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Research in Microbiology xx (2016) 1–9

www.elsevier.com/locate/resmic

Microbial community profiling of the Chinoike Jigoku ("Blood Pond Hell") hot spring in Beppu, Japan: isolation and characterization of Fe(III)-reducing *Sulfolobus* sp. strain GA1

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Received 26 February 2016; accepted 27 April 2016
Available online ■ ■ ■

Abstract

Chinoike Jigoku ("Blood Pond Hell") is located in the hot spring town of Beppu on the southern island of Kyushu in Japan, and is the site of a red-colored acidic geothermal pond. This study aimed to investigate the microbial population composition in this extremely acidic environment and to isolate/characterize acidophilic microorganism with metal-reducing ability. Initially, PCR (using bacteria- and archaea-specific primers) of environmental DNA samples detected the presence of bacteria, but not archaea. This was followed by random sequencing analysis, confirming the presence of wide bacterial diversity at the site (123 clones derived from 18 bacterial and 1 archaeal genera), including those closely related to known autotrophic and heterotrophic acidophiles (*Acidithiobacillus* sp., *Sulfobacillus* sp., *Alicyclobacillus* sp.). Nevertheless, successive culture enrichment with Fe(III) under micro-aerobic conditions led to isolation of an unknown archaeal organism, *Sulfolobus* sp. GA1 (with 99.7% 16S rRNA gene sequence identity with *Sulfolobus shibatae*). Unlike many other known *Sulfolobus* spp., strain GA1 was shown to lack sulfur oxidation ability. Strain GA1 possessed only minor Fe(II) oxidation ability, but readily reduced Fe(III) during heterotrophic growth under micro-aerobic conditions. Strain GA1 was capable of reducing highly toxic Cr(VI) to less toxic/soluble Cr(III), demonstrating its potential utility in bioremediation of toxic metal species.

Keywords: Microbial diversity; Extreme environment; Acidophile; Fe(III) reduction; Cr(VI) reduction; *Sulfolobus*

1. Introduction

Japan lies on the "Ring of Fire" and its volcanic nature has endowed the country with a number of geothermal hot springs. These abundant extreme environments in the country are a precious resource for extremophiles [e.g., [1–3]]. Some of the many highly acidic hot springs in Japan are located on the southern island of Kyushu. These hot springs are a potential source of extremely acidophilic microorganisms with various applications in biohydrometallurgy.

Metal-polluted aqueous systems, including acid mine drainages (AMDs) and metal refinery process waters, are often highly acidic. When some toxic metal pollutants such as Cr(VI), V(V) and U(VI) are reduced to lower oxidation states, their toxicity and solubility can be decreased, facilitating their removal from the aqueous phase. Since Fe(III)-reducing microorganisms are often capable of reducing other metals (e.g., *Acidocella aromatic* [4], *Acidiphilum cryptum* [5], *Shewanella* spp. [6,7]), the search for Fe(III)-reducing extreme acidophiles may enable isolation of useful microorganism for metal bioremediation purposes.

Another potential use of Fe(III)-reducing acidophiles in biohydrometallurgical application lies in their reductive dissolution of Fe(III)-minerals (e.g., nickel laterites where Ni is intimately associated with goethite (α-FeO•OH)) [8].

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http://dx.doi.org/10.1016/j.resmic.2016.04.011
0923-2508/

Acidithiobacillus ferrooxidans, *Sulfobacillus benefaciens*, *Acidicaldus organivorans*, and *Acidiphilum* sp. SJH have been used to reduce soluble Fe(III), causing disequilibrium between Fe(III) in the mineral phase and the solution phase, thereby accelerating the mineral dissolution rate. *A. ferrooxidans* was shown to dissolve over 70% of Ni from the ore within 14 days [8].

The genus *Sulfolobus* is known to be a group of obligately aerobic, thermo-acidophilic sulfur-oxidizing archaea. They have been isolated from a number of sulfur-rich geothermal environments around the world [9–12]. Either autotrophically or heterotrophically, utilization of sulfur is widely recognized in *Sulfolobus* spp. (*Sulfolobus tokodaii* [2], *Sulfolobus metallicus* [10], *Sulfolobus yangmingensis* [11], *Sulfolobus shibatae* [13] and *Sulfolobus tengchongensis* [14]). Some *Sulfolobus* spp. such as *S. tokodaii* and *S. metallicus* were reported to possess Fe(II) oxidation ability, and *S. metallicus* was used as an aniron/sulfur oxidizer in bioleaching application of chalcopyrite [15–19]. On the other hand, Fe(III) reduction ability in *Sulfolobus* is much less clear.

This study aimed to reveal the microbial community structure of this geothermal hot spring and to enrich/isolate Fe(III)-reducing acidophiles potentially useful in biohydrometallurgical applications.

2. Materials and methods

2.1. Environmental sample collection and analysis

Chinoike Jigoku is located in the hot spring town of Beppu on the southern island of Kyushu (33° 33′ N; 131° 48′ E), and features its distinctively red-colored, highly acidic pond (Fig. 1).

Samples of surface water and 70 cm-deep bottom sediment were collected at the identical location of the pond on July 1, 2013. In the sampling tube, red sediment occupied the upper layer 20 cm thick, below which was found a much denser gray sediment (70 cm). Parameters such as temperature, pH, Eh and dissolved oxygen (DO) were measured on-site for surface water sample. Elemental concentrations in surface water sample were determined by ICP-AES (Vista-MPX, Seiko). Total organic carbon (TOC) content of sediment samples was analyzed using TOC-VCHS (Shimazu), after a hydrothermal pre-treatment in which teflon vessels containing a known amount of each sediment sample and 60% HNO_3 solution were placed in the microwave digestion system (Ethos Plus, Milestone) and heated to 230 °C with 7 °C/min increments, kept for 15 min at 230 °C, and finally allowed to cool to room temperature. Sediment samples were freeze-dried overnight for X-ray fluorescence (XRF; ZSX Primus II, Rigaku) and X-ray diffraction (XRD; Ultima IV, Rigaku; CuKα 40 mA, 40 kV) analyses. To estimate copy numbers of the 16S rRNA gene in the samples, real-time PCR (TaqMan Mater; LightCycler 1.5, Roche) was conducted on genomic DNAs extracted from each sample (Ultra Clean Water DNA isolation kit and Power Soil DNA isolation kit, MOBIO) using bacterial primers 341f (5′-TCTACGGAAGGCTGCAG-3′) and 517r (5′-ATTACCGCGGCTGCTGG-3′) (94 °C-30 s, 52 °C-30 s, 72 °C-30 s in 20 μl). *Escherichia coli* 16S rRNA genes

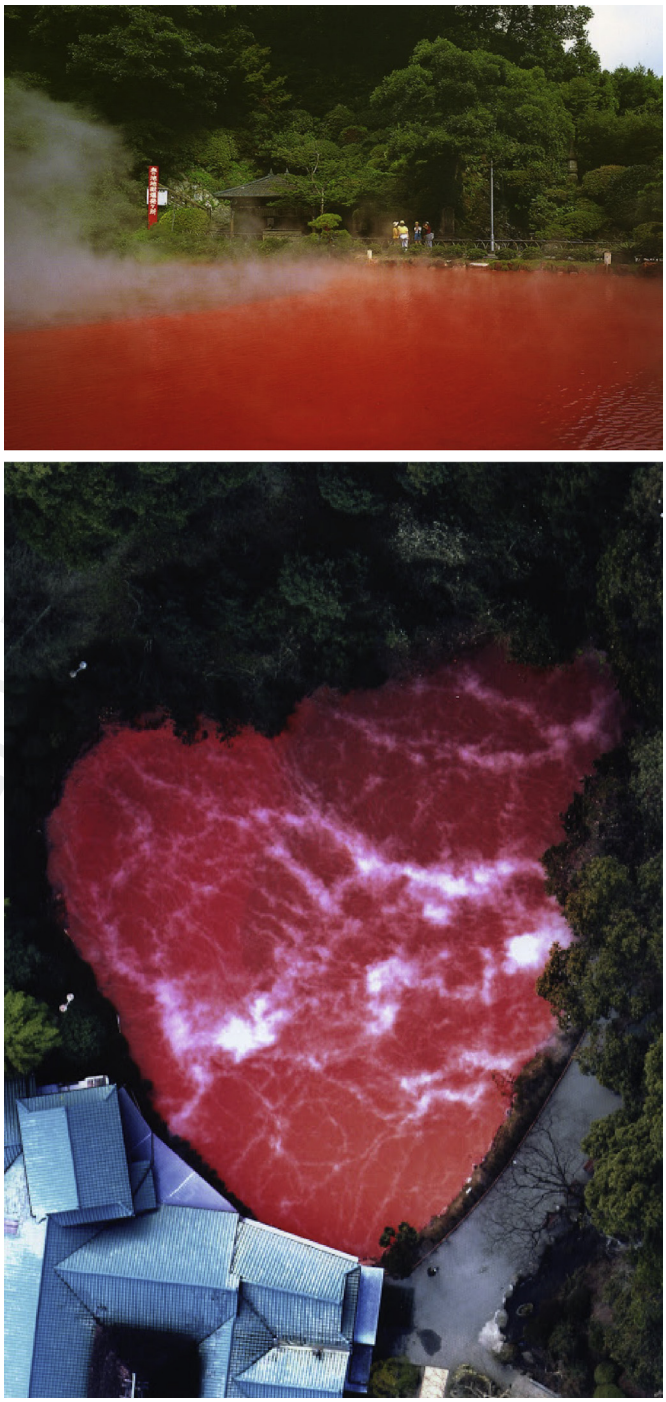

Fig. 1. Photographic images of the Chinoike Jigoku ("Blood Pond Hell") hot spring: Ground view (upper) and aerial view (lower). (Photos courtesy of Mr. Masafumi Kudo, Chinoike Jigoku).

(ranging from 2.7×10^4 to 2.7×10^7 copies) were used as standard.

2.2. Microbial community profiling

The presence of bacteria and archaea in the samples was investigated by conducting PCR on genomic DNA extracted from red sediment using bacterial (27f and 1492r; [20]) and archaeal (Arch21f (5′-TTCCGGTTGATCCYGCCGGA-3′:

Please cite this article in press as: Masaki Y, et al., Microbial community profiling of the Chinoike Jigoku ("Blood Pond Hell") hot spring in Beppu, Japan: isolation and characterization of Fe(III)-reducing *Sulfolobus* sp. strain GA1, Research in Microbiology (2016), http://dx.doi.org/10.1016/j.resmic.2016.04.011