Taxonomic Significance of Electrophoretic Comparison of Enzymes in the Genera Rhodotorula and Rhodosporidium

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We used polyacrylamide slab gel electrophoresis with specific staining for seven enzymes to compare 108 strains belonging to the genera Rhodotorula and Rhodosporidium. The patterns obtained allowed us to divide the strains of Rhodotorula glutinis and its varieties into six groups. The strains of Rhodotorula rubra and Rhodotorula pilimanae examined had identical patterns. Rhodotorula minuta var. minuta and Rhodotorula minuta var. texensis clearly differed from Rhodotorula glutinis and Rhodotorula rubra in their hexokinase and 6-phosphogluconate dehydrogenase patterns. Rhodotorula marina, Rhodotorula palida, and Rhodotorula lactosa each had a characteristic pattern. We found three haploid mating type strains to mating type a of Rhodosporidium toruloides, mating type a of Rhodosporidium sphaerocarpum, and mating type a of Rhodosporidium diobovatum among the strains of Rhodotorula glutinis var. rufusa, Rhodotorula glutinis var. salinaria, and Rhodotorula glutinis var. glumatis by performing conjugation tests to confirm the relationships that were revealed when the enzyme patterns were compared. In the genus Rhodosporidium, compatible mating type strains showed similar enzymatic patterns. Strains of Rhodosporidium toruloides which could not assimilate potassium nitrate had the same enzymatic patterns as those that could.

Morphological and physiological characteristics have been used to classify and identify the yeasts and yeastlike organisms, but the problems of using such properties have been described by Phaff and Price (38). Recently, information useful in yeast taxonomy has been reported in the areas of serology (47), proton magnetic resonance spectra of cell wall mannans (12), base compositions of deoxyribonucleic acids (DNA) (30), coenzyme Q systems (51), and DNA-DNA hybridizations (7).

The electrophoretic patterns (zymograms) of various enzymes are considered to be useful tools in the taxonomy of plants (5), animals (25, 36, 42), and microorganisms (4, 35, 53). It is thought that zymograms reflect minor structural differences at the molecular level and that similarities in enzyme systems reflect the relatedness of organisms. However, this technique has been used little in yeast taxonomy until now. Electrophoretic analyses of enzymes have been reported for some Candida species (6, 40) and for Cryptococcus laurentii (3).

The sexual state of a Rhodotorula species was first reported by Banno (1) in 1963; he observed a basidiomycetous life cycle in strains of Rhodotorula glutinis after conjugation of two strains. Banno (2) described the genus Rhodosporidium and the species Rhodosporidium toruloides for this teleomorph. Later, Newell and Fell (33) described Rhodosporidium sphaerocarpum, and six Rhodosporidium species were described by Fell (10), Newell and Hunter (34), and Fell et al. (11).

Nakase and Komagata (31) reported that the guanine-plus-cytosine (G+C) contents of the DNAs of Rhodotorula strains range from 50.0 to 68.5 mol%, and they classified these strains into four groups on the basis of their G+C contents. Yamada and Kondo (52) reported that the coenzyme Q systems of Rhodotorula species are Q9, Q8, or Q10. This study revealed that these yeasts should be related to heterobasidiomycetes rather than to ascomycetes and that cytological and biochemical characteristics would be useful in the classification of asporogenous yeasts.

In this investigation we compared the enzymes of Rhodotorula strains with the enzymes of Rhodosporidium strains and applied the results to the classification of these genera.

MATERIALS AND METHODS

Yeast strains. We used 64 strains representing 12 species and six varieties in the genus Rhodotorula and 44 strains representing eight species in the genus Rhodosporidium. The sources of these strains are shown in Table 1. We confirmed the identities of all of these strains by the methods of van der Walt (48). The designations for the mating types are the designations used in the original reports.

361
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*Abbreviations: IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan; CCY, Czechoslovak Collection of Yeasts, Bratislava, Czechoslovakia; AJ, Central Research Laboratories, Ajinomoto Co., Inc., Kawasaki, Japan; CBS, Centraalbureau voor Schimmel-cultures, Delft, The Netherlands; ATCC, American Type Culture Collection, Rockville, Md.; RIFY, Research Institute of Fermentation, Yamashita University, Kofu, Japan.

**Cultivation and harvest of cells.** The culture medium used contained 3 g of yeast extract, 20 g of glucose, 5 g of (NH₄)₂SO₄, 0.5 g of K₂HPO₄, 0.5 g of Na₂HPO₄, 1 g of MgSO₄·7H₂O, and 1,000 ml of deionized water; the pH of this medium was 7.2. The yeasts were cultivated in 500-ml flasks containing 200 ml of medium; incubation was for 24 h at 20 or 27°C with shaking. The cells were harvested by centrifugation at 4,500 × g for 5 min at a low temperature and were washed with 0.05 M tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 7.8). The harvested cells were stored in a freezer at −20°C until they were used.

**Preparation of cell-free extracts for electrophoresis.** Cells were disrupted with a mechanical cell homogenizer (B. Braun, Melsungen, Germany). The frozen cells of each culture were suspended in 1 to 2 volumes of 0.05 M tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 7.8) and were transferred to a 50- or 70-ml glass vessel containing glass beads.
yses and was stored in a freezer at \(-20^\circ C\) until just before use.

**Gel slab electrophoresis.** A type SJ-1060 SDH electrophoretic apparatus (ATTO Co., Ltd.) was used for vertical gel slab electrophoresis; this apparatus provided a slab 2 mm thick. A 3.0% large-pore upper gel and a 7.5% small-pore separation gel were prepared by the method of Davis (9), and 12 individual slots were available at one time. Tris(hydroxymethyl)-aminoethane-glycine buffer (pH 8.3) was used as the electrode buffer, and bromophenol blue was used as the tracking dye. Usually, an enzyme preparation from *Rhodotorula glutinis* var. *glutinis* YK 105 (IAM 12263) was used as a reference. Gel electrophoresis was performed at a regulated current of 15 to 20 mA/gel slab for 6 to 8 h at \(5^\circ C\). After electrophoresis, the gel slab was removed from the gel mold and was stained to visualize the enzymes.

**Staining procedures.** We studied the following seven enzymes: fructose-1,6-bisphosphate aldolase (FA; EC 4.1.2.13), 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.41), malate dehydrogenase (MDH; EC 1.1.1.37; nicotinamide adenine dinucleotide-dependent), hexokinase (HK; EC 2.7.1.1), phosphoglucomutase (PGm; EC 2.7.5.1), glucose-6-phosphate dehydrogenase (6PDH; EC 1.1.1.49), and glutamate dehydrogenase (GDH; EC 1.4.1.4). These enzymes were chosen because they play important roles in the metabolism of glucose by yeasts. The staining procedures used for these enzymes have been described by Siciliano and Shaw (41). After staining, the gels were dried under a vacuum with warming, and the relative mobilities \(R_m\) of the enzyme bands were calculated as the ratio of the distance that the enzyme moved from the origin to the distance that the tracking dye moved.

**RESULTS**

Figure 1 shows 6PGDH electrophoretic patterns for some strains of *Rhodotorula* and *Rhodosporidium* species, and Fig. 2 shows patterns for representative strains of these two genera diagrammatically. Tables 2 through 5 present data on the \(R_m\) values for seven enzymes from the yeasts tested. The FA, 6PGDH, MDH, and HK patterns were characteristic for each species. Several species had identical PGm and 6PDH patterns. GDH showed small \(R_m\) values, and clear differences between species were not detected. MDH produced several enzymatic bands, HK, PGm, and G6PDH produced one or two bands, and FA, 6PGDH, and GDH produced a single band.

**Electrophoretic comparison of *Rhodosporidium* species.** The *Rhodosporidium toruloides* strains tested produced two different zymograms for each enzyme; that is, FA had an \(R_m\) of 0.43 or 0.48, G6PDH had an \(R_m\) of 0.24 or 0.26, GDH had an \(R_m\) of 0.12 or 0.13, and MDH had \(R_m\) values of 0.16, 0.20, 0.22, and 0.25 or 0.16, 0.20, and 0.31. However, 6PGDH produced only one band (\(R_m\) 0.33 to 0.34) (Table 2). FA with an \(R_m\) of 0.48 was found in strains YK 214, YK 215, and YK 216, which produced pseudomycelia. Strain YK 216 was once named *Rhodotorula glutinis* var. *rufusa* (24). It is interesting that the only strains producing FA with an \(R_m\) of 0.48 produced pseudomycelia. Strains YK 217, YK 218, and YK 219 were received as self-sporulating strains of *Rhodosporidium toruloides* (27). The enzymes of YK 217 were similar to the enzymes of strains of both mating types A and a, but the enzymes of YK 218 and YK 219 were not. However, on the whole, the strains of mating types A and a showed similar patterns for their respective enzymes.

Three *Rhodosporidium sphaerocarpum* strains, including the self-sporulating strain, produced identical patterns for FA, 6PGDH, HK, PGm, G6PDH, and GDH (Table 2). Of the four *Rhodosporidium diobovatum* strains tested, YK 223, YK 225, and YK 226 showed identical patterns for FA, 6PGDH, MDH, HK, G6PDH, and GDH, and YK 224 was
similar to the above-mentioned strains with respect to FA and PGm but showed slight variations in its MDH, HK, and GDH patterns and a different G6PDH pattern (Table 2).

Both mating type strains of Rhodosporidium malvinellum tested produced identical patterns for FA, 6PGDH, MDH, PGm, G6PDH, and GDH but a different pattern for HK (Table 2). Four Rhodosporidium bisporidiis strains, each representing one of the four mating types, showed identical patterns for all seven enzymes and were similar to Rhodosporidium diobovatum strains YK 223, YK 224, YK 225, and YK 226 with respect to their FA, 6PGDH, MDH, PGm, and GDH patterns. In addition, strain YK 224 was similar to four strains of Rhodosporidium bisporidiis with respect to HK and G6PDH (Table 2).

![Diagram of FA and 6PGDH patterns for representative Rhodotorula and Rhodosporidium strains](image)

**Fig. 2.** Diagrammatic illustration of the FA and 6PGDH patterns for representative Rhodotorula and Rhodosporidium strains. Asterisks indicate self-sporulating strains. Solid lines, FA; dashed lines, 6PGDH.
<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
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<th>DNA base composition (mol%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Coenzyme Q&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
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<td>0.52 0.54 0.26 0.12</td>
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</table>

<sup>a</sup> Data from Yamazaki and Komagata (1983).
Of the five *Rhodosporidium dacryoidum* strains tested, four (each representing one of the four mating types) showed identical patterns for all seven enzymes, but *YK* 235, which was believed to be a mutant, differed from the other strains in its *FA*, *GPGDH*, *MDH*, *HK*, and *PGm* patterns (Table 2).

Of four *Rhodosporidium infirmo-miniatum* strains, (strains *YK* 240, *YK* 241, and *YK* 242; representing the three known mating types; multiallelic bipolar) produced exhibited similar *FA*, *GPGDH*, *MDH*, *GGPDH*, and *GDH* patterns, but there were slight variations in the *HK* and *PGm* patterns. Strain *YK* 243, whose mating type was not confirmed, showed a pattern which was similar to the patterns of above-mentioned strains with respect to *GDH* but different with respect to *FA*. The patterns of the other enzymes showed slight variations (Table 2).

The two strains of *Rhodosporidium capitatum* tested exhibited identical patterns for all enzymes (Table 2).

Of the *Rhodotorula* species, strains of *Rhodotorula glutinis* and its varieties were classified into six groups on the basis of their *FA* and *GPGDH* patterns (Table 3). The strains in group 1 produced FA with an *Rm* of 0.53 and 6PGDH with an *Rm* of 0.25; *Rhodotorula minuta* var. *minuta* strains *YK* 126 and *YK* 127 and *Rhodotorula minuta* var. *minuta* strains *YK* 133 and *YK* 134 were included in this group. The strains in group 2 produced FA with an *Rm* of 0.57 to 0.58 and 6PGDH with an *Rm* of 0.41 to 0.42; *Rhodotorula glutinis* var. *halophila* *YK* 117 belonged to group 2. The strains in group 3 produced FA with an *Rm* of 0.57 to 0.58 and 6PGDH with an *Rm* of 0.41 to 0.42; *Rhodotorula glutinis* var. *halophila* strains *YK* 110 and *YK* 111 were included in this group. The strains in group 4 produced FA with an *Rm* of 0.57 to 0.58 and 6PGDH with an *Rm* of 0.41 to 0.42; *Rhodotorula minuta* var. *minuta* strains *YK* 126 and *YK* 127 and *Rhodotorula minuta* var. *minuta* strains *YK* 133 and *YK* 134 were included in this group. The strains in group 5 produced FA with an *Rm* of 0.57 to 0.58 and 6PGDH with an *Rm* of 0.41 to 0.42; *Rhodotorula minuta* var. *minuta* strains *YK* 126 and *YK* 127 and *Rhodotorula minuta* var. *minuta* strains *YK* 133 and *YK* 134 were included in this group. The strains in group 6 produced FA with an *Rm* of 0.57 to 0.58 and 6PGDH with an *Rm* of 0.41 to 0.42; *Rhodotorula minuta* var. *minuta* strains *YK* 126 and *YK* 127 and *Rhodotorula minuta* var. *minuta* strains *YK* 133 and *YK* 134 were included in this group. The strains in group 7 produced FA with an *Rm* of 0.57 to 0.58 and 6PGDH with an *Rm* of 0.41 to 0.42; *Rhodotorula minuta* var. *minuta* strains *YK* 126 and *YK* 127 and *Rhodotorula minuta* var. *minuta* strains *YK* 133 and *YK* 134 were included in this group. The strains in group 8 produced FA with an *Rm* of 0.57 to 0.58 and 6PGDH with an *Rm* of 0.41 to 0.42; *Rhodotorula minuta* var. *minuta* strains *YK* 126 and *YK* 127 and *Rhodotorula minuta* var. *minuta* strains *YK* 133 and *YK* 134 were included in this group.
<table>
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<th>Species or variety</th>
<th>Strain</th>
<th>Group</th>
<th>FA</th>
<th>6PGDH</th>
<th>MDH</th>
<th>HK</th>
<th>PGm</th>
<th>G6PDH</th>
<th>GDH</th>
<th>DNA base composition (mol%)</th>
<th>Coenzyme Q&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup> From Nakase and Komagata (31).
<sup>b</sup> From Yamada and Kondo (52).
Table 4. Grouping of Rhodotorula minuta var. minuta and Rhodotorula minuta var. texensis strains on the basis of enzymatic patterns

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<th>Strain</th>
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<th>FA</th>
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<th>MDH</th>
<th>HK</th>
<th>PGm</th>
<th>G6PDH</th>
<th>GDH</th>
<th>DNA base composition (mol%)</th>
<th>Coenzyme Q&lt;sub&gt;v&lt;/sub&gt;</th>
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<td>0.26</td>
<td>0.43</td>
<td>0.24</td>
<td>0.15</td>
<td>50.2</td>
</tr>
</tbody>
</table>

*From Nakase and Komagata (31).  
†From Yamada and Kondo (52).

rula minuta var. minuta YK 129 and YK 130 and Rhodotorula minuta var. texensis YK 137 were included in this group. Members of group 3 produced FA with an R<sub>m</sub> of 0.49 and 6PGDH with an R<sub>m</sub> of 0.29; Rhodotorula minuta var. texensis YK 135 and YK 136 were included in this group. Rhodotorula marina YK 133 and YK 137 were reidentified as Rhodotorula minuta var. texensis in this study. The one strain in group 4, strain YK 128, produced FA with an R<sub>m</sub> of 0.53 and 6PGDH with an R<sub>m</sub> of 0.30.

Rhodotorula aurantiaca YK 100 and YK 101 were nearly identical with respect to their 6PGDH, MDH, HK, G6PDH, and GDH patterns, but the PGm patterns of these two strains differed (Table 5).

Of the three Rhodotorula lactosa strains tested, YK 122 and YK 123 showed identical patterns, but YK 124 differed from both of these strains in all enzymes (Table 5).

Of the three Rhodotorula graminis strains tested, YK 119 and YK 120 exhibited identical patterns in all enzymes, but YK 121 showed patterns different from the patterns of YK 119 and YK 120 (Table 5).

Of 16 Rhodotorula rubra strains, 14 exhibited patterns which were similar with respect to FA, 6PGDH, MDH, HK, and PGm but showed variations in G6PDH (R<sub>m</sub>, 0.31, 0.33, or 0.35) and GDH (R<sub>m</sub>, 0.13, 0.15, or 0.16) (Table 5). Of the other two strains, YK 155 produced characteristic patterns for 6PGDH (R<sub>m</sub>, 0.36, 0.38, and 0.42) and G6PDH (R<sub>m</sub>, 0.24, 0.26, and 0.30) but produced the same patterns for the other enzymes as the other strains. YK 156 produced characteristic patterns for 6PGDH (R<sub>m</sub>, 0.36, 0.39, and 0.42) and MDH (R<sub>m</sub>, 0.29, 0.32, 0.34, 0.37, 0.39, and 0.42) but the same patterns for the other enzymes as the other strains.

Rhodotorula pilimanae strains produced the same patterns as Rhodotorula rubra strains for FA, 6PGDH (R<sub>m</sub>, 0.36), MDH, HK, PGm, G6PDH (R<sub>m</sub>, 0.31), and GDH (R<sub>m</sub>, 0.16) (Table 5).

Rhodotorula marina YK 125 produced peculiar FA, 6PGDH, and HK patterns and resembled Rhodotorula pallida YK 138 and YK 159 in its FA, 6PGDH, HK, and GDH patterns (Table 5).

Of the three Rhodotorula pallida strains tested, YK 138 and YK 159 showed identical patterns for all enzymes, but YK 160 was different from these two strains in all enzymes (Table 5).

Rhodotorula araucariae YK 161 and YK 162 differed from each other in their FA, 6PGDH, HK, PGm, and G6PDH patterns (Table 5).

Rhodotorula acheniorum YK 163 and YK 164 produced identical patterns for all enzymes (Table 5).

Rhodotorula sinensis YK 165 produced a characteristic pattern for FA and 6PGDH (Table 5).

Interrelationships between the genus Rhodotorula and the genus Rhodosporidium on the basis of the enzymatic patterns. Rhodotorula glutinis var. glutinis YK 102 and YK 103 (group 1) were similar to Rhodosporidium toruloides YK 218 and YK 219 (which were received as self-sporulating strains) with respect to FA, 6PGDH, MDH, and GDH.

Rhodotorula glutinis var. rufusa YK 117 produced a pattern identical to the patterns of the mating type strains of Rhodosporidium toruloides in their respective enzymes.

Rhodotorula glutinis var. salinaria YK 118 produced a pattern identical to the patterns of Rhodosporidium sphaerocarpum strains for all enzymes.

Rhodotorula glutinis var. glutinis YK 104
<table>
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<th>Species</th>
<th>Strain</th>
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<th>6PGDH</th>
<th>MDH</th>
<th>HK</th>
<th>PGm</th>
<th>G6PDH</th>
<th>GDH</th>
<th>DNA base composition (mol%)</th>
<th>Coenzyme Q&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>0.13</td>
<td>60.0&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.16</td>
<td>60.0&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.48, 0.50</td>
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<td>0.48, 0.50</td>
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<td>0.16</td>
<td>60.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.48, 0.50</td>
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<td>0.16</td>
<td>60.7&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.48, 0.50</td>
<td>0.31</td>
<td>0.16</td>
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</table>

*<sup>a</sup>*:**YK** means the Yamanashi University, Kofu, Japan; **<sup>b</sup>** indicates the values for Rhodotorula species.
Rhodotorula glutinis group 1 was similar to three strains of Rhodosporidium diobovatum (YK 223, YK 225, and YK 226) for all enzymes.

In Rhodotorula glutinis group 4, strains YK 108 and YK 111 were similar to Rhodosporidium bisporidiis strains with respect to FA, HK, PGm, and G6PDH but not with respect to 6PGDH and MDH.

Rhodotorula graminis YK 121 resembled Rhodosporidium diobovatum strains with respect to FA, MDH, PGm, and GDH, but other Rhodotorula graminis strains did not.

Rhodotorula pallida YK 160 was similar to Rhodosporidium dacryoidum YK 235 with respect to FA, 6PGDH, MDH, and GDH, but other Rhodotorula pallida strains showed no similarity to any Rhodosporidium strain.

Rhodotorula araucariae YK 162 was similar to all Rhodosporidium dacryoidum strains except YK 235 with respect to 6PGDH, MDH, and HK.

Rhodotorula sinensis YK 165 was similar to Rhodosporidium infirmo-miniatum YK 240 with respect to 6PGDH, MDH, HK, 6PGDH, and GDH, to YK 241 and YK 242 with respect to 6PGDH, MDH, PGm, G6PDH, and GDH, and to YK 243 with respect to FA, MDH, HK, and GDH.

A total of 16 Rhodotorula rubra strains, 2 Rhodotorula pilimanae strains, 1 Rhodotorula marina strain, 5 Rhodotorula minuta var. minuta strains, 7 Rhodotorula minuta var. texensis strains, 3 Rhodotorula lactosa strains, and 2 Rhodotorula aurantiaca strains were not similar to any of the known strains of the genus Rhodosporidium.

Conjugation tests between strains showing similar enzymatic patterns. Compatible mating strains of each Rhodosporidium species had identical enzymatic patterns. Based on this observation, we expected that Rhodotorula strains would be able to conjugate with Rhodosporidium strains having similar patterns. Therefore, we performed conjugation tests between such strains.

The strains used were inoculated onto yeast extract-malt extract agar slants and incubated for 2 days at 25°C. Two loopfuls of cells (one loopful of each of two strains) were mixed together and inoculated at the center of a corn meal agar plate. Cells of the two strains were also inoculated separately at other points on the same agar plate. The plate was incubated at 20°C, and the growth of the mixed cells was observed with the naked eye and microscopically at intervals.

Table 6 shows the combinations of strains tested and the results obtained. Conjugation be-
tween cells, mycelial development with clamp connections, and teliospore formation were observed with the following combinations: Rho-

table 6. Results of the cell conjugation test which was suggested by the electrophoretic comparison of enzymes

<table>
<thead>
<tr>
<th>Anamorph</th>
<th>Teleomorph</th>
<th>Mating type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodotorula glutinis var. glutinis</td>
<td>Rhodosporidium diobovatum</td>
<td>α</td>
</tr>
<tr>
<td>Rhodotorula glutinis var. rufusa</td>
<td>Rhodosporidium toruloides</td>
<td>α</td>
</tr>
<tr>
<td>Rhodotorula glutinis var. salinaria</td>
<td>Rhodosporidium sphaerocarpum</td>
<td>a</td>
</tr>
<tr>
<td>Rhodotorula sinensis YK 165</td>
<td>Rhodosporidium infirmo-miniatum</td>
<td>?</td>
</tr>
</tbody>
</table>

These results clearly indicated that Rhodotorula glutinis var. rufusa YK 117 is the haploid mating type α of Rhodosporidium toruloides, Rhodotorula glutinis var. salinaria YK 118 is the haploid mating type α of Rhodosporidium sphaerocarpum, and Rhodotorula glutinis var. glutinis YK 104 is the haploid mating type α of Rhodosporidium diobovatum.

In the combination of Rhodotorula sinensis YK 165 and Rhodosporidium infirmo-miniatum, when strains YK 165 and YK 242 (mating type A2) were mixed, mycelial development and resting spores were observed, but conjugation and clamp connections were not (Fig. 6).

Fig. 3. Conjugation between Rhodotorula glutinis var. rufusa YK 117 and Rhodosporidium toruloides YK 201 (mating type A). (A) Conjugated cells, showing a copulation tube. (B) Teliospores and hyphae, showing a clamp connection. (C) Germinated teliospore.
DISCUSSION

As discussed above, the electrophoretic patterns of enzymes are considered to be useful in the taxonomy of the genera *Rhodosporidium* and *Rhodotorula*.

*Rhodosporidium.* Almost all *Rhodosporidium toruloides* strains, irrespective of the mating type, produced identical electrophoretic patterns for the enzymes tested. However, we did find a few exceptional strains. For example, strains YK 214, YK 215, and YK 216 each conjugated with YK 201, YK 207, YK 208, YK 209, and YK 210, but the $R_e$ values of six enzymes (but not 6PGDH) differed to some extent. It is not clear why strains with different patterns conjugated. Further genetic and biochemical investigations will be required to answer this question. Moreover, of three self-sporulating strains, YK 217 showed the same pattern as typical haploid mating type strains, and YK 218 and YK 219 differed from YK 217 in the shape of the teliospores. Strains YK 218 and YK 219 would be included in *Rhodosporidium sphaerocarpum* on the basis of the shapes of their teliospores (Fig. 7), but they would be excluded from this species on the basis of the differences in their enzymatic patterns (Table 2).

Keller and Wegener (26) reported that cell conjugation and teliospore formation were ob-
served in matings between *Rhodotorula glutinis* and *Rhodotorula rubra*. Strains YK 207, YK 208, YK 209, and YK 210 were received as haploid strains of mating type A of *Rhodosporidium toruloides*. It has been reported that these strains do not assimilate potassium nitrate. Therefore, it is reasonable that these strains were identified as *Rhodotorula rubra* according to the conventional identification methods, using only physiological and morphological features. When they were mixed with *Rhodosporidium toruloides* YK 211 cells of haploid mating type a, conjugation, mycelial development with clamp connections, and teliospore formation were observed. These results confirmed those of Keller and Wegener (26). The strains

**Fig. 5.** Conjugation between *Rhodotorula glutinis* var. *glutinis* YK 104 and *Rhodosporidium diobovatum* YK 223 (mating type a). (A) Conjugated cells. (B) Teliospores and hyphae, showing a clamp connection.

**Fig. 6.** Resting spores of *Rhodotorula sinensis* YK 165 mixed with *Rhodosporidium infirmo-miniatum* YK 242 (mating type A2).
received as *Rhodotorula rubra* showed the same enzymatic pattern as *Rhodosporidium toruloides*. From these results, we concluded that these strains had lost the ability to assimilate potassium nitrate. In the anamorph, strains of *Rhodosporidium toruloides*, *Rhodosporidium sphaerocarpum*, and *Rhodosporidium diobovatum* that do not assimilate nitrate might be included in *Rhodotorula rubra*. We believe that these findings make the identification of such strains troublesome by the criteria commonly used, and comparing the electrophoretic patterns of the enzymes is a useful solution.

The DNA base composition (32) and coenzyme Q system (52) of *Rhodosporidium malvinellum* were reported to be the same as those of *Rhodotorula minuta* and *Rhodotorula pallida*. However, *Rhodosporidium malvinellum* strains produced an enzyme pattern similar to the patterns *Rhodotorula minuta* and *Rhodotorula pallida* only with respect to HK. Therefore, we believe that the *Rhodotorula minuta* and *Rhodotorula pallida* strains examined in this study are not the anamorph of *Rhodosporidium malvinellum*.

Of the four *Rhodosporidium diobovatum* strains tested, YK 224 differed from the other three strains in its zymogram and rather resembled *Rhodosporidium bisporidis*. It did not mate with the mating type strains of either

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**Fig. 7.** Teliospores of two self-sporulating strains. (A) *Rhodosporidium toruloides* YK 218. (B) *Rhodosporidium sphaerocarpum* YK 222.
species. Therefore, further investigations will be required for a better understanding of this strain from a biochemical standpoint.

Of the five Rhodosporidium dacryoidum strains examined, the self-sporulating strain YK 235 produced a pattern similar to that of Rhodotorula pallida YK 160. Strain YK 235 was isolated from a cross between Rhodosporidium dacryoidum YK 231 and YK 232 and is believed to be a mutant similar to the Amut Bx heterokaryon mimics of Coprinus lagopus and Schizophyllum commune (11). Other information will be necessary to reveal the correct taxonomic position of this strain.

Rhodosporidium infimo-miniatum is a peculiar species which possesses coenzyme Q₉ (52) and is bipolar with three known alleles (11). The anamorph of this species has been classified in the genus Cryptococcus because of its assimilation of inositol. Conjugation between strain YK 243 and other Rhodosporidium infimo-miniatum strains was not observed. We could not determine whether strain YK 243 belongs to Rhodosporidium infimo-miniatum.

Rhodosporidium capitatum is a self-sporulating species which has a life cycle similar to that described for a self-sporulating strain of Rhodosporidium sphaerocarpum (11), but the enzymatic patterns of these two strains were not similar.

All of the Rhodosporidium species produced different enzymatic patterns. Nakase and Komagata (32) reported that in the genus Rhodosporidium the G+C contents of the DNAs ranged from 50.5 to 67.3 mol%, and Yamada and Kondo (52) reported that the coenzyme Q systems of these species were Q₆, Q₈, or Q₁₀. Based on these results and those reported by other workers, this genus will have to be divided into several taxa in the near future.

Rhodotorula. Hasegawa et al. (21, 22) divided the genus Rhodotorula into two subgenera, Rubrotorula and Flavotorula, mainly on the basis of the absorption spectra of pigments. These authors further divided the subgenus Rubrotorula into three groups on the basis of cell morphology, requirements for vitamins, and assimilation of lactose. Nakase and Komagata (31) determined the G+C contents of the DNAs and reported four groups in this genus. Yamada and Kondo (52) determined the coenzyme Q systems and reported the presence of Q₈, Q₆, or Q₁₀. Table 7 shows the results obtained by these workers.

Strains of Rhodotorula glutinis and its varieties were divided into six groups on the basis of FA and 6PGDH patterns because these two enzymes showed specific patterns within the species of Rhodotorula and Rhodosporidium and other enzymes did not. Rhodotorula glutinis was considered to be heterogenous with respect to enzymatic pattern. Rhodotorula glutinis var. glutinis strains YK 102 and YK 103, with G+C contents of 67 mol%, were similar to

<table>
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<tr>
<th>Group</th>
<th>Species</th>
<th>DNA base composition (mol%)</th>
<th>Coenzyme Q</th>
<th>Requirement for biotin</th>
<th>Requirement for p-aminobenzoic acid</th>
<th>Grouping by Hasegawa</th>
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<td>1</td>
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<td>8</td>
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<td>10</td>
<td>-</td>
<td>-</td>
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<td>9</td>
<td>-</td>
<td>-</td>
<td>I</td>
</tr>
<tr>
<td></td>
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<td>10</td>
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</tbody>
</table>

* This table summarizes the data of Hasegawa (20), Nakase and Komagata (31), and Yamada and Kondo (52).
Rhodotorula glutinis var. rufusa YK 117 was isolated from the water (depth, 15 m) of Lake Vanda in Antarctica and was identified by Goto et al. (16). This strain was found to be the haploid mating type a of Rhodotorula glutinis by the conjugation test which was performed as a result of the similarity found after a comparison of the enzymes. Yamada and Kondo (52) reported that a strain of Rhodotorula glutinis var. rufusa (IFO 1698, RJ-5) was the haploid mating type a of Rhodotorula toruloides on the basis of its coenzyme Q system and a conjugation test. Rhodotorula toruloides strains have not been found yet in Antarctica; therefore, YK 117 is considered to be interesting from an ecological point of view.

Rhodotorula glutinis var. salinaria YK 118 was isolated as a halophilic yeast from a salt farm at Yashima along the shore of Seto Inland Sea in Japan and was described by Hirosawa and Takada (23). This strain was found to be the haploid mating type a of Rhodotorula sphaerocarpum by the conjugation test which was suggested by an electrophoretic comparison of enzymes. An anamorph strain of Rhodotorula sphaerocarpum has not been found among the strains which have been preserved in culture collections up to this time. Therefore, this strain is also considered to be of interest from an ecological viewpoint.

Nakase and Komagata (31) divided Rhodotorula glutinis var. glutinis strains into two groups on the basis of the G+C contents of their DNAs (60 and 67 mol%). The strains of group 2 (60 mol%) were assigned to Rhodotorula toruloides. Yamada and Kondo (52) further divided this strain into two subgroups on the basis of coenzyme Q systems. The first subgroup possessed Q10, whereas the second, which included the haploid mating strains of Rhodotorula toruloides, had Q9. Nakase and Komagata (32) mentioned that group 1 (67 mol%) might be the anamorph of Rhodotorula diobovatum because the DNAs of these strains have the same G+C contents (about 67 mol%) and that another teleomorph might be found among the strains of group 2 which showed the presence of Q10 and 60.7 to 61.2 mol% G+C. Strains YK 102, YK 103, and YK 108 possessed 66.8 to 67.3 mol% G+C (31), but the enzymatic patterns of these strains were different from those of Rhodotorula diobovatum strains. Therefore, a new teleomorph might be expected for these strains. The teleomorph of YK 106, which has Q10 and 61.2 mol% G+C, was not found in this study.

The haploid mating type strains of three Rhodotorula species were found among the strains of Rhodotorula glutinis and its varieties as the result of conjugation tests which were suggested by electrophoretic comparisons of enzymes. Furthermore, new teleomorphs were suggested by the results of the enzymatic pattern determinations.

According to Phaff and Ahearn (37), Rhodotorula minuta var. texensis differs from Rhodotorula minuta var. minuta by the strong assimilation of lactose, but these varieties are very similar in all other respects. All strains of both varieties produced unique 6PGDH (Rm, 0.25 or 0.29) and HK (Rm, 0.26 or 0.29) patterns, which
were not detected in other Rhodotorula species. Rhodotorula minuta var. minuta YK 126 and Rhodotorula minuta var. texensis YK 131 are the type strains of their varieties, and these two strains have the same coenzyme Q system, identical enzyme patterns, and similar G+C contents of their DNAs. From these results, we believe that differentiation between Rhodotorula minuta var. minuta and Rhodotorula minuta var. texensis on the basis of assimilation of lactose alone is not sound. These two varieties should be joined because of their enzymatic patterns and the above-mentioned considerations.

Rhodotorula aurantiaca was once described as a variety of Rhodotorula glutinis (21), but it was elevated to species status by Phaff and Ahearn (37) on the basis of its physiological properties. Tsuchiya et al. (46) reported that Rhodotorula glutinis and Rhodotorula aurantiaca possess the same antigenic structures, but Nakase and Komagata (31) pointed out that Rhodotorula aurantiaca was considered to be a species distinct from Rhodotorula glutinis because a difference of more than 5 mol% in the DNA base compositions of the two species was found. Furthermore, Rhodotorula aurantiaca differs from Rhodotorula glutinis with respect to enzymatic patterns. Therefore, we believe that Rhodotorula aurantiaca should be a species distinct from Rhodotorula glutinis.

Rhodotorula graminis YK 119 and YK 120 were once identified as Rhodotorula rosa by Goto and Yokotsuka (18) and as Sporobolomyces coprophilus by Sugiyama and Goto (44); later, these two strains were identified as Rhodotorula graminis by Phaff and Ahearn (37). The enzymatic patterns of these strains are identical. On this basis, the identification of these strains by Phaff and Ahearn was confirmed. The enzymatic pattern of YK 121, which is the type strain of Rhodotorula graminis, was not similar to the patterns of YK 119 and YK 120 but was similar to the patterns of Rhodospiridium diobovatum strains. Fell (10) stated that Rhodotorula graminis may consist of two distinct species, namely, Rhodospiridium malvinellum and Sporidiobolus ruinenii. Moreover, all three of these taxa grew on the same carbon compounds, but the color of the type strain of Rhodotorula graminis was different from that of Rhodospiridium malvinellum. Rhodotorula graminis and Rhodospiridium diobovatum strains did not mate. Nakase and Komagata (32) pointed out that the G+C content of the DNA of Rhodospiridium malvinellum was 50.0 mol% and thus was about 20 mol% lower than that of the type strain of Rhodotorula graminis, as reported by Storck et al. (43). The enzymatic pattern of the type strain of Rhodotorula graminis differed from the patterns of Rhodospiridium malvinellum strains. We believe that Rhodotorula graminis strains have phylogenetic relationships to Rhodotorula glutinis and Rhodospiridium diobovatum on the basis of the G+C contents of their DNAs and their enzymatic patterns.

Rhodotorula lactosa appeared to be an interesting species in the genus Rhodotorula, and the strains of this species were divided into two groups on the basis of the G+C contents of their DNAs (31) and their coenzyme Q systems (52). Rhodotorula lactosa YK 124 originated from Rhodotorula aurantiaca ATCC 9536, which was identified as Rhodotorula lactosa by Hasegawa (20), but its pattern of assimilation of carbon compounds was not similar to the patterns of Rhodotorula lactosa YK 122 and YK 123 (Table 8). In addition, Yamada et al. (50, 52) found that the assimilation patterns of these strains were not similar, with the exception of the assimilation of potassium nitrate and maltose. The assimilation pattern of Rhodotorula lactosa YK 124 was similar to that of Rhodotorula aurantiaca YK 100, but the DNA base compositions of those two strains were dissimilar. With respect to enzymatic patterns, Rhodotorula lactosa YK 124 was not similar to other Rhodotorula lactosa strains. Consequently, we believe that YK 124 may be a new species in the genus Rhodotorula or an anamorph of an unknown teleomorphic species.

Of four Rhodotorula marina strains, YK 136 and YK 137 were identified as Rhodotorula minuta var. texensis, although they assimilated lactose strongly but not raffinose. Strain YK 154 was identified as Rhodotorula rubra because of the strong assimilation of maltose. According to Phaff and Ahearn (37), Rhodotorula marina is a physiologically unstable species. The enzymatic patterns of strains YK 136, YK 137, and YK 154 indicated similarities to strains of Rhodotorula minuta and Rhodotorula rubra. The remaining Rhodotorula marina strain, YK 125, is the type strain, and its enzymatic pattern was different from the patterns of all of the other Rhodotorula species except Rhodotorula pallida.

Of three Rhodotorula pallida strains, YK 169 showed a pattern similar to that of Rhodospiridium dacryoidum YK 235 but did not mate with the mating types of this species. Fell et al. (11) reported that Rhodospiridium dacryoidum showed the same assimilation pattern as Rhodotorula minuta and Rhodotorula pallida but that it did not mate with the type strain of either species. The G+C contents of the DNAs, the coenzyme Q systems, and the requirements for vitamins are similar in Rhodotorula minuta and Rhodotorula pallida, but with the exception of
YK 160, neither species had an enzyme pattern similar to that of *Rhodosporidium dacryoidum*. Storck et al. (43) reported that the G+C contents of the DNAs of two strains of *Rhodotorula pallida* were 54.5 and 63.5 mol%. Thus, *Rhodotorula pallida* is a heterogeneous species and includes strains related to *Rhodosporidium dacryoidum*.

*Rhodotorula rubra*, a species commonly found in nature, includes morphologically different strains which were once recognized as separate species. Strains YK 142, YK 146, YK 150, YK 151, and YK 152 were once named *Rhodotorula mucilaginosa*, but they were combined with *Rhodotorula rubra* by Hasegawa (20) because of instability of their cell shapes. *Rhodotorula rubra* strains and the strains once named *Rhodotorula mucilaginosa* showed similar enzymatic patterns and could not be separated from each other. Strain YK 154 was received as *Rhodotorula marina* but was identified as *Rhodotorula rubra* in this study because of its strong assimilation of maltose. This strain had an enzyme pattern similar to that of other *Rhodotorula rubra* strains for six enzymes but not for G6PDH. Strains YK 155 and YK 156 had three 6PGDH bands. On this basis, these strains might be separated from *Rhodotorula rubra*. *Rhodotorula rubra* differs from *Rhodotorula glutinis* by not assimilating potassium nitrate, but the carbon assimilation spectra of the two species are nearly identical. *Rhodotorula glutinis* and *Rhodotorula rubra* showed clear differences in the electrophoretic patterns of their enzymes. Therefore, comparisons of the electrophoretic patterns of enzymes are useful for differentiating these two species.

*Rhodotorula pilimanae* differs from *Rhodotorula rubra* by not assimilating maltose and melezitose, but these two species have identical G+C contents in their DNAs (31) and identical coenzyme Q systems (52). *Rhodotorula pilimanae* could not be differentiated from *Rhodotorula rubra* on the basis of enzymatic patterns. Phaff and Ahearn (37) point out that *Rhodotorula pilimanae* resembles *Rhodotorula minuta* in its physiological properties. However, there was no resemblance between these two species in their enzymatic patterns and the G+C contents of their DNAs (31). As the electrophoretic mobilities of enzymes reflect even slight variations in enzyme structures, we believe that *Rhodotorula pilimanae* should be included in *Rhodotorula rubra*.

*Rhodotorula araucariae*, which was isolated from a rotting Araucaria araucana tree in Llaima, Chile, was described by Grinbergs and Yarrow (19). This species closely resembles *Rhodotorula pallida* in its physiological properties but differs from it in the assimilation of potassium nitrate as the sole source of nitrogen. However, none of the enzyme patterns of the type strain (*Rhodotorula araucariae* YK 161) were similar to the enzyme patterns of *Rhodotorula pallida*, but they did resemble those of *Rhodosporidium dacryoidum*. Mating was not observed between strain YK 161 and any of the mating types of *Rhodosporidium dacryoidum*. *Rhodotorula acheniorum* was isolated from strawberries and was described by Buhagiar and Barnett (8) under the name *Sterigmatomyces acheniorum*. It was transferred to the genus *Rhodotorula* by Rodrigues de Miranda (39) because sterigmata were not observed. The assimilation patterns of the carbon sources and the enzymatic patterns were similar to those of *Rhodo-

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**Table 8. Some physiological and biochemical properties of Rhodotorula lactosa and Rhodotorula aurantiaca strains**

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Sucrese</th>
<th>Maltose</th>
<th>Lactose</th>
<th>Melibiose</th>
<th>Raffinose</th>
<th>Melezitose</th>
<th>NO₃⁺</th>
<th>DNA base composition (mol%)</th>
<th>Coenzyme Q⁻⁺</th>
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<td>+</td>
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<td>50.0 10 (H-10)</td>
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<td>-</td>
<td>-</td>
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<td>50.0 10 (H-10)</td>
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<td>57.3 9</td>
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<td>+</td>
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<td></td>
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</tr>
</tbody>
</table>

* Potassium nitrate.
* From Nakase and Komagata (31).
* From Yamada et al. (50, 52).
R. Rhodotorula glutinis groups 1 and 4. Thus, Rhodotorula acheniorum can be regarded as a synonym of Rhodotorula glutinis.

Rhodotorula sinensis, which was isolated from diseased pears taken from cold storage in Peking, China, was named and described by Lee (28). von Arx et al. (49) stated that this species could be Rhodospiridium capitatum. The enzymatic patterns of Rhodotorula sinensis were not similar to those of Rhodospiridium capitatum, but they resembled those of Rhodospiridium infirmo-miniatum. However, mating occurs between Rhodotorula sinensis YK 165 and each of the alleles in Rhodospiridium infirmo-miniatum is not yet clear.

Implication of electrophoretic patterns of enzymes in yeast taxonomy. We found that comparisons of the electrophoretic patterns of enzymes provided a reliable method for identifying and classifying species of yeasts. Clearly, identical enzyme mobility is not always proof of structural similarity. However, differences in mobility are likely to be reflections of primary structural differences in enzymes. Therefore, in comparing strains of yeasts, the electrophoretic patterns of many enzymes should be examined.

It is interesting that possible haploid mating partners were detected by comparing the enzymatic patterns of various strains and that such strains did indeed cross with the known haploid mating type strains. We conclude that this zymogram technique can contribute to the chemotaxonomy of yeasts. Moreover, we believe that further investigations between anamorphs and teleomorphs will be required in the genera Sporobolomyces and Cryptococcus, which are related to the basidiomycetes on the basis of their enzymatic patterns.

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REPRINT REQUESTS

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