한국환경위생학회지, 제26권 제4호(2000)
Kor. J. Env. Hlth. Soc., Vol. 26, No. 4, pp 65~74(2000)

Effect of Cd^{2+} and Cu^{2+} on the Growth of a Methanogen and a Sulfate-Reducing Bacterium isolated from sea-based landfill

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(Received 5 November 2000 ; Accepted 8 December 2000)

해안폐기물매립지로부터 분리한 메탄생성균과 황산염 환원균의 Cd^{2+} 및 Cu^{2+}에 대한 감수성 검토

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ABSTRACT

The sensitivity of a methanogen and sulfate-reducing bacterium isolated from a sea-based landfill site to Cd^{2+} and Cu^{2+} was studied. Methanogens and sulfate-reducing bacteria in leachates of the waste disposal site were enumerated using the MPN method. *Methanobacterium thermoautotrophicum* KHT, isolated from the leachate, could not grow at 0.5 mM Cd^{2+} or 1.0 mM Cu^{2+}. *Desulfotomaculum* sp. RHT, isolated from the same leachate, was able to insolubilize 3.0 mM Cd^{2+} or 2.0 mM Cu^{2+} by production of hydrogen sulfide. When strains KHT and RHT were cultured together in the presence of the heavy metals, strain KHT could grow at high heavy metal concentrations after insolubilization of the metals by strain RHT.

Keywords : *Methanobacterium thermoautotrophicum*, *Desulfotomaculum* sp., waste disposal site, heavy metal

요 약

해안폐기물매립지에서 분리한 메탄생성균과 황산염환원균의 Cd^{2+} 및 Cu^{2+}에 대한 감수성을 검토했다. 대상으로 한 매립지는 해상매립지로서 그 침출수중의 메탄생성균과 황산염환원균의 존재를 MPN법에 의해 확인하였다. 매립지 침출수로부터 분리한 Methanobactacterium *thermoautotrophicum* KHT는 0.5 mM의 Cd^{2+} 및 Cu^{2+}의 존재하에서는 생육이 불가능하였다. 한편 분리한 *Desulfotomaculum* sp. RHT는 생성하는 황화수소의 작용으로 3.0 mM의 Cd^{2+} 또는 2.0 mM의 Cu^{2+}를 불용화하였다. KHT균주가 증식억제를 받는 중금속농도하에서 RHT균주와 함께 배양하면 RHT균주에 의한 중금속의 불용화 후 KHT 균주는 생육가능하였다.

I. Introduction

Landfill is the traditional disposal method in waste management and is widely used all over the world due to its high degree of efficiency and simplicity. With consideration given to the safety of environments, suitable sites are selected by local governments for the purpose of waste dumping. If landfill sites are properly managed, the sites could be used as parking places or public spaces for building. Although the construction and dumping methods, as well as the controlling and monitoring, of landfill sites have been studied in detail and are well developed, there are few studies on microorganisms for the stabilization of wastes. Moreover, mechanisms for the decomposition of

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organics and chemicals as well as the fate of metals in wastes have not been elucidated yet.

A large quantity of waste containing metals dumped in landfill sites,[1,2] for example an average 6.5% of waste is metal as determined over for 13 years for municipal solid wastes,[3] and there is 0.01% cadmium, 0.1-0.68% copper and 0.45-0.96% zinc in incineration ash.[4] Leaching of heavy metals from incineration ash depends on the characteristics of the ash and environmental conditions, *i.e.*, pH and temperature. The chemical form of metals in dumped wastes and in landfill sites is not clear.

The emission of methane, a well-known phenomenon in landfill sites, is the result of reaction between methanogens and organics in that site.[5,6] Methanogens play an important role in the degradation of organics, but this group is generally sensitive to heavy metals. In fact, only *Methanobacterium bryantii* BKYH, a copper-resistant methanogen has been reported.[7] According to Jarrell and Saulnier,[8] zinc and copper have a strong inhibitory effect on methanogenesis (*Methanobacterium thermoautotrophicum* Marburg, *Methanobacterium formicicum*, *Methanospirillum humgatei* JF1 and *Methanosaricna barkeri* MS) at 1-10 m*l*/*l*. Although a large amount of waste containing heavy metals is dumped in landfill sites, methanogenesis in the sites increases continually up to approximately the 7th year from the start of dumping and reduces gradually thereafter.[9]

Sulfate-reducing bacteria, which inhabit in the same environment as methanogens, are widely distributed,[10,11] and produce hydrogen sulfide in waste disposal sites.[12] Experimentally, it has been reported that sulfate-reducing bacteria insolubilize the heavy metal ions in leachates by the production of hydrogen sulfide thus contributing to the stabilization of metals.[11] It has also been reported that the number of sulfate-reducing bacteria is inversely proportional to the copper concentration in leachates.[10]

These findings suggest that hydrogen sulfide produced by sulfate-reducing bacteria makes heavy metals insoluble, and consequently reduces their toxicity to methanogens which can them produce. In this study, we isolated a methanogen and a sulfate-reducing bacterium from a waste disposal site and examined the effects of heavy metals on the activities of these two bacteria. Furthermore, when these strains were cocultured, the effect on methanogenesis in the presence of heavy metals was investigated.

The purpose of the above works are to investigate the effect of heavy metals on methanogen and Sulfate-reducing bacterium which are widely distributed in landfill at the same time and find out the relationship among heavy metals, methanogen and sulfate-reducing bacterium under the actual landfill environment where wastes containing heavy metals have been disposed.

II. Materials And Methods

1. Sampling, enumeration of methanogens and sulfate-reducing bacteria, and analysis of water

Leachate samples were taken from five pipes used for the monitoring of sinkage and water quality at a sea-based solid waste disposal site in the port of Osaka in Japan. The site was located in the sea and was an artificial island.[13,14] The site was being filled with waste mainly consisting of incineration ash and methane was generated from gas-exhaust pipes at the time of sampling in July, 1996.

Methanogens and sulfate-reducing bacteria in the leachate were measured using the MPN method. One milliliter of leachate of which initial pH was 7.5, diluted serially, was cultivated at 50°C using autoclaved medium A containing sodium formate (5 g/*l*), sodium acetate (5 g/*l*) and methanol (5 m*l*/*l*) as growth substrates and medium B for the determination of methanogens and sulfate-reducing bacteria, respectively. The media were prepared according to the method described by Sowers and Schreier.[15] Medium A contained (/*l*): 0.75 g K_2HPO_4, 0.75 g KH_2PO_4, 0.90 g NH_4Cl, 0.36 g $MgCl_2 \cdot 6H_2O$, 20.0 g NaCl, 2.0 g yeast extract (Oriental Yeast Co. Ltd., Tokyo), 2.0 g trypticase peptone (BBL, Maryland, USA), 0.001 g resazurin, 10 m*l* trace elements solution[16] and 10 m*l* DSM 141 vitamin solution.[15] Cultivation was performed using 20-m*l* vials sealed with butyl-rubber stoppers and aluminum caps. Twenty-m*l* vials containing 10 m*l* of medium A were flushed with N_2 (101.29 kPa) and observed at 70°C. Under this condition, the serial dilution method for the isolation was attempted in the presence of ampicillin (1000 μg/m*l*) and streptomycin (100 μg/m*l*).[19] Of the serial dilution vials, the culture receiving 10^{-3} exhibited methanogenesis and this culture was subsequently diluted serially in medium A. The purity of the isolate was verified by microscopic observation of cultures in medium

A containing 10 mM glucose or 20 mM lactate + 10 mM Na_2SO_4 at 37, 50 and 70°C. The following compounds, H_2/CO_2, sodium formate (20 mM), sodium acetate (20 mM) and methanol (20 mM) were evaluated as growth substrates. Growth was examined at temperatures ranging from 37 to 75°C, and pHs ranging from 6 to 9 in medium A containing 5 g/*l* sodium formate.

2. Isolation of sulfate-reducing bacterium

One milliliter of leachate was inoculated into liquid medium B containing 0.02% $FeSO_4 \cdot 7H_2O$ as a primary enrichment for sulfate-reducing bacteria. After 3 d of incubation at 50°C, we observed precipitation of FeS. The culture receiving 10^{-5}-10^{-6} showed colony formation in medium B containing 1.5% agar and 0.02% $FeSO_4 \cdot 7H_2O$ in a Weinberg tube (6 mm in diameter, 300 mm in length)[17] after 3 d, and a colony was transferred to liquid medium B. For examination of the physiological characteristics, H_2/CO_2, sodium formate (20 mM), sodium acetate (20 mM), sodium pyruvate (20 mM), sodium malate (20 mM), sodium lactate (20 mM), methanol (20 mM) and ethanol (20 mM) were evaluated as electron donors using medium B not containing sodium lactate. Sodium sulfate (20 mM), sodium sulfite (20 mM), sodium thiosulfate (20 mM), sodium nitrate (20 mM) autoclaved at 121°C, 1 kg/m^3 for 20 min. Before inoculation, the pH was adjusted with 10% (w/v) Na_2CO_3 solution, and 0.50 g g/*l* $Na_2S \cdot 9H_2O$ and 0.50 g/*l* cysteine · HCl (final concentration) were added as reductants. Methane production of serially diluted leachate after 4 weeks of cultivation was measured for the detection of methanogens. Medium B contained (/*l*): 0.4 g KH_2PO_4, 1.0 g K_2HPO_4, 2.8 g Na_2SO_4, 2.2 g sodium lactate, 0.1 g $MgCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 2.0 g yeast extract, 10 m*l* trace elements solution[16] and 10 m*l* DSM 141 vitamin solution.[15] Before cultivation, 0.02 g/*l* ascorbic acid (final concentration) was added as a reductant. Culture conditions were the same as described above. In the MPN procedure, 0.02% $FeSO_4 \cdot 7H_2O$ was added to medium B. The precipitation of the FeS after 1 week of cultivation, as a result of the production of H_2S from $FeSO_4 \cdot 7H_2O$, was used for the detection of sulfate-reducing bacteria.[17]

The pH was measured using a pH meter B-212 (Horiba, Kyoto) and the COD was determined by the standard method.[18]

3. Isolation of methanogen

The same vials and anaerobic techniques described above were used. One milliliter of leachate from one pipe was inoculated into medium A containing sodium formate (5 g/*l*), sodium acetate (5 g/*l*) and methanol (5 m*l*/*l*) as a primary enrichment for methanogens. After incubation for 4 weeks at 50 and 70°C, methanogenesis occurred. We then determined the optimum conditions for the isolation of methanogen. When formate (5.0 g/*l*) was used as the sole substrate, significant growth of rod-shaped microbes exhibiting autofluorescence was eventually and sodium nitrite (20 mM) were evaluated as electron acceptors using medium B not containing sodium sulfate. Growth was examined at temperatures ranging from 30 to 70°C, and pHs ranging from 5 to 9 in medium B.

4. Microscopy

An Olympus BHS microscope equipped with a UV lamp was used to determine the purity of isolates and for routine observation.

5. 16S rRNA gene analysis of the isolates

Cells were harvested from 500 m*l* of culture by centrifugation at 4°C. The cell pellet was suspended in Tris-EDTA buffer (pH 8.0), and extraction and purification of the DNA were carried out according to the methods of Marmur[20] and Wang-Iverso and Bonit.[21] The 16S rRNA gene of the methanogen was amplified using the following primers: forward primer 5'-CGTTTGATCCTGGCGGAGG -3' (*E. coli* positions, 10-28) and reverse primer 5'-ACGGGCGGTGTGTRC-3' (*E. coli* positions, 1392-1378).[15,22] The 16S rRNA gene of the sulfate-reducing bacterium was amplified using the following primers: forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' (*E. coli* positions, 8-27) and the same reverse primer used for the methanogen (15, 23). The reaction mixture (100 μ*l*) contained: 10 pmol of each primer, 250 μmol of each dNTP, PCR buffer containing 15 mM $MgCl_2$ and 1.25 U AmpliTaq Gold™ (Applied Biosystems, Foster City, USA). PCR was performed as follows: 95°C for 9 min, followed by 35 cycles consisting of 95°C for 1 min, 50°C for 1 min and 72°C for 2 min. The PCR products were sequenced using an ABI 310 automated sequencer (Applied Biosystems). Four primers (the two PCR primers, 5'-CAGCCTCTCCCGACCTC-3' and 5'-ATAAGGGTTCC-

GCTGGTAAT-3') were used for sequencing the methanogen 16S rRNA gene and three primers (the two PCR primers and 5'-GCAGCAGTGGGGAATCTT-3') were used for sequencing the sulfate-reducing bacterium 16S rRNA gene. Approximately 500 bp of the 16S rRNA genes were initially sequenced using the PCR primers. Finally, 1327 and 1348 bp of the 16S rRNA genes of the methanogen and sulfate-reducing bacterium, respectively, were sequenced by primer walking. Sequences were compared using the BLAST program of the National Center for Biotechnology Information (NCBI). An evolutionary tree was constructed and sequence similarities were calculated using the Clustal W program and the Tree View package.[24,25]

5. Effect of heavy metals on the growth of isolates

Medium A (pH 7.0) containing 5.0 g/*l* sodium formate was used for the growth of methanogen, and incubation was at 60°C. $Na_2S \cdot 9H_2O$ was not added to medium A to avoid its reaction with metals, and cysteine Cl was the sole reductant. There was no effect on the growth of the methanogen due to a lack of $Na_2S \cdot 9H_2O$. To determine heavy metal sensitivity, one milliliter of a culture of the methanogen, which was grown to exponential phase in medium A, was transferred to 20-m*l* vials containing 10 m*l* medium supplemented with 0-1 mM $CdCl_2$ or $CuSO_4$. Methane production was measured to determine the sensitivity. Medium B (pH 7.0) was used for the growth of the sulfate-reducing bacterium and incubation was at 50°C. In the experiment to determine heavy metal sensitivity, to prevent carryover of HS^- from the initial culture, the cells were harvested by centrifugation (15000 rpm, 15 min and 4°C) and suspended in the same volume of fresh medium B. One milliliter of suspension was transferred to 20-m*l* vials containing 10 m*l* medium supplemented with 0-5 mM $CdCl_2$ or $CuSO_4$. The concentration of heavy metal ions was measured to determine the degree of insolubilization by the sulfate-reducing bacterium.

6. Methane production profiles of sulfate-reducing bacterium in the presence of heavy metals

Coculture of the sulfate-reducing bacterium and methanogen was performed in medium C which had the following composition (/*l*): 0.4 g KH_2PO_4, 1.0 g K_2HPO_4, 0.36 g $MgCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.9 g NH_4Cl, 2.8 g Na_2SO_4, 3 g sodium formate, 1.1 g sodium lactate, 2.0 g yeast extract, 2.0 g trypticase peptone, 0.001 g resazurin, 10 m*l* trace elements solution[16] and 10 m*l* DSM 141 vitamin solution.[15] Cultivation was performed at 60°C using autoclaved 120-m*l* vials sealed with butyl-rubber stoppers and aluminum caps containing 80 m*l* of medium C flushed with N_2 (101.29 kPa). The initial pH was adjusted to 7.0 with 10% Na_2CO_3 solution, and 0.5 g/*l* cysteine Cl was added before inoculation. Eight milliliters of each culture of the methanogen and sulfate-reducing bacterium were inoculated simultaneously into medium C containing Cd^{2+} or Cu^{2+}. Prior to inoculation, the sulfate-reducing bacterium was washed as described above. Methane and hydrogen sulfide production, and concentration of heavy metal ions were measured.

7. Analytical method

Methane in the gas phase was analyzed by gas chromatography using a Shimadzu GC-4C equipped with a Unibeads C60/80 column (Shimadzu, Kyoto) and a thermal conductivity detector.[26] Hydrogen sulfide in the gas phase was analyzed by gas chromatography using a Shimadzu GC-9A equipped with a 1.2.3.Tris(2-cyanoethoxy) propane column (Shimadzu) and a flame photometric detector. The temperatures of the oven, injection port, and detector were 70, 140 and 140°C, respectively. Nitrogen was used as a carrier gas at a flow rate of 65 m*l* min^{-1}.

Metal ions in the liquid medium were measured using an atomic absorption spectrophotometer AA-640-12 (Shimadzu).[18] In order to remove the sulfide precipitate, the liquid sample was passed through a cellulose acetate membrane (pore size 0.22 μm; Adovantec, Tokyo).

Optical density was measured at 660 nm using 20-m*l* vials that fit in a UV-1200 spectrophotometer (Shimadzu).

III. Results and Discussion

1. Leachate qualities and presence of methanogens and sulfate-reducing bacteria

We analyzed the leachate from 5 pipes at a waste disposal site in Osaka. In situ temperatures were 43-57°C and the pH was approximately 8.0. The CODs were 236-916 mg/*l*. Methanogens and sulfate-reducing bacteria in all the leachate samples were counted by the MPN method. The

numbers of methanogens and sulfate-reducing bacteria were in the range of 12-1100> MPN m*l* and 210-2100 MPN m*l*, respectively. Methanogens and sulfate-reducing bacteria were widely distributed in the site.

2. Isolation and identification of a methanogen

After the serial dilution experiment was repeated ten times, a thermophilic methanogen, designated strain KHT was isolated. No contamination was observed under a microscope in the purity check.

The cells of strain KHT were rod-shaped, 3.0 to 4.0 μm by 0.5 μm (Fig. 1 A), and used H_2/CO_2 and formate as carbon sources. The ranges of temperature and initial pH for growth were 50-70°C and 7.0-8.5, respectively. Almost the entire 16S rRNA gene (1327 bases) was sequenced for strain KHT. Phylogenetic analysis showed that the sequence was the same as that of *M. thermoautotrophicum* HN4, which was previously identified as *M. thermoformicicum* HN4 (sequence similarity 100%).[27,28] According to the characterization and 16S rDNA analysis results, strain KHT was identified as *M. thermoautotrophicum*.

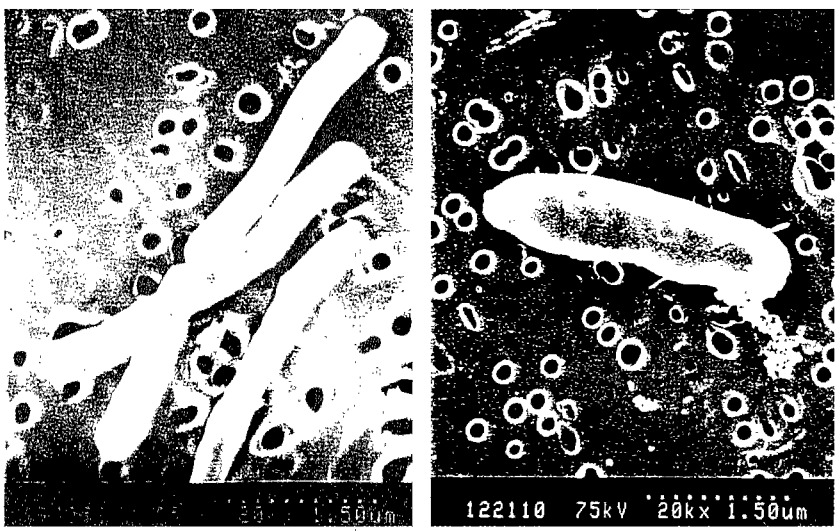
3. Isolation and identification of a sulfate-reducing bacterium

After five repetitions of the isolation procedure, the bacterial cells were morphologically uniform and a thermophilic sulfate-reducing bacterium, designated strain RHT was isolated.

The cells of strain RHT were rod-shaped, 2.3 to 3.3 μm by 0.8 to 1.0 μm(Fig. 1 B) and spore-forming. The isolate used H_2/CO_2, formate (poorly utilized), pyruvate and lactate as electron donors, and SO_4^{2-}, SO_3^{2-} and $S_2O_3^-$ as electron acceptors. The ranges of temperature and initial pH for growth were 45-60°C and 6.0-7.5, respectively. Almost the entire 16S rRNA gene (1348 bases) was sequenced for strain RHT. Phylogenetic analysis revealed that strain RHT belongs to a clade of the genus *Desulfotomaculum*, and sequence similarities to *D. nigrificans*, *D. aeronauticum* and *D. putei* were 96.6, 95.3 and 95.0%, respectively. The characteristics of these species are not the same as those of strain RHT. *D. aeronauticum* is mesophilic and the optimum growth temperature is 37°C[29] and *D. nigrificans* and *D. putei* are capable of using ethanol as an electron donor.[30,31] Therefore, we designated the isolate *Desulfotomaculum* sp. RHT.

4. Effect of heavy metals on *M. thermoautotrophicum* KHT

The growth curve of strain KHT measured by optical density was similar to the methane production profile (Fig. 2). Hence, we used methane production as a growth



(A) (B)

Fig. 1. Scanning electron micrograph of *M. thermoautotrophicum* KHT(A) and *Desulfotomaculum* sp. RHT(B) isolated form sea-based landfill.

indicator thereafter.

The effects of Cd^{2+} and Cu^{2+} on the growth of strain KHT are presented in Figs. 3 (A) and (B), respectively. For 0.1 mM or less Cd^{2+} or Cu^{2+}, methane production was not inhibited. In the presence of 0.5 mM or more of Cd^{2+} or Cu^{2+}, methane production was strongly inhibited. Kim *et al.* reported that *Methanobacterium* species were significantly affected by 0.2 to 0.5 mM Cu^{2+}, except for *Methanobacterium bryantii* BKYH which is a copper-resistant methanogen, and *M. thermoautotrophicum* Marburg and ΔH could not grow at concentration of Cu^{2+} higher than 0.2 mM.[7] Some bacteria can tolerate high levels of Cu (2.5-12.5 mM), *e.g. Escherichia coli*, *Proteus vulgaris* and *Pseudomonas syringae*.[32,33] Although Cu^{2+} is required as a trace element for many organisms, it can exert an inhibitory effect on bacterial growth at relatively low concentrations. For example, 0.4 mM Cu^{2+} inhibits the respiratory chain of *E. coli*.[34] Cadmium is also known to be toxic to microorganisms, but some bacteria are tolerant to Cd^{2+}[35] such as *Pseudomonas putida* GAM-1 which is able to grow at 7 mM Cd^{2+}.[36] Hence, it is concluded that *M. thermoautotrophicum* KHT is sensitive to Cd^{2+} and Cu^{2+}.

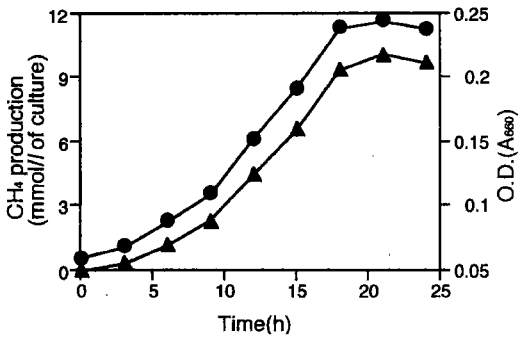


Fig. 2. The growth curve of *M. thermoautotrophicum* KHT. Medium A(see text) containing 5 g/l formate was used for cultivation at 60°C and pH 7.5. Symbols: optical density(●) and CH_4 production(▲).

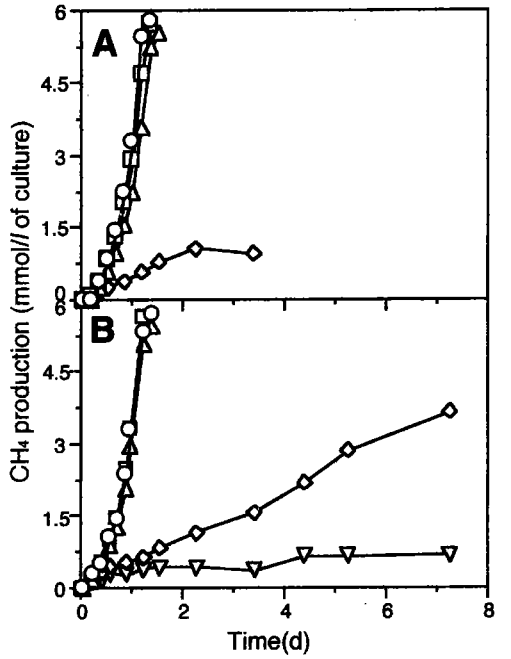


Fig. 3. Effect of Cd^{2+} (A) and Cu^{2+} (B) on the methane production of *M. thermoautotrophicum* KHT. Medium A (see text) containing 5 g/l formate was used for cultivation at 60°C and pH 7.5. Symbols: CH_4 production under 0 mM (○), 0.05 mM (□), 0.1 mM (△), 0.5 mM (◇) and 1.0 mM (▽) of heavy metal.

5. Insolubilization of heavy metals by *Desulfotomaculum* sp. RHT

The growth profile and hydrogen sulfide emitted in the gas phase by strain RHT are shown in Fig. 4. Hydrogen sulfide production was correlated with the growth of strain RHT. To determine the sensitivity and degree of insolubilization of heavy metals, strain RHT was cultured in the presence of Cd^{2+} or Cu^{2+}. The time courses of heavy metal insolubilization at various concentrations of Cd^{2+} and Cu^{2+} are shown in Figs. 5 (A) and (B), respectively. When high concentrations of heavy metals were added to the medium before cultivation, precipitation occurred due

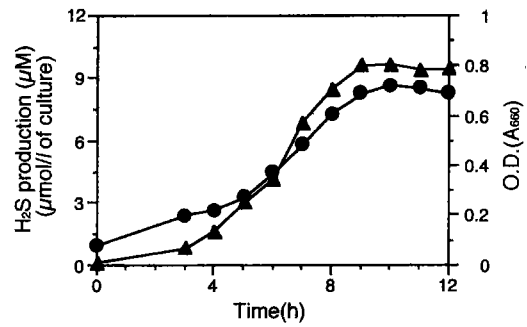


Fig. 4. The growth curve of *Desulfotomaculum* sp. RHT. Medium B (see text) was used for cultivation at 50°C and pH 7.0. Symbols: optical density (●) and H_2S production (▲).

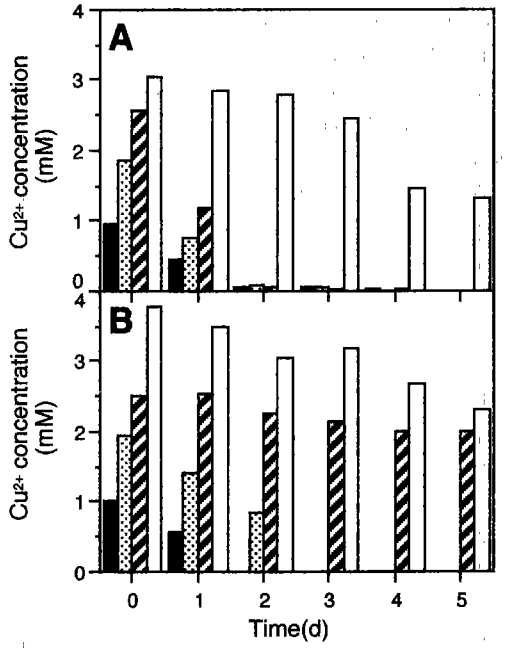


Fig. 5. Heavy metal insolubilization by *Desulfotomaculum* sp. RHT. Medium B (see text) containing Cd^{2+} (A) or Cu^{2+} (B) was used for cultivation at 50°C and pH 7.0. Symbols: heavy metal ions in the medium at 1 mM (■), 2 mM (□), 3 mM (▨) and 5 mM (□) as initial concentrations.

to chemical reaction. Hence, we could not precisely evaluate the degree of insolubilization at concentrations higher than 3 mM. When strain RHT was inoculated into the medium and cultured, precipitation of heavy metals as a result of CdS and CuS formation was observed, and for concentraion of Cd^{2+} up to 3 mM, Cd^{2+} was insolubilized within 2 days. A similar pattern was found at initial Cu^{2+} concentration of 2 mM or less, but the rate of decrease in concentration of Cu^{2+} ions in the medium was slower than that of Cd^{2+} ions. A sulfate-reducing consortium became incapable of sulfate reduction at higher than 10 mM Cd^{2+} or 5 mM Cu^{2+}.[37] Our results indicated a similar pattern for Cd^{2+} and Cu^{2+} but the isolate was more sensitive to heavy metals than a sulfate-reducing consortium.

6. Effect of heavy metals on methane production by *M. thermoautotrophicum* KHT in coculture with *Desulfotomaculum* sp. RHT

We determined whether it was possible to recover the growth and methanogenesis of *M. thermoautotrophicum*

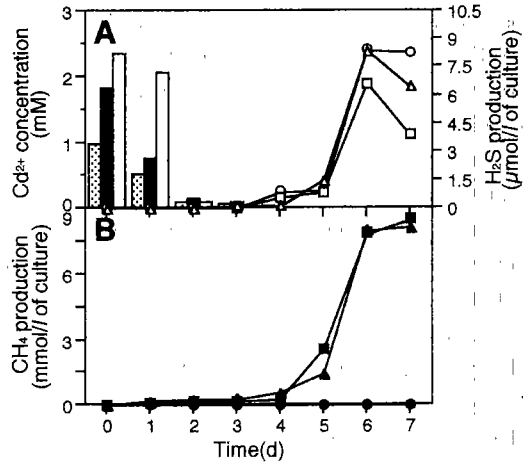


Fig. 6. The profiles of Cd^{2+} insolubilizatin (A) and methane production (B) by the coculture of *Desulfotomaculum* sp. RHT and *M. thermoautotrophicum* KHT. in the Medium C (see text) at 60°C and pH 7.0. Symbols: H_2S production at 1 mM (△), 2 mM (□) and 3 mM (○); CH_4 production at 1 mM (▲), 2 mM(■); Cd^{2+} concentration in the medium at 1 mM (▭), 2 mM (▭) and 3 mM (▭) as initial concentrations.

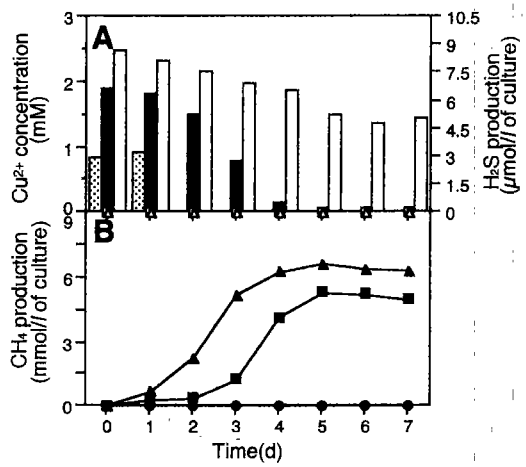


Fig. 7. The profiles of Cu^{2+} insolubilizatin (A) and methane production (B) by the coculture of *Desulfotomaculum* sp. RHT and *M. thermoautotrophicum* KHT. in the Medium C (see text) at 60°C and pH 7.0. Symbols: H_2S production at 1 mM (△), 2 mM (□) and 3 mM (○); CH_4 production at 1 mM (▲), 2 mM(■); Cu^{2+} concentration in the medium at 1 mM (▭), 2 mM (▭) and 3 mM (▭) as initial Cd^{2+} concentrations.

KHT after decreasing the concentration of heavy metal ions to a low level using *Desulfotomaculum* sp. RHT.

Strains KHT and RHT were cocultured with Cd^{2+} or Cu^{2+}. As shown in Figs. 6 and 7, with 1 and 2 mM Cd^{2+} as initial concentrations, the concentrations of Cd^{2+} decreased to 0.09 and 0.15 mM within 2 d, respectively, and methane production began from the 4th day. Although Cd^{2+} removal was observed at 3 mM, methane was not produced, indicating inactivation of the strain at high Cd^{2+} concentrations. Hydrogen sulfide in the gas phase was detected in all cases after the removal of Cd^{2+}.

The removal of Cu^{2+} in the coculture occurred more slowly than in the case of Cd^{2+}, and the Cu^{2+} concentration hardly decreased for an initial Cu^{2+} concentration of 3 mM (Fig. 7). In the case of 1 mM Cu^{2+}, Cu^{2+} was completely removed from the medium within 2 d, and methane production began from the first day. On the other hand, the concentration decreased to 0.14 mM within 4 d when the initial Cu^{2+} concentration was 2 mM, and methane production was observed from the second day.

In a pure culture of strain RHT containing 1 mM CuS, few cells were counted under a microscope and little production of hydrogen sulfide was observed (data not shown). CuS precipitation may have inhibited the growth of strain RHT and thus hydrogen sulfide could not be detected (Fig. 7). CdS precipitation (<3 mM) had no serious effect on the growth of strain RHT, resulting in hydrogen sulfide production (Fig. 6).

These results indicate that high concentrations of heavy metals (2 mM of Cd^{2+} or Cu^{2+}) did not fatally damage strain KHT, and methanogenesis can be recovered after the insolubilization of heavy metal ions. *M. thermoautotrophicum* KHT may be able to survive even if high concentrations of heavy metals exist in the environment.

M. thermoautotrophicum and strains of the genus *Desulfotomaculum* have been isolated from many anaerobic environments, *i.e.*, anaerobic sewage digestors, hot springs, compost, ground water and oil reservoirs, but we do not know whether our strains are dominant in the site from which they were isolated.

It is well known that many wastes contain high amounts of heavy metals, *e.g.*, 27-118 mg Cd/kg and 570-8500 mg Cu/kg.[38,39] The potential release percentages of heavy metals from wastes determined by the Availability Test are 15-110% for Cd and 0.3-64% for Cu.[38,39] However, we do not know the actual heavy metal ion concentrations in the site. We only know the concentrations in the leachates or final effluents as a result of chemical and/or biological reactions in the site.

In general, Cd and Cu ions seldom leach out at waste disposal sites.[40] Therefore, sulfate-reducing bacteria, which are distributed widely in waste disposal sites,[10,11] may function to insolubilize heavy metals, thus maintaining the activity of metal-sensitive methanogens.

IV. Conclusion

The result of the effect of heavy metals on a methanogen and sulfate-reducing bacterium were as follow

1. *M. thermoautotrophium* KHT seems to be sensitive to Cd^{2+} and Cu^{2+}. In the presence of 0.5 mM or more of Cd^{2+} and Cu^{2+}, the growth of *M. thermoautotrophium* KHT and methane production was strongly inhibited.

2. When *Desulfotomaculum* sp. RHT was Inoculated Into the medium and cultured, precipitation of heavy metals as a result of CdS and CuS formation was observed.

3. When M. *thermoautotrophicum* KHT and *Desulfotomaculum* sp. RHT were cocultured with Cd^{2+} and Cu^{2+} at high metal concentrations, methane production began after Cd^{2+} and Cu^{2+} concentration decreased due to Insolubilization of the heavy metal by strain RHT.

4. M. *thermoautotrophicum* KHT may be able to survive even If high concentration of heavy metals exist In the environment. These results Indicate that high concentration of heavy metals did not fatally strain KHT and methanogensis can be recovered after the Insolubilization of heavy metal Ions by sulfate-reducing bacterium.

Acknowledgements

We thank Dr. Kazuhiro Takamizawa, Gifu University, for performing the 16S rRNA analysis and Dr. Benedict C. Okeke, California University, for critical reading of the manuscript.

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