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Isolation and Identification of Tetramethylammonium-Biodegrading Bacteria

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Tetramethylammonium hydroxide (TMAH)-using bacteria were isolated from soils in a factory which has produced similar C_1 compounds with TMAH for 30 years. Four Gram-positive strains (TH-6, TH-30, TH-31 and TH-35) use TMAH easily, and grow in up to 8 wt% TMAH. Two Gram-negative strains (TH-3 and TH-15) use TMAH weakly, and grow in up to 3 wt% TMAH. The Gram-positive strains belong to the *Mcobacterium*, Gram-negative TH-3 resembles *Pseudomonas aminovorans*, and TH-15 belongs to the *Methylobacterium* from its morphological and chemotaxonomic characteristics. *Mycobacterium* sp. TH-30 biodegrades 1% TMAH in a continuous culture with a dilution rate below 0.06 h^{-1} completely. This bacterium seems to be useful for the biodegradation of waste fluids containing TMAH.

Tetramethylammonium hydroxide (TMAH) is synthesized chemically, and ultrapurified TMAH solution (2.38%) is used widely as a posi-resist developer of integrated circuits at electronics factories. Almost the whole quantity of TMAH used as a posi-resist developer leaves the factories as waste fluid, but the biodegradation of waste fluid contained TMAH is usually difficult in activated sludge treatment, and this waste fluid has been degraded by combustion and so on. Therefore, TMAH-degrading microorganisms are desired (1, 2). Bacterium 5H (1) and *Pseudomonas* strains (3) were reported as TMAH-using bacteria. However, not much taxonomical study of these bacteria or the biodegradation of TMAH were done.

This paper deals with the isolation of TMAH-using bacteria, and the biodegradation of TMAH by batch and continuous cultures.

MATERIALS AND METHODS

Isolation of TMAH-using bacteria TMAH-using bacteria were isolated by the enrichment culture technique at 30°C from soils from the Niigata Factory of Mitsubishi Gas Chemical Company (Niigata). This factory produced large amounts of methanol, formaldehyde, *N,N*-dimethylformamide, methylamine, and other chemicals from approximately 30 years ago. TMAH seems to be dealt with the same metabolic pathway as these C_1 compounds (1, 3). The TMAH-containing medium (TMAH medium) was used for the isolation of TMAH-using bacteria. TMAH basal medium was composed of $(NH_4)_2SO_2$, 3.0 g; KH_2PO_4, 1.4 g; Na_2HPO_4, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; ferric citrate, 30 mg; $CaCl_2 \cdot 2H_2O$, 30 mg; $MnCl_2 \cdot 4H_2O$, 5.0 mg; $ZnSO_4 \cdot 7H_2O$, 5.0 mg; $CuSO_4 \cdot 5H_2O$, 0.5 mg; yeast extract, 0.2 g; vitamin solution, 0.1 ml; tetramethylammonium chloride, 10 g; and distilled water, 1,000 ml; the pH was adjusted to 7.0. The vitamin solution was composed of biotin, 2 mg; calcium pantothenate, 400 mg; pyridoxine HCl, 400 mg; thiamine HCl, 400 mg; *p*-aminobenzoic acid, 200 mg; folic acid, 2 mg; inositol, 2 g; nicotinic acid, 400 mg; riboflavin, 200 mg; and distilled water 1,000 ml. Pure cultures were obtained by repeating plate culture on the TMAH basal medium solidified with 2% agar. By this procedure, 6 strains were isolated.

Identification methods The TMAH basal medium and PYG agar medium containing 0.5% peptone, 0.5% yeast extract, 0.5% glucose, and 2.0% agar (this medium was adjusted to pH 7.0 with 1 M NaOH) were used for the preculture and the basal media.

Cell form, Gram reaction, motility, and acid-fast stain were investigated by the methods reported previously (4, 8). Growth in nutrient broth, peptone water, and 1% Ogawa medium "Eiken" (Eiken, Tokyo) were examined. Usage of trimethylamine (TMA), trimethylamine-*N*-oxide (TMA-*N*-oxide), dimethylamine (DMA), monomethylamine (MoMA), or methanol was observed in the TMAH basal medium after 3 weeks of cultivation, where TMAH was replaced with these carbon compounds at 0.15 wt%.

DNA base composition, principal amino acids in the cell wall, quinone and quinone homologs, cellular fatty acid composition, hydroxy fatty acid composition, and mycolic acid composition were identified as described previously (4–6).

Culture system Batch cultures by flask were done in 1-*l* flasks containing 200 ml of the TMAH basal medium on a rotary shaker (220 rpm) at 30°C. Growth inhibition by TMAH of the TMAH-using bacteria isolated was examined using the TMAH basal medium at 0.5, 1.0, 2.0, 3.0, 5.0, 8.0, or 10.0 wt% TMAH.

Batch cultures by jar were done in a 3-*l* jar fermentor containing 1.5 *l* of medium at an aeration rate of 1.5 *l*/min and an agitation rate of 1500 rpm to keep the dissolved oxygen concentration between 2 and 8 ppm during cultivations. The composition of the medium used was modified TMAH medium where yeast extract and vitamin solution were omitted and 1 wt% TMAH was added. The culture temperature was controlled at 30°C, and the culture pH was controlled at 7.0 by adding 10 wt% ammonia water. For the batch cultures in flask and jar, a 1.0% inoculum was used.

Chemostat cultures were done in a 3-*l* jar fermentor with temperature and pH controllers to maintain 30°C and pH 7.0 by ammonia water. The composition of the feed medium used was as follows: KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$,

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0.5 g; $CaCl_2 \cdot 2H_2O$, 30 mg; ferric citrate, 30 mg; $MnCl_2 \cdot 4H_2O$, 5.0 mg; $ZnSO_4 \cdot 7H_2O$, 5.0 mg; $CuSO_4 \cdot 5H_2O$, 0.5 mg; TMAH solution (10% w/w, pH 7.0), 100 ml; and distilled water 900 ml (pH 5.0). Cultures were done for 5 d or more at each dilution rate, where they were changed from low to high.

Analysis In the batch cultures, growth and generation time were estimated from the optical density at 610 nm, and the concentrations of TMAH, MoMA, DMA, TMA, and NH_4^+ in the broth were analyzed. In the chemostat cultures, the dry cell weight, the concentration of TMAH, MoMA, DMA, TMA, NH_4^+, Chemical Oxygen Demand (COD_{Mn}), and Total Organic Carbon (TOC) in the supernatant of the broth were measured in a number of steady state conditions after 5 d or more of cultivation at each dilution rate. For the estimation of the dry cell weight, samples of cell suspension were centrifuged at 10,000 *g* for 10 min, washed twice with distilled water, dried at 105°C for 24 h, and weighed. The concentrations of TMAH, MoMA, DMA, TMA, and NH_4^+ were measured by an Ion Chromatography (Toso, Tokyo), and column of TSK gel IC-Cation (Toso). The column temperature was 40°C. The solvent used was 2 mM HNO_3 in methanol—2-methyl-2-propanol—water (1.5 : 3.5 : 95, vol/vol). The flow rate was 1 ml/min. The content of extracellular carbon compounds in the supernatant of the broth was shown as COD and TOC. COD was measured by the method of Mn described in our previous report (7). TOC was measured using a Total Organic Carbon Analyzer TOC-10B (Shimadzu, Kyoto).

RESULTS AND DISCUSSION

Phenotypic characteristics of TMAH-using bacteria TMAH-using bacteria, strains TH-3, TH-6, TH-15, TH-30, TH-31 and TH-35 were isolated on the basal TMAH medium. Four strains (TH-6, TH-30, TH-31, and TH-35) were Gram-positive, non-motile, rod-shaped, acid-fast, and white-colony-forming bacteria. One strain (TH-3) was a Gram-negative, subpolar flagellated, rod-shaped, and white-colony-forming bacterium, and another strain (TH-15) was a Gram-negative, polar flagellated, rod-shaped, and pink-colony-forming bacterium. All 6 strains used TMAH, TMA, TMA-*N*-oxide, DMA, and MoMA as a sole carbon source. Furthermore, 5 srains other than TH-3 used methanol.

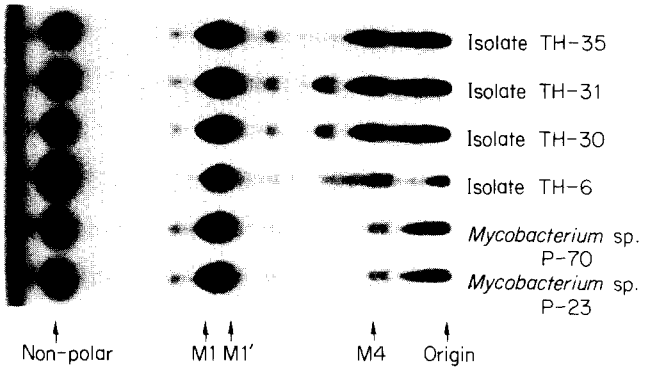

FIG. 1. Thin layer chromatographic patterns of fatty acid methyl esters of Gram-positive TMAH-using bacteria and Gram-positive methanol-using bacteria. Non-polar: Nonpolar fatty acid methyl esters, M1: α-mycolic acid methyl esters, M1': α'-mycolic acid methyl esters, M4: dihydroxy mycolic acid methyl esters.

Chemotaxonomic characteristics of TMAH-using bacteria DNA base composition, cellular fatty acid composition, hydroxy fatty acid composition, quinone type, major cell wall diamino acid type, and occurence of mycolic acid

TABLE 1. Cellular fatty acid composition of TMAH-using bacteria

Strains	Composition of cellular fatty acid (%)								
	Straight-chain acids					10-Methyl acid	3-Hydroxy acids		Unknown acid
	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{19:0}$	$C_{12:0}$	$C_{14:0}$	
Isolate TH-6	5.4	32.5	12.4	2.3	34.0	13.4	ND	ND	ND
Isolate TH-30	5.5	34.1	12.8	2.3	31.0	14.3	ND	ND	ND
Isolate TH-31	6.2	33.4	13.1	1.7	31.6	14.0	ND	ND	ND
Isolate TH-35	6.3	34.7	13.1	2.2	32.2	11.5	ND	ND	ND
Isolate TH-3	ND	10.1	5.9	4.3	70.1	ND	0.7	0.2	8.7
Isolate TH-15	ND	2.3	3.7	6.4	86.0	ND	ND	1.6	ND

ND: Not detected.

TABLE 2. DNA base composition, cell wall type, mycolic acid, and hydroxy fatty acid composition of TMAH-using bacteria

Strains	G+C content (mol%)	Major cell wall diamino acid	Mycolic acid	3-Hydroxy acids (%)	
				$C_{12:0}$	$C_{14:0}$
Isolate TH-6	65.9	meso-A_2Pm	M1 (M1', M4)	ND	ND
Isolate TH-30	66.0	meso-A_2Pm	M1 (M1', M4)	ND	ND
Isolate TH-31	67.2	meso-A_2Pm	M1 (M1', M4)	ND	ND
Isolate TH-35	66.4	meso-A_2Pm	M1 (M1', M4)	ND	ND
Isolate TH-3	63.5	—	—	78.8	21.2
Isolate TH-15	67.3	—	—	ND	100

ND: Not detected.
mso-A_2Pm: meso-Diaminopimelic acid.
M1: α-Mycolic acid.
M1' : α'-Mycolic acid.
M4: Dihydroxy mycolic acid.

TABLE 3. Quinone types of TMAH-using bacteria

Strains	Quinone homologs (mol%)								
	Q-9	Q-10	Q-11	MK-8	MK-8 (H_2)	MK-9	MK-9 (H_2)	MK-10	MK-10 (H_2)
Isolate TH-6	ND	ND	ND	0.2	2.9	0.3	96.2	ND	0.4
Isolate TH-30	ND	ND	ND	ND	2.7	0.1	97.0	ND	0.2
Isolate TH-31	ND	ND	ND	0.9	3.4	0.1	95.1	ND	0.5
Isolate TH-35	ND	ND	ND	1.7	3.0	ND	95.1	ND	0.2
Isolate TH-3	0.3	99.7	ND	ND	ND	ND	ND	ND	ND
Isolate TH-15	0.9	98.8	0.3	ND	ND	ND	ND	ND	ND

ND: Not detected.

Q-n: Q, ubiquinone; n, a specified number of isoprenoid units in the side chain.

MK-n (Hm): MK, menaquinone; n, a specified number of isoprenoid units in the side chain; m, the number of hydrogen atoms saturating the isoprenoid chain.

are shown in Fig. 1 and Tables 1 to 3. Four Gram-positive strains (TH-6, TH-30, TH-31, and TH-35) had 65–68 mol% G+C (DNA), major cellular fatty acid of $C_{16:0}$, $C_{16:1}$, $C_{18:1}$ and 10 methyl $C_{19:0}$, a menaquinone system of MK-9(H_2), major cell wall diamino acid of meso-diaminopimelic acid (A_2Pm), and mycolic acid. Mycolic acid was composed of a large amount of α-mycolic acid (M1), and a small amount of α'-mycolic acid (M1′) and dihydroxy mycolic acid (M4), and was the same as those of *Mycobacterium* sp. P-23 and P-70 (6) used as reference strains. Isolate TH-3 had 63.5 mol% G+C (DNA), major cellular fatty acid of $C_{18:1}$, major hydroxy fatty acid of 3-hydroxy $C_{12:0}$, and an ubiquinone system of Q-10. Isolate TH-15 had 67.3 mol% G+C (DNA), major cellular fatty acid of $C_{18:1}$, major hydroxy fatty acid of 3-hydroxy $C_{14:0}$, and an ubiquinone system of Q-10. Isolates TH-3 and TH-15 did not contain mycolic acid.

Four strains (TH-6, TH-30, TH-31, and TH-35) of TMAH-using bacteria seem to resemble methanol-utilizing *Mycobacterium* strains reported previously (6), strain TH-3 does *Pseudomonas aminovorans* (5), and strain TH-15 does *Methylobacterium* strains (8–11) on the basis of phenotypic and chemotaxonomic characteristics. Our isolates were distinguished from the Gram-negative, nonmotile bacterium 5H reported by Hampton and Zatman (1) on the basis of Gram stain and motility. But the relationship of Gram-negative isolates (TH-3 and TH-15) and *Pseudomonas* strains of Ghisalba *et al.* (3) was not clear. Correct naming of the isolates would require further study.

Flask cultivation Four Gram-positive strains (TH-6, TH-30, TH-31, and TH-35) used TMAH strongly, and TMAH in the supernatant of the broth was degraded completely after 40 h of cultivation at 0.5% TMAH. Two Gram-negative strains (TH-3 and TH-15) used TMAH slowly, and TMAH in the supernatant of the broth was not degraded completely, as shown in Fig. 2. Furthermore, 4 Gram-positive strains grew in the medium containing 8.0 wt% TMAH, but the other two strains grew only below 3.0 wt% TMAH (Fig. 3).

Batch culture by a jar fermentor The batch culture of strain TH-30, which was one strain of four strains (TH-6, TH-30, TH-31, and TH-35) selected by the use of TMAH (Fig. 2 and 3) and the resistant to TMAH, were done (Fig. 4). Cells grew with an approximately 4.5-h generation time, and the growth was over after 44 h of cultivation. On the other hand, TMAH in the broth decreased with growth, and was not detected at 44 h of cultivation. Furthermore, the intermediate compounds of TMAH metabolism (1, 12), MoMA, DMA, and TMA,

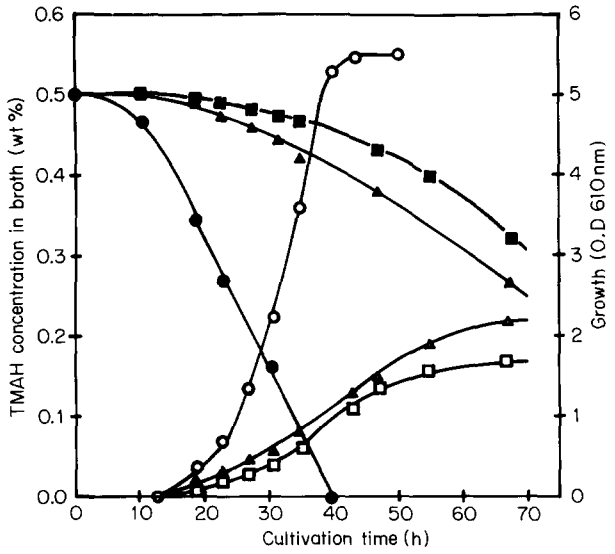

FIG. 2. Flask culture of TMAH-using bacteria using 0.5 wt% TMAH as a carbon source. TMAH concentration in broth (wt%): ●, strain TH-30; ▲, strain TH-3; ■, strain TH-15. Growth ($O.D_{610\,nm}$): ○, strain TH-30; △, strain TH-3; □, strain TH-15.

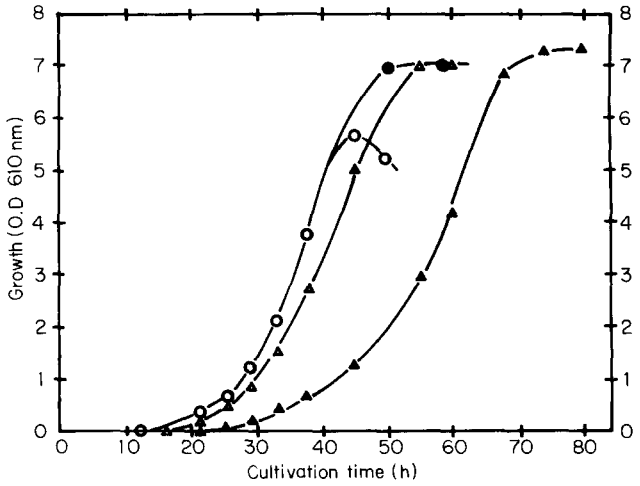

FIG. 3. Growth curve of *Mycobacterium* sp. TH-30 by flask culture using various concentrations of TMAH as a carbon source. TMAH concentrations: ○, 0.5 wt%; ●, 1.0 wt%; △, 2.0 wt%; ▲, 5.0 wt%.

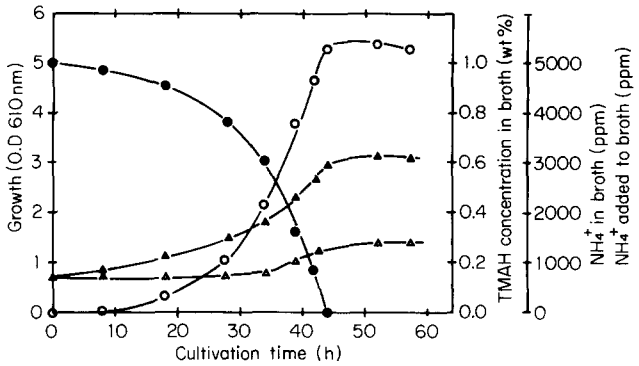

FIG. 4. Batch culture of *Mycobacterium* sp. TH-30 using TMAH as a carbon source. Initial concentration of TMAH: 1.0 wt%; Temperature: 30°C; pH: 7.0. Symbols: ○, growth ($O.D_{610\,nm}$); ●, TMAH concentration in broth (wt%); ▲, NH_4^+ in broth (ppm); △, total amounts of NH_4^+ added to broth.

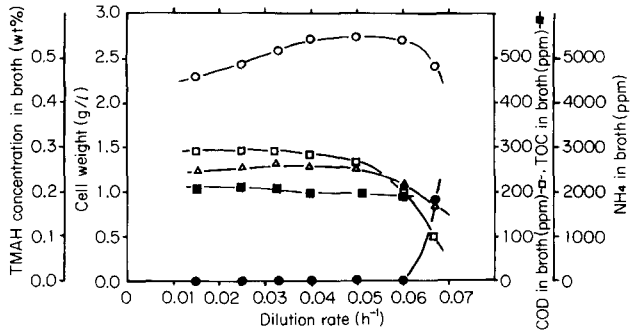

FIG. 5. Continuous culture of *Mycobacterium* sp. TH-30 using TMAH as a carbon source. TMAH concentration of the feed medium: 1.0 wt%; Temperature: 30°C; pH: 7.0. Symbols: ○, cell weight (g/*l*); ●, TMAH concentration in broth (wt%); ■, COD in broth (ppm); □, TOC in broth; △, NH_4^+ in broth (ppm).

were not detected in the broth. NH_4^+ concentration in the supernatant of the broth was increased above the added NH_4^+. This should be caused by NH_4^+ production by the biodegradation of TMAH.

Chemostat culture TMAH-limiting chemostat cultures of strain TH-30 were done at various dilution rate (Fig. 5). TMAH and intermediate compounds (MoMA, DMA, and TMA) were not detected in the supernatant of the broth at a dilution rate below 0.06 h^{-1}. The cell concentration in the broth was 2.2 to 2.8 g/*l*. TOC values decreased to approximately 200 ppm in the chemostat culture. TOC values in the feeding medium were approximately 5000 ppm. On the other hand, COD values increased to approximately 300 ppm in the chemostat culture. COD values in the feeding medium were approximately 70 ppm. The cause of this was that TMAH was detected as TOC, but not as COD_{Mn}, and unknown extracellular carbon compounds except TMAH were produced. The NH_4^+ concentration in the broth was approximately 2500 ppm, and increased from the amounts of NH_4^+ added in the jar. Strain TH-30 should biodegraded TMAH completely from the lack of detection of TMAH, MoMA, DMA and TMA, decrease of TOC values, and increase of NH_4^+. All organic compounds are detected as TOC, but some including TMAH are not detected as COD_{Mn}. And the degradation of waste fluid should be confirmed by TOC.

Mycobacterium sp. TH-30 seems to be useful for the biodegradation of waste fluids containing TMAH.

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