A TAXONOMIC STUDY OF THE BASIDIOMYCETOUS YEAST GENERA *RHODOSPORIDIUM* BANNO AND *RHODOTORULA* HARRISON BASED ON WHOLE-CELL PROTEIN PATTERNS, DNA BASE COMPOSITIONS AND COENZYME Q TYPES

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A numerical analysis was performed on one-dimensional whole-cell protein electrophoretic fingerprints of 107 strains belonging to the basidiomycetous yeast genera *Rhodosporidium* and *Rhodotorula*. This technique allowed evaluation of taxonomic relationships at the species level. In particular for the anamorphic genus *Rhodotorula*, the electrophoretic groupings did not correspond in all cases with the existing species. Heterogeneity of strains within the anamorphic species *Rhodotorula acheniorum*, *Rt. aurantiaca*, *Rt. araucariae*, *Rt. foliorum*, *Rt. glutinis*, *Rt. graminis* and *Rt. minuta* was found. There was a good correlation between the grouping obtained by numerical analysis of protein patterns, the mol% G+C content and the coenzyme Q type. Furthermore, the results obtained with the different techniques used suggest possible close interspecific and/or intergeneric relationships. Most *Rhodotorula glutinis* strains, including the type strain, and the type strain of *Rhodotorula graminis* were highly similar to strains of *Rhodosporidium diobovatum*. For other investigated strains of *Rhodotorula glutinis*, a high similarity was found with strains of *Rhodosporidium kratochvilovae*, *Rs. sphaerocarpum*, *Rs. toruloides* and *Rhodotorula mucilaginoso*, respectively. Most of the *Rhodotorula graminis* strains could not be differentiated from *Rhodosporidium paludigenum* strains.

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A natural system of yeast classification should be based on the coordinate use of morphological, physiological, biochemical and molecular information (17). Molecular approaches based on the DNA base composition (2,9-12,14), DNA-DNA homology (3), electrophoretic patterns of enzymes (24), rDNA sequencing results (19,22,23), the ubiquinone system (14,21) and the cellular carbohydrate composition (14) documented the heterogeneity of the genera *Rhodosporidium* and *Rhodotorula*. Based on these newly developed criteria, species of the genus *Rhodosporidium* have been reclassified in the genera *Cystofilobasidium* and *Kondoa* (5,13,22). Weijman et al. (18) emended the anamorphic yeast genus *Rhodotorula* because of the reassignment of the *Candida* species of basidiomycetous affinity to the genera *Cryptococcus* and *Rhodotorula*. In particular, the taxonomy of the anamorphic yeasts remains controversial by a lack of the characteristics of the sexual reproduction and many genera will have to be reclassified in the future.

Although there has been much progress in the taxonomy and diagnosis of yeast species, there is a continuing need for simple and reliable laboratory procedures to differentiate these organisms. With an increasing number of species, it became obvious that the conventional diagnostic tests frequently provided insufficient phenotypic characterization to permit unambiguous assignment at the species level.

In previous papers, sodium dodecyl sulphate-polyacrylamide gel electrophoresis of whole-cell proteins (SDS-PAGE) proved to be a valuable criterion for classifying yeasts at the species level because of its high levels of sensitivity and selectivity (15,16). Moreover, the ready availability and reproducibility of SDS-PAGE and the possibility of computerized processing of data suggest a method useful for analytical characterization of yeasts.

In this paper, the results of comparative protein electrophoresis in the genera *Rhodosporidium* and *Rhodotorula* species are presented. To evaluate the taxonomic validity of this technique additional chemotaxonomical criteria such as the DNA base composition and the type of the ubiquinone system are used. Relying on these results and the literature data, the possible relationships among different strains and species in the genera *Rhodosporidium* and *Rhodotorula* are discussed.

MATERIALS AND METHODS

Strains examined. A total of 107 strains were studied. These included 23 strains representing 6 species in the genus *Rhodosporidium*, 82 strains belonging to 23 different *Rhodotorula* species and two *Rhodotorula* strains for which the species assignments were not known. The strains used are listed in Table 1.

Electrophoretic pattern of whole-cell proteins. All the strains were analyzed by electrophoresis of whole-cell proteins. The methods used for cultivation of cells and the preparation and electrophoresis of the SDS-protein extracts have been described elsewhere (16). The densitometric analysis, normalization and interpolation of the protein profiles, numerical analysis, and construction of a database were performed using the GelCompar 1.3 software package (Helix C. V., Biotechnology Business

Table 1. List of strains studied, the source of isolation, the major coenzyme Q type and the DNA base composition (superscripts c to m indicate literature data; T, NT and AUT indicate type strains, neo-type strains and authentic strains, respectively).

Species and strain	Source	CoQ	mol% G+C
MUCL^a CBS^b			
<i>Rhodosporidium dacryoidum</i> Fell, Hunter & Tallman			
30319 6353 J. W. Fell; Antarctic Ocean; T of <i>Rhodosporidium dacryoidum</i> Fell, Hunter & Tallman, mating type A1B1		Q10 ^e	58.4 ^d -57.3 ^k
30357 6356 J. W. Fell; Antarctic Ocean; mating type A1B2		Q10 ^e	58.9 ^d
30364 7142 J. W. Fell; sea; mating type A2B2			
<i>Rhodosporidium diobovatum</i> Newell & Hunter			
30314 6085 J. W. Fell; sea, USA; T of <i>Rhodosporidium diobovatum</i> Newell & Hunter, mating type A1		Q10 ^m	65.0 ^d -67.3 ^l
30329 994 O. Verona; soil, Italy; mating type A1			
30335 4213 R. A. Kilpatrick; clover <i>Trifolium repens</i> , USA; mating type A2		Q10 ^e	64.8 ^d -66.7
30612 6084 J. W. Fell; sea, USA; mating type A2		Q10 ^m	65.5 ^d -67.1 ^l
30682 5540 J. Villoutreix; cherry blossom, France; mating type A2		Q10	67.2
<i>Rhodosporidium kratochvilovae</i> Hamamoto, Sugiyama & Komagata			
31974 7436 J. Sugiyama, T of <i>Rhodosporidium kratochvilovae</i> Hamamoto, Sugiyama & Komagata, self fertile		Q10 ^d	64.7 ^d -65.0
<i>Rhodosporidium paludigenum</i> Fell & Tallman			
30312 6567 J. W. Fell; mangrove <i>Rhizophora</i> mangle swamp, USA; mating type A2		Q10	62.5
30313 6565 J. W. Fell; mangrove <i>Rhizophora</i> mangle swamp, USA; mating type A1		Q10	62.8
30336 4477 D. G. Ahearn; water, USA; mating type A2			
31973 6566 J. W. Fell; black-rush <i>Juncus roemerianus</i> marsh, USA; T of <i>Rhodosporidium paludigenum</i> Fell & Tallman, mating type A1			63.0
<i>Rhodosporidium sphaerocarpum</i> Newell & Fell			
30298 5939 J. W. Fell; water, Antarctica; T of <i>Rhodosporidium sphaerocarpum</i> Newell & Fell, mating type A2		Q10 ^m	64.9 ^l -62.7 ^d -62.5
30306 5940 J. W. Fell; water, Antarctica; mating type A2		Q10 ^m	65.1 ^l -62.5 ^d
30351 5941 J. W. Fell; water, Antarctica; self fertile			62.7 ^d -63.0
30455 6985 J. W. Fell; salt farm, Japan; T of <i>Rhodotorula glutinis</i> (Fresenius) Harrison var. <i>salinaria</i> Hirose & Takada			62.0
<i>Rhodosporidium toruloides</i> Banno			
11965 no information			
28631 6016 IFO; conjugated strain between IFO 0559 and IFO 0880; T of <i>Rhodosporidium toruloides</i> Banno			
30249 14 E. Rennerfelt; conifer pulp, Sweden; T of <i>Rhodotorula gracilis</i> Rennerfelt, mating type A1		Q9 ^m	59.9 ^d -60.79
30250 349 K. Okunuki; soil, Japan; AUT of <i>Torula koishikawensis</i> Okunuki, T of <i>Rhodotorula longissima</i> Lodder, mating type A2		Q9 ^{k,m}	59.9 ^d -60.79-59.9
30328 350 E. Rennerfelt; conifer pulp, Sweden; mating type A1		Q9 ^{k,m}	60.1 ^d
30347 5745 S. Goto; soil, Japan; T of <i>Rhodotorula glutinis</i> (Fresenius) Harrison var. <i>rufusa</i> Iizuka & Goto, mating type A2			
<i>Rhodotorula acheniorum</i> (Buhagiar & Barnett) Rodrigues de Miranda			
30451 6384 R. W. M. Buhagiar; strawberries, UK		Q10 ^k	
30452 6386 R. W. M. Buhagiar; strawberries, UK; T of <i>Sterigmatomyces acheniorum</i> Buhagiar & Barnett			53.6 ^d -52.8
30460 8078 J. F. Brown; leaf of <i>Callistemon viminalis</i> , Australia		Q10	55.7
<i>Rhodotorula araucariae</i> Grinbergs & Yarrow			
30439 5143 J. Swieringa; bronchial secretion, The Netherlands		Q10 ^k -Q10	48.5 ^d -49.5
30447 6031 J. Grinbergs; rotting bark, Chile; T of <i>Rhodotorula araucariae</i> Grinbergs & Yarrow		Q10 ^k -Q10	64.6 ^k -63.9 ^d
<i>Rhodotorula armeniacae</i> Shivas & Rodrigues de Miranda			
30459 8076 J. F. Brown; leaf of <i>Callistemon viminalis</i> , Australia; T of <i>Rhodotorula armeniacae</i> Shivas & Rodrigues de Miranda		Q10	
<i>Rhodotorula aurantiaca</i> (Saito) Lodder			
30404 317 K. Saito; atmosphere, Tokyo; T of <i>Torula aurantiaca</i> Saito		Q10 ^m -Q10	55.49-54.0 ^d
30410 348 T. Castelli; human colostrum; T of <i>Mycotorula colostri</i> Castelli		Q9	57.6
30422 2029 H. J. Phaff; bark beetle, USA, California; T of <i>Rhodotorula crocea</i> Shiffrine & Phaff		Q10	59.3
30624 2786 J. P. van der Walt; soil, South Africa		Q10	61.3
30625 5950 J. P. van der Walt; Bantu beer, South Africa			58.4
30645 8074 J. F. Brown; leaf of <i>Callistemon viminalis</i> , Australia		Q10	55.1
30646 2201 A. Montemartini		Q9	57.9

Table 1. (continued)

Species and strain	Source	Co Q	mol% G+C
MUCL ^a CBS ^b			
<i>Rhodotorula auriculariae</i> (Nakase) Rodrigues de Miranda & Weijman			
29804 6379 T. Nakase; fruiting body of fungus <i>Auricularia auricula-judae</i> ; T of <i>Torulopsis auriculariae</i> Nakase	Q10	54.4 ^c	
<i>Rhodotorula bacarum</i> (Buhagiar) Rodrigues de Miranda & Weijman			
27857 6526 R. W. M. Buhagiar; black currants <i>Ribes nigrum</i> , UK; T of <i>Rhodotorula bacarum</i> Buhagiar	Q10	52.4 ^f	
<i>Rhodotorula bogoriensis</i> (Deinema) von Arx & Weijman			
11976 4101 M. H. Deinema; leaf of <i>Randia malleifera</i> , Indonesia; T of <i>Candida bogoriensis</i> Deinema	Q10 ^l	56.6 ⁱ	
<i>Rhodotorula diffluens</i> (Ruinen) von Arx & Weijman			
29960 5233 J. Ruinen; <i>Tillandsia usneoides</i> , Paramaribo; T of <i>Candida diffluens</i> Ruinen	Q10		
<i>Rhodotorula foliorum</i> (Ruinen) Rodrigues de Miranda & Weijman			
29828 5234 J. Ruinen; leaf of <i>Drymoglossum piloselloides</i> , Indonesia; T of <i>Candida foliorum</i> Ruinen	Q10 ^k	55.5	
30358 6370 M. R. Bardin; leaves	Q10	52.0	
<i>Rhodotorula fragaria</i> (Barnett & Buhagiar) Rodrigues de Miranda & Weijman			
29963 6254 J. A. Barnett; strawberries, UK; T of <i>Torulopsis fragaria</i> Barnett & Buhagiar	Q10		
30355 6256 J. A. Barnett; strawberries, UK			
<i>Rhodotorula glutinis</i> (Fresenius) Harrison			
27808 J. Zimmermann; phenol-containing water	Q9	60.0	
30253 20 E. Pringsheim; atmosphere; T of <i>Cryptococcus glutinis</i> Fresenius	Q10 ^m -Q10	67.1 ^d -67.1	
30409 332 R. Ciferri; sputum; T of <i>Torulopsis bronchialis</i> Ciferri & Redaelli	Q10	64.9	
30602 322 H. Will; water supply of brewery, Germany		66.9	
30603 347 K. Okunuki; atmosphere, Japan	Q10 ^m	67.8 ^g -66.4	
30604 324 J. Marcilla; T of <i>Rhodotorula glutinis</i> (Fresenius) Harrison var. <i>lusitanica</i> Marcilla et al.	Q10 ^m		
30605 2371 J. L. Etchells; cucumbers pickled in 10% brine, USA	Q10	62.8	
30606 321 K. Saito; atmosphere, Japan; T of <i>Torula rutula</i> Saito	Q10	64.2	
30607 4406 T. Hasegawa; T of <i>Rhodotorula glutinis</i> (Fresenius) Harrison var. <i>dairenensis</i> Hasegawa & Banno	Q10 ^m -Q10	61.2 ^g -60.3	
30632 2202 R. Ciferri; soil, USA, Minnesota; T of <i>Torulopsis minuta</i> (Saito) Ciferri & Redaelli var. <i>americana</i> Ciferri	Q10	60.7	
30633 2367 ATCC; fruit of <i>Phyllodendron</i> sp., Italy; T of <i>Mycotorula rosea-coralina</i> Scaramella			
30634 2366 K. Saito; atmosphere, Japan; T of <i>Torulopsis saitoi</i> Ciferri & Redaelli and <i>Rhodotorula rubra</i> (Saito) Harrison			
30683 4476 D. G. Ahearn; Biscayne Bay, USA, Florida	Q10	68.9	
30684 6020 S. Goto; soil from drill core, Japan; T of <i>Rhodotorula terrestra</i> Sugiyama & Goto		66.9	
<i>Rhodotorula graminis</i> di Menna			
11961 3043 M. H. Deinema; citrus leaves, Indonesia		63.0	
11962 4648 M. H. Deinema; cacao	Q10	63.0	
30431 2826 M. E. di Menna; grass, New Zealand; T of <i>Rhodotorula graminis</i> di Menna	Q10 ^{k,m} -Q10	67.0 ^d -66.9	
30436 5016 D. G. Ahearn; Atlantic Ocean, Bahamas	Q10	63.0	
30618 5711 R. E. Simard; baneberry, Canada	Q10		
30685 5624 S. Goto; atmosphere, Japan; T of <i>Pichia rosa</i> Nishiwaki	Q10 ^m	62.0 ^d -61.3	
<i>Rhodotorula hordea</i> Rodrigues de Miranda & Weijman			
30689 6403 H. G. Diem; barley leaf <i>Hordeihevastichi</i> ; T of <i>Rhodotorula hordea</i> Rodrigues de Miranda & Weijman	Q10		
<i>Rhodotorula hylophila</i> (van der Walt et al.) Rodrigues de Miranda & Weijman			
30692 6226 J. P. van der Walt; tunnels of <i>Xylopsocus capucinus</i> in <i>Rapanea melanophloeos</i> , South Africa; T of <i>Candida hylophila</i> van der Walt et al.	Q9		
<i>Rhodotorula ingensiosa</i> (di Menna) von Arx & Weijman			
30594 4240 M. E. di Menna; grass, New Zealand; T of <i>Torulopsis ingensiosa</i> di Menna	Q10 ^l -Q10	55.6 ^h -54.8 ^d -55.4	
<i>Rhodotorula javanica</i> (Ruinen) von Arx & Weijman			
29971 5236 J. Ruinen; leaf of <i>Ixora coccinea</i> , Indonesia; T of <i>Candida javanica</i> Ruinen	Q9 ^k		
30315 4977 J. Ruinen			
<i>Rhodotorula lactosa</i> Hasegawa			
30445 5826 IFO; atmosphere, Japan; T of <i>Rhodotorula lactosa</i> Hasegawa	Q9 ^m -Q9	57.3 ^g -56.1 ^d -56.6	
30446 5827 IFO; atmosphere, Japan	Q9 ^m	57.6 ^g -56.9 ^d	

Table 1. (continued)

Species and strain	Source	CoQ	mol% G+C
MUCL^a CBS^b			
<i>Rhodotorula minuta</i> (Saito) Harrison			
30405 319 K. Saito; atmosphere, Japan; T of <i>Torula minuta</i> Saito		Q10 ^e -Q10	50.0 ^d -51.0 ^e -49.8
30424 2172 J. Swieringa; sputum, The Netherlands			48.6
30425 2177 H. J. Phaff; shrimp, Gulf of Mexico; T of <i>Rhodotorula texensis</i> Phaff et al.		Q10 ^m -Q10	50.1 ^d -50.2 ^e -49.0
30621 8132 J. P. van der Walt; <i>Euphorbia ingens</i> , South Africa		Q7	41.8
30622 2179 J. P. van der Walt; decaying tooth, South Africa			
30623 2622 W. Zischa; blood			
30635 2178 S. Fjølervold; throat swab, Norway			
30636 2408 J. L. Etchells; cucumbers pickled in 10% brine, USA		Q10	
30637 2365 H. J. Phaff; shrimp, Mexico; T of <i>Rhodotorula marina</i> Phaff et al.		Q10 ^k -Q10	52.4 ^d -53.9 ^k -53.2
30638 4407 IFO; atmosphere, Japan; T of <i>Rhodotorula tokyoensis</i> Kobayashi ex Hasegawa		Q10 ^e	50.1 ^d -51.0 ^e
30639 4478 D. G. Ahearn; sea, USA			
30687 5706 E. K. Novak; throat swab, Hungary; T of <i>Rhodotorula sloofiae</i> Novak & Voros-Felkai		Q10 ^m	48.2 ^d -50.7 ^e
30688 8018 B. Norkrans; sea, Sweden		Q10	47.0
<i>Rhodotorula mucilaginosa</i> (Jørgensen) Harrison			
29272 E. Paligot; maple slime flux			
30397 17 G. Pollacci; NT of <i>Saccharomyces ruber</i> Demme		Q10 ^e -Q10	59.0 ^d -59.6
30403 316 NCTC; NT of <i>Torula mucilaginosa</i> Jørgensen			60.5 ^g
30408 329 A. Castellani; sputum; T of <i>Cryptococcus parosaeus</i> Castellani			
30416 1011 P. Negroni; ulcers, Madagascar			
30428 2397 M. Giovannozzi; tobacco			
30586 2382 R. Ciferri; faeces; T of <i>Torulopsis nitrotophila</i> Ciferri & Ashford			
30587 992 F. Zach; nail, Austria; T of <i>Torulopsis aurantia</i> Zach			
30588 333 R. Ciferri; tubercular lung, Italy; T of <i>Torulopsis sanniei</i> Ciferri & Redaelli			
30590 330 A. Sartory; hair; T of <i>Cryptococcus corallinus</i> Sartory et al. and <i>Rhodotorula mucilaginosa</i> (Jørgensen) Harrison var. <i>plicata</i> Lodder			
30591 327 H. Will; pasteurized beer, Germany; T of <i>Torula sanguinea</i> Schimon			
30592 326 Laboratoire de Parasitologie Paris; atmosphere; T of <i>Rhodotorula rubra</i> (Demme) Lodder var. <i>longa</i> Lodder			
30593 328 R. Ciferri; T of <i>Blastodendron carboni</i> Ciferri & Redaelli			
30630 2381 R. Ciferri; lung abscess, Italy; T of <i>Mycotorula pulmonalis</i> Ciferri & Redaelli			
30631 2376 H. Kufferath; water, France; T of <i>Rhodotorula acclotiana</i> Harrison			
<i>Rhodotorula muscorum</i> (di Menna) von Arx & Weijman			
30320 6921 M. E. di Menna; rotting sphagnum moss, New Zealand; T of <i>Candida muscorum</i> di Menna		Q10 ^k	46.1 ^f
<i>Rhodotorula philyla</i> (van der Walt et al.) Rodrigues de Miranda & Weijman			
29986 6272 J. P. van der Walt; tunnels of <i>Xyleborus ferrugineus</i> in <i>Harpephyllum caltrum</i> , South Africa; T of <i>Torulopsis philyla</i> van der Walt et al.		Q10 ^k	62.9 ^f
<i>Rhodotorula pustula</i> (Buhagiar) Rodrigues de Miranda & Weijman			
27861 6527 R. W. M. Buhagiar; black currants <i>Ribes nigrum</i> , UK; T of <i>Torulopsis pustula</i> Buhagiar		Q10	51.0 ^f
30359 6528 R. W. M. Buhagiar; black currants <i>Ribes nigrum</i> , UK		Q10	
<i>Rhodotorula sonckii</i> (Hopsu-Havu et al.) Rodrigues de Miranda & Weijman			
30044 6713 V. K. Hopsu-Havu; washing pool, Finland; T of <i>Candida sonckii</i> Hopsu-Havu et al.		Q10	
30365 8115 V. K. Havu; arm of patient, Finland			
<i>Rhodotorula</i> sp.			
30345 5652 E. Friedrich; dust, FRG		Q10	58.8
30617 5778 L. de Montemayor; petroleum, Venezuela		Q9	60.0

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Development, Gent, Belgium). The profiles were recorded and stored on a PC-AT computer. The similarity between all pairs of traces (using points 10 to 285 of the 400 points of each interpolated trace) was expressed by the Pearson product moment correlation coefficient *r*.

Isolation and purification of DNA and determination of DNA base composition.

Yeast strains were inoculated in 100 ml GYPP (per liter distilled water: 20 g glucose, 5 g yeast extract, 10 g peptone, 0.025 M phosphate buffer, pH 7.5) in 500 ml Erlenmeyer flasks and incubated at room temperature for 24 h. Each liquid culture was transferred to 900 ml GYPP broth in a 5 l Erlenmeyer flask and incubated for a further period of 24–48 h while shaking. Cells were washed twice in 0.02 M EDTA (pH 8.0). High molecular weight DNA was isolated by grinding the lyophilized yeast cells (1–3 g) in a mortar in the presence of liquid nitrogen. The ground cells were then suspended in extraction buffer (0.2 M Tris-HCl, 0.25 M NaCl, 0.025 M EDTA, pH 8.5). Proteinase K (50 µg/ml) and SDS (20 g/l) were added and the lysed cell suspension was incubated for 3 h at 37°C. The subsequent procedure for isolation and purification of DNA followed the method described by Marmur (7) with minor modifications. The DNA base composition was calculated from the thermal denaturation temperature of the DNA (T_m) using the formula of Marmur and Doty (8). The T_m value was determined in $0.5 \times$ SSC solution using a Gilford spectrophotometer.

Extraction, purification and identification of ubiquinones. The organisms were grown as shaking cultures, as described above for the determination of the DNA base composition. Cells were harvested by centrifugation after 48 h and washed three times with distilled water. The washed cells (3–6 g) were transferred to a 500 ml flask. For saponification 50 ml distilled water, 100 ml methanol, 20 g sodium hydroxide and 5 g pyrogallol were added and the mixture was heated at 90°C during 1 h in a water bath. Ubiquinones were extracted with hexane from intact cells after saponification according to the method described by Yamada and Kondo (21), and isolated by preparative thin-layer chromatography using Kieselgel 60F₂₅₄ (Merck, Darmstadt, Germany) plates and benzene as a solvent system. Ubiquinone isoprenologues were identified by high performance liquid chromatography (HPLC) by comparing then retention times with those of standard ubiquinones from Q₆ to Q₁₀ (Sigma Chemical Co., St. Louis, MO, USA). HPLC was performed on a liquid chromatograph (Waters, Milford, MA, USA) equipped with a Zorbax ODS column (4.6 mm $\times$ 25 cm; Chrompack International B. V., Middelburg, The Netherlands). Ubiquinone isoprenologues were eluted with a mobile phase of methanol–isopropanol (3 : 1, v/v) at 1 ml/min, and monitored at 275 nm.

RESULTS AND DISCUSSION

A dendrogram obtained after numerical analysis of protein electrophoregrams of 107 *Rhodospiridium* and *Rhodotorula* strains is shown in Fig. 1. The overall reproducibility of the electrophoresis technique averaged $r = 0.94$. Also a good reproducibility ($r \geq 0.94$) was found between the protein patterns from duplicated

Fig. 1. Dendrogram based on average linkage cluster analysis of SDS-PAGE protein patterns of 107 strains of the basidiomycetous genera *Rhodospiridium* and *Rhodotorula* (*Rs.*, *Rhodospiridium*; *Rt.*, *Rhodotorula*).

Fig. 1

cultures (data not shown). Eighteen clusters are delineated at a correlation level of approximately $r = 0.90$. These clusters group visually highly similar protein profiles (Fig. 2). Within each cluster only minor quantitative differences are observed. Data concerning the DNA base composition and the ubiquinone type (Table 1) are used to evaluate the grouping obtained by numerical analysis of the protein profiles. In the present study, determination of the mol% G+C content is repeated for several strains for which the value was reported earlier. It allows to evaluate in some cases the somewhat aberrant data reported by different authors. The relative standard error of the thermal denaturation method in this work is less than 1.0% (data not shown).

The genus Rhodosporidium

The delineation and intraspecific homogeneity of species of the basidiomycetous genus *Rhodosporidium* based on morphological and phenotypical characteristics (1, 6) are confirmed by numerical analysis of whole-cell protein patterns (16, Fig. 1), determination of the DNA base compositions and the coenzyme Q systems (Table 1). Except for *Rhodosporidium toruloides*, all *Rhodosporidium* species possess the coenzyme Q₁₀ system (3, 21, Table 1).

Three *Rhodosporidium dacryoidum* strains constitute the protein electrophoretic cluster 12 ($r \geq 0.92$; Fig. 1) and are characterized by a mol% G+C of 57.3–58.9% (2, 14).

Five *Rhodosporidium diobovatum* strains belong to SDS-PAGE cluster 8 ($r \geq 0.88$; Fig. 1). Data of different authors demonstrate a considerable variation in the mol% G+C content. A different range from 67.1 to 67.3% and from 64.8 to 65.5% is reported by Nakase and Komagata (12) and Hamamoto et al. (2), respectively. Results from the present study show a DNA base composition of 66.7 and 67.2 mol% G+C for *Rhodosporidium diobovatum* MUCL 30335 and MUCL 30682, respectively. Similar DNA base compositions are detected for representative strains of other species within cluster 8 (see below).

The type strain of *Rhodosporidium kratochvilovae* groups in SDS-PAGE cluster 9 ($r \geq 0.93$; Fig. 1) and is characterized by a DNA base composition of 65.0 mol% G+C, which confirms the value of 64.7% obtained by Hamamoto et al. (2, 4).

Four *Rhodosporidium paludigenum* strains group in SDS-PAGE cluster 5 ($r \geq 0.90$; Fig. 1). Representative strains have a DNA base composition of 62.5–63.0 mol% G+C (Table 1).

Four *Rhodosporidium sphaerocarpum* strains group in SDS-PAGE cluster 2 ($r \geq 0.91$; Fig. 1) and a DNA base composition is found in the range of 62.0–63.0

Fig. 2. SDS-protein electrophoregrams of 58 representative strains from the clusters obtained by numerical analysis (*Rs.*, *Rhodosporidium*; *Rt.*, *Rhodotorula*). The positions of the molecular weight markers (track labeled MWM) are indicated from left to right: lysozyme, 14,500; trypsin inhibitor, 20,100; carbonic anhydrase, 29,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; egg albumin, 45,000; bovine albumin, 66,000; β -galactosidase, 116,000.

MUCL	Species name	SDS-PAGE cluster
30397	<i>Rt. mucilaginos</i>	1
30632	<i>Rt. glutinis</i>	
30298	<i>Rs. sphaerocarpum</i>	2
30605	<i>Rt. glutinis</i>	
30249	<i>Rs. toruloides</i>	3
27808	<i>Rt. glutinis</i>	
30617	<i>Rt. sp.</i>	
29971	<i>Rt. javanica</i>	4
30315	<i>Rt. javanica</i>	
30436	<i>Rt. graminis</i>	5
31973	<i>Rs. paludigenum</i>	
27861	<i>Rt. pustula</i>	6
30359	<i>Rt. pustula</i>	
11976	<i>Rt. bogoriensis</i>	
30624	<i>Rt. aurantiaca</i>	7
30685	<i>Rt. graminis</i>	
30447	<i>Rt. araucariae</i>	
30314	<i>Rs. diobovatum</i>	8
30253	<i>Rt. glutinis</i>	
30431	<i>Rt. graminis</i>	
31974	<i>Rs. kratochvilovae</i>	9
30409	<i>Rt. glutinis</i>	
29963	<i>Rt. fragaria</i>	10
30355	<i>Rt. fragaria</i>	
30320	<i>Rt. muscorum</i>	
30594	<i>Rt. ingeniosa</i>	
30451	<i>Rt. acheniorum</i>	11
30452	<i>Rt. acheniorum</i>	
30621	<i>Rt. minuta</i>	
30683	<i>Rt. glutinis</i>	
30319	<i>Rs. dacryoidum</i>	12
30364	<i>Rs. dacryoidum</i>	
30422	<i>Rt. aurantiaca</i>	13
30625	<i>Rt. aurantiaca</i>	
30404	<i>Rt. aurantiaca</i>	14
30645	<i>Rt. aurantiaca</i>	
30637	<i>Rt. minuta</i>	
30424	<i>Rt. minuta</i>	15
30405	<i>Rt. minuta</i>	
30445	<i>Rt. lactosa</i>	16
30446	<i>Rt. lactosa</i>	
30439	<i>Rt. araucariae</i>	
30688	<i>Rt. minuta</i>	
30044	<i>Rt. sonckii</i>	17
30365	<i>Rt. sonckii</i>	
30689	<i>Rt. hordea</i>	
27857	<i>Rt. bacarum</i>	
29804	<i>Rt. auriculariae</i>	
30410	<i>Rt. aurantiaca</i>	18
30646	<i>Rt. aurantiaca</i>	
29960	<i>Rt. diffluens</i>	
30358	<i>Rt. foliorum</i>	
29828	<i>Rt. foliorum</i>	
29986	<i>Rt. phillyla</i>	
30345	<i>Rt. sp.</i>	
30460	<i>Rt. acheniorum</i>	
30459	<i>Rt. armeniaca</i>	
30692	<i>Rt. hylophila</i>	
MWM		

Fig. 2

mol% G+C (Table 1). A similar DNA base composition of 62.5–62.7 mol% G+C was detected by Hamamoto et al. (2). Nakase and Komagata (12) reported a somewhat higher mol% G+C of 64.9–65.1%.

The strains of *Rhodospiridium toruloides*, possessing the ubiquinone Q₉ type (21), belong to the SDS-PAGE group 3 ($r \geq 0.91$; Fig. 1) and show a DNA base composition of 59.9–60.7 mol% G+C (2, 9). In this study a DNA base composition of 59.9 mol% G+C is found for strain MUCL 30250.

As discussed below, some *Rhodospiridium* species show similar protein patterns with strains of the anamorphic species *Rhodotorula glutinis* and *Rt. graminis* (Fig. 1). One *Rhodotorula glutinis* strain groups with *Rhodospiridium sphaerocarpum* strains in SDS-PAGE cluster 2, one *Rhodotorula glutinis* strain groups with *Rhodospiridium toruloides* strains in cluster 3, seven *Rhodotorula glutinis* strains, including the type strain, group with *Rhodospiridium diobovatum* strains in cluster 8, and two *Rhodotorula glutinis* strains group with the type strain of *Rhodospiridium kratochvilovae* in cluster 9. Concerning *Rhodotorula graminis*, the type strain groups with *Rhodospiridium diobovatum* strains in cluster 8 and four other strains group in cluster 5 with *Rhodospiridium paludigenum* strains. Other data (DNA base composition and ubiquinone type) also suggest that the protein electrophoretic clusters contain closely related strains (see below).

The genus Rhodotorula

Unlike the teleomorphic genus *Rhodospiridium*, species of the anamorphic genus *Rhodotorula* lack the morphological characteristics of the sexual reproduction and are delineated by classical phenotypical tests (1, 6). The results of this study indicate that the groupings obtained by the whole-cell protein patterns do not always support the existing classification. We have evaluated the SDS-PAGE technique by determining the DNA base composition and the ubiquinone type from representative strains.

Only the type strain is included for the following ten species: *Rhodotorula armeniaca*, *Rt. auriculariae*, *Rt. bacarum*, *Rt. bogoriensis*, *Rt. diffluens*, *Rt. hordea*, *Rt. hylophila*, *Rt. ingeniosa*, *Rt. muscorum* and *Rt. philyla*. All these type strains demonstrate a qualitatively well-characterized protein profile (Figs. 1, 2), which might indicate that each of these species occupies a unique and separate taxonomic position within the genus *Rhodotorula*. For several of these strains, the DNA base composition and the ubiquinone type is given in Table 1.

The protein profiles of the strains studied belonging to each of the following five species are highly similar and constitute a separate cluster: *Rhodotorula fragaria* (cluster 10; $r \geq 0.95$), *Rt. javanica* (cluster 4; $r \geq 0.97$), *Rt. lactosa* (cluster 16; $r \geq 0.97$), *Rt. pustula* (cluster 6; $r \geq 0.98$) and *Rt. sonckii* (cluster 17; $r \geq 0.92$). Data concerning the DNA base composition and the ubiquinone type of strains of these clusters are indicated in Table 1.

Intraspecific heterogeneity is demonstrated within the following seven species: *Rhodotorula acheniorum*, *Rt. araucariae*, *Rt. aurantiaca*, *Rt. foliorum*, *Rt. glutinis*,

Rt. graminis and *Rt. minuta*. For each of these species, the observed protein electrophoretic heterogeneity is evaluated below, relying on the DNA base composition and the ubiquinone type.

Two strains of *Rhodotorula acheniorum*, the type strain MUCL 30452 and strain MUCL 30451, exhibit highly similar protein patterns ($r=0.95$) and group in cluster 11. A third strain MUCL 30460 displays a distinct protein profile (a correlation level $r=0.65$ with cluster 11). The difference between the latter strain and the strains from cluster 11 is confirmed by a difference in mol% G+C. For the type strain MUCL 30452 a DNA base composition of 52.8 mol% G+C is determined, which is analogous with the value of 53.6% mentioned by Hamamoto et al. (2). A significant different DNA base composition of 55.7 mol% G+C is observed for strain MUCL 30460. The presence of the coenzyme Q₁₀ system is reported for a representative strain of cluster 11 (14) and for strain MUCL 30460 (Table 1).

Two *Rhodotorula araucariae* strains produce highly distinct protein profiles ($r=0.73$). Although both strains possess the coenzyme Q₁₀ system (14), the observed heterogeneity in protein pattern is confirmed by a difference in DNA base composition. Strain MUCL 30439 is characterized by a mol% G+C of 49.5%, which confirms the value of 48.5% obtained by Hamamoto et al. (2), while the type strain MUCL 30447 has a much higher DNA base composition of 63.9–64.6 mol% G+C (2, 14).

Seven *Rhodotorula aurantiaca* strains yield four qualitatively different protein profiles. Cluster 13 groups the strains MUCL 30422 and MUCL 30625 ($r=0.93$), two strains characterized by the coenzyme Q₁₀ system and a DNA base composition of 58.4–59.3 mol% G+C (Table 1). Cluster 14 groups the type strain of *Rhodotorula aurantiaca* MUCL 30404 and strain MUCL 30645 ($r=0.96$). Besides a highly similar protein pattern, both strains are characterized by the coenzyme Q₁₀ system and an analogous DNA base composition of 54.0–55.4 mol% G+C (2, 9, Table 1). In protein cluster 18, *Rhodotorula aurantiaca* strains MUCL 30410 and MUCL 30646 group at a correlation level $r=0.95$. In contrast with other strains studied of this species, both strains show the coenzyme Q₉ system (Table 1). These strains share a homologous DNA base composition of 57.6–57.9 mol% G+C (Table 1). Strain MUCL 30624 shows a similar protein profile with *Rt. graminis* MUCL 30685 and groups in cluster 7 ($r=0.90$; Fig. 1). Both strains are characterized by a similar DNA base composition of 61.3 mol% G+C and the coenzyme Q₁₀ system (Table 1).

Two *Rhodotorula foliorum* strains studied, the type strain MUCL 29828 and MUCL 30358, show a protein electrophoretic similarity of $r=0.87$. This low correlation level is confirmed by a different DNA base composition of 55.5 and 52.0 mol% G+C, respectively. Both strains possess the coenzyme Q₁₀ system.

The *Rhodotorula glutinis* strains studied are in terms of protein electrophoretic similarity heterogeneous and are scattered over six taxonomic groups (Fig. 1). This grouping is confirmed by the results of the determination of the DNA base

composition and the ubiquinone type (Table 1). Two strains of the species, MUCL 30607 and MUCL 30632, show a similar electrophoretic protein pattern with the *Rhodotorula mucilaginosa* strains and belong to cluster 1 ($r \geq 0.88$) (Figs. 1 and 2). All strains of this group are characterized by the coenzyme Q₁₀ system (Table 1). A similar DNA base composition varying between 59.0 and 60.5 mol% G + C for *Rhodotorula mucilaginosa* strains (2, 9, Table 1) and 60.3 and 60.7% for the strains MUCL 30607 and MUCL 30632, respectively, confirms that all these strains could be closely related. The inadequacy of discrimination between *Rhodotorula glutinis* and *Rhodotorula mucilaginosa* strains based on the ability to assimilate nitrate is already suggested by Hamamoto et al. (3) and is confirmed here by SDS-PAGE.

One strain of *Rhodotorula glutinis* MUCL 30605 groups in SDS-PAGE cluster 2 with all strains investigated of *Rhodospiridium sphaerocarpum* ($r \geq 0.91$). These strains are characterized by the coenzyme Q₁₀ system (21, Table 1). The DNA base composition of *Rhodotorula glutinis* MUCL 30605 is 62.8 mol% G + C and is analogous to the values of 62.0–63.0% for *Rhodospiridium sphaerocarpum* strains. The latter data are in accordance with those of Hamamoto et al. (2). The data presented in this study suggest that strain MUCL 30605 might be an anamorphic strain of *Rhodospiridium sphaerocarpum*.

Another strain of *Rhodotorula glutinis* MUCL 27808 and the unidentified *Rhodotorula* strain MUCL 30617 show highly similar protein profiles with *Rhodospiridium toruloides* strains and group in cluster 3 ($r \geq 0.91$). Both strains are characterized by the coenzyme Q₉ system and a DNA base composition of 60.0 mol% G + C. Similar characteristics for strains of *Rhodospiridium toruloides* are reported (2, 21). This suggests that *Rhodotorula glutinis* MUCL 27808 and the unidentified *Rhodotorula* strain MUCL 30617 might be anamorphic strains of *Rhodospiridium toruloides*.

The majority of the *Rhodotorula glutinis* strains, the type strains MUCL 30253 and six other strains studied (MUCL 30602, MUCL 30603, MUCL 30604, MUCL 30633, MUCL 30634 and MUCL 30684) show similar protein profiles with the *Rhodospiridium diobovatum* strains studied and belong to cluster 8 ($r \geq 0.88$). All these strains possess the ubiquinone Q₁₀ type (21, Table 1). They also share a similar DNA base composition varying between 66.4 and 67.1 mol% G + C for the *Rhodotorula glutinis* strains and ranging from 66.7 to 67.3% for the *Rhodospiridium diobovatum* strains (12, Table 1). A possible anamorph-teleomorph relationship between some strains of *Rhodotorula glutinis* and *Rhodospiridium diobovatum* is suggested by Hamamoto and coworkers (3). Our results confirm this statement for the strains grouping in cluster 8.

Two strains of *Rhodotorula glutinis* MUCL 30409 and MUCL 30606 group with the type strain of *Rhodospiridium kratochvilovae* MUCL 31974 in the SDS-PAGE cluster 9 ($r \geq 0.93$). The latter strain is characterized by the coenzyme Q₁₀ system (21) and a DNA base composition of 64.7–65.0 mol% G + C (2, Table 1). *Rhodotorula glutinis* MUCL 30409 and MUCL 30606 also possess the ubiquinone Q₁₀ type and have a DNA base composition of 64.9 and 64.2 mol% G + C,

respectively (Table 1). These data suggest that MUCL 30409 and MUCL 30606 might be considered as anamorphic strains of the self-sporulating species *Rhodospiridium kratochvilovae*. Hamamoto et al. (4) already classified two *Rhodotorula glutinis* strains in the species *Rhodospiridium kratochvilovae* based on similar chemotaxonomic data.

In comparison with other *Rhodotorula glutinis* strains studied, strain MUCL 30683 demonstrates a unique protein pattern (Fig. 1) and a significantly higher DNA base composition of 68.9 mol% G+C. The strain is characterized by the coenzyme Q₁₀ system (Table 1).

Six *Rhodotorula graminis* strains are investigated. The type strain of this species groups in cluster 8 together with strains of *Rhodotorula glutinis*, including the type strain, and all strains investigated of *Rhodospiridium diobovatum*. The observed DNA base composition of 66.9 mol% G+C is analogous with the reported value of 67.0% (2), and is furthermore similar to the values of other strains of cluster 8 (see above). In addition, all strains studied of this protein electrophoretic group possess the coenzyme Q₁₀ system (Table 1). These data indicate that the type strain of *Rhodotorula graminis* might be an anamorphic strain of *Rhodospiridium diobovatum*, as is suggested in the present study and by Hamamoto et al. (3) for the majority of the strains of *Rhodotorula glutinis*.

Four *Rhodotorula graminis* strains (MUCL 11961, MUCL 11962, MUCL 30436 and MUCL 30618) group in the protein electrophoretic cluster 5 with all *Rhodospiridium paludigenum* strains investigated ($r \geq 0.90$). A possible close relatedness between strains of this cluster is confirmed by an analogous DNA base composition of 63.0 mol% G+C for representative *Rhodotorula graminis* strains and a range of 62.5 to 63.0% for *Rhodotorula paludigenum* strains. Moreover, the coenzyme Q₁₀ system is found in representative strains of cluster 5.

Rhodotorula graminis MUCL 30685 groups in the protein electrophoretic cluster 7 with *Rt. aurantiaca* MUCL 30624 ($r = 0.90$). Both strains are characterized by a similar DNA base composition of 61.3 mol% G+C (Fig. 1). An analogous value of 62.0% is reported for strain MUCL 30685 (2). Both strains possess the coenzyme Q₁₀ system (21). Relying on the partial sequences of the 18S and 26S rRNA, Yamada et al. (23) have demonstrated that this strain is related to *Rhodospiridium sphaerocarpum* and *Rs. paludigenum*.

Thirteen strains of the species *Rhodotorula minuta* are investigated. Ten strains group in protein cluster 15 and are characterized by a DNA base composition varying between 48.2 and 51.0 mol% G+C (2, 19, Table 1). These strains possess the coenzyme Q₁₀ system (3, 21).

Two other coenzyme Q₁₀-possessing *Rhodotorula minuta* strains, MUCL 30637 and MUCL 30688, show a different protein pattern with correlation levels of $r = 0.84$ and $r = 0.82$ versus cluster 15 strains, respectively. The heterogeneity of strains of this species is confirmed by determination of the DNA base composition which reveals a mol% G+C content of 53.2% for strain MUCL 30637 and 47.0% for strain MUCL 30688.

Rhodotorula minuta MUCL 30621 displays a unique protein profile, a much lower DNA base composition of 41.8 mol% G+C and an aberrant coenzyme Q system. The major ubiquinone component is Q₇ and significant amounts (approximately 30% of the total amount) of the minor component coenzyme Q₈ are observed (Table 1).

Conclusions

Previous work demonstrated that numerical analysis of one-dimensional protein electrophoretic patterns is extremely useful for the identification of yeast species. Furthermore, it allows a rapid and reliable grouping of a large number of strains (15, 16). Within several anamorphic *Rhodotorula* species (*Rt. acheniorum*, *Rt. aurantiaca*, *Rt. araucariae*, *Rt. foliorum*, *Rt. glutinis*, *Rt. graminis* and *Rt. minuta*), intraspecific differences in banding patterns are observed. The protein electrophoretic heterogeneity within these species is confirmed by differences in DNA base composition and/or the ubiquinone type of representative strains. These data suggest a very close relatedness between several of the *Rt. glutinis* strains studied, including the type strain, and the type strain of *Rhodotorula graminis* with strains of *Rhodosporidium diobovatum*. Other investigated strains of *Rhodotorula glutinis* show a close relationship with strains of *Rhodosporidium kratochvilovae*, *Rs. sphaerocarpum*, *Rs. toruloides* and *Rhodotorula mucilaginoso*, respectively. Most of the *Rhodotorula graminis* strains studied show a close relatedness to *Rhodosporidium paludigenum* strains. The application of additional taxonomic approaches relying on interfertility tests and DNA-DNA homology will be necessary to confirm these statements.

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