Enumeration of Sulfur-Oxidizing Microorganisms on Deteriorating Stone of the Angkor Monuments, Cambodia

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Annual change in the density of sulfur-oxidizing microorganisms on sandstone was enumerated to know the effects on the deterioration of stone materials of the Angkor monuments in Cambodia. Samples were obtained from total 12 stations at the Angkor Wat, Bayon, and Phnom Krom temples between 1998 and 2007. Sulfur-oxidizing microorganisms enumerated in a mineral salts medium supplemented with elemental sulfur as the sole energy source had a density of 10³–10⁵ MPN (g sample)⁻¹. The sulfur-oxidizing microorganisms of the samples collected at Angkor Wat have tended to decrease in density since 2002; on the other hand, relatively constant values have been recorded in the samples of Bayon and Phnom Krom. These results suggest that the sulfur-oxidizing microorganisms on the stone play an important role in the decay of the building blocks by excreting sulfuric acid.

Key words: Angkor monuments, sulfur-oxidizing microorganisms, chemoorganotrophs, biodeterioration, stone weathering

The Angkor monuments in Cambodia were constructed between the 9th and 13th centuries. Sandstone and laterite were the main building materials, and the outer surface of the buildings was usually covered with grey to yellowish brown sandstone that has higher porosity than red sandstone and greenish greywacke. The Angkor monuments were cataloged in the UNESCO World Heritage List in 1992, and international cooperation has taken place because of severe deterioration. The Angkor monuments have been in the care of the Authority for the Protection and Management of Angkor and the Region of Siem Reap (APSARA) since 1997.

Weathering of stone is a combination of physical, chemical, and biological processes. While biological agents such as lichens, bryophytes, and higher plants are easily identified, the proliferation of microorganisms including bacteria, fungi, and algae is difficult to estimate. Biodeterioration of stone involves the production of inorganic and organic acids, the formation of biofilms, and pigmentation.

The microorganisms on stone have been analyzed using various procedures including microscopic observation, enrichment and cultivation, and measurements of community metabolism. Chemolithoautotrophic organisms of sulfur-oxidizing and nitrifying bacteria have been detected on stone surfaces of monuments. These bacteria excrete corrosive acids such as sulfuric acid and nitric acid which solubilize the minerals in stone. Sulfur-oxidizing bacteria of Thiobacillus, important causative microorganism in the deterioration of concrete, were isolated from stone by Gugliandolo.

Surveys of organisms at the Angkor site made by the Japanese Government Team for Safeguarding Angkor, UNESCO/Japan Trust Fund (JSA) since 1994, showed that cyanobacteria, algae, and lichens seem to cause the biodeterioration of sandstone. However, quantitative research on the biological deterioration has not yet been reported. The authors joined the JSA team in 1998 to specify the biodeterioration caused by bacteria. In this study, we focused on enumerating the cell density of sulfur-oxidizing microorganisms on the surface of stone materials because sulfuric acid, the metabolic product of these microbes, is a strong corrosive agent.

Material and Method

Site description

Cambodia has a typical monsoon climate where the direction of wind changes seasonally. Average rain fall is 200 mm per month in the wet season from May to October, and less than 50 mm per month in the dry season from November to April. Annual rainfall at the Angkor site is 1300–1500 mm per year, and the average temperature is 25°C. The average pH of the rainfall observed in 1995 was 6.29, not in the range of the definition for acid precipitation. Large animals that can be seen at the Angkor site are bats. In the evening, about 1000 bats per day in Angkor Wat, and about 500 bats per day in Bayon were counted flying away in Oct. 2000. Although the monuments are managed well at present and the number of bats seems to be decreasing, bat droppings were still observed on the floor of the monuments in 2007.

Sampling

Samples were taken at 12 stations on the surface of walls, the base of pillars near the ground, and the entrance of monuments (Fig. 1). Stone materials of Angkor Wat (samples AW1, AW2, AW3, AW5, and AW7) and Bayon (samples BY2-1, and BY2-2) were obtained mainly in the wet season from 1998 to 2004, 2006, and
2007. All samples except AW3, which was collected from a deteriorated wall, were collected from pillars near the ground (Fig. 2). Some samples of Phnom Krom (samples PK2 and PK3) and Bayon (samples BYb, BYc, and BYd) were obtained from 2002 to 2004, 2006, and 2007. Samples for the dry season were obtained at AW1, AW3, and AW5 in Jan. and at AW5 in Apr. 2002.

A small amount of surface material (approximately 0.4 g) was obtained from a severely deteriorated area without significant cultural or artistic value. Samples were collected aseptically using a sterilized spatula onto medical paper which was sterilized in advance under UV, and then placed in a sterilized test tube. Incubation for the enumeration of microorganisms was started within 3 days after the sampling.

**Enumeration of sulfur-oxidizing microorganisms**

An aliquot of sample was pulverized with a sterile spatula, and then diluted (1:10) in a sterilized 0.85% NaCl solution. The suspension was mixed thoroughly and then diluted ten-fold to $10^{-3}$ for inoculation. The pH of the suspension was measured in the samples obtained in 2006 and 2007. In the Angkor Wat and Bayon samples, the pH was in the range of 6.8–7.9 and in the Phnom Krom samples, 6.4–6.8. Sulfur-oxidizing microorganisms were enumerated based on the MPN method. The liquid medium used here was for chemolithoautotrophic sulfur bacteria with minor modifications: KH$_2$PO$_4$, 3.0 g; (NH$_4$)$_2$SO$_4$, 0.2 g; MgSO$_4$·7H$_2$O, 0.5 g; FeSO$_4$·7H$_2$O, 0.01 g; CaCl$_2$·2H$_2$O, 0.25 g; elemental sulfur, 10 g in 1000 mL of deionized water, pH 5.0 (WS5 medium). Elemental sulfur was sterilized separately by intermittent steaming for one hour every 24 hours three times. The diluted sample (0.5 mL) was inoculated to a 15 mL-volume test tube containing 4.5 mL of WS5 medium. The cultivation was conducted with reciprocal shaking at 30°C. Growth of sulfur-oxidizing microbes was confirmed by checking the decrease in the pH of the medium using a meter (Twin pH, Type B-212, Horiba Ltd., Kyoto, Japan) every week for two months. When the pH decreased from 5.0 to less than 4.4, the culture was considered positive.

**Enumeration of chemoorganotrophs**

Chemoorganotrophs were enumerated by the CFU method using one-tenth diluted Tryptosoy agar medium (E-MC83, Eiken Chemical, Tokyo, Japan) containing tryptophan, 1.5 g; soy peptone, 0.5 g; NaCl, 0.5 g; Bactoagar (Bekton Dickinson, Sparks, MD, USA), 15 g in 1000 mL of deionized water, pH 7.3. The diluted sample (0.1 mL) was spread onto the agar plate and cultivated at 30°C. Colonies formed on the plate were counted during 2 to 4 weeks and the CFU values were obtained from the mean for three plates.
Amount of total organic carbon (TOC) and water content

The TOC of surface materials of stone ground into powder with a mortar was measured with a non-dispersive infrared gas analyzer using 1.0 g of potassium peroxodisulfate and a modified oxidation vessel. Water contents of some samples collected in Aug. 2007 were determined in our lab as follows: sample AW1, 0.73%; sample AW2, 1.14%; sample BYd, 1.39% and sample PK3, 1.65%. Water contents of other samples were not determined because of the minimal amount of sample taken, however almost all samples obtained seemed similar to the samples for which water content was determined. Therefore, all densities were recognized as dry.

Results

Angkor Wat

Most of the lower part of pillars, up to about 40 cm from the base, have seriously deteriorated showing thinning especially the pillars on the inner side of the gallery (Fig. 2). No obvious biofilm was observed on the surface at any of the sampling stations.

Colonies of bacteria and fungi were observed on the agar medium for samples collected since 1998. The density of the chemoorganotrophs varied from $10^3$ to $10^6$ CFU (g sample)$^{-1}$ in the wet season (Fig. 3a). On the other hand, the cell density of sulfur-oxidizing microorganisms varied with each year and/or each station. In the case of sample AW1, sulfur-oxidizing microorganisms have been detected each year at a density of $10^1$–$10^5$ MPN (g sample)$^{-1}$. However in the case of samples AW5 and AW7, $10^1$–$10^5$ MPN (g sample)$^{-1}$ was measured from 1998 to 2001 and in 2007, and have not been detected in other years (Fig. 3b).

In the dry season, the density of chemoorganotrophs was $10^1$–$10^4$ CFU (g sample)$^{-1}$ for samples AW1, AW3, and AW5 collected in Jan. 2002, and $10^2$ CFU (g sample)$^{-1}$ for sample AW5 collected in Apr. 2002. Samples AW1 and AW3 collected in Jan. 2002 showed sulfur-oxidizing microorganisms at a density of $10^2$ MPN (g sample)$^{-1}$.

The TOC of the samples collected at Angkor Wat in 2007 was as follows: 2.2 mg C g$^{-1}$ for AW1 and 2.1 mg C g$^{-1}$ for AW2.

Bayon

The presence of biofilms of microorganisms was observed by naked eyes at all locations except BY2-1 and BY2-2. Similar to Angkor Wat, bat droppings were seen at the sampling stations.

BY2-1 and BY2-2 were on the lower part of the pillars at the entrance of the tower. A density of $10^2$–$10^6$ CFU (g sample)$^{-1}$ was enumerated in all samples, higher than the values for Angkor Wat (Fig. 4a). The density of sulfur-oxidizing microorganisms in Bayon was in the range of $10^1$–$10^5$ MPN (g sample)$^{-1}$ (Fig. 4b). The TOC of BY2-1 and BY2-2 was 2.2 mg C g$^{-1}$, and 0.5 mg C g$^{-1}$, respectively.

Samples BYb, BYc and BYd were collected from the wall of the inner gallery where both biofilms with various pig-
meltations and serious deterioration were observed. The TOC of BYc and BYd was 9.6 mg C g\(^{-1}\), and 7.8 mg C g\(^{-1}\), respectively. Sulfur-oxidizing microorganisms were in the range of 10\(^2\)–10\(^4\) MPN (g sample\(^{-1}\)) (Fig. 4a, b).

**Phnom Krom**

The stone materials of Phnom Krom showed exfoliation, and most of the relief was detached. A large number of bats have been observed flying away from one of the buildings in the evening, and many bat droppings were found inside, indicating the building is a roost. Sample PK2 was collected from lower part of a pillar at the entrance of a small temple located close to the east gate. Sample PK3 was collected from the surface of the inner wall of the temple.

The density of chemoorganotrophs was in the range of 10\(^4\)–10\(^6\) CFU (g sample\(^{-1}\)) and numbers were relatively stable. The density of sulfur-oxidizing microorganisms was in the range of 10\(^2\)–10\(^4\) MPN (g sample\(^{-1}\)) (Fig. 4a, b). Both of them were detected every sampling year, and no significant difference was seen between the two samples. The TOC of PK2 and PK3 was 0.9 mg C g\(^{-1}\), and 3.0 mg C g\(^{-1}\), respectively.

**Discussion**

In this study, we investigated the annual change in the cell density of sulfur-oxidizing microorganisms inhabiting the surface of deteriorated sandstone and producing sulfate. Freshly quarried sandstone obtained as a negative control from the Angkor site in 1998 showed no decrease in pH in the WS5 medium. Therefore, the decrease in pH of sandstone in the WS5 medium was considered to be caused by the deteriorated sample. Uchida and Suda\(^{18}\) reported 46.8 mg g\(^{-1}\) of sulfate in deteriorated sandstone at the Angkor site, about 400 times higher than the level in freshly quarried sandstone (0.119 mg g\(^{-1}\)). Arroyo et al.\(^3\) showed that a considerable amount of sulfate found on the decayed stone was produced by sulfur-oxidizing bacteria. Therefore, the high sulfate concentration in deteriorated sandstone at the Angkor site was considered to be produced by sulfur-oxidizing microorganisms.

The source of sulfur may be bat droppings at the Angkor site. Hosono et al.\(^8\) used an isotope to study the sandstone of the Angkor monuments, and found that the S (sulfur) and P (phosphorus) components of sandstone are mainly from bat droppings. As mentioned above, the average pH of rain water at the Angkor site was 6.29, not in the range of acid precipitation. Therefore, the high concentration of sulfate found in deteriorated stone was considered to originate from biological change and not from a reaction of sulfide contained in the atmosphere. These results indicated that the bat droppings on the monuments are one of the sources of salts containing sulfur.

Our results indicated relatively constant cell densities of sulfur-oxidizing microorganisms in Bayon and Phnom Krom. However, sulfur-oxidizing microorganisms in sample AW5 at Angkor Wat had tended to decrease in density since
The density of sulfur-oxidizing microorganisms on the stone surface was considered to have a relationship with the cleaning of bat droppings at the Angkor site. Till 1999, there were many bats living at the Angkor site and bat droppings were observed in the temples. With the increasing number of visitors, APSARA that constructed in 1995 conducted a thorough cleaning of Angkor Wat. The bat droppings on the ground were swept away and new droppings were cleaned quickly. This may explain the decreasing trend seen in Angkor Wat especially at stations AW5 from 2001. The density of sulfur-oxidizing microorganisms in the samples collected in Bayon increased after 2002 and remained relatively stable at high levels [around 10^3 MPN (g sample)^−1], different from the density values of Angkor Wat. Bat droppings in Bayon were cleared by APSARA similar to Angkor Wat from 2003 to 2007. The TOC was approximately 4 times higher in the Bayon (samples BYb, BYc, and BYd) than Angkor samples. The high TOC value may be due to chemolithoautotrophs, chemoorganotrophs, algae and lichen on the surface of the stone, providing organic and inorganic substances and water and supporting the sulfur-oxidizing microorganisms. In Phnom Krom, a large number of bats have been observed flying away in the evening from one of the buildings where samples were not collected. However, no obvious bat droppings were observed on the floor of the sampling location. In addition, algae and lichen were not observed at the sampling site. No clear reason for the continuous detection of sulfur-oxidizing microorganisms at Phnom Krom was found.

There was little or no significant change in the density of chemoorganotrophs at the Angkor site, with values in the range of 10^3–10^6 CFU (g sample)^−1. Tayler and May(17) reported a similar density of chemoorganotrophs, 10^3–10^6 cells g^−1, at Portchester Castle in southern England, although the medium used was different.

In the dry season, the density of chemoorganotrophs was lower than that in the wet season although no obvious difference was found in the density of sulfur-oxidizing microorganisms. Uchida et al.(19) showed the water content of sandstone to be up to 14% in the wet season and 3% in the dry season. Water is an important environmental factor both for the growth of microorganisms and for the diffusion of salts by capillary action. Therefore, the low water content of sandstone in the dry season was correlated to the low density of microorganisms. Tayler and May(17) studied the seasonal variation of microorganisms on decayed stone and reported higher numbers of bacteria in the wetter winter than dry summer. However, the lower water content in the dry season was also considered to cause the concentration of sulfuric acid in stone and lead to much more serious deterioration.

A novel species of sulfur-oxidizing bacteria using thiosulfate as an energy source was isolated from volcanic deposits in Miyake-Jima (TOC content, 4 mg g^−1; water content, 3.6%)10; water content, 3.6%)10; Moser and Olson14 and Badawy15 reported 10^3 cells g^−1 of autotrophic sulfur-oxidizing bacteria in soils. Chapman15 also reported that the density of sulfur-oxidizing bacteria (neutrophilic Thiobacillus spp.) was in the range of 10^2–10^5 cells g^−1, the bacteria being found in 84% of agricultural soil samples. Therefore, the sulfur-oxidizing microorganisms can be detected easily in many kinds of soils and even in volcanic environment. However, on the sandstone surface the density of sulfur-oxidizing microorganisms was considered to differ according to location. For example, sulfur-oxidizing microorganisms have been detected in Bayon and Phnom Krom, but varied by location in Angkor Wat. This may have a relationship with environmental factors such as the infiltration of rain water through crannies in the wall, changes in humidity and temperature caused by sunlight, and the supply of organic materials on the stone. However at this time, details are still unknown.

Our data on the annual change in microbial density clearly showed that microorganisms with sulfur-oxidizing activity inhabited the stone and should be considered to contribute to the deterioration.

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