TAXONOMIC STUDY OF METHANOL-ASSIMILATING YEASTS

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Thirty-one freshly isolated strains of methanol-assimilating yeasts were identified as *Pichia cellobiosa* (3 strains), *Candida boidinii* (20 strains), *Candida cariosilignicola* (1 strain), *Candida succiphila* (6 strains), and *Torulopsis molischiana* (1 strain). *Kloeckera* sp. No. 2201 and *Candida methanolica*, which were reported as methanol-assimilating yeasts, and *Candida koshuensis* were reidentified as *C. boidinii* on the basis of biological and chemotaxonomic characteristics. *Torulopsis methanolovescens* showed characteristics similar to those of *Pichia lindnerii*, and the chemotaxonomic characteristics of the two were in good agreement. The DNA base composition (GC-content of DNA) of these yeasts was distributed in a broad range from 29.2% to 50.2%. The ubiquinone system, proton magnetic resonance spectra of cell-wall mannans, and electrophoretic patterns of methanol oxidase were characteristic for each species. These methanol-assimilating yeasts were divided into four groups: 1) the *C. boidinii* group, 2) methanol-assimilating *Hansenula* and its closely related yeasts, 3) the *P. cellobiosa* group, and 4) the *Hansenula capsulata* group.

Since microbial utilization of methanol was first reported by LOEW (1) in 1892, C₁ compound-utilizing microorganisms have been studied from the viewpoint of biochemical interest. On the other hand, methanol is a valuable and attractive substrate for the production of single-cell protein from microbial cells, and a number of methanol-assimilating yeasts have been described (2–15). Especially, in 1972, HAZEU *et al.* (16) examined methanol assimilation by the type strains preserved at the Culture Collection of Centraalbureau voor Schimmelcultures, and reported the assimilation by the type strains of 15 species in the genera *Hansenula*, *Pichia*, *Candida*, and *Torulopsis*. However, in spite of the fact that methanol-assimilating yeast is one of the most important microorganisms for production

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Table 1. Methanol-assimilating yeasts used in this study.

Species	Strain ^a	Source ^b
<i>Hansenula</i>		
<i>H. capsulata</i>	IFO 0721	NRRL Y-1842
	IFO 0984	CBS 1993
	MG 117	CBS 1993
<i>H. glucozyma</i>	IFO 1472	NRRL YB-2185
<i>H. henricii</i>	IFO 1477	NRRL YB-2194
	MG 58	IFO 1477 (=NRRL YB-2194)
<i>H. minuta</i>	IFO 0975	NRRL Y-411
	MG 60	IFO 0975 (=NRRL Y-411)
<i>H. nonfermentans</i>	IFO 1473	NRRL YB-2203
	MG 59	IFO 1473 (=NRRL YB-2203)
<i>H. philodendra</i>	MG 75	CBS 6075
<i>H. polymorpha</i>	IFO 1024	IBL 2692
	IFO 1071	NRRL Y-2423
	IFO 1475	NRRL Y-2214
	IFO 1476	NRRL Y-5445
	MG 70	CBS 4732 (=NRRL Y-5445)
	NRRL Y-1798	
<i>H. wickerhamii</i>	NRRL Y-7560	
	MG 73	CBS 4307
<i>Pichia</i>		
<i>P. cellobiosa</i>	KL 1, KL 2, KL 5	LEE and KOMAGATA, 1980 (26)
<i>P. haplophila</i>	CBS 2028	
	IFO 0947	YCC, 52-11 (H. J. Phaff)
<i>P. lindnerii</i>	DSM 70718	HENNINGER and WINDISCH, 1975 (11)
<i>P. pastoris</i>	IFO 0948	YCC, K-214 (H. J. PHAFF)
	IFO 1013	IBL 2466
	MG 65	CBS 704
<i>P. pinus</i>	MG 66	CBS 744
<i>P. trehalophila</i>	MG 76	CBS 5361
<i>Candida</i>		
<i>C. boidinii</i>	MG 69	CBS 2428
	AJ 4779	NAKASE <i>et al.</i> 1977 (30)
	KL 4, KL 6, KL 7, KL 8, KL 9, KL 10, KL 11, KL 12, KL 18, KL 19, KL 25, KL 26	Isolates (from soil)
	KL 15	Isolate (from sap of a cherry tree)
	KL 17, KL 23, KL 33	Isolates (from waste water of a wine factory)

Table 1. (Continued)

Species	Strain ^a	Source ^b
<i>C. cariosilignicola</i>	KL 20, KL 27, KL 28	Isolates (from a pine tree)
	KL 29	Isolate (from lake water)
	KL 24	LEE and KOMAGATA, 1980 (26)
<i>C. koshuensis</i>	AJ 4939	YOKOTSUKA and GOTO, 1955 (29)
<i>C. methanolica</i>	ATCC 26175	OKI <i>et al.</i> , 1972 (6)
<i>C. succiphila</i>	KL 14, KL 16, KL 21, KL 22, KL 30, KL 31	LEE and KOMAGATA, 1980 (26)
<i>Kloeckera</i>		
<i>Kloeckera</i> sp. No. 2201	AKU 4705	OGATA <i>et al.</i> , 1969 (2)
<i>Torulopsis</i>		
<i>T. glabrata</i>	IFO 0005	(<i>Cryptococcus glabratus</i>) HUT
	IFO 0622	CBS L-138
	IFO 0861	RIFY 7726
	IFO 1085	FMJ 4030
<i>T. methanolovescens</i>	ATCC 26176	OKI <i>et al.</i> , 1972 (6)
<i>T. molischiana</i>	KL 3	Isolate (from lake water)
<i>T. nemodendra</i>	MG 72	CBS 6280
<i>T. nitratophila</i>	MG 68	CBS 2027
<i>T. pinus</i>	IFO 1327	IBL 2807 (=CBS 970)
	MG 67	CBS 970
<i>T. sonorensis</i>	71-148, 76-201 A, 78-508 C	MILLER <i>et al.</i> , 1978 (12)
<i>Pichia saitoi</i>	IAM 4945	LKB 060
<i>Saccharomyces cerevisiae</i>	IAM 4512	ATU (Jozo-kyokai No. 6)
<i>Candida lipolytica</i>	IAM 4964	NRRL YB-423-12
<i>Candida utilis</i>	IAM 4961	ATCC 9226

^{a, b} AJ, Central Research Laboratories, Ajinomoto Co., Inc., Kawasaki, Japan; AKU, Faculty of Agriculture, Kyoto University, Kyoto, Japan; ATCC, American Type Culture Collection, Rockville, U.S.A.; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; DSM, Deutsche Sammlung von Mikroorganismen, München, West Germany; FMJ, Faculty of Medicine, Juntendo University, Tokyo, Japan; HUT, Faculty of Engineering, Hiroshima University, Hiroshima, Japan; IAM, Institute of Applied Microbiology, University of Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan; LKB, Laboratory of Kodama Brewery, Akita, Japan; MG, Mitsubishi Gas Chemical Co., Niigata, Japan; NRRL, ARS Culture Collection, Northern Regional Research Center, U. S. Department of Agriculture, Peoria, U.S.A.; RIFY, Research Institute of Fermentation, Yamanashi University, Kofu, Japan; YCC, Department of Food Science and Technology University of California, Davis, U.S.A.

For the origins of these cultures, see the references (List of Cultures, Institute for Fermentation, Osaka, 6th Edition, 1978; List of Cultures, Centraalbureau voor Schimmelcultures, Delft, 29th Edition, 1978; Catalogue of Strains I, American Type Culture Collection, Rockville, 13th Edition, 1978).

of microbial cells, its systematics has not been studied in detail. Therefore, we attempted to isolate methanol-assimilating yeasts from a wide variety of natural sources in Japan for taxonomic comparison with the presently described yeasts. Furthermore, we reidentified the known methanol-assimilating yeast strains which were received from researchers and type culture collections.

This paper deals with the identification of freshly isolated methanol-assimilating yeasts and chemotaxonomic comparison of the known methanol-assimilating yeasts, and discusses their relationship to other yeast species.

MATERIALS AND METHODS

Isolation of yeasts. Yeasts were isolated from 150 sources by the use of a methanol medium composed of $(\text{NH}_4)_2\text{SO}_4$, 3 g; KH_2PO_4 , 4 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; NaCl , 0.2 g; ferric citrate, 3 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 3 mg; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.5 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.05 mg; yeast extract, 0.2 g; methanol, 10 ml; chloramphenicol, 50 mg; distilled water, 1,000 ml. The pH was 4.5 or was adjusted to 6.6 with Na_2HPO_4 . About 1 g of source material was inoculated into 10 ml of the medium and incubated at 30° with shaking. The enrichment-culture technique was used for isolation, and pure cultures were obtained by the conventional streaking technique. Assimilation of methanol was tested in yeast nitrogen base (Difco) which contained 1% methanol as the sole carbon source.

Microorganisms. Thirty-one isolates and 44 strains of known methanol-assimilating yeasts were used. They represented 27 species belonging to five genera. *Saccharomyces cerevisiae*, *Pichia saitoi*, *Candida lipolytica*, and *Candida utilis* were used as controls. Sources of the cultures are listed in Table 1.

Characterization of yeasts. The methods used to characterize the methanol-assimilating yeasts were primarily those reported by WICKERHAM (17) and LODDER (18). Vitamin requirements were determined by the method of KOMAGATA and NAKASE (19). Deoxyribonucleic acid (DNA) was isolated and purified by the procedure of CRYER *et al.* (20), and the DNA base composition (GC-content of DNA) was calculated from the thermal denaturation temperature (T_m) according to the formula of MARMUR and DOTY (21). The ubiquinone system was determined by the method of YAMADA and KONDO (22). The isolation and purification of mannans from yeast cells were carried out as reported by GORIN and SPENCER (23). The proton magnetic resonance (PMR) spectra of the mannans were obtained, by the use of a JNM-4H 100 Spectrometer equipped with a temperature control unit, from 0.4 ml of a D_2O solution containing 80 mg of mannan at 70° , with 3-(trimethylsilyl) propane sulfonic acid Na salt as an internal standard.

Electrophoretic pattern of methanol oxidase. Cultivation and harvest. The culture medium consisted of $(\text{NH}_4)_2\text{SO}_4$, 5 g; KH_2PO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g; NaCl , 0.1 g; yeast extract, 0.1 g; methanol, 12 ml; and distilled water, 1,000 ml. The pH was adjusted to 6.0 to 6.2. Microorganisms were grown at 27° with shaking. Cultures in the logarithmic phase were harvested by

centrifugation at a low temperature (5°), and washed with phosphate buffer (pH 7.2).

Disruption of cells and preparation of crude enzyme solution. Cells were disrupted by Zymolyase (Kirin Brewery Co., Tokyo) digestion which was used for the isolation of DNA (20). Sorbitol-phosphate buffer (1 M sorbitol, pH 7.2) was used in place of sorbitol-EDTA during spheroplast formation. The spheroplasts which were formed by Zymolyase digestion were washed twice with sorbitol-phosphate buffer and disrupted by dilution in phosphate buffer. Cell debris was removed by centrifugation at $23,000 \times g$ (5°) for one hour. The resulted cell-free extracts were stored at 5° until used.

Electrophoresis and staining of methanol oxidase. Electrophoresis for methanol oxidase was performed in 7.5% polyacrylamide gels as described by DAVIS (24). The procedure of GREGORY and FRIDOVICH (25) for staining catalase was used for visualization of methanol oxidase activity, with 1% methanol instead of H_2O_2 as substrate. When the gels contained a small amount of methanol oxidase, a thin dark brown band marked the position of the oxidase after 30 to 90 min in methanol solution.

RESULTS

Isolation and identification of yeasts

Thirty-one strains of methanol-assimilating yeasts were isolated, and found to belong to five species in the genera *Pichia*, *Candida*, and *Torulopsis*. Strains of *Candida boidinii* were isolated most frequently and accounted for 20 strains among the 31 isolates, and 21 strains belonged to the known yeast species. Ten strains identified as new species had previously been reported as *Pichia cellobiosa*, *Candida cariosilignicola*, and *Candida succiphila* (26).

Furthermore, we reidentified 44 known methanol-assimilating yeast strains which were received from researchers and type culture collections for the purpose of taxonomic study. Almost all of the strains showed the same characteristics as those in the original descriptions, but some exhibited different characteristics. Biochemical and physiological characteristics of the isolates and the known methanol-assimilating yeasts are shown in Tables 2–7. We selected typical strains from the isolates and their chemotaxonomic characteristics were investigated. The GC-content of their DNA and their ubiquinone systems are shown in Table 8, and the PMR spectra of their cell-wall mannans in Figs. 1 and 2.

Methanol-assimilating yeast species

Ascosporogenous yeasts:

Hansenula capsulata Wickerham. Strains: IFO¹ 0721, IFO 0984, and MG

¹ Abbreviations of the culture collections are shown in Table 1.

Table 2. Biochemical and physiological characteristics of freshly isolated methanol-assimilating yeasts.

Species	Strain		Fermentation								Vitamines required	Maximum growth temperature (°C)	Liquefaction of gelatin	Production of starch	Growth in 50% glucose	NaCl tolerance (%)	Assimilation of nitrate	Assimilation of methanol	Assimilation of pectin
			Glucose	Galactose	Sucrose	Maltose	Cellobiose	Lactose	Melibiose	Raffinose									
<i>P. cellobiosa</i>	KL	1	+	—	—	—	+	—	—	—	B ^a	35–36	—	—	—	9–10	—	+	+
		2	+	—	—	—	+	—	—	—	NT	35–36	—	—	—	9–10	—	+	NT
		5	+	—	—	—	+	—	—	—	NT	35–36	—	—	—	7–8	—	+	NT
<i>C. boidinii</i>	KL	4	+	—	—	—	—	—	—	—	B	33–35	—	—	—	9–10	+	+	+
		6	+	—	—	—	—	—	—	—	NT	33–35	—	—	—	9–10	+	+	+
		7	+	—	—	—	—	—	—	—	NT	33–35	—	—	—	9–10	+	+	NT
		8	+	—	—	—	—	—	—	—	NT	33–35	—	—	—	9–10	+	+	NT
		9	+	—	—	—	—	—	—	—	NT	33–35	—	—	—	9–10	+	+	NT
		10	+	—	—	—	—	—	—	—	NT	33–35	—	—	—	9–10	+	+	NT
		11	+	—	—	—	—	—	—	—	NT	33–35	—	—	—	9–10	+	+	NT
		12	+	—	—	—	—	—	—	—	NT	33–35	—	—	—	9–10	+	+	NT
		15	+	—	—	—	—	—	—	—	B	33–35	—	—	—	9–10	+	+	+
		17	+	—	—	—	—	—	—	—	B	33–35	—	—	—	9–10	+	+	+
		18	+	—	—	—	—	—	—	—	NT	33–35	—	—	—	9–10	+	+	NT
		19	+	—	—	—	—	—	—	—	NT	33–35	—	—	—	9–10	+	+	NT
		20	+	—	—	—	—	—	—	—	NT	33–35	—	—	—	9–10	+	+	NT
		23	+	—	—	—	—	—	—	—	NT	33–35	SW	—	—	9–10	+	+	NT
		25	+	—	—	—	—	—	—	—	NT	33–35	—	—	—	9–10	+	+	NT
		26	+	—	—	—	—	—	—	—	NT	33–35	—	—	—	9–10	+	+	NT
		27	+	—	—	—	—	—	—	—	NT	33–35	—	—	—	9–10	+	+	NT
		28	+	—	—	—	—	—	—	—	NT	33–35	—	—	—	9–10	+	+	NT

Table 2. (Continued)

		29	+	—	—	—	—	—	—	NT	33-35	SW	—	—	9-10	+	+	NT
		33	+	—	—	—	—	—	—	NT	33-35	—	—	—	9-10	+	+	NT
<i>C. cariosilignicola</i>	KL	24	+	—	+	—	—	—	+	B	41-42	—	—	—	6-7	+	+	+
<i>C. succiphila</i>	KL	14	+	+	—	—	+	—	—	NT	35-36	—	—	—	7-8	—	+	NT
		16	+	+	—	—	L	—	—	NT	35-36	—	—	—	7-8	—	+	NT
		21	+	+	—	—	+	—	—	NT	35-36	—	—	—	6-7	—	+	NT
		22	+	+	—	—	+	—	—	NT	35-36	—	—	—	6-7	—	+	NT
		30	+	+	—	—	L	—	—	B	35-36	—	—	—	7-8	—	+	+
		31	+	+	—	—	+	—	—	NT	35-36	—	—	—	6-7	—	+	NT
<i>T. molischiana</i>	KL	3	+	—	—	—	—	—	—	B, P, T ^b	42-43	—	—	—	4-5	+	+	NT
<i>P. saitoi</i>	IAM	4945	+	—	—	—	—	—	—			+	—	—		—	—	
<i>S. cerevisiae</i>		4512	+	+	+	—	—	—	+			—	—	—		—	—	
<i>C. lipolytica</i>		4964	—	—	—	—	—	—	—			+	—	—		—	—	
<i>C. utilis</i>		4961	+	—	+	—	—	—	+			—	—	—		+	—	

L, latent; SW, slow and weak; NT, not tested.

^a B, biotin; P, pyridoxine; T, thiamin.^b stimulatively required.

Table 3. Assimilation of carbon compounds by freshly isolated methanol-assimilating yeasts.

Species	Strain		Glucose	Galactose	L-Sorbose	Sucrose	Maltose	Cellobiose	Trehalose	Lactose	Melibiose	Raffinose	Melezitose	Inulin	Soluble starch	D-Xylose	L-Arabinose	D-Arabinose	D-Ribose	L-Rhamnose	Ethanol	Glycerol	Erythritol	Ribitol	Galactitol	D-Mannitol	D-Glucitol	α -Me-D-Glucoside	Salicin	DL-Lactic acid	Succinic acid	Citric acid	Inositol	
<i>P. cellobiosa</i>	KL	1	+	+	+	-	-	+	+	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-	-		
		2	+	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	-	+	-	-	-		
		5	+	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	-	+	-	-	-		
<i>C. boidinii</i>	KL	4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	W	-	-	
		6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	W	-	-	
		7	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	W	-	-
		8	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	W	W	-	-	
		9	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	W	-	-
		10	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	W	-	-
		11	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	W	-	-
		12	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	W	W	-	-	
		15	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	W	-	-
		17	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	W	-	-
		18	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	W	W	-	-	
		19	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	W	W	-	-	
		20	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	W	W	-	-	
		23	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	W	-	-
		25	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	W	-	-
26	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	W	-	-		
27	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	W	-	-		
28	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	W	-	-		
29	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	W	-	-		
33	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	W	-	-		

Table 4. Biochemical and physiological characteristics of the known methanol-assimilating yeasts (1).

Species	Strain	Fermentation								Vitamines required	Maximum growth temperature (°)	Liquefaction of gelatin	Production of starch	Growth in 50% glucose	NaCl tolerance (%)	Assimilation of nitrate	Assimilation of methanol	Assimilation of pectin
		Glucose	Galactose	Sucrose	Maltose	Cellobiose	Lactose	Melibiose	Raffinose									
<i>C. boidinii</i>	MG 69	+	—	—	—	—	—	—	—	B ^a	34–35	—	—	—	9–10	+	+	+
	AJ 4779	+	—	—	—	—	—	—	—	B	34–35	—	—	—	9–10	+	+	+
<i>C. koshuensis</i>	AJ 4939	+	—	—	—	—	—	—	—	B	34–35	—	—	—	9–10	+	+	+
<i>C. methanolica</i>	ATCC 26175	+	—	—	—	—	—	—	—	B	35–36	—	—	—	9–10	+	+	+
<i>Kloeckera</i> sp. No. 2201	AKU 4705	+	—	—	—	—	—	—	—	B	35–36	—	—	—	9–10	+	+	+
<i>P. lindnerii</i>	DSM 70718	L	—	—	—	—	—	—	—	B	35–36	—	—	—	7–8	—	+	+
<i>T. methanolovescens</i>	ATCC 26176	—	—	—	—	—	—	—	—	B	35–36	—	—	—	7–8	—	+	+

L, latent.

^aB, biotin.

Table 5. Assimilation of carbon compounds by the known methanol-assimilating yeasts (*I*).

Species	Strain																																	
			Glucose	Galactose	L-Sorbose	Sucrose	Maltose	Cellobiose	Trehalose	Lactose	Melibiose	Raffinose	Melezitose	Inulin	Soluble starch	D-Xylose	L-Arabinose	D-Arabinose	D-Ribose	L-Rhamnose	Ethanol	Glycerol	Erythritol	Ribitol	Galactitol	D-Mannitol	D-Glucitol	α -Me-D-Glucoside	Salicin	DL-Lactic acid	Succinic acid	Citric acid	Inositol	
<i>C. boinii</i>	MG	69	+	—	—	—	—	—	—	—	—	—	—	—	—	+	+	—	+	—	+	+	+	+	—	+	+	—	—	+	—	—	—	
	AJ	4779	+	—	—	—	—	—	—	—	—	—	—	—	—	+	+	—	+	—	+	+	+	+	—	+	+	—	—	+	W	—	—	
<i>C. koshuensis</i>	AJ	4939	+	—	—	—	—	—	—	—	—	—	—	—	—	+	+	—	+	—	+	+	+	+	—	+	+	—	—	+	W	—	—	
<i>C. methanolica</i>	ATCC	26175	+	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	+	—	+	+	+	+	—	+	+	—	—	+	W	—	—	
<i>Kloeckera</i> sp. No. 2201	AKU	4705	+	—	—	—	—	—	—	—	—	—	—	—	—	+	S	—	+	—	+	+	+	+	—	+	+	—	—	+	W	—	—	
<i>P. lindnerii</i>	DSM	70718	+	—	—	—	—	—	+	+	—	—	—	—	—	+	—	—	+	+	+	+	—	+	—	+	+	—	—	+	—	—	—	—
<i>T. methanolovescens</i>	ATCC	26176	+	—	—	—	—	—	+	+	—	—	—	—	—	+	—	—	W	+	+	+	—	+	—	+	+	—	—	+	—	—	+	—

S, slow; W, weak

Table 6. Biochemical and physiological characteristics of the known methanol-assimilating yeasts (2).

Species	Strain	Fermentation											
		Glucose	Galactose	Sucrose	Maltose	Cellobiose	Lactose	Raffinose	Growth in vitamin-free medium	Growth at 37°	Assimilation of nitrate	Assimilation of methanol	Assimilation of pectin
<i>H. capsulata</i>	IFO 0721	+	—	—	—	—	—	—	—	—	+	+	NT
<i>H. capsulata</i>	0974	+	—	—	—	—	—	—	—	—	+	+	NT
<i>H. capsulata</i>	0984	+	—	—	—	—	—	—	—	—	+	+	NT
<i>H. capsulata</i>	MG 117	+	—	—	—	—	—	—	—	—	+	+	+
<i>H. glucozyma</i>	IFO 1472	+	—	—	—	—	—	—	—	—	+	+	NT
<i>H. glucozyma</i>	MG 74	—	—	—	—	—	—	—	—	—	+	+	W
<i>H. henricii</i>	IFO 1477	—	—	—	—	—	—	—	—	—	+	+	NT
<i>H. henricii</i>	1478	—	—	—	—	—	—	—	—	—	+	+	NT
<i>H. henricii</i>	MG 58	—	—	—	—	—	—	—	—	—	+	+	S
<i>H. minuta</i>	IFO 0975	+	—	—	—	—	—	—	—	—	+	+	W
<i>H. minuta</i>	MG 60	+	—	—	—	—	—	—	—	—	+	+	W
<i>H. nonfermentans</i>	IFO 1473	—	—	—	—	—	—	—	—	—	+	+	NT
<i>H. nonfermentans</i>	MG 59	—	—	—	—	—	—	—	—	—	+	+	+
<i>H. philodendra</i>	MG 75	—	—	—	—	—	—	—	—	—	NT	+	+
<i>H. polymorpha</i>	IFO 0799	+	—	—	—	—	—	—	—	—	+	+	NT
<i>H. polymorpha</i>	1024	+	—	—	—	—	—	—	—	—	+	+	NT
<i>H. polymorpha</i>	1071	+	—	—	—	—	—	—	—	—	+	+	NT
<i>H. polymorpha</i>	1475	+	—	—	—	—	—	—	—	—	+	+	NT
<i>H. polymorpha</i>	1476	+	—	—	—	—	—	—	—	—	+	+	NT
<i>H. polymorpha</i>	MG 70	+	—	—	—	—	—	—	—	—	+	+	NT
<i>H. polymorpha</i>	NRRL Y-1798	+	—	—	—	—	—	—	—	—	+	+	NT
<i>H. polymorpha</i>	Y-7560	+	—	—	—	—	—	—	—	—	+	+	NT

Table 7. Assimilation of carbon compounds by the known methanol-assimilating yeasts (2).

Species	Strain	Glucose	Galactose	L-Sorbose	Sucrose	Maltose	Cellobiose	Trehalose	Lactose	Melibiose	Raffinose	Melezitose	Inulin	Soluble starch	D-Xylose	L-Arabinose	D-Arabinose	D-Ribose	L-Rhamnose	Ethanol	Glycerol	Erythritol	Ribitol	Galactitol	D-Mannitol	D-Glucitol	α -Me-D-Glucoside	Salicin	DL-Lactic acid	Succinic acid	Citric acid	Inositol	
<i>H. capsulata</i>	IFO 0721	+	-	-	-	+	+	+	-	-	-	L	-	+	+	+	-	+	+	+	+	+	+	-	+	+	L	+	-	-	-	-	
	0974	+	-	-	-	+	+	+	-	-	-	W	-	+	+	+	-	+	+	+	+	+	+	-	+	+	L	+	-	-	-	-	
	0984	+	-	-	-	+	+	+	-	-	-	W	-	+	+	+	-	+	+	+	+	+	+	-	+	+	L	+	-	-	-	-	
	MG 117	+	-	-	-	+	+	+	-	-	-	-	-	+	+	+	S	+	+	+	+	+	+	-	+	+	L	+	-	-	-	-	
<i>H. polymorpha</i>	IFO 0799	+	-	-	+	+	-	+	-	-	-	+	-	-	L	-	S	+	-	+	+	+	+	+	L	+	+	L	+	-	-	W	-
	1024	+	-	-	+	+	+	+	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+	L	+	+	-	+	-	-	+	-	
	1071	+	-	-	+	+	+	+	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+	L	+	+	-	+	-	-	+	-	
	1475	+	-	-	+	+	+	+	-	-	-	+	-	-	L	-	-	+	-	+	+	+	+	+	+	+	L	+	-	+	+	-	
	1476	+	-	-	+	+	W	+	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+	L	+	+	-	+	-	-	+	+	-
	MG 70	+	-	-	+	+	W	+	-	-	-	+	-	-	L	-	-	+	-	+	+	+	+	L	+	+	W	+	-	-	-	-	
	NRRL Y-1798	+	-	-	+	+	-	+	-	-	-	+	-	+	+	-	S	+	-	+	+	+	+	S	+	+	+	+	-	-	W	-	
	NRRL Y-7560	+	W	-	+	+	+	+	-	-	-	+	-	+	+	-	-	-	+	+	+	+	+	S	+	+	+	+	+	-	-	+	-
<i>H. glucozyma</i>	IFO 1472	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	W	+	+	L	+	+	-	+	+	-	W	-	-	W	-	
	MG 74	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	W	+	+	L	+	+	-	+	+	-	W	-	-	-	-	
<i>H. henricii</i>	IFO 1477	+	-	-	-	-	W	+	-	-	-	-	-	-	+	-	-	+	+	+	L	+	+	-	+	+	-	+	-	-	-	-	-
	1478	+	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+	-	+	+	-	+	-	-	-	-	
	MG 58	+	-	-	-	-	W	+	-	-	-	-	-	-	+	-	-	+	+	S	S	+	+	-	+	+	-	+	-	-	-	-	
<i>H. minuta</i>	IFO 0975	+	-	-	-	-	+	+	-	-	-	-	-	-	W	-	-	+	-	W	+	-	+	-	+	+	-	+	-	-	-	-	-
	MG 60	+	-	-	-	-	+	+	-	-	-	-	-	-	W	-	-	+	-	W	+	-	+	-	+	+	-	+	-	-	-	-	-
<i>H. nonfermentans</i>	IFO 1473	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	W	+	-	+	-	+	+	-	+	-	-	-	-	-
	MG 59	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	W	+	-	+	-	+	+	-	+	-	-	-	-	-
<i>H. philodendra</i>	MG 75	+	-	+	-	-	-	+	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	-	-	W	+	-	
<i>H. wickerhamii</i>	MG 73	+	-	+	-	-	-	+	-	-	-	-	-	-	+	S	+	+	+	+	S	+	+	-	+	+	-	-	-	-	-	-	-

Table 8. The GC-content in DNA and the ubiquinone system in methanol-assimilating yeasts.

Species	Strain		T _m (°)	GC Content (mol. %)	Ubiquinone system
<i>Hansenula philodendra</i>	MG	75	90.0	49.7	Q7
<i>Pichia cellobiosa</i>	KL	1	84.7	36.9	Q8, (Q7)
	KL	2	—	—	Q8, (Q7)
	KL	5	—	—	Q8, (Q7)
<i>Pichia lindnerii</i>	DSM	70718	89.3	48.0	Q7
<i>Candida boidinii</i>	MG	69	81.6	29.2	Q7
	KL	4	82.0	30.3	Q7
	KL	15	82.0	30.2	Q7
	KL	17	81.9	29.9	Q7
	AJ	4779	82.4	31.2	Q7
<i>Candida cariosi-</i> <i>lignicola</i>	KL	24	84.0	35.1	Q7
<i>Candida koshuensis</i>	AJ	4939	82.5	31.5	Q7
<i>Candida methanolica</i>	ATCC	26175	83.1	32.9	Q7
<i>Candida succiphila</i>	KL	21	86.8	41.9	Q7
	KL	30	86.4	40.9	Q7
<i>Kloeckera</i> sp. No. 2201	AKU	4705	83.0	32.7	Q7
<i>Torulopsis</i> <i>methanolovescens</i>	ATCC	26176	89.9	49.4	Q7
<i>Torulopsis molischiana</i>	KL	3	89.0	47.2	Q8
<i>Torulopsis nemodendra</i>	MG	72	85.8	39.5	Q7
<i>Torulopsis sonorensis</i>		71-148	—	36.1 (12)	Q7
<i>Candida parapsilosis</i>	IFO	1396	85.8	39.4	Q9 (38)
Calf thymus			86.8	42.0	

Hansenula glucozyma Wickerham. Strain: IFO 1472

Hansenula henricii Wickerham. Strains: IFO 1477 and MG 58

Hansenula minuta Wickerham. Strains: IFO 0975 and MG 60

Hansenula nonfermentans Wickerham. Strains: IFO 1473 and MG 59

Hansenula philodendra van der Walt et Scott. Strain: MG 75

Hansenula polymorpha de Moraes et Maia. Strains: IFO 1024, IFO 1071, IFO 1475, IFO 1476, MG 70, NRRL Y-1798, and NRRL Y-7560

Hansenula wickerhamii Capriotti. Strain: MG 73

Methanol assimilation of the above species was reported by HAZEU *et al.* (16), and all the strains used assimilated methanol as the sole carbon source. They showed the same characteristics as those in the standard descriptions (18, 27).

Pichia cellobiosa Lee et Komagata. Strains: KL 1, KL 2, and KL 5

This species forms hat-shaped ascospores, and assimilates galactose, L-sorbose, cellobiose, and a considerable number of sugars (Table 3). This species shows a GC-content of 36.9%, a characteristic ubiquinone system (Table 8), and a characteristic PMR spectrum of the cell-wall mannan (Fig. 2). The description

Fig. 1. PMR spectra of cell-wall mannans in the species of *Candida boidinii* strains and related species.

of this species was given in the previous paper (26).

Pichia lindnerii Henninger et Windisch. Strain: DSM 70718

This species was described by HENNINGER *et al.* (11) as a methanol-assimilating yeast, and the strain used showed the same characteristics as those in the standard description. The GC-content of the DNA was 48.0%, and the ubiquinone system was Q7 (Table 8). The PMR spectrum of the cell-wall mannan is shown in Fig. 2.

Pichia pastoris (Guilliermond) Phaff. Strains: IFO 0948, IFO 1013, and MG 65

Pichia pinus (Holst) Phaff. Strain: MG 66

Pichia trehalophila Phaff, Miller, et Spencer. Strain: MG 76

Methanol assimilation of these species was reported by HAZEU *et al.* (16), and all the strains used assimilated methanol as the sole carbon source. They showed the same characteristics as those in the standard descriptions. The PMR spectra of the cell-wall mannans of *P. pastoris* (IFO 0984 and MG 65), which are the same as those reported by GORIN and SPENCER (23) are shown in Fig. 2.

Asporogenous yeasts:

Candida boidinii Ramirez. Strains: KL 4, KL 6, KL 7, KL 8, KL 9, KL 10, KL 11, KL 12, KL 15, KL 17, KL 18, KL 19, KL 20, KL 23, KL 25, KL 26, KL 27, KL 28, KL 29, KL 33, MG 69, AJ 4779, *Candida kosuensis* (AJ 4939), *Kloeckera* sp. No. 2201 (AKU 4705), and *Candida methanolica* ATCC 26175

Fig. 2. PMR spectra of cell-wall mannans in methanol-assimilating yeasts.

The morphological, biochemical, and physiological characteristics of these strains resemble those of the type strain of *C. boidinii* (MG 69=CBS 2428). All the strains studied assimilated methanol as the sole carbon source. The GC-content of their DNA ranged from 29.9% to 32.9%, and the ubiquinone system and the PMR spectra of the cell-wall mannans were the same as those of the type strain of *C. boidinii* (Table 8 and Fig. 1).

C. koshuensis was described by YOKOTSUKA and GOTO (29) as a film-forming yeast of wine, and its similarity to *C. boidinii* strains (AJ 4778 and AJ 4779) which were isolated from a water-soluble fraction from defatted soybean flakes was reported by NAKASE and KOMAGATA (30). This similarity was confirmed in this study. The species was reidentified as *C. boidinii* because of the priority of the latter name

Table 9. Grouping of methanol-assimilating yeasts on the basis of chemotaxonomic characteristics.

Group	Species	GC Content (%)	Ubiquinone system	PMR Spectrum (Group)	Methanol oxidase
1	<i>Candida boidinii</i>	29.2–32.9	Q7	18-p	Type II
	<i>Candida succiphila</i>	40.9	7	9-a	Type III
	<i>Hansenula glucozyma</i>	45.1 (32)	7 (35)	9-b (23)	Type II
	<i>henricii</i>	49.8–50.2 (32)	7 (35)	9-b (23)	Type II
	<i>minuta</i>	46.8–47.3 (32)	7 (35)	9-b (23)	Type II
	<i>nonfermentans</i>	45.6 (32)	7 (35)	9-b (23)	Type II
	<i>polymorpha</i>	47.8–48.3 (32)	7 (35)	9-b (23)	Type II
	<i>wickerhamii</i>	45.4 (32)	7 (35)	9-b (23)	Type II
2	<i>Pichia pinus</i>	44.4 (34)	7 (35)	9-b (23)	Type II
	<i>Candida cariosilignicola</i>	35.1	7	9-b	Type II
	<i>Hansenula philodendra</i>	49.7	7	9-b	Type I
	<i>Pichia lindnerii</i>	48.0	7	9-b	Type I
	<i>Torulopsis methanolovescens</i>	49.4	7	9-b	Type I
	<i>pinus</i>	37.3 (37)	7 (38)	9-b (23)	Type I
	<i>nitratophila</i>	36.6 (37)	7 (38)	9-b (23)	Type III
	<i>Pichia trehalophila</i>	34.6 (34)	7 (35)	9-c (23)	Type II
	<i>Torulopsis nemodendra</i>	39.5	7	9-c	Type I
	<i>Torulopsis sonorensis</i>	36.1 (12)	7	6-f	Type I
3	<i>Pichia cellobiosa</i>	36.9	8, 7	9-d	Type II
	<i>Hansenula capsulata</i>	46.8–47.1 (32)	8 (35)	11-h (23)	Type III
4	<i>Torulopsis molischiana</i>	47.2	8	11-h (23)	Type III
	<i>Pichia pastoris</i>	40.2–41.0 (34)	8 (35)	6-b	Type III

Fig. 3. Cells of *Kloeckera* sp. No. 2201 (AKU 4705).

A, Vegetative cells grown in glucose-yeast extract-peptone water for 3 days at 25° ;
B, pseudomycelium grown on a slide culture for 7 days at 25°. Bar indicates 10 μ m in
both micrographs.

(31). *Kloeckera* sp. No. 2201 was isolated by OGATA *et al.* (2) as the first methanol-assimilating yeast, but until now the identification of this strain had not been clear. OGATA *et al.* identified it temporarily as a new species of *Kloeckera* on the basis of its lemon or oval cell shape and nonformation of pseudomycelium. But the strain had a long-oval or cylindrical cell shape in glucose-yeast extract-peptone water after three days at 25°, and formed an abundant *Candida*-type pseudomycelium on a slide culture (Fig. 3). From the morphological and chemotaxonomical characteristics, this strain was identified as *C. boidinii*. *C. methanolica* is a methanol-assimilating yeast which was isolated and described by OKI *et al.* (6). They identified it as a new species of *Candida* on the basis of the different assimilation pattern of sugars in spite of the similarity in cell morphology to *C. boidinii*. But the strain of the species used in this study had the same assimilation pattern as that of *C. boidinii*, which was different from that in the original description (Tables 4 and 5). From these characteristics, the species was reidentified as *C. boidinii*.

Candida cariosilignicola Lee et Komagata. Strain: KL 24

The GC-content of DNA was 35.1% in strain KL 24, and the ubiquinone system was Q7 (Table 8). This species showed the same PMR spectrum of the

cell-wall mannan as that of *H. philodendra*, *P. lindnerii*, and *T. methanoloescens* (Fig. 2). The description of this species was given in the previous paper (26). *Candida succiphila* Lee et Komagata. Strains: KL 14, KL 16, KL 21, KL 22, and KL 30

The GC-content of DNA was 40.9% in strain KL 30, and 41.9% in strain KL 21, and the ubiquinone system was Q7 (Table 8). The PMR spectrum of the cell-wall mannan is shown in Fig. 2. The description of this species was given in the previous paper (26).

Torulopsis methanoloescens Oki et Kouno. Strain: ATCC 26176

This species was reported as a methanol-assimilating yeast along with *C. methanolica* by OKI *et al.* (6). But it showed a very different sugar assimilation pattern from that in the original report, and the pattern seemed to be rather similar to that of *P. lindnerii* (Tables 4 and 5). The GC-content of DNA was 49.4%, which is similar to that of *P. lindnerii* (Table 8). Furthermore, this strain showed the same ubiquinone system and PMR spectrum of the cell-wall mannan as those of *P. lindnerii* (Table 8 and Fig. 2). These findings suggest a close relationship between *T. methanoloescens* and *P. lindnerii*.

Torulopsis molischiana (Zikes) Lodder. Strain: KL 3

This strain showed methanol assimilation and the same characteristics as those of *H. capsulata* (Tables 2 and 3), but did not form ascospores on various ascospore-forming media. The GC-content of DNA was 47.2% (Table 8), which was similar to that of *H. capsulata* (32). Furthermore, the strain showed the same ubiquinone system and PMR spectrum of the cell-wall mannan as those of *H. capsulata* (Table 8 and Fig. 2). Therefore, this strain was identified as *T. molischiana*, the imperfect form of *H. capsulata*, on the basis of these characteristics.

Torulopsis nemodendra van der Walt, van der Klift, et Scott. Strain: MG 72

Methanol assimilation of this species was reported by HAZEU *et al.* (16), and the strain used assimilated methanol as the sole carbon source. It showed the same characteristics as those of the standard description of *T. nemodendra* (33). The GC-content of DNA was 39.5%, and the ubiquinone system was Q7 (Table 8). The PMR spectrum of the cell-wall mannan is shown in Fig. 2.

Torulopsis nitratophila Shifrine et Phaff. Strain: MG 68

Torulopsis pinus Lodder et Kreger-van Rij. Strain: IFO 1327 and MG 67

Methanol assimilation by these species was reported by HAZEU *et al.* (16), and the strains used assimilated methanol as the sole carbon source. They showed the same characteristics as those in the standard descriptions of the species (18). *Torulopsis sonorensis* Miller, Phaff, Miranda, Heed, et Starmer.

Strains: 71–148, 76–201 A, and 78–508 C

Methanol assimilation of this species was reported by MILLER *et al.* (12), and the strains studied assimilated methanol as the sole carbon source. The ubiquinone system was Q7 (Table 8), and the species showed a characteristic PMR spectrum of the cell-wall mannan (Fig. 2).

Yeast species for which assimilation of methanol has been reported but was not confirmed in this study

Ascosporogenous yeasts:

Pichia haplophila Shifrine et Phaff. Strains: CBS 2028 and IFO 0947

Methanol assimilation of this species was reported by OKUMURA *et al.* (28), but the strains studied did not assimilate methanol as the sole carbon source. The strains studied showed the same characteristics as those in the standard description (18).

Asporogenous yeasts:

Torulopsis glabrata (Anderson) Lodder et de Vris. Strains: IFO 0005, IFO 0622, IFO 0861, and IFO 1085

In 1972, ASTANA *et al.* (3) isolated a methanol-assimilating yeast and identified it as *T. glabrata*. The strains used in this study showed the same characteristics as those in the standard description (18), but they did not assimilate methanol as the sole carbon source.

Fig. 4. Diagrammatic illustration of zymograms of methanol-oxidase in methanol-assimilating yeasts. ^a Relative mobility.

Electrophoretic patterns of methanol oxidase

We examined the electrophoretic pattern of methanol oxidase in yeast strains selected from those listed in Table 1, and the methanol oxidase was divided into three types by the zymograms as shown in Fig. 4.

The type strain of *C. boidinii* (MG 69=CBS 2428) and seven other *C. boidinii* strains which included *C. methanolica*, *Kloeckera* sp. No. 2201, and *C. koshuensis* had completely identical patterns (Fig. 5-A). This finding suggests again that they belong to the same species.

P. lindnerii DSM 70718, *T. methanolovescens* ATCC 26176, and *H. philodendra* MG 75 had a rather high GC-content of DNA (Table 8), and showed similar PMR spectra of their cell-wall mannans (Fig. 2). Furthermore, similar electrophoretic patterns of methanol oxidase were obtained (Fig. 4).

It is noteworthy that without exception all of the strains in the same species showed identical electrophoretic patterns of methanol oxidase, as indicated, for example, by the results with six strains of *H. polymorpha* shown in Fig. 5-B.

Fig. 5. Electrophoretic patterns of methanol oxidase.

A, *Candida boidinii* strains and related species; B, *Hansenula polymorpha* strains.

DISCUSSION

The methanol-assimilating yeasts freshly isolated by the present authors and those reported by other workers represented four genera, *Hansenula*, *Pichia*, *Candida*, and *Torulopsis*. *Hansenula* and *Pichia* are considered to be similar to each other in ascosporeogenous yeasts, and separated only by the utilization of potassium nitrate. They are similar not only in their cultural and physiological characteristics, but also in their chemotaxonomic characteristics, namely the GC-content of DNA (32, 34), the ubiquinone system (35, 36), and the PMR spectra of

cell-wall mannans (23). Methanol-assimilating yeasts in the genera *Candida* and *Torulopsis* are considered to be the imperfect forms of ascosporogenous yeasts, especially *Hansenula* and *Pichia*, on the basis of the GC-content of DNA (30, 37), the ubiquinone system (38), and the PMR spectra of the cell-wall mannans (23), as well as other characteristics. These facts suggest a close relationship among the methanol-assimilating yeasts, although they are distributed in the four genera by the present taxonomic system.

Starting with *C. boidinii*, almost all of the isolates tested and the known methanol-assimilating yeast species examined assimilated pectin from which methanol is formed by hydrolysis of its methyl esters, as shown in Tables 2, 4, and 6. From this point of view, we believe that methanol-assimilating yeasts play an important role in decomposition of methanol which originated from plant materials.

The GC-content of DNA of all methanol-assimilating yeasts showed a rather broad distribution from 29.2% (*C. boidinii*) to 50.2% (*H. henricii*). But their ubiquinone systems were Q7, with the exception of Q8 in *H. capsulata* and *P. pastoris* and Q8 (Q7) in *P. cellobiosa*, according to the present study and the results reported by YAMADA *et al.* (35, 36, 38). Furthermore, on the basis of the PMR spectra of the cell-wall mannans (Figs. 1 and 2), the methanol-assimilating yeasts belong to Group 9 in the grouping of GORIN and SPENCER (23). These facts suggest that methanol-assimilating yeasts have a close relationship with one another. As mentioned above, in our studies *P. haplophila* and *T. glabrata* did not assimilate methanol although their assimilation was reported by OKUMURA *et al.* (28) and ASTANA *et al.* (4). The ubiquinone systems of these species were Q9 and Q6 (36, 38), and they belong to Group 16 and Group 12 in the grouping of GORIN and SPENCER (23). Therefore, we consider that they have no or only a little relationship with the methanol-assimilating yeasts discussed above.

On the basis of chemotaxonomic characteristics, methanol-assimilating yeasts are divided into four groups as shown in Table 9.

1) *Group 1.* This group consists only of *C. boidinii* strains. *C. boidinii* has a lower GC-content of DNA than any other methanol-assimilating yeasts. The PMR spectra of the cell-wall mannans showed characteristic peaks with δ 4.94, δ 4.97, δ 5.05, and δ 5.19.

2) *Group 2.* Almost all of the methanol-assimilating yeasts which have been reported up to now, except *C. boidinii*, *H. capsulata*, and *P. pastoris*, belong to this group. The GC-content of DNA of this group has a broad distribution from 34.6% to 50.2%. The ubiquinone system is Q7, and the PMR spectra of the cell-wall mannans showed main peaks with δ 5.05 and δ 5.19, although they differ in minor peaks. The electrophoretic patterns of their methanol oxidases are Type I, Type II, and Type III, and vary within the group. This group will be separated into three or four sub-groups by their chemotaxonomic characteristics.

It is interesting that all of the methanol-assimilating yeasts of the genus *Hansenula*, except for *H. capsulata*, belong to this group. They are on line 5 or

near where the line is separated on Wickerham's phylogenetic tree (39). The GC-content of DNA is distributed in a rather narrow range from 45.1% to 50.2%, and the electrophoretic patterns of methanol oxidase show Type II, except for *H. philodendra*. These facts suggest a close relationship among the methanol-assimilating *Hansenula* yeasts.

3) *Group 3*. Only *P. cellobiosa* belongs to this group. It has a PMR spectrum of the cell-wall mannan and an electrophoretic pattern of methanol oxidase similar to those of group 2, but it showed a characteristic ubiquinone system (Q8 and Q7) which is different from any other methanol-assimilating yeasts.

4) *Group 4*. *H. capsulata* (*T. molischiana*) and *P. pastoris* belong to this group. Their ubiquinone systems are Q8 and differ from those of any other methanol-assimilating yeasts. The PMR spectra of their cell-wall mannans and the electrophoretic patterns of their methanol oxidase are different from those of the other groups.

As shown above, we could clearly characterize phenotypically similar methanol-assimilating yeasts on the basis of the GC-content of their DNA, the ubiquinone system, the PMR spectra of their cell-wall mannans, and the electrophoretic pattern of their methanol oxidase. Therefore, these chemotaxonomic characteristics should give useful information for yeast systematics.

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