Isolation from Soil of Aerobic Chemoheterotrophic Bacteria Capable of Decomposing Methanethiol and Hydrogen Sulfide

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(Received August 17, 1998—Accepted April 9, 1999)

Twenty-eight strains of aerobic chemoheterotrophic bacteria capable of decomposing methanethiol and hydrogen sulfide were isolated from soil samples obtained from mountains, forests, farm fields, parks, hot springs, riversides, the seashore, pigpens, cowsheds, henhouses and other areas in Japan. Among the strains isolated, TOS-35 and TOS-55 had the greatest activities against both methanethiol and hydrogen sulfide. Based on physiological traits, strain TOS-35 was identified as a member of the genus *Alcaligenes* and strain TOS-55 as that of the genus *Pseudomonas*. Some authentic strains such as *Pseudomonas putida* IFO 3738, *Comamonas testosteroni* IFO 12048, *Brevundimonas diminuta* IFO 14213 and *Burkholderia cepacia* IFO 14595 were found to be capable of oxidizing methanethiol and hydrogen sulfide. Our results showed that many species of chemoheterotrophic bacteria in nature are capable of decomposing organic and inorganic sulfur compounds.

Key words: heterotrophs, methanethiol, hydrogen sulfide

Chemoautotrophic bacteria capable of metabolizing organic and inorganic sulfur compounds have been reported to belong to the genus *Thiobacillus*. For example, Kanagawa and Mikami^{7,14)} reported the removal of methanethiol, dimethylsulfide and hydrogen sulfide from malodorous air by *Thiobacillus thioparus* TK-m. Ongcharit *et al.*¹⁰⁾ and Dalsgaard and Bak⁴⁾ described the oxidation of hydrogen sulfide by *Thiobacillus denitrificans* in continuous cultures. Wada *et al.*¹⁵⁾ reported the isolation from a peat biofilter of a *Thiobacillus intermedius* strain capable of oxidizing hydrogen sulfide.

However, few reports have dealt with chemoheterotrophic bacteria capable of oxidizing hydrogen

sulfide and organic sulfur compounds. Suylen *et al.*¹²⁾ showed that methylmercaptan oxidase was a key enzyme in the metabolism of methylated sulfur compounds in *Hyphomicrobium* EG. Zhang *et al.*¹⁶⁾ demonstrated the removal of dimethylsulfide, methanethiol and hydrogen sulfide by *Hyphomicrobium* sp. I55. Cho *et al.*^{2,3)} reported the oxidation of hydrogen sulfide to polysulfide by *Xanthomonas* sp. DY44.

Recently, we¹³⁾ isolated aerobic chemoheterotrophs from various soil samples capable of decomposing organic sulfur compounds by the enrichment culture method. This report describes the isolation and partial characterization of the heterotrophs capable of oxidizing organic sulfur compounds.

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Materials and Methods

Chemicals

Dimethylsulfide (DMS) and sodium methylsulfide were purchased from Tokyo Kasei Kogyo Co. Ltd., Tokyo. Methanethiol (MT) and hydrogen sulfide (HS) were purchased as 1000 ppm standard gases in N₂ from Japan Air Liquid Co. Ltd., Tokyo. DTNB (5,5'-dithio-bis (2-nitrobenzoic acid)) was obtained from Sigma Chemical Co., St. Louis USA. All other chemicals used were commercially available and of chemically pure grade.

Enrichment culture and isolation procedure

Soil was collected from 71 grounds in 11 areas of Japan (Table 1). Approximately 20 mg (wet weight) of soil was suspended in 15 ml of sterile 10 mM phosphate buffer (pH 7.5) and vortexed for 3 min. After being allowed to stand for 5 min, 5 ml of the supernatant was inoculated into 50 ml of enrichment culture medium containing the following nutrients: 2 g of Na₂HPO₄ · 2H₂O, 2 g of K₂HPO₄, 0.4 g of NH₄Cl, 0.2 g of CaCl₂, 0.2 g of MgCl₂, 2 g of glucose and 740 µl of DMS, per liter. Cultivation was done at room temperature for two weeks with gentle

shaking. During the cultivation, 37 µl of DMS was added on days 4 and 11.

Following the DMS-enrichment culture, 0.1 ml of the supernatant was spread on tryptic soya (TS) agar medium and incubated for 1 to 3 days at 30°C, and then colonies were isolated from the plate.

Determination of MT- and HS-decomposing activities

The isolated bacteria were cultured in TS liquid medium at 30°C for 12 h with shaking and then 5 ml of the culture medium was transferred into 50 ml of TS liquid medium and cultivated for a further 12 h. After being washed, each strain was suspended and diluted in 100 mM phosphate buffer (pH 7.0) containing 0.85% NaCl to give an absorbance at 660 nm of approximately 0.2 (ca. 2×10^8 cells) in a serum-capped vial. The vial was continuously bubbled with air containing 100 ppm MT or HS at a flow rate of 100 ml/min and incubated at 30°C for 10 min with gentle shaking.

The concentration of MT in the liquid phase was determined using Ellman's method modified by Grassetti and Murray⁵⁾, and Ishiguro⁶⁾. The reaction mixture in the vial was filtered through a Millipore filter (pore size: 0.45 µm), and 20 µl of 10 mM DTNB solution in 100 mM phosphate buffer (pH 7.0) was added to 3 ml of the filtrate and vortexed. The absorbance of the mixture at 412 nm was measured with a HITACHI MPS-2000 spectrophotometer. A quantitative curve was obtained using sodium methylsulfide as a standard. A linear relationship was observed between 0 and 2.5 mM MT under the above analytical conditions.

The concentration of HS in the liquid phase was directly determined using an ORION EA920 ionmeter for the first 10 min and 6 h after the addition of 10 ml of 1 M NaOH to 10 ml of reaction mixture that had been incubated.

MT in the gas phase of the vial was measured, for the first 10 min and 24 h later by gas chromatography with a Hewlett Packard 5890 system equipped with a DB-17 column (J&W, supplied by Yokogawa Analytical Systems, Tokyo) and a FPD detector (200°C). HS in the gas phase was measured during the first 10 min and

Table 1. Occurrence of heterotrophs tolerant to DMS and capable of decomposing MT in soil samples obtained from various places.

Areas	Number of tested samples	DMS	MT
Mountain	4	32	2
Forest	3	15	1
Farm field	6	33	5
Park	8	48	3
Hot spring	9	23	4
Riverside	3	16	6
Seashore	1	5	1
Pigpen	3	6	0
Cowshed	22	76	0
Henhouse	9	0	0
Others	3	18	6
Total	71	272	28

Abbreviations for activities are as follows: DMS, number of DMS-tolerant and/or -decomposing strains; MT, number of MT-decomposing strains.

again 6 h later. The flow rate of the carrier gas was 1 ml/min and the injection temperature was 150°C. MT- and HS-decomposing activity are indicated by the residual amount of MT and HS in the gas phase.

Identification and Characterization of MT- and HS-decomposing heterotrophs

All isolates were examined by using a commercial Pile tube No. 2 identification kit (Nissui Pharmaceutical Co. Ltd., Tokyo) and BiOLOG MicroStation System™ (BiOLOG Inc., USA., supplied by Gunze Sangyo, Tokyo). In addition, the physiological characteristics of the two strains with the most potent MT- and HS-decomposing activities (TOS-35 and TOS-55) were examined in more detail. TOS-35 was examined by using a commercial ID test NF-18 identification kit (Nissui Pharmaceutical Co. Ltd., Tokyo). The characterization was based on Bergey's Manual of Determinative Bacteriology⁸⁾.

Assay of Guanine + Cytosine (G + C) contents

Genomic DNA was isolated and purified from bacterial cells by the method of Birnboim and Doly¹⁾. The G + C content of the DNA was determined by the HPLC method of Mesbah *et al.*⁹⁾. The HPLC system used was :column, a STR ODS-H column (150 × 4 mm, Shimazu), previously equilibrated with 0.2 M NH₄HPO₄-acetonitrile (40:1 v/v), at a flow rate of 1 ml/min and a column temperature of 40°C.

Results and Discussion

Isolation of MT-decomposing heterotrophs

As shown in Table 1, a total of 272 heterotrophic bacterial strains were isolated from 71 soil samples by DMS-enrichment culture. The MT-decomposing activity of the isolated strains was examined and 28 strains exhibited this activity.

None of the heterotrophic bacteria isolated from pigpen, cowshed and henhouse were capable of decomposing MT. Since the above three areas were located within institutions of stock raising, it was thought that antibacterial agents were frequently scattered on these grounds both to prevent epidemics and for deodorization.

Table 2 shows the MT- and HS-decomposing activities and generic assignment of the 28 strains. Sixteen strains were identified as *Bacillus*, 10 strains as *Pseudomonas*, one strain as *Alcaligenes* and one strain as *Enterococcus*. Five strains of *Bacillus* and one *Enterococcus* had strong MT-decomposing activity, but showed weaker HS-decomposing activity. In the case of *Bacillus* strains TOS-64 and TOS-182, however, the activities were reversed. Most of the *Pseudomonas* and *Alcaligenes* strains tested showed strong

Table 2. MT- and HS-decomposing activities and generic assignment of the isolated heterotrophic bacteria.

Strain	MT	HS	generic assignment
TOS-1	+++	+	<i>Bacillus</i>
TOS-3	+++	++	<i>Bacillus</i>
TOS-5	+++	+	<i>Bacillus</i>
TOS-7	+++	+	<i>Bacillus</i>
TOS-32	++	+	<i>Bacillus</i>
TOS-60	+	+	<i>Bacillus</i>
TOS-64	+	+++	<i>Bacillus</i>
TOS-72	+	+	<i>Bacillus</i>
TOS-83	+	+	<i>Bacillus</i>
TOS-93	+++	++	<i>Bacillus</i>
TOS-182	+	+++	<i>Bacillus</i>
TOS-207	++	+	<i>Bacillus</i>
TOS-211	+	+	<i>Bacillus</i>
TOS-213	++	+	<i>Bacillus</i>
TOS-230	++	+++	<i>Bacillus</i>
TOS-254	+	+	<i>Bacillus</i>
TOS-38	+++	+	<i>Pseudomonas</i>
TOS-52	+++	++	<i>Pseudomonas</i>
TOS-54	+++	++	<i>Pseudomonas</i>
TOS-55	+++	+++	<i>Pseudomonas</i>
TOS-101	++	+	<i>Pseudomonas</i>
TOS-104	+	+++	<i>Pseudomonas</i>
TOS-133	+++	+++	<i>Pseudomonas</i>
TOS-227	++	++	<i>Pseudomonas</i>
TOS-276	++	++	<i>Pseudomonas</i>
TOS-283	++	++	<i>Pseudomonas</i>
TOS-35	+++	+++	<i>Alcaligenes</i>
TOS-130	+++	+	<i>Enterococcus</i>

Abbreviations for activities are as follows: MT, methanethiol decomposition; HS, hydrogen sulfide decomposition. Symbols indicate the decomposing activities (%): +, less than 49%; ++, 50–89%; +++, more than 90%.

MT- and HS-decomposing activities. The difference of the activities between bacterial genera may be of interest.

Cho *et al.*^{2,3)} reported the isolation of a *Xanthomonas* strain capable of decomposing HS from peat. Wada *et al.*¹⁵⁾ reported the isolation of HS-oxidizing bacteria from a peat biofilter. From the results in Tables 1 and 2, however, it can be concluded that chemoheterotrophic bacteria capable of decomposing MT and HS occur widely in the environment.

MT-decomposing activity versus HS-decomposing activity of each isolate

The MT- and HS-decomposing activities measured in the gas phase of our isolates and some of the authentic strains obtained from the IFO culture collection are shown in Table 3.

All the isolates and authentic strains tested exhibited stronger MT-decomposing activities than *Thiobacillus thioparus* TK-m, which is a well-known MT-oxidizing chemoautotroph⁷⁾. Strains TOS-35, TOS-55, and 4 authentic strains showed particularly strong MT-decomposing activities. The comparison

between MT-decomposing and HS-decomposing activity after 10 min revealed two types of heterotrophic bacteria. In the first group (group I), the MT-decomposing activity is higher than the HS-decomposing activity, as observed for strain TOS 35, *Pseudomonas putida*, *Comamonas testosteroni*, and *Brevundimonas diminuta*. We could not find any report on the MT- and HS-decomposing activity of *Pseudomonas putida*, *Comamonas testosteroni*, and *Brevundimonas diminuta*. In the second group (group II), both activities are almost the same as observed in strains TOS-38, TOS-52, TOS-54, TOS-55, TOS-101, TOS-130 and *Burkholderia cepacia*.

Most of the isolates and authentic strains but not TOS-101 decomposed MT in 24 h. However, most isolates and authentic strains belonging to group II were unable to decompose HS in 6 h although strain TOS-55 could.

Smith and Kelly¹¹⁾ proposed that MT and HS are intermediates in the metabolic pathway for DMS oxidation by *Thiobacillus thioparus*. According to them, potent MT-decomposing bacteria should also have potent HS-oxidizing activity. From Table 3, how-

Table 3. MT- and HS-decomposing activities of the isolated and IFO strains.

Strain	Residual conc.* (ppm) after			
	MT		HS	
	10 min	24 h	10 min	6 h
Blank	98.9	88.0	101.0	91.0
<i>Thiobacillus thioparus</i> TK-m	60.0	22.2	ND**	ND**
group I				
TOS-35	6.8	0.0	60.2	9.1
<i>Pseudomonas putida</i>	16.0	0.0	74.4	0.0
<i>Comamonas testosteroni</i>	8.7	0.0	64.5	0.0
<i>Brevundimonas diminuta</i>	0.0	0.0	39.0	0.6
group II				
TOS-38	48.1	5.0	63.0	60.0
TOS-52	50.7	3.4	60.5	43.9
TOS-54	46.3	1.8	61.5	47.9
TOS-55	53.0	0.0	55.7	1.1
TOS-101	105.7	11.5	70.6	69.8
TOS-130	92.4	2.4	69.4	60.0
<i>Burkholderia cepacia</i>	55.7	0.0	85.4	85.1

* The concentration in the gas phase.

** ND: Not determined.

Table 4. Bacteriological characteristics of strain TOS-35.

	TOS-35
1. Morphological and cultural characteristics:	
Shape	short rod
Gram stain	—
Motility	+
Endospore	—
Color of colonies on TS agar	white without soluble pigment
Growth temperature	5–30°C
Growth pH	5.0–11.5
2. Biochemical characteristics:	
Litmas milk	alkaline (blue)
Oxygen requirement	obligate aerobe
GC mol% of DNA	54.1–57.8
Poly- β -hydroxybutyrate accumulation	+
Growth on MacConkey agar	+
Setriamide agar	+
OF test	—
Voges-Proskauer test	—
Methyl red test	—
Hemolysis	+
Pyocyanin production	—
Pyoverdine production	—
Oxidase	+
Catalase	+
Urease	—
Lipase (Tween 80 hydrolysis)	+
Protease (gelatin liquefying)	—
β -Galactosidase	—
Phenylalanine deaminase	—
Lysine decarboxylase	—
Arginine dihydrolase	—
Ornithine decarboxylase	—
Nitrate reduction	—
Nitrite reduction	+
Anaerobic culture	—
Penicillin tolerance	+
Acid production from:	
L-arabinose	—
D-xylose	—
Utilization of:	
DL-lactate	+
glutarate	+ w
phenyl acetate	+
malonate	+
azelate	—
D-malate	—
citrate	+
acetate	+
trans-aconitate	—
DL-4-aminobutyrate	—
pelargonate	—
L-phenylalanine	+
L-histidine	—
L-aspartate	+
L-leucine	+
L-tyrosine	+
ethanol	+
2,3-butanediol	—

Symbols indicate as follows: —, negative; + w, weak positive; +, positive; ND, Not determined.

ever, it can be seen that the MT-decomposing activities of the strains are not directly proportional to their HS-decomposing activities, particularly in the cases of TOS-38, TOS-101, TOS-130 and *Burkholderia cepacia* IFO 14595. This result indicates that the metabolic pathway for MT oxidation in aerobic chemoheterotrophic bacteria is different from that in autotrophic bacteria. Further biochemical study of each isolate will be required for the clarification of the decomposing pathway of MT and HS.

Physiological traits of strains capable of MT- and HS- decomposition

The taxonomical traits of strains TOS-35 and TOS-55, both of which had the strongest MT- and HS-decomposing activities, were investigated (Table 4). The colonies of strain TOS-35 on nutrient agar (NA) were white without soluble pigment, and emitted a strawberry-like odor. The strain was a Gram-negative obligately aerobic motile rod, lacked endospores, produced oxidase and catalase and was negative for oxidation-fermentation (OF) test. Strain TOS-35 could not utilize any of the sugars tested; including glucose, fructose, maltose, galactose, xylose, mannitol, sucrose, lactose, and esculin.

According to Bergey's manual⁸⁾, the properties of strain TOS-35 were consistent with the genus *Alcaligenes* except for the hydrolysis of Tween 80. The absolute likelihood score with the ID kit showing that strain TOS-35 is *Alcaligenes fecalis* was 0.829. The characteristics of strain TOS-35 coincided with those of *A. fecalis* JCM 2503 (the type strain) including the Tween 80 hydrolysis and the GC content. Strain TOS-35, therefore, was identified as *Alcaligenes fecalis*.

The colonies of strain TOS-55 on nutrient agar (NA) were pale cream-colored without soluble pigment. It was a Gram-negative, obligately aerobic motile rod, lacked endospores, produced oxidase and catalase, and was positive for OF (oxidation). Other characteristics were as follows: growth temperature, 10–30°C; growth pH, 4.0–10.5; Litmus milk, alkaline; oxygen requirement, obligate aerobe; growth on MacConkey agar, positive; poly- β -hydroxybutyrate accumulation, positive; hemolysis, positive; arginine

dihydrolase, positive; penicillin tolerance, positive; acid from L-arabinose, D-xylose, positive; utilization-positive, glucose, 2-ketogluconate, β -alanine, L-arginine, DL-lactate, glutarate, D-malate, trans-aconitate, DL-4-aminobutyrate, L-phenylalanine, L-leucine, L-tyrosine, ethanol, 2,3-butanediol, malonate, L-histidine; Voges-Proskauer test, negative; methyl red test, negative; anaerobic culture, negative; denitrification, negative; trehalose, meso-inositol, azelate utilization-negative.

According to Bergey's manual⁸⁾, these properties of TOS-55 were similar to those of *Pseudomonas* sp. In addition, TOS-55 showed high similarity to *Pseudomonas putida* based on the data from the BiOLOG MicroStation SystemTM, which evaluates 95 carbon utilization tests (similarity score 0.862). Although a more precise study is needed to identify strain TOS-55, it is highly possible that TOS-55 belongs to the genus *Pseudomonas*. More detailed systematic studies of strains TOS-35 and TOS-55 are in progress and will be described elsewhere.

A section of the results presented here was announced at the Annual Meeting of the Japanese Society for Culture Collection, 1995.

Acknowledgements

The author is grateful to Professor Kenji Watanabe and Dr. Yukihiko Nakashima (Fukuoka University, Faculty of Pharmaceutical Sciences) for kind advice.

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