Biological Studies of Spring Deposits Situated in Kuroiwa Spring, Kyushu

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Introduction

Hot spring deposits are formed from dissolved matter in spring waters undergoing physical and chemical changes in high-pressure or hot water erupting from underground. The first report of chemical analysis of hot spring deposits was published by Ohashi R. (1920) on radioactive deposits at Tamagawa Hot Springs, Akita Prefecture in Japan¹. After that, many papers have been published on hot spring deposits²⁻⁷, and deposits were classified according to chemical components of the waters; the categories sulfur, sulfides, hydrous ferric oxide, hydrous silica and carbonates²).

Until now, hot spring deposits have been studied only chemically. But at this time we are focusing on the formation of the deposits, which were observed with the naked eyes and also involves the participation of microorganisms. Therefore we undertook a biological study, that is, light and electron microscopic observations and biological characteristics of microorganisms of Kuroiwa Spring, Fukuoka Prefecture. The many kinds of microorganisms observed and isolated are reported herein.

Materials and Methods

1. Samples

The spring deposit samples used in this study were collected from Kuroiwa Spring (No.1) in Sedaka-Cho, Fukuoka Prefecture on May 5th, 1988 (Fig. 1). The deposits were located at the bottom of a small puddle. The deposits and spring waters were collected in polycarbonate bottles (250ml). The temperature and pH value of the water were measured at that time.

2. Light microscopic observation of deposits

The samples were observed under light microscopy, and representative photographs were taken. The methods of observation were as follows: a) fresh samples were used for observation by phase-contrast microscope; b) Gram’s method was used for the preparation of fresh samples;
c) samples that were fixed with formalin and no staining, were observed by direct observation method. Identification of microorganisms was performed using the following textbooks: "Illustration of the fresh water plankton of Japan"; "Illustration of the fresh water algae in Japan"; "Encyclopaedia zoologica illustrated in colours" and "Photomicrographs of the fresh-water algae".

3. Electron microscopic observation

First, samples deposits were washed with 0.1M PBS buffer, and fixed by 2.5% glutaldehyde (in 0.1M PBS buffer). The thin cover glass was coated with 0.2% poly-L-lysine, and the fixed sample placed on the coated glass (slide), and used for after staining (1% osmic acid and conductive staining method 2% tannic acid and 1% osmic acid). A scanning electron microscope (SEM) was used for observation of the samples.

4. Isolation and identification of bacteria from deposits

a) Medium

Nutrient broth (Nissui), Nutrient agar (Nissui) and Thiobacillus identification medium were used for isolation of bacteria living in the spring deposits. The components of Thiobacillus identification medium were as follows: MgSO₄•7H₂O: 0.20g, K₂HPO₄: 1.00g, FeSO₄•7H₂O: 0.05g, CaCl₂: 0.02g, NH₄Cl: 1.00g, MnCl₂•4H₂O: 0.002g, Na₂MoO₄•2H₂O: 0.001g, Na₂S₂O₃•5H₂O; 7.00g, purified agar (Oxoid) (if necessary); 12g in 1000ml of distilled water (The pH of medium will be 7.4 after mixture).

b) Isolation

Spring deposit/spring water suspension was added to each medium, at a volume of 10%, and incubated at 30°C for 1 week. After that, the turbidity of the medium was confirmed, and medium was streaked on a medium plate, as mentioned above.
c) Characterization of bacteria

Bacteria colonies were observed for color, form and diameter. Gram stained bacteria were observed by light microscope, and the following tests were carried out on heterotrophic bacteria: catalase test, oxidase test, motility test, growth condition test, oxidation or fermentation of glucose test, acid from glucose test, inositol and saccharose test, growth in 6% NaCl or without NaCl test, lysine decarboxylase test, ornithine decarboxylase test, gelatine hydrolysis test and starch hydrolysis test. Genetic names were decided. Autotrophic bacteria were grown in Thiobacillus identification medium, and identified as *Thiobacillus spp*..

### Results and discussion

White-color deposits were observed at the bottom of a small puddle of Kuroiwa Spring (Photo 1). The chemical components of the spring waters are presented in Table 1. It was recognized that the spring waters belonged to Ca-SO$_4$ type simple cold waters. The temperature and pH value of the spring waters were 21.7°C and 7.2, respectively.

In both light microscopic and electron microscopic observations, many kinds of microorganisms were observed in the deposits.

In the protozoa group, *Trinema sp.*, *Euglypha sp.*, and *Paramecium sp.* were identified from their morphological characteristics (Photo 2). Especially, *Paramecium sp.*, which showed active movement with a peritrichal flagella was observed in the deposits, using the phase-contrast microscopic method (Photo 2-3).

<table>
<thead>
<tr>
<th>Chemical components</th>
<th>K</th>
<th>Na</th>
<th>Ca</th>
<th>Mg</th>
<th>Fe</th>
<th>Mn</th>
<th>Cl</th>
<th>SO$_4$</th>
<th>HCO$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.9</td>
<td>4.2</td>
<td>52.4</td>
<td>12.5</td>
<td>tr</td>
<td>0.30</td>
<td>4.6</td>
<td>168.0</td>
<td>17.0</td>
</tr>
</tbody>
</table>

*tr*: trace

Photo 1 Sampling point of Kuroiwa Spring
(near flow out point)
In the blue-green algae group, *Chroococcus minutus var. minutus*, *Phormidium lepidum var. amorphum*, *Scytonema sp.*, *Calothrix braunii* and *Anabaena sp.* were identified from their remarkably morphological characteristics (Photo 3). In *Scytonema sp.*, false branches arose in unilateral pairs (Photo 3-5-e). In *Calothrix braunii*, trichomes were tapered from basal heterocyst to a fine point, abruptly ending in the others (Photo 3-3). Among these algae, *Scytonema sp.* and *Calothrix braunii* occupied the majority of the deposits. *Anabena sp.* was identified by their remarkable heterocyst, akinete and tricone cell (Photos 3-1 and 2). On the other hand, by the use of tram staining, heterocysts and akinetes were stained to gram positive, and vegetative cells were stained to gram negative, as shown in Photo 4.

In the diatomaceae group, *Navicula cuspidata*, *Nitzschia capitalla*, *Pinnularia microstauron*, *Eunotia arcus*, *Eunotia sp.* were identified by their morphological characteristics under electron microscopic observation (Photo 5). Rostrum were slightly inflated at the apex in *N. capitalla* (Photo 5-1), and this diatom was reported to be rarely identified in Japan. So, it is considered very significant that *N. capitalla* was found in the springs in Kyusyu.

In the bacteria group, short rod and rod shape were observed in the deposits by electron microscopic observation. Characterization of the bacteria, revealed that 3 different types of bacteria were isolated from the deposits (Table 2). As for, short rod bacteria, these strains grew in the autotorophic medium containing thiosulfate as the only energy source. Therfore, the strain was determined to be *Thiobacillus spp.*. As for the rod-shape bacteria, remaining 2 strains showed heterotrophic growth. According to the results of characterization of heterotrophic bacteria, these strains were determined to be *Aeromonas spp.* and *Alcarigenes spp.*
Photo 3 Photographs (light microscopic observation) of blue-green algae detected from the deposits
1 and 2) Anabaena sp. (a and c: heterocyst, b: akinete)
3) Calothrix braunii BORNET et FLAHAULT (d: heterocyst)
4) Pholmidium lepideum GEITLER var. amorpham EMOTO
   et HIROSE
5) Scytonema sp. (e: false branching)
6 and 7) Chroococcus minutus (KÜTZING) NAEGELI var.
   minutus

Photo 4 Gram stained cells of Anabaena sp.
(a: akinete, b: heterocyst)
Photo 5 Photographs (electron microscopic observation) of diatom detected from the deposits
1) Nitzschia capitata HUSTEDT
2) Eunotia arcus EHRENBERG
3) Eunotia sp.
4) Pinnularia microstauron (EHRENBERG) CLEVE
5) Navicula cuspidata KÜTZING

Table 2 Characteristics of 3 kind of bacteria isolated from the deposits

<table>
<thead>
<tr>
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<th>Isolated strains</th>
</tr>
</thead>
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<tr>
<td></td>
<td>88 – 1</td>
</tr>
<tr>
<td>Colony color</td>
<td>W</td>
</tr>
<tr>
<td>shape</td>
<td>S</td>
</tr>
<tr>
<td>diameter (mm)</td>
<td>1.5</td>
</tr>
<tr>
<td>Shape</td>
<td>Rod</td>
</tr>
<tr>
<td>Gram staining</td>
<td>–</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
</tr>
<tr>
<td>Growth in aero.</td>
<td>+</td>
</tr>
<tr>
<td>Growth in anaero.</td>
<td>–</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
</tr>
<tr>
<td>O/F test</td>
<td>–</td>
</tr>
<tr>
<td>Growth in: thiosulfate only</td>
<td>+</td>
</tr>
<tr>
<td>42°C</td>
<td></td>
</tr>
<tr>
<td>6% NaCl</td>
<td>–</td>
</tr>
<tr>
<td>Lysine decarboxylase</td>
<td>–</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>–</td>
</tr>
<tr>
<td>Acid from: Inositol</td>
<td>–</td>
</tr>
<tr>
<td>Saccharose</td>
<td>+</td>
</tr>
<tr>
<td>Gelatine hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>+</td>
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</table>

W: White, S: Smooth, R: Rough
In all, 3 different types of protozoa, 5 different types of blue-green algae, 5 different types of diatoma and 3 different types of bacteria were observed and isolated in the soft type deposits of Kuroiwa Spring (Table 3). It seems that the deposits are mainly composed of those microorganisms, and especially blue-green algae was at the most important constituent of the deposits. This opinion is based on the observations under electron microscope and light microscope. The three photographs in Photo 6 show that many kinds of blue-green algae clung together, and materials seeming to be nonorganic existed on or around the algae. Thus, it seems that the deposits in this study visible with the naked eye were made up of blue-green algae growth. In detail, small deposits that could not be seen with the naked eye were made up of "chemical deposits", which, when setting on the algae, grew bigger with more and more chemical deposits. After that, the deposit became a "house-like composer" for protozoa, diatoma and bacteria, thus the small free-living organisms

<table>
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<th>Table 3</th>
<th>Species name of protozoa, blue-green algae, diatoma and bacteria detected from the deposits</th>
</tr>
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<tr>
<td><strong>Protozoa</strong></td>
<td>Trinema sp. &lt;br&gt; Euglypha sp. &lt;br&gt; Paramecium sp.</td>
</tr>
<tr>
<td><strong>Blue-green algae</strong></td>
<td>Chroococcus minutus (KÜTZING) NAEGELI var. minutus &lt;br&gt; Calothrix braunii BORNET et FLAHAULT var. amorphum EMOTO et HIROSE &lt;br&gt; Anabaena sp. &lt;br&gt; Scytonema sp.</td>
</tr>
<tr>
<td><strong>Diatoma</strong></td>
<td>Navicula cuspidata KÜTZING &lt;br&gt; Nitzschia capitella HUSTEDT &lt;br&gt; Pinnularia microstauron (EHRENBERG) CLEVE &lt;br&gt; Eunotia arcus EHRENBERG &lt;br&gt; Eunotia sp.</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td>Alcaligenes sp. &lt;br&gt; Aeromonas sp. &lt;br&gt; Thiobacillus sp.</td>
</tr>
</tbody>
</table>

Photo 6 Photographs (1 and 2: electron microscopic observation, 3: light microscopic observation) of deposits: many kinds of blue-green algae were clinging together
were thought to live in it (Photo 6). Although a report by Yamazaki Y. (1973), mentions some leaves, seeds and spiral shells being detected in the hot spring deposits in Shiobara Area with the substance microscopy. This paper was the first to show that many kinds of microorganisms actually thrive in hot spring deposits.

Acknowledgements

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福岡県黒岩湧水にて採取した
沈澱物の生物学的検討

杉森賢司，御船政明，湯原浩三

温泉沈澱物に関しては現在まで化学的なアプローチが主に行われてきが、生物学的研究は行われていないのが現状である。しかし、その成因については生物の関与も大きいと考え、黒岩湧水の湧出口付近にみられた沈澱物について生物学的考察を行った。その結果、沈澱物中から細菌類（3種），藻類（5種），原生動物（3種）が検出された。さらに沈澱物の電子顕微鏡観察により、その沈澱物は藍藻類からみあってその核をなしているのが観察され、検出された藻類類，細菌類，原生動物はあたかもそこに宿しているかのごとく生息していることがわかった。さらにそのまわりに無生物と思われる（いわゆる化学的な物質と考えられる）付着物が確認された。それが徐々に成長し大きくなって目に入れる沈澱物になると考えられる。この様に、一見化学的に出来たと考えられる湧水沈澱物であっても、その成因には実際に生物が関与しており、これらのことから、沈澱物を考える上で生物学的ファクターは非常に重要であると考える。

Key words: Spring deposits, Blue-green algae, Bacteria, Diatoms, Protozoa