ISOLATION OF FREE-LIVING NITROGEN-FIXING BACTERIA FROM THE RHIZOSPHERE OF RICE

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Bacterial flora of rhizosphere of the rice plant was examined to determine the relationship of nitrogen-fixing bacteria to rice plants. Of 7,344 bacterial strains from the rhizosphere, 1,005 strains (13.7%) reduced acetylene. Representative 74 strains with acetylene reduction activity were selected for identification. All of these strains were gram negative, aerobic or facultatively anaerobic, and most had ubiquinone (Q-10) and cellular fatty acid composition, mainly with straight-chain fatty acids. Twenty-two strains were assigned to the following known species: *Azospirillum brasilense* Tarrand, Krieg and Döbereiner (1 strain), *Azospirillum lipoferum* Tarrand, Krieg and Döbereiner (1 strain), *Enterobacter cloacae* (Jordon) Hormaeche and Edwards (4 strains), *Erwinia herbicola* (Löhnis) Dye (2 strains), *Klebsiella oxytoca* (Flügge) Lautrop (6 strains), and *Xanthobacter autotrophicus* (Baumgarten, Reh and Schlegel) Wiegand, Wilke, Baumgarten, Opitz and Schlegel (8 strains). Fifty-two strains could not be assigned to any known species.

Since YOSHIDA and ANCAJAS(1) reported the association of free-living nitrogen-fixing bacteria with rice plants, several workers have isolated such bacteria and investigated the interrelation between bacteria and rice (2–17). Until recently, *Azospirillum lipoferum* (11–14), *Azospirillum brasilense* (12–14), *Enterobacter cloacae* (12, 13, 15), *Klebsiella oxytoca* (12, 13), *Pseudomonas paucimobilis* (12, 13), *Pseudomonas* sp. (16, 17), and *Clostridium* sp. (5) have been isolated. SANO et al. (18) reported a seasonal variation of nitrogen-fixation in the rice plant and a possible symbiotic relation between bacteria and the plants. We have collaborated with them to investigate the relation between the nitrogen-fixing bacteria and the rice plant.

This paper deals with the characterization and identification of the free-living nitrogen-fixing bacteria isolated from rice roots and paddy soil.

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MATERIALS AND METHODS

Materials. Plant materials of *Oryza sativa* strains C5444 and T65 and paddy soils were surveyed as isolation sources of free-living nitrogen-fixing bacteria. These materials were obtained from the National Institute of Genetics, Mishima, Japan during 3 years (1980–1982) by the courtesy of Dr. Fujii. Rice plants were grown in a greenhouse in plastic pots (7.2 cm diam.). Each pot contained 180 g of soil (pH 6.5) and fertilizers (urea 0.043 g, superphosphate of lime 0.267 g, KCl 0.04 g, rotted soil 2 g).

Isolation medium. Three kinds of media were used for isolation: 1) The malate medium reported by VON BULOW and DÖBEREINER (19) was composed of 0.4 g of KH_2PO_4 , 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g of NaCl, 10 mg of FeCl_3 , 2 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.1 g yeast extract, 2 ng of biotin, and 1,000 ml of distilled water. The pH of the medium was adjusted with KOH to 6.8, and Bromothymol Blue (BTB) was used as an indicator. This medium was designated as M medium. 2) The nitrogen-free glucose medium of STANIER et al. (20) was composed of 10 g of glucose, 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g of K_2HPO_4 , 50 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg of CaCl_2 , 2 mg of $\text{MnSO}_4 \cdot 4\text{--}6\text{H}_2\text{O}$, 1 mg of $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 5 g of CaCO_3 , 0.1 g of yeast extract, and 2 ng of biotin, and 1,000 ml of distilled water. The pH of the medium was adjusted with NaOH to 7.0. The CaCO_3 was heat-sterilized separately. This medium was designated as NFG medium. 3) The nutrient medium was composed of 10 g of beef extract (Difco Laboratories, Michigan, U.S.A.), 10 g of Polypepton (Daigo, Osaka, Japan), 5 g of NaCl, and 1,000 ml of distilled water. The pH of the medium was adjusted with NaOH to 7.0. Two grams of agar were added to 1,000 ml of medium to prepare a semisolid medium and 16 g for a solid medium.

Isolation procedure. (1) Paddy soil: One gram of paddy soil was suspended in 10 ml of sterile water, and 0.1 ml of the suspension was spread on NFG agar plate. The plate was incubated for 7 days at 30°C anaerobically using the Gaspack anaerobic system (BBL, Cockeysville, U.S.A.). Colonies from each plate were picked up on agar slant with the same composition and cultivated at 30°C aerobically and anaerobically, then acetylene activity (AR activity) was tested. (2) Rice materials: For the isolation from rice plants, rice stubbles were taken out of the pots, and soil adhering to the roots was removed by gently shaking in tap water. Then the roots were washed three times with 200 ml each of sterile water. One gram of the root was ground in 9 ml of sterile water in a mortar with pestle, and a series of 10-fold dilutions was prepared. From these samples, 0.1 ml was spread on M, NFG, and nutrient agar plates. The plates were incubated aerobically for 5 days at 30°C or anaerobically for 7 days using the Gaspack anaerobic system (BBL). Colonies from each plate were picked up on agar slants with the same composition, and the AR activity of isolates was tested. Purified isolates were maintained on nutrient agar slants.

Assay of acetylene reduction. A culture grown on nutrient agar was stabbed into both M and NFG semisolid medium test tubes. After 2 days of incubation at

30°C, a paper stopper was replaced with a tight rubber stopper, then 10% of the air in the test tube was replaced with acetylene. In the NFG test tube, air was replaced with argon first, and then 10% of the argon was replaced with acetylene. After 24 h of incubation at 30°C, ethylene formed by reduction of acetylene was detected by gas chromatography (Shimadzu GC-mini 2, Shimadzu, Kyoto, Japan) with an H₂-flame ionization detector, using 1.8-m column containing Porapak N (80–100 mesh) and N₂ gas as the carrier. These gas compositions used to assay acetylene reduction were the best composition for the most effective selection of nitrogen-fixing bacteria.

Identification methods. From the isolates 74 strains with high AR activities were selected for identification. Cell morphology, gram reaction, motility, and flagellar morphology were investigated in cells grown on nutrient agar or M semisolid agar at 30 or 37°C after one day's incubation. Gram staining was carried out by HUCKER and CONN's modification (21). Motility was tested by the hanging drop, and flagellar morphology was determined by the staining method of TODA (22). In addition, preparations negatively stained with a 1% solution of phosphotungstic acid were examined by electron microscopy. Cell wall and cell shape were also studied by the electron microscopy. The fixative used was a 3% glutaraldehyde solution in potassium phosphate buffer (pH 7.0). Fixed cells were washed several times in the buffer and postfixed in 2% OsO₄ for 2 h at room temperature. After the cells were washed with distilled water, they were stained in 0.5% aqueous uranyl acetate for 2 h and embedded in agar blocks. They were dehydrated by passage through a series of increasing concentrations of ethanol and absolute acetone, and embedded in SPURR's resin (23). Sections were cut with a Du Pont diamond knife on a Sorvall MT-2 ultramicrotome (Du Pont Co., Wilmington, Delaware, U.S.A.). They were stained with uranyl acetate and lead citrate (24), then viewed in a JEOL 200CX electron microscope (JEOL, Akishima, Japan) at 100 kV. Poly- β -hydroxybutyrate (PHB) granules were studied by the methods of BURDON (25) and OSTLE and HOLT (26). Spore formation was investigated by the method described by COWAN (27). Cyst formation was investigated by the method of STEVENSON and SOCOLOFSKY (28).

Biochemical characters were investigated basically according to the methods described by COWAN (27) and TARRAND et al. (29). Nutrient agar was used for preculture and as a basal medium. Bacterial strains were cultivated at 30 or 37°C without shaking. Production of fluorescent pigments was tested on King A and B media (Nissui Seiyaku, Tokyo, Japan) and mannitol yeast-extract medium (27). Production of pigment from gluconate was investigated on the medium described by KORTH et al. (30). Oxidation and fermentation (OF) basal medium (Difco 0688) was used to determine the production of acid from sugars and sugar alcohols. For spirals, the medium of TARRAND et al. (29) was used. Hydrolysis of esculin was tested as described by COWAN (27) and HYLEMON et al. (31). Hydrolysis of arginine was tested as described by THORNLEY (32). Nitrate reduction was tested in nitrate broth and succinate-nitrate broth (33) after 1, 3, and 7 days' incubation. Nitrate respiration was tested by the method of IIZUKA and KOMAGATA (34). Production of

hydrogen sulfide was tested on TSI agar (Nissui Seiyaku) for 2 weeks. Tetrazolium chloride reduction was investigated by the method described by SAKAZAKI (35). Christensen medium (Nissui Seiyaku) and Simmons medium (Nissui Seiyaku) were used for the citrate utilization. Malonate medium (Nissui Seiyaku) was used for the malonate utilization and phenylalanine deaminase. Decarboxylase base (Difco 0890) was used to examine lysine and ornithine decarboxylases and arginine dihydrolase. Cytochrome-oxidase reagent-"Nissui" (Nissui Seiyaku) was used to detect oxidase. Extracellular DNase activity was determined on DNase test agar (Difco 0632) plates after 1, 3, and 7 days' incubation. For spirals, urease was investigated by the method of TARRAND et al. (29). Production of 3-ketolactose from lactose was examined as described by BERNAERTS and DE LEY (36). Assimilation of carbon compounds was tested according to the method described by STANIER et al. (37) and URAKAMI and KOMAGATA (38) at 30°C for 3 weeks with some modification. Carbohydrates, organic acids, and alcohols were used at a concentration of 0.2% or 1%, but formaldehyde, formamide, and phenol were used at 0.02% because of their toxicity. Assimilation of polygalacturonate and pectin was tested according to the method of EDWARDS and EWING (39). Utilization of hydrogen was tested by growth under an atmosphere of hydrogen, oxygen, and carbon dioxide (85: 5: 10) in N-free mineral medium (40).

DNA base composition. DNAs were isolated by the method of SAITO and MIURA (41) and the mol% guanine + cytosine (G + C) values were calculated from the thermal denaturation temperature of DNA (T_m) by the formula of MARMUR and DOTY (42). Reference DNA was extracted from *Escherichia coli* IAM 1268 (= ATCC 11303 = strain B), and its DNA base composition was presumed to be 50.9%.

DNA-DNA hybridization. Purified DNA was labeled with (1',2',5-³H) cytidine 5'-triphosphate in vitro by the nick translation technique described by RIGBY et al. (43), using the ³H-Nick Translation kit (Code TRK 625 and Code N5000, Amersham International plc, Buckinghamshire, England). The DNA-DNA hybridization was carried out by the procedure described by KANEKO (44). The radioactivity was determined with a PACKARD TRI-CARB 300 model scintillation spectrometer (Packard Instrument Co., Downers Grove, Illinois, U.S.A.). The relative binding was calculated as follows: Relative binding (%) = ((cpm of heterologous filters)/(cpm of homologous filters)) × 100.

Quinone system. The quinone system was determined by the method of YAMADA et al. (45).

Cellular fatty acid composition and sphingolipid. The cellular fatty acid composition was determined by the method of IKEMOTO et al. (46). Cells cultivated in nutrient broth at 30°C for 24 h with shaking were used to analyze cellular fatty acids. Sphingolipid was determined by the method described by YAMAMOTO et al. (47).

Visible absorption spectrum of yellow-pigment. Yellow-pigment was extracted from bacterial cells by the method described by YABUCHI et al. (48). The visible

absorption spectrum of yellow-pigment in acetone-extract was determined with a Shimadzu Recording Spectrophotometer Model A300 (Shimadzu, Kyoto, Japan).

Reference strains. Reference strains used in this study are listed below. The superscript "T" indicates the type strain of the species. The abbreviations for culture collections were as follows: ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.; CDC, Centers for Disease Control, Atlanta, Georgia, U.S.A.; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, West Germany; IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; KM, Kansai Medical School, Osaka, Japan; NCIB, National Collection of Industrial Bacteria, Aberdeen, U. K. *Agrobacterium radiobacter* IAM 12048^T, *Agrobacterium tumefaciens* IAM 1037, *Alcaligenes denitrificans* IAM 12370, *Alcaligenes eutrophus* IAM 12368^T, *Alcaligenes faecalis* IAM 12369, *Alcaligenes latus* IAM 12599, *Alcaligenes paradoxus* DSM 30034, *Azomonas agilis* DSM 375^T, *Azotobacter chroococcum* IAM 12666^T, *Azotobacter vinerandii* DSM 87^T, *Azospirillum brasilense* IAM 12400^T, *Azospirillum lipoferum* IAM 12399^T, *Beijerinckia indica* NCIB 8712^T, *Derxia gummosa* NCIB 9064^T, *Enterobacter cloacae* IAM 12349, *Erwinia herbicola* IAM 1562 and IAM 1584, *Klebsiella oxytoca* RH 1586 (=ATCC 13182), *Klebsiella pneumoniae* CDC 2551-63 and IAM 12351^T, *Protomonas extroquens* TK 0001^T (=NCIB 9399), *Pseudomonas paucimobilis* KM 2395^T and KM 2397, *Pseudomonas* sp. strain H3 (17), *Xanthobacter autotrophicus* DSM 431 and 432^T, and *Xanthobacter flavus* NCIB 10071^T.

RESULTS AND DISCUSSION

Grouping and identification of isolates

Of the 7,344 isolates from rice materials and paddy soil, 1,005 strains showed AR activity. From these strains 74 strains with high AR activity were selected for identification. The strains studied were divided into 10 groups by morphological, biochemical, and chemotaxonomic characters as shown in Tables 1 and 2.

Group 1. Azospirilla

Azospirillum brasilense Tarrand, Krieg and Döbereiner 1979

Strain: SL-6

The strain was gram negative and formed spiral or vibrio-like cells. The strain had a polar flagellum and exhibited high spirillum-like motility. Cells were 1.0 μm by 1.5 to 5.0 μm . Old cells became longer than 10 μm . PHB granules were accumulated in cells. Colonies on nutrient agar plate were white and dull. On semisolid medium cultures they produced whitish rings 1–2 cm below the surface.

Strain SL-6 did not produce acid from glucose aerobically or anaerobically and did not use glucose as a sole carbon source determined by the method of TARRAND et al. (29). Strain SL-6 did not produce acid from carbon compounds except ribose and fructose as shown in Table 3. Strain SL-6 did not require biotin.

The chemotaxonomic characters of strain SL-6 are shown in Table 2. Strain

Table 1. Phenotypic characters of nitrogen-fixing bacteria isolated from rice.

Group	Number of strains tested	Cell shape	Pleomorphism	Motility	Flagellation	PHB granules	Color of colonies	Extracellular slime	Fluorescent pigment	OF test (glucose)	Hydrolysis of: Gelatin	Starch	Esculin	Esculin (Hylemone)	Tween 80 (Sierra)	Growth in bile	Growth on tellurite agar	VP	MR	Indole
Group 1 Azospirilla																				
<i>A. brasilense</i>	1	S	-	+	Po	+	-	-	-	NA	-	-	-	+	-	-	-	-	-	-
<i>A. lipoferum</i>	1	S	-	+	Po	+	-	-	-	F	+	-	-	+	-	-	-	-	-	-
<i>Azospirillum</i> sp.	5	S	-	+	Po	+	-	-	-	F	-	-	+	+	-	V	-	-	-	-
Group 2 Xanthobacters																				
<i>X. autotrophicus</i>	8	R	+	-	-	+	yellow	+	-	NA	-	-	-	-	-	-	-	-	-	-
Group 3 Enterobacteria																				
<i>Enterobacter cloacae</i>	4	R	-	+	Pe	-	-	-	-	F	+	+	+	-	-	-	-	+	-	-
<i>Erwinia herbicola</i>	2	R	-	+	Pe	-	yellow	-	-	F	+	+	+	-	-	-	-	+	-	+
<i>Klebsiella oxytoca</i>	6	R	-	-	-	-	-	-	-	F	V	-	+	-	-	-	-	+	-	+
Group 4 Gram negative spiral-shaped bacteria	7	S	-	+	Po	-	-	-	-	NA	-	-	-	-	-	-	-	-	-	-
Group 5 Gram negative pleomorphic bacteria	6	R	+	-	-	+	-	+	-	NA	-	-	-	-	-	-	-	-	-	-
Group 6 Gram negative fermentative pleomorphic bacteria	8	R	+	-	-	+	-	-	-	F	-	-	-	-	-	-	-	-	-	+
Group 7 <i>Alcaligenes</i> sp.	4	R	-	+	Pe	+	yellow	-	-	O	V	+	+	-	+	-	-	-	-	-
Group 8 <i>Protomonas</i> -like bacteria	8	R	-	+	Po	-	red	-	-	NA	-	-	-	-	-	-	-	-	-	-
Group 9 <i>Azotobacter</i> -like bacteria	5	R	-	+	Po	+	-	-	-	NA	-	-	-	-	-	-	-	-	-	-
Group 10 Others	9	R	-	V	Po	-	-	-	-	NA or O	-	V	V	-	V	-	-	-	-	-

Table 1. (continued)

Group	Citrate as C source: Christensen	Simmons	β -Galactosidase	H ₂ S production on TSI agar	Gas production on TSI agar	Malonate	Glucanate	Phenylalanine	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Nitrate reduction:	Nitrate broth	Succinate broth	Nitrate respiration	Tetrazolium chloride reduction	Production of: reduction	Catalase	Oxidase	Urease (Christensen)	Urease (BES)	Tyrosinase	Phosphatase	DNase
Group 1 Azospirilla																								
<i>A. brasilense</i>		+																+	+	+	+	+	+	+
<i>A. lipoferum</i>		+																+	+	+	+	+	+	+
<i>Azospirillum</i> sp.		+	+															+	+	+	+	+	+	+
Group 2 Xanthobacters																								
<i>X. autotrophicus</i>		+																+	+	+	+	+	+	+
Group 3 Enterobacteria																								
<i>Enterobacter cloacae</i>		+	+						+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Erwinia herbicola</i>		+	+						+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Klebsiella oxytoca</i>		+	+						+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Group 4 Gram negative spiral- shaped bacteria																								
Group 5 Gram negative pleo- morphic bacteria																								
Group 6 Gram negative fer- mentative pleomorphic bacteria		+																						
Group 7 <i>Alcaligenes</i> sp.	V																							
Group 8 <i>Protonomas</i> -like bacteria																								
Group 9 <i>Azotobacter</i> -like bacteria																								
Group 10 Others																								

S, spiral; R, rod; +, positive reaction; -, negative reaction; Po, polar; Pe, peritrichous; F, fermentative; NA, not attacked; O, oxidative; V, variable.

Group of strains	GC content (%)	Quinone system	Straight-chain acids										Cellular
			12:0	14:0	14:1	15:0	16:0	16:1	17:0	18:0	18:1	19:0	
Group 1	SL-6	66.3	Q-10	0.87	0.79		0.12	6.80	13.35	0.08	0.30	59.31	
	COC 8	66.8	Q-10	1.03	0.70	1.66	1.94	5.46	17.34	0.12	0.32	44.14	0.37
	SL-1	65.6	Q-10	0.60	0.69	0.04	0.18	12.81	12.08		0.36	60.78	
	SL-2	65.5	Q-10	0.58	0.66	0.05	0.14	8.25	14.01	0.31	0.34	61.66	
	SL-9	67.5	Q-10										
	SL-13	65.5	Q-10	0.13	1.25	0.24	0.30	11.82	9.61	0.49	6.41	56.49	
	SL-18	67.5	Q-10										
Group 2	Y-29	67.5	Q-10	0.42	0.03			2.99	0.54	0.64	7.71	79.47	
	Y-30	64.3	Q-10	1.15	0.17		0.25	2.54	0.55		4.44	77.33	
	Y-31	63.1	Q-10					3.28	0.56	0.57	4.52	79.17	
	Y-32	65.1	Q-10	0.02	0.18	T	0.34	3.89	0.48	0.74	3.52	70.66	
	Y-33	64.3	Q-10	0.20	0.07	0.04	0.02	2.98	0.99	0.52	4.62	74.11	
	Y-34	63.1	Q-10	0.06	0.10			3.03	0.89	0.44	4.68	81.78	
	Y-35	67.5	Q-10		T			3.41	0.64	0.64	4.47	77.71	
	Y-36	66.3	Q-10					3.33	0.67	0.69	4.74	79.22	
Group 3	Fe-6		Q-8	5.77	5.43	0.03	1.05	41.28	4.36	0.88	0.65	8.32	
	Fe-3		Q-8										
	Fe-4		Q-8	6.72	7.77	0.06	1.53	27.25	9.80	0.74	0.39	15.62	
	Fe-10		Q-8	3.42	6.02	0.05	3.40	29.50	9.11	3.01	1.38	20.12	
	NG 13		Q-8	5.32	6.55		0.16	21.97	6.81	T	0.36	11.44	
Group 4	SS-20	63.1	Q-8	0.38	2.29		0.18	34.06	47.91		0.30	14.52	
	SS-22	63.1	Q-8	0.33	1.50	0.24	0.05	24.36	46.66		0.56	25.88	
	SS-25		Q-8	0.21	1.62	0.05		32.72	47.70		0.37	16.97	
	SS-28	65.1	Q-8	T	2.77			36.65	46.45		0.32	13.57	
	SS-29	63.1	Q-8	0.46	3.08	0.32	0.10	31.16	48.38		0.29	15.89	
	SS-32	65.3	Q-8					32.36	47.70		0.42	17.16	
Group 5	B-26	61.1	Q-10	0.51	0.06	0.02	0.04	3.52	0.74	0.19	4.34	29.33	
	B-45	61.6	Q-10										
	B-40	61.9	Q-10					3.26	0.83	0.25	7.21	59.16	
	B-46	60.2	Q-10		0.04	0.04	0.08	1.44	0.56	0.05	3.57	62.76	
	B-47	62.2	Q-10				0.06	2.58	1.22	0.09	2.32	45.62	
	B-51	59.9	Q-10	0.62	0.07	0.03	0.03	3.60	0.84	0.08	4.08	31.05	
Group 6	F-4	62.1	Q-10		0.32	T	0.09	12.94	1.14	1.41	9.21	56.96	
	F-7	63.1	Q-10	0.39	0.38		0.15	12.79	0.56	1.12	11.63	26.34	
	B-2	62.8	Q-10		0.65	0.08	0.23	13.77	1.01	1.52	12.92	36.36	

nitrogen-fixing bacteria and reference strains.

fatty acid composition

Cyclopropane acids		Hydroxy acids							Unknown peaks				
17:0	19:0	3-OH 10:0	2-OH 12:0	3-OH 12:0	2-OH 14:0	3-OH 14:0	3-OH 16:0	3-OH 18:0	1	2	3	4	Others
1.08	0.86					6.30	3.36	1.97	1.77	1.29			1.74
0.63	0.65					11.92	6.16	3.45	1.45	1.51			1.14
0.60	0.63					5.66	3.07	1.61	1.52	1.28			1.28
0.93	0.94					4.51	3.08	1.33	1.19	0.73			1.30
0.76	0.26					3.59	2.25	0.42	0.71	3.31			2.22
0.18	1.47						1.76		0.67				4.77
0.32	4.73						4.16						4.35
0.21	4.03						3.76						3.90
0.16	7.32						5.70		5.62				1.66
0.30	5.02						4.03		0.89				6.18
0.26	2.58						2.93		0.40				2.40
0.16	4.50						4.18		T				4.28
0.22	4.19						3.34						3.63
9.95	7.98					8.05							2.41
6.81	7.77					13.81							1.04
7.27	5.34					8.04							1.37
2.25	8.50					32.42							4.02
0.10					0.26								
0.07					0.27								
0.03					0.23								
					0.25								
					0.26								
					0.24								
0.06	18.91					4.59	4.67	5.38			14.63		13.01
0.03	4.48					5.03	1.92	5.48			6.28		6.07
0.45	5.04					4.49	3.19	4.48			5.27		8.54
0.67	5.06					11.71	4.02	11.75			10.97		3.93
0.08	17.35					3.86	4.56	6.16			14.60		12.99
0.15	5.17					3.43	1.09	0.77			4.52		2.80
0.32	15.53					2.83	3.62	1.38			12.57		10.39
0.28	9.70					3.05	2.56	1.28			9.09		7.50
0.88	8.50					3.49	2.40	1.18			8.50		6.08
0.24	9.50					3.76	2.22	1.20			8.12		5.48
0.29	5.51					3.91	4.94	1.11			0.17		3.97
0.28	8.34					2.35	2.08	0.76			8.54		7.73
0.31	10.22					6.27	3.24	2.53			10.66		6.55
0.57	0.52				8.36							8.90	3.44
0.69	1.12				14.43							11.11	
0.28	0.31				16.05							8.65	1.70
0.34	0.34				16.16							9.22	
0.28						2.20						6.66	1.38
0.24						3.96						2.20	1.46
0.24						2.11						8.16	0.99

Table 2.

Group of strains	GC content (%)	Quinone system	Cellular										
			Straight-chain acids										
			12:0	14:0	14:1	15:0	16:0	16:1	17:0	18:0	18:1	19:0	
Group 8 R-11	64.1	Q-10	0.06	0.99			15.01	3.33	0.41	13.35	56.41	0.46	
R-12	64.3	Q-10	0.28	1.08			15.61	3.22	0.46	14.45	57.82	0.36	
R-13	63.6	Q-10	0.17	0.76			13.43	2.22	0.23	13.83	60.63		
R-15	62.6	Q-10	0.27	0.95			13.91	3.17	0.40	11.22	56.56	0.52	
Group 9 A-2	64.1	Q-8	2.14	0.71	0.60	0.31	29.28	54.98		1.24	7.27		
A-3	65.1	Q-8	1.92	0.92	0.37	0.49	27.89	54.13		2.56	8.70		
A-7	65.1	Q-8	1.82	0.48	0.25		26.22	58.00		T	9.26		
A-24		Q-8	2.34	0.58	0.22	0.22	24.09	58.04		1.31	8.40		
<i>Agrobacterium radiobacter</i> IAM 12048 ^T	59.2	Q-10	0.02	1.50	0.22	0.21	30.33	0.11	0.04	0.50	8.49	3.18	
<i>A. tumefaciens</i> IAM 1037	59.4	Q-10		0.50	0.06	0.44	30.65		0.09	0.54	5.32	1.69	
<i>Alcaligenes denitrificans</i> IAM 12370	67.5	Q-8					0.18	34.23		0.76	1.45	14.22	
<i>A. eutrophus</i> IAM 12368 ^T	65.8	Q-8	0.20	7.14			1.35	38.15	22.44		0.44	6.75	2.09
<i>A. faecalis</i> IAM 12369	57.2	Q-8	0.04	1.64			0.68	36.21	3.87	0.86	0.95	12.80	9.79
<i>A. latus</i> IAM 12599	69.2	Q-8	1.70	3.68	0.05	0.08	22.86	40.33	0.09	5.96	24.85		
<i>A. paradoxus</i> DSM 30034	68.0	Q-8	3.11	1.50			9.81	25.82	7.27	6.13	0.54	8.56	0.23
<i>Azomonas agilis</i> DSM 375		Q-9		3.12			67.72	12.48				6.52	
<i>Azospirillum brasilense</i> IAM 12400	69.0	Q-10	1.96	0.98	0.11	0.10	3.73	14.59		T	40.21		
<i>A. lipoferum</i> IAM 12399 ^T	69.0	Q-10	0.88	0.52	1.00	1.02	6.40	9.11		0.35	49.60	0.18	
<i>Azotobacter vinelandii</i> DSM 87		Q-8		3.48	0.14	T	41.48	28.97	0.82			8.88	
<i>Beijerinckia indica</i> MCIB 8712		Q-10											
<i>Derrxia gummosa</i> NCIB 9064	69.2	Q-8	4.57	0.52		0.07	15.37	31.76		T	33.74		
<i>Klebsiella oxytoca</i> RH 1586		Q-8	4.08	5.21	8.24	0.10	27.92	11.33	T	1.03	13.98		
<i>Protomonas extroquens</i> TK 0001	65.6	Q-10					4.3a	13.1		2.1	72.7		
<i>Pseudomonas paucimobilis</i> KM 2395 ^T	64.3	Q-10		9.90			16.35	2.09		T	65.68		
KM 2397	65.8	Q-10	0.33	4.97			9.27	6.99		0.49	58.76		
<i>Xanthobacter autotrophicus</i> DSM 431	67.2	Q-10		0.25			4.70	4.56	0.14	4.28	79.24		
DSM 432 ^T	67.0	Q-10		0.24			4.93	4.81	0.13	3.83	80.52		
<i>X. flavus</i> NCIB 10071		Q-10	0.06	1.19			3.19	0.25	0.17	5.58	85.59		

Symbols: a, This value was obtained from ref. 63; b, Numbers are percentages of total acids; T, acid

(continued)

fatty acid composition

Cyclopropane acids		Hydroxy acids							Unknown peaks				
17:0	19:0	3-OH 10:0	2-OH 12:0	3-OH 12:0	2-OH 14:0	3-OH 14:0	3-OH 16:0	3-OH 18:0	1	2	3	4	Others
0.26						2.18						5.73	1.81
0.30						1.80						3.99	
						2.19						6.55	
0.31						2.21						9.65	0.83
	0.11			2.64	0.28								
	0.18			2.28	0.24								
				3.73	0.25								
				2.37	0.19								
3.46	20.40					4.48	5.49				12.23	5.89	3.45
3.53	22.74					7.27	8.33				12.61	2.77	3.46
20.50	1.75					16.74							10.15
7.48					4.43	8.56							
21.31			0.61			0.92							10.35
0.34		0.06											
15.13	4.09	0.30		11.24			0.28						5.99
	0.81	T		1.05									8.30
0.84						16.57	8.80	0.51	0.31	10.25			1.04
9.88	0.99					6.91	4.91	1.96	2.89	2.02			0.93
5.46	2.91			2.32	2.89	0.84							1.81
0.32				5.94	3.68	2.29							1.74
6.03	4.12					7.06							10.90
						4.8							
						5.71							
0.31						18.47							
0.34	0.86							1.28					4.36
0.42	0.94							2.73					1.45
1.49	1.14							1.30					

present at less than 0.01%.

Table 3. Production of acid from carbon compounds by strains of group 1 (*Azospirilla*) and reference strains.

Carbon compound	Strain								<i>A. brasilense</i> ^a	<i>A. lipoferum</i> ^a
	SL-6	COC 8	SL-1	SL-2	SL-9	SL-13	SL-18			
L-Arabinose	—	+	—	+	+	—	+	V	+	
D-Xylose	—	+	+	+	+	+	+	—	V	
D-Ribose	+	+	+	+	+	+	+	+	+	
D-Glucose	—	+	+	+	+	+	+	V	+	
D-Fructose	+	+	+	+	+	+	+	+	+	
D-Galactose	—	+	+	+	+	+	+	V	+	
Lactose	—	—	—	—	—	—	—	—	—	
Maltose	—	—	—	—	—	—	—	—	—	
L-Rhamnose	—	+	—	—	—	—	—	—	—	
Cellobiose	—	—	+	+	—	—	—	—	—	
Sorbitol	—	—	—	—	—	—	—	V	—	
Mannitol	—	—	+	—	+	+	—	—	V	
Inositol	—	—	+	+	—	+	+	—	V	

Symbols: +, positive reaction; —, negative reaction; V, variable.

^a These data were taken from Bergey's Manual of Systematic Bacteriology, Vol. 1 (51).

SL-6 had 66.3 mol% G + C and the ubiquinone system (Q-10) as shown in Table 2. Straight-chain unsaturated C_{18:1} fatty acid was the major fatty acid, and 3-hydroxy C_{14:0} (3-OH C_{14:0}), 3-OH C_{16:0}, and 3-OH C_{18:0} were also found.

On the basis of the above characters the strain SL-6 belongs to the genus *Azospirillum*. This genus includes 4 species, *A. amazonense*, *A. brasilense*, *A. halopraeferens*, and *A. lipoferum* (29, 49, 50). *Azospirillum lipoferum* differed from *A. brasilense* in the production of acid from glucose and mannitol, utilization of glucose as a sole carbon source for growth in nitrogen-free semisolid medium, requirement of biotin, and DNA-DNA hybridization (29), from *A. amazonense* in GC content, cell size, and the presence of the pili (49), and from *A. halopraeferens* in the tolerance of NaCl (50). Chemotaxonomic characters of *A. lipoferum* IAM 12399^T and *A. brasilense* IAM 12400^T are also shown in Table 2. *Azospirillum lipoferum* could not be differentiated from *A. brasilense* in the chemotaxonomic characters of the GC content, fatty acid composition, and quinone system.

Strain SL-6 was identified as *A. brasilense* Tarrand, Krieg and Döbereiner 1979 because it did not produce acid from glucose, did not utilize glucose as a sole carbon source, and did not require biotin.

Source: Strain SL-6 was isolated in 1982 from the root of *Oryza sativa* strain C5444.

Azospirillum lipoferum Tarrand, Krieg and Döbereiner 1979

Strain: COC 8

The morphological characters of strain COC 8 are similar to those of strain SL-

Fig. 1. Photomicrographs of strains of groups 1 and 4.

A: *Azospirillum* sp. strain SL-1 (group 1) grown on M semisolid agar medium for 1 day at 30°C (optical microscopy, stained with crystal violet). B: *Azospirillum lipoferum* strain COC 8 (group 1) grown on nutrient agar medium for 1 day at 30°C (optical microscopy, stained with crystal violet). C: PHB granules in *Azospirillum lipoferum* strain COC 8 stained with Nile blue A and observed under fluorescent light. D: Gram negative spiral-shaped bacterial strain SS-22 (group 4) grown on nutrient agar medium for 1 day at 30°C (optical microscopy, stained with crystal violet). Bars indicate 10 μ m.

6. The strain formed spiral or vibrio-like cells as shown in Figs. 1B, 1C, and 2. It had a polar flagellum as shown in Fig. 2.

The strain produced acid from glucose aerobically and anaerobically, and used

Fig. 2. Electron micrograph of a negatively stained cell of *Azospirillum lipoferum* strain COC 8 showing polar flagellum.

Bar indicates 1 μ m. Cell grown on nutrient medium for 1 day at 30°C.

glucose as a sole carbon source by the method of TARRAND et al. (29). Strain COC 8 produced acid from L-arabinose, D-xylose, D-ribose, D-glucose, D-fructose, D-galactose, and L-rhamnose but not from sorbitol, lactose, maltose, mannitol, inositol, cellobiose, and sucrose. The strain required biotin. It utilized hydrogen.

Strain COC 8 had 66.8 mol% G + C and a ubiquinone system (Q-10) as shown in Table 2. The fatty acid composition was similar to those of strains SL-6, *A. brasilense* IAM 12400^T, and *A. lipoferum* IAM 12399^T.

Consequently strain COC 8 was identified as *A. lipoferum* Tarrand, Krieg and Döbereiner 1979 from the characters mentioned above.

Source: Strain COC 8 was isolated in 1980 from the roots of *Oryza sativa* strain C5444.

Azospirillum sp.

Strains: SL-1, SL-2, SL-9, SL-13, and SL-18

The morphological and biochemical characters of these strains were similar to those of strain COC 8. The cell shape of strain SL-1 is shown in Fig. 1A. The production of acid from L-arabinose, D-xylose, D-glucose, D-galactose, L-rhamnose, cellobiose, mannitol, and inositol differed among strains as shown in Table 3. The utilization of hydrogen was investigated in strains SL-1, SL-2, and SL-18. These three strains utilized hydrogen.

The chemotaxonomic characters of isolates are shown in Table 2. The GC content of the strains ranged from 65.5 to 67.5%. The fatty acid composition was similar to that of strain COC 8, and the quinone system was Q-10.

The strains studied belong to the genus *Azospirillum*. They utilized glucose as a sole carbon source for growth and produced acid from glucose and fructose aerobically and anaerobically, although they did not require biotin. From these characters, these strains were not identified as belonging to a known species in the genus *Azospirillum*.

Source: Strains SL-1, SL-2 and SL-13 were isolated in 1982 from the roots of *Oryza sativa* strain C5444. Strain SL-18 was isolated in 1981 from the roots of *Oryza sativa* strain C5444. Strain SL-9 was isolated in 1982 from the roots of *Oryza sativa* strain T65.

Group 2. *Xanthobacters*

Xanthobacter autotrophicus (Baumgarten, Reh and Schlegel 1974) Wiegell, Wilke, Baumgarten, Opitz and Schlegel 1978

Strains: Y-29, Y-30, Y-31, Y-32, Y-33, Y-34, Y-35, and Y-36

These strains had non-motile, pleomorphic, gram negative cells as shown in Fig. 3. The cells were 0.5 to 1.0 μm by 1.0 to 5.0 μm . PHB granules were accumulated in the cells. Colonies were yellow due to a water-insoluble pigment. The strains did not produce acid from glucose oxidatively or fermentatively. They did not grow on Tellurite agar medium. Nor did they hydrolyze gelatin or starch. They reduced nitrate and tetrazolium chloride, and produced catalase, oxidase, and phosphatase. They did not produce urease, tyrosinase, or DNase. Utilization of hydrogen was investigated for strains Y-29, Y-31, and Y-32. These strains grew at the expense of hydrogen. Table 4 shows the results of assimilation of 55 carbon compounds. Assimilation of L-arabinose, D-xylose, D-ribose, maltose, mannose, glyoxylate, β -alanine, and L-valine differed among the strains as shown in Table 4.

The GC content of the strains studied ranged from 63.1 to 67.5% as shown in Table 2. They had the ubiquinone system Q-10. The isolates contained a large amount of straight-chain unsaturated fatty acid of $\text{C}_{18:1}$ (67–82%), and only a small amount of 3-OH $\text{C}_{16:0}$ acid.

From these characters the isolates apparently belong to the genus *Xanthobacter*. The genus *Xanthobacter* includes two species, *X. autotrophicus* and *X. flavus*. *Xanthobacter autotrophicus* differs from *X. flavus* in requiring the growth

Fig. 3. Electron micrographs of *Xanthobacter autotrophicus* strains of group 2.
A: strain Y-30. B: strain Y-34. These cells were grown on nutrient agar medium for 1 day at 30°C. Bars indicate 1 μ m.

Table 4. (continued)

	Glutamate	2-Oxoglutarate	Glyoxylate	Pimelate	Adipate	Suberate	Citrate	DL-Lactate	DL-Malate	DL-Tartrate	Pyruvate	DL-Mandelate	Gluconate	DL- β -Hydroxybutyrate	Propionate	2-Ketoglutarate	Iaconate	n-Propanol	Methanol	Ethanol	Sorbitol	Mannitol
Group 2	+	+	+	-	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+
Group 5	+	+	-	-	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+
Group 6	-	+	-	-	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+
Group 7	-	+	-	+	-	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+
Group 8	+	+	+	+	-	+	+	-	-	-	-	-	(3/4)	+	+	-	-	+	+	(3/4)	-	-
<i>Agrobacterium radiobacter</i> IAM 12048 ^T																						
<i>A. tumefaciens</i> IAM 1037																						
<i>Alcaligenes denitrificans</i> IAM 12370																						
<i>A. eutrophus</i> IAM 12368																						
<i>A. faecalis</i> IAM 12369																						
<i>A. latus</i> IAM 12599																						
<i>A. paradoxus</i> DSM 30034																						
<i>Protomonas extroquens</i> TK 0001 ^T																						
<i>Pseudomonas paucimobilis</i> KM 2395 ^T																						
<i>P. paucimobilis</i> KM 2397																						
<i>Xanthobacter autotrophicus</i> DSM 432 ^T	+	+	-	-	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	-	-
<i>X. autotrophicus</i> DSM 431	+	+	-	-	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	-	-

Table 4. (continued)

	Inositol	Glycerol	Benzoate	Mucate	Phenol	m-Hydroxybenzoate	Betaine	Formaldehyde	Formamide	Methylamine	Ethylamine	Glycine	β -Alanine	L-Threonine	L-Serine	L-Arginine	L-Glutamine	L-Aspartate	L-Leucine	L-Lysine	L-Ornithine	L-Methionine	L-Valine	L-Phenylalanine	β -Aminobutyrate	L-Histidine	L-Proline	L-Cysteine
Group 2	+	+	+				+						V	+	+	+	+	+	+	+	+	+	V	+	+	+	+	+
Group 5	+	+	+				+							+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Group 6	+	+	+				+							+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Group 7	+	+	+				+							+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Group 8	+	+	+				+							+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Agrobacterium radiobacter</i> IAM 12048 ^T		+	+				+	+	+	+	+	W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>A. tumefaciens</i> IAM 1037		+	+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Alcaligenes denitrificans</i> IAM 12370		+	+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>A. eutrophus</i> IAM 12368		+	+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>A. faecalis</i> IAM 12369		+	+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>A. latus</i> IAM 12599		+	+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>A. paradoxus</i> DSM 30034		+	+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Protomonas extroquens</i> TK 0001 ^T		+	+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Pseudomonas paucimobilis</i> KM 2395 ^T		+	+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>P. paucimobilis</i> KM 2397		+	+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Xanthobacter autotrophicus</i> DSM 432 ^T		+	+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>X. autotrophicus</i> DSM 431		+	+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Symbols: V, variable; + W, assimilated weakly.

Table 5. Assimilation of carbon compounds by strains of group 2 (*Xanthobacters*) and *X. autotrophicus* (DSM 431 and DSM 432^T).

Carbon compound	Y-29	Y-30	Y-31	Y-32	Y-33	Y-34	Y-35	Y-36	DSM 431	DSM 432 ^T
L-Arabinose	—	+	—	+	+	—	+	—	—	—
D-Xylose	+	+	+	+	+	+	+	+	—	—
D-Ribose	—	+	—	+	+	—	+	—	—	—
D-Glucose	—	—	—	—	—	—	—	—	—	+W
D-Galactose	—	—	—	—	—	—	—	—	—	+W
Trehalose	—	—	—	—	—	—	—	—	—	+W
Maltose	+	+	+	—	+	—	—	—	—	—
Mannose	+	—	—	—	—	—	—	—	—	—
DL- β -Hydroxybutylate	+	+	+	+	+	+	+	+	—	—
Glyoxylate	—	—	—	—	—	—	+	—	—	—
β -Alanine	+	+	+	+	—	+	—	—	—	—
L-Valine	—	+	—	—	+	—	—	—	—	—

Symbols: +, assimilated; +W, assimilated weakly; —, not assimilated.

factor. Like *X. autotrophicus* DSM 431 and DSM 432^T, the strains studied did not require the growth factor. Moreover the strains of this group resemble *X. autotrophicus*. The strains produced catalase, oxidase, and phosphatase and did not produce DNase, urease, tyrosinase. The strains of this group and the *X. autotrophicus* reference strains (DSM 431 and DSM 432^T) assimilated D-fructose, sucrose, acetate, succinate, fumarate, glutamate, 2-oxo-glutarate, citrate, DL-lactate, DL-malate, pyruvate, gluconate, *n*-propanol, methanol, ethanol, L-glutamine, and L-proline, but did not assimilate L-rhamnose, sorbose, lactose, cellobiose, salicin, formate, oxalate, malonate, DL-tartrate, DL-mandelate, sorbitol, mannitol, inositol, glycerol, benzoate, L-threonine, L-serine, L-arginine, L-leucine, L-lysine, L-ornithine, L-methionine, L-phenylalanine, β -aminobutyrate, L-histidine, and L-cysteine. However, the strains of this group assimilated DL- β -hydroxybutyrate but *X. autotrophicus* DSM 431 and 432^T did not. The strains of this group and *X. autotrophicus* DSM 431 did not assimilate D-glucose, D-galactose, and trehalose but *X. autotrophicus* DSM 432^T did so weakly.

Consequently, strains of group 2 were identified as *X. autotrophicus* (Baumgarten, Reh and Schlegel 1974) Wiegell, Wilke, Baumgarten, Opitz and Schlegel 1978.

This is the first report of the isolation of *X. autotrophicus* from the roots of rice.

Source: Strains Y-29, Y-30, and Y-31 were isolated in 1982 from the roots of *Oryza sativa* strain C5444. Strains Y-32, Y-33, Y-34, Y-35, and Y-36 were isolated in 1981 from the roots of *Oryza sativa* strain T65.

Group 3. Enterobacteria

Enterobacter cloacae (Jordon 1890) Hormaeche and Edwards 1960

Strains: OO 13a, X-1, X-7, and Fe-6

Fig. 4. Photomicrographs of nitrogen-fixing bacteria grown on nutrient agar medium for 1 day at 30°C.

A: *Enterobacter cloacae* strain OO 13a (group 3). B: *Protomonas*-like bacteria strain R-5 (group 8). C: *Azotobacter*-like bacteria strain A-2 (group 9). D: *Alcaligenes* sp. strain OSG 47 (group 7). Bars indicate 10 μ m.

Strains OO 13a, X-1, X-7, and Fe-6 were fermentative gram negative rods, and were motile with peritrichous flagella as shown in Fig. 4A. They formed circular, smooth, convex, and entire colonies on nutrient agar medium. Colonies on nutrient agar plate were light brown. The morphological and phenotypic characters of the strains studied are shown in Tables 1 and 6. Strains OO 13a, X-1, X-7, and Fe-6 liquefied gelatin and esculin. They showed positive reactions for VP-reaction, nitrate respiration, production of gas on TSI agar, β -galactosidase, catalase, arginine dihydrolase, ornithine decarboxylase, and oxidation of gluconate. They did not produce the pigment on gluconate medium, indole, hydrogen sulfide, phenylalanine deaminase, oxidase, urease, and DNase. They did not decarboxylate lysine. Strains OO 13a, X-1, and X-7 did not produce acid from L-arabinose but strain Fe-6 did.

Strain Fe-6 contained a large amount of straight-chain $C_{16:0}$, $C_{18:0}$, and 3-OH

Table 6. Phenotypic characters of strains of group 3 (Enterobacteria) and reference strains.

Strain	Indole production	Hydrolysis of gelatin	20°C	30°C	Pigmentation on gluconate medium	Assimilation of:	Polygalacturonate	Inulin	Acid from:	l-Arabinose	D-Xylose	D-Rhamnose	D-Glucose	Trehalose	Sucrose	Lactose	Maltose	Cellobiose	Melibiose	Raffinose	Salicin	Amygdalin	Inulin	Polygalacturonate	Sorbitol	Inositol	Adonitol	D-Mannitol	Dulcitol	Glycerol
Group 3																														
OO 13a	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
X-1	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
X-7	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fe-6	+	+	+	+	NT	+	NT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fe-3	+	+	+	+	NT	+	NT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fe-4	+	+	+	+	NT	+	NT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OSG 51	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OG 9	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NO 17	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NSG 3	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NG 13	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fe-10	+	+	+	+	NT	+	NT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Enterobacter cloacae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IAM 12349	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Erwinia herbicola	-	-	+	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
IAM 1562	-	-	+	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
IAM 1584	-	-	+	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Klebsiella oxytoca	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RH 1586	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K. pneumoniae	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CDC 2551-63	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IAM 12351	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Symbols: +, positive reaction; -, negative reaction; + W, produced acid weakly; NT, not tested.

C_{14:0} acids. The fatty acid composition of strain Fe-6 was similar to that of *Klebsiella oxytoca* RH 1586 as shown in Table 2. Strain Fe-6 had the ubiquinone system Q-8.

The strains differed from *Enterobacter cloacae* IAM 12349 in the assimilation of polygalacturonate and hydrolysis of gelatin at 20°C. However, the strains were identified as *Enterobacter cloacae* (Jordon 1890) Hormaeche and Edwards 1960 on the basis of motility with peritrichous flagella, and they showed oxidation of gluconate, production of arginine dihydrolase and ornithine decarboxylase, and liquefaction of gelatin at 30°C. They did not produce lysine decarboxylase, urease, indole, and the pigment from gluconate medium, and did not assimilate inulin. They did not produce acid from dulcitol, adonitol, and inulin.

Source: Strains OO 13a, X-1, and X-7 were isolated from paddy soil. Strain Fe-6 was isolated in 1981 from the roots of *Oryza sativa* strain T65.

Erwinia herbicola (Löhnis 1911) Dye 1964

Strains: Fe-3 and Fe-4

The strains studied were fermentative gram negative rods, motile with peritrichous flagella. They formed smooth, convex, and yellow colonies on nutrient agar medium.

The biochemical characters of the strains are shown in Tables 1 and 6. They hydrolyze gelatin and esculin. They showed positive VP-reaction, and produced indole, β -galactosidase, catalase and DNase. They did not produce hydrogen sulfide, oxidase, and urease. They did not show phenylalanine deaminase, oxidation of gluconate, lysine decarboxylase, arginine dihydrolase, ornithine decarboxylase, and nitrate respiration.

The fatty acid composition of strain Fe-4 was similar to that of strain *K. oxytoca* RH 1586 as shown in Table 2. Strains Fe-3 and Fe-4 had the ubiquinone system Q-8.

The strains differed from *Erwinia herbicola* IAM 1562 and IAM 1584 in producing indole. However, strains Fe-3 and Fe-4 were identified as *E. herbicola* (Löhnis 1911) Dye 1964 on the basis of motility with peritrichous flagella, and pigmentation of colonies. They liquefied gelatin and showed DNase but did not produce urease, arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase. They did not have nitrate respiration. They did not produce acid from dulcitol, adonitol, and inulin.

Klebsiella oxytoca and *E. cloacae* are the common nitrogen-fixing bacteria associated with a number of rice varieties (12, 13, 15, 52, 53). A nitrogen-fixing *E. herbicola* strain was isolated from water and a compost of paper mill process (54), and it was found in strains preserved at the Centers for Disease Control (CDC) (Atlanta, Georgia, U.S.A.) (55). However, this is the first report of isolation of nitrogen-fixing *E. herbicola* from the roots of rice.

Source: Strains Fe-3 and Fe-4 were isolated in 1981 from *Oryza sativa* strain C5444.

Klebsiella oxytoca (Flugge 1886) Lautrop 1956

Strains: OSG 5I, OG 9, NO 17, NSG 3, NG 13, and Fe-10

These strains were fermentative gram negative and non-motile rods. They formed smooth and convex colonies on solid medium. Colonies on nutrient agar plate were light brown.

Their phenotypic characters are shown in Tables 1 and 6. They produced brown pigment from gluconate medium and assimilated pectin and polygalacturonate as shown in Table 6. Strains OG 9, NSG 3, NG 13, and Fe-10 did not liquefy gelatin but strain NO 17 did. Strain OSG 5I liquefied gelatin at 30°C but not at 20°C. Strains NG 13 and NSG 3 did not utilize hydrogen.

The fatty acid composition of the isolates was similar to that of *Klebsiella oxytoca* RH 1586 as shown in Table 2. Strain NG 13 contained a large amount of straight-chain C_{16:0}, C_{18:1}, and 3-OH C_{14:0} acids. Like *K. oxytoca* RH 1586 strain NG 13 had the ubiquinone system Q-8.

Consequently, all of the strains were identified as *Klebsiella oxytoca* (Flugge 1886) Lautrop 1956 on the basis of positive reactions for indole, hydrolysis of urea, β -galactosidase, lysine decarboxylase, and production of pigment from gluconate medium, and negative reactions for arginine dihydrolase and ornithine decarboxylase, assimilation of polygalacturonate, and production of acid from a number of sugars.

Klebsiella pneumoniae CDC 2551-63 was used as a reference strain for a nitrogen-fixing *Klebsiella* strain in this study. Strain *K. pneumoniae* CDC 2551-63 differed from strain *K. pneumoniae* IAM 12351^T in the production of indole, pigmentation on gluconate medium, production of acid from inulin and polygalacturonate as shown in Table 6. On the other hand, strain *K. pneumoniae* CDC 2551-63 was similar to the isolates in the characters mentioned above, although it did not liquefy gelatin at 20 and 30°C. Further detailed examination of the characters of strain CDC 2551-63 is needed for taxonomy in the future.

Strains *Klebsiella oxytoca* RH 1586 and *K. pneumoniae* IAM 12351 did not fix nitrogen.

Source: Strains OSG 5I, OG 9, NO 17, NSG 3, and NG 13 were isolated from paddy soil. Strain Fe-10 was isolated in 1981 from a root of *Oryza sativa* strain T65.

Group 4. Gram negative, spiral-shaped bacteria

Strains: SS-18, SS-20, SS-22, SS-25, SS-28, SS-29, and SS-32

These strains had spiral-shaped cells as shown in Fig. 1D. The cells were 0.5 to 1.0 μ m by 1.0 to 3.0 μ m. They were gram negative. They had a polar flagellum and showed *Spirillum*-like motility. They did not accumulate PHB granules. Colonies were opaque, convex, and light brown plaques on nutrient agar plate.

The strains did not produce acid from glucose oxidatively and fermentatively. Phenotypic characters are shown in Table 1. Production of acid from carbon compounds is shown in Table 7. Strains SS-25, SS-28, and SS-29 were different from the other strains in the production of acid from ribose (SS-28), sorbitol (SS-29), and

Table 7. Production of acid from carbon compounds by strains of group 4 (gram negative spiral-shaped bacteria) and group 7 (*Alcaligenes* sp.), and reference strains.

	Number of strains tested	D-Arabinose	L-Arabinose	D-Xylose	D-Ribose	D-Glucose	D-Fructose	Mannose	D-Galactose	Trehalose	Sucrose	Lactose	Maltose	Cellobiose	Melezitose	Melibiose	Inulin	Ethanol	Sorbitol	Inositol	Adonitol	D-Mannitol	Dulcitol
Group 4	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group 7	4	-	+	+	(6/7)	+	+	+	+	+	(3/4)	+	+	+	-	+	-	-	(6/7)	-	-	(6/7)	-
<i>Agrobacterium radiobacter</i>																							
IAM 12048 [†]	1	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+	+	+	+	+
<i>A. tumefaciens</i>																							
IAM 1037	1	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+	+	+	+	+
<i>Alcaligenes denitrificans</i>																							
IAM 12370	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
<i>A. eutrophus</i>																							
IAM 12368 [†]	1	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
<i>A. faecalis</i>																							
IAM 12369	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
<i>A. paradoxus</i>																							
DSM 30034	1	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
<i>Pseudomonas paucimobilis</i>																							
KM 2395 [†]	1	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-
<i>P. paucimobilis</i>																							
KM 2397	1	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-

Symbols: +, produced acid; -, not produced acid; + W, produced acid weakly.

mannitol (SS-25).

The chemotaxonomic characters of the strains are shown in Table 2. The GC content of strains studied ranged from 63.1 to 65.3%. They had the ubiquinone system Q-8. They had straight-chain $C_{16:0}$, $C_{16:1}$, and $C_{18:1}$ acids as major components, and 2-OH $C_{14:0}$ acid.

Although the strains had spiral-shaped cells and fixed nitrogen, they differed from the known species of the genus *Azospirillum*. The cells of the strains are smaller than those of the genus *Azospirillum*. The strains did not reduce nitrate, did not hydrolyze esculin, and did not produce urease and phosphatase, but *Azospirillum* species did. Further, their chemotaxonomic characters indicate that this group is different from the genus *Azospirillum* as shown in Table 2. Their GC contents were also 5% lower than those of *Azospirillum brasilense* IAM 12400^T and *A. lipoferum* IAM 12399^T. The ubiquinone systems and the fatty acid composition of the strains were clearly different from those of *A. brasilense* IAM 12400^T and *A. lipoferum* IAM 12399^T.

Source: Strains SS-18, SS-29, and SS-32 were isolated in 1982 from the roots of *Oryza sativa* strain T65. Strains SS-20, SS-22, and SS-25 were isolated in 1982 from the roots of *Oryza sativa* strain C5444.

Group 5. Gram negative pleomorphic bacteria

Strains: B-26, B-40, B-45, B-46, B-47, and B-51

These strains produced branched or pleomorphic, gram negative cells on agar and semisolid media as shown in Figs. 5A, 5B, and 5D. Old cultures showed many ovoid cells. Cells were 0.5 to 1.0 μm by 1.0 to 5.0 μm . Nonmotile. PHB granules were accumulated in the cells. Neither water soluble nor water insoluble pigments were produced.

None of the strains produced acid from glucose oxidatively and fermentatively. Their phenotypic characters are shown in Table 1. All of the strains grew at the expense of a number of carbon compounds, especially sugars (Table 4). Strains B-26, B-45, and B-47 utilized hydrogen.

The GC content of the strains ranged from 59.9 to 62.2%. The strains had the ubiquinone system Q-10. They had straight-chain $C_{16:0}$, $C_{16:1}$, and $C_{18:1}$ acids as major components, and 3-OH $C_{14:0}$, 3-OH $C_{16:0}$, 3-OH $C_{18:0}$ acids as shown in Table 2.

From these findings this group apparently belongs to *Xanthobacter*. However, they were different from *Xanthobacter* strains in the formation of colorless colonies, nitrate reduction, production of urease and DNase, and assimilation of sugars. They grew at the expense of L-arabinose, D-xylose, D-ribose, L-rhamnose, D-glucose, D-fructose, mannose, D-galactose, trehalose, lactose, maltose, cellobiose, salicin, malonate, sorbitol, mannitol, inositol, glycerol, L-threonine, L-serine, L-arginine, L-leucine, L-lysine, L-ornithine, L-valine, L-phenylalanine, L-histidine, and L-proline, but *X. autotrophicus* DSM 431 and DSM 432^T did not (Table 4). Further, their GC contents were 6% lower than those of *X. autotrophicus* DSM 431 and DSM 432^T.

Fig. 5. Photomicrographs (A–C) and an electron micrograph (D) of strains of groups 5 (gram negative pleomorphic bacteria) and 6 (gram negative fermentative pleomorphic bacteria).

These cells were grown on nutrient agar medium for 1 day at 30°C. A and B: strain B-46 (group 5) stained with crystal violet. C: strain B-2 (group 6) stained with crystal violet. D: strain B-46 negative stain, electron microscopy. Bars indicate 1 μ m.

Table 8. DNA-DNA homologies among strains of group 5 and 6 and related organisms.

Source of unlabeled DNA	GC mol%	% relative binding of DNA from			
		B-26	B-47	DSM 432	IAM 12399
Group 5					
B-26	61.1	100	98.1	12.1	9.1
B-47	62.2	99.8	100	20.7	14.2
Group 6					
F-7	63.1	4.4		28.7	14.9
<i>Xanthobacter autotrophicus</i>					
DSM 431	67.2	14.2		75.8	7.4
DSM 432 ^T	67.0	3.3		100	20.0
<i>Azospirillum lipoferum</i>					
IAM 12399 ^T	69.0	17.3		30.5	100
<i>A. brasilense</i>					
IAM 12400	69.0	17.8		22.1	24.6
<i>Azotobacter chroococcum</i>					
IAM 12666		7.3			
H3 (from Dr. Watanabe)	61.1	99.9		48.3	24.6

(Table 2). The cellular fatty acid composition of the strains resembled those of *X. autotrophicus* DSM 431 and DSM 432^T (Table 2). But the amount of straight-chain unsaturated C_{18:1} acid was less than those of *Xanthobacter* strains, and the isolates had 3-OH C_{14:0} and C_{18:0} acids, and showed an unknown peak (5–10%) which *Xanthobacter* species did not have.

Table 8 shows the results of DNA-DNA hybridization obtained with strains B-26, B-47, F-7 (group 6), *Xanthobacter autotrophicus* DSM 431 and DSM 432^T, *Azospirillum brasilense* IAM 12400^T, *Azospirillum lipoferum* IAM 12399^T, *Azotobacter chroococcum* IAM 12666^T, and H3 from Dr. Watanabe. With the DNA probe from strain B-26, strain B-47 showed a very high degree of relative binding (99.8%) but the other strains, except strain H3, showed low degrees of relative binding (3.3 to 17.3%). Strains B-26 and B-47 showed low degrees of relative binding (14.2 to 28.7%) with the DNA probes from *X. autotrophicus* DSM 432^T and *A. lipoferum* IAM 12399^T. These results indicate that these strains do not belong to the genus *Xanthobacter*. Strain B-26 showed a high degree of relative binding (99.9%) with strain H3 from Dr. Watanabe. Strain H3 was isolated from the rhizosphere of rice, and identified as *Pseudomonas* sp. by BARRAQUIO et al. (17).

Source: Strain B-26 was isolated in 1982 from the roots of *Oryza sativa* strain T65. Strains B-46 and B-47 were isolated in 1981 from the roots of *Oryza sativa* strain T65. Strains B-40 and B-45 were isolated in 1981 from the roots of *Oryza sativa* strain C5444. Strain B-51 was in 1982 from the roots of *Oryza sativa* strain C5444.

Group 6. Gram negative fermentative pleomorphic bacteria

Strains: F-4, F-7, B-2, B-4, B-7, B-18, B-25, and B-32

The strains studied resembled the group 5 strains in many respects. They produced nonmotile, gram negative, pleomorphic cells as shown in Fig. 5C. The cells were 0.5 to 1.0 μm by 1.0 to 5.0 μm . Old cells became ovoid as the group 5 strains did. PHB granules were accumulated in cells. Neither water-soluble nor water-insoluble pigments were produced.

All of strains produced acid from glucose fermentatively. Their phenotypic characters are shown in Table 1. The strains did not hydrolyze gelatin, starch, and esculin. They did not grow on tellurite agar medium. The indole reaction was positive. They reduced nitrate but not tetrazolium chloride. They produced catalase, oxidase, urease, and tyrosinase but not phosphatase and DNase. They assimilated a number of carbon compounds (Table 4). Strains F-4, B-2, and B-32 did not utilize hydrogen.

The chemotaxonomic characters of the strains are shown in Table 2. The GC content of the strains ranged from 62.1 to 63.1%. They had the ubiquinone system Q-10. They had straight-chain $\text{C}_{16:0}$, $\text{C}_{16:1}$, and $\text{C}_{18:1}$ acids as major compounds, and 3-OH $\text{C}_{14:0}$, 3-OH $\text{C}_{16:0}$, and 3-OH $\text{C}_{18:0}$ acids as did the strains of group 5.

Although they had pleomorphic cells, they were different from *Xanthobacter* in the formation of colorless colonies, production of acid from glucose by fermentative metabolism, hydrogen utilization, and nitrate reduction. They produced indole, hydrolyzed urea, and decomposed tyrosine. They did not reduce tetrazolium chloride and did not produce phosphatase. The average value of the GC contents of the strains was 4% lower than that of *X. autotrophicus*.

Group 6 was different from group 5 in the reduction of nitrate and tetrazolium chloride, and production of indole, phosphatase and DNase. The strains did not assimilate maltose, succinate, malonate, glutamate, 2-oxoglutarate, citrate, DL-lactate, β -hydroxybutyrate, *n*-propanol, methanol, ethanol, β -alanine, L-threonine, L-arginine, L-leucine, L-lysine, L-ornithine, L-valine, L-phenylalanine, or L-histidine, but the group 5 strains did. Oxalate was assimilated by the strains of this group but not by the group 5 strains.

Strain F-7 showed a low degree of relative binding with the DNA probes from strain B-26 (group 5) (4.4%), *X. autotrophicus* DSM 432^T (28.7%), and *A. lipoferum* IAM 12399^T (14.9%) (Table 8).

Source: Strains F-4, F-7, B-2, B-4, B-7, and B-18 were isolated in 1982 from the roots of *Oryza sativa* strain C5444. Strains B-25 and B-32 were isolated in 1982 from the roots of *Oryza sativa* strain T65.

Group 7. *Alcaligenes* sp.

Strains: OSG 47, Y-22, Y-23, and Y-39

Small rod-shaped cells measuring 0.5 to 1.0 μm by 1.0 to 3.0 μm were produced on agar and semisolid media. Four strains of this group were gram negative, non-spore forming, and motile with peritrichous flagella as shown in Fig. 4D. PHB granules were accumulated in the cells. Colonies were circular, smooth, convex, entire, opaque, and yellow-orange on agar medium.

Table 9. Characters of strains of group 7 (*Alcaligenes* sp.) and reference strains.

Strain	N ₂ -fixation	Motility	Flagellation	Hydrolysis of:	Gelatin	Starch	Esculin	Tween 80	Chitin	Citrate as C source	Christensen	Simmons	Nitrate reduction	β -Galactosidase	Production of:	Catalase	Oxidase	Urease	Tyrosinase	Phosphatase	DNase	Quinone system	GC content (%)	Hydroxy fatty acids	3-OH	2-OH
Group 7																										
OSG-47	+	+	Pe	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Q-10	66.3	14:0		14:0
Y-22	+	+	Pe	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Q-10	66.0	14:0		14:0
Y-23	+	+	Pe	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Q-10	66.8	14:0		14:0
Y-39	+	+	Pe	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Q-10	68.0	14:0		14:0
<i>Agrobacterium radiobacter</i>																										
IAM 12048 ^T	—	+	Pe	—	—	—	+	—	+	+	+	+	+	+	+	+	+	+	+	—	—	Q-10	59.2	14:0, 16:0		
<i>A. tumefaciens</i>																										
IAM 1037	—	+	Pe	—	—	—	+	—	+	+	+	+	+	+	+	+	+	+	—	—	—	Q-10	59.4	14:0, 16:0		
<i>Alcaligenes denitrificans</i>																										
IAM 12370	—	+	Pe	—	—	—	—	—	—	+	+	+	+	—	—	+	+	+	+	—	—	Q-8	67.5	14:0		
<i>A. eutrophus</i>																										
IAM 12368 ^T	—	+	Pe	—	—	—	—	—	+	+	+	+	+	—	—	+	+	+	+	—	—	Q-8	65.8	14:0		14:0
<i>A. faecalis</i>																										
IAM 12369	—	+	Pe	—	—	—	+	—	+	+	+	+	—	—	—	+	+	+	+	—	—	Q-8	57.2	14:0		14:0
<i>A. latus</i>																										
IAM 12599	+	+	Pe	+	+	+	—	—	—	+	+	+	+	—	—	+	+	+	+	—	—	Q-8	69.2	10:0		
<i>A. paradoxus</i>																										
DSM 30034	—	+	Pe	—	—	—	+	+	—	+	+	+	+	—	—	+	+	+	+	—	—	Q-8	68.0	10:0		14:0
<i>P. paucimobilis</i>																										
KM 2395 ^T	—	+	Po	—	—	+	+	+	—	+	+	—	—	+	+	+	+	+	—	+	+	+	Q-10	64.3		14:0
KM 2397	—	+	Po	+	+	+	+	+	—	—	—	—	—	+	+	+	+	+	—	+	+	+	Q-10	65.8		14:0

Symbols: +, positive reaction; —, negative reaction; Pe, peritrichous; Po, polar.

As shown in Table 9, these strains hydrolyzed starch, esculin, and Tween 80 but not chitin. They produced catalase, β -galactosidase, oxidase, phosphatase, and DNase but not produce indole, H_2S , and arginine dihydrolase. They did not utilize citrate on Simmons medium and malonate. They did not reduce nitrate. Although not listed in Table 9, all isolates grew at 25, 30, and 37°C but not grow at 42°C and in 2.5, 5, 7.5, and 10% NaCl. They did not produce 3-ketolactose from lactose. The responses to hydrolysis of gelatin, utilization of citrate on Christensen medium, production of urease and tyrosinase differed among stains as shown in Table 9. Acid was produced from L-arabinose, D-xylose, D-glucose, D-fructose, mannose, D-galactose, trehalose, lactose, maltose, cellobiose, and melibiose, but not from D-arabinose, D-ribose, melezitose, inulin, ethanol, sorbitol, inositol, adonitol, D-mannitol, and dulcitol (Table 7). Strain Y-22 did not produce acid from sucrose and melibiose but the others did. The assimilation of 40 carbon compounds is shown in Table 4. All strains grew at the expense of L-arabinose, D-xylose, D-glucose, D-fructose, trehalose, sucrose, lactose, maltose, cellobiose, raffinose, starch, acetate, succinate, DL-malate, pyruvate, DL- β -hydroxybutyrate, L-glutamine, and L-aspartate, but not at the expense of malonate, adipate, citrate, DL-tartrate, DL-mandelate, gluconate, 2-ketogluconate, itaconate, methanol, glycerol, benzoate, mucate, phenol, *m*-hydroxybenzoate, betaine, β -alanine, β -aminobutyrate, and L-proline. Utilization of the other five carbon compounds, i.e., pimelate, suberate, citrate, gluconate, and ethanol differed among the strains. Strain Y-22 assimilated citrate weakly. Strain Y-23 did not assimilate suberate and ethanol. Strain Y-39 assimilated gluconate but did not pimelate. Strain OSG 47 did not utilize hydrogen.

The GC content of the strains ranged from 66.0 to 68.0%, and all strains of this group had the ubiquinone system Q-10 (Table 2). They had straight-chain $C_{16:0}$, $C_{16:1}$, $C_{18:1}$ acids as major components, and 2-OH $C_{14:0}$ acid was found but there were no 3-hydroxy acids. Strains OSG 47 and Y-22 had sphingolipid as shown in Fig. 6. The visible absorption spectrum of acetone extract of yellow pigment had 2 peaks in 452 and 480 nm (Fig. 7).

The strains resemble the members in the genus *Alcaligenes* in having gram negative rod-shaped cells with peritrichous flagella (56), but they were different from the genus *Alcaligenes* in nitrate reduction, assimilation patterns of carbon compounds (57), quinone system, and fatty acid composition. *Alcaligenes* species had Q-8 and straight-chain $C_{16:0}$, $C_{16:1}$, and $C_{18:1}$ acids and cyclopropane acids of $C_{17:0}$ and $C_{19:0}$ as major components and contained 3-OH $C_{10:0}$ or 3-OH $C_{14:0}$ acids (Table 2).

In the assimilation of sugars the isolates seem to be close to the members of the genus *Agrobacterium*, which are motile with peritrichous flagella and are well known as plant pathogens (58). *Agrobacterium radiobacter* IAM 12048^T and *A. tumefaciens* IAM 1037 had Q-10 (Table 2). However, the isolates did not produce 3-ketolactose from lactose but *A. radiobacter* IAM 12048^T and *A. tumefaciens* IAM 1037 did. The isolates did not assimilate gluconate and 2-ketogluconate but *A. radiobacter* IAM 12048^T and *A. tumefaciens* IAM 1037 did. The GC content of

Fig. 6.

Fig. 6. Thin-layer chromatography of original and alkaline stable lipid from *P. paucimobilis* KM 2395^T and strain OSG 47.

(1) Original lipid from *P. paucimobilis* KM 2395^T, (2) Lipid obtained after alkaline hydrolysis (0.5N KOH methanol-chloroform (1:1 by vol.) for 6 h at room temperature) from *P. paucimobilis* KM 2395, (3) Original lipid from strain OSG 47, (4) Lipid obtained after alkaline hydrolysis from strain OSG 47. a, fatty acids; b, unknown lipid; c, unknown phospholipid; d, unknown glycolipid (alkaline stable); e, unknown phospholipid. TLC solvent: chloroform-methanol-acetic acid-water (100:20:12:5, by vol.).

Fig. 7.

Fig. 7. Visible absorption spectra of yellow pigments in acetone-extract.

A: strain OSG 47 (group 7). B: *Pseudomonas paucimobilis* KM 2395^T.

Agrobacterium radiobacter IAM 12048^T (59.2%) and *A. tumefaciens* IAM 1037 (59.4%) was 7% lower than the isolates. The fatty acid composition of *Agrobacterium* differed from those of the isolates (Table 2). *Agrobacterium* contained straight-chain C_{16:0} and cyclopropane of C_{19:0} acids as major components, and 3-OH C_{14:0} and 3-OH C_{10:0} acids but not 2-hydroxy acid.

On the other hand, the isolates closely resemble *Pseudomonas paucimobilis* in phenotypic and chemotaxonomic characters except for their flagellation, acid production from arabinose and ethanol, and assimilation of galactose. The strains had PHB granules in the cells and the same visible absorption spectrum of yellow pigments in acetone-extract as *P. paucimobilis* KM 2395^T (Fig. 7). They hydrolyzed starch, esculin, and Tween 80, produced phosphatase and DNase, as *P. paucimobilis* did. Also, the isolates had only 2-OH C_{14:0} acid and did not contain 3-hydroxy acid as *P. paucimobilis* did (59–61). The strains had sphingolipid (Fig. 6) and ubiquinone system Q-10, as *P. paucimobilis* did. Table 10 shows the DNA-DNA hybridization between isolates (OSG 47 and Y-39), *P. paucimobilis* KM 2395^T and KM 2397, *Alcaligenes faecalis* IAM 12369, *Alcaligenes latus* IAM 12599, *Agrobacterium*

Table 10. DNA-DNA homologies among strains of group 7 (*Alcaligenes* sp.) and related organisms.

Source of unlabeled DNA	GC mol%	% relative binding of DNA from		
		OSG 47	KM 2397	IAM 12369
Group 7				
OSG 47	66.3	100	24.8	1.7
Y-39	68.0	99.7	19.2	2.6
<i>Pseudomonas paucimobilis</i>				
KM 2395 ^T	64.3	55.7	92.9	3.8
KM 2397	65.8	34.0	100	2.7
<i>Alcaligenes faecalis</i>				
IAM 12369	57.2	3.3	1.6	100
<i>A. latus</i>				
IAM 12599	69.2	1.0	2.1	1.0
<i>Agrobacterium tumefaciens</i>				
IAM 1037	59.4	18.3	9.1	5.3
<i>Azospirillum lipoferum</i>				
IAM 12399 ^T	69.0	23.3	5.2	2.9
H3 from Dr. Watanabe	61.1	13.4		

tumefaciens IAM 1037, *Azospirillum lipoferum* IAM 12399^T, and strain H3. The degree of relative binding between strains OSG 47 and Y-39 was high (99.7%). With the DNA probe from strain OSG 47, *P. paucimobilis* KM 2395^T and KM 2397 showed relatively high degrees of the relative binding (34.0–55.7%), but the other five strains gave low degrees of the relative binding (1.0–23.3%). Isolates showed 19.2–24.8% relative bindings with the DNA probe from *P. paucimobilis* KM 2397. With respect to the DNA-DNA hybridization, the isolates are not the same species as *P. paucimobilis* but appeared to be very close to *P. paucimobilis*.

Recently nitrogen-fixing *P. paucimobilis* was isolated from rice (12,13), *Deschampsia caespitosa* (62), and soil (63). The acetylene reduction activity of the type strain of *P. paucimobilis* KM 2395^T was tested but no activity was found.

Source: Strains OSG 47 and Y-22 were isolated from paddy soil. Strains Y-23 and Y-39 were isolated in 1982 from the roots of *Oryza sativa* strain C5444.

Group 8. *Protomonas*-like bacteria

Strains: R-2, R-4, R-5, R-7, R-11, R-12, R-13, and R-15

All strains of group 8 were gram negative, motile with single polar flagella (Fig. 4B). The cells were 0.5 to 1.0 μm by 1.0 to 3.0 μm . PHB granules were not accumulated in the cells. Colonies were circular, smooth, convex, entire, opaque and red on agar medium. The strains studied did not produce fluorescent and water soluble pigments in King A and B media.

Acid was not produced from glucose oxidatively and fermentatively. Strains studied did not grow in more than 2.5% NaCl and at 42°C. They hydrolyzed Tween

80 but did not hydrolyze gelatin, starch, and esculin. Nitrate reduction and production of tyrosinase, phosphatase, and DNase were negative but tetrazolium chloride reduction (TTC reduction) and production of oxidase, catalase, and urease were positive. Table 4 shows the results of assimilation of 33 carbon compounds by isolates. Strains studied assimilated acetate (weakly), oxalate (weakly), succinate, malonate (weakly), glutarate, glyoxylate, propionate (weakly), *n*-propanol, methanol (weakly), ethanol, formaldehyde, ethylamine, glycine (weakly), and β -alanine but not sugars. Strains R-2, R-5, and R-12 did not utilize hydrogen.

The GC content of the strains ranged from 62.2 to 65.1% and, they had ubiquinone system Q-10. They had straight-chain fatty acids and straight-chain hydroxy fatty acid of 3-OH C_{14:0} (Table 2).

URAKAMI and KOMAGATA (64) proposed the genus *Protomonas*, for pink-pigmented facultatively methylotrophic bacteria that produce carotenoids and bacteriochlorophyll *a* in the cells grown aerobically in intermittent light. On the basis of presence of the red pigment, ubiquinone system Q-10, and fatty acid composition, isolates seem to be closely related to this genus. However, the GC content of the isolates ranged from 61.2 to 62.3%, and were 5% lower than that of *Protomonas*. Isolates had straight-chain C_{12:0}, C_{14:0}, C_{19:0} and cyclopropane of C_{17:0} acids but *P. extroquens* TK 0001^T did not, and the amounts of straight-chain C_{16:0}, C_{16:1}, C_{18:0}, and C_{18:1} acids were different from those of *P. extroquens*. Further, the isolates did not assimilate DL-lactate, DL-tartrate, formate, formamide, and methylamine, and did not accumulate PHB granules. They assimilated methanol very weakly compared to *P. extroquens* TK 0001^T. *Protomonas extroquens* TK 0001^T did not fix nitrogen.

Some pink-pigmented bacteria motile with subpolar flagella, and having 60–64% GC contents and the ubiquinone system Q-10 were studied by SHIBA and SHIMIDZU (65), and for those organisms a new genus *Erythrobacter* was established. However their habitat is marine environments.

Source: Strains R-2, R-4, R-7, and R-11 were isolated in 1982 from the roots of *Oryza sativa* strain T65. Strains R-12 and R-15 were isolated in 1981 from the roots of *Oryza sativa* strain T65. Strain R-5 was isolated in 1982 from the roots of *Oryza sativa* strain C5444. Strain R-13 was isolated in 1981 from the roots of *Oryza sativa* strain C5444.

Group 9. *Azotobacter*-like bacteria

Strains: A-2, A-3, A-4, A-7, and A-24

These strains were gram negative and motile with a polar flagellum as shown in Fig. 4C. The cells were 0.5 to 0.7 μ m by 1.5 to 2.0 μ m. Old cells became ovoid. They did not form cysts. They grew slowly and produced characteristic wrinkled hard colonies on agar medium, but did not produce viscid slime and water soluble fluorescent pigments in King A and B media and in mannitol yeast-extract medium. They accumulated PHB granules in their cells.

They did not hydrolyze gelatin, starch, or Tween 80. They reduced nitrate.

Acid was not produced from glucose oxidatively and fermentatively. Catalase and oxidase were produced but DNase was not. Strains A-2, A-3, and A-7 did not utilize hydrogen.

The GC content of the strains studied ranged from 64.1 to 65.1% as shown in Table 2. They had the ubiquinone system Q-10 and straight-chain C_{16:0}, C_{16:1}, and C_{18:1} acids as major components, and 2-OH C_{14:0} and 3-OH C_{12:0} acids as minor components.

The isolates were considered to belong to the *Azotobacteriaceae* in cell size and the presence of the PHB granules in the cells, and the cells becoming ovoid. The isolates appear to be related to the genus *Azotobacter* or *Derxia* in their ubiquinone system Q-8, straight-chain C_{16:0}, C_{16:1}, and C_{18:1} acids as major components, and 3-OH C_{12:0} and 2-OH C_{14:0} acids as minor components. The GC contents of the strains of *Azotobacteriaceae* were reported by DE LEY (66) to be 64.8–66.5% for *Azotobacter*, 57–58.6% for *Azomonas*, 54.7–59% for *Beijerinckia*, 70% for *Derxia*. The isolates resemble the genus *Derxia* more than the genus *Azotobacter* in their polar flagellation, but are different from in the reaction of catalase, utilization of hydrogen, and GC content. However, the isolates were different from *Azotobacter* in their cyst formation and flagellation.

Source: Strain A-2 was isolated in 1982 from the roots of *Oryza sativa* strain T65. Strains A-3, A-4, A-7, and A-24 were isolated in 1982 from the roots of *Oryza sativa* strain C5444.

Group 10. Others

Strains: O-1, O-3, O-5, O-6, O-9, O-11, O-15, O-16, and OSG 53III

The nine strains of this group were different from those of the other nine groups in biochemical and chemotaxonomic characters. All of these strains were gram negative rods and did not produce fluorescent pigments in King A and B media. Some strains hydrolyzed starch (O-3) and Tween 80 (O-5 and OSG 53III) but others did not. Some strains produced tyrosinase (O-6 and OSG 53III) and DNase (O-3 and OSG 53III) but others did not. Some strains did not produce urease (O-3 and OSG 53III) but others did. Strains O-3, O-6, O-9, and O-11 had the ubiquinone system Q-8 and OSG 53III had the ubiquinone system Q-10.

For the identification of these strains more precise characterization is needed.

Source: Strains O-1, O-6, O-9, and O-11 were isolated in 1982 from the roots of *Oryza sativa* strain C5444. Strains O-15 and O-16 were isolated in 1981 from the roots of *Oryza sativa* strain T65. Strain OSG 53III was isolated from paddy soil.

Döberiner's malate semisolid medium has been used most frequently to isolate nitrogen-fixing bacteria from plants and soil (2, 4, 8, 9, 11–14, 18, 67–69). The most frequently isolated nitrogen-fixing bacteria in this medium were species of *Azospirillum*. This uniformity seems to reflect the carbon source (malate) of selective medium and semisolid agar culture. Nevertheless, a large number and many kinds of nitrogen-fixing bacteria were isolated from the rhizosphere of rice and it was

found that more than 10% of the bacteria around the rhizosphere of rice fixed nitrogen (details will be reported in another paper).

Of these strains isolated from rice some were identified as *Azospirillum brasilense*, *A. lipoferum*, *Enterobacter cloacae*, *Erwinia herbicola*, *Klebsiella oxytoca*, and *Xanthobacter autotrophicus* but the others were not assigned to any known species. Unidentified strains were spirals (group 4), gram negative pleomorphic bacteria (group 5), gram negative fermentative pleomorphic bacteria (group 6), *Alcaligenes* sp. (group 7), *Protomonas*-like bacteria (group 8), *Azotobacter*-like bacteria (group 9), and others (group 10). These findings may suggest that the more improvement of the procedure and the selective medium for isolation advance the more unknown nitrogen-fixing bacteria will be found around the rhizosphere of rice. Using chemotaxonomic methods for identification may make clear the characters of nitrogen-fixing bacteria, as mentioned in our experiments. But there are still a large number of unidentified nitrogen-fixing bacteria. To identify them, more detailed characterization is needed.

The strains of groups 5 and 6 had pleomorphic cells, as *Xanthobacter* species have. Further, the strains of group 5 utilized hydrogen as *Xanthobacter* species did. However the strains of group 5 were different from *Xanthobacter* phenotypically and chemotaxonomically and in the results of the DNA-DNA hybridization. Although more detailed characterization is needed to identify those group 5 strains, they are considered to constitute a new genus. On the other hand, the strains of groups 1 and 5 utilized hydrogen. The ability to utilize hydrogen was found in *A. lipoferum* (70). WATANABE et al. (16) reported the predominance of hydrogen-utilizing bacteria among nitrogen-fixing bacteria in wetland rice roots. Since a large number of nitrogen-fixing bacteria occur around the rhizosphere of rice, hydrogen-utilizing and nitrogen-fixing bacteria appear to have some relationships with rice.

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REFERENCES

- 1) T. YOSHIDA and R. R. ANCAJAS, *Proc. Soil Sci. Soc. Am.*, **35**, 156 (1970).
- 2) Y. DOMMERGUES, J. BALANDREAU, G. RINAUDO, and P. WEIHARD, *Soil Biol. Biochem.*, **5**, 83 (1973).
- 3) T. YOSHIDA and R. R. ANCAJAS, *Soil Biol. Biochem.*, **5**, 153 (1973).
- 4) J. P. BALANDREAU, C. R. MILLIER, and Y. R. DOMMERGUES, *Appl. Environ. Microbiol.*, **27**, 662 (1974).
- 5) Y. HIROTA, T. FUJII, Y. SANO, and S. IYAMA, *Nature*, **276**, 416 (1978).
- 6) I. WATANABE and D. R. CABRERA, *Appl. Environ. Microbiol.*, **37**, 373 (1979).
- 7) I. WATANABE, W. L. BARRAQUIO, M. R. de GUZMAN, and D. A. CABRERA, *Appl. Environ. Microbiol.*, **37**, 813 (1979).
- 8) M. HABTE and M. ALEXANDER, *Appl. Environ. Microbiol.*, **40**, 507 (1890).
- 9) J. F. BALDENSBERGER, *Plant Soil*, **57**, 439 (1980).

- 10) A. A. APP, I. WATANABE, M. ALEXANDER, W. VENTURA, C. DAEZ, T. SANTIAGO, and S. K. DATTA, *Soil Sci.*, **130**, 283 (1980).
- 11) M. L. KUMARI, S. K. KAVIMANDAN, and N. S. SUBBA-RAO, *Indian J. Exp. Biol.*, **14**, 638 (1976).
- 12) D. THOMAS-BAUZON, P. WEINHARD, T. HEULIN, and J. BALABDREAU, *Can. J. Microbiol.*, **28**, 922 (1982).
- 13) R. BALLY, D. THOMAS-BAUZON, T. HEULIN, J. BALANDREAU, C. RICHARD, and L. DE LEY, *Can. J. Microbiol.*, **29**, 881 (1983).
- 14) R. B. LAMM and C. A. NEYRA, *Can. J. Microbiol.*, **27**, 1320 (1981).
- 15) J. K. LADHA, W. L. BARRAQUIO, and I. WATANABE, *Can. J. Microbiol.*, **29**, 1301 (1983).
- 16) I. WATANABE, W. L. BARRAQUIO, and M. L. DAROV, *Can. J. Microbiol.*, **28**, 1051 (1982).
- 17) W. L. BARRAQUIO, J. K. LADHA, and I. WATANABE, *Can. J. Microbiol.*, **29**, 867 (1983).
- 18) Y. SANO, T. FUJII, S. IYAMA, Y. HIROTA, and K. KOMAGATA, *Crop. Sci.*, **21**, 758 (1981).
- 19) J. F. W. VON BULOW and J. DÖBEREINER, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 2389 (1975).
- 20) R. Y. STANIER, M. DOUDOROFF, and E. A. ADELBERG, *The Microbial World*, 1st ed., Prentice-Hall, Inc., Englewood Cliffs, N. J. (1957), p. 287.
- 21) G. J. HUCKER and H. J. CONN, *Tech. Bull. N. Y. St. Agric. Exp. Stn.*, No. 93 (1923).
- 22) T. TODA, *Nippon Iji Shinpo*, **283**, 113 (1928), in Japanese.
- 23) A. R. SPURR, *J. Ultrastruct. Res.*, **26**, 31 (1969).
- 24) E. S. REYNOLDS, *J. Cell. Biol.*, **17**, 208 (1963).
- 25) K. L. BURDON, *J. Bacteriol.*, **52**, 665 (1946).
- 26) A. G. OSTLE and G. HOLT, *Appl. Environ. Microbiol.*, **44**, 238 (1982).
- 27) S. T. COWAN, *Cowan and Steel's Manual for the Identification of Medical Bacteria*, 2nd ed., Cambridge Univ. Press, Cambridge (1974), pp. 137–180.
- 28) L. H. STEVENSON and M. D. SOCOLOFSKY, *J. Bacteriol.*, **91**, 304 (1969).
- 29) J. J. TARRAND, N. R. KREIG, and J. DÖBEREINER, *Can. J. Microbiol.*, **24**, 967 (1978).
- 30) H. KORTH, I. ORSKOV, and G. PULVERER, *Zentralbl. Bakteriell. Infektionskr. Hyg., Abt. 1, Orig. A*, **211**, 105 (1969).
- 31) P. B. HYLEMON, J. S. WELLS, JR., N. R. KREIGE, and H. W. JANNASCH, *Int. J. Syst. Bacteriol.*, **23**, 340 (1973).
- 32) M. J. THORNLEY, *J. Appl. Bacteriol.*, **23**, 37 (1960).
- 33) K. KOMAGATA, *Nippon Nōgeikagaku Kaishi*, **35**, 981 (1961), in Japanese.
- 34) H. IIZUKA and K. KOMAGATA, *J. Gen. Appl. Microbiol.*, **9**, 73 (1963).
- 35) R. SAKAZAKI, *Shin Saikin Baichi Gaku Koza, Kindaishuppan*, Tokyo (1978), in Japanese.
- 36) M. L. BERNARTS and J. DE LEY, *Nature*, **197**, 406 (1963).
- 37) R. Y. STANIER, N. J. PALLERONI, and M. DOUDOROFF, *J. Gen. Microbiol.*, **13**, 159 (1966).
- 38) T. URAKAMI and K. KOMAGATA, *J. Gen. Appl. Microbiol.*, **25**, 343 (1979).
- 39) P. R. EDWARDS and W. H. EWING, *Identification of Enterobacteriaceae*, 3rd ed., Burgess Publ. Co., Minn. (1972).
- 40) K. A. MALIK and D. CLAUS, *Int. J. Syst. Bacteriol.*, **29**, 183 (1979).
- 41) H. SAITO and K. MIURA, *Biochim. Biophys. Acta*, **72**, 619 (1963).
- 42) J. MARMUR and P. DOTY, *J. Mol. Biol.*, **5**, 109 (1962).
- 43) P. W. J. RIGBY, M. DECKMANN, C. RHODES, and P. BERG, *J. Mol. Biol.*, **113**, 239 (1977).
- 44) T. KANEKO, *In Biseibutu no Kagakubunruihou*, ed. by K. KOMAGATA, Gakkai Shuppan Center, Tokyo (1983), p. 255, in Japanese.
- 45) Y. YAMADA, K. AIDA, and T. UEMURA, *J. Gen. Appl. Microbiol.*, **15**, 181 (1969).
- 46) S. IKEMOTO, K. KATOH, and K. KOMAGATA, *J. Gen. Appl. Microbiol.*, **24**, 41 (1978).
- 47) A. YAMAMOTO, I. YANO, M. MASUI, and E. YABUCHI, *J. Biochem.*, **83**, 1213 (1978).
- 48) E. YABUCHI, E. TANIMURA, A. OHYAMA, I. YANO, and A. YAMAMOTO, *J. Gen. Appl. Microbiol.*, **25**, 95 (1979).
- 49) E. C. FALK, J. DÖBEREINER, J. L. JOHNSON, and N. R. KREIG, *Int. J. Syst. Bacteriol.*, **35**, 117 (1985).

- 50) B. REINHOLD, T. HUREK, I. FENDRIK, B. POT, M. GILLIS, K. KERSTERS, S. THIELEMANS, and J. DE LEY, *Int. J. Syst. Bacteriol.*, **37**, 43 (1987).
- 51) N. R. KREIG and J. DÖBEREINER, Genus *Azospirillum*, In *Bergey's Manual of Systematic Bacteriology*, Vol. 1, ed. by N. R. KREIG and J. G. HOLT, Williams & Wilkins, Baltimore (1986), p. 94.
- 52) S. A. KULASOORIYA, P. A. ROGER, W. L. BARRAQUIO, and I. WATANABE, *Soil Sci. Plant Nutr. (Tokyo)*, **27**, 19 (1981).
- 53) B. SENGUPTA, A. S. NANDI, R. K. SAMANTA, P. PAL, D. N. SENGUPTA, and S. P. SEN, *Ann. Bot.*, **48**, 705 (1981).
- 54) A. H. NELSON and L. SPARELL, *Appl. Environ. Microbiol.*, **32**, 197 (1976).
- 55) H. PAPAN and D. WERNER, *Arch. Microbiol.*, **120**, 25 (1979).
- 56) K. KERSTERS and J. DE LEY, Genus *Alcaligenes*, In *Bergey's Manual of Systematic Bacteriology*, Vol. 1, ed. by N. R. KREIG and J. G. HOLT, Williams & Wilkins, Baltimore (1986), p. 361.
- 57) K. YAMASATO, M. AKAGAWA, N. OISHI, and H. KURAISHI, *J. Gen. Appl. Microbiol.*, **28**, 195 (1982).
- 58) K. KERSTERS and J. DE LEY, Genus *Agrobacterium*, In *Bergey's Manual of Systematic Bacteriology*, Vol. 1, ed. by N. R. KREIG and J. G. HOLT, Williams & Wilkins, Baltimore (1986), p. 244.
- 59) B. HOLMES, R. J. OWEN, A. EVANS, H. MALNICK, and W. R. WILLCOX, *Int. J. Syst. Bacteriol.*, **27**, 133 (1977).
- 60) H. OYAIZU and K. KOMAGATA, *J. Gen. Appl. Microbiol.*, **29**, 17 (1983).
- 61) K. KAWAHARA, K. UCHIDA, and K. AIDA, *Biochim. Biophys. Acta*, **712**, 571 (1982).
- 62) K. HAAHTELA, K. KARIZ, and V. SUNDMAN, *Appl. Environ. Microbiol.*, **45**, 563 (1983).
- 63) R. J. RENNIE, *Can. J. Microbiol.*, **27**, 8 (1981).
- 64) T. URAKAMI and K. KOMAGATA, *Int. J. Syst. Bacteriol.*, **34**, 188 (1984).
- 65) T. SHIBA and U. SHIMIDZU, *Int. J. Syst. Bacteriol.*, **32**, 211 (1982).
- 66) J. DE LEY, *Antonie van Leeuwenhoek J. Microbiol. Serol.*, **34**, 66 (1968).
- 67) Y. OKON, S. L. ALBRECHT, and R. H. BURRIS, *Appl. Environ. Microbiol.*, **33**, 86 (1977).
- 68) M. E. TYLER, J. R. MILAM, R. L. SMITH, S. C. SCHANK, and D. A. ZUBERER, *Can. J. Microbiol.*, **25**, 693 (1979).
- 69) I. NUR, Y. OKON, and Y. HENIS, *Can. J. Microbiol.*, **26**, 714 (1980).
- 70) P. P. WONG, N. E. STENBERG, and L. EDGAR, *Can. J. Microbiol.*, **26**, 291 (1980).