfate and sulfite in WSS-S (sulfate-free WSS) medium containing KH₂PO₄, 3.0 g; NH₄Cl, 0.16 g; MgCl₂·6H₂O, 0.41 g; FeCl₃·4H₂O, 0.007 g; CaCl₂·2H₂O, 0.25 g; elemental sulfur, 0.2 g dissolved in 1,000 mL of deionized water, pH 5.0. For a plate culture, 30 g of agar was supplemented in 1,000 mL of the medium. At the center of WSS-S agar medium, THF01 was inoculated and incubated until clear zone was observed around the colony. Agar of both inside and outside of the clear zone were cut off to avoid
contamination of mycelium, and then thiosulfate and sulfite were extracted with MIHQ water (2.0 mL, water to 0.5 mL of agar sample). The medium without inoculation was used as a negative control. The supernatant was injected to an ion chromatograph as described below after filtration (Millipore GV, 0.22 μm pore size, Millipore, USA).

Germination of chlamydospore and subsequent elongation of the hyphae was examined in liquid WS-S medium inoculated with the rinsed chlamydospore collected from the 3 days old colony grown on agar medium of WS-S. To avoid carryover of trace amounts of elemental sulfur, THF01 was inoculated at the center of a nitrocellulose filter (0.45 μm pore size, ADVANTEC, Tokyo, Japan) placed on the agar medium. After 4 days of incubation, spores were collected from the mycelium on the filter and suspended in small amounts of MIHQ water, centrifuged at 1,300×g for 5 min, and then suspended in MIHQ water. Then the spores were inoculated to fresh WS-S medium at a density of 8.3×10^7 mL. Numbers of germinated chlamydospore and the length of germ tube were estimated by using microscope BX-8000 (Keyence, Osaka, Japan). When bipolar germination from single chlamydospore was observed, length of each hyphae was summed up.

Thiosulfate and sulfite ions were measured by ion chromatography of WS-S output (IC, Metrohm Ltd., Switzerland) consisted of Shodex IC-524A column (4.6 mm in diameter×100 mm in length) with a IC IA-G guard column (4.6 mm in diameter×10 mm in length); 2.5 mL of pycticate acid as eluant (pH 4.0) with a flow rate of 1.2 mL per min, a pressure of 5.5 MPa, and column temperature at 40°C.

Degradation of Carbonyl Sulfide by Strain THF01

Degradation of carbonyl sulfide (COS) by strain THF01 was examined according to the procedures described by Kato et al. [16]. When the mycelium of THF01 developed on slant medium of PD, a cap of the slant culture was changed from a silicone sponge to a butyl rubber, and then 11.5 mL COS gas [105,000 parts per million by volume (ppmv)] as N2 as the balance gas (Nissan Tanaka Corp., Saitama, Japan) was added to the headspace to make a final COS mixing ratio of 30 ppm. Then 50 mL of headspace gas of the test tube was injected into a gas chromatograph (GC-14B, Shimadzu, Kyoto, Japan) equipped with a flame photometric detector for COS quantification [16].

For degradation of the ammonium level of COS, a 5 L gas-sampling bag (aluminized polyethylene bag, AAK-5, GL Sciences, Tokyo, Japan) was used. Strain THF01 was pre-inoculated in malt extract liquid medium containing malt extract, 20 g; peptone, 1 g; KH2PO4, 0.1 g; and distilled water up to 1,000 mL of deionized water, pH 6.0, for 24 h, collected by centrifugation, and then rinsed with sterile 25 mM phosphate buffer (pH 7.2) for three times to clean outside the mycelium. After the third centrifugation, aqueous phase was discarded, and fresh 25 mM phosphate buffer (pH 7.2) was added to prepare the suspension. Then the suspension was filtered onto a GLASS FIBER FILTER (GF-75, pore size: 0.3 μm, ADVANTEC, Tokyo Japan) and then placed in a sterile Petri dish (8.8 cm L x 2.2 cm W) where thick filter paper pre-moistened with the same buffer was set on the bottom dish. Then the bottom dish was placed in a 5 L gas-sampling bag, the inside air (300 mL) was taken, and then concentrated under liquid oxygen to measure COS as described [16].

Results and Discussion

Isolation of Fungus Capable to Grow in Inorganic Medium with Sulfur

Fungi-like microorganisms were often observed in MPN medium prepared for the enumeration of chemoautotrophic sulfur-oxidizing microorganisms on the deteriorated sandstone in Angkor monuments [20]. Therefore, we tried to isolate microorganisms-like microorganisms from the culture by using agar medium of WS-S which contained powder sulfur as the sole source of energy. Strain THF01 was isolated from the culture inoculated with the deteriorated stone sample of AW1 sulfur-fixation (that which was collected from a pillar close to the floor level in the Northern Library of Angkor Wat [20]).

Sulfur-Oxidizing Fungi in Angkor Monuments

Although the growth was poor, strain THF01 was able to grow in mineral salts medium supplemented with elemental sulfur, and decreased the culture pH gradually from 5 to 4 during the cultivation. It was unexpected that fungi were isolated, therefore fungi having sulfur-oxidizing activity distributing in Angkor monuments were enumerated by counting colonies with clearing zone on agar medium containing powder sulfur. Both thiosulfate and sulfite were the oxidizing metabolites of sulfur detected in the clearing zone on the agar.

Enumeration of fungi is difficult because of the fragmentation of mycelium and dispersion of spores during handling of the samples. Thus, we treated the samples for plating under identical condition as much as possible so that comparisons among the samples could be made. Sulfur-oxidizing fungi were found widely in temples examined except for AW3, AW5, and AW7 which were located in the inner wall on the first floor of the main building of Angkor Wat (Fig. 1). The density in samples collected at Bayon temple was slightly

higher than the others, suggesting different community structure of microorganisms and/or difference of circumstance of these temples [20]. Total 18 fungal strains were all isolated from the mycelial colonies producing clearing zone on agar plate. Then, ITS regions of these fungi were sequenced for the phylogenetic identities. A phylogenetic tree has been made for the 13 strains belong to the genus Penicillium and Aspergillus, and the rest were close to the genus Pseudoc- mocystis, Fungiunc, Bipolaris, and Pselurospora (Fig. 2). Although participation of these fungi in stone deterioration is still unclear, the current results indicate that fungi having sulfur-oxidizing activity are widely distributed on the surface of deteriorated sandstones. Furthermore, our results suggest that sulfur-oxidation can be found in much diverse taxonomic groups. For further experiments, strain THF01 was used as the representative of these fungi.

Identification of Strain THF01

Mycelia of strain THF01 consisted of septated hyphae, and conidia were formed as asexual reproduction. Telomorph has not yet been found. These characteristics indicate that strain THF01 as a member of Anamorphic fungi. Macroc- conidium was observed however with very low frequencies (Fig. 3a). Acrochlamydium consisting of many microconidium was formed at the end of septate hyphae (2 to 3 septate) (Fig. 3b). Microconidium and macroconidium were observed in mycelial grown in nutrient-rich medium such as CMA and PDA. Smooth-walled chlamydospore were
formed during mycelia grown in a basal salts medium supplemented with elemental sulfur (Fig. 3c). Morphological observation indicated that strain THF01 belonged to the genus Fusarium and considered as Fusarium solani based on the conidial morphology [25].

Identification of strain THF01 as a member in the genus Fusarium was further confirmed by sequence data of ITS and the 18S rRNA gene and ITS (Fig. 4). These results indicate that the closest relative of strain THF01 is F. solani F. sp. radicicola [35], phytopathogenic ability which has been known in F. solani F. sp. radicicola has not yet been examined in strain THF01. On the basis of these results, THF01 was identified as F. solani.

Chlamydospore is known to be formed during mycelia grown under stressful conditions, suggesting utilizing elemental sulfur is not favorable substrate for the growth of THF01.

**Bradyrhizobium sp.** as an Endobacterium of Strain THF01

There are several reports on association of fungi and bacterium, therefore genomic DNA extracted from THF01 was subjected to PCR amplification of 16S rRNA gene. Agarose electrophoresis of the amplified product showed presence of the targeted DNA. In total, 1,377 bp of 16S rRNA gene was sequenced, and the phylogenetic analysis showed the closest relatives were *Bradyrhizobium* sp. Aceti2.3 (EF569645, 98%) and *Bradyrhizobium* sp. R144 (CP000494, 99%). THF01 has been re-inoculated on PDA supplemented with chloramphenicol, however the bacterial cells survived suggesting proliferation of the bacteria inside fungal hyphae. Specific participation of the endobacterium on the activity of sulfur oxidation is not yet fully determined.

**Oxidation of Elemental Sulfur by Strain THF01**

To examine production of sulfate by the oxidation of elemental sulfur on agar medium supplemented with sulfur, water soluble metabolite was extracted from the medium after 7 days of inoculation. For this experiment, sulfate-free medium of WSS-S was used to measure sulfate produced by fungi. Powder sulfur impregnated in agar underneath of the colony became clear because of the dissolution of sulfur. During the cultivation of strain THF01, pH of the culture medium decreased from an initial pH 5.8 to 4.6 in 3 days, and both thiosulfate and sulfate were detected from the extract obtained on the agar located inside of the clearing zone (Fig. 5a). These results suggested that strain THF01 oxidized elemental sulfur to sulfate through thiosulfate during metabolism.

Effects of Elemental Sulfur on Chlamydospore Germination

Chlamydospores of THF01 that were prepared from agar medium of WSS-S grown mycelium were inoculated to the liquid medium to confirm the growth and oxidation of sulfur in the mineral salts medium. The culture pH after THF01 inoculation decreased gradually from initial pH 5.0 to 4.0 over a period of 60 days. According to the results, concentrations of thiosulfate and then sulfate increased. WSS-S medium without THF01 showed negligible or no decrease of medium pH, and the production of thiosulfate or sulfate was not detectable (Fig. 5b, c). Microscopic observation of the culture after 30 days of inoculation showed germination of chlamydospore and growth of hyphae. Germination of spores inoculated in sulfur-free WSS liquid medium was 1.0% (2 out of 201), and the length of the hyphae was 3.0 to 6.0 µm. On the other hand, the germination in the culture supplemented with elemental sulfur was 60.2% (121 out of 201), and the length of hyphae was in the range of 2 to 109 µm. These results indicated that strain THF01 harboring elemental sulfur-oxidizing activity in an autotrophic medium and that chlamydospore germination need presence of elemental sulfur.

Strain THF01 isolated from the deteriorated sandstone showed elemental sulfur-oxidizing activity and was capable of growth in chemolithothrophic medium, although the growth of hyphae were limited in length compared with those grown in heterotrophic medium. Growth of fungi under autotrophic condition lacks available carbon sources has been reported in *Fusarium* sp. and *Cephalosporium* sp. [24] and to fungi isolated from soil [28]. Thus this study is the first report on the sulfur oxidation by fungi in medium without any organic carbon source.

**Degradation of COS by Strain THF01**

COS is the most abundant sulfur compound in the troposphere at a mixture ratio reported to be around 500 parts per trillion by volume (pptv) [3, 8, 39]. To obtain the information of possible sulfur source from atmosphere for strain THF01, COS degradation was examined at 30 ppmv and the ambient level. As shown in Fig. 6, strain THF01 degraded COS at both high concentration of 30 ppmv and approximately 600 ppmv suggesting COS in atmosphere could be a source of sulfur for the fungi growing on stone surface.

![Figure 5](image-url) Oxidation of elemental sulfur by THF01 in WSS-S medium. Concentrations of thiosulfate and sulfate in the agar of inside and outside of the clearing zone (a). Control indicates agar obtained from non-inoculated plate. Change of culture pH (b) and concentration of thiosulfate and sulfate (c) in liquid WSS-S medium. Symbols: (b) Empty circles, + THF01; filled circles, + THF01; *c* Empty, + THF01; Filled, + THF01; filled triangles, thiosulfate; empty squares and filled squares, sulfate

![Figure 6](image-url) Degradation of 30 ppmv (a) and ambient concentration (b) of COS by strain THF01. Empty circles, + THF01; filled circles, + THF01