FURTHER TAXONOMIC STUDY OF METHANOL-ASSIMILATING YEASTS WITH SPECIAL REFERENCES TO ELECTROPHORETIC COMPARISON OF ENZYMES

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An electrophoretic comparison was made of 13 enzymes in 53 strains of methanol-assimilating yeasts. Nine strains of *Candida boidinii* were divided into two closely related interspecific clusters. The methanol-assimilating *Hansenula* yeasts showed electrophoretic patterns of enzymes very similar to one another, suggesting a close interrelationship. *Pichia lindnerii* and *Candida methanolovescens* showed similar physiological and chemotaxonomic characteristics, but they differed in the electrophoretic patterns of their enzymes. *Pichia methanolica* and three strains of *Pichia cellobiosa* showed the same electrophoretic patterns and chemotaxonomic characteristics. Eleven strains of *Hansenula capsulata* and its supposed anamorph *Candida molischiana* showed different electrophoretic patterns of enzymes and DNA base composition. They were divided into 5 clusters on the basis of these characteristics. The facts suggest the heterogeneity of these species. It would not be appropriate to regard *C. molischiana* simply as an anamorph of *H. capsulata*. The electrophoretic patterns of the enzymes exhibited a good correlation with the groups of methanol-assimilating yeasts, based on the DNA base composition, the coenzyme Q systems, and the proton magnetic resonance spectra of cell-wall mannans.

In an earlier paper (1), we examined the freshly isolated and the known methanol-assimilating yeasts with regard to their deoxyribonucleic acid (DNA) base compositions, coenzyme Q systems, proton magnetic resonance (PMR) spectra of cell-wall mannans, and the electrophoretic patterns of methanol oxidases.

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They were divided into the following four groups: 1) The *Candida boidinii* group, 2) the group of methanol-assimilating *Hansenula* species and its related yeast species, 3) the *Pichia cellobiosa* group, and 4) the *Hansenula capsulata* and related species group. *Candida methanolica* (2), *Candida kosshuensis* (3), and *Kloeckera* sp. No. 2201 (4) were reidentified as *Candida boidinii*, and a relationship between *Candida methanolovescens* (2) and *Pichia lindnerii* (5) was suggested on the basis of phenotypic and chemotaxonomic characteristics.

The electrophoretic patterns of enzymes in microorganisms have been examined by researchers as a useful taxonomic tool (6-11). Recently, YAMAZAKI and KOMAGATA (12) reported the electrophoretic comparison of seven enzymes in 108 strains belonging to the genera *Rhodotorula* and *Rhodospiridium*, and they clarified the interrelationship of *Rhodotorula glutinis* and its varieties to *Rhodospiridium* species. Moreover, YAMAZAKI and KOMAGATA (13) carried out an electrophoretic comparison of enzymes on some asporogenous yeasts and their supposed ascosporeogenous states, and reported the close relationships between 14 teleomorphs and their anamorphic counterparts.

In this study, we compared the electrophoretic patterns of 13 enzymes of methanol-assimilating yeast strains. Furthermore, we investigated the DNA base compositions, the coenzyme Q systems, and the PMR spectra of cell-wall mannans of 14 newly added strains of methanol-assimilating yeasts, and confirmed the previous grouping (1).

This paper deals with the relationships among the methanol-utilizing yeasts on the basis of electrophoretic comparisons of enzymes and chemotaxonomic characteristics.

MATERIALS AND METHODS

Yeast strains. Fifty-three methanol-assimilating yeast strains belonging to 30 species in the genera *Hansenula*, *Pichia*, and *Candida* were used. The identity of the 14 newly added strains was confirmed by the methods described in the monograph, "The Yeasts, a Taxonomic Study" 2nd ed. (14). The methanol-assimilating yeasts in the genus *Torulopsis* were transferred to the genus *Candida* in accordance with the amendment of the concept of the genus *Candida* proposed by YARROW and MEYER (15). The sources of the strains tested are shown in Table 1.

The DNA base compositions. DNAs were isolated and purified by the procedure of CRYER et al. (17), 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 (18).

The coenzyme Q systems. The coenzyme Q was purified as reported by YAMADA and KONDO (19), and determined by the method of NATORI and NAGASAKI (20). Samples were analyzed by a Shimadzu LC-3A high performance liquid

chromatograph with 4.6×250 mm column of Zorbax ODS. The solvent system consisting of methanol-isopropyl ether (85:15) was used, and the flow rate was 1 ml/min. Detection was made with a Shimadzu spectrophotometric detector SPD-2A at 275 nm.

The PMR spectra of cell-wall mannans. Isolation and purification of mannans from yeast cells were carried out as reported by GORIN and SPENCER (21). PMR spectra of the mannans were obtained by the use of JNM-FX 400 Spectrometer, from 0.4 ml of D₂O solution containing 30–40 mg of mannan at room temperature (25°). In this paper, the groups of cell-wall mannans reported by GORIN and SPENCER (21) were expressed as “Type” in order to avoid confusion with our grouping of methanol-assimilating yeasts.

Electrophoretic patterns of enzymes.

1. *Cultivation and harvest of cells.* The culture medium contained 3 g, yeast extract; 3 g, malt extract; 10 g, glucose; 5 g, peptone; and 1,000 ml of deionized water (YM medium); the pH was not adjusted. The yeasts were cultivated and harvested by the method of YAMAZAKI and KOMAGATA (12). For methanol oxidase, the culture medium contained 5 g, (NH₄)₂SO₄; 1 g, K₂HPO₄; 0.5 g, MgSO₄·7H₂O; 0.1 g, CaCl₂·2H₂O; 0.1 g, NaCl; 0.5 g, yeast extract; 12 ml of methanol; and 1,000 ml of distilled water; the pH was adjusted to 6.5. Yeasts were cultivated and harvested by the same method as previously reported (1).

2. *Preparation of cell-free extract for electrophoresis.* Disruption of cells and preparation of cell-free extracts were the same as those of YAMAZAKI and KOMAGATA (12). In the case of methanol oxidase, cells were suspended in 0.05 M potassium phosphate buffer (pH 7.5), and were disrupted for 15 to 18 min by a mechanical cell homogenizer (B. Braun, Melsungen, Germany).

3. *Gel slab electrophoresis.* A type SJ-1060 SDH electrophoretic apparatus (ATTO Co., Ltd.) was used for vertical gel slab electrophoresis. A 3.0% large-pore upper gel and a 7.4% small-pore separation gel were prepared by the method of DAVIS (22). Electrophoresis was carried out by the method of YAMAZAKI and KOMAGATA (12).

4. *Staining procedure.* The following 13 enzymes were examined: Fructose-1, 6-bisphosphate aldolase (FA; EC 4.1.2.13), hexokinase (HK; EC 2.7.1.1), phosphoglucomutase (PGm; EC 2.7.5.1), glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.41), malate dehydrogenase (MDH; EC 1.1.1.37; NAD dependent), glutamate dehydrogenase (GDH; EC 1.4.1.4), alcohol dehydrogenase (ADH; EC 1.1.1.1), lactate dehydrogenase (LDH; EC 1.1.1.27), fumarase (Fmase; EC 4.2.1.2), catalase (Cat; EC 1.11.1.6), esterase (Est; EC 3.1.1.1), and methanol oxidase (MO; EC 1.1.3.13). The staining procedures for these enzymes except for methanol oxidase were those described by SICILLIANO and SHAW (23). Methanol oxidase was stained by the method previously reported (1). After staining, the gels were dried under vacuum with warming, and the relative mobilities (*R_m*) of the enzyme bands

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

Species	Strain ^a	Source ^b
<i>Candida</i>		
<i>C. boidinii</i>	MG 69	CBS 2428
	KL 4	soil, LEE and KOMAGATA, 1980 (1)
	KL 15	sap of cherry tree, LEE and KOMAGATA, 1980 (1)
	KL 17	waste water of a wine factory, LEE and KOMAGATA, 1980 (1)
	UCD 78-508D	Identified by PHAFF et al.
	AJ 4779	NAKASE et al., 1977 (32)
	AJ 4939	YOKOTSUKA and GOTO 1955 (3)
<i>C. boidinii</i> (Syn., <i>C. kosshuensis</i>)		
<i>C. boidinii</i> (Syn., <i>Kloeckera</i> sp. No. 2201)	AKU 4705	OGATA et al., 1970 (4)
<i>C. boidinii</i> (Syn., <i>C. methanolica</i>)	ATCC 26175	OKI et al., 1972 (2)
<i>C. cariosilignicola</i>	KL 24	IAM 12484, LEE and KOMAGATA, 1980 (16)
<i>C. maris</i> (Syn., <i>Torulopsis maris</i>)	CBS 5151	IGC 3398
<i>C. methanosorbosa</i> (Syn., <i>Torulopsis methanosorbosa</i>)	CBS 6853	YOKOTE et al., 1974 (33)
<i>C. methanolovescens</i> (Syn., <i>Torulopsis methanolovescens</i>)	ATCC 26176	OKI et al., 1972 (2)
<i>C. molischiana</i> (Syn., <i>Torulopsis molischiana</i>)	KL 3	soil, LEE and KOMAGATA, 1980 (1)
	CBS 136	NRRL Y-2237
	CBS 836	NRRL Y-2238
	CBS 837	NRRL Y-2234
	CBS 4327	
<i>C. nemodendra</i> (Syn., <i>Torulopsis nemodendra</i>)	MG 72	CBS 6280
<i>C. nitratophila</i> (Syn., <i>Torulopsis nitratophila</i>)	MG 68	CBS 2027
<i>Hansenula</i>		
<i>H. capsulata</i>	MG 117	CBS 1993
	IFO 0721	NRRL Y-1842
	IFO 0974	NRRL Y-1889 (= ATCC 16753)
	IFO 1768	
	IFO 1769	
	IFO 1770	
<i>H. glucozyma</i>	IFO 1472	NRRL YB-2185
<i>H. henricii</i>	IFO 1477	NRRL YB-2194
<i>H. minuta</i>	IFO 0975	NRRL Y-411
<i>H. nonfermentans</i>	IFO 1473	NRRL YB-2203
<i>H. philodendra</i>	MG 75	CBS 6075
<i>H. polymorpha</i>	MG 70	CBS 4732 (= NRRL Y-5445)
	IFO 1475	NRRL Y-2214

Table 1. (Continued)

Species	Strain ^a	Source ^b
	NRRL Y-1798	
	NRRL Y-7560	
<i>H. wickerhamii</i>	MG 73	CBS 4307
<i>Pichia</i>		
<i>P. cellobiosa</i>	KL 1	IAM 12481, LEE and KOMAGATA, 1980 (16)
	KL 2	IAM 12482, LEE and KOMAGATA, 1980 (16)
	KL 5	IAM 12483, LEE and KOMAGATA, 1980 (16)
<i>P. lindnerii</i>	DSM 70718	HENNINGER and WINDISCH, 1975 (5)
<i>P. membranaefaciens</i>	IAM 4987	IFO 0460
<i>P. methanolica</i>	CBS 6515	KATO et al., 1973 (26)
<i>P. naganishi</i>	IFO 1670	KODAMA and KYONO 1974 (34)
<i>P. pastoris</i>	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. pignaliae</i>	CBS 6071	
(Syn., <i>Torulopsis pignaliae</i>)		
<i>C. pinus</i>	IFO 1327	IBL 2807 (=CBS 970)
(Syn., <i>Torulopsis pinus</i>)		
<i>C. sonorensis</i>	UCD 71-148	MILLER et al., 1978 (35)
(Syn., <i>Torulopsis sonorensis</i>)	UCD 76-201A	MILLER et al., 1978 (35)
	UCD 78-508C	MILLER et al., 1978 (35)
<i>C. succiphila</i>	KL 21	IAM 12487, LEE and KOMAGATA, 1980 (16)
	KL 30	IAM 12489, LEE and KOMAGATA, 1980 (16)

^{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, Göttingen, West Germany; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; IBL (IGC), Laboratório de Microbiologia, Centro de Biologia, Instituto Gulbenkian de Ciência, Oeiras, Portugal; IFO, Institute for Fermentation, Osaka, 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.; UCD, Department of Food Science and Technology, University of California, Davis, U.S.A.

were calculated as the ratio of the distance that the enzyme moved from the origin to the distance that the tracking dye moved. The boldfaced *Rms* in Tables 4 through 7 are the most deeply stained bands.

RESULTS

The GC-contents of DNAs, the coenzyme Q systems, and the PMR spectral types of cell-wall mannans of the newly added 14 strains are shown in Table 2.

All strains of *Hansenula capsulata* showed the same coenzyme Q systems (Q-8) and PMR spectra of cell-wall mannans (Type 11-h), as far as examined. The GC-contents of the DNAs of the two strains, IFO 1768 and IFO 1769, were 46.2% and 46.6%, but the GC-content of DNA of the strain IFO 0974 was 51.2%, which was 5% higher than those of above-mentioned two strains.

Candida molischiana CBS 136 (type strain) and CBS 4327 showed the same coenzyme Q systems (Q-8) and PMR spectra of cell-wall mannans (Type 11-h), and these results accorded with those of the *H. capsulata* strains mentioned above, but the GC-content of CBS 4327 was 45.6%, which was 5% lower than the other *C. molischiana* strains.

Grouping of methanol-assimilating yeasts including 14 newly added strains

The previous grouping of methanol-assimilating yeasts based on the GC-content of DNA, the coenzyme Q systems, and the PMR spectra of cell-wall mannans (1) was confirmed by adding results from 14 newly added strains. The electrophoretic patterns of methanol oxidase was not used for this grouping. The confirmed grouping of methanol-assimilating yeasts is shown in Table 3. The methanol-yeasts were divided into 4 groups, as previously reported (1). However, in the present study, Group 2 was divided into 4 sub-groups, and Group 4 was divided into 2 sub-groups by the PMR spectra of cell-wall mannans.

Table 2. The GC-contents, the coenzyme Q systems, the PMR spectra of cell-wall mannans of the newly added strains.

Species	Strain	T_m (°)	GC-content (mol %)	Co-Q system	PMR spectrum (Type)
<i>C. boidinii</i>	UCD78-508D	83.10	33.3	Q7	18-p
<i>C. maris</i>	CBS 5151	88.03	40.1	Q7	9-b
<i>P. naganishi</i>	IFO 1670	87.10	43.3	Q7	9-c
<i>P. methanolica</i>	CBS 6515	84.71	37.4	Q8, 7	9-c
<i>C. methanosorbosa</i>	CBS 6853	83.50	34.3	Q7	9-c
<i>C. pignaliae</i>	CBS 6071	85.80	40.1	Q7	9-c
<i>H. capsulata</i>	IFO 0721	—	—	Q8	11-h
	IFO 0974	90.25	51.2	Q8	11-h
	IFO 1768	88.25	46.2	Q8	—
	IFO 1769	88.40	46.6	—	—
<i>C. molischiana</i>	CBS 136	89.80	50.1	Q8	11-h
	CBS 836	90.15	51.0	—	—
	CBS 837	89.50	49.3	—	—
	CBS 4327	88.00	45.6	Q8	11-h
<i>C. parapsilosis</i>	IFO 1396	85.77	40.00		
Calf thymus		86.82	42.63		

Table 3. Grouping of methanol-assimilating yeasts on the basis of the GC-contents, the coenzyme Q systems, and the PMR spectra of cell-wall mannans.

Group	Sub-group	Species	GC-content (%)	Co-Q system	PMR spectrum (Type)
1		<i>C. boidinii</i>	29.5–33.3	Q-7	18-p
	A	<i>P. membranaefaciens</i>	(41.7–42.4)*	(7)**	(9-a)***
		<i>C. succiphila</i>	41.5–42.5	7	9-a
	B	<i>C. cariosilignicola</i>	35.5	7	9-b
		<i>C. nitratophila</i>	(36.6)	(7)	(9-b)
		<i>C. pinus</i>	(37.3)	(7)	(9-b)
		<i>C. maris</i>	40.1	7	9-b
		<i>P. pinus</i>	44.4	(7)	(9-b)
		<i>H. glucozyma</i>	(45.1)	(7)	(9-b)
		<i>H. wickerhamii</i>	(45.4)	(7)	(9-b)
2		<i>H. nonfermentans</i>	(45.6)	(7)	(9-b)
		<i>H. minuta</i>	(46.8–47.3)	(7)	(9-b)
		<i>H. polymorpha</i>	(47.8–48.3)	(7)	(9-b)
		<i>P. lindnerii</i>	48.7	7	9-b
		<i>C. methanolovescens</i>	50.2	7	9-b
		<i>H. henricii</i>	(49.8–50.2)	(7)	(9-b)
		<i>H. philodendra</i>	50.5	7	9-b
	C	<i>C. methanosorbosa</i>	34.3	7	9-c
		<i>P. trehalophila</i>	(34.6)	(7)	(9-c)
		<i>C. nemodendra</i>	40.0	7	9-c
		<i>C. pignaliae</i>	40.1	7	9-c
		<i>P. naganishi</i>	43.3	7	9-c
	D	<i>C. sonorensis</i>	(36.1)	7	6-f
3		<i>P. methanolica</i>	37.4	7, 8	9-c
		<i>P. cellobiosa</i>	36.2	7, 8	9-c
	A	<i>H. capsulata</i>	46.2–51.2	8	11-h
4		<i>C. molischiana</i>	45.6–51.0	8	11-h
	B	<i>P. pastoris</i>	(40.2–41.0)	(8)	6-b

The values in parenthesis are quoted from references (*36–38, **39, 40, ***21).

Candida boidinii UCD 78-508D belongs to the Group 1 (the *C. boidinii* group).

Candida maris (CBS 5121) belongs to the sub-group B of the Group 2, and *Pichia naganishi*, *Candida methanosorbosa*, and *Candida pignaliae* belong to the sub-group C of the Group 2 on the basis of their characteristics mentioned above. Four strains of *Hansenula capsulata* and 4 strains of *Candida molischiana* belong to sub-group A of Group 4 (*H. capsulata* and related species group) without exception.

Electrophoretic patterns of enzymes

Tables 4 through 7 show the *Rm* values of 13 enzymes. Activities of G6PDH, MDH, GDH, LDH, and Fmase were found in all the strains tested, and their

Table 4. Comparison of the electrophoretic *Rm*

Species	Strain	FA	HK	PGm	G6PDH	6PGDH
<i>C. boidinii</i>	NG 69 (CBS 2428)	0.70	0.82	0.52	0.30	—
	KL 4	"	"	"	"	—
	KL 15	"	"	"	"	—
	KL 17	"	"	"	"	—
<i>C. boidinii</i>	UCD 78-508 D	"	"	0.63	0.32	—
	AJ 4779	"	"	"	"	—
	AJ 4939	"	"	0.60	"	—
	ATCC 26175	"	"	0.55	"	—
	AKU 4705	"	"	0.63	"	—
				0.60 0.63		

patterns were characteristic for each species throughout all groups. Activities of HK, FA, and PGm seemed to be comparatively weak, as the staining procedures were two-step reactions (23), and in several species they were not detected. It was difficult to detect catalase bands. Methanol oxidases showed small *Rm* values, and clear differences between species were not detected. MDH, ADH, LDH, and Est produced several enzymatic bands. HK, PGm, G6PDH produced one or two bands. FA, 6PGDH, GDH, and MO produced a single band.

Electrophoretic comparison of the species in the Group 1

The electrophoretic patterns of enzymes in nine *C. boidinii* strains were highly similar to each other, and distinct from those of other methanol-assimilating yeasts. These 9 strains formed two electrophoretically different sub-groups as shown in Table 4. PGm, G6PDH, MDH, ADH, LDH, Fmase, and Est were found in the strains tested. 6PGDH was not detected in any strains tested, and Cat was not detected in 4 strains. FA, HK, and MO produced single bands with *Rms* of 0.7, 0.82, and 0.10, respectively (Table 4).

From these results, they were divided into two clusters. The type strain MG 69 (CBS 2428) and three isolates (KL 4, KL 15, and KL 17) were of the cluster I, and UCD 78-508 D, AJ 4779, AJ 4939, ATCC 26175, and AKU 4705 were of the cluster II. Figure 1 shows the electrophoretic pattern of HK, PGm, Fmase, and GHD diagrammatically, and Fig. 2 shows those of Est, MDH, G6PDH, and MO.

Electrophoretic comparison of the species in Group 2

Six species (*H. glucozyma*, *H. wickerhamii*, *H. nonfermentans*, *H. minuta*, *H.*

values of enzymes from strains of Group 1.

MDH	GDH	ADH	LDH	Fmase	Cat	MO	Est
0.46	0.19	0.30, 0.44, 0.45, 0.46, 0.57	0.15, 0.28, 0.42, 0.57	0.37	—	0.10	0.68
"	"	"	"	"	—	"	"
"	"	"	"	"	—	"	"
"	"	"	"	"	—	"	"
0.41	"	0.22, 0.24, 0.37	0.16, 0.22, 0.35, 0.55	0.34	0.16	"	0.64, 0.68
"	"	0.22, 0.24, 0.30, 0.37	0.16, 0.22, 0.35, 0.54	"	0.17	"	"
"	"	0.22, 0.24, 0.37	"	"	"	"	"
"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	0.64, 0.75

polymorpha, and *H. philodendra*) of the genus *Hansenula* belonging to sub-group B of Group 2 showed similar patterns in FA, HK, PGm, LDH, and Fmase, and identical patterns in MO, but slight variations were found in other enzymes (Table 5). Four strains of *H. polymorpha* (strains MG 70, NRRL-1798, IFO 1475, and NRRL-7560) showed identical patterns in PGm, GDH, and MO, and very similar patterns in FA, HK, G6PDH, 6PGDH, and Est. Slight variations were found in the MDH, ADH, LDH, Fmase, and Cat patterns (Table 5). However, *H. henricii* showed different patterns in HK, G6PDH, GDH, and MDH from those of the above 6 species of the genus *Hansenula*, and the activity bands of FA, PGm, and ADH were not detected (Table 5).

Three strains of *Candida sonorensis* belonging to sub-group D of Group 2 showed identical patterns in PGm, G6PDH, MDH, GDH, ADH, Fmase, Cat, and MO, and very similar patterns in other enzymes tested (Table 5). *Candida nemodendra*, *Candida pignaliae*, and *Pichia naganishi* belonging to the sub-group C of the Group 2 and *Candida nitratoiphila* and *Candida maris* belonging to sub-group B of Group 2 showed patterns in many enzymes comparatively similar to each other and to those of *C. sonorensis* (Table 5).

The other species (*Pichia pinus*, *Pichia lindnerii*, *Candida methanolovescens*, *Hansenula henricii*, and *Candida pinus*) belonging to sub-group B of Group 2, *Pichia membranaefaciens* and *Candida succiphila* (two strains; KL 21, KL 30) belonging to the sub-group A of the Group 2, and *Candida methanosorbasa* and *Pichia trehalophila* belonging to the sub-group C of the Group 2 did not show significant similarities to the above-mentioned species in most all of the enzymes tested. *P. lindnerii* and *C. methanolovescens* showed comparatively similar patterns in HK, PGm, MDH, and LDH, and identical patterns in GDH and

Table 5. Comparison of the electrophoretic *R_m*

Sub-group	Species	Strain	FA	HK	PGm	G6PDH	6PGDH
A	<i>P. membranaefaciens</i>	IAM 4987	0.44	0.28 0.63	0.40	0.38	0.35
	<i>C. succiphila</i>	KL 30	0.62	0.95	0.51	0.28	0.29
		KL 21	"	"	0.52	"	"
B	<i>C. cariosilignicola</i>	KL 24	0.70	0.43 0.56	—	0.38 0.41	—
	<i>C. nitratophila</i>	MG 68	0.57	0.56 0.61	0.68	0.33	—
	<i>C. pinus</i>	IFO 1327	—	0.47 0.87	—	0.38	0.67
	<i>C. maris</i>	CBS 5151	—	0.38 0.69	—	0.33 0.35	—
	<i>P. pinus</i>	MG 66	0.55	0.46 0.74	0.69	0.37	—
	<i>H. glucozyma</i>	IFO 1472	0.53	0.33 0.72	0.65	0.32 0.34	0.45
	<i>H. wickerhamii</i>	MG 73	0.54	0.34 0.68	0.57	0.30 0.32	0.56
	<i>H. nonfermentans</i>	IFO 1473	0.47	0.36 0.70	0.59	0.39 0.43	0.41
	<i>H. minuta</i>	IFO 0975	0.57	0.38 0.72	0.59	0.45 0.48	0.34 0.36
	<i>H. polymorpha</i>	MG 70	0.51	0.40 0.69	0.58	0.25 0.28	0.34
		NRRL-1798	0.48	0.38 0.69	"	"	"
	<i>H. polymorpha</i>	IFO 1475	0.51	0.38 0.69	0.58	0.29 0.32	0.33
		NRRL-7560	"	0.36 0.66	"	"	"
	<i>P. lindnerii</i>	DSM 70718	—	0.79	0.71	0.30 0.32	—
	<i>C. methanolovescens</i>	ATCC 26176	—	0.49 0.82	0.68	0.38 0.40	—
	<i>H. henricii</i>	IFO 1477	—	0.44 0.81	—	0.39 0.40	0.41
	<i>H. philodendra</i>	MG 75	0.56	0.32 0.69	0.53	0.29 0.30 0.32	0.32
C	<i>C. methanosorbosa</i>	CBS 6853	0.71	0.45 0.80	0.48	0.37 0.39	—
	<i>P. trehalophila</i>	MG 76	0.66	0.56 0.68	0.62	0.38 0.40 0.45	—

values of enzymes from strains of Group 2.

MDH	GDH	ADH	LDH	Fmase	Cat	MO	Est
0.34	0.11	—	0.14 , 0.53	0.30	—	0.08	0.67
0.46, 0.50	0.19	0.31, 0.41	0.20, 0.25 0.51, 0.58	0.36	0.19	0.08	0.50, 0.73 , 0.75
"	"	"	"	"	"	"	0.60 , 0.64
0.35 , 0.50	0.16	—	0.22 , 0.58	0.32	0.20 0.25	0.10	0.62, 0.67 , 0.70
0.30 , 0.32	0.16	0.32, 0.45 0.50, 0.53	0.45 , 0.58	0.39	0.28	0.08	0.62, 0.67 , 0.70, 0.72
0.35 , 0.62	0.16	—	0.25 , 0.51	0.36	0.20	0.12	0.45 , 0.63
0.32 , 0.57	0.12	0.34 , 0.47	0.34, 0.45 , 0.56	0.30 0.33 0.45	0.19 0.24	0.12	0.50, 0.65 0.68
0.46 , 0.50	0.16	0.34, 0.42	0.55	0.43	—	0.10	0.40
0.38, 0.42 , 0.44	0.14	0.28, 0.40	0.22 , 0.48	0.35	0.23	0.10	0.40, 0.54 0.67 , 0.70
0.42 , 0.54, 0.56	0.155	0.25, 0.28	0.21 , 0.25 0.51	0.24	0.15	"	0.60, 0.63 , 0.75
0.35, 0.38 , 0.42, 0.52 0.44	0.16 0.14	0.22, 0.31 0.18, 0.22, 0.27 , 0.52	0.20 , 0.57 0.20, 0.56	0.35 0.39	0.16 —	0.10 "	0.46, 0.54 0.38, 0.52 0.54 , 0.58
0.30 , 0.40	0.20	0.22 , 0.31, 0.35	0.17 , 0.51	0.30	0.17	"	0.38 , 0.48, 0.53
0.32	"	0.24 , 0.31, 0.38	0.18 , 0.58	0.22 0.30	"	"	0.40 , 0.48
0.38 , 0.41 0.43	0.20	0.15, 0.25, 0.31	0.15 , 0.57,	0.23	0.20	0.10	0.39 , 0.52, 0.59
0.34 , 0.41	"	0.24 , 0.29, 0.35	0.15 , 0.59	0.22 0.30	"	"	0.40 , 0.48, 0.52
0.44 , 0.58 0.60	0.135	0.38	0.22 , 0.51	0.34	0.21	0.12	0.40, 0.52 , 0.55
0.44 , 0.52 0.54, 0.56 0.45	0.135 0.12	0.42 —	0.20 , 0.56 0.26 , 0.45	0.38 0.35	— 0.20	" 0.10	0.35, 0.58, 0.66 0.68 , 0.71
0.27 , 0.30	0.11	0.22 , 0.32, 0.35	0.15 , 0.51	0.28	0.15 0.20	0.11	0.42, 0.75
0.43	0.15	0.31	0.20 , 0.31	0.32	0.19 0.24	0.10	0.65
0.35 , 0.50	0.19	0.32, 0.47	0.18 , 0.48	0.34	0.22	0.11	0.65, 0.68

Table 5.

Sub-group	Species	Strain	FA	HK	PGm	G6PDH	6PGDH
D	<i>C. nemodendra</i>	MG 72	0.57	0.40	—	0.33	—
				0.66		0.35	
	<i>C. pignaliae</i>	CBS 6071	0.58	0.32	—	0.38	—
				0.66		0.42	
	<i>P. naganishi</i>	IFO 1670	0.55	0.45	0.52	0.45	0.35
					0.59	0.47	
	<i>C. sonorensis</i>	UCD 71-148	0.76	0.53	0.60	0.33	—
				0.78		0.35	
						0.37	
		76-201 A	"	0.40	"	"	—
				0.58			
		78-508 C	"	"	"	"	—

Fig. 1. Diagrammatic illustration of the HK, PGM, Fmase, and GDH patterns in the strains of *C. boidinii*.

A, MG 90; B, KL 4; C, KL 15; D, KL 17; E, UCD 78-508 D; F, AJ 4779; G, AJ 4939; H, ATCC 26175; I, AKU 4705.

MO, but variations occurred in the other enzymes. FA and 6PGDH were not detected in either species, and Cat was not detected in *C. methanolovescens* (Table 5).

Electrophoretic comparison of the species in Group 3

Three strains (KL 1, KL 2, and KL 5) of *Pichia cellobiosa* and *Pichia methanolica* showed identical patterns in the enzymes tested except for slight variations in FA, LDH, Cat, and G6PDH (Table 6).

Continued.

MDH	GDH	ADH	LDH	Fmase	Cat	MO	Est
0.35, 0.37	0.16	—	0.57	0.23	0.14	0.12	0.38, 0.50
0.32, 0.55	0.20	—	0.22, 0.55	0.32	0.14 0.26	0.11	0.35, 0.39, 0.42, 0.57, 0.59
0.27, 0.52	0.08	0.35, 0.37, 0.56	0.26, 0.51, 0.61, 0.62	0.32	0.22 0.26	0.08	0.50, 0.52, 0.68
0.30, 0.60 0.62	0.20	0.22, 0.27, 0.45	0.22, 0.33, 0.57	0.31	0.26	0.10 0.12	0.58, 0.62, 0.64
"	"	"	"	"	"	"	0.64, 0.67
"	"	"	0.22, 0.33, 0.61	"	"	"	0.46, 0.64, 0.70

Fig. 2. Diagrammatic illustration of the Est, MDH, G6PDH, and MO patterns in the strains of *C. boidinii*.

A, MG 69; B, KL 4; C, KL 15; D, KL 17; E, UCD 78-508 D; F, AJ 4779; G, AJ 4939; H, ATCC 26175; I, AKU 4705.

Electrophoretic comparison of the species in Group 4

Eleven strains of *Hansenula capsulata* and *Candida molischiana* belonging to sub-group A tended to be classified into 5 clusters on the basis of their enzymatic patterns, especially in the patterns of G6PDH, MDH, GDH, and LDH (Table 7). The strains in the *cluster I* produced G6PDH with *Rm* values of 0.42 to 0.44, MDH with *Rm* values of 0.43 to 0.44 or 0.34 and 0.42, GDH with an *Rm* value of 0.19, and LDH with *Rm* values of 0.21 and 0.60; the type strain (MG 117) and 4 strains (IFO 1721, IFO 1768, IFO 1769, and IFO 1770) of *H. capsulata* were included in this type. Members of *cluster II* produced G6PDH with an *Rm* value

Fig. 3. Diagrammatic illustration of the Est, MDH, and GDH patterns in the strains of *H. capsulata* and *C. molischiana*.

H. capsulata: A, MG 117; B, IFO 0721; C, IFO 1768; D, IFO 1769; E, IFO 1770; F, IFO 0974. *C. molischiana*: G, KL 3; H, CBS 136; I, CBS 836; J, CBS 837; K, CBS 4327.

Fig. 4. Diagrammatic illustration of the G6PDH, Fmase, and MO patterns in the strains of *H. capsulata* and *C. molischiana*.

H. capsulata: A, MG 117; B, IFO 0721; C, IFO 1768; D, IFO 1769; E, IFO 1770; F, IFO 0974. *C. molischiana*: G, KL 3; H, CBS 136; I, CBS 836; J, CBS 837; K, CBS 4327.

of 0.48, MDH with R_m values of 0.34 and 0.44 to 0.51, GDH with an R_m value of 0.18, and LDH with R_m values of 0.23 and 0.53; the type strain (CBS 136) and 2 strains (CBS 836 and CBS 837) of *C. molischiana* were included in this cluster. *H. capsulata* IFO 0974 produced G6PDH with an R_m value of 0.48, MDH with R_m values of 0.42 and 0.44, GDH with an R_m value of 0.16, and LDH with R_m values of 0.23 and 0.56, and belonged to *cluster III*. *C. molischiana* KL 3 produced G6PDH with an R_m value of 0.46, MDH with R_m values of 0.34 and 0.53,

Table 7. Comparison of the electrophoretic *Rm*

Species	Strain	FA	HK	PGm	G6PDH	6PGDH	MDH
<i>H. capsulata</i>	MG 117	—	—	0.64	0.44	—	0.43
	IFO 0721	—	—	—	"	—	0.44
	IFO 1768	—	—	—	0.42	—	"
	IFO 1769	—	—	—	"	—	"
	IFO 1770	—	—	—	0.44	—	0.34, 0.42
	IFO 0974	0.55	0.57	0.68	0.48	—	0.42, 0.44
<i>C. molischiana</i>	CBS 136	0.52 0.65	0.57	0.86	0.48	—	0.34, 0.51
	CBS 836	"	"	"	"	—	"
	CBS 837	"	"	—	"	—	0.34, 0.44
	KL 3	0.65	—	—	0.46	—	0.34, 0.53
	CBS 4327	"	0.55	0.68	0.53	—	0.38, 0.50
<i>P. pastoris</i>	MG 65	0.55	0.40	0.61	0.35	0.39	0.46

GDH with an *Rm* value of 0.16, and LDH with *Rm* values of 0.23 and 0.53, and belonged to *cluster IV*. *C. molischiana* CBS 4327 produced G6PDH with an *Rm* value of 0.53, MDH with *Rm* values of 0.38 and 0.50, GDH with an *Rm* value of 0.175, and LDH with *Rm* values of 0.24 and 0.53, and belonged to *cluster V*. Figure 3 shows Est, MDH, and GDH, and Fig. 4 shows electrophoretic patterns of G6PDH, Fmase, and MO in these strains diagrammatically.

Pichia pastoris MG 65 belonging to the sub-group B showed patterns in many of the enzymes tested very different from those of the strains belonging to the sub-group A (Table 7).

DISCUSSION

Candida boidinii (Group 1)

This species is characterized by the low GC-content of DNA (about 30%) and the PMR spectra of cell-wall mannans (Type 18-p). Therefore, this species was differentiated from other methanol-assimilating yeast species and classified in Group 1 (Table 3). *C. boidinii* was described by RAMIREZ in 1953 (24), and the strains of this species were isolated from many natural sources not related to methanol (16). *C. boidinii* showed comparatively broad distribution in natural sources (3, 4, 14, 25), and a large number of isolates in our previous study (1) were included in this species. Among the 9 strains tested in this study, 3 strains, AJ 4779 (= *Candida koshuensis* Yokotsuka et Goto), ATCC 26175 (= *Candida methanolica* Oki et al.), and AKU 4705 (= *Kloeckera* sp. No. 2201 Ogata et al.),

values of enzymes from strains of Group 4.

GDH	ADH	LDH	Fmase	Cat	MO	Est
0.19	—	0.21, 0.60	0.34	—	0.08	0.75
"	—	"	0.36	—	"	0.45, 0.60, 0.68
"	—	"	"	—	"	0.50, 0.72
"	—	"	"	—	"	"
"	—	"	0.37	—	"	0.45, 0.60, 0.68
0.16	0.22, 0.32, 0.47	0.23, 0.56	0.32	—	0.09	0.45, 0.50, 0.67
0.18	—	0.23, 0.53	0.37	0.17	0.08	0.67, 0.75
"	—	"	0.38	"	"	"
"	—	"	0.37	"	"	"
0.16	—	"	0.38	0.15	"	0.55, 0.58, 0.71
0.175	0.22, 0.32, 0.49	0.24, 0.53	0.32	"	"	0.60, 0.68
0.18	0.42	0.25, 0.57	0.34	0.17	"	0.71

were reidentified as *Candida boidinii* Ramirez on the basis of morphological, biological, and chemotaxonomic characteristics in our previous study (1).

As mentioned above, 9 strains of *C. boidinii* were classified into two clusters by their electrophoretic patterns of enzymes. Further, the GC-contents of the DNAs of these strains showed a good correlation with these results; the strains (CBS 2428, KL 4, KL 15, and KL 17) of the *cluster I* showed the GC-contents of 29.5–30.7%, and the strains (UCD 78-508 D, AJ 4779, ATCC 26175, AKU 4705) of *cluster II* showed GC-contents of 31.6–33.3%. From this, we can deduce the existence of two chemovarieties.

The species of Group 2

The close interrelationships in *Hansenula* methanol-assimilating yeasts species except *H. capsulata* were discussed in the previous study (1), and were confirmed on the basis of electrophoretic patterns of enzymes in this study. *H. henricii* showed comparatively different patterns in some enzymes tested from other *Hansenula* species mentioned above, and showed slightly higher GC-contents of DNAs (49.8–50.2%) than those of other species (45.1–48.3%). Therefore, it will be necessary to study more detailed taxonomic characteristics.

C. methanolovescens was considered to be an anamorph of *P. lindnerii* in the previous study (1) because of high similarities in morphological and biochemical characteristics. However, in this study, they did not show such high similarity in the electrophoretic patterns of the enzymes, and we could not confirm that *C. methanolovescens* is an anamorph of *P. lindnerii*.

C. sonorensis had been classified into sub-group D according to the characteristic PMR spectra of cell-wall mannans (Table 3), but this species showed similarities to three other species (*C. nemodendra*, *C. pignaliae*, and *P. naganishi*) of sub-group C and two other species (*C. nitratophila* and *C. maris*) of sub-group B. These species showed a relatively narrow range in GC-content (36.6–43.3%).

C. succiphila which had been classified in sub-group A according to the PMR spectra of cell-wall mannans showed similarity to two species (*H. henricii* and *C. pinus*) of sub-group B and *P. pastoris* (the sub-group B of Group 4) on the basis of electrophoretic comparison of enzymes. These species showed a very narrow range of GC-contents of DNAs (40.2–44.4%).

The slightly different trends, as mentioned above, of the PMR spectra of cell-wall mannans and the electrophoretic patterns of enzymes are supposed to come from the following: The PMR spectra of cell-wall mannans mainly express the differences in exterior cell structure. On the other hand, the electrophoretic patterns of enzymes express the differences in the base arrangement of DNA. These assumptions are based on the facts that the GC-content of DNA was correlated with the electrophoretic patterns rather than the PMR spectra of the cell-wall mannans.

P. methanolica and *P. cellobiosa* (Group 3)

P. methanolica was isolated and identified by KATO et al. (26) as a new species of the genus *Pichia* on the basis of non-assimilation of nitrate and the formation of spherical or hemispherical ascospores. In this study, however, *P. methanolica* showed assimilation and fermentation patterns different from those in the original description (26), and had the same patterns as those of *P. cellobiosa*, which was reported as a new methanol-assimilating yeast species by LEE and KOMAGATA in 1980 (16). Table 8 indicates characteristics of *P. methanolica* different from those of KATO et al. (26), and some other chemotaxonomic characteristics of these species. *P. cellobiosa* and *P. methanolica* showed not only exactly identical patterns in most all of the enzymes, but also had similar GC-contents of DNAs and the same coenzyme Q systems, PMR spectra of cell-wall mannans, and biochemical characteristics (Table 3). From this, we confirmed the synonymous relation between *P. methanolica* and *P. cellobiosa*.

The species of Group 4

H. capsulata was isolated from frass from a coniferous tree and described by WICKERHAM in 1951 (27). This species is supposed to be the most primitive homothallic species, and was distinguished from other methanol-assimilating *Hansenula* species, especially by the coenzyme Q systems (Q-8) and the PMR spectra of cell-wall mannans (Type 11-h). *C. molischiana* was first described by ZIKES (28) in 1911 with *Torula molischiana*, and later LODDER (29) transferred it to the genus *Torulopsis* in 1934, while SKINNER (30) classified it as *Cryptococcus*

Table 8. Some characteristics of *P. methanolica* and *P. cellobiosa* strains.

Species	Strain	GC-content (%)	Co-Q system	PMR spectrum (Type)	Fermentation of maltose	Fermentation of cellobiose	Assimilation of L-sorbose	Assimilation of D-arabinose	Assimilation of galactitol	Assimilation of DL-lactate	Shape of ascospore	References
<i>P. methanolica</i>	CBS 6515	NT	NT	NT	+	NT	—	—	NT	+	Spherical or hemispherical	KATO et al. (26)
<i>P. methanolica</i>	CBS 6515	37.4	Q 7, 8	9-c	—	L	+	+	+	—	Hat-shape	
<i>P. cellobiosa</i>	KL 1	36.2	Q 7, 8	9-c	—	L	+	+	+	—	Hat-shape	
	KL 2	NT	Q 7, 8	NT	—	L	+	+	+	—	Hat-shape	
	KL 5	NT	Q 7, 8	NT	—	L	+	W	W	—	Hat-shape	

NT, not tested; L, latent; W, weak.

Table 9. Some characteristics of *H. capsulata* and its anamorph *C. molischiana* strains.

Species	Strains	GC-content (%)	Co-Q system	PMR spectrum (Type)	Assimilation of maltose	Growth at 37°	Colony characteristics
<i>H. capsulata</i>	MG 117	47.1	Q 8	11-h	+	—	Slightly mucoid
	IFO 0721	NT	Q 8	NT	+	—	Mucoid
	IFO 1768	46.2	Q 8	NT	—	—	Mucoid
	IFO 1769	46.6	NT	NT	—	—	Mucoid
	IFO 1770	NT	NT	NT	+	—	Mucoid
	IFO 0974	51.2	Q 8	11-h	+	+	Smooth
<i>C. molischiana</i>	CBS 136	50.1	Q 8	NT	+	+	Mucoid
	CBS 836	51.0	NT	NT	+	+	Smooth
	CBS 837	49.3	NT	NT	+	+	Smooth
	KL 3	48.0	Q 8	11-h	+	+	Mucoid
	CBS 4327	45.6	Q 8	11-h	+	+	Slightly mucoid

molischianus in 1947. However, this species has very similar characteristics to those of *H. capsulata* except for the formation of ascospore, and was regarded as the anamorph of the homothallic species, *H. capsulata*.

In this study, 11 strains of *H. capsulata* and *C. molischiana* produced different electrophoretic patterns of enzymes, and they showed variation in the assimilation of maltose, growth at 37°, colonial characteristics, and the GC-contents of DNAs, but they showed the same coenzyme Q systems and PMR spectra of cell-wall mannans, as shown in Table 9. These facts suggest the heterogeneity of these species even though *C. molischiana* was regarded as an anamorph of *H. capsulata* by VAN UDEN and VIDAL-LEIRIA (31). The strains in both species were divided into several clusters based upon the electrophoretic patterns of enzymes and the GC-contents of DNAs. The type strain (MG 117) and 4 strains (IFO 0721, IFO 1768, IFO 1769, and IFO 1770) of *H. capsulata* are in *cluster I* (GC-content of DNA: 46.2–47.1%); the type strain (CBS 136) and 2 strains of *C. molischiana* are in *cluster II* (GC-content of DNA: 49.3–50.1%); *H. capsulata* IFO 0974 is in *cluster III* (GC-content of DNA: 51.2%); *C. molischiana* KL 3 is in *cluster IV* (GC-content: 48.0%); and *C. molischiana* CBS 4327 is in *cluster V* (GC-content: 45.6%).

From this, we can conclude that *H. capsulata* and *C. molischiana* are very closely related compared to any other species, but it is difficult to say that *C. molischiana* is an anamorph of *H. capsulata* as reported by VAN UDEN and VIDAL-LEIRIA (31). More detailed taxonomic studies are needed to clarify the varieties of these species.

Methanol-assimilating yeasts are composed of species which have similar phenotypic characteristics, and difficulties are encountered in distinguishing these

species from one another and from the other non-methanol-assimilating yeasts by the conventional methods of yeast classification. As shown above, we can clarify their taxonomic relationships on the basis of chemotaxonomic characteristics. Methanol-assimilating yeasts are composed of some closely related species which are distributed in the genera *Hansenula* and *Pichia* of ascosporogenous yeasts and the genus *Candida* (especially the *Candida* species which are supposed to be the anamorphs of the species of *Hansenula* and *Pichia*). The electrophoretic comparison of enzymes is very useful for differentiation and identification of micro-organisms, especially for the clarification of inter-relationships among the species, and the strains of the same species.

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