Microscopic observation of microorganisms involved in methane production and oxidation in paddy field soil

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Introduction

Methane is major greenhouse gas, next to carbon dioxide, and about 20% of methane in the atmosphere is released from paddy fields (1). One of the characteristics of paddy field soil is that the supply of oxygen is limited under submersion, resulting in the lowering of redox potential. Therefore, organic matter in flooded paddy field soil is decomposed anaerobically into methane and carbon dioxide. Three microorganism groups are involved in the process by which organic matter is decomposed into methane and carbon dioxide. At the beginning, high molecular compounds such as cellulose and starch are hydrolyzed by fermentative bacteria and converted to alcohols such as ethanol and butanol and organic acids such as lactate and butyrate. Then, butyrate and ethanol are converted to acetate, hydrogen and carbon dioxide by the syntrophic association with a hydrogen-producing, acetate-producing bacterium and a hydrogen-utilizing methanogen. Acetate, hydrogen and carbon dioxide are converted to methane and carbon dioxide by methanogens in the last stage. With respect to anaerobes in paddy field soil, fermentative bacteria, sulfate-reducing bacteria and methanogens have been isolated from paddy field soil so far. However, until now, there have been few examinations of how fatty acids are degraded in a syntrophic association with hydrogen-utilizing methanogens, and how sulfate-reducing bacteria degrade lactate in syntrophic association with hydrogen-utilizing methanogens under the condition without sulfate. In paddy field soil, methane-oxidizing bacteria, which convert methane to carbon dioxide, live in paddy field soil. We describe here Type Ⅱ methane-oxidizing bacteria that can grow without copper dominant over the Type I species in paddy field soil. This report shows that the cell morphology of anaerobes involved in methane production and intracytoplasmic membranes of methane-oxidizing bacteria were observed with a photo and an electron microscope.

Materials and Methods

Microorganisms and media

The microorganisms used in this experiment were as follows: *Methanobacterium formicicum* strain TM-8 (2), *Methanobacterium bryantii* strain H5-1 (3), *Methanospirillum* sp. strain TM20-1 (4), *Methanosarcina strains* J-6 and 2-P (5,13), *Methanosaeta* sp. strain K-5 (6), *Desulfovibrio* sp. strain SK3-4 (7), *Syntrophomonas* sp. strain TB-6 (2), *Methylosinus* strains D2-11 and W3-6 (8). The microorganisms were isolated from the Kanagi paddy field soil of the teaching and research center for bio-coexistence at Hirosaki University.

Media used were the same media as the culture for the microorganisms described above. The bacterial growth was measured by optical density (400 nm). CH_4 and H_2, acetate and butyrate were measured with gas chromatography (2).

Photomicroscopy

Cells were observed with a photomicroscope. Phase contrast and UV-epifluorescence micrographs were taken using wet mount with an Olympus model BX50 photomicroscope. The fluorescence of cells was observed with a V (BP400-410) excitation filter.

Electron microscopy

For scanning electron microscopy, cells were collected on a filter paper and fixed in 1% glutaraldehyde in 0.01 M cacodylate buffer (pH 7.0) for 2 hr at room temperature and left overnight at 4°C. The filter paper was dehydrated, using a graded acetone series. The sample was subsequently dried to the critical point and then coated with gold for observation with a scanning electron microscope (JSM 5000/LV) operated at 20 kV.

Flagella and fimbriae were observed with a transmission electron microscope. Cells were negatively stained with 0.5% phosphotungstic acid (pH 7.0) for 30 seconds and observed with an electron microscope (JOEL model JEM-2000EX) operated at 80 kV.

For thin-section electron microscopy, cells were placed in a Kellenberger buffer containing 1% OsO_4 for prefixing cells. The cells were harvested by centrifugation for 15 min at 10,000 rpm and fixed in the same buffer for 2 hr at room temperature and then left overnight at 5°C. The fixed samples were rinsed three times in buffer, and twice in deionized water, and then soaked in 0.5% uranyl acetate in deionized water for 2 hr. The cells were embedded in small agar blocks, and dehydrated in increasing concentrations of ethanol, transferred to acetone, and infiltrated with Spurr's low viscosity resin. Thin sections were obtained with glass knives on an LKB2188 Ultrotome NOVA, and post-stained with uranyl acetate and lead citrate. Sections were examined with an electron microscope (JOEL model JEM-2000EX) operated at 80 kV.

Results and Discussion

Hydrogen-utilizing methanogens in paddy field soil

The number of hydrogen-utilizing methanogens in the Kanagi paddy soil was 6.7×10^5 MPN/g dry soil. *Methanobacterium formicicum* was the most abundant in hydrogen-utilizing methanogens isolated from the soil. Growth substrates of *Methanobacterium formicicum* strain TM-8 were hydrogen-carbon dioxide and formate. The cells were 2.0 to 6.6 μm in length and 0.5 to 1.0 μm in width as shown in Fig. 1-a. Though many cells were single cells, filament cells were also observed. The cells had the fluorescence of coenzyme F_{420} peculiar to methanogens (Fig. 1-b). The cells were Gram-negative, and spore was not formed. Though there was no motility on the strain, a large number of fimbriae were observed by the transmission electron microscope (Fig. 1-c and d). Fimbriae were 5 nm thick.

Methanobacterium bryantii strain H5-1 and *Methanospirillum* sp. strain TM20-1 were isolated in addition to *M. formicicum* as hydrogen-utilizing methanogens isolated from the Kanagi paddy field soil. These 2 strains are not numerically dominant hydrogen-methanogens, because they were isolated from the enrichment culture. *Methanobacterium bryantii* strain H5-1 was grown on hydrogen-carbon dioxide, but did not grow on formate and methanol. The strain was a non-motile, Gram-negative rod. The strain was different in Gram staining, because *Methanobacterium bryantii* is Gram-positive. Strain H5-1 was the same as *M. bryantii* when using a secondary alcohol such as isopropanol and isobutanol as the hydrogen donor. The cells were 2 to 7 μm in length and 0.5 to 0.8 μm in width as shown in Fig. 2-a. Though many cells were single cells, 10 to 15 μm of filament cells were also observed. The cells had the fluorescence of coenzyme F_{420} peculiar to methanogens (Fig. 2-b).

Methanospirillum sp. strain TM20-1 grew on hydrogen-carbon dioxide and formate as a growth substrate. Strain TM20-1 was a motile, Gram-negative spiral-shaped rod. As the methanogen grew in the isopropanol

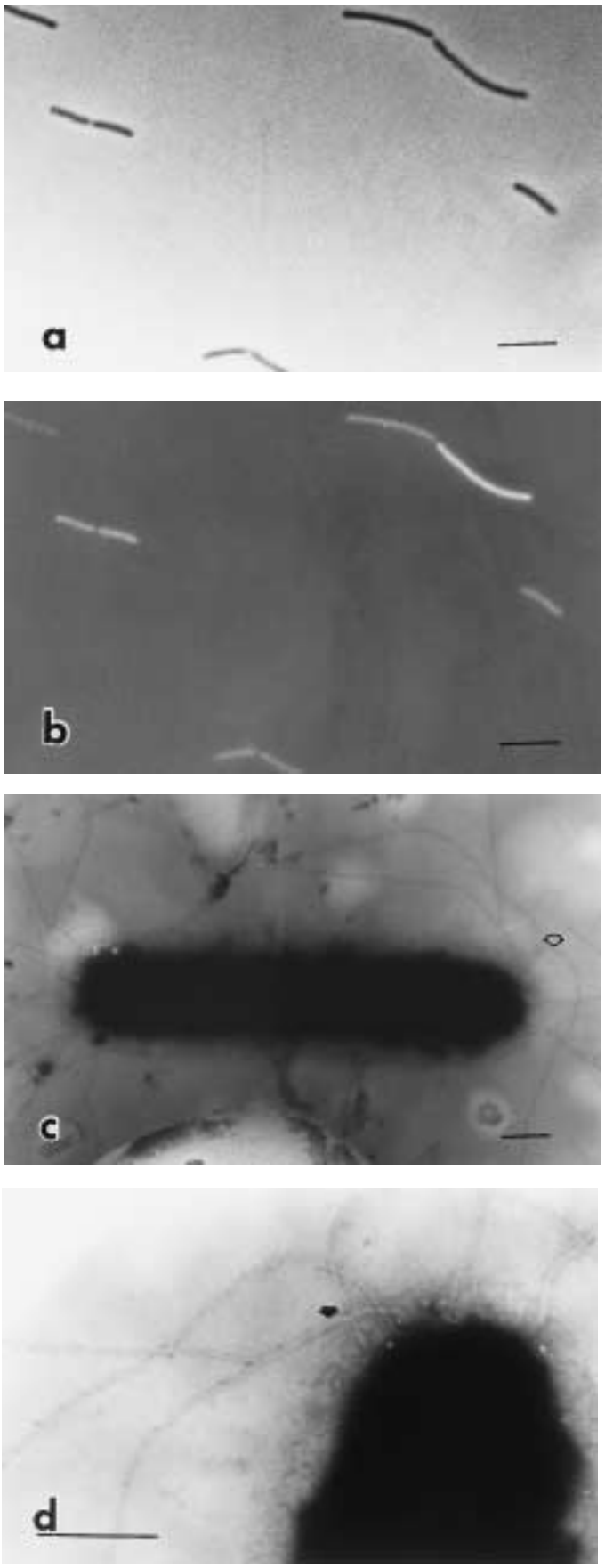

Fig. 1. Phase-contrast Photomicrograph (a), Epifluorescence Photomicrograph (b) and Transmission Electron Micrographs (c, d) of Hydrogen-utilizing methanogen, *Methanobacterium formicicum* strain TM-8 grown on H_2-CO_2. Arrows (c and d) showing fimbriae negatively stained with phosphotungstic acid. Bars (a and b) indicate 5μm, and c and d indicate 0.25μm.

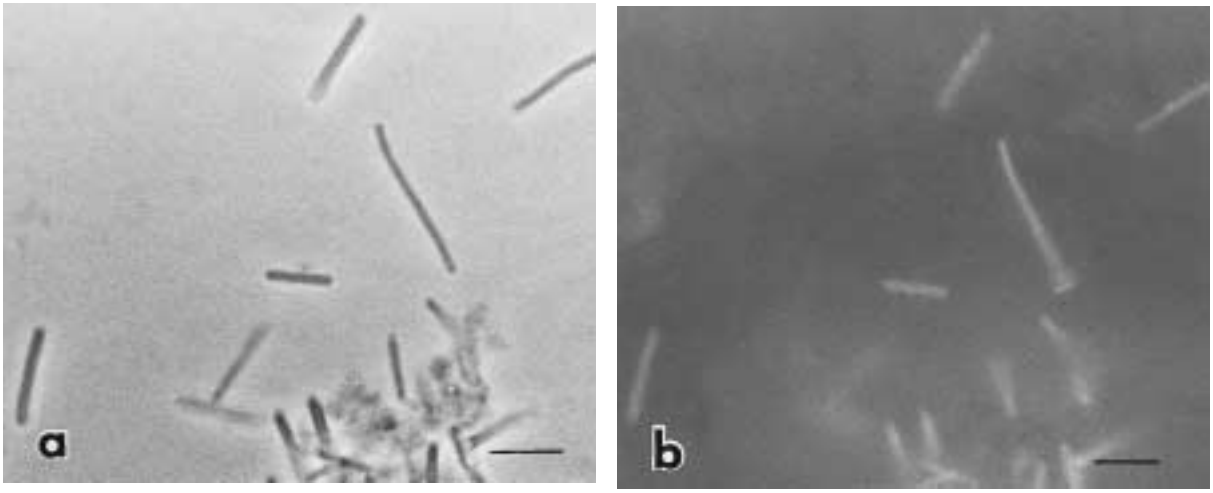

Fig. 2. Phase-contrast Photomicrograph (a) and Epifluorescence Photomicrograph (b) of *Methanobacterium bryantii* strain H-5 grown on H_2-CO_2. Bars indicate 5 μm.

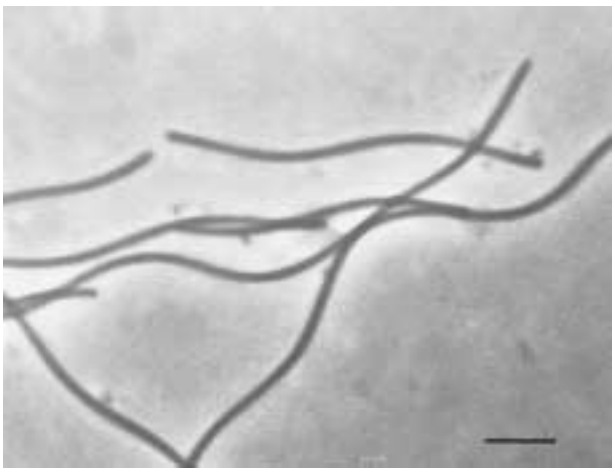

Fig. 3. Phase-contrast Photomicrograph of *Methanospirillum* sp. strain TM20-1 grown on H_2-CO_2. Bar indicates 5 μm.

-carbon dioxide medium, it was the same as *Methanospirillum bryantii* when using the secondary alcohol as the hydrogen donor. The cells were 2.0 to 7.0 μm in length and 0.4 μm in width as shown in Fig. 3. Though many cells were single cells, 13 μm of long spiral cells were observed by the fluorescence microscope.

Acetate-utilizing methanogens in paddy field soil

Acetate-utilizing methanogens were isolated in two genus of *Methanosarcina* and *Methanosaeta* from paddy field soil. *Methanosarcina* was isolated from the paddy soil of Italy by Fitzer et al. in 1993 (9). Asakawa et al. (10) isolated *Methanosarcina mazei* TMA from the paddy soil of a Kyushu agriculture laboratory and examined the microbial characteristics in 1995.

Methanosarcina strain 2-P and strain J-6 were isolated from the Kanagi paddy field soil. These methanogens were Gram-negative coccus. The cells were 1.5 to 2 μm in diameter, and both cells were single cells or aggregate cells (sarcina) as shown in Fig. 4-a and b (strain J-6) and c (strain 2-P). The aggregate cells were 50 to 60 μm. The cells had the fluorescence of coenzyme F_{420} peculiar to methanogens (Fig. 4-a and b). Strains 2-P and J-6 were methanogens, which could grow on hydrogen-carbon dioxide, trimethylamine and methanol as substrates in addition to acetate. The growth of both strains was not good for acetate. Trimethylamine, methanol and hydrogen-carbon dioxide were more suitable growth

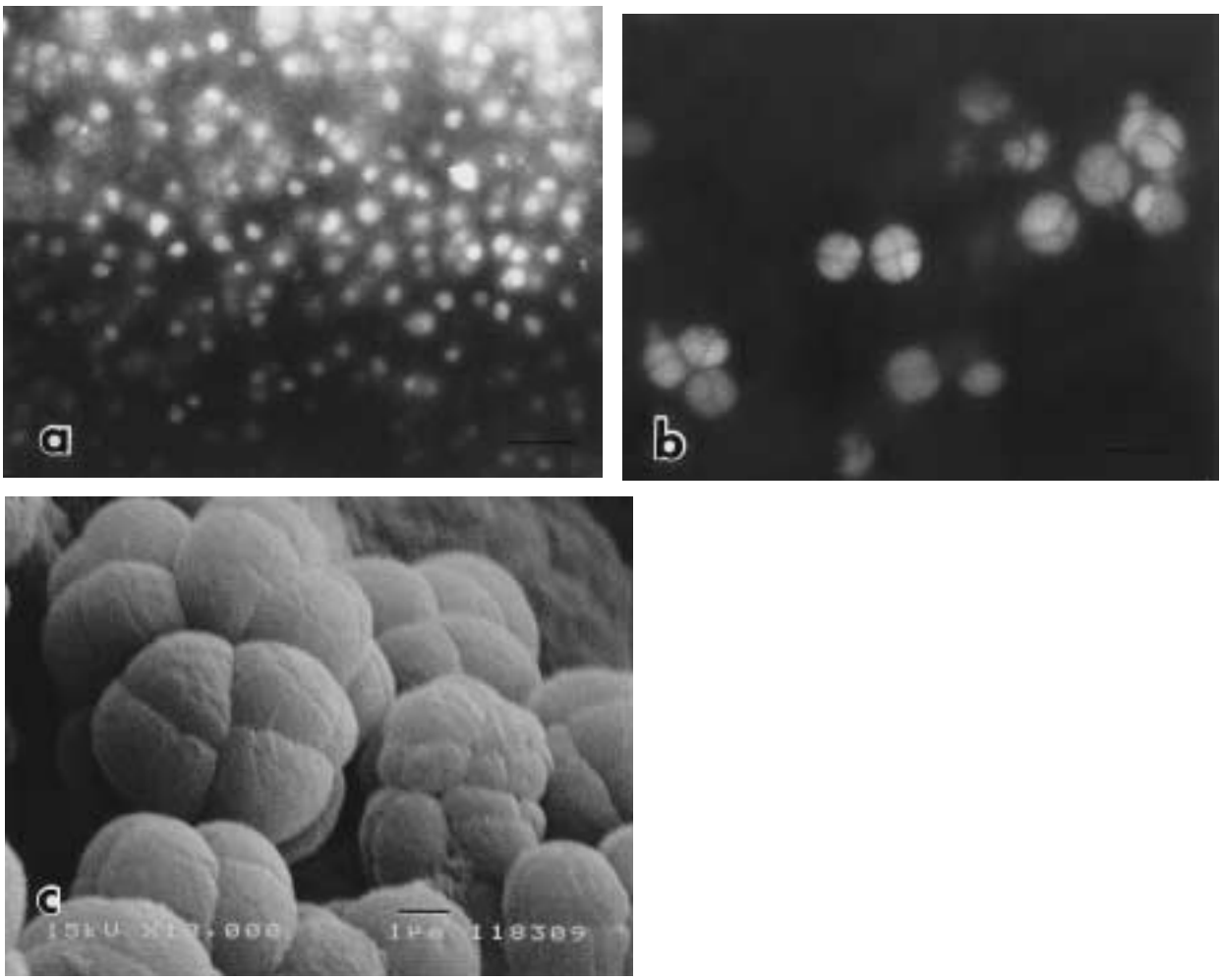

Fig. 4. Epifluorescence Photomicrographs (a and b) of *Methanosarcina* strain J-6 and Scanning Electron Micrograph (c) of *Methanosarcina* strain 2-P grown on acetate. b and c showing sarcina shaped cells. Bars (a and b) indicate 5μm and c indicates 1μm.

substrates than acetate.

Filamentous acetate-utilizing methanogens are dominant for methane fermentation sludges, and have been studied in detail. However, *Methanosaeta* was not isolated from paddy soil because it did not make a colony. *Methanosaeta* was isolated from the paddy soil of Italy by Groβkopf et al. in 1998 (11), but they obtained the pure culture by the dilution. Detailed archeal characteristics were not described, though they reported a 16S rRNA gene sequence and acetate utilization.

Mizukami et al. succeeded in producing the *Methanosaeta* colony (12). Since they isolated *Methanosaeta* from paddy field soils in each place in Japan, filamentous acetate-utilizing methanogens are considered to widely inhabit the paddy field. *Methanosaeta* sp. strain K-5 isolated from Kanagi paddy field soil was a Gram-negative, non-motile rod. The cells were 0.8 x 2.0 to 3.0μm as shown in Fig. 5-a and b. The cells had the weak fluorescence of coenzyme F_{420} peculiar to methanogens. The strain formed short filaments (3.0 to 15.7μm) that consisted of 2-7 rod-shaped cells in the sheath. A photograph of the expanded cell membrane and sheath is shown in Fig. 5-c. The sheath is shown on the outside of the cell membrane. The cell wall was not observed. The strain grew only on acetate as a substrate.

Syntrophic association with sulfate-reducing bacteria and methanogens

The number of sulfate-reducing bacteria in Kanagi paddy field soil was 2 x 10^5/g dry soil. The colonies were isolated from the high diluted tube after counting. *Desulfovibrio* sp. strain SK3-4 isolated

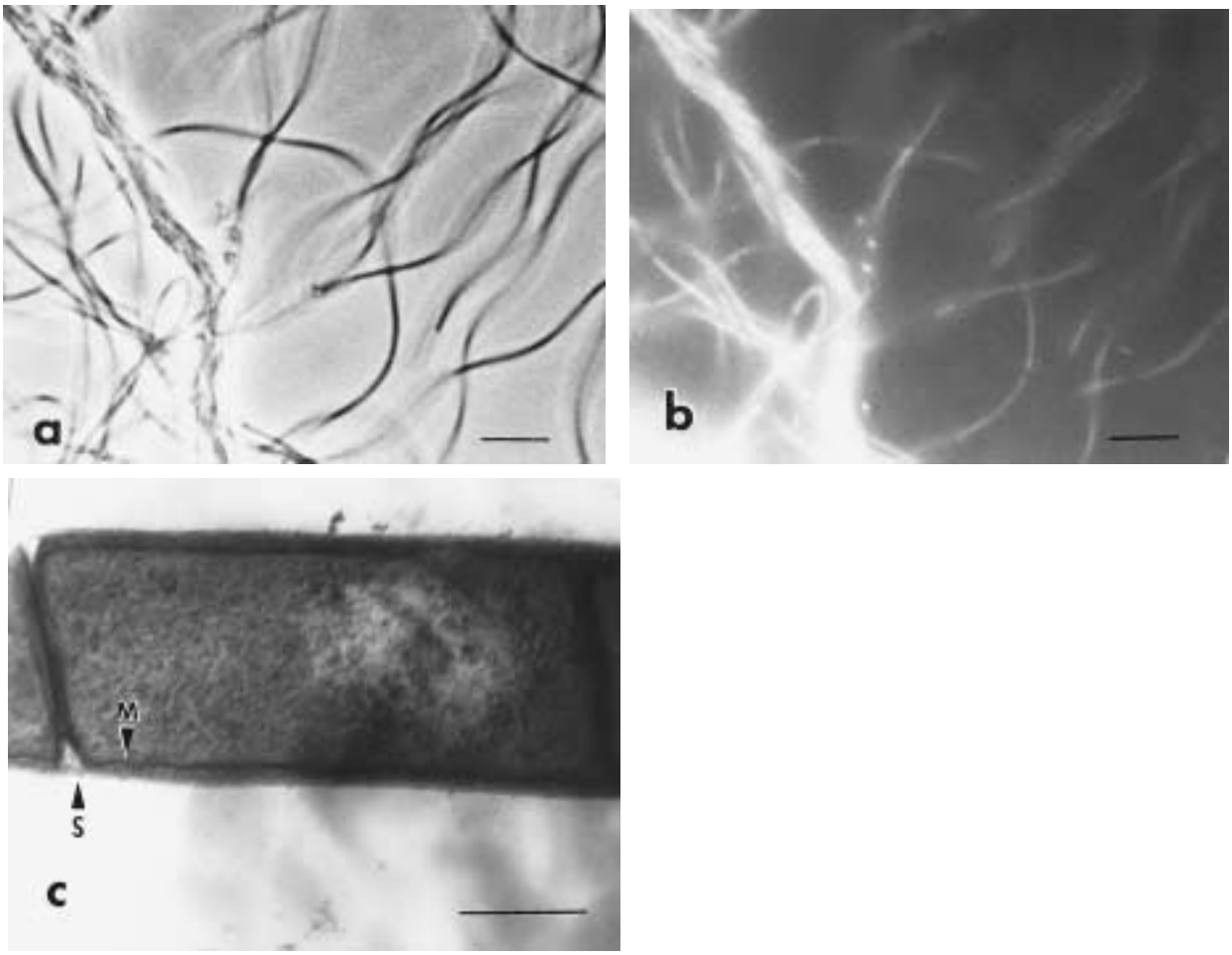

Fig. 5. Phase-contrast Photomicrograph (a), Epifluorescence Photomicrograph (b) and Transmission Electron Micrographs (c) of *Methanosaeta* strain K-5 grown on acetate. M and S showing cell membrane and sheath, respectively. Bars (a and b) indicate 5μm and c is 0.25μm.

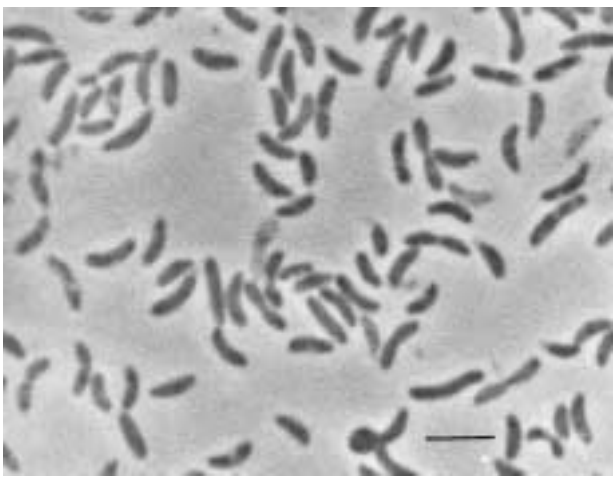

Fig. 6. Phase-contrast Photomicrograph of *Desulfovibrio* strain SK-3-4 grown on lactate medium containing sulfate. Bar indicates 5μm.

grew in sulfate medium containing lactate, or ethanol. *Desulfovibrio* sp. strain SK3-4 cells were 1.0 to 1.5 μm x 2.0 to 4.5 μm as shown in Fig. 6. The strain was a Gram-negative vibrio rod. Strain SK3-4 grew in lactate medium without sulfate when it was co-cultured with *Methanobacterium formicicum* strain TM-8, and produced acetate, carbon dioxide and methane. The lactate medium did not contain sulfate,

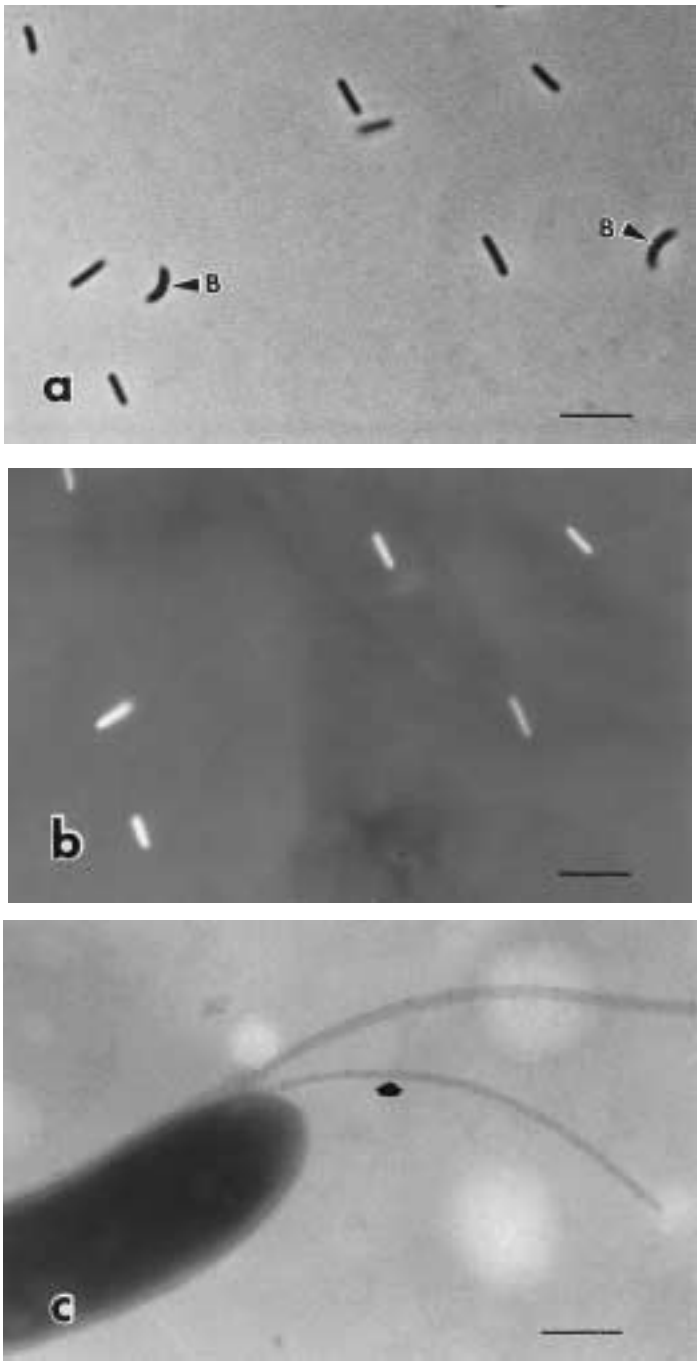

Fig. 7. Phase-contrast Photomicrograph (a) and Epifluorescence Photomicrograph (b) of *Syntrophomonas* strain TB-6 grown on butyrate in syntrophic association with *Methanobacterium formicicum* strain TM-8. B showing *Syntrophomonas* strain TB-6 cells. Transmission Electron Micrographs (c) of *Syntrophomonas* strain TB-6 grown on crotonate. Arrow showing two subpolar flagella. Bars (a and b) indicate 5μm and c indicates 0.25μm.

and hydrogen was not included in the atmosphere in the test tube. Therefore, neither strain could grow singly in the medium. Sulfate-reducing bacteria could grow on lactate in syntrophic association with a hydrogen-utilizing methanogen in the sulfate deficient paddy soil.

Syntrophic association with butyrate-degrading bacteria and a methanogen

The number of butyrate-degrading bacteria in Kanagi paddy field soil was 1.7 x 10^3 MPN/g dry soil. *Syntrophomonas* sp. strain TB-6 isolated from the Kanagi paddy soil degraded butyrate to acetate in co-

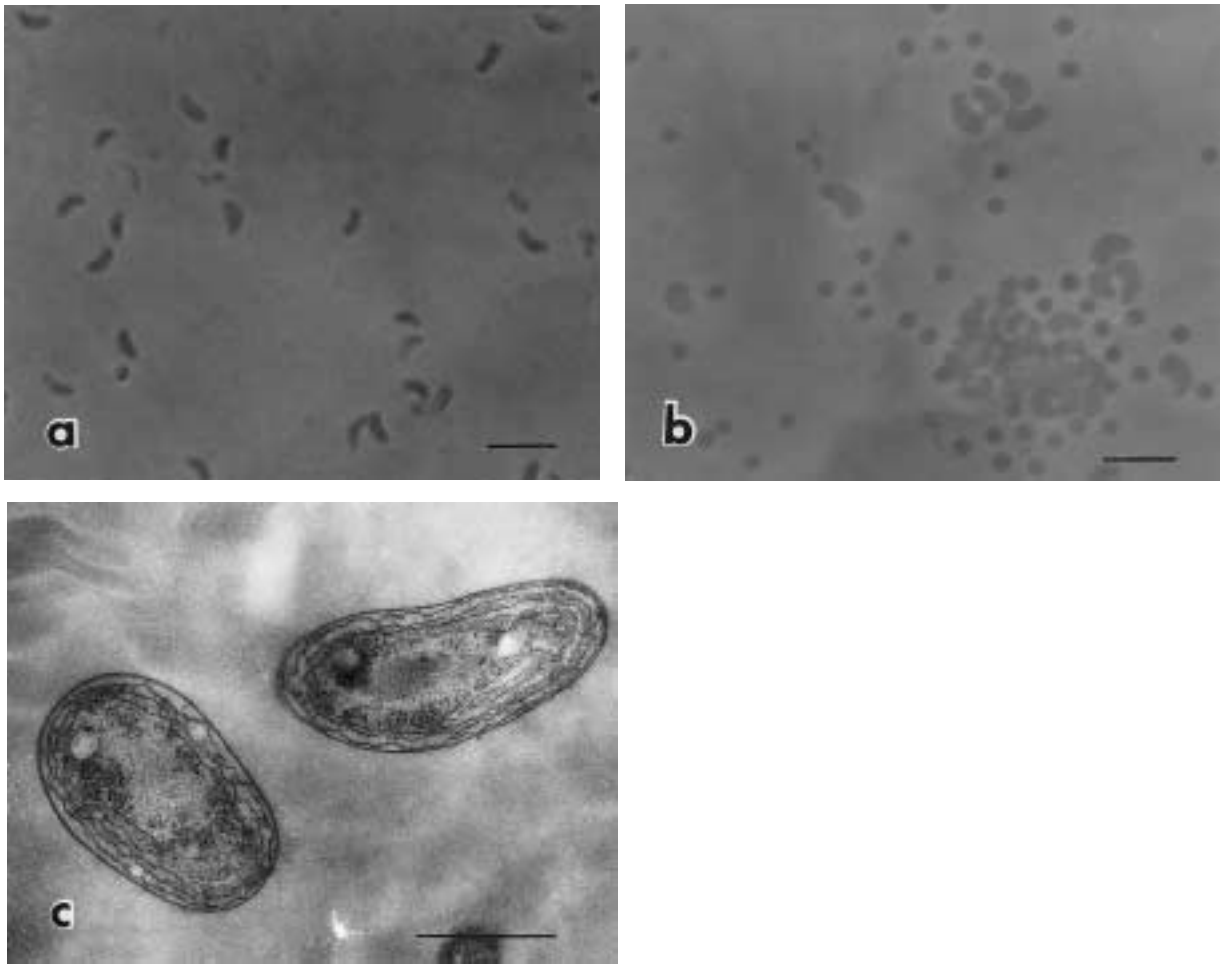

Fig. 8. Phase-contrast Photomicrographs of *Methylosinus* strains D2-11 (a) and strain W3-6 (b) grown on methane. Transmission Electron Micrograph (c) showing intracytoplasmic membranes. Bars (a and b) indicate 5μm and c indicates 0.5μm.

culture with *Methanobacterium formicicum* strain TM-8. A hydrogen-utilizing methanogen also played an important role in the degradation of butyrate. The photographs in Fig. 7-a and b show the co-culture of strain TB-6 and *Methanobacterium formicicum* strain TM-8. Strain TB-6 was a non-fluorescent curved rod. The cells were 0.8 to 1.0 x 2.0 to 4.5μm. The strain TB-6 cells were Gram-negative curved rods. Though the motility was not confirmed in the photomicroscopy, two polar or subpolar flagella (20 nm thick) were observed (Fig. 7-c). The strain could grow on valerate, hexanoate heptanoate and caprylate in addition to butyrate. Strain TB-6 was able to grow on crotonate without a syntrophic partner, *Methanobacterium formicicum* strain TM-8.

Methane-oxidizing bacteria in paddy field soil

Methane-oxidizing bacteria, which are strictly aerobes, live in flooded paddy soil. The number of methane-oxidizing bacteria in paddy field soil was 1.3×10^6MPN/g dry soil. The numbers were almost the same under conditions of flooded paddy soil and drainage paddy soil.

Methylosinus strain D2-11 (a) and *Methylosinus* strain W3-6 (b) isolated from the Kanagi paddy field soil were Gram-negative, oxidase positive and catalase positive rods. The strains were unique in cell morphology. The cells were curved rods and comma-shaped rods and globular exospores were formed. The cells were 2.0 to 3.0μm in length and 0.5μm in width as shown in Fig. 8-a and b. The methane-oxidizing bacteria possessed intracytoplasmic membranes along the cell periphery as shown in Fig. 7-c. The membrane system of both strains belongs to Type II. The intracytoplasmic membranes were composed

of a paired membrane with a total thickness of 20 to 25 nm; two unit membranes 7 nm thick were separated by an interspace of 10 nm. The strains grew on methane and methanol as sources of carbon and energy, and grew irrespective of the presence or absence of copper.

Summary

The shape, size, flagella and intracytoplasmic membranes of the microbial cells involved in methane production and oxidation in paddy field soil were observed using a phase-contrast microscope, a fluorescence microscope and a scanning and transmission electron microscope. *Methanobacterium formicicum* was the numerically dominant hydrogen-utilizing methanogen in paddy field soil. The methanogens possessed 7 to 12 polar fimbriae. When fatty acid-degrading bacteria converted fatty acids to acetate, *Methanobacterium formicicum* played an important role as a syntrophic partner. In sulfate deficient conditions, sulfate-reducing bacteria degraded lactate to acetate in syntrophic association with *M. formicicum*. *M. formicicum* promotes anaerobic degradation of organic matter in paddy soil by removing the hydrogen and formate. When paddy soil was incubated with an acetate medium, a large number of *Methanosarcina* was observed. However, acetate was not a good growth substrate for *Methanosarcina*. Seventy percent of methane production in paddy fields is made from acetate. Whether either species of *Methanosarcina* and *Methanosaeta* produce methane from acetate in the paddy field is an item for future study. Different shaped cells of methane-oxidizing bacteria, *Methylosinus*, were isolated from the Kanagi paddy field soil. These bacteria possessed the same intracytoplasmic membrane system as Type II. Type II methane-oxidizing bacteria is dominant in paddy field soil.

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水田土壌に生息するメタン生成・酸化に関わる微生物の顕微鏡像

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水田のメタン生成・酸化に関わる微生物細胞の形態，大きさ，蛍光，鞭毛，線毛や細胞内膜組織を位相差顕微鏡，落射蛍光顕微鏡，走査型電子顕微鏡と透過型電子顕微鏡を使って明らかにした。水田の水素利用メタン生成菌は3菌株分離された。*Methanobacterium* の2菌株は棒状桿菌，*Methanospirillum* はらせん状桿菌であった。いずれの菌株もメタン生成菌特有の F_{420} の蛍光を有した。*Methanobacterium formicicum* は水田に生息する水素利用メタン生成菌の中で菌数が最も多かった。このメタン生成菌は細胞両末端に7～12本の線毛を持っていた。*M. formicicum* は脂肪酸を嫌気的に分解する際に脂肪酸分解細菌の栄養共生のパートナーとしての役割を演じた。水田にはビブリオ状の *Desulfovibrio* が多数生息する。*Desulfovibrio* は硫酸塩のない所で *M. formicicum* と栄養共生により乳酸を酢酸に分解した。*M. formicicum* は水素と蟻酸を除去することにより水田の有機物の嫌気分解を促進していると考えられる。球状細胞の *Methanosarcina* と糸状細胞の *Methanosaeta* は水田の酢酸利用メタン生成菌である。これらいずれも F_{420} の蛍光を有した。酢酸で水田土壌を培養すると前者が多く観察される。しかし水田から分離された *Methanosarcina* J-6 菌株と 2-P 菌株の増殖に対して酢酸は良い基質ではなかった。他方，*Methanosaeta* は酢酸が唯一の増殖基質であった。水田から放出されるメタンの70%が酢酸由来であると言われているが，*Methanosarcina* と *Methanosaeta* のどちらの酢酸利用メタン生成菌が主要な酢酸からのメタン生成菌かは今後に残された課題である。水田には絶対好気性細菌であるメタン酸化細菌が生息している。分離された *Methylosinus* D2-11 菌株と W3-6 菌株は細胞内膜構造から Type Ⅱに属するメタン酸化細菌であった。これらの菌株は増殖に銅を要求しなかった。水田には銅を増殖に要求する Type Ⅰの菌種よりは Type Ⅱの菌種が優占することを示唆している。

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