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Sulfur-metabolizing bacterial populations in microbial mats of the Nakabusa hot spring, Japan

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ABSTRACT

At the Nakabusa hot spring, Japan, dense olive-green microbial mats develop in regions where the slightly alkaline, sulfidic effluent has cooled to 65 °C. The microbial community of such mats was analyzed by focusing on the diversity, as well as the *in situ* distribution and function of bacteria involved in sulfur cycling. Analyses of 16S rRNA and functional genes (*aprA*, *pufM*) suggested the importance of three thermophilic bacterial groups: aerobic chemolithotrophic sulfide-oxidizing species of the genus *Sulfurihydrogenibium* (Aquificae), anaerobic sulfate-reducing species of the genera *Thermodesulfobacterium*/*Thermodesulfatator*, and filamentous anoxygenic photosynthetic species of the genus *Chloroflexus*. A new oligonucleotide probe specific for *Sulfurihydrogenibium* was designed and optimized for catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH). *In situ* hybridizations of thin mat sections showed a heterogeneous vertical distribution of *Sulfurihydrogenibium* and *Chloroflexus*. *Sulfurihydrogenibium* dominated near the mat surface (50% of the total mat biovolume), while *Chloroflexus* dominated in deeper layers (up to 64% of the total mat biovolume). Physiological experiments monitoring *in vitro* changes of sulfide concentration indicated slight sulfide production by sulfate-reducing bacteria under anoxic-dark conditions, sulfide consumption by photosynthetic bacteria under anoxic-light conditions and strong sulfide oxidation by chemolithotrophic members of Aquificae under oxic-dark condition. We therefore propose that *Sulfurihydrogenibium* spp. act as highly efficient scavengers of oxygen from the spring water, thus creating a favorable, anoxic environment for *Chloroflexus* and *Thermodesulfobacterium/Thermodesulfatator* in deeper layers.

Introduction

Microbial mats develop in a wide range of aquatic habitats, such as geothermal hot springs, hypersaline ponds, marine cold seeps or hydrothermal vents. On the deep sea floor, light is absent and filamentous mat-forming chemoautotrophic sulfur bacteria develop, while microbial mats from terrestrial hot springs are also often composed of phototrophic bacteria [9]. In this environment, two types of phototrophs contribute to the formation of mats: (i) oxygenic phototrophs (cyanobacteria) growing autotrophically with water and carbon dioxide as the sole electron donor and carbon source, respectively, and (ii) anoxygenic phototrophs growing by photosynthesis without producing oxygen.

One of the best investigated hot springs is the slightly alkaline, sulfidic hot spring at Nakabusa, Nagano Prefecture, Japan. This site is well known for the formation of dense, colorful microbial mats (Fig. 1). Due to high temperatures of up to 70 °C, the thermophilic microorganisms in the mats are protected from grazing by higher organisms like insects (Matsuura, personal communication) [4]. The spring water is of volcanic origin and contains various reduced sulfur compounds in high concentrations, which can be used as electron donors for microbial growth [27]. The temperature and sulfide concentration in Nakabusa spring water are the key factors structuring the microbial community [27]. Close to the source, the temperature is approximately 75 °C, which is beyond the tolerance of any cyanobacteria. However, at this point, streamers extend from gray-colored mats, and 16S rRNA gene sequences from sulfide-oxidizing (*Aquifex* spp., *Sulfurihydrogenibium* spp.) and sulfate-reducing bacteria (*Thermodesulfobacterium*-affiliated species) have been retrieved from the streamers [27]. At low sulfide concentrations (<0.1 mM), filamentous *Aquifex*-like bacteria dominated, while at high sulfide concentrations (>0.1 mM) large sausage-shaped *Sulfurihydrogenibium*-like bacteria dominated the

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Fig. 1. Sampling site. Nakabusa hot spring in Nagano prefecture, Japan. Microbial mats from two contrasting sites were sampled. Site 1 consisted of grayish mats which developed close to the spring source under flowing water of about 75 °C, whereas at site 2 olive-green mats developed on an almost vertical concrete wall overflowing with spring water of 65 °C. Mats were growing to a thickness of approximately 4 mm.

microbial mat community, as determined by DGGE analysis [27]. Fluorescence *in situ* hybridization (FISH) of the streamer confirmed the presence of *Aquifex* spp., a dominance of sulfate-reducing *Thermodesulfobacterium*-related species (82% of the total DAPI counts) at high sulfide concentration [27] and the presence of numerous sausage-shaped bacteria. Thus, Nakagawa and Fukui [27] proposed active sulfur cycling in the streamers. Species of *Sulfurihydrogenibium* were also assumed to dominate the so called sulfur-turf microbial mats in many other neutral to alkaline hot springs [13,18,19,41].

A major change in the microbial community structure of Nakabusa mats and streamers occurs further down when the spring water flowing down a wall has cooled to a temperature of approximately 70 °C [26]. At this point, the mats turn olive-green, indicating the growth of photosynthetic organisms. Based on pigment analysis, Sugiura et al. [36] suggested a dominance of anoxygenic photosynthetic bacteria related to the green non-sulfur bacterial group of Chloroflexi. Similar mats were also observed in several other alkaline hot springs in Japan [9], Italy [29], in Yellowstone National Park, USA [28], and Iceland [34]. For Icelandic hot springs, Skirnisdottir et al. [34] reported that *Chloroflexus* spp. were the dominant mat organisms in a low-sulfide spring (0.030 mM) below 70 °C, whereas Aquificae were dominant in a high-sulfide spring (0.364 mM) of a similar temperature.

In this study, the olive-green microbial mat of the Nakabusa hot spring was analyzed in more detail. This is the first study linking diversity and community structure with the function of key microbial populations. The focus was on sulfur cycling inside the mat using a combination of molecular methods (DGGE, comparative 16S rRNA gene sequence analysis, FISH) and physiological experiments. The diversity of sulfur-metabolizing bacteria and photosynthetic bacteria was further studied by the analysis of the key genes for dissimilatory adenosine 5′-phosphosulfate reductase (*aprA*) and subunit M of the photosynthesis reaction center (*pufM*). Using newly developed specific oligonucleotide FISH probes, the spatial distribution and interactions of key populations were shown for the first time in intact mat sections. Furthermore, the biomass of key populations was estimated.

Materials and methods

Study site and microbial mat sampling

The Nakabusa hot spring is located in the Nagano Prefecture, Japan (36°23′ 15″N, 137°45′ 00″E). The pH of the spring water was slightly alkaline (pH 8.5–9.0). Earlier studies reported a sulfate concentration of 0.019–0.246 mM, a total organic carbon content of 0.393–0.415 mg L^{-1}, and moderate sulfide concentrations of 0.046–0.123 mM [26,27]. However, these chemical characteristics can vary between different sampling sites and seasons.

In September 2006 and October 2007, mats from two contrasting sites were sampled. Site 1 consisted of grayish mats which developed close to the spring source under flowing water with a temperature of approximately 75 °C (called "microbial streamer"), whereas at site 2 olive-green mats developed on an almost vertical concrete wall with overflowing spring water of 65 °C (Fig. 1). All mats had grown to approximately 4 mm thickness.

Microbial mat samples were kept in sterile plastic tubes (for molecular analysis) or glass bottles completely filled with hot spring water to avoid oxidation. Samples were transferred to the laboratory on ice and used for physiological experiments within 8 h.

Sulfide consumption measurements

Artificial hot spring water (~0.5 mM S^{2-}, 1 mM Cl^-, 1 mM PO_4^{3-}, 0.6 mM SO_4^{2-}, 2 mM K^+, ~4.1 mM Na^+, pH 8.5) amended with 1 mM HCO_3^- was used to measure the CO_2-dependent change of the sulfide concentration. An aliquot (60 mL) of spring water was added to a clean sterilized glass bottle (70 mL), bubbled with H_2O-vapor-saturated N_2 gas for 20 min, and preheated to 65 °C in a water bath. Approximately 1 g of the mat from site 2 grown at 65 °C was placed in the bottle, which was subsequently sealed with a butyl rubber stopper, under a headspace of N_2 gas and incubated at 65 °C. Bottles were wrapped in aluminum foil for dark treatment, and were illuminated with incandescent light (approximately 200 W/m^2). For oxic incubations the butyl rubber stopper was removed and the water was stirred at approximately 500–600 rpm to incorporate air. Subsamples of 250 μL of spring water were collected with a gas-tight syringe (Hamilton, Nevada, USA) in several minute intervals. The subsamples were immediately fixed with 500 μL 0.4% zinc acetate and alkalized with 500 μL 0.04 N NaOH. Dissolved sulfide concentrations were measured colorimetrically by the methylene blue formation method [5].

DNA extraction

Approximately 0.5 g (wet weight) of the mat pieces were placed in a 1.5 mL tube containing 1 mL of extraction buffer (100 mM Tris–HCl, 10 mM EDTA, 100 mM NaCl, 0.5% SDS [pH 8.0]). Initially, three different protocols for mechanical cell lysis were applied: (i) homogenization with a pestle, (ii) homogenization with a pestle