Hydrocarbon mineral formation by micrpprganisms in tsukiokaoily hot springs in Niigata, Japan -A clus to bioremediation Part ?-

HYDROCARBON MINERAL FORMATION BY MICROORGANISMS IN TSUKIOKA OILY HOT SPRINGS IN NIIGATA, JAPAN

- A CLUE TO BIOREMEDIATION PART II -

Rie WAKIMOTO[1] and Kazue TAZAKI[2]

[1] *Graduate School of Natural Science&Ttechnology, Kanazawa University, Kanazawa, Ishikawa 920-1192, JAPAN; e-mail: wacky7@earth.s.kanazawa-u.ac.jp*

[2] *Department of Earth Sciences, Kanazawa University, Kanazawa, Ishikawa 920-1192, JAPAN; e-mail:kazuet@kenroku.kanazawa-u.ac.jp*

ABSTRACT

Bacterial hydrocarbon mineral formation was found in the green colored microbial mats adhered on the source of well at the Tsukioka Hot Springs, Niigata Prefecture in Japan. Water quality of hot springs showed neutral pH (7.3 ~ 7.8) and anaerobic condition (Eh: -184 ~ -167 mV). The green colored microbial mats and crude oil have been analyzed and observed by XRD, ED-XRF, optical microscope, SEM-EDX and TEM to clarify the bacterial hydrocarbon mineral formation. Crude oil of the Tsukioka Hot Springs, which contains a high concentration of S and traces of Si and Cu, gleams orange fluorescence light under ultraviolet-ray. Optical and epifluorescence microscopic observations show that filamentous bacteria inhabit at the green microbial mats with orange colored oil. Amorphous hydrocarbon minerals were covered with the biofilm formed by the bacteriolytic process. EDX spectrum of hydrocarbon minerals indicated the high concentrations of P, Si, and S associated with Na, Mg, Al, Cl, K, Ca, Fe, and Cu. The green microbial mats accumulated a large amount of Cu ion to form Cu-minerals, such as covellite (CuS), covering with filamentous bacteria around oil droplets. TEM observations of filamentous bacteria showed the double membrane of gram-negative bacteria adhere to oil droplets. The results imply that the bacterial hydrocarbon mineral formation can be used remediation of petoroleum hydrocarbons at oil-polluted area and Cu-contaminated mining area.

INTRODUCTION

Biodegradation is an important and potentially ubiquitous process affecting both the chemical composition and physical properties of crude oil. The influences of biodegradation on molecular composition and physical properties of crude oil are empirically well-known from many studies of in-reservoir biodegradation, laboratory degradation experiments, and crude oil spills in the field (e. g. Oldenburg *et al.*, 2000). The development of petroleum industry into new frontiers, the apparent inevitable spillage, which usually occur during routine operations, and records of acute accidents during transportation, has needed for more studies into oil pollution problems. These pollution problems have been prevalent all around the world since the 1950s (e. g. Okoh *et al.*, 2001). Remediation of polluted systems could be achieved by physical, chemical or biological methods. However, the attendant negative consequences of the physicochemical methods make the biological alternative or bioremediation more attractive (Okoh, 2003).

Furthermore, petroleum hydrocarbon contamination of aquifers presents a serious threat to ground water resources. While most hydrocarbon compounds are known to be degraded under aerobic conditions, many petroleum-contaminated aquifers contain large areas where anaerobic processes are dominant (Anderson *et al.*, 1998). On the other hands, Yushkin (1998) reported a concept of hydrocarbon crystallization of life is initially from reworking biogenic hydrocarbons with various shape of hydrocarbon minerals showing SEM micromorphology. Most of heavy metals and toxic materials are accumulated by microbes and formation of biominerals, after precipitation of insoluble metals (e. g. Lloyd and Macaskie, 2000). However, there are few reports of mineral formation of hydrocarbon under the specific environment where crude oil mixed with hot spring water.

In this study, hydrocarbon mineral formation by filamentous bacteria was found at the Tsukioka Hot Springs in Niigata, Japan. We focus on investigations of the hydrocarbon mineral formation by microorganisms especially in oily hot spring water to get an idea for bioremediation at oil-polluted area.

LOCATION AND GEOLOGY

The Tsukioka Hot Springs is located at the northwestern part of Niigata prefecture in Japan (Fig. 1). Most of oil and gas fields in Japan are concentrated in the Neogene Niigata and Akita Basins, located along the eastern margin of the Sea of Japan. The Niigata and Akita Basins were generated as rift basins related to the opening of the Sea of Japan around 16 Ma, and were inverted into compressional basins around 6.5 Ma due to a change of plate tectonic condition. This basin history brought a scenario to make a good combination not only between source and reservoir rocks, but also between reservoir rocks and trap structures (Waseda and Omogawa, 1988). The geology of Tsukioka consists of Tertiary and Quaternary sedimentary rocks (Hirai *et.al.*, 1995). The Tsukioka Hot Springs pumps up water from fracture zone of mudstone of Pleiocene Taira formation, 280 m in depth. The well is used for pumping crude oil, therefore the hot spring water is mixed with oil associated with fossil seawater (Shinmazu, 2001).

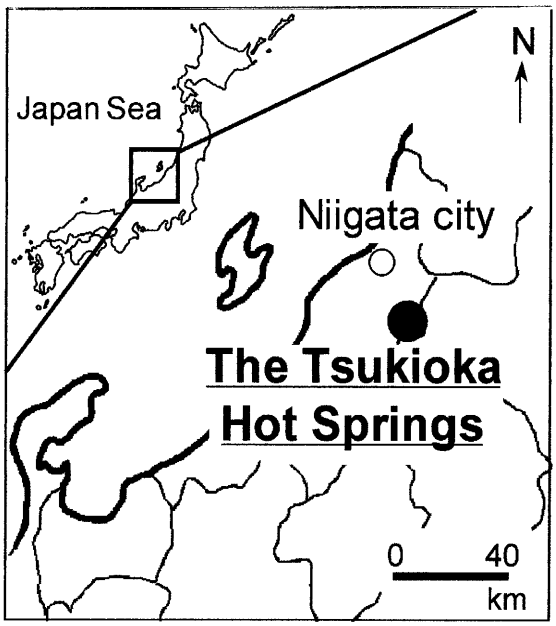


Figure 1 Locality map of the Tsukioka Hot Springs in Niigata Prefecture, Japan.

MATERIALS AND METHODS

Materials

Crude oil, oily hot spring water and microbial mats were collected from the Tsukioka Hot Springs on May 25, 2003. The hot springs is gushed out together with crude oil and water (Fig. 2A). Green colored microbial mats formed on the well wall (Fig. 2B, arrow ①) of black crude oil (Fig. 2B, arrow ②). After filtering hot spring water, the color changed to transparent green without oil droplets (Fig. 2C).

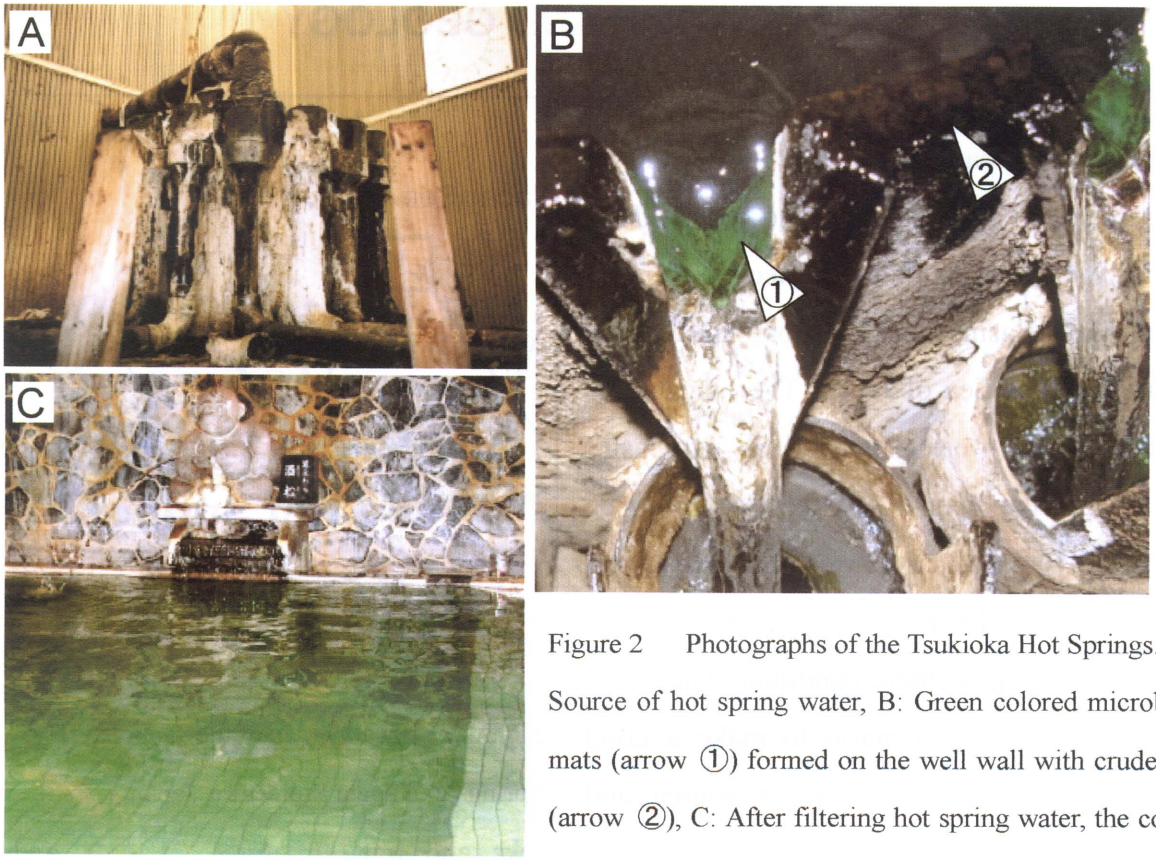


Figure 2 Photographs of the Tsukioka Hot Springs. A: Source of hot spring water, B: Green colored microbial mats (arrow ①) formed on the well wall with crude oil (arrow ②), C: After filtering hot spring water, the color changed to transparent green.

Water chemistry

Water qualities of the hot springs were measured in the field on May 25, 2003. The pH was measured using a pH meter HORIBA D-12, Electrode potential versus the standard hydrogen electrode (Eh) was measured using HORIBA D-13. Electrical conductivity (EC) was measured using HORIBA ES-12, Dissolved oxygen (DO) and Water temperature (WT) were measured using HORIBA OM-12 equipments. The concentration of Cu was analyzed by atomic absorption photometer (SEIKO, SAS-727). Measuring points are shown in Fig. 2B (the source of hot spring water) and Fig. 2C (after filtering water).

X-ray powder diffraction analysis (XRD)

The mineralogical properties of green colored microbial mats were analyzed by X-ray powder diffractometer (XRD), using Rigaku RINT 2000 with CuKα radiation, and generating at 40 kV and 30 mA using the $2\theta / \theta$ method with a scan speed of 1° /min. Microbial mats were washed with deionized water for removal the halite (NaCl), air-dried at room temperature, and ground to fine powder. The powder samples were fixed on the square concavity of glass slide and set up on the stage of XRD for analyses.

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

The samples were analyzed by two methods of ED-XRF. One method of ED-XRF used for bulk samples of crude oil and microbial mats were used. The crude oil sample (50 μl) for ED-XRF analysis was set up between double Mylar films, whereas, the microbial mat samples were air-dried at room temperature and ground to fine powder for ED-XRF analysis. The powder samples were pressed to make pellets and mounted on Mylar film. Chemical analyses of both crude oil and microbial mats bulk samples were carried out by an energy dispersive X-ray fluorescence spectrometer (JEOL JSX-3201), using Rh Kα, operated at an accelerating voltage of 30 kV under vacuum condition. Other method of ED-XRF makes it possible to image the chemical distribution of elements in the specimen. The point analysis with visual images can be possible by ED-XRF (JEOL JSX-3600) using Mo Kα, which operated at an accelerating voltage of 30 kV under vacuum condition.

Optical light and epifluorence microscopy of microbial mats

To identify the presence and variety of microorganisms in microbial mats, optical microscopic observation was carried out. The microbial mats were fixed with 2.5 % glutaradehyde. The fixed samples were stained with DAPI (5 mg/ml) for 3 minutes for observation under epifluoresence microscope (Nikon EFD-3). The DNA of bacterial cell and of the crude oil resulted in the fluorescence blue or orange under ultraviolet-ray (365 nm).

Scanning electron microscopy (SEM)

Freeze-dried method was used for microbial mats, referred to Suzuki *et al.* (1995). One drop of fixed microbial mats with 1 % glutaraldehyde was mounted onto a JEOL filter, washed by deionized water, and fixed with t-butyl alcohol. The frozen samples in liquid nitrogen were dried in low-vacuum SEM. After freeze-drying, the sample was transferred on carbon stub with double-sided adhesive carbon tape. After carbon coating, the sample was observed with a scanning electron microscope (JEOL JSM-5200LV), equipped with an energy dispersive X-ray spectrometer (Philips-EDX PV9800 STD).

Transmission electron microscopy (TEM)

Transmission electron microscopy (JEOL JEM-2000EX) were used for the observation of bacterial cells. One drop of the suspension of microbial mats was mounted on micro

grids. After air-drying, the uncoated samples were observed at an accelerating voltage of 120 kV. Ultramicrotomy method was used for sample preparation referred to Horita (1992). The cells in the suspension were also concentrated by centrifugation. The concentrated cells were included in agar and cut into 1 mm cubes for handling. The cubes were fixed with 2.5 % glutaraldehyde solution for 5 hours, rinsed 4 times with phosphate buffer, post-fixed with 1 % osmium tetroxide for 2 hours, and again rinsed 4 times with phosphate buffer (0.2 M, pH 7.4). The cubes were dehydrated in a graded series of ethanol solutions (50, 70, 80, 90, 95, 100 %), at room temperature, with 15 minutes at each step. The dehydrated samples were substituted 2 times with propylene oxide for 30 min each, and were impregnated with mixtures (1:2, 1:1, and 2:1) of Epon 812 epoxy resin and polymerization at 35, 45 and 60 ℃ for 12, 12 and 48 hours, respectively. Ultra thin sections 70 nm in thickness were cut with a diamond knife on a ultramicrotome (Lica ULTRACUT UCT), mounted on carbon-coated copper specimen grids, and contrasted with uranyl acetate and lead citrate. The ultra thin sections were observed at an accelerating voltage of 100 kV and 120 kV.

RESULTS AND DISCUSSION

Water chemistry

The characteristics of the hot spring water before and after filtering are shown in Table 1. Both samples were quite similar quality showing neutral anaerobic condition, suggest that the gush of spring water from deep geological strata. The concentrations of Cu in both water samples were not detected by atomic absorption analyses.

Table 1. Chemical characteristics of the Tsukioka Hot Spring

	pH	Eh (mV)	EC (mS/cm)	DO (mg/l)	WT (℃)	Cu (ppm)
The source of hot spring water	7.3	−184	7.3	0.8	49.2	n.d.
After filtering water	7.8	−167	7.0	1.6	43.2	n.d.

n.d. ; not detected

ED-XRF chemical analysis of oil and microbial mats and XRD mineralogical analysis of microbial mats

The ED-XRF analyses of the crude oil showed high concentration of S and traces of Na,

Si and Cu except hydrocarbon (Fig. 3A). On the other hand, the green microbial mats contained high concentration of Cu (53.2 wt%), S (27.9 wt%) and Fe (6.2 wt%) with traces of Na, Si, P, K, and Ca (Fig. 3B). XRD patterns of microbial mats showed the existence of pyrite (FeS_2) (2.71, 2.42, and 1.73 Å) and covellite (CuS) (3.05, 2.81, and 1.89 Å) with a broad back-ground, suggesting that organic materials or amorphous materials are present (Fig. 3C). Covellite usually exists in small quantities in oxidizing zone of copper deposits associated with other copper sulfides such as chalcocite (Cu_2S) and digenite (Cu_9S_5). Stability of covellite and digenite depend on the reduction condition. Under the neutral pH, covellite is stable in a condition of Eh over –250 mV, whereas digenite is stable in a range of Eh under –250 mV (Mcneil and Little, 1999). Digenite occurs as copper sulfide minerals under the reduction condition. When the Eh go up to -250 mV, digenite is transformed to covellite. The results of ED-XRF chemical analysis agreed with XRD mineralogy that the Cu and S of covellite are originated from crude oil.

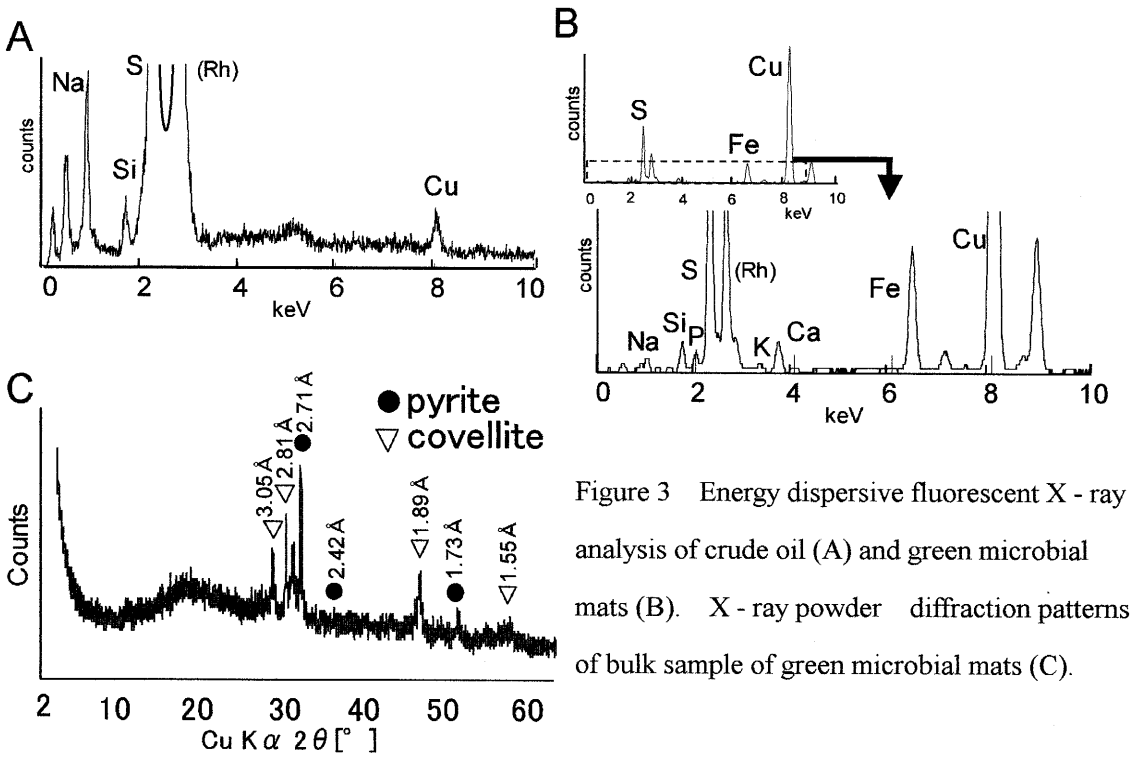
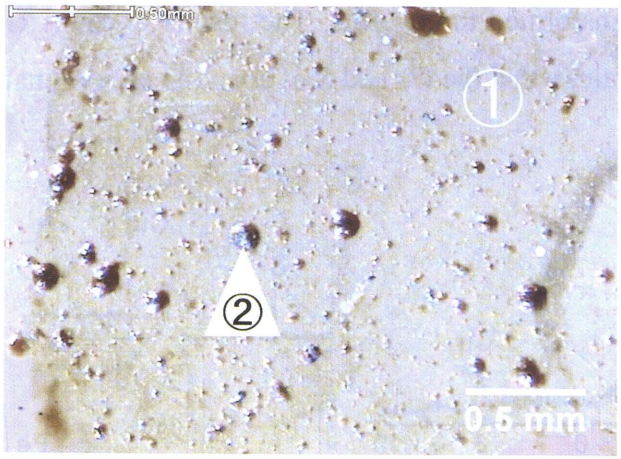


Figure 3 Energy dispersive fluorescent X - ray analysis of crude oil (A) and green microbial mats (B). X - ray powder diffraction patterns of bulk sample of green microbial mats (C).

ED-XRF point analytic microphotograph of non-treatment microbial mats shows two different colored distributions of green and black parts. The microbial sheets appear smooth and flat green surface whereas the black parts are oil droplets, 0.1 mm in diameter.

The point analyses of green parts of microbial sheets contain high Na (39.0 wt%), Mg (17.1 wt%), S (13.9 wt%), and Ca (10.1 wt%) cation (Fig. 4 ①). These elements reflect the composition of hot spring water. On the other hand, the black parts of oil droplets contain high S ion (48.8 wt%), suggesting mainly oil components, while P ion (30.3 wt%) coexist, suggesting component of microorganisms. Only Cu concentration of green parts (0.5 wt%) clearly different from black parts (4.1 wt%) (Fig. 4, arrow ②). The result suggests that elemental composition of green part originated from hot spring water showing high concentration of Na, Ca, Mg and Cl indicate characteristics of fossil seawater, whereas black parts of oil droplets are oil component itself. The P and S elements are originated from microorganisms, which have the ability to use the oil droplets as source of energy.



	Na	Mg	Si	P	S	Cl	K	Ca	Fe	Cu
Green part of microbial mats ①	39.0	17.1	1.6	9.2	13.9	5.1	2.2	10.1	n.d.	0.5
Black part of oil droplets ②	n.d.	7.5	n.d.	30.3	48.8	n.d.	n.d.	8.7	0.5	4.1

n.d. ; Not detected (wt%)

Figure 4 Points analyses by energy dispersive fluorescent X – ray of non-treatment of green microbial mats. Analytical point ① indicate green part of microbial sheets, and point ② indicate black part of oil droplet on the surface of microbial mats.

Optical microscopic, SEM and TEM observations of microbial mats

Filamentous bacteria and oil droplets are found in the green microbial mats under the ultraviolet-ray (Fig. 5A). Oil droplets are recognized as black patches in Fig. 4, while they gleam with orange fluorescence light under the ultraviolet-ray. Filamentous bacterial colonies were aggregated together with the yellow colored mineral particles, 10 μm in

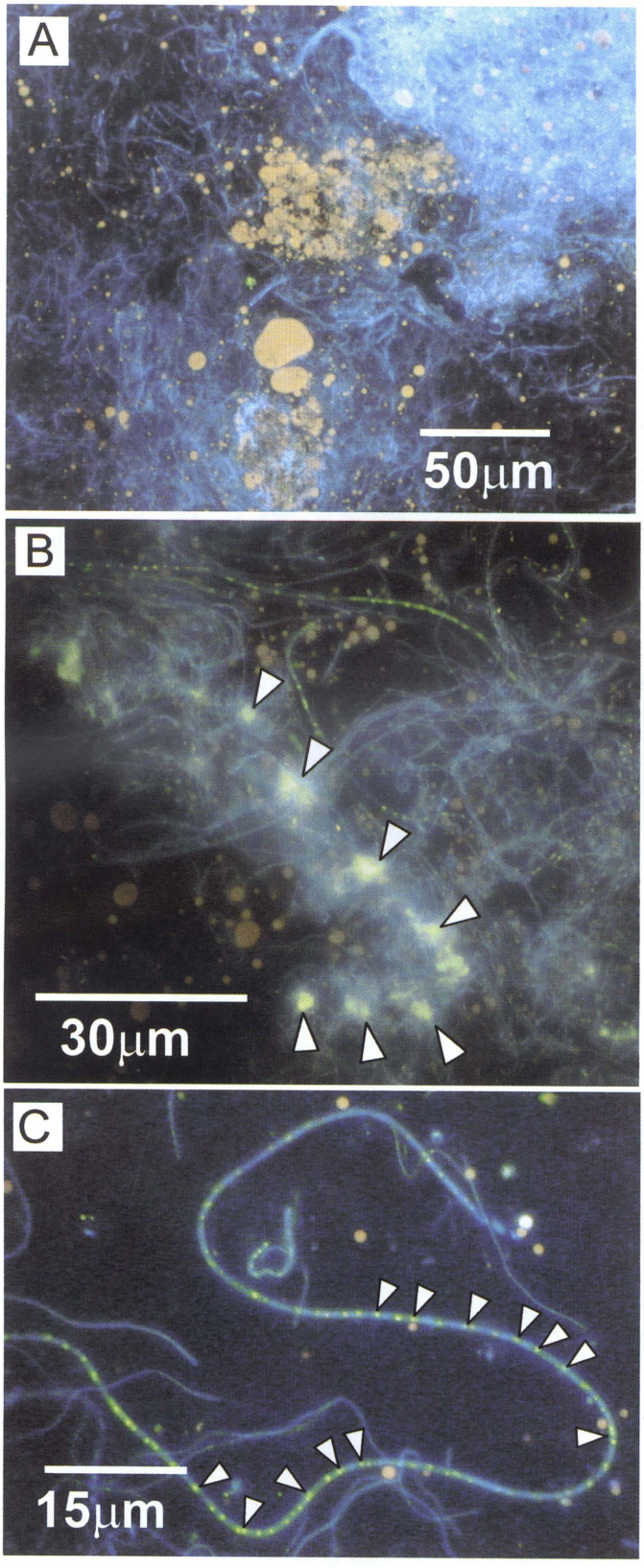


Figure 5 Epifluorescence micrographs of the green microbial mats showing filamentous bacteria associated with orange colored oil droplets (A). Filamentous bacteria aggregated with bacteria and yellow colored mineral particles suggesting covellite (arrows B). Yellow colored fine spherical particles in filamentous bacterial cells (arrows C).

diameter, suggesting covellite as shown in Fig. 5B (arrows). Yellow colored fine spherical particles were recognized inside of bacterial cells (arrows in Fig. 5C).

SEM images of the filamentous bacterial colonies were shown in Fig. 6A. Filamentous bacteria gather around a crude oil droplet. High-density bacterial agglutination of filamentous cell is not distinguishable, suggesting bacteriolytic process (Fig. 6B). EDX spectrum of filamentous bacteria (arrow in Fig. 6B) shows high concentration of P and S associated with Si, Ca, Cl, Mg, Na, and Fe with a hilly background, suggesting the presence of organic materials.

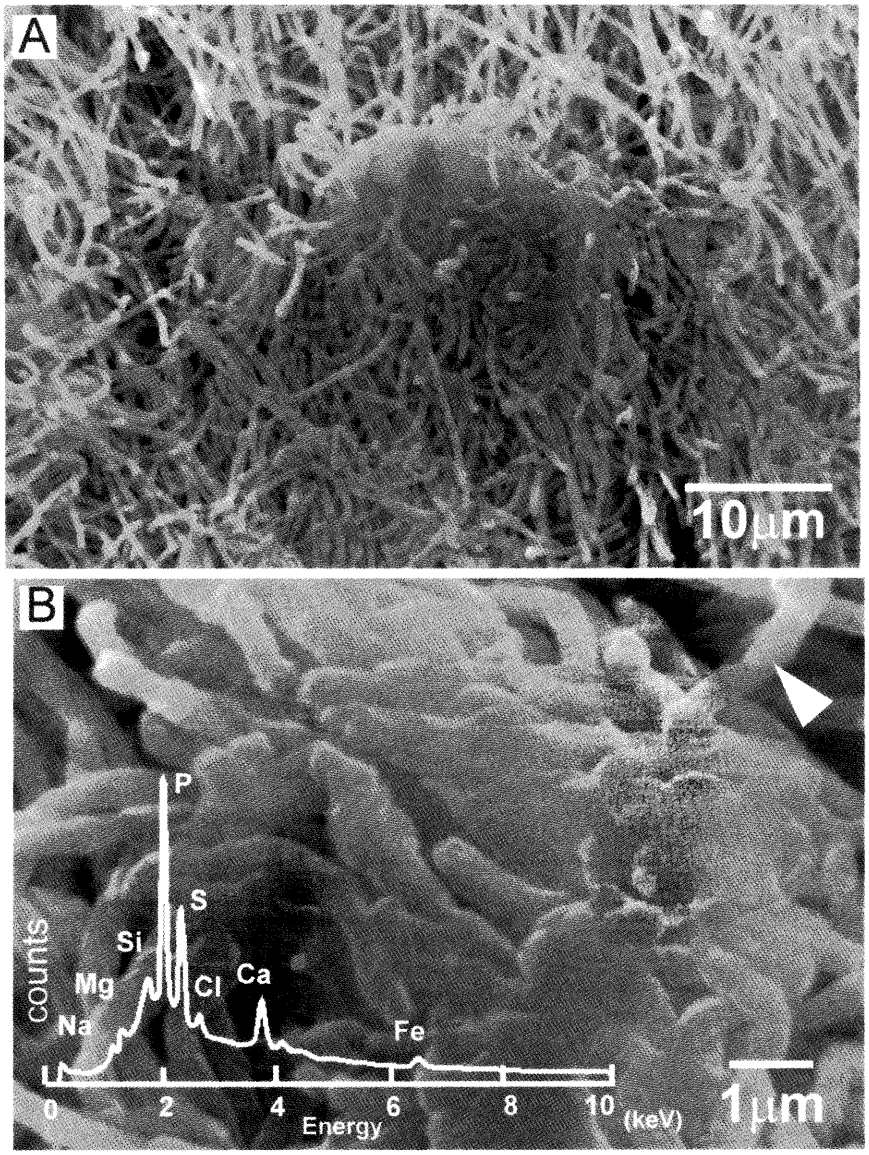


Figure 6 SEM micrograph showing agglutination of filamentous bacterial colony (A). The bacteriolytic processes made for biofilms around a crude oil droplet (B). EDX spectrum of filamentous bacteria (arrow : analytical point) showed strong P and S peaks associated with Na, Mg, Si, Cl, Ca, and Fe.

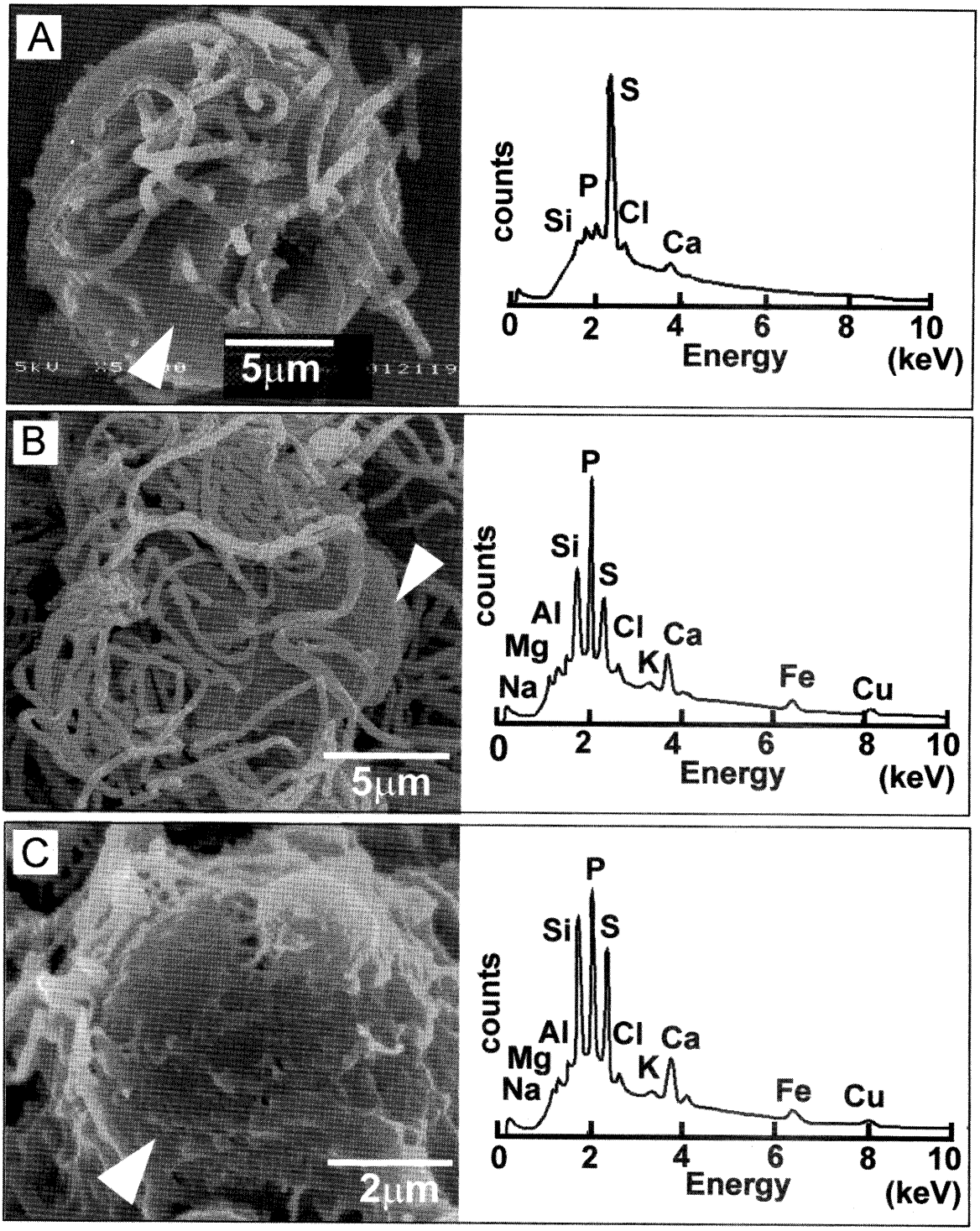


Figure 7 SEM micrographs of oil droplet within green microbial mats showing a successive process of hydrocarbon mineral formation. Oil droplet contains mainly S with the traces of Si, P, Cl and Ca (A). The filamentous bacteria gather around oil droplet (B). EDX spectrum indicated high concentration of P, Si and S associated with Na, Mg, Al, Cl, K, Ca, Fe and Cu. The oil droplet is completely covered with biofilm (C). EDX spectrum shows high concentration of P, Si and S associated with Na, Mg, Al, Cl, K, Ca, Fe and Cu. Analytical points are marked by arrows.

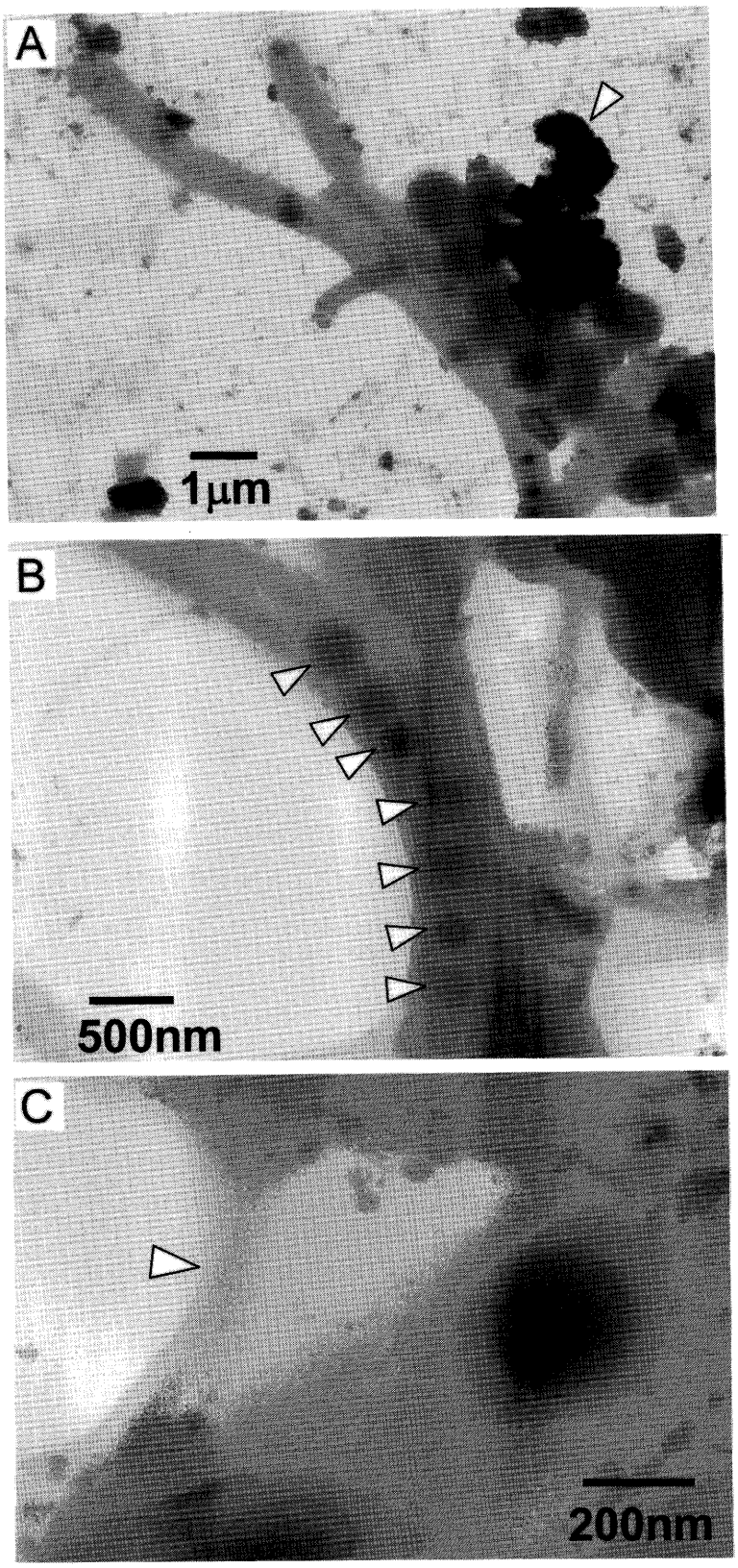


Figure 8 TEM micrographs of filamentous bacteria in green microbial mats . The high-density materials suggesting caked oil droplets attached with filamentous bacteria (arrows in Fig. 8A). The high-density spherical materials exist internal cell (arrows in Fig. 8B). The cell is covered with the sticky films (arrow in Fig. 8C).

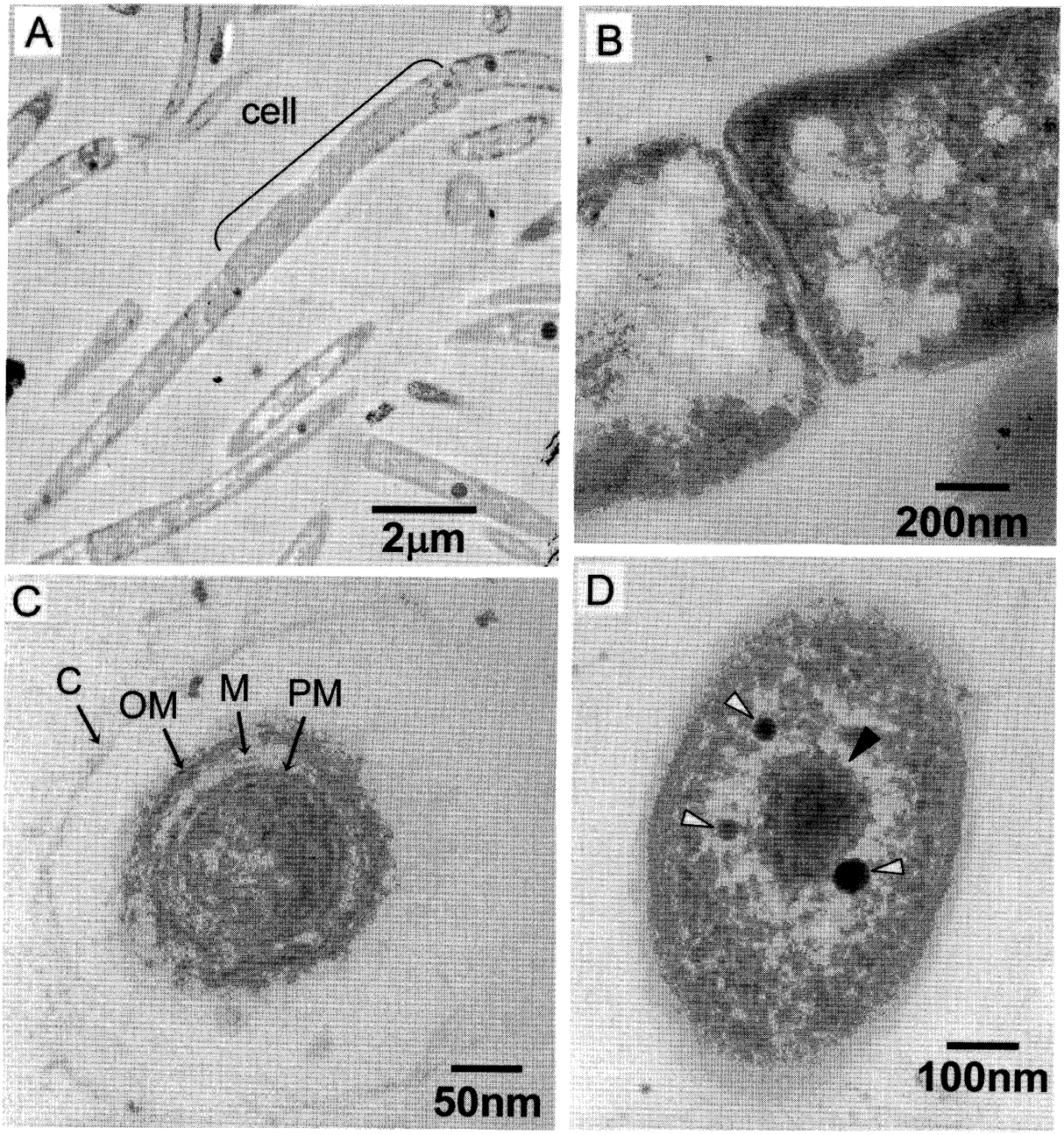


Figure 9 TEM micrographs of vertical ultra thin-section revealed that filamentous bacteria consist of joints of cell about 4 μm in length (A). The jointed section of each cells are intercepted by double membrane (B). TEM image of cross ultra thin-section of filamentous bacteria showing the double membrane surrounding each bacterial cell. Abbreviation: C; capsule, OM; outer membrane, M; murein (peptidoglycan) layer in periprasm space, PM; plasma membrane (C). Agglomerate chromosome was found in the center of the cell (pointed by black arrow in Fig. 9D) with high densed spherical materials (pointed by white arrows in Fig. 9D).

Amorphous hydrocarbon mineral formation processes by microorganisms in green microbial mats are shown in Fig. 7. At the first stage, filamentous bacteria gather around the crude oil droplet which contains high S (Fig. 7A, arrow). The bacteria crowd around crude oil droplet is coating with the bacteriolitic material (Fig. 7B arrow). The EDX spectrum of the coating material indicated high concentrations of P, Si, and S associated with Na, Mg, Al, Cl, K, Ca, Fe, and Cu (arrow). High concentration of P was due to biological components. Finally, the crude oil droplet is completely covered with the bio-film, resulting in high concentrations of P, Si, and S associated with Na, Mg, Al, Cl, K, Ca, Fe, and Cu (Fig. 7C, arrow). The concentrations of Si, P, and S increased step by step according to the development of hydrocarbon mineral formation.

TEM image of the green microbial mats shows filamentous bacteria attached with high-density materials indicating the caked oil droplets because the materials evaporated under vacuum condition (arrow in Fig. 8A). The high-density spherical materials, 200 nm in diameter, forming a line existed internal filamentous bacterial cells (Fig. 8B, arrows). The cells including the high-density spherical materials were covered with sticky cell membrane as capsule (Fig. 8C, arrow). The spherical materials line up farther apart from each other.

TEM observation of vertical ultra thin-section revealed that filamentous bacteria consist of cell joints about 4 μm in length. The each bacterial cells were lined up to form long chain structure (Fig. 9A). The combined section of each cell is intercepted by double membrane (Fig. 9B). TEM image of cross ultra thin-section of filamentous bacteria showed that the bacterial cell was surrounded by the double membrane and the capsule, which protected the cell from toxic environment (Osborn and Wu, 1980) (Fig. 9C). The cross ultra thin-section of filamentous bacteria clearly showed the cell structure of the outer membrane (OM), murein (peptidoglycan) layer in periplasm space (M) and plasma membrane (PM). The double membrane reveals that the filamentous bacteria belong to gram-negative bacteria. Furthermore, agglomerate chromosome was found in the center of the cell (black arrow in Fig. 9D) with high-density spherical materials 20～100 nm in diameter (white arrows in Fig. 9D). These spherical materials are considered to the same materials in Fig. 5C (arrows) and Fig. 8B (arrows).

Pallasser (2000) has reported that the biodegradation of crude oil was active under anaerobic condition. The Tsukioka Hot Springs show the quite anaerobic condition (Eh -280 mV), suggesting that active anaerobic bacterial biodegradation of crude oil expedites hydrocarbon mineral formation. Moreover, periplasm space in gram-negative bacteria, which can protect cell chemically, and hydrolytic enzyme and protein which has high affinity with various nutrition (Osborn and Wu, 1980). The filamentous bacteria in green microbial mats have the ability to use petroleum hydrocarbons as a source of energy by degrading harmful substances in hydrocarbon with enzyme inside periplasm space. The filamentous bacterial mineral formation of hydrocarbon can be used for remediation of petroleum hydrocarbons at oil-polluted area. Furthermore, the bacteria in oily hot spring water might be used for remediation at Cu-contaminated mining area.

CONCLUSIONS

The filamentous bacterial mineral formation of hydrocarbon has been found on the Tsukioka Hot Springs, Niigata, which forms green microbial mats. Water quality of hot springs showed neutral pH (7.3 ~ 7.8) and anaerobic condition (Eh: -184 ~ -167 mV). The green microbial mats contain a large amount of Cu ion to form Cu-minerals, such as covellite (CuS) coexisting with pyrite. The crude oil contains a high S and traces of Si and Cu, gleam the orange colored fluorescence light under ultraviolet-ray, suggesting Cu and S are originated from covellite in crude oil.

Optical and epifluorescence microscopic observations show that filamentous bacteria formed colonies aggregating together with the yellow colored mineral particles and fine spherical particles internal cells. Hydrocarbon minerals were covered with filamentous bacteria in the biofilm formed on bacteriolytic process. EDX spectrum of hydrocarbon minerals indicated high concentrations of P, Si, and S associated with Na, Mg, Al, Cl, K, Ca, Fe, and Cu. The SEM images suggest that covellite form nucleate amorphous hydrocarbon minerals which covered with biofilms. TEM observation revealed that filamentous bacteria have double membrane suggesting the ability to use petroleum hydrocarbons as a source of energy by degrading enzyme in periplasm space. The

filamentous bacteria have a great role of the mineral formation of hydrocarbon and covellite. The result implies that filamentous bacterial mineral formation of hydrocarbon is an effective remediation of petroleum hydrocarbons at oil-polluted area and at Cu-contaminated mining area.

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