［原著］

Fossilization of bacteria in the Kotelnikovsky Hot Springs located on the northwest coast of Lake Baikal, Russia

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Abstract

Electron microscopic observations of green biomats and deposits from the Kotelnikovsky Hot Springs (pH 8.0) located on the northwest coast of Lake Baikal, Russia, showed variety of fossilized microorganisms. The energy dispersive X-ray fluorescence (ED-XRF), X-ray diffraction (XRD), Fourier transform infrared (FT-IR) microspectroscopy, and scanning electron microscopy equipped with energy dispersive X-ray (SEM-EDX) analyses revealed that the biomats and deposits were rich in silicon and calcium suggesting fossilization processes. The XRD analysis identified the amorphous silica with diffused peak at 4.1Å associated with quartz (SiO_2) with main peaks at 3.34 and 4.25Å, calcite ($CaCO_3$) with main peak at 3.04Å, and fluorite (CaF_2) with main peaks at 3.15, 1.92－1.93, and 1.65Å. The SEM-EDX analyses successfully detected fossilized microorganisms in the hot spring biomats and deposits. The content of sulfur as well as Ca/K and P/S ratios considerably differed between the bacterial cells and amorphous particles. The SEM-EDX analyses of green biomats showed Ca/K ratio could be shifted during fossilization process, because the ratio was found to be 0.7 for the bacterial cells and 2.1 for fossilized microorganisms. The FT-IR results indicated the fossilization process of bacteria from green biomats to deposits, decreasing organic compounds (C-H, C-N-H, N-H bonds) and increasing silica-organic bonding (Si-O-Si and Si-O). Silicification and calcification of the microorganisms extensively appeared in the biomats and deposits under pH 8.0 hydrothermal conditions.

Key words : bacteria calcification, fossil, hot springs, silicification

Introduction

The role of microorganisms in the formation of modern hydrothermal deposits is important (Duhig *et al*., 1992 ; Ehrlich, 1996 ; Allen *et al*., 2000 ; Kasama and Murakami, 2001 ; Hamade *et al*., 2003). By examining microbial fossil assemblages, Ferris *et al*. (1986) suggested that microfossils formed as a result of mineral precipitation were probably best preserved if they had been previously embedded in a fibrous silica matrix. Whereas thermal spring microbes are apparently preserved poorly in carbonates or iron (hydro)oxides, fossilization by silica can provide enduring evidence of life (Allen *et al*., 2000). Preservation of microbial fossils by silicification has been documented in ancient thermal springs (Walter *et al*., 1972; Oehler, 1976 ; Duhig *et al*., 1992 ; Renaut *et al*., 1998).

Direct observation of fossilized microorganisms is difficult in natural environments, because traditional microbiological methods are ineffective for detecting and identifying individual species in mixed natural

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populations. Modern techniques have been developed to allow the identification of lithobiontic microorganisms and to recognize them when they are alive inside the deposits, but it is still difficult to recognize fossilized microorganisms (Chang *et al*., 1986 ; Muliukin *et al*., 2002). Nevertheless, it is possible to recognize biomarkers without mineralized cell remnants if marks in a form of mechanical or chemical alterations over mineral, biomineralizations or organic molecules are found. Frequently, some of the ultrastructural features characteristic of alive microorganisms are also observable in fossilized samples. A scanning electron microscopy with energy dispersive X-ray permits the study of the fossilization processes. To properly identify a fossilized cell, it is necessary to recognize mineralized cellular structures such as cell walls and membranes. However, it is often difficult to distinguish microbial cells from abiotic particles in environmental samples by using only electron microscopy. The content of sulfur, phosphorus, calcium, and potassium as well as Ca/K and P/S ratios considerably differ in cells with different physiological state (e.g., vegetative cells, resting cells, endospores, and dead cells) and abiotic particles (Chang *et al*., 1986; Nikitin *et al*., 1998; Goldberg *et al*., 2001; Muliukin *et al*., 2002). In such cases, energy dispersive X-ray (EDX) analysis may allow to reveal statistically significant differences in these parameters as markers for the direct detection of microbial cells in natural habitats. The absence of phosphorus and sulfur peaks in EDX spectra in most cell-like particles is regarded as non-living entities. Among some other particles that were preliminary recognized as putative microbial cells based on chemical composition, are resting forms of microorganisms. They had the increased intracellular level of calcium, high Ca/K ratio and low P/S ratio. The distinction of cells with different physiological states is important for ecological monitoring of the environments. The EDX analysis can be regarded as a promising tool for a primary detection of microbial cells *in situ* in their environments and to predict their physiological states.

In this study, an *in situ* visualization of the fossilized microorganisms in the green biomats and deposits of the Kotelnikovsky Hot Springs located on the northwest coast of Lake Baikal, Russia, was performed to show the important role of microorganisms during fossilization processes of the modern hydrothermal deposits. The Kotelnikovsky Hot Springs were chosen for this study because the water had a high discharge temperature (80℃) and showed Na-F-HCO_3-type with high content of silicic acids ranging from 120 to 130 mg/l. The salinity and fluoride concentration were 0.32 g/l and about 0.02 g/l, respectively (Imekhtenov and Kartushin, 1993). Modern hydrothermal deposits and green biomats were collected from the drilled well for energy dispersive X-ray fluorescence, X-ray diffraction, scanning electron microscopy with energy dispersive X-ray, and Fourier transform infrared microspectroscopy analyses.

Materials and methods

Description of sampling area

Lake Baikal, the world's deepest (1637 m) and perhaps oldest lake, occupies an active continental rift in southeastern Siberia. The seismically active Baikal Rift Zone separates the Siberian Platform to the northwest from the Mongolian fold belt to the southeast (Colman *et al*., 2003). More than 30 hot and mineral springs are located on the coasts of Lake Baikal. The water chemistry of most of these springs was studied extensively in the 1960s for medical exploration purposes, where they were grouped according to the chemical composition of main ions (Tkachuk and Tolstikhin, 1963; Lomonosov and Volkova, 1963). Microbial communities growing in the stream of hot springs, which are located around Lake Baikal, have been studied over the last several decades (Namsaraev *et al*., 2000). Namsaraev *et al*. (2003) reported that, in an alkaline sulfide Bol'sherechinskii Hot Springs, the cyanobacteria were the most adapted to the alkaline conditions of the spring within the community. Few studies have been done on chemical, microscopical, and mineralogical analyses of hot springs around Lake Baikal. Biogenic strontium-rich aragonite from Zhemchug Hot Springs was investigated, and the findings suggested that the laminated structure of the biomats was formed due to the photosynthetic activity of cyanobacteria (Tazaki *et al*., 2001). The present analyses of Kotelnikovsky Hot Springs were focused on the chemical, mineralogical, and microscopic nature of the water as well as green biomats and deposits.

Field measurement of water quality

Samples of green biomats and deposits were collected in July 2001 and August 2002 from the Kotelnikovsky Hot Springs on the northwest coast of Lake Baikal, Siberia, Russia (Fig. 1). The chemical characteristics of the water (WT = water temperature; pH ; ORP = oxidation-reduction potential ; EC = electrical conductivity; DO = dissolved oxygen) were measured in the field.

Energy dispersive X-ray fluorescence (ED-XRF) analysis

The samples of green biomats and deposits were air-dried at room temperature and ground to fine powder for ED-XRF analysis. The powder samples were mounted on Mylar film and analyzed by an ED-XRF spectrometer (JEOL JSX 3201 using Rh Kα), which operated at an accelerating voltage of 30 kV in a vacuum condition.

Scanning electron microscopy (SEM)

Green biomats were fixed with glutaraldehyde solution (2.5% final concentration) and stored at 4℃ until further microscopic observation. Freeze-drying method was used for the sample preparation (Suzuki *et al*., 1995). A drop of fixed biomats was mounted onto a filter with a pore diameter of 0.45 μm. The sample was washed and fixed with *t*-butyl alcohol, frozen in liquid nitrogen, and dried with low-vacuum SEM. After the samples were freeze-dried, they were transferred onto cupper stubs with a double-sided adhesive carbon tape, coated with carbon, and observed with SEM (JEOL JSM-5200 LV) at an accelerating voltage of 15 kV. The SEM was equipped with an EDX spectrometer (Philips EDAX PV9800 STD) and the chemical composition of bacterial cells and fossilized microorganisms was analyzed.

Fourier transform infrared (FT-IR) microspectroscopy

A drop of biomats or deposits was mounted on a fluorite disk (CaF_2, 0.5 mm in thickness) and air-dried. Measurements of infrared spectra were taken for individual cell-like structures under a micro-FT-IR system consisting of an FT-IR spectrometer (JASCO FTIR-610) and an infrared microscope (JASCO MICRO -20) with a ×38 objective and with a transmission method in the range of ν = 600 − 4000 cm^{-1}.

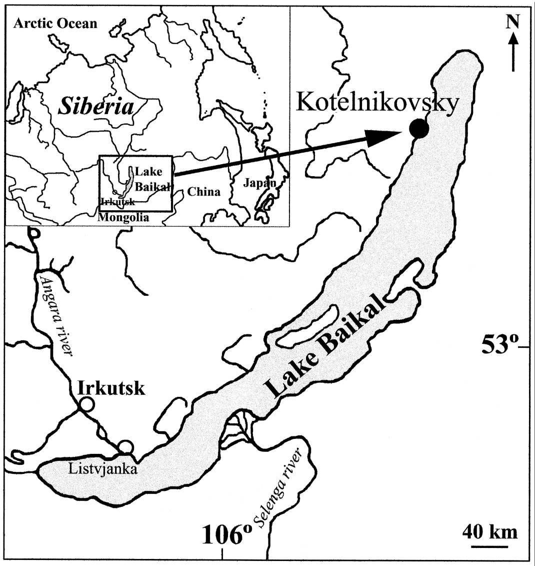

Fig. 1. Locality map of the sampling area at Kotelnikovsky Hot Springs, northwest coast of Lake Baikal, Siberia, Russia.

Results and discussion

Chemical and mineralogical analyses of hot spring water, biomats, and deposits

The water of the Kotelnikovsky Hot Springs showed the alkaline pH (8.6), ORP of -350 mV, EC of 490 μS/cm, and DO of 2.7 mg l^{-1}. The water temperature was 76℃.

The results of ED-XRF analysis of green biomats and deposits of the Kotelnikovsky hot springs showed high concentrations of silicon and calcium(Table 1). The ratio of Si/Ca was 2.1 in green biomats. Calcium content in deposits was 21.0 wt%, but the Si/Ca ratio was 5.0 indicating an extensive silicification process.

Table 1. Energy dispersive X-ray fluorescence analysis of green biomats and deposits from the Kotelnikovsky Hot Springs, northwest coast of Lake Baikal, Siberia, Russia.

(wt%)

Elements	Mg	Al	Si	P	S	K	Ca	Mn	Fe	Sr	Ti	Si/Ca	Ca/K	P/S
Green biomats	1.5	4.1	44.4	2.6	1.5	5.8	21.0	0.6	16.9	0.2	1.4	2.1	3.6	1.7
Deposits	−	−	80.9	−	1.0	0.2	16.3	−	1.2	0.3	0.1	5.0	81.0	0

− : not detected

On the other hand, Ca/K ratio varied from 3.6 in the green biomats to 81.0 in the deposits. Additionally, the green biomats were enriched in biological elements, such as phosphorus, sulfur, magnesium, and manganese. Interestingly, the concentrations of bivalent and multivalent ions, such as calcium, aluminum, and iron, characteristically increased in the green biomats with diminished calcium as well as the absence of aluminum and iron in the deposits. Previous studies (Urrutia and Beveridge, 1994, Schultze-Lam *et al.*, 1996) indicate that in the environments with high content of silicon at neutral or slightly alkaline pH, the silicification process of cells might occur on the bacterial surfaces through a cationic bridging mechanism. Silicate ions with an electronegative charge can be bound to bacterial surfaces, which have a net electronegative charge, through multivalent metal ions called "bridges," and electrostatic interaction (Urrutia and Beveridge, 1994 ; Ehrlich, 1996 ; Fortin *et al*., 1998). These silicate minerals are mostly amorphous (Fortin *et al*., 1998). Thus, the high content of multivalent cations, such as calcium, iron, and aluminum found in the green biomats may have resulted from the geochemical activity of the microorganisms.

Observed XRD patterns (Fig. 2) indicated the presence of amorphous silica with the diffused peak at 4.1Å in both samples. In addition, quartz (3.34, 4.25 Å) and fluorite (3.15, 1.92, 1.65Å) were found in the biomats, whereas fluorite (3.15, 1.93, 1.65Å) and calcite (3.04, 2.09, 1.87Å) were detected in the deposits. Thus, we infer that silicification and calcification of microorganisms occurred in the microbial mats resulting in the concentration of silicon, calcium, and aluminum.

Morphological structure of green biomats and deposits

A large variety of microorganisms was found in green biomats showing short or curved rods, spherical and filamentous bacteria (Figs. 3-A, B, D, E). The EDX spectrum obtained from the spherical bacteria showed strong peaks of silicon and calcium associated with magnesium, aluminum, phosphorus, and iron (Figs. 3-B, C). The same spectra were obtained from rod-shaped and filamentous bacteria that were free from the mineral encrustation and had transparent structures (data not shown). The content of potassium was higher than that of calcium, and the Ca/K ratio was 0.7. The same ratio was reported for alive microorganisms (Chang *et al*., 1986 ; Nikitin *et al*., 1998 ; Goldberg *et al*., 2001 ; Muliukin *et al*., 2002). For the resting forms of bacteria, the content of calcium and Ca/K ratio was high, but P/S ratio was low (Muliukin *et al*., 2002). The green biomats consisted mainly of fossilized bacteria-like structures, which had a size and shape similar to those of bacteria (Figs. 3-D, F). The EDX analyses obtained from filamentous particles showed a high content of calcium, a high Ca/K ratio (2.1), and a small phosphorus peak, indicating the presence of organic matter (Figs. 3-F, E). On the other hand, the P/S ratio was low indicating physiological state of dead

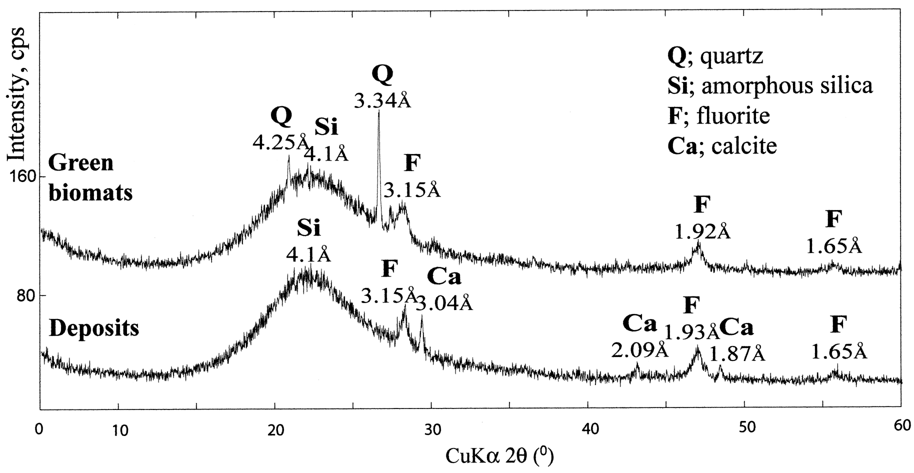

Fig. 2. XRD spectra of green biomats and deposits from Kotelnikovsky Hot Springs, showing the presence of amorphous silica associated with quartz, calcite, and fluorite.

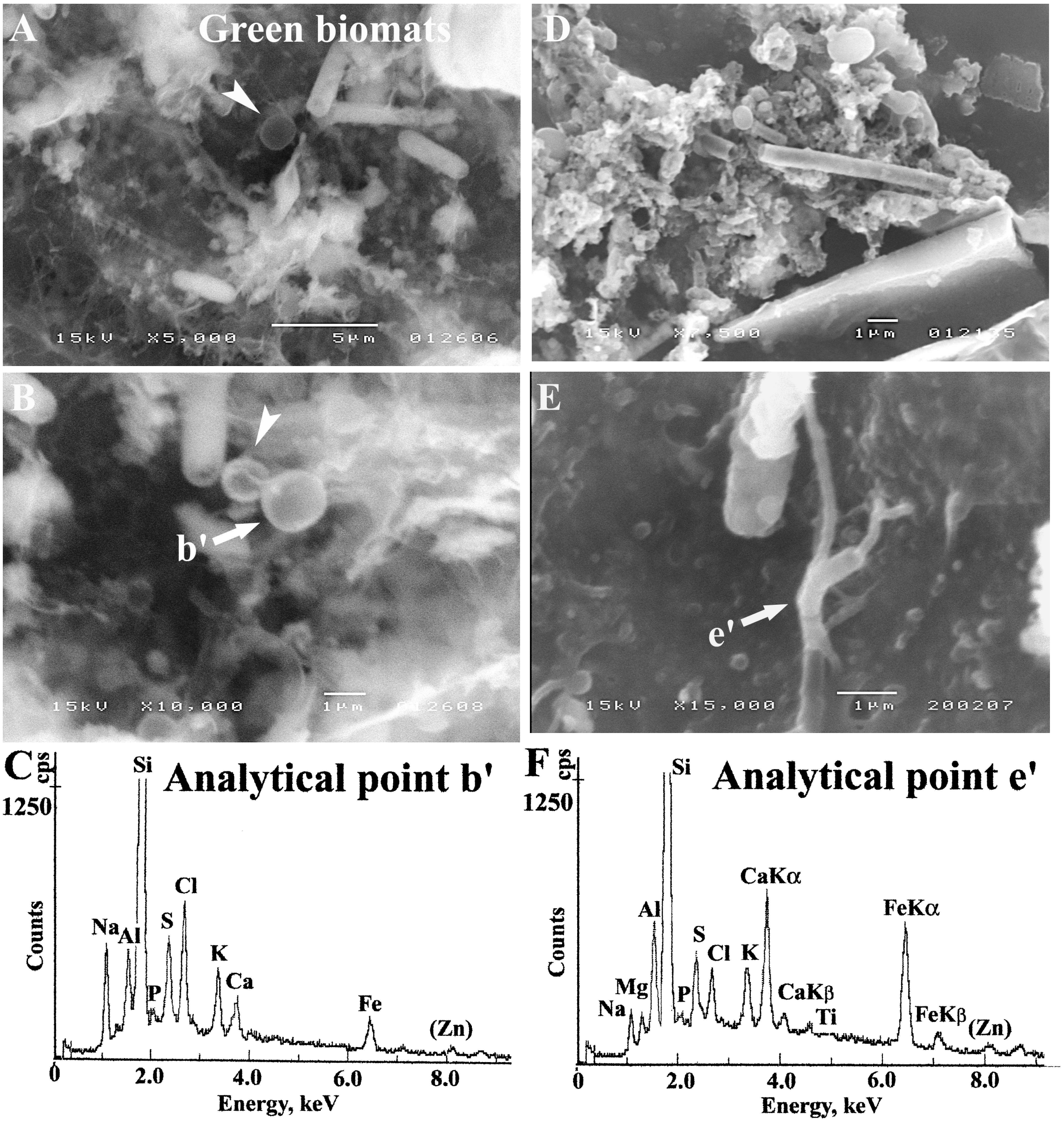

Fig. 3. Scanning electron micrographs of microorganisms from green biomats of Kotelnikovsky Hot Springs. A, B ; abundant spherical sheathless bacterial cells in the green biomats (marked with arrows). C ; EDX spectrum derived from bacteria (b' , arrow) showing strong peaks of Si and S with traces of P, and low Ca/K ratio (0.7). D, E ; variety of mineralized bacterial cells. F ; EDX spectrum derived from fossilized microorganisms (e', arrow) showing strong peaks of Si and S with traces of P, and high Ca/K ratio (2.1).

or/and resting bacterial forms. Additionally, the Si/Ca ratio detected from bacterial cells of green biomats varied from 2 to 1 on average.

A variety of fossilized microorganisms was observed in the hot spring deposits (Figs. 4-A-C). The EDX analyses obtained from fossilized microorganisms of deposits showed the spectra with the same pattern that resulted from bacterial cells from green biomats (data not shown). On the other hand, the EDX spectra of amorphous particles showed the average Si : Ca ratio varying from 5 : 1 to 10 : 1, a high calcium content, a high Ca/K ratio, and the absence of phosphorus and

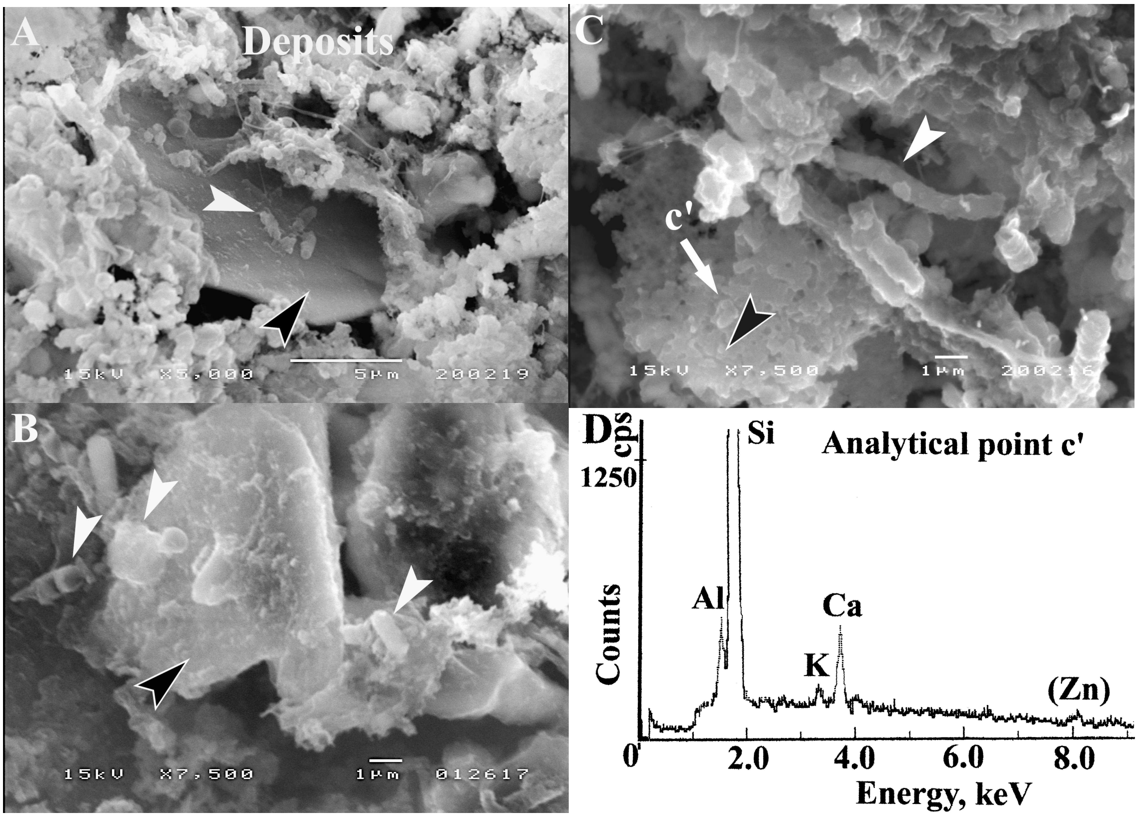

Fig. 4. Scanning electron micrographs of fossilized bacteria-like particles from deposits of Kotelnikovsky Hot Springs. A ; general view of the sample, B, C ; close-up view of spherical, rod-shaped and filamentous bacteria-like structures (white arrows) are associated with amorphous particles (black arrows), D ; EDX spectrum derived from mineral particle (c', arrow) showing strong peaks of Si, absence of P and S, and high Ca/K ratio (2.0).

sulfur (Figs. 4-C, D).

Bacterial silicification and calcification are widespread phenomena, but both processes are not common in geothermal systems. Biogenic amorphous silica, Fe-silica minerals, calcite, and stromatolite deposition have been reported (Schultze-Lam *et al*., 1995, Asada and Tazaki, 2001; Kasama and Murakami, 2001; Yasuda *et al*., 2000). Microorganisms can enhance the rate of metal mobilization due to a high surface-to-volume ratio and specific properties of cell walls, such as the presence of metal-binding domains and anionic sites (Beveridge, 1989). Calcium binds to the bacterial surfaces through electrostatic interaction (Schultze-Lam *et al*., 1995) and dedicated calcium-binding domains (Rodrigues *et al*., 1997 ; Rigden *et al*., 2003) and serves as a nucleation site for further metal complexation (Ehrlich *et al*., 1996). In this study, both silicification and calcification of bacteria under pH 8.0 condition were found during fossilization.

FT-IR microspectroscopical analysis of green biomats and deposits

Recently, FT-IR spectroscopy is developed for bacterial identification (Filip and Hermann, 2001; Oberreuter *et al*., 2002). The intraspecific diversity of 31 strains of *Brevibacterium linens*, 27 strains of *Corynebacterium glutamicum* and 29 strains of *Rhodococcus erythropolis* was determined by partial 16S rDNA sequence analysis and FT-IR spectroscopy (Oberreuter *et al*., 2002). Oberreuter *et al*. (2002) demonstrated that FT-IR spectroscopy is a rapid and reliable screening method for similar isolates and for identifying these actinomycetes at the species level. There was no correlation of FT-IR spectral similarity and 16S rDNA sequence similarity below the species level. Therefore, Oberreuter *et al*. (2002) suggested that diversification

Table 2. Infrared transmission bands of the mineralized particles in the green biomats and deposits from the Kotelnikovsky Hot Springs.

Band (cm^{-1})		Type of chemical bonds	Possible assignment*
Green biomats	Deposits		
–	819	Si-O-Si bending	Silica-minerals
1010	1067	Si-O stretching	Silica-minerals
1102	1100	P-O stretching vibration of the phosphate group and C-O stretching vibration of the ribose or desoxyribose	Nucleic acids
1398	–	Terminal C-H bending vibration	Fatty acids
1543	–	Coupled C-N stretching and N-H in-plane bending (C-N-H combination)	Polypeptides
1638	1635	C=O stretching	Polypeptides
2925	–	Aliphatic C-H stretching	Fatty acids
3396 – 3545	3391	N-H stretching vibrations in adenine, quinine, cytosine and O-H stretching	Nucleic acids and H_2O (air)

– : not detected

*Possible assignments after Rao (1963), Lyon (1967), Filip and Hermann (2001), and Choo-Smith *et al*. (2001).

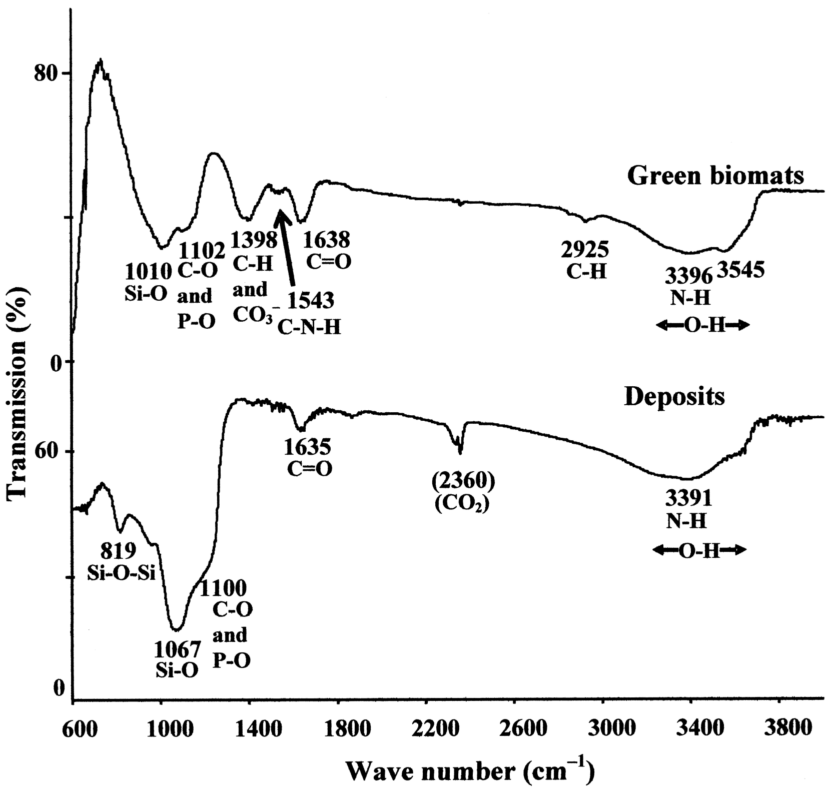

Fig. 5. FT-IR spectra of individual particles from green biomats and deposits showing bands at 819 cm^{-1} (Si-O-Si bending), at 1010/1067 cm^{-1} (Si-O stretching), at 1100/1102 cm^{-1} (C-O and P-O stretching in nucleic acids), at 1398 cm^{-1} (terminal C-H bending in fatty acids and carbonate CO_3^- group in calcite), at 1543 cm^{-1} (C-N-H combination in polypeptides), at 1635/1638 cm^{-1} (C=O stretching in polypeptides), at 2925 cm^{-1} (aliphatic C-H stretching in fatty acids), and at 3391—3545 cm^{-1} (N-H stretching in nucleic acids and O-H stretching).

of 16S rDNA sequences and microevolutionary changes of the cellular components detected by FT-IR spectroscopy are de-coupled. We used FT-IR technique to distinguish between biotic and abiotic mineralized particles that were found in the biomats and deposits. In addition, we carried out an analysis of individual particle (Fig. 5). The wave numbers (cm^{-1}) of the chemical bonds are presented in Table 2. The database of infrared bonds on silicon-organic and calcium-organic bonding is not well known. We assign the peaks at 819 cm^{-1} and 1010/1067 cm^{-1} to the Si-O-Si and Si-O bonds after Lyon (1967). Both of these peaks were evident in the infrared spectrum of particles from deposits, whereas particles from green biomats delivered only one of them at 1010 cm^{-1} (Fig. 5). Commonly, carbonates show strong spectral peaks at about 1400 cm^{-1}, which corresponds to CO_3^- group bands (Lyon, 1967). This peak is detected in the particles from biomats.

The other peaks and shoulders (Fig. 5) resulted mainly from the organic matters of the microorganisms (Rao, 1963 ; Filip and Hermann, 2001 ; Choo-Smith *et al*., 2001). The spectral region around 900 to 1200 cm^{-1} could be assigned to carbonyl groups of cell-wall glycopeptides, which absorb strongly at 1080 cm^{-1}. Additionally, the vibration of phosphate group and C-O stretching vibration of the nucleic acid ribose or deoxyribose moieties overlaps at the same region (Filip and Hermann, 2001 ; Choo-Smith *et al*., 2001). The FT-IR spectrum of particles from biomats indicated more organic-specific bands in comparison with deposits, where only one shoulder could be determined at 1100 cm^{-1}. The shoulder at 1102 cm^{-1}, which is clearly distinguished in the spectrum of mineral particles from biomats, appears to correspond to nucleic acids. The peaks and shoulders corresponding to the C-H vibrations occurred at 1396－1389 cm^{-1} (terminal C-H bending), at 1455－1467 cm^{-1} (C-H deformations in CH_2 and CH_3), and at 3000－2800 cm^{-1} (typical region for C-H stretching) (Filip and Hermann, 2001). In general, cell wall, capsular lipids, and to some extent polysaccharides, could be responsible for these features. Two of these bands, namely the peak at 1398 and the shoulder at 2925 cm^{-1}, are visible in the spectrum of amorphous particles from biomats. Cell proteins are typically indicated by a number of amide bands. They dominate at 1658－1656 cm^{-1} and show additional bands at 1537－1535 cm^{-1} and 1240－1235 cm^{-1} (Filip and Hermann, 2001). An enhanced infrared transmission at 1638 cm^{-1} and a small shoulder at 1543 cm^{-1} were observed in the infrared spectrum of biomats. The absence of a third peak at 1240-1235 cm^{-1} (C-N stretching) in both samples is caused by diagenesis of hydrothermal deposits.

A strong band at 2360 cm^{-1} and a wide band at 3391-3396 cm^{-1} (Fig. 5) could be attributed to some moisture and CO_2 content in the air. However, because a free water band at 1613 cm^{-1} is not present in the spectra, and because there are bands at 1100/1102 cm^{-1} that correspond to the vibrations in nucleic acids, the shoulder at 3391-3396 cm^{-1} could also be attributed to the N-H stretching vibration in adenine, guanine and/or cytosine of nucleic acids. Therefore, we can propose the biogenic provenance for the mineralized particles observed in the green biomats from the Kotelnikovsky Hot Springs.

Summary

The field measurements of water quality showed an alkaline pH (8.6), ORP of -350 mV, EC of 490 μS/cm, DO of 2.7 mg l^{-1}, and water temperature of 76℃ of the Kotelnikovsky Hot Springs. The ED-XRF analysis revealed that the hot spring biomats and deposits were rich in silicon and calcium suggesting fossilization processes. The bulk X-ray diffraction analyses of hot spring biomats and deposits identified the amorphous silica, quartz, calcite, and fluorite. The SEM-EDX analyses of fossilized microorganisms showed Ca/K ratio could be changed during fossilization process. Fourier transform infrared (FT-IR) microspectroscopy results indicated the fossilization process of bacteria from green biomats to deposits, decreasing organic compounds (C-H, C-N-H, N-H bonds), and increasing Si-O-Si and Si-O bonding.

Our findings indicate that the SEM-EDX microscopic observation combined with FT-IR analysis allows to detect fossilized microorganisms in the hot spring biomats and deposits. We can conclude that silicification and calcification of microorganisms appeared simultaneously and extensively in the biomats and deposits under pH 8.0 hydrothermal conditions. Fossilization of microorganisms is an essential process in the formation of modern hydrothermal deposits.

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