Bacterial Fe-As mineralization

Kaori NAGAI [1], ABM Rafiqul ISLAM [1], Kazue TAZAKI [2]

1 Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa, Ishikawa 920-1192 Japan
2 Department of Earth Sciences, Faculty of Science, Kanazawa University, Kakuma, Kanazawa 920-1192 Japan

Abstract : Bacterial Fe-As mineralization was found in the reddish brown biomats adhered on the wall near to the water drainage of Masutomi mineral springs at Yamanashi Prefecture in Japan. The reddish brown biomats have been analyzed and observed by XRD, FT-IR, optical microscope, SEM-EDX complimented with TEM and ED pattern to clarify the bacterial biomineralization. The reddish brown biomats mainly composed of bacillus and coccus types of bacteria, those can adsorb Fe and As associated with other trace elements forming biominerals. The process of adsorption of Fe and As is evidenced by SEM-EDX and TEM. Also, the ED pattern of capsule identified lollingite ($FeAs_2$) and calcite ($CaCO_3$) around the cell wall. The significant occurrence of organic components C-H, C=O, CNH, -COOH and N-H in FT-IR data emphasized for metal binding character of bacteria. While XRD data shows the poor crystalline character mainly of hydrous iron oxides (2.7Å). Formation processes of Fe-As biominerals take place by adsorption onto the bacterial cell wall evidenced from the microscopic direct observation and spectroscopic analysis. It was indicated that bacteria in the reddish brown biomats could have the ability to form biominerals with heavy metals and toxic metalloids like Fe and As. Bacterial Fe-As mineralization might also be considered to clean up the environmental geo-aquatic ecosystem, as having their endurance ability in toxic environment.

Key words : bacteria, Fe-As mineralization, accumulation, microbial mats, lollingite, calcite.

1. Introduction

Bacteria in microbial mat have controlled biotransfer process and the biogeochemical cycles during most of the history of life on the Earth (Krumbein, 1979). Bacteria can grow in some of the most extreme environments on the Earth such as high temperature and high pressure even in the strong acidic condition (Fyfe, 1997). Heavy metals, such as Fe, Mn, Cu, Zn, Cd, Pb, and As, can be accumulated by bacteria in most of the aquatic environment including hot and mineral springs. Bacteria selectively accumulate heavy metals as having their own niche in the geo-aqua-ecosystem (Ariza, 1998). The accumulation takes place in

different forms based on various mechanisms such as adsorption, complexation and active transport into the cell along with physicochemical parameters like pH and ionic compositions (Tazaki, 1999 ; Ledin, 2000). Adsorption of dissolved constituents from the aquatic environment is considered to contribute significantly to the mineral formation processes (Tazaki and Ishida, 1996). Most of the heavy metals and toxic materials are accumulated by bacteria and forming biominerals, after precipitation of insoluble metals (Nagai et al., 1999 a, b ; Lloyd and Macaskie, 2000).

The toxic metalloid, As is a hazardous material for its widespread carcinogenic character (Lena et al., 2001), responsible for many diseases such as lung, skin and bladder cancer, gangrene and placental problem for unborn children (Fuji and Karim, 1998 ; Banfield and Nealson, 1999). Most of these kinds of cancer can eventually be developed in those people, who drink water from tinted sources including As polluted underground water (Kaiser, 2000). Recently the evidence of arsenical chronic poisoning has been reported in many parts of the world and has become a serious public health problem in some Asian countries like Bangladesh, India and Taiwan (Asia Arsenic Network, RGAG, DOEH, and NIPSM, 1999 ; Islam and Tazaki, 2000 a).

Arsenic is ubiquitous, found at trace levels in the biosphere including organisms (Newman et al., 1998). Naturally it occurs in many chemical forms on different environmental conditions (Jack et al., 1998). Consequently, it may be considered that geological sources of arsenic are environmental problem worldwide (Rosen, 1999). Having high toxicity in ionic-form is very harmful for the human beings rather than As minerals in geo-aqueous-environmental ecosystem.

However biochemical activities like bacterial mineralization has not been focused for bacterial Fe-As mineralization in the natural environment. In most of the cases, it is found that arsenic concentration is corresponding to Fe, while it is rich in hot springs or underground water (Akai, 1997). The present study is specifically designed to clarify the mechanism of Fe-As biomineralization that can take part in the bioremedial activity for cleaning As polluted areas.

In this paper, the presence of Fe-As minerals both on the bacterial intra and extracellular wall has been revealed by transmission electron microscopy (TEM) through a direct observation.

2. Materials and Methods

Mineral spring water and reddish brown biomats were collected from Masutomi mineral springs located at the northwestern part of Yamanashi prefecture in Japan (Fig. 1). The area is granite-bodied basement in Neogene penetrating to Shimanto belt, which is composed of sandstone, mudstone, and alternating shales. Yaita et al. (1991) pointed out that Masutomi mineral spring water is related to the plutonic rocks in Neogene.

The reddish brown biomats were picked up from the mineral spring water drainage of Furokaku at Masutomi (Fig. 2 A). Biomats were formed over an area of about 3 m in

height and 0.5 m in width with the thickness of about 10 cm from the substrate. Underground water was the main source of Masutomi mineral spring. It contains mainly Cl^-(3953 mg/kg), Na^+ (2960 mg/kg), HCO_3^- (1553.0 mg/kg), SO_4^{2-}(549.8 mg/kg), K^+ (336.0 mg/kg), Ca^{2+} (331.1 mg/kg), H_2SiO_3 (140.3 mg/kg), Mg^{2+} (26.3 mg/kg), Fe^{2+} (9.0 mg/kg), $HAsO_4^{2-}$(7.4 mg/kg), Al^{3+} (3.1 mg/kg), Mn^{2+} (0.4 mg/kg) (The central spring lab., 1972). The spring water shows pH 6.5～7.7, Eh - 90～38 mV, EC 11.9～12.9 mS/cm, DO about 4.2 mg/l, and WT 26～32℃. That was measured by a portable water quality

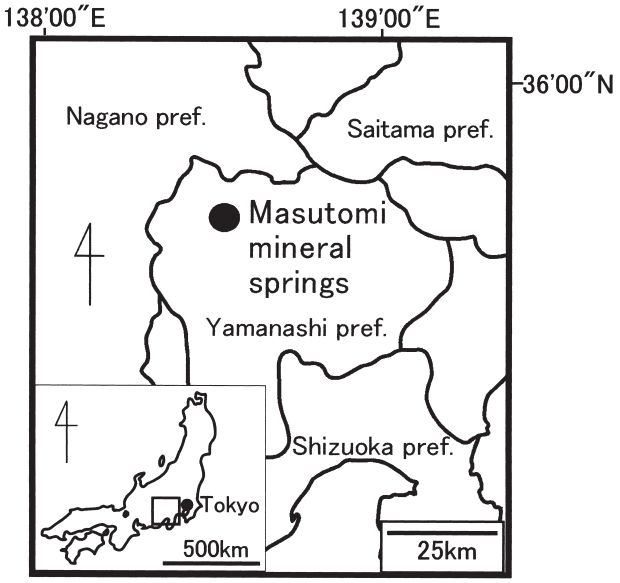

Fig. 1. Locality map of Masutomi mineral springs at Yamanashi Prefecture in Japan.

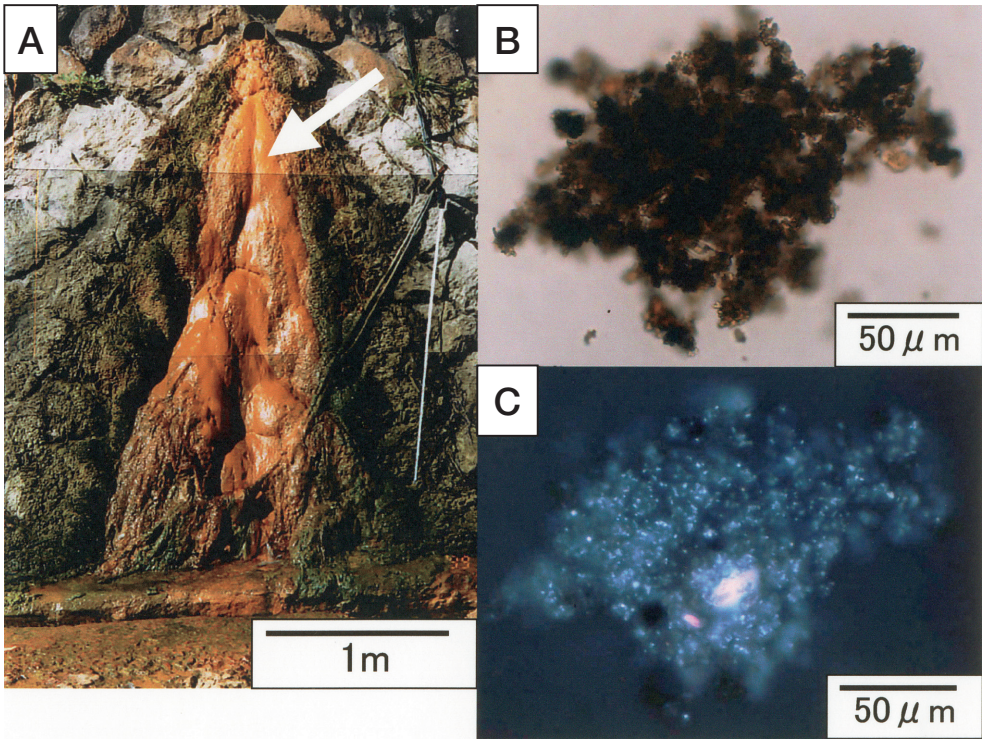

Fig. 2. Reddish brown biomats (arrow) at Masutomi mineral springs (A). Optical micrograph of the reddish brown biomats shows highly densed brown materials (B), and DAPI (4', 6-diamidino-2-phenylindole) stained epifluorescence micrograph shows the fluorescent blue parts that indicate the presence of DNA in bacterial cells (C).

inspection meter (pH ; D-12, Eh ; D-13, EC ; ES-12, DO ; OM-12, made by HORIBA). The measurement was carried twice in the field, on the December 12, 1998 and July 30, 1999.

Analyses for the chemical and mineralogical characterization of the reddish brown biomats, as follows.

Fourier-Transform Infrared absorbance spectroscopy (FT-IR)

The organic compounds associated with minerals and organo-metalic complexes in the reddish brown biomats were analyzed by Fourier-Transform Infrared absorbance spectroscopy (FT-IR ; Jasco FT/IR-610). The reddish brown biomats were air-dried and ground for fine powder to carry out chemical analyses. 30 μg of powdered biomats and 10 mg of amorphous potassium bromide (KBr) were taken in an mortar and mixed them very well. After grinding all to fine particle, tablets were prepared by MP-1 micro tablet maker and MT-1 model mini press for measuring the transmission of IR light by the spectrometer. IR frequency ranges between 400～4000 cm^{-1}.

X-ray powder diffractometer (XRD)

The mineralogical properties of the reddish brown biomats were also analyzed by X-ray powder diffractometer (XRD), using a Rigaku RINT 2000 with a CuKα radiation. It was generated at 40 kV and 30 mA using the 2θ/θ method with a scan speed of 2°/min. Dried biomats powder was taken on the square concavity of the slide and fixed them up. After fixation the slide was set up on the stage of XRD for analysis.

Optical and electron microscopic observations for the identification of microorganisms and their habitat in the reddish brown biomats, as follows.

Optical microscopy

To identify the presence and variety of bacteria optical microscopic observation was carried out. Hand picked reddish brown biomats, washed with distilled water were mounted on and spread over the slide simultaneously. Both of the episcopic and DAPI (4', 6-diamidino-2-phenylindole) stained samples were observed through episcopic fluorescence microscope (Nikon OPTIPHOT-2 /LABOPHOT-2). The DNA of bacterial cell in DAPI staining sample shows the fluorescence blue under the ultra violet ray (365 nm).

Scanning electron microscope (SEM-EDX)

The scanning electron microscope equipped with an energy dispersive X-ray spectroscopy (SEM-EDX ; JEOL JSM-5200 LV and PHILLIPS EDAX PV 9800 EX) was used in order to observe the micro morphological surface of bacteria and its chemical composition. Pipet drawn biomats was mounted on a carbon tape pasted stub and dried in air. Dehy-

drated sample was coated by carbon carried out in 15 and 25 kV with different magnifications.

Transmission electron microscopy (TEM)

Complementary techniques of transmission electron microscopy (TEM ; JEOL JEM-2000 EX) were used for the observation of extra and intra cellular condition of bacteria. This was also equipped with electron diffraction (ED) analysis, which can identify minerals through the diffraction pattern of the reddish brown biomats. The sample was set into the ultra sonic separator (JINMEIDAI Ind. sine sonic 100) for residual separation for about 3 minutes. One drop of the suspension was taken by pipet and mounted on the micro grid for observation. The accelerating voltage ranged between 120 and 200 kV with different magnifications.

3. Results

3.1. Optical microscopy

The reddish brown biomats were collected from the water drainage of Masutomi mineral springs for microscopic observation (Fig. 2 A arrow). Optical micrograph of the reddish brown biomats showed the presence of high-density brown materials in the microbial colonies (Fig. 2 B). Epifluorescence micrographs revealed numerous bacterial cells absorbed the fluorescent blue in DAPI stained biomats (Fig. 2 C). It was indicated that the reddish brown biomats contain granular materials with living bacteria.

3.2. SEM-EDX

SEM observation recognized the bacterial colonies in the reddish brown biomats. The entire colony is composed of bacillus typed bacteria, about 1.0 μm in size (Fig. 3 A) and surrounded by granular particles of about 0.5 μm in size (Fig. 3 B arrow). EDX spectrum of a bacillus type of bacterium in reddish brown biomats shows in Fig. 3 C. The arrow in Fig. 3 B indicated analytical point for EDX showing the presence of Fe, Ca, Si, P, Cl, K and As with a hilly back ground, which suggest the presence of organic materials. Furthermore, SEM elemental mapping showed that the association of Fe and As on the bacterial surface within a specific area, while Ca and Si were observed although the cell surface (Fig. 4). It was indicated that the bacterial cells are capable to accumulate Fe and As with Si and Ca from the mineral spring water.

3.3. FT-IR and XRD

The spectra in Fig. 5 A imply that organic components are present in the reddish brown biomats. The most intense contributions consist of two doublets one at 2853-2923 cm^{-1} arising from stretching of C-H, C-H groups and the others at 1653-1744 cm^{-1} stretching of C=O and C=O groups. Additionally the presence of peptide linkage functional groups are notable, such as N-H, -COOH and CNH at 3287, 3068 and 1546 cm^{-1} bands

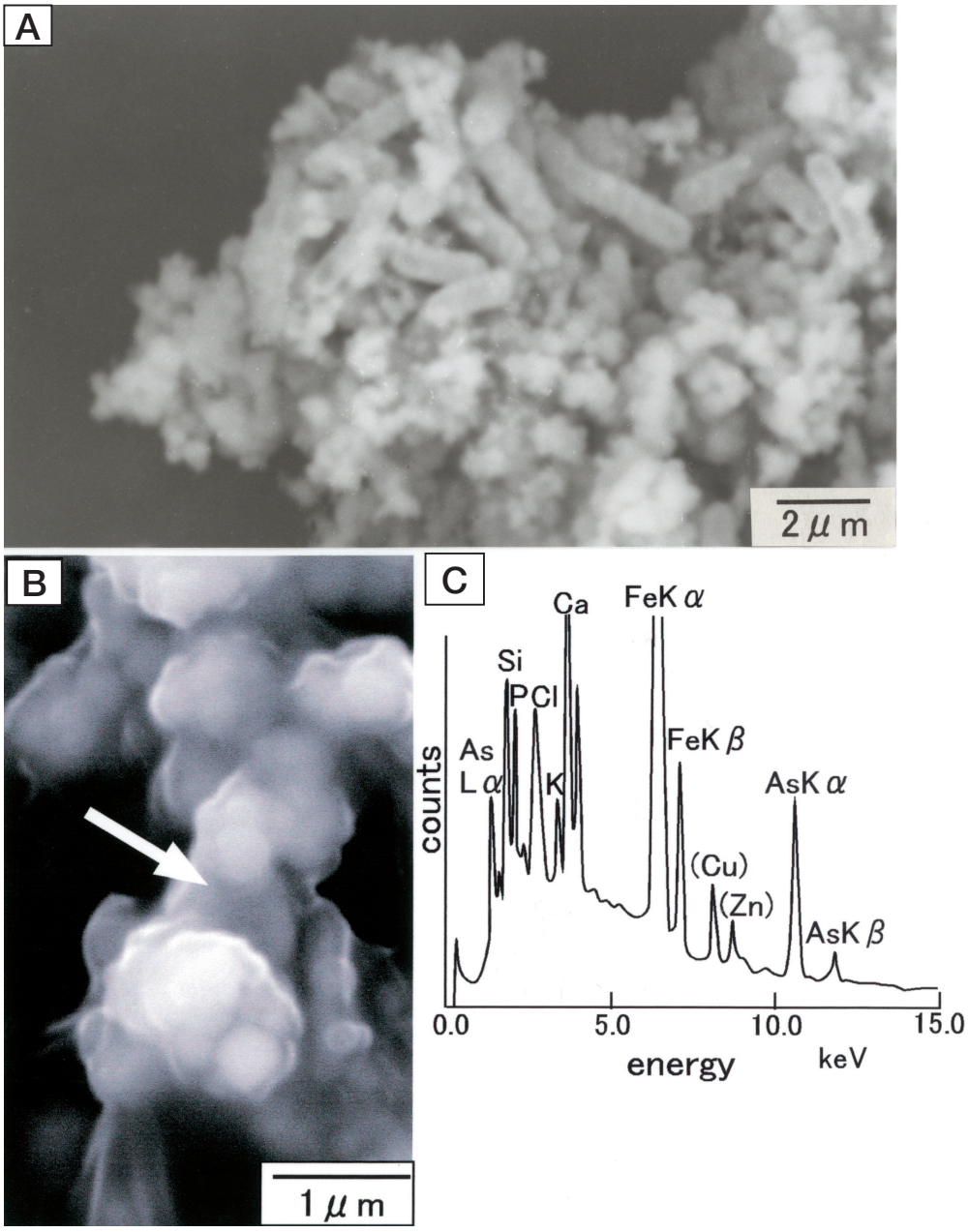

Fig. 3 SEM image of the reddish brown biomats, showing the bacterial colony (mainly consisted of bacillus types of bacteria) aggregated with granular particles (A). Closed up bacillus cell (indicated by arrow) surrounded by granular particles (B). The EDX spectrum of a bacterium (analyzed in the arrow pointed B) indicating the presence of Fe, Ca, Si, P, Cl, K and As (C).

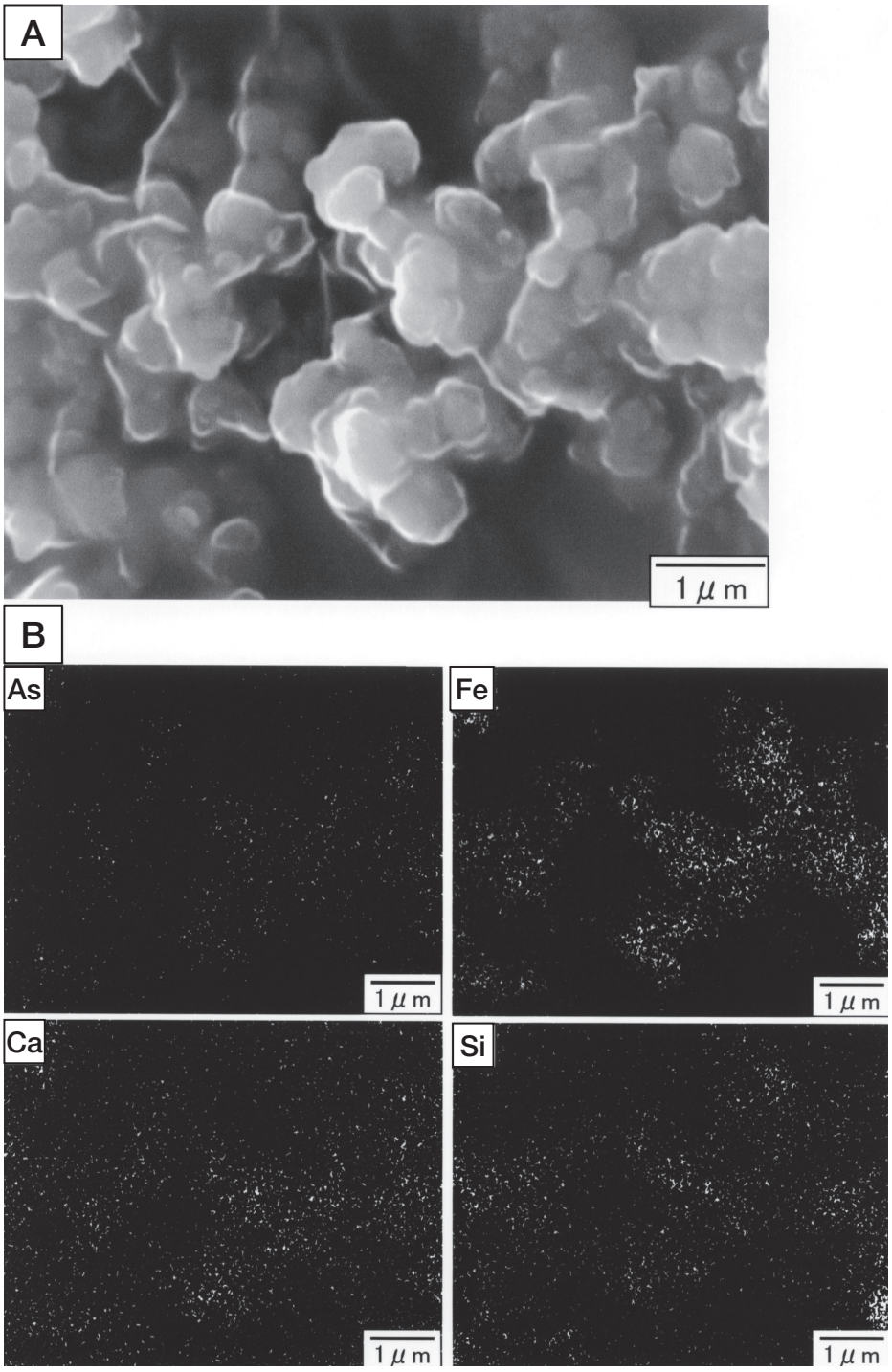

Fig. 4 SEM image (A) and the EDX maps (B) of the reddish brown biomats, showing the association of Fe and As in the same bacterial surface whereas Ca and Si are observed at almost all the cell surface (B).

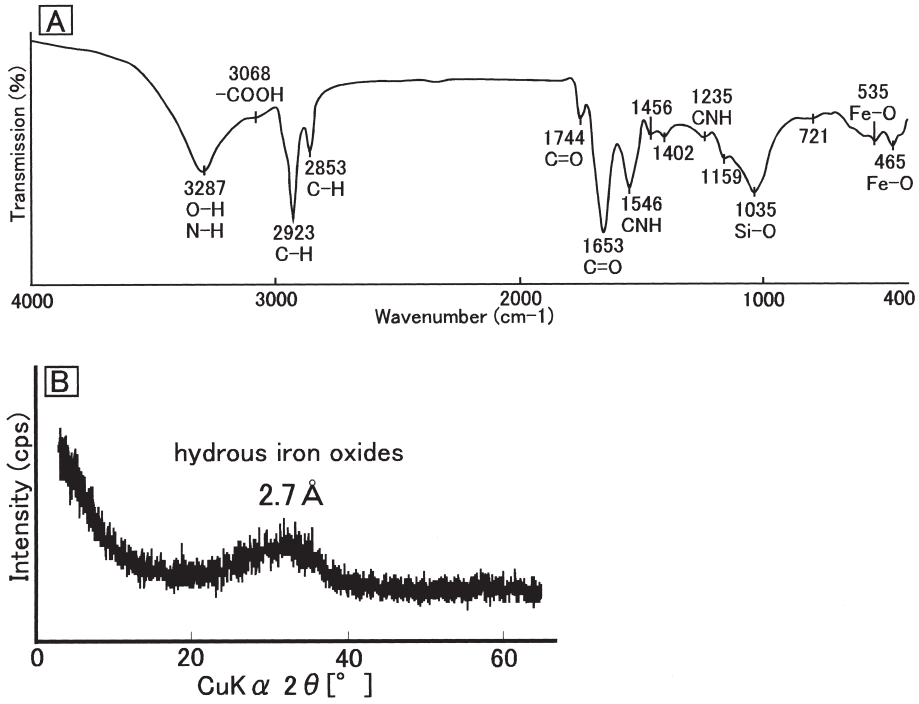

Fig. 5 FT-IR spectrum of the reddish brown biomats showing the presence of organic and inorganic substances. The C-H, N-H, C=O, and CNH bands are derived from peptides contained in bacterial cells. The Si-O and Fe-O bands are derived from biominerals (A). The XRD pattern at about 2.7 Å shows the presence of amorphous or poorly crystalline materials, suggesting hydrous iron oxides (B).

respectively. Furthermore, the stretching peaks at 465, 535 cm^{-1} and 1035 cm^{-1} suggest the presence of Fe-O and Si-O. The XRD patterns of the reddish brown biomats from Masutomi mineral springs reproduced in Fig. 5 B showed the hilly peak at about 2.7 Å suggesting the presence of hydrous iron oxides.

3.4. TEM

The microbial mats, collected from Mastomi mineral springs showed the dominance of coccus and bacillus types of bacteria that formed colonies (Fig. 6 A). The size of coccus is about 100 nm in diameter. As far as bacillus type of bacteria is about 200～1000 nm in length and 100～200 nm in width (Fig. 6 B, C and D). Most of them have a crust of about 50 nm in thickness, which made a capsule. Electrical high-density crust formation process has been seen in Fig. 6 B, C and D. In the later stage, bacillus type bacteria formed spherules range between 100～200 nm in diameter. These are also electrical high-density (Fig. 6 D). At the final stage, both of the extra and intra cellular surface of bacillus type cell was covered with numerical cohesive spherules (Fig. 7 A, B). The coccus typed

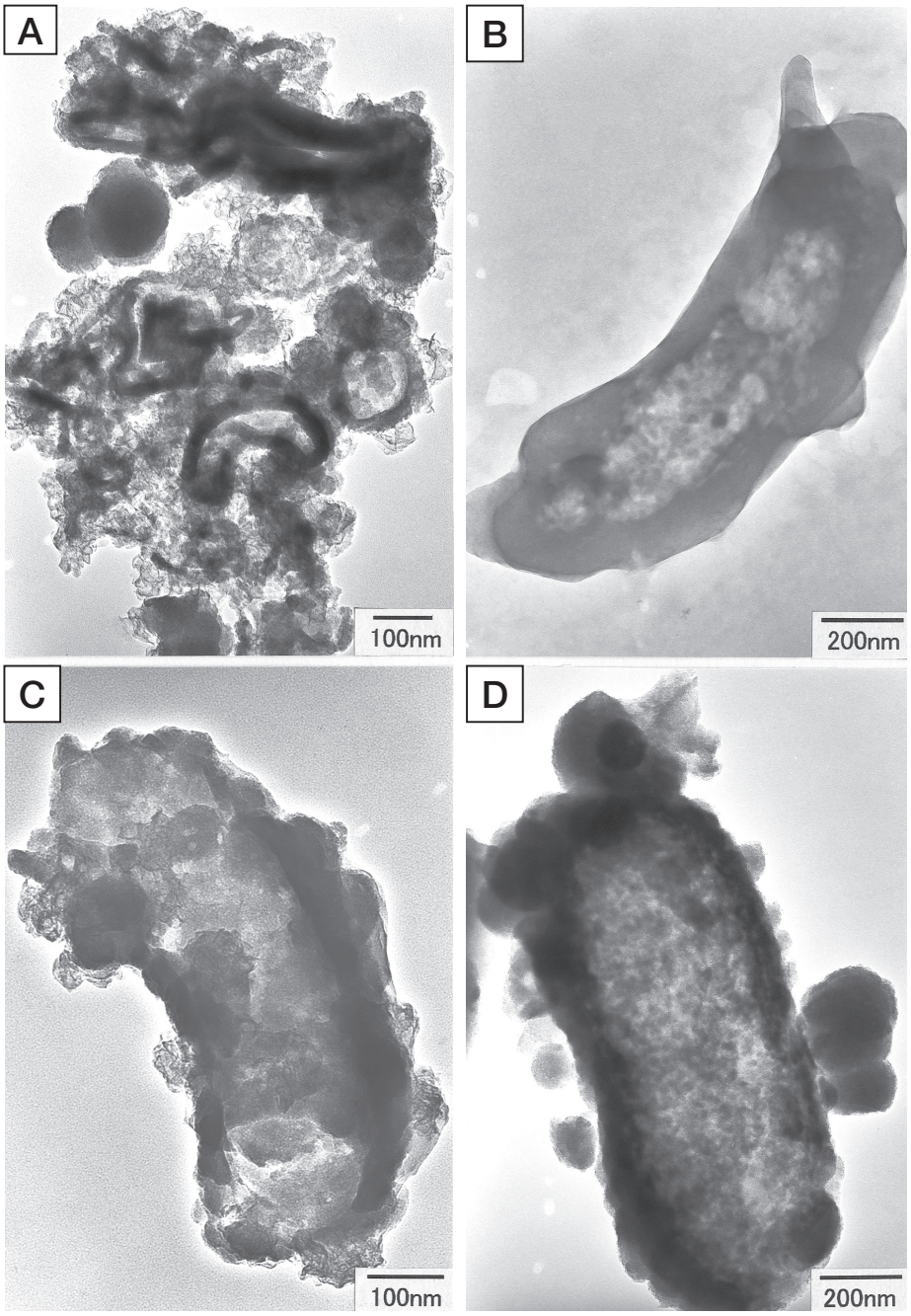

Fig. 6 TEM micrographs of bacterial cells in the reddish brown biomats indicating the dominance of bacillus and coccus types of bacteria encrusted with granular particles (A). Image of a bacillus typed cell about 200～1000 nm in length and 100～200 nm in width showing the successive process of crust formation by cohesive materials around their cell wall (B). Electrically highly densed bacillus types of bacteria undergo encrustation (C). High densed nanoparticulate materials encapsulate the cell. Colloidally aggregated nanoparticles are also existence (D). Biomineralization processes can be followed as B, C, and D.

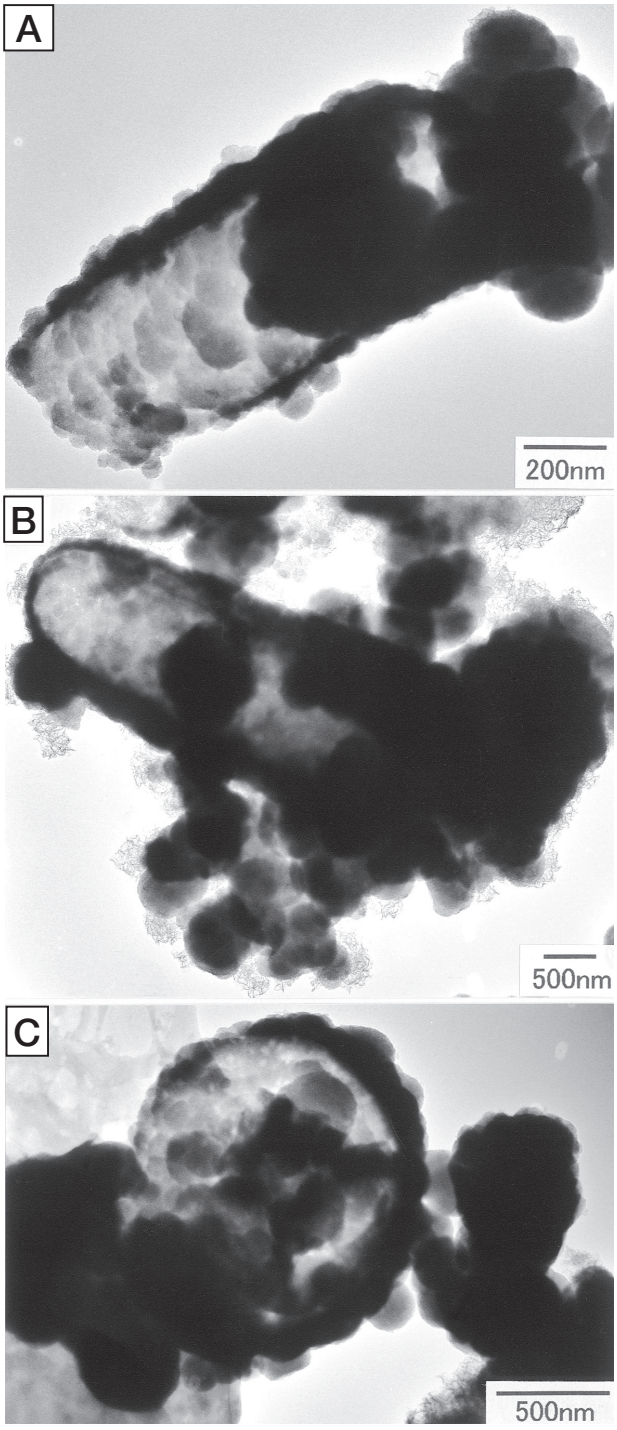

Fig. 7 TEM micrographs of the bacillus (A, B) and coccus (C) types of cells covered with abundant spherules around 10～100 nm in diameter. Thin flaky materials are formed around spherules (B).

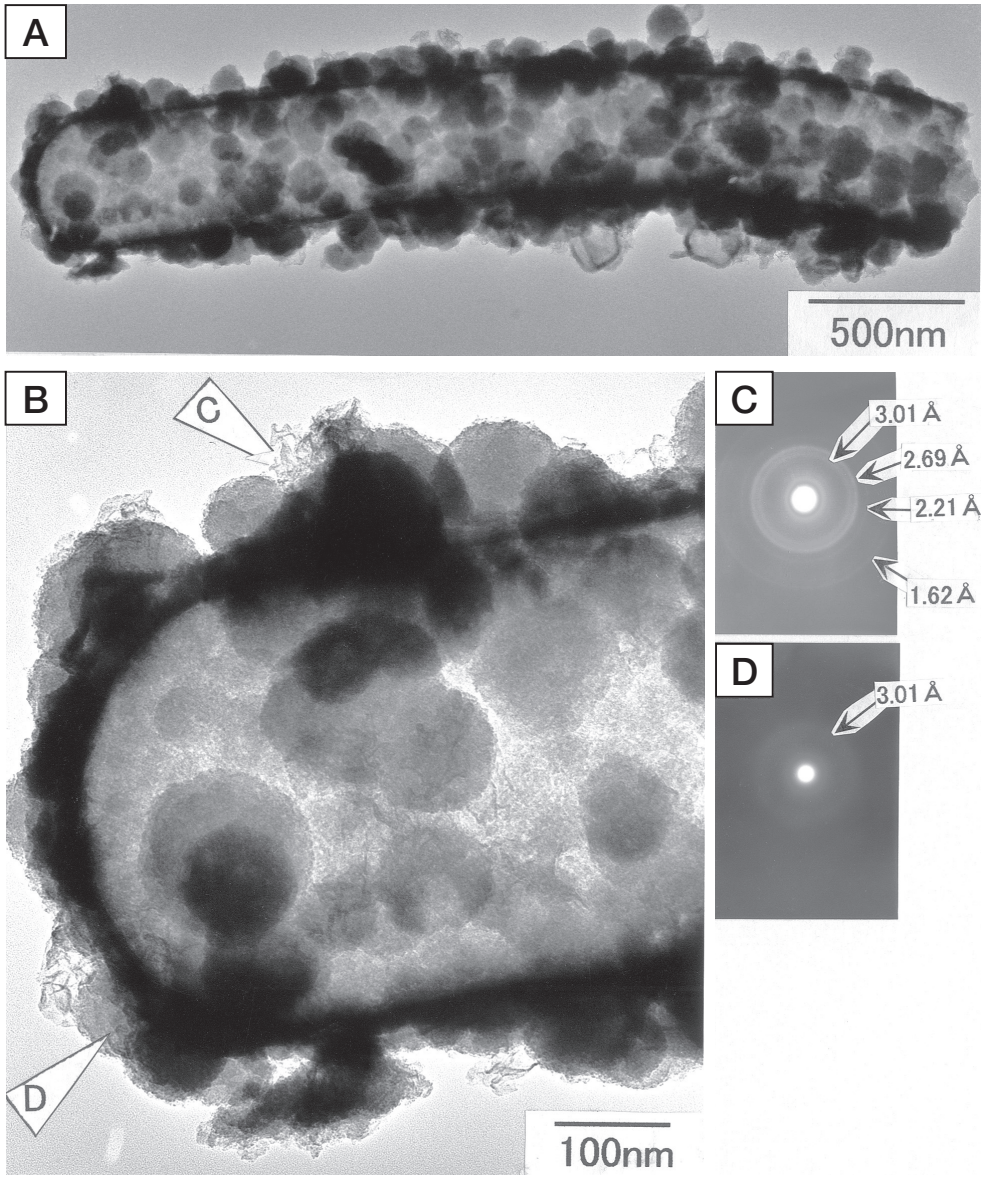

Fig. 8 TEM micrographs of the bacillus typed cell covered with numerical spherules around 50～100 nm in diameter (A, B). Oriented lattice images are also can be seen in the cell (1.2～3.9 nm)(B). Electron diffraction (ED) pattern of the capsule around the bacterial cell wall indicates amorphous (D) or poorly crystallized (C) lollingite ($FeAs_2$) showing diffraction at 1.62, 2.21, 2.69 and 3.01 Å (C). The 3.01 Å diffraction is identified as calcite ($CaCO_3$) underneath of lollingite.

bacteria also heavily encapsuled with spherules (Fig. 7 C). Numerous spherules are formed on cellular surface of bacillus type bacteria (Fig. 8 A). The spherules are composed of Fe-As, which show the defused electron diffraction pattern at 1.62Å, 2.21Å, 2.69Å and 3.01Å (Fig. 8 C, D) suggesting lollingite ($FeAs_2$) and calcite ($CaCO_3$). This is also consistent with the SEM-EDX data. The highly magnified TEM image of Fig. 8 B shows lattice images on the cell surface. The space of lattice image ranges between 1.2～3.9 nm on the bacterial surface suggesting the presence of calcite underneath of lollingite spherules (Fig. 8 B). Extra and intra cellular surfaces of both bacillus and coccus types of cells were covered with numerical cohesive spherules around 10～100 nm in diameter.

4. Discussion

In this study the results revealed that bacillus and coccus types of bacteria in the reddish brown biomats of Masutomi mineral springs are capable of adsorb Fe and As forming lollingite by microbial processes in an As contained spring water system. It is ambiguously identified that both of the bacillus and coccus types of bacteria are enable to accumulate Fe and As around their extra and intra cellular surface. Generally the cell wall of microbes contains more or less polysaccharides, proteins, amines, or polyamines (Oshima, 1995 ; Asada and Tazaki, 2001). Bacteria can produce macromolecules outside of their cell wall, commonly consisting of polysaccharides together with some protein, DNA and RNA (Geesey and Jang, 1990). The cellular systems actively transport ion through their membrane in order to maintain osmotic stability (Simkiss and Wilbur, 1989). The reasons resemble with this study basing on the optical microscope, SEM, TEM observation and FT-IR analyses. The heavy metals and toxic materials can be accumulated by bacteria after precipitation or accumulation processes of insoluble metals (Lloyd and Macaskie, 2000). Microorganisms in different color of biomats, (e.g. green, black, and reddish brown) could accumulate heavy metals and toxic elements in the geo-aquatic environments through adsorption, precipitation, complexation or transportation (Gadd, 1992 ; Nagai and Tazaki, 2000 a, 2000 b, 2001 ; Islam and Tazaki, 2000 b). Ledin (2000) reported that metal ion was accumulated by bacteria in presence of other ions and showed that *Scenedesmus pannonicus* can accumulate As, which is consistent to Demon et al. (1998). In addition Labrenz et al. (2000) also reported some selective microorganism (e.g. the bacteria of *Disulfobacteriacea* family) can survive in highly toxic environment and can reduce aqueous Zn, As, and Se concentration to well below the acceptable level by the geochemical and microbial processes. Hence, it is reasonable to assume that bacteria can clean up As polluted water, because recently technologies for As removal and environmental remediation will be considered in a combination of geology with microbiology (Fyfe et al., 2000). Besides this *Pseudomonas aeruginosa* can accumulate Fe (II) and Fe (III), Cu (II), Pb (II) with U (Hu et al., 1996). *Zoogloea ramigera* also showed the capability of Fe (III) with Pb (II), Ni (II), and Cu (II) (Sag and Kutsal, 1995). The similarity has been found in the present study that bacteria can adsorb Fe and As to form lollingite ($FeAs_2$).

The presence of various functional groups on the bacterial surface can interact with metals. The importance of-COOH (carboxylic) and NH_2 (amino) groups has been emphasized for binding metals (Beveridge and Murry, 1976, 1980 ; Beveridge, 1981, 1989). For an instance, when As enters the human-body once by drinking or through membrane also, it can not comes out easily as it might be forming a arsenic-protein complex (Minato, 1989 ; Islam and Tazaki, 2001) and remains in the body. It might also be considered for the microorganisms, as having high molecular polymeric peptide bonded protein compound in their cell surface (Jones, 1997). Polysaccharides of bacterial capsule can also be influenced by the presence of metals, because metals and metalloids can serve as cofactors in polysaccharides synthesis (Geesey et al., 1988). For example, the mucoid substance (polysaccharides) of some oxidizing bacteria such as *Toxothrix sp*. is very effective for adhesion of Fe^{3+} producing iron hydroxide $Fe(OH)_3$ biominerals (Tashiro and Tazaki, 1999). The iron hydroxides are expected to have a key role in the geochemical behavior of toxic heavy elements or harmful radionuclides by their accumulative or incorporative character on or into the cell surface (Schwertmann, 1988 ; Schwertmann and Taylor, 1989). Ca^{2+} can also be adhered to the bacterial surface forming calcite ($CaCO_3$) (Yasuda et al., 2000). Asada and Tazaki (2000) reported that silica biomineralization also takes place in microorganisms and forming Si crust over their cell surface, even under the strong acidic condition.

In our study it might be suggested that polysaccharides of bacteria in microbial mats could adsorb Si and Ca first, from the spring water to form a complex minerals containing calcite in the preliminary stage. In the secondary stage Fe-As adsorption takes place through cohesive method to form spherules, and finally producing Fe-As biominerals (lollingite) over the calcite that encapsuled the bacterial cell surface (Fig. 9). Goldberg and Glaubig (1988) reported that calcite is capable to adhere As strongly for adsorption when pH value ranges between 6～10. It is notable that pH value of mineral spring water ranges between 6.5～7.7. It might be the reason of lollingite formation in the secondary stage. However, microbial mineralization of metals or metalloids can clean up the environmental geo-aquatic ecosystem (Lovley, 2000), having their (microorganism) endurance ability in the toxic environment as in As or Ti polluted area (Tazaki et al., 1998).

Consequently, bacteria in microbial mats certainly play an important role in the formation of biominerals with heavy metals or toxic metalloids like Fe and As. Stabilized As in minerals is less harmful for human beings rather than its ionic form. That's why it also might be considered that selective bacteria can play an important role in the natural bioremediation process of As polluted geo-aqua ecosystem.

5. Conclusion

Bacteria in the reddish brown biomats show the formation of Fe-As biominerals (lollingite $FeAs_2$). TEM on direct observations show the processes of Fe-As encrustation over the bacterial capsule by adhering and cohering materials. Bacillus and coccus types of bacteria in the reddish brown biomats play an important role for cleaning the As polluted

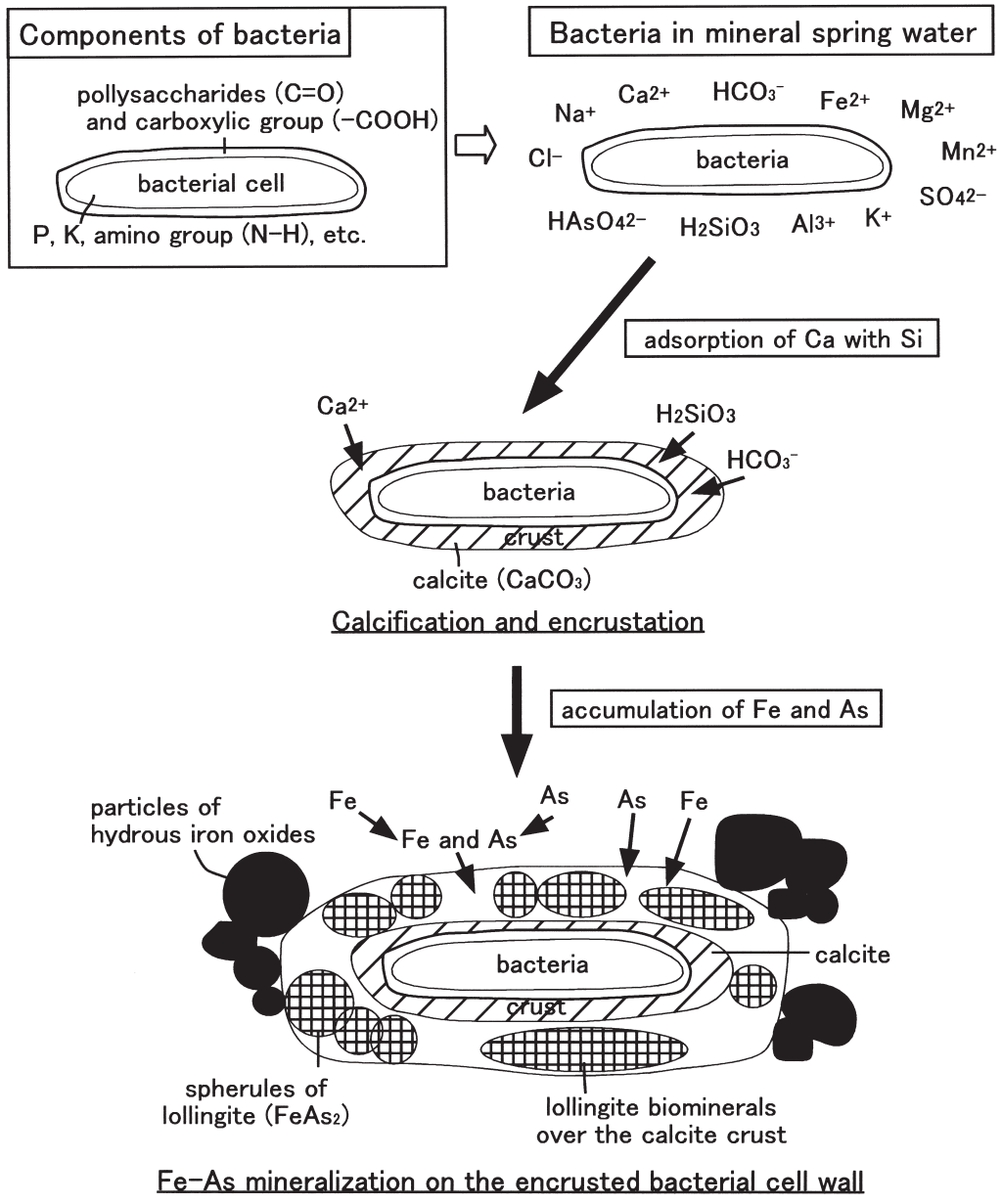

Fig. 9 The schematic sectional diagram of bacterial cell shows preliminary and secondary crust formations over their cell surface with the spherules of hydrous iron oxides.

geo-aqueous system. Bacteria have the metal accumulating character even in the toxic environment as they can endure there.

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