New method of observing *in situ* microbe-metal-mineral interaction by μ-XAFS-FISH technique

Microorganisms in the environment critically impact global geochemical cycles and redox reactions of various elements. Many geochemical important redox reactions are largely associated with microbial activity and are energy sources for microorganisms. In addition, microbes can mediate mineral formation by a process called biomineralization. For instance, recent studies suggest a significant relationship between Fe(II)-oxidizing bacteria and ancient banded iron formation (BIF), one of the large geochemical events in Earth's history [1]. Biominerals have unique morphologies and characteristics such as nanoparticles, high surface area, and reactivity. The biominerals could be important sorbents for a range of metals and often play a critical role as natural catalysts in oxidation-reduction reactions for the metals [2].

The general ecological importance of environmental microbial reaction and biomineralization has been well recognized; however, the specific factors of the reactions in the environments, such as the reaction rate, spatial dynamics, and controlling factors, are poorly understood, even in sediments and soils. For example, in sediments and soils, which have heterogeneous components (e.g., water, organic materials, microorganisms, and primary and authigenic minerals), the local chemical profiles (e.g., pH and abundance of oxygen and nutrients) drastically change at the micrometer scale. Depending on such profiles, microbial reactions and habitability vary locally and form a complex geochemical network in the environments. Hence, it is necessary to develop new analytical techniques that allow the simultaneous determination of both microbial community composition and elemental characteristics in high spatial resolutions, in order to understand the linkage between microbial activity/reaction and biomineralization.

Here, we directly coupled a synchrotron microprobe analysis (μ-XAFS) and an *in situ* phylogenetic analysis, fluorescence *in situ* hybridization (FISH), to determine simultaneously the chemical species and distributions of microbial groups at the micrometer scale (Fig. 1). Coupling of μ-XAFS and FISH provides more direct information on the identity and localization of microbially catalyzed redox processes and the associated minerals, which leads to a better understanding of the role of microorganisms in the geochemical cycling of elements. In this study, we applied the "μ-XAFS-FISH" technique to one of the most ubiquitous and important environmental biomineralizations, Fe(III) mineral deposition by Fe(II)-oxidizing microbes.

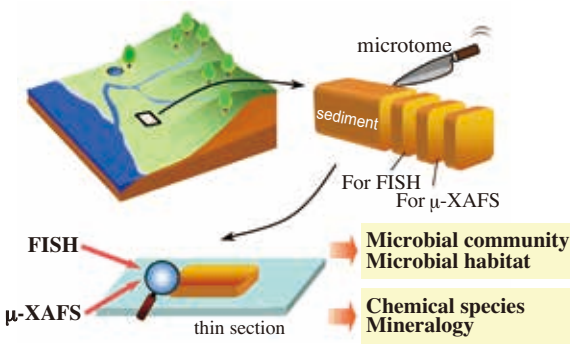

Fig. 1. Schematic figure showing coupled XAFS-FISH technique.

Natural Fe(III) oxyhydroxide mats were collected near the Sambe hot spring in Shimane Pref., Japan. Orange Fe microbial mats cover the floor of the spring flow path, which extend to tens of meters from the spring site (Figs. 2(a) and 2(b)). For μ-XAFS and FISH analyses, we prepared a thin section of the Fe mat sample. Fe *K*-edge μ-XRF-XAFS experiments were performed at **BL37XU** at SPring-8 and at BL4A at KEK-PF. The incident beam was monochromatized with a Si(111) double-crystal monochromator and focused to 0.9 (V) μm × 1.3 (H) μm.

The 16S rRNA gene phylogenetic analysis indicated that neutrophilic chemolithotrophic Gallionella-related bacteria of Betaproteobacteria was involved in the biogenically mediated oxidation of Fe(II) in the Sambe mats. First, we attempted to visualize the potential Fe(II) oxidizers in thin-sectioned mats by the FISH technique using a specific probe for Betaproteobacteria. Bright-field and FISH images obtained with a Betaproteobacteria probe for thin sections are given in Figs. 2(c) and 2(d), respectively. The FISH analysis of the Fe mat thin section showed the localization of Betaproteobacteria (presumably related to *Gallionella* and involved in Fe oxidation) in the upper 10–15 μm of the mat. Assuming that *Gallionella* was involved in Fe oxidation in this layer, we would expect that larger amounts of biogenic Fe oxides should also be found in this layer owing to microbial activity. Furthermore, we expected that the Fe oxides would carry a biogenic signature, as has been previously observed in the stalks produced by Fe(II)-oxidizing bacteria [3]. To address these hypotheses, we characterized the Fe chemical speciation and mineralogy in the mat by Fe μ-EXAFS with high spatial resolutions.

Fe k^3-weighted EXAFS spectra of reference materials, Sambe spots 1–3, and bulk mats are given in Fig. 3(b). The spectral features of spots 1–3

and the bulk sample were more similar to those of ferrihydrite than to those of goethite or lepidocrocite, which indicates that the Fe mats are mainly composed of short-ordered Fe(III) (oxyhydr)oxides, such as ferrihydrite. However, a small peak at $k = 7.0-7.5$ Å^{-1} was observed in ferrihydrite, the Sambe bulk sample, and spot 3 (dotted line box in Fig. 3(b)), whereas this peak was not found in spots 1 and 2 of the Fe mats. This implies that the coordination environment for Fe-Fe linkages in spots 1 and 2 (*Gallionella*-accumulating parts) was different from the others, because the observed small peak is dominantly derived from the Fe-Fe coordination in the Fe(III) (oxyhydr)oxides. EXAFS simulation analysis indicated that Fe(III) oxyhydroxides in spot 1 (*Gallionella*-accumulating part) are dominantly composed of edge-sharing linkages of Fe-O_6 octahedra [4], showing the Fe(III) oxyhydroxides with secondary structures.

In the present study, we developed a novel method using μ-XAFS combined with FISH to determine directly the microbial communities and chemical speciation of elements with high spatial resolutions (1–5 μm), and applied it to bacteriogenic Fe deposition. Our novel approach has many merits for investigating the relationship between microbes and chemical species: (i) simultaneous analysis by FISH and μ-XAFS allows us to characterize directly the biomineral associated with a targeted microbe, while most of the previous studies lack *in situ* phylogenetic information on a specific microbe; (ii) nondestructive analytical techniques, such as μ-XAFS with high sensitivity, elemental specificity, and spatial resolution, are useful in tracing various biogenic reactions in natural environments and characterizing resultant biominerals; and (iii) both FISH and μ-XAFS are cultivation-independent methods. These advantages enable us to obtain direct information on a specific biogenic reaction mediated by its target microorganism in environmental samples by the μ-XAFS-FISH technique.

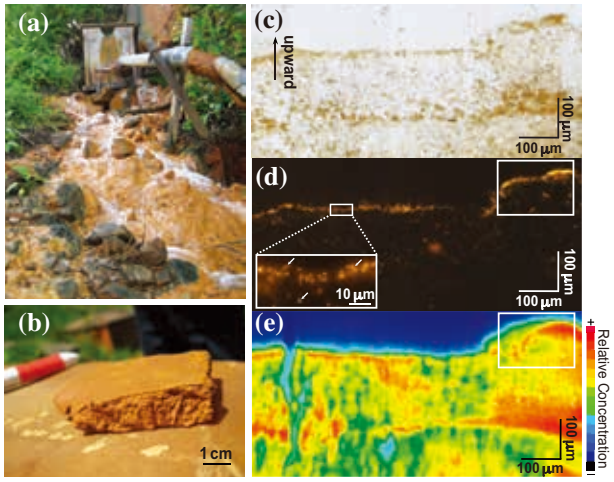

Fig. 2. **(a, b)** Photographs of sampling location and collected Fe mats at Sambe hot spring, respectively. **(c)** Bright-field image of thin-sectioned Sambe Fe mat. **(d)** FISH image of the same field stained by *Gallionella*-specific FISH probe and the magnification of high population area. Arrows in the magnified image stand for rod-shaped cells typical of *Gallionella* relatives. **(e)** Fe concentration map of the same field collected by μ-XRF. White-line boxes in (d) and (e) show the area analyzed by μ-XAFS in Fig. 3

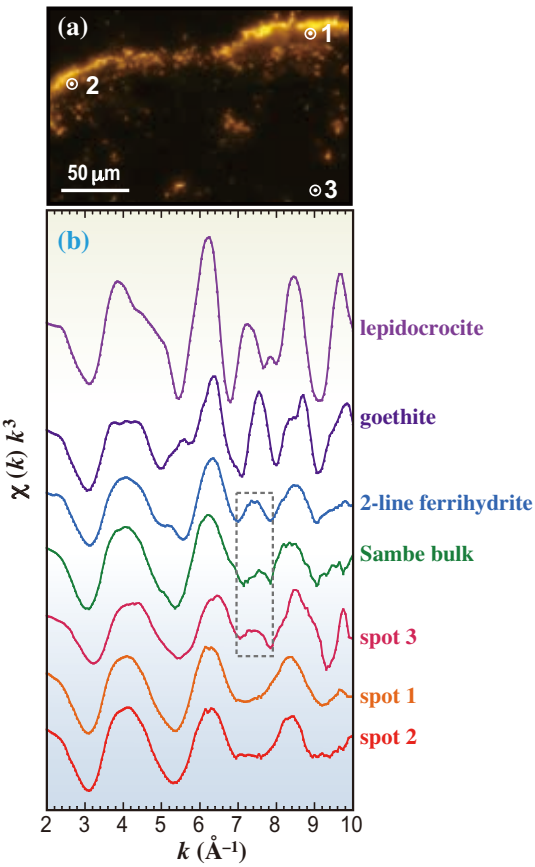

Fig. 3. **(a)** FISH image of area analyzed by Fe μ-XAFS. White circles in the image (spots 1–3) indicate points of interest in Fe μ-XAFS measurements. **(b)** Micro- and bulk-Fe *K*-edge EXAFS spectra of the points in Sambe thin section, bulk sample, and reference materials (lepidocrocite, goethite, and 2-line ferrihydrite).

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