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Identification of Dimethyl Disulfide-Forming Bacteria Isolated from Activated Sludge

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Twenty-four strains with high dimethyl disulfide (DMDS)-forming ability were isolated from activated sludge and identified to the genus level. These bacteria were classified into four groups (A, B, C, and D) by the API ZYM System (API System S.A., Montalieu, France). Group A (three strains) was identified as genus *Lactobacillus* by the API 20B System, by the method of Cowan and Steel, and by production of lactic acid as confirmed by gas-liquid chromatography. Group B (eight strains) was identified as genus *Corynebacterium* by API 20B and the Cowan and Steel method. Group C (one strain) was suggested to belong to genus *Corynebacterium* by the API 20B System. Group D (12 strains) was identified as genus *Pseudomonas* or *Alcaligenes* by the API 20B System, as genus *Alcaligenes* by the Cowan and Steel method, and as *Achromobacter* group Vd by the API 20NE System. However, on the basis of guanine-plus-cytosine contents in DNA and form of flagella, these strains were identified as genus *Pseudomonas*. Formation of DMDS from DL-methionine and *S*-methyl-L-cysteine was tested. DMDS-forming bacteria isolated from activated sludge formed DMDS from both precursors. In genus *Pseudomonas*, *P. aeruginosa* could not form DMDS from either precursor, but *P. acidovorans*, *P. alcaligenes*, *P. pseudoalcaligenes*, and *P. testosteroni* formed DMDS. In genus *Alcaligenes*, *A. denitrificans* subsp. *xylosoxydans*, *A. denitrificans* subsp. *denitrificans*, *A. faecalis*, and *A. odorans* formed DMDS from both precursors. *Achromobacter* group Vd formed DMDS from *S*-methyl-L-cysteine, but could not from DL-methionine.

In a previous paper (23), we reported that when activated sludge was abnormally conditioned at high biochemical oxygen demand (BOD) loading, bacteria capable of forming dimethyl disulfide (DMDS) from methionine increased in the aeration tank.

The formation of DMDS from methionine by the following several organisms has been reported: *Pseudomonas* spp. (7), *Streptomyces* and *Bacterium* spp. (21), *Aspergillus* spp. (19), *Clostridium sporogenes* (11), *Alteromonas putrefaciens*, *Pseudomonas fluorescens* and *Achromobacter* spp. (14), *Pseudomonas putida* (5), *Proteus* spp. (4) and *P. fluorescens*, *Proteus vulgaris*, and *Serratia marcescens* (18). These bacteria were isolated from soil, wastewater, fish, and so on. However, DMDS-forming bacteria isolated from activated sludge have not been reported.

When activated sludge was cultured at high BOD loading (5.1 to 5.4 kg of BOD/m^3 per day) with synthetic wastewater (23), the conditions of activated sludge changed abnormally. From such abnormally conditioned activated sludge (cultures II-Ab and III-Ab) we isolated seven strains with high DMDS-forming ability (23). Further, when the abnormally conditioned activated sludge (culture III-Ab) was cultured under normal BOD loading (0.59 kg of BOD/m^3 per day), the normal conditions of activated sludge were gradually restored, and 17 strains with high DMDS-forming ability were isolated from this convalescent activated sludge.

In this paper, isolated bacteria (24 strains) with high DMDS-forming ability were identified to the genus level.

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MATERIALS AND METHODS

Tested bacteria. (i) DMDS-forming bacteria isolated from activated sludge. Seven strains (II-Ab-39, -40, -41, -42, and -43 and III-Ab-5 and -28) were isolated from abnormally conditioned activated sludge (23). The abnormally conditioned activated sludge (III-Ab) was cultured under normal BOD loading (0.59 kg of BOD/m^3 per day), and convalescent activated sludge (III-R) was obtained. From this convalescent activated sludge, 17 strains (III-R-3, -4, -5, -7, -8, -16, -18, -22, -23, -31, -32, -44, -49, -51, -54, -57, and -60) were isolated.

(ii) Reference strains. The following 24 strains were used as reference strains. *Achromobacter* group Vd biotype 1 (strains 5174, 4892, 4813, 4492, 4464, and 3968) and biotype 2 (strains 5085, 4532, 4494, 4491, 4229, and 1256) were obtained from the Hospital for Joint Diseases and Medical Center (New York, N.Y.) *Alcaligenes odorans* (Laboratory Research, API System [LRA] 41-02-82), *Pseudomonas aeruginosa* (ATCC 27853), and *Flavobacterium multivorum* (LRA 26-07-76) were obtained from Aska-junyaku (Tokyo, Japan). *Alcaligenes faecalis* (Institute for Fermentation, Osaka [IFO] 13111 and ATCC 8750), *P. aeruginosa* (IFO 12689, ATCC 10145), *Pseudomonas acidovorans* (IFO 13582, ATCC 9355), *Pseudomonas alcaligenes* (IFO 14159, ATCC 14909), *Pseudomonas pseudoalcaligenes* (IFO 14167, ATCC 17440), and *Alteromonas putrefaciens* (IFO 3908, ATCC 8071) were obtained from IFO (Osaka, Japan). *Pseudomonas testosteroni* (Japan Collection of Microorganisms [JCM] 5505 and ATCC 11996) and *Alcaligenes denitrificans* subsp. *denitrificans* (JCM 5490, ATCC 15173) were obtained

from JCM (Saitama, Japan). *Alcaligenes denitrificans* subsp. *xylosoxydans* (Gifu Anaerobic Institute [GAI] 10678, ATCC 27061) was provided by the Institute of Anaerobic Bacteriology, Gifu University School of Medicine (Gifu, Japan).

Classification and identification of DMDS-forming bacteria by the API systems. The API ZYM System (API System S.A., Montalieu, France) was used to classify DMDS-forming bacteria. The API 20B System was used for the presumptive identification of DMDS-forming bacteria at genus level. The API 20NE System was used to identify glucose-nonfermenting gram-negative rod bacteria.

Identification of DMDS-forming bacteria by conventional methods. Biological and biochemical characteristics of DMDS-forming bacteria (24 strains) were tested, and the identification of these bacteria at genus level was performed by the method of Cowan and Steel (2). The characteristics of the glucose-nonfermenting gram-negative rods (12 strains; group D) were compared with those of reference strains (21 strains) by the methods described in the *Manual of Clinical Microbiology* (13). The characteristics listed in Tables 1 and 2 were tested by conventional methods (2, 13, 22), and two items (oxidation-fermentation and acid production from carbohydrate) were tested by two methods each. That is, oxidation-fermentation was tested in Hugh-Leifson semisolid medium (Eiken, Tokyo, Japan) with 1% glucose and 7 days of incubation at 30°C, according to Cowan and Steel, as well as in OF basal medium (Difco Laboratories) with 1% glucose and 30 days of incubation at 30°C according to the *Manual of Clinical Microbiology*. Acid production from carbohydrate was tested in Andrade peptone water (Oxoid, England) with 1% glucose and incubated for 30 days at 30°C, according to Cowan and Steel, and also in OF basal medium (Difco) with 1% carbohydrate, incubated for 30 days at 30°C, according to the *Manual of Clinical Microbiology*. Flagella were stained by the method of Leifson (10) and with Victoria blue B (15).

Determination of metabolic products by gas-liquid chromatography. To identify the genus *Lactobacillus*, it is necessary to confirm that the strains in question produce lactic acid and do not produce acetic acid from glucose. Therefore, lactic acid and acetic acid in metabolic products were determined as follows. GAM medium with 1% glucose (Nissui, Tokyo, Japan) was sterilized in an autoclave for 15 min at 115°C. Strains II-Ab-39, -40, and -41 were inoculated and cultured on a Monodo shaker for 7 days at 30°C. Volatile and nonvolatile fatty acid fractions in the culture medium were separated. From each fraction, lactic acid and acetic acid were determined by gas-liquid chromatography (flame ionization detector) by the method of Ueno et al. (25). Gas-liquid chromatographic conditions were as follows: FID-GC (Shimadzu GC-6A); Reoplex 400 10% support Chromosorb W AW-DMCS, 80/100 mesh; glass column (3 m by 3-mm internal diameter); injection temperature 250°C; column temperature, 155°C; carrier gas (N_2) at 40 ml/min; air at 1.0 kg/cm^2; H_2 at 0.6 kg/cm^2; sensitivity of 10^2 MΩ; range, 16 × 0.01 V; chart speed, 5.0 mm/min; injected volume 0.2 μl.

Determination of G+C content in DNA. To identify to which genus strains of group D belong, guanine-plus-cytosine (G+C) contents in DNA were determined.

Volumes of 200 ml of nutrient broth medium (Eiken, Tokyo, Japan) were placed in 500-ml flasks and sterilized in the autoclave for 15 min at 121°C. The strains of group D (12 strains: III-Ab-5, and III-R-3, -4, -5, -7, -8, -31, -32, -44, -51, -54, and -60) and reference strains (14 strains) were inoculated and cultured for 4 days at 30°C on a shaker (type SKL-1-F; Nihon-ikaki, Osaka, Japan). DNA from each cultured medium was prepared by the method of Saito et al. (20). The DNA was broken down to deoxyribonucleotides, and each deoxyribonucleotide was determined by high-performance liquid chromatography by the method of Katoh et al. (8, 9).

DMDS formation from DL-methionine and *S*-methyl-L-cysteine. To investigate DMDS formation from DL-methionine and *S*-methyl-L-cysteine by each strain, two culture media (A and B) were prepared. Culture medium A was prepared as follows: 3.0 g of glucose, 4.5 g of peptone, 0.25 g of $Na_2HPO_4 \cdot 12H_20$, 0.20 g of KCl, 0.25 g of $MgCl_2 \cdot 6H_20$, and 100 mg of DL-methionine were dissolved in about 950 ml of distilled water, the pH was adjusted to 7.3 with 2% NaOH, and the volume was made up to 1 liter. For culture medium B, 90.6 mg of *S*-methyl-L-cysteine was added instead of DL-methionine. Each culture medium was sterilized by passing through a membrane filter (Sterifil-GV, 0.22-μm pore size; Millipore), and each 10.0-ml aliquot was placed in a 40-ml test tube. Each isolated strain (24 strains) and reference strain (24 strains) was inoculated into a test tube, and the tubes were plugged with double rubber stoppers covered with a Teflon sheet. After culturing on a Monodo shaker for 48 h at 30°C, the content of DMDS formed (nanograms per tube) was determined by the headspace gas-liquid chromatographic method (23).

RESULTS AND DISCUSSION

Classification of DMDS-forming bacteria by the API ZYM System. Nineteen kinds of enzyme activity of DMDS-forming bacteria isolated from activated sludge (24 strains) were determined by the API ZYM System.

The DMDS-forming bacteria were classified into four groups, A, B, C, and D. Three strains (II-Ab-39, -40, and -41) were assigned to group A, 8 strains (II-Ab-42 and -43; III-R-16, -18, -22, -23, -49, and -57) to group B, 1 strain (III-Ab-28) to group C, and 12 strains (III-Ab-5 and III-R-3, -4, -5, -7, -8, -31, -32, -44, -51, -54, and -60) to group D. Among 19 kinds to enzymes, some enzymes showed different activity among the groups. That is, in group A α-glucosidase, β-glucosidase, and *N*-acetyl-β-glucosaminidase showed strong activity; in group B, β-galactosidase, α-glucosidase, β-glucosidase, and *N*-acetyl-β-glucosaminase were strongest; in group C, α-glucosidase and α-fucosidase were prominent; and in group D, alkaline phosphatase, trypsin, acid phosphatase, and phosphoamidase showed strongest activity.

Presumptive identification of genus of DMDS-forming bacteria by the API 20B System. The isolates within each group (as classified by API ZYM System) showed the same characteristics, and the following numerical profiles were obtained by the API 20B System: 077770121 for group A, 477770131 for group B, 577770071 for group C, and 200000650 for group D. Thus, the classification by API 20B agreed with that by API ZYM.

Presumptive identification was performed according to the recommendations of the manufacturer. Based on the results, strains of group A were presumptively identified as genus *Lactobacillus*, those of groups B and C were identified as genus *Corynebacterium*, and those of group D were identified as genus *Pseudomonas* or *Alcaligenes*.

Identification of DMDS-forming bacteria by the Cowan and Steel method. Biological and biochemical characteristics of DMDS-forming bacteria were tested, and identification at genus level was performed by the method of Cowan and Steel (Table 1).

TABLE 1. Characteristics of DMDS-forming bacteria by the Cowan and Steel method[a]

Group	No. tested	Gram stain	Cell morphology	Spore	Acid fast	Oxidase	Catalase	Motility	Oxidation of glucose	Fermentation of glucose	Aerobic growth	Anaerobic growth	Acid from glucose
A	3	3	R[b]	0	0	0	0	0	3	3	3	3	3
B	8	8	R	0	0	0	8	0	8	8	8	8	8
C	1	1	R	0	0	0	1	1	1	1	1	0	1
D	12	0	R	0	—[c]	12	12	12	0	0	12	0	0

[a] Number positive.
[b] R, Rod.
[c] —, Not tested.

The strains within each group showed the same characteristics, and grouping by this method agreed with the results with the API ZYM and API 20B systems.

From these characteristics, identification at genus level was performed by the method of Cowan and Steel. It was suggested that the strains of group A belonged to genus *Lactobacillus*, and those of group B were identified as genus *Corynebacterium*. These results agreed with those of the API 20B System. The strain of group C could not be identified by the Cowan and Steel method. The strains of group D were identified as genus *Alcaligenes*.

Identification of group A strains by determination of metabolic products. To confirm the strains of group A as belonging to genus *Lactobacillus*, the metabolic products of these strains (II-Ab-39, -40, and -41) were investigated by gas-liquid chromatography. Lactic acid was recognized, at a retention time of about 3.0 min, from all these strains, and acetic acid was not recognized. From these results, the strains of group A were identified as genus *Lactobacillus* in agreement with the API 20B System.

Identification of DMDS-forming bacteria of group D by the API 20NE System. The strains of group D (12 strains) were identified by API 20NE. All the strains of group D showed almost the same characteristics by this system; the numerical profiles were 1047775 (eight strains), 1047765 (three strains), and 1047764 (one strain). These strains were identified by the API Computer Service as *Achromobacter* group Vd at high identification percentage (above 97.1%).

Group A (three strains) was identified as genus *Lactobacillus* by the API 20B System and the method of Cowan and Steel and on the basis of production of lactic acid from glucose. Group B (8 strains) was identified as genus *Corynebacterium* by the API 20B System and the method of Cowan and Steel. Group C (1 strain) was suggested to be genus *Corynebacterium* by the API 20B System. However, group D (12 strains) was identified as genus *Pseudomonas* or *Alcaligenes* by API 20B, as genus *Alcaligenes* by the Cowan and Steel method, and as *Achromobacter* group Vd by the API 20NE System. Thus, each method showed different results of identification.

Identification of strains of group D. To clarify to which genus, *Pseudomonas*, *Alcaligenes*, or *Achromobacter* group Vd, the isolates of group D (12 strains) (glucose-nonfermenting gram-negative rods) belonged, we obtained reference strains of *Pseudomonas* (5 strains), *Alcaligenes* (4 strains), and *Achromobacter* group Vd (12 strains), which may show almost identical characteristics, according to other reports (1–3, 12, 13, 26; G. L. Gilardi, Clin. Microbiol. Newsl. **6**:111–113, 1984; G. L. Gilardi, Clin. Microbiol. Newsl. **6**:126–129, 1984). The biological and biochemical characteristics of these isolates were compared with those of reference strains by methods outlined in the *Manual of Clinical Microbiology* (13). These results are shown in Table 2.

The sludge isolates (12 strains) and reference strains (21 strains) showed the same characteristics, as follows: motility; acid production from lactose; catalase and oxidase; growth on MacConkey agar; indole acid production on TSI agar; hydrolysis of DNA; growth at 25 and 35°C; ornithine decarboxylase and lysine decarboxylase activities; growth in nutrient broth without NaCl; and 3-ketolactose production.

Though the isolates in group D showed extremely weak positive reactions, acid production from glucose is an important item for their identification. Of the reference strains, *Achromobacter* group Vd showed an apparent positive reaction. Among the *Pseudomonas* strains, *P. aeruginosa* showed an apparent positive reaction, but *P. acidovorans*, *P. alcaligenes*, *P. pseudoalcaligenes*, and *P. testosteroni* showed alkaline reactions. *A. denitrificans* subsp. *xylosoxydans* showed a weak positive reaction, but *A. faecalis*, *A. odorans*, and *A. denitrificans* subsp. *denitrificans* showed alkaline reactions. Thus, on the basis of this reaction, generic identification of the group D strains was difficult. Inconsistent results were obtained in the following determinations: action on blood agar; oxidation-fermentation; acid production from xylose, mannitol, sucrose, maltose, and fructose; growth on SS and cetrimide agars; utilization of citrate; urease; nitrate reduction; gas from nitrate; H_2S production; hydrolysis of gelatin, acetamide, and esculin; pigment production; growth at 42°C; arginine dihydrolase activity; growth in nutrient broth with 6% NaCl; and phenylalanine deaminase. No reference strain corresponded with all characteristics of the group D strains.

The genus of the group D strains could not be identified by biological and biochemical characteristics. Therefore, we determined the G+C contents in the DNA of these strains, which is an important index for the identification of bacteria (Table 3).

The G + C contents of strains of group D ranged from 67.8 to 69.2 mol%. It was confirmed that the group D strains all belonged to the same genus, because the G+C contents of these strains were almost identical. It was evident that the group D strains did not belong to *Acromobacter* group Vd, as the G+C values were quite different. In the case of *Pseudomonas*, *P. aeruginosa* and *P. acidovorans* showed values similar to those of group D, but the values in *P.*

TABLE 2. Characteristics of glucose-nonfermenting gram-negative rod bacteria (group D)[a]

Strains	Cell morphology[b]	Motility	Polar flagella	Peritrichous flagella	Action on blood agar	Fermentation or oxidation[c]	Acid from: (OF basal medium) Glucose	Xylose	Mannitol	Lactose	Sucrose	Maltose	Fructose	Catalase	Oxidase	Growth on: MacConkey agar	SS agar	Cetrimide agar
Isolates from activated sludge																		
III-Ab-5	R	+	+	−	−	(O)	(±)	−	−	−	−	−	(±)	+	+	+	−	−
III-R-3	R	+	+	−	−	(O)	(±)	−	−	−	−	−	(±)	+	+	+	−	−
III-R-4	R	+	+	−	−	(O)	(±)	−	−	−	−	−	(±)	+	+	+	−	−
III-R-5	R	+	+	−	−	(O)	(±)	−	−	−	−	−	(±)	+	+	+	−	−
III-R-7	R	+	+	−	−	(O)	(±)	−	−	−	−	−	(±)	+	+	+	−	−
III-R-8	R	+	+	−	−	(O)	(±)	−	−	−	−	−	(±)	+	+	+	−	−
III-R-31	R	+	+	−	−	(O)	(±)	−	−	−	−	−	(±)	+	+	+	−	−
III-R-32	R	+	+	−	−	(O)	(±)	−	−	−	−	−	(±)	+	+	+	−	−
III-R-44	R	+	+	−	−	(O)	(±)	−	−	−	−	−	(±)	+	+	+	−	−
III-R-51	R	+	+	−	−	(O)	(±)	−	−	−	−	−	(±)	+	+	+	−	−
III-R-54	R	+	+	−	−	(O)	(±)	−	−	−	−	−	(±)	+	+	+	−	−
III-R-60	R	+	+	−	−	(O)	(±)	−	−	−	−	−	(±)	+	+	+	−	−
Achromobacter group Vd biotype 1																		
5174	R	+	−	+	−	O	+	+	+	−	+	+	+	+	+	+	+	−
4892	R	+	−	+	−	O	+	+	+	−	+	+	+	+	+	+	+	−
4813	R	+	−	+	−	O	+	+	±	−	+	+	+	+	+	+	+	−
4492	R	+	−	+	−	O	+	+	+	−	+	+	+	+	+	+	+	−
4464	R	+	−	+	−	O	+	+	+	−	+	+	+	+	+	+	+	−
3968	R	+	−	+	−	O	+	+	+	−	+	+	+	+	+	+	+	−
biotype2																		
5085	R	+	−	+	−	O	+	+	+	−	+	+	+	+	+	+	+	−
4532	R	+	−	+	−	O	+	+	±	−	+	+	+	+	+	+	+	−
4494	R	+	−	+	−	O	+	+	+	−	+	+	+	+	+	+	+	−
4491	R	+	−	+	−	O	+	+	+	−	+	+	+	+	+	+	+	−
4229	R	+	−	+	−	O	+	+	+	−	+	+	+	+	+	+	+	−
1256	R	+	−	+	−	O	+	+	+	−	±	±	+	+	+	+	+	−
Pseudomonas aeruginosa ATCC 27853	R	+	+	−	+	O	+	+	+	−	−	−	+	+	+	+	+	+
P. acidovorans ATCC 9355	R	+	+	−	−	I	−	−	+	−	−	−	+	+	+	+	+	−
P. alcaligenes ATCC 14909	R	+	+	−	+	I	−	−	−	−	−	−	−	+	+	+	−	+
P. pseudoalcaligenes ATCC 17440	R	+	+	−	−	I	−	+	−	−	−	−	+	+	+	+	+	+
P. testosteroni ATCC 11996	R	+	+	−	−	I	−	−	−	−	−	−	−	+	+	+	+	−
Alcaligenes faecalis ATCC 8750	R	+	−	+	−	I	−	−	−	−	−	−	−	+	+	+	+	+
A. odorans LRA 41-02-82	R	+	−	+	−	I	−	−	−	−	−	−	−	+	+	+	+	+
A. denitrificans subsp. *xylosoxydans* ATCC 27061	R	+	−	+	−	(O)	(±)	+	−	−	−	−	−	+	+	+	+	+
A. denitrificans subsp. *denitrificans* ATCC 15173	R	+	−	+	−	I	−	+	−	−	−	−	−	+	+	+	+	+

Continued on following page

TABLE 2—*Continued*

					Acid production on TSI agar:		H_2S production on:		Hydrolysis of:				Pigment formation on King medium:		Growth at:						Growth in nutrient broth plus:			
Utilization of citrate (Simmons)	Urease (Christensen)	Nitrate reduction	Gas from nitrate	Indole (SIM)[d]	Slant	Butt	TSI butt	Lead acetate paper	Gelatin (20°C, 30 days)	Acetamide	DNA	Esculin	A	B	25°C	35°C	42°C	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	0% NaCl	6% NaCl	3-Ketolactose production	Phenylalanine deaminase
−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	+	+	+	−	−	−	+	−	−	−
−	+	+	−	−	−	−	−	(±)	−	−	−	−	−	−	+	+	+	−	−	−	+	−	−	−
−	+	+	−	−	−	−	−	(±)	−	−	−	−	−	−	+	+	+	−	−	−	+	−	−	−
−	(+)	+	−	−	−	−	−	(±)	−	−	−	−	−	−	+	+	−	−	−	−	+	−	−	−
−	(+)	+	−	−	−	−	−	(+)	−	−	−	−	−	−	+	+	+	−	−	−	+	−	−	−
−	(+)	+	−	−	−	−	−	+	−	−	−	−	−	−	+	+	±	−	−	−	+	−	−	−
−	(+)	+	−	−	−	−	−	+	−	−	−	−	−	−	+	+	±	−	−	−	+	−	−	−
−	+	+	−	−	−	−	−	(±)	−	−	−	−	−	−	+	+	±	−	−	−	+	−	−	−
−	+	+	−	−	−	−	−	(±)	−	−	−	−	−	−	+	+	±	−	−	−	+	−	−	−
−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	+	+	+	−	−	−	+	−	−	−
−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	+	+	±	−	−	−	+	−	−	−
−	+	+	−	−	−	−	−	(±)	−	−	−	−	−	−	+	+	+	−	−	−	+	−	−	−
+	+	+	+	−	−	−	−	+	−	−	−	+	−	−	+	+	+	−	−	−	+	+	−	+
+	+	+	+	−	−	−	−	+	−	−	−	+	−	−	+	+	+	−	+	−	+	+	−	+
−	+	+	+	−	−	−	−	+	−	−	−	+	−	−	+	+	±	−	±	−	+	+	−	+
−	+	+	−	−	−	−	−	+	−	−	−	+	−	−	+	+	+	−	−	−	+	+	−	+
−	+	+	+	−	−	−	−	+	−	−	−	+	−	−	+	+	−	−	−	−	+	+	−	+
+	+	+	+	−	−	−	−	+	−	−	−	+	−	−	+	+	+	−	−	−	+	+	−	+
+	+	+	+	−	−	−	−	+	−	−	−	+	−	−	+	+	±	−	+	−	+	+	−	+
+	+	+	+	−	−	−	+	+	−	−	−	+	−	−	+	+	+	−	+	−	+	+	−	−
+	+	+	+	−	−	−	−	+	−	−	−	+	−	−	+	+	±	−	−	−	+	+	−	+
−	+	+	−	−	−	−	−	+	−	−	−	+	−	−	+	+	±	−	−	−	+	+	−	+
+	+	+	+	−	−	−	+	+	−	−	−	+	−	−	+	+	±	−	(+)	−	+	+	−	+
+	+	+	−	−	−	−	−	+	−	−	−	+	−	−	+	+	+	−	+	−	+	+	−	+
+	+	+	+	−	−	−	+	+	+	+	−	−	+	+	+	+	+	−	+	−	+	+	−	−
+	−	+	−	−	−	−	−	+	−	+	−	−	−	−	+	+	−	−	−	−	+	−	−	−
−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	+	+	+	−	−	−	+	−	−	−
−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	+	+	+	−	−	−	+	−	−	−
+	−	+	−	−	−	−	−	+	−	−	−	−	−	−	+	+	−	−	−	−	+	−	−	−
+	−	−	−	−	−	−	−	+	−	+	−	−	−	−	+	+	±	−	−	−	+	+	−	−
+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	+	+	−	−	−	+	+	−	−
+	−	+	+	−	−	−	−	±	−	+	−	−	−	−	+	+	+	−	−	−	+	+	−	−
+	−	+	+	−	−	−	−	+	−	+	−	−	−	−	+	+	−	−	−	−	+	+	−	−

[a] Cultures were incubated at 30°C except where indicated. +, Positive for test; ±, weakly positive for test; −, negative for test; ( ), reactions delayed.
[b] R, Rod.
[c] O, Oxidation; I, inactive.
[d] SIM, Sulfide-indole-motility.

TABLE 3. G+C contents of DNA[a]

Strains	Mol% G+C
Isolates from activated sludge	
III-Ab-5	67.8
III-R-3	68.8
III-R-4	67.9
III-R-5	67.9
III-R-7	68.2
III-R-8	68.2
III-R-31	68.3
III-R-32	68.0
III-R-44	68.2
III-R-51	69.2
III-R-54	68.4
III-R-60	69.1
Achromobacter group Vd	
biotype 1 5174	57.2
biotype 2 5085	57.7
Pseudomonas aeruginosa ATCC 27853	67.6
P. aeruginosa ATCC 10145	66.6
P. acidovorans ATCC 9355	67.0
P. alcaligenes ATCC 14909	64.8
P. pseudoalcaligenes ATCC 17440	62.9
P. testosteroni ATCC 11996	62.8
Alcaligenes faecalis ATCC 8750	59.2
A. odorans LRA 41-02-82	63.1
A. denitrificans subsp. *xylosoxydans* ATCC 27061	68.2
A. denitrificans subsp. *denitrificans* ATCC 15173	68.1
Flavobacterium multivorum LRA 26-07-76	40.6
Alteromonas putrefaciens ATCC 8071	45.4

[a] High-pressure liquid chromatography conditions: pump, Shimadzu LC-3A; column, Develosil ODS, 150 mm by 4.6-mm internal diameter; detector, Shimadzu SPD-2A, UV 270 nm; mobile phase, 10 mM Na_2HPO_4 (pH 7.0); flow rate, 0.8 ml/min.

alcaligenes, *P. pseudoalcaligenes*, and *P. testosteroni* were a little lower. *A. denitrificans* subsp. *xylosoxydans* and *A. denitrificans* subsp. *denitrificans* showed almost the same G+C values as group D, but the *A. faecalis* and *A. odorans* values were different. Thus, on the basis of G+C content, it was difficult to decide whether group D, belonged to *Pseudomonas* or *Alcaligenes*. However, *Alcaligenes* and *Pseudomonas* spp. differ significantly in flagellar form; i.e., *Alcaligenes* sp. have peritrichous flagella, but *Pseudomonas* sp. have polar flagella (Table 2). All the strains of group D had polar flagella, and thus they were assumed to represent new species in the genus *Pseudomonas*. These 12 strains were numbered as *Pseudomonas* sp. strain III-Ab-5 and III-R-3, -4, -5, -7, -8, -31, -32, -44, -51, -54, and -60.

DMDS formation from DL-methionine and *S*-methyl-L-cysteine. Previously, we have reported that methylmercaptan is formed from methionine by activated sludge, and the methylmercaptan is in turn changed to DMDS in the presence of dissolved oxygen (24). Further, we noted that when activated sludge was conditioned abnormally and DMDS was detected, high contents of methionine were found in the mixed liquor of aeration tanks, and a small amount of *S*-methyl-L-cysteine was detected (23). *S*-Methyl-L-cysteine was reported as a precursor of methylmercaptan, in addition to methionine (5, 6, 16, 17). Therefore, we tested for DMDS formation from both precursors by isolated DMDS-forming bacteria and reference strains (Table 4).

All isolates from activated sludge formed DMDS from both DL-methionine and *S*-methyl-L-cysteine. In the case of *Pseudomonas* strains, *P. aeruginosa* could not form DMDS

TABLE 4. Formation of DMDS from DL-methionine and *S*-methyl-L-cysteine[a]

Strains	DMDS formed (ng per tube) from:	
	DL-Methionine	*S*-Methyl-L-cysteine
Isolates from activated sludge		
II-Ab-39	6,260 ± 60	4,190 ± 2,540
II-Ab-40	3,830 ± 0	2,790 ± 1,130
II-Ab-41	9,050 ± 1,070	14,800 ± 800
II-Ab-42	10,900 ± 570	19,000 ± 9,000
II-Ab-43	13,900 ± 2,700	31,400 ± 6,100
III-Ab-5	5,440 ± 490	5,510 ± 2,460
III-Ab-28	4,110 ± 1,040	6,900 ± 2,100
III-R-3	5,020 ± 110	6,290 ± 130
III-R-4	6,460 ± 450	4,510 ± 730
III-R-5	5,540 ± 280	5,020 ± 570
III-R-7	5,140 ± 60	5,620 ± 280
III-R-8	5,820 ± 230	5,700 ± 740
III-R-16	5,500 ± 340	5,380 ± 3,660
III-R-18	5,140 ± 280	7,800 ± 7,080
III-R-22	5,620 ± 1,970	5,380 ± 3,660
III-R-23	6,180 ± 1,070	11,100 ± 5,500
III-R-31	4,110 ± 60	4,700 ± 110
III-R-32	5,300 ± 60	5,900 ± 680
III-R-44	5,580 ± 340	5,780 ± 850
III-R-49	8,090 ± 960	7,100 ± 1,010
III-R-51	4,940 ± 0	3,390 ± 280
III-R-54	4,070 ± 110	3,910 ± 1,240
III-R-57	5,900 ± 110	3,310 ± 400
III-R-60	5,140 ± 280	4,710 ± 1,010
Achromobacter group Vd		
Biotype 1		
5174	(−)	38,500 ± 16,000
4892	(−)	25,300 ± 800
4813	(−)	21,900 ± 18,600
4492	(−)	18,300 ± 5,700
4464	(−)	43,900 ± 18,000
3968	(−)	44,000 ± 26,400
Biotype 2		
5085	(−)	44,700 ± 20,100
4532	(−)	13,500 ± 7,900
4494	(−)	51,700 ± 23,400
4491	(−)	53,400 ± 27,000
4229	(−)	3,730 ± 1,610
1256	(−)	18,400 ± 10,100
Pseudomonas aeruginosa ATCC 10145	(−)	(−)
P. aeruginosa ATCC 27853	(−)	(−)
P. acidovorans ATCC 9355	13,600 ± 1,400	14,800 ± 300
P. alcaligenes ATCC 14909	1,620 ± 700	638 ± 226
P. pseudoalcaligenes ATCC 17440	9,000 ± 2,420	1,700 ± 700
P. testosteroni ATCC 11996	10,400 ± 3,000	10,600 ± 8,000
Alcaligenes faecalis ATCC 8750	20,200 ± 5,400	17,400 ± 2,500
A. odorans LRA 41-02-82	22,800 ± 3,000	17,500 ± 3,400
A. denitrificans subsp. *xylosoxydans* ATCC 27061	15,800 ± 1,500	31,900 ± 9,100
A. denitrificans subsp. *denitrificans* ATCC 15173	12,800 ± 3,700	30,700 ± 12,400
Flavobacterium multivorum LRA 26-07-76	738 ± 253	5,580 ± 570
Alteromonas putrefaciens ATCC 8071	3,340 ± 480	1,340 ± 420

[a] Strains were incubated at 30°C for 48 h in each culture medium.

from either amino acid, but other reference strains of *Pseudomonas* sp. formed DMDS. The reference strains of *Alcaligenes* sp. formed DMDS from both amino acids. The reference strains of *Achromobacter* group Vd could not form DMDS from DL-methionine, but produced it in significant amounts from *S*-methyl-L-cysteine.

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LITERATURE CITED

1. **Chester, B., and L. H. Cooper.** 1979. *Achromobacter* species (CDC group Vd): morphological and biochemical characterization. J. Clin. Microbiol. **9:**425–436.
2. **Cowan, S. T.** 1974. Cowan and Steel's manual for the identification of medical bacteria, 2nd ed. Cambridge University Press, England.
3. **Gilardi, G. L.** 1971. Characterization of nonfermentative nonfastidious Gram negative bacteria encountered in medical bacteriology. J. Appl. Bacteriol. **34:**623–644.
4. **Hayward, N. J., T. H. Jeavons, A. J. C. Nicholson, and A. G. Thornton.** 1977. Methyl mercaptan and dimethyl disulfide production from methionine by *Proteus* species detected by headspace gas-liquid chromatography. J. Clin. Microbiol. **6:**187–194.
5. **Ito, S., T. Nakamura, and Y. Eguchi.** 1976. Purification and characterization of methioninase from *Pseudomonas putida*. J. Biochem. **79:**1263–1272.
6. **Kadota, H., and Y. Ishida.** 1972. Production of volatile sulfur compounds by microorganisms. Annu. Rev. Microbiol. **26:**127–138.
7. **Kallio, R. E., and A. D. Larson.** 1955. Methionine degradation by a species of *Pseudomonas*, p. 616–634. *In* W. D. McElroy and H. B. Glass (ed.), A symposium on amino acid metabolism. Johns Hopkins University Press, Baltimore.
8. **Katoh, K., T. Ohta, M. Kumagai, and M. Fujimoto.** 1986. Microbiological identification of single cell proteins based on DNA GC contents. III. Accuracy of HPLC determination of DNA GC contents by modified chromatography. Rept. Natl. Food Res. Inst. **48:**70–78.
9. **Katoh, K., A. Suzuki-Onozaki, T. Ohta, H. Ebine, M. Kumagai, M. Fujimoto, and A. Kuninaka.** 1983. Microbiological identification of single cell proteins based on DNA-GC contents. II. Chemical determination of DNA-GC contents. Rept. Natl. Food Res. Inst. **43:**79–89.
10. **Leifson, E.** 1951. Staining, shape, and arrangement of bacterial flagella. J. Bacteriol. **62:**377–389.
11. **Kreis, W., and C. Hession.** 1973. Isolation and purification of L-methionine-α-deamino-γ-mercaptomethane-lyase (L-methioninase) from *Clostridium sporogenes*. Cancer Res. **33:**1862–1865.
12. **Krieg, N. R. (ed.).** 1984. Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins, London.
13. **Lennette, E. H., A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.).** 1985. Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
14. **Miller, A., III, R. A. Scanlan, J. S. Lee, and L. M. Libbey.** 1973. Volatile compounds produced in sterile fish muscle (*Sebastes melanops*) by *Pseudomonas putrefaciens*, *Pseudomonas fluorescens*, and an *Achromobacter* species. Appl. Microbiol. **26:**18–21.
15. **Minamide, W., and K. Nakajima.** 1985. Usefulness of new simple method for fragella staining. San-in Kansensyo Zashi **7:**60–64. (In Japanese.)
16. **Moore, D. P., and J. F. Thompson.** 1967. Methionine biosynthesis from S-methylcysteine by thiomethyl transfer in *Neurospora*. Biochem. Biophys. Res. Commun. **28:**474–479.
17. **Nomura, J., Y. Nishizuka, and O. Hayaishi.** 1963. S-Alkylcysteinase: enzymatic cleavage of S-methyl-L-cysteine and its sulfoxide. J. Biol. Chem. **238:**1441–1446.
18. **Pohl, M., E. Bock, M. Rinken, M. Avdin, and W. A. König.** 1984. Volatile sulfur compounds produced by methionine degrading bacteria and the relationship to concrete corrosion. Z. Naturforsch. Teil C **39:**240–243.
19. **Ruiz-Herrera, J., and R. L. Starkey.** 1969. Dissimilation of methionine by fungi. J. Bacteriol. **99:**544–551.
20. **Saito, H., and K. Miura.** 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta **72:**619–629.
21. **Segal, W., and R. L. Starkey.** 1969. Microbial decomposition of methionine and identity of the resulting sulfur products. J. Bacteriol. **98:**908–913.
22. **Tokyo-daigaku ikagaku kenkyukai (Institute of Medical Science, Tokyo University).** 1981. Handbook of practical methods for microbiology. Maruzen, Tokyo. (In Japanese.)
23. **Tomita, B., N. Hamamura, A. Nakamura, M. Yamada, H. Inoue, and Y. Ose.** 1986. Studies on the control index of activated sludge. VII. Relation between bacteria flora and dimethyl disulfide formation in activated sludge. Eisei Kagaku **32:**267–275.
24. **Tomita, B., N. Hamamura, and Y. Ose.** 1981. Studies on the control index of activated sludge. V. The mechanism on the formation of dimethyl disulfide in mixed liquor of activated sludge. Eisei Kagaku **27:**98–104. (In Japanese.)
25. **Ueno, K., K. Watanabe, and M. Isono.** 1982. Determination of end metabolic products by gas liquid chromatography, p. 45–62. *In* K. Ueno (ed.), Procedures for isolation and identification of anaerobic bacteria. Saikon Publishing, Tokyo. (In Japanese.)
26. **Weaver, R. E., and D. G. Hollis.** 1983. Gram-negative organisms: an approach to identification (guide to presumptive identification). U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.