Clinical Isolates of *Candida guilliermondii* Include *Candida fermentati*

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Clinical isolates of *Candida guilliermondii* that were investigated by isoenzyme and randomly amplified polymorphic DNA analyses represented two distinct species. The two species were distinguished on the basis of delayed fermentation of galactose. The larger group of isolates was closely related to the anamorph *C. guilliermondii* ATCC 6260^T (T = type strain) and its teleomorph, *Yamadazyma* (= *Pichia*) *guilliermondii* ATCC 46036^T. The remaining group, whose members fermented galactose, was very similar to *Candida fermentati* CBS 2022, which had for many years been placed in synonymy with *C. guilliermondii*. Three additional groups were represented by individual strains; these strains included *C. guilliermondii* var. *soya* ATCC 20216, which was found to represent *Yamadazyma ohmeri*. The type strain of *Y. guilliermondii* is redefined.

Candida guilliermondii (Castellani) Langeron et Guerra was described by Castellani as a yeast that was obtained in culture from expectorated material associated with tea factory cough (bronchomycosis) in Sri Lanka (8). It was first named *Endomyces guilliermondii* and was discriminated from other yeasts on the basis of a physiological profile which included failure to ferment fructose, maltose, and galactose. *Endomyces negrii*, which was later brought into synonymy (18), was also obtained from individuals with bronchomycosis but differed from *E. guilliermondii* by weakly fermenting galactose (8, 9). Mating of isolates showed that the teleomorph of *C. guilliermondii* was an ascomycetous yeast, *Yamadazyma* (= *Pichia*) *guilliermondii* (41). This relationship was confirmed by the demonstration of DNA complementarity between the type strains representing the anamorph and the teleomorph (17).

Since *C. guilliermondii* was first described, a considerable number of yeasts with somewhat similar phenotypes have been reported and, in many cases, have been assigned new names. These yeasts include isolates obtained from clinical and environmental sources and isolates that are being investigated for the production of riboflavin or citric acid (16). Taxa which have been brought into synonymy with *C. guilliermondii* have been listed previously (2), and over the years the taxonomic relationships of these taxa to *C. guilliermondii* have been summarized in a variety of monographs (13, 25, 26, 39). In some cases, cultures of the type or authentic strains no longer exist; taxa which fall into this category include *E. negrii* (see above), *Monilia pseudoguilliermondi* (= *Monilia guilliermondi* var. *pseudoguilliermondi*) (which differed in its ability to clot milk and was not accepted as a distinct species by Diddens and Lodder [13]), *Candida paranensis*, *Monilia muhira*, and *Monilia lustigi*. Other taxa that have been brought into synonymy are represented by living cultures.

Recently, Bai studied a limited selection of isolates identified as *C. guilliermondii* and was able to identify two groups of organisms which differed in their electrophoretic karyotypes and DNA base compositions (1). Bai used the differences in these characteristics to define *Candida fermentati* (Saito) Bai as a species distinct from *C. guilliermondii*. However, he was not

able to detect phenotypic characteristics that were useful for distinguishing the two species, and he did not examine all of the taxa that had been brought into synonymy with *C. guilliermondii*.

In this study, we used isoenzyme and randomly amplified polymorphic DNA (RAPD) profiles to analyze a panel of clinical and environmental isolates that have been named *C. guilliermondii*. We found that there is substantial genetic heterogeneity within this set of organisms and that *C. fermentati* is not unusual in clinical specimens. The synonyms of *C. guilliermondii* and *C. fermentati* are defined below, and a phenotype that is based on fermentation of D-galactose appears to be useful for distinguishing these species. In addition, we describe additional groups within the panel of strains identified as *C. guilliermondii*.

(Some of the results of this study have been presented previously [34].)

MATERIALS AND METHODS

Fungi. Isolates that were originally identified as *C. guilliermondii* are listed in Table 1. These organisms were obtained from the American Type Culture Collection (ATCC), the Centraalbureau voor Schimmelcultures, the Agricultural Research Service Culture Collection at the National Center for Agricultural Utilization Research, U.S. Department of Agriculture, Peoria, Ill., the collection at the New York State Department of Health, Albany, and Donald G. Ahearn, Georgia State University, Atlanta. The basic methods used to confirm the identities of the strains were analysis of morphology following growth on Sabouraud and cornmeal agar media and growth in a API 20C kit (bioMérieux Vitek, Hazelwood, Mo.) to obtain a carbohydrate assimilation profile (7).

Only isoenzyme profile analyses, API 20C profile analyses, and fermentation tests with glucose and galactose were performed for strains MCO 1557 through MCO 1563^T (T = type strain). The remaining strains were investigated for these characteristics and for additional properties by using the methods described below.

Isoenzyme analysis. An isoenzyme analysis in which native discontinuous polyacrylamide gel electrophoresis was used was performed by previously described procedures (15, 19, 20). Briefly, isolates were inoculated into 250-ml Erlenmeyer flasks containing 100 ml of broth (yeast nitrogen base without amino acids [YNB] [Difco Laboratories, Detroit, Mich.] supplemented with 0.1 M sucrose and 0.03 M mannitol) and incubated in a rotatory shaker for 3 days at 28°C and 150 rpm. Washed yeast cells were then broken with glass beads in the presence of 2.0 mM phenylmethylsulfonyl fluoride and centrifuged at 13,000 × g. The supernatants were used as the sources of enzymes, and their protein contents were assayed by the method of Bradford (5).

The chromogenic reagents used to detect enzyme activity were those listed by Lin et al. (24). The enzyme activities analyzed by using 7.5% (wt/vol) polyacrylamide gels were malate dehydrogenase (EC 1.1.1.37) (amount of protein loaded, 350 µg/gel lane), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (200 µg/gel lane), α-glucosidase (EC 3.2.1.20) (200 µg/gel lane), β-glucosidase (EC 3.2.1.21) (350 µg/gel lane), alkaline phosphatase (EC 3.1.3.1) (350 µg/gel lane), acid phosphatase (EC 3.1.3.2) (350 µg/gel lane), and α-esterase (EC 3.1.1.1) (350

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TABLE 1. Strains of *C. guilliermondii* used in this study

Strain	Other designation ^a	Taxon	Origin	API 20C profile
Group I strains				
MCO 1442 ^T	ATCC 6260 ^T	<i>C. guilliermondii</i>	Expectoration from bronchomycosis	6772 373
MCO 1459	ATCC 9058		Acidophilous milk	6776 373
MCO 1460	ATCC 20283	<i>C. parapsilosis</i> var. <i>tokyoensis</i>	?	6776 373
MCO 1461	ATCC 22017 ^T	<i>C. guilliermondii</i> var. <i>japonica</i>	Soil, Japan	6776 373
MCO 1462	ATCC 26547 ^T	<i>C. parapsilosis</i> var. <i>tuxtlensis</i>	Sugar- and grain-based beverage, Mexico	6776 371
MCO 1463	ATCC 56822		Human urethra, Poland	6772 273
MCO 1467	ATCC 42715 ^T	<i>C. mamillae</i>	Nipple of nursing bottle, Japan	6776 373
MCO 1525	ATCC 58070	<i>Y. guilliermondii</i>	?	6776 373
MCO 1536 ^T	ATCC 46036 ^{Tb}	<i>Y. guilliermondii</i>	<i>Ulmus americana</i> frass	6776 373
MCO 1558	NRRL Y-2083 ^{Tc}	<i>Blastodendron artzi</i>	Blood	6776 373
MCO 1562	CBS 2024 ^{Tc}	<i>Blastodendron krausi</i>	Ulcer of horse	6776 373
MCO 1560	NRRL Y-17140 ^{Tc}	<i>C. amidevorans</i>	Soil, Italy	6776 373
MCO 1557	NRRL Y-2081 ^{Tc}	<i>Candida melibiosi</i> var. <i>melibiosi</i> (= <i>Mikroanthomyces alpinus</i>)	<i>Gentiana imbricata</i> flower	6776 373
MCO 1559	NRRL Y-12774 ^c	<i>Torulopsis xylinus</i>	Mulberry bush	6776 373
MCO 1539	NYDOH 267-83		Clinical	6776 373
MCO 1540	NYDOH 374-84		Clinical	6776 373
MCO 1541	NYDOH 607-84		Clinical	6776 373
MCO 1542	NYDOH 244-85		Clinical (leg ulcer)	6776 373
MCO 1543	NYDOH 693-85		Clinical (ear)	6776 373
MCO 1544	NYDOH 819-85		Clinical	6774 373
MCO 1545	NYDOH 821-85		Clinical	6776 373
MCO 1546	NYDOH 1306-85		Clinical	6776 373
MCO 1547	NYDOH 146-86		Clinical (sputum)	6776 373
MCO 1548	NYDOH 1036-86		Clinical (wound)	6776 373
MCO 1549	NYDOH 1058-86		Clinical	6776 373
MCO 1551	NYDOH 277-90		Clinical	6776 373
MCO 1553	NYDOH 711-92		Clinical (eye)	6776 373
MCO 1554	NYDOH 160-95		Clinical (blood)	6776 373
Group II strains				
MCO 848	GSU Atl. GD-17		?	6776 373
MCO 1439	ATCC 22995 ^T	<i>Torulopsis kestoni</i>	Air in sugar refinery, United Kingdom	6776 (1,3)73
MCO 1441	ATCC 28873 ^T	<i>Trichosporon appendiculare</i>	Human appendix, Brazil	6776 373
MCO 1464	ATCC 20296	<i>C. guilliermondii</i> subsp. <i>galactosa</i>	?	6776 373
MCO 1550	NYDOH 1015-88		Clinical (sputum)	6776 373
MCO 1552	NYDOH 488-92		Clinical (vitreous)	6776 373
MCO 1561 ^T	CBS 2022 ^{Tc}	<i>Torula fermentati</i>	Air, Japan	6776 373
Group III strain				
MCO 1440	ATCC 22949		Pigeon	67(5,7)6 373
Group IV strain				
MCO 1538	ATCC 20216	<i>C. guilliermondii</i> var. <i>soya</i>	?	6356 372
Group V strain				
MCO 1563 ^T	CBS 5256 ^{Tc}	<i>C. guilliermondii</i> var. <i>carphophila</i>	Fig wasp in fruit of <i>Ficus</i> sp.	6776 362

^a ATCC, American Type Culture Collection, Rockville, Md.; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, U.S. Department of Agriculture, Peoria, Ill.; NYDOH, Collection at the New York State Department of Health, Albany; GSU, Donald G. Ahearn, Georgia State University, Atlanta.

^b Redefined type strain of *Y. guilliermondii* and type strain of *P. guilliermondii*.

^c A RAPD pattern was not obtained; the group to which the strain belonged was determined by an isoenzyme pattern analysis.

µg/gel lane). The enzyme activities analyzed by using 5.0% (wt/vol) polyacrylamide gels were α-mannosidase (EC 3.2.1.24) (350 µg/gel lane), catalase (EC 1.11.1.6) (100 µg/gel lane), and superoxide dismutase (EC 1.15.1.1) (350 µg/gel lane).

RAPD analysis. Yeasts that were cultured as described above for the isoenzyme analysis were centrifuged at 2,000 × g for 10 min, and 200 µl of each cell pellet was transferred to a 1.5-ml microcentrifuge tube. The yeasts were washed once with 100 mM Tris hydrochloride (pH 8.0), and then the DNA was extracted and purified. The DNA was prepared for RAPD analysis as described previously (21).

The following primers used for PCR were obtained from J. R. Hobbs, University of British Columbia, Vancouver, British Columbia, Canada: UBC32 (5'-GGGGCCTTAA), UBC134 (5'-AACACACGAG), UBC143 (5'-TCGCAGAA CG), UBC147 (5'-GTGCGTCCTC), UBC709 (5'-CCTCCTCCTC), UBC710 (5'-GGTGGTGGGT), UBC711 (5'-CCCTCTCCCT), UBC712 (5'-GGGTGTG

GGT), UBC713 (5'-CCCTCCCTCT), UBC714 (5'-GGGTGGGTGT), UBC715 (5'-CCACCACCCA), UBC716 (5'-GGAGGAGGGA), UBC718 (5'-GGGAG AGGGA), UBC720 (5'-GGGAGGGAGA), UBC721 (5'-CCCTTCCCTC), UBC722 (5'-CCTCTCCCTC), UBC723 (5'-CCCTCTCCTC), UBC725 (5'-GG GTTGGGTG), UBC726 (5'-GGTGTGGGTG), UBC727 (5'-GGGTGTGGT G), UBC728 (5'-GTGGGTGGTG), UBC731 (5'-CCCACACCAC), UBC733 (5'-GGGAAGGGAG), UBC734 (5'-GGAGAGGGAG), UBC736 (5'-GAGGG AGGAG), UBC738 (5'-GGTGGGTGGT), UBC739 (5'-GGAGGGAGAG), UBC740 (5'-GGAGGGAGGA), UBC741 (5'-CCTCCCTCTC), UBC742 (5'-C CTCCCTCTC), UBC743 (5'-CCACCCACAC), UBC744 (5'-CCACCCACCA), UBC745 (5'-GGGAAGAGGG), UBC747 (5'-CCACCAACCC), and UBC754 (5'-GGGTGGTGGT).

When the RAPD analysis was performed, a single 10-mer primer was used with *C. guilliermondii* DNA in a PCR having a 94°C-36°C-72°C cycle; the PCR products were then analyzed by agarose gel electrophoresis (21).

FIG. 1. Isoenzyme patterns produced by members of *C. guilliermondii* groups I through IV. Lanes 1 through 3 and 6 through 8, group I strains MCO 1442^T, MCO 1459, MCO 1536, MCO 1461, MCO 1462, and MCO 1463, respectively; lane 4, group III strain MCO 1440; lanes 5 and 9, group 2 strains *C. fermentati* MCO 1441 and MCO 1464, respectively; lane 10, group IV strain MCO 1538. α -Man, α -mannosidase; α -Glu, α -glucosidase; β -Glu, β -glucosidase; CAT, catalase; EST, α -esterase; MDH, malate dehydrogenase.

Slime production. Slime production was determined by using a modification of the test established for coagulase-negative staphylococci (11, 12) as described by Branchini et al. (6). Briefly, loopfuls of yeast cells from the surfaces of plates containing Sabouraud dextrose agar (20 g of D-glucose per liter, 10 g of neopeptone [Difco] per liter, 15 g of agar per liter) were inoculated into tubes containing 10 ml of Sabouraud dextrose broth, Sabouraud dextrose broth supplemented with glucose (final concentration, 80 g/liter), YNB (Difco) containing 8% (wt/vol) glucose, or YPD broth supplemented with glucose (10 g of yeast extract [Difco] per liter, 10 g of peptone [Difco] per liter, 80 g of D-glucose per liter). The tubes were incubated at 37°C for 24 h without movement and then in a reciprocal shaker at ~140 rpm, after which the broth was removed and the walls of the tubes were stained with safranin. Staining was scored as negative to weak, moderate, or heavy.

Proteinase detection. Secretion of proteinase by *C. guilliermondii* on bovine serum albumin (BSA) agar was tested by using a semiquantitative screening procedure (24) similar to that described by Rùchel et al. (31). The inocula were prepared from 24-h cultures on Sabouraud dextrose agar. Colonies were resuspended in sterile distilled water to a turbidity equivalent to a no. 0.5 McFarland standard. Yeast suspension samples (5 μ l) were inoculated onto BSA agar plates, and the cultures were incubated at 24°C for 3, 6, 9, 12, 15, and 20 days. Proteolysis of BSA was visualized by staining with 0.1% naphthol blue black (Sigma) in 3.5 M acetic acid and was scored in the manner described by Lin et al. (24).

Esterase detection. To detect esterase activity, modified substrate agar plates were used (32, 37). The polyoxyethylene sorbitan substrates Tween 20, Tween 40, Tween 60, Tween 80, and Tween 85 (Sigma Chemical Co., St. Louis, Mo.) were added separately to Sabouraud liquid modified medium (Difco) to a final concentration of 1% (vol/vol). Overnight cultures were transferred to the substrate test medium, and the plates were incubated at 37°C in a humid environment and observed at various times for up to 20 days. Lipolytic activity was detected by inspecting the plates for zones of precipitation around the colonies.

Killer activity. The *C. guilliermondii* isolates were tested for killer activity against either *Candida albicans* MCO 261, an adenine auxotroph (SGY 126: *ade2*) obtained from D. R. Kirsch, Squibb Institute for Medical Research, Princeton, N.J., or *Saccharomyces cerevisiae* MCO 182, a uracil auxotroph (TH2-9A: *mnn2 ura3*) obtained from C. E. Ballou, University of California, Berkeley. The target yeasts were grown on Sabouraud dextrose agar and then suspended in sterile distilled water, and the turbidity was adjusted to a McFarland standard of ~2. Then 1-ml portions of the suspensions were spread onto petri dishes containing SD agar (6.7 g of YNB [Difco] per liter, 20 g of glucose per liter, 15 g of agar per liter), which was buffered at pH 4.5 with 100 mM phosphate-citrate (30), and the excess liquid was removed after 5 min. Each strain of *C. guilliermondii* was streaked onto the dry agar as described previously (30). The plates were incubated at 24°C for 48 h to allow growth of *C. guilliermondii*, and then adenine (1.2 mg/ml) or uracil (2.4 mg/ml) was added by filling six or seven wells (diameter, 8 mm) that had been cut into the agar. Addition of these nutrients enabled the auxotrophic target yeasts to grow. Following an additional 24 to 48 h of incubation, the plates were inspected for killer activity (30).

Physiological tests. A number of physiological tests were used mainly because their results had been reported to be variable in previous studies on *C. guilliermondii* (2, 26). To test for assimilation, yeasts were inoculated into broth containing D-glucose, melibiose, galactitol, glycerol, D-ribose, or D-arabinose as a

sole source of carbon. For fermentation studies, D-glucose, D-galactose, methyl- α -D-glucopyranoside, and raffinose were used, and the cultures were incubated at 30°C (38). To test for assimilation of compounds as sole sources of nitrogen, we used an auxanographic method in which a solution of uracil, adenine, potassium nitrate, potassium nitrite, D-glucosamine, L-lysine, thiourea, or creatinine was added to a well cut into yeast carbon base (Difco) agar on which *C. guilliermondii* had been plated as a lawn (38).

CHROMagar. Isolates were incubated at 30°C for 24 to 72 h on CHROMagar (CHROMagar, Palm Harbor, Fla.). The medium was prepared by mixing CHROMagar powder with distilled water and heating the preparation to boiling, which melted the agar; then the medium was poured into petri dishes. The color of the resulting colonies was recorded (29).

Antifungal agent susceptibility test. An antifungal agent susceptibility test was performed by a macrodilution method described by Fromtling et al. (14), with the minor modifications described by Lin et al. (24). The tubes were incubated without shaking at 30°C for 48 h. The lowest concentration of an antifungal agent associated with no visible growth in a tube was considered the MIC.

Rapid identification kits. The RapID Yeast Plus System (Innovative Diagnostics Systems, Norcross, Ga.) and the MicroScan Rapid Yeast Identification Panel (Baxter Diagnostics, Deerfield, Ill.) were used according to the manufacturers' instructions.

RESULTS

All of the strains produced yeast cells and filaments, usually clearly pseudohyphae, on corn meal agar, although in some cases the filaments were sparse. Initial studies in which isoenzyme analysis was used demonstrated the presence of apparently unrelated organisms among the *C. guilliermondii* strains obtained from the ATCC. While all of the strains were tested, the results obtained for only a selection of the strains and enzymes employed are shown in Fig. 1, in which the ATCC isolates represent four groups. Several strains resembled the type cultures of *C. guilliermondii* and *Y. guilliermondii*, but the patterns obtained for several enzymes, including α -mannosidase, α -glucosidase, catalase, α -esterase, and malate dehydrogenase, placed MCO 1441 and MCO 1464 in a separate group (group II). The strong β -glucosidase activity visible in Fig. 1 was not observed for all of the isolates in group II. Another distinctive group, group IV, was readily detected by using the isoenzyme patterns obtained for MCO 1538. The API 20C biotype of this strain was quite different from the API 20C biotypes of the other strains. The single strain in group III, MCO 1440, had features of both group I and group II in some of its enzyme patterns but could not be clearly assigned to one

FIG. 2. Isoenzyme patterns produced by members of *C. guilliermondii* groups I and II. Lanes 1 through 5, group I strains MCO 1442^T, MCO 1525, MCO 1541, MCO 1544, and MCO 1545, respectively; lanes 6 through 10, group II strains MCO 1439, MCO 1464, MCO 1550, MCO 1552, and MCO 848, respectively. CAT, catalase; α -Man, α -mannosidase; α -Glu, α -glucosidase; MDH, malate dehydrogenase; EST, α -esterase; ALP, alkaline phosphatase; GDH, glucose-6-phosphate dehydrogenase.

of these groups. Another isoenzyme-defined group, not shown in Fig. 1, was represented by *C. guilliermondii* var. *carphophila* MCO 1563^T (Table 1).

The clinical isolates, many of which had been sent for identification to the New York State Department of Health, belonged to *C. guilliermondii* groups I and II. Most of these strains had isoenzyme profiles consistent with group I, but the profiles of three of the clinical isolates in the panel, MCO 1441, MCO 1550, and MCO 1552, were group II profiles (Table 1). The distinctive isoenzyme and cellular protein patterns that distinguished group I from group II were produced by both clinical isolates and some non-clinical reference strains (Fig. 2).

The same groups were detected when RAPD profiles were used. The somewhat intermediate nature of group III, which had been noted when the isoenzyme patterns were examined, was confirmed by the RAPD analyses; MCO 1440 (group III) exhibited some similarity to group II when primers UBC710 and UBC739 were utilized, but it did not resemble group II when primer UBC740 was used (Fig. 3). However, when the remaining 31 primers listed in Materials and Methods were used to obtain RAPD patterns for the group III isolate, all but two patterns separated MCO 1440 from strains representing groups I and II (MCO 1459, MCO 1460, MCO 1441, and MCO 1464). The results obtained with primer UBC713 suggested that MCO 1440 has affinity to both group I and group II, and the results obtained with primer UBC715 suggested that MCO 1440 has affinity to group II (data not shown). The group IV strain, MCO 1538, was clearly discriminated from groups I, II, and III in all cases.

A number of different *Candida* species were compared by RAPD analyses in which primers UBC710, UBC739, and UBC740 (Fig. 4) and primer UBC32 (data not shown) were used. None of these species exhibited a clear resemblance to any *C. guilliermondii* group.

Phenotypic characteristics of groups. A number of tests were performed to distinguish the *C. guilliermondii* groups phenotypically and to investigate characteristics that have been suggested as characteristics that play a role in determining the virulence of pathogenic yeasts. These tests included tests for production of slime and formation of extracellular proteinases.

(i) Slime production. Initially, four media were tested for the ability to detect slime production by *C. guilliermondii*. Slime production was inhibited by shaking the cultures in Sabouraud and YNB broth media that contained 8% (wt/vol) glucose. In contrast, slime production was more pronounced in shaken cultures than in unshaken cultures when Sabouraud broth containing 2% glucose was used. Glucose-enriched YPD broth was quite variable in its support of slime production, both in still cultures and in shaken cultures. Given the well-defined medium provided by unshaken, glucose-enriched YNB broth, this medium was used to screen the strains.

Table 2 shows the slime production results. The majority of the strains in groups I and II produced slime, and slime production was associated with both clinical and environmental isolates. Neither the group III strain nor the group IV strain produced slime.

(ii) Formation of proteinases. Proteinase production was generally weak or not detectable. After 12 days, only MCO 1467 produced a distinct zone, and after 20 days an additional

FIG. 3. RAPD patterns obtained for members of *C. guilliermondii* groups and other *Candida* species by using primers UBC710 (A), UBC739 (B), and UBC740 (C). Lanes 1 through 6, group I strains MCO 1525, MCO 1442^T, MCO 1461, MCO 1541, MCO 1544, and MCO 1545, respectively; lanes 7 through 12, group II strains MCO 1464, MCO 1439, MCO 1441, MCO 1550, MCO 1552, and MCO 848, respectively; lane 13, group III strain MCO 1440; lane 14, group IV strain MCO 1538; lane 15, *Candida famata* ATCC 62894; lane 16, *Candida lusitanae* ATCC 42720; lane 17, *Candida maltosa* R-42 (obtained from D. G. Ahearn, Georgia State University); lane 18, *C. haemulonii* CDC 86-041135 (group I); lane 19, *C. haemulonii* CDC 90-003593 (group II) (23).

4 of the 23 group I strains appeared to be positive (score, 1+) (24). None of the strains belonging to the other groups were positive after 12 days, although a single group II strain, MCO 1464, showed evidence of weak activity after 20 days of incubation. In contrast to the limited activity associated with *C. guilliermondii*, after 12 days both *Candida parapsilosis* MCO 846 and MCO 847 exhibited obvious proteinase activity (score, 2+) (24).

Esterase. Minimal esterase activity was detected on agar media containing Tween 20, Tween 60, or Tween 80. Thus, this test did not allow us to distinguish *C. guilliermondii* groups I and II.

Killer activity. Both of the target strains used were auxotrophic mutants. This allowed us to suspend the growth of these organisms for 2 days in an attempt to increase the assay's sensitivity for inhibitory compounds. Weak inhibitory activity was produced by group IV isolate MCO 1538 (*Pichia ohmeri*) when it was tested against *C. albicans* and *S. cerevisiae*. Weak inhibitory activity was observed with *C. guilliermondii* MCO

1459, MCO 1460, and MCO 1525 (group I strains) when these organisms were tested against *C. albicans*, but no activity was observed when *S. cerevisiae* was the target. The activity was detectable in media buffered at pH 4.0, 4.5, and 5.0 but not in media buffered at pH 6.0 or 7.0. These results did not establish a useful phenotype for discriminating *C. guilliermondii* groups I and II.

Physiological tests. In the sugar assimilation and fermentation tests done in broth that were performed to distinguish *C. guilliermondii* group I from group II, the only difference found was a difference in the ability of the strains to ferment D-galactose after prolonged incubation. After 21 days at 30°C, all of the group II strains had produced gas, as had *C. guilliermondii* MCO 1538 (group IV) and MCO 1563^T (group V), whereas no *C. guilliermondii* group I or III strain had this phenotype. Fermentation of D-glucose was detected within 21 days for all strains belonging to groups I through V.

In tests performed with all of the strains except strains MCO 1557 through MCO 1563^T (see Materials and Methods),

FIG. 4. Comparison of RAPD patterns obtained for different species by using primers UBC710 (A), UBC739 (B), and UBC740 (C). Lane M, 100-bp DNA ladder with the lowest bright band at 600 bp; lane 1, *C. guilliermondii* group I strain MCO 1525; lane 2, *C. guilliermondii* group II strain MCO 1439; lane 3, *C. guilliermondii* group III strain MCO 1440; lane 4, *C. guilliermondii* group IV strain MCO 1538; lane 5, *C. parapsilosis* group I strain ATCC 22019; lane 6, *C. parapsilosis* group II strain MCO 471; lane 7, *C. parapsilosis* group III strain MCO 429 (24); lane 8, *C. haemulonii* group I strain CDC 86-041135; lane 9, *C. haemulonii* group II strain CDC 90-003593; lane 10, *Candida famata* ATCC 62894; lane 11, *Candida intermedia* R-666 (obtained from M. G. Rinaldi, University of Texas Health Sciences Center, San Antonio); lane 12, *Candida lusitanae* ATCC 42720; lane 13, *Candida zeylanoides* ATCC 7251; lane 14, *Candida ernobii* ATCC 60118; lane 15, *Candida rhagii* ATCC 22983; lane 16, *Candida holmii* ATCC 22034; lane 17, *Candida inconspicua* ATCC 16783; lane 18, *Candida maltosa* R-42.

D-raffinose was fermented by all of the strains except two group I strains (MCO 1463 and MCO 1525), and gas was detected after 6 to 18 days. None of the strains fermented methyl- α -D-glucopyranoside. All of the strains assimilated D-glucose, glycerol, D-arabinose, and dulcitol. The majority of the group I strains (20 of the 23 strains tested) and the group III and IV strains were positive for assimilation of glycerol after 3 days, and the remainder of the group I strains were positive after 6 days. In contrast, the group II strains were not positive for glycerol assimilation until after 6 or more days. Assimilation of D-arabinose was typically more rapid with group II strains than with group I strains. Five of the six group II strains were positive after 3 days, and the remaining strain (MCO 1550) was positive after 10 days. Only 2 of the 23 group I strains (MCO 1459 and MCO 1467) were positive for arabinose assimilation after 6 days, and the remainder of the group I strains were positive after 10 to 15 days.

API 20C profiles and characteristics on CHROMagar. Most of the API 20C profiles (Table 1) appeared to be very similar, and typically these profiles had biocode 6776 373; the only exception was the profile for *C. guilliermondii* var. *soya* MCO 1538 (group IV). This strain also appeared to be quite distinct

TABLE 2. Number of strains producing slime

Group	No. of strains tested	No. of strains with the following slime production:		
		Negative to weak	Moderate	Heavy
I	23	3	16	4
II	6	1	3	2
III	1	1		
IV	1	1		

TABLE 3. MICs of amphotericin B, ketoconazole, and 5-fluorocytosine

Antifungal agent	Group	No. of strains tested	No. of strains with an MIC of:						
			0.015 µg/ml	0.03 µg/ml	0.06 µg/ml	0.125 µg/ml	0.25 µg/ml	0.50 µg/ml	2.0 µg/ml
Amphotericin B	I	23		1	4	6	11	1	
	II	6		1	2		1	1	1
	III	1						1	
	IV	1				1			
Ketoconazole	I	23	6	12	5				
	II	6		3	1	2			
	III	1					1		
	IV	1		1					
5-Fluorocytosine	I	23	6	10	7				
	II	6	2	3	2				
	III	1	1						
	IV	1			1				

on CHROMagar, on which it produced blue colonies similar to *Candida tropicalis* colonies. All group I, II, and III strains produced pink colonies.

Antifungal agent susceptibilities. The numbers of strains that were inhibited by different MICs of antifungal agents are shown in Table 3. The pronounced overlap of MICs that characterized the *C. guilliermondii* group I strains and the group II strains did not allow discrimination of the two yeast groups on the basis of antifungal agent susceptibility.

Rapid identification kits. The results obtained with rapid identification kits are shown in Table 4. Only 10 group I strains were used in these tests. We found no obvious characteristics that differentiated the biotypes of *C. guilliermondii* belonging to groups I, II, and III. MCO 1538 (group IV; *P. ohmeri*) had a distinct MicroScan Rapid Yeast Identification Panel biotype with unique scores in the third and fifth positions which reflected a lack of production of esterases hydrolyzing glycyl-L-arginine 4-methoxy- β -naphthylamide and L-histidine β -naphthylamide, and the group V strain had a unique RapID Yeast Plus score in the third position of the biotype that reflected its failure to produce α -glucosidase and its production of an *N*-acetyl- β -D-galactosaminidase. Otherwise, the biotypes for all strains were similar, and, notably, groups I and II were indistinguishable.

Taxonomy of *C. guilliermondii* group II. The demonstration that there were distinct genetic groups among the strains led us to investigate which of the taxa that had been brought into synonymy with *C. guilliermondii* were found in each of the groups. Table 1 shows that many of the synonyms suggested in *The Yeasts: a Taxonomic Study* (26) and *Yeasts: Characteristics and Identification* (2) appeared in the same genetic group as the type strain (*C. guilliermondii* ATCC 6260). However, *Torulopsis kestoni*, *Torula fermentati*, *Trichosporon appendiculare*, and *C. guilliermondii* subsp. *galactosa* (a name applied to a strain deposited for patent purposes) represented *C. guilliermondii* group II. *Torula fermentati* Saito 1922 (33) represents the earliest description of an extant group II culture; the type strains of *E. negrii* Castellani 1912, *M. lustigi* Castellani et Chalmers 1913, and *M. pseudoguilliermondii* Castellani et Chalmers 1919 apparently are not extant.

Candida negrii was considered a valid name for *C. guilliermondii* group II, for the original description of *E. negrii* included weak fermentation of galactose as a characteristic (8). We chose not to use this name because we found that in order for fermentation of galactose to be useful for distinguishing *C. guilliermondii* groups I and II, it is necessary to use a temper-

ature of 30°C for the test. The use of either a higher temperature or a lower temperature does not give clear-cut results (22). It is not clear whether an incubation temperature of 30°C was used in the fermentation tests used to describe *E. negrii* (8). Other potential difficulties associated with the use of fermentation tests with D-galactose are discussed below. Given our incomplete understanding of the yeast described as *E. negrii*, a taxon for which no type strain appears to have been deposited, we recommend that yeast isolates which are identified as *C. guilliermondii* and which have RAPD and isoenzyme profiles that place them in group II should be named *C. fermentati* (Saito) Bai (1).

Status of the type strain of *Y. guilliermondii*. Strain CBS 2031 (= MCO 1525 = ATCC 58070) was defined as the type strain

TABLE 4. Biotypes of *C. guilliermondii* strains as determined with rapid identification kits

Strain	Group	Biotype as determined with ^a :	
		RYPY kit	MRYIP kit
MCO 1442 ^T	I	502307	556070654
MCO 1462	I	522317	717070040
MCO 1463	I	506137	756072064
MCO 1536 ^T	I	502237	776072044
MCO 1539	I	502337	774272644
MCO 1540	I	502137	754270640
MCO 1541	I	502337	777072640
MCO 1542	I	502137	774272646
MCO 1543	I	502337	774270044
MCO 1544	I	502337	774372646
MCO 848	II	506507	756072666
MCO 1439	II	502337	756272646
MCO 1441	II	502537	755370644
MCO 1464	II	506537	746070262
MCO 1550	II	506117	746370646
MCO 1552	II	506517	756370666
MCO 1561 ^T	II	502137	775372666
MCO 1440	III	502307	756170640
MCO 1538	IV	502107	542060640
MCO 1563 ^T	V	505537	775370666

^a RYPY, RapID Yeast Plus System; MRYIP, MicroScan Rapid Yeast Identification Panel.

of *Y. guilliermondii* (4), and we wish to correct an error in this assignment. Strain CBS 2030 (= MCO 1536 = ATCC 46036) is the original type strain deposited as *Pichia guilliermondii* by Wickerham (40), and so this strain should have been considered the type strain of *Y. guilliermondii*.

DISCUSSION

The use of molecular genetic procedures has resulted in detection of several groups of organisms among the organisms identified as *C. guilliermondii*. Group I is represented by the type strains of *Y. (= P.) guilliermondii* and *C. guilliermondii*. The synonyms for this taxon include *C. parapsilosis* var. *tuxtlensis*, *C. parapsilosis* var. *tokyoensis*, *C. guilliermondii* var. *japonica*, *Candida mamillae*, and *Candida amidevorans*. This group is common among clinical isolates and has also been detected in the environment (Table 1). No group I strain has been found to ferment galactose within 21 days at 30°C.

Group II also includes strains that were obtained from clinical specimens. In contrast to the group I strains, the group II strains produced gas during fermentation of galactose; however, this fermentation could be slow and might not become apparent before 21 days of incubation. The type strain of this group is the recently named organism *C. fermentati* CBS 2022 (= MCO 1561), which was originally obtained from the air in Japan (33). Another strain, strain ATCC 22995 (= MCO 1439), which was originally named *Torulopsis kestoni*, was also obtained from air samples (35). Other synonyms for *C. fermentati* are *Trichosporon appendiculare*, which was obtained from a clinical source (3), and *C. guilliermondii* subsp. *galactosa* (nomen nudum), which was applied to a strain deposited for patent purposes (16). In addition, the original description of *C. paransensis* stated that the species fermented galactose (28); therefore, this name should be considered a synonym of *C. fermentati*.

The distinction between *C. guilliermondii* (group I) and *C. fermentati* (group II) based on delayed fermentation of galactose may not be absolutely reliable. Wickerham reported, in his analysis of 16 sexually reactive strains of *C. guilliermondii* that were presumably group I strains, that galactose was "seldom" fermented (40). Furthermore, in contrast to our findings, other researchers working with group II strains have failed to detect fermentation of galactose despite incubation for 15 or 21 days (3, 33, 35); however, fermentation of galactose was a significant property that was used in the naming of *C. guilliermondii* subsp. *galactosa* (16). It is difficult to explain the differences between our results and those of other workers; however, over the years, there may have been slight changes in medium components, or strains may have developed altered phenotypes after being held in culture. Such changes have been recognized commonly; indeed, Castellani stated, "It must be remembered that a certain number of fungi of the genus *Monilia* have not permanent sugar reactions and can be trained to ferment sugars which they do not ferment when recently isolated, and therefore for purposes of classification and comparison such fungi should be investigated using only recently isolated strains" (10). Similarly, we noted changes in the morphology of the type strain of *Candida haemulonii* when a laboratory culture was compared to its original description (23).

Groups III through V were studied by using single strains (Table 1). Group III exhibited some similarities in its RAPD and isoenzyme patterns to the group I strain and the group II strain. The taxonomic status of this group requires further investigation. Group IV is represented by a single strain that has been misidentified; the phenotype of this strain is clearly that of *Yamadazyma (= Pichia) ohmeri*, and its identity has

been confirmed by an isoenzyme analysis performed with a panel of authentic strains which included the type culture of *Y. ohmeri*, NRRL Y-1932 (22).

In view of the reported DNA homology shown by *C. guilliermondii* var. *carpophila* (group V) and *C. guilliermondii* group I (27), it is surprising that the isoenzyme patterns of groups I and V are so different (data not shown). Indeed, these patterns suggest that the two groups may represent distinct species, and this could be supported by the rather different G+C contents of the group I and V DNAs (27).

Our research shows the value of protocols in which genotypic characteristics are used for classifying yeasts. As we have reported for other *Candida* species (42), many taxa appear to contain genetically distinct organisms; therefore, descriptions of a taxon's properties can represent combinations of characteristics associated with different species. Genotypic characteristics clarify the number of species present in a taxon and also can be used to validate the application of selected phenotypes to taxonomy under conditions where few phenotypes are available to separate species. In the current example, *C. fermentati* is distinguished from *C. guilliermondii* group I only by the fermentation of galactose, a test that can require up to 3 weeks of incubation before a result is obtained. Although these species are genetically distinct, the results obtained for markers with potential clinical significance, such as production of extracellular proteinase or slime and susceptibility to antifungal agents, showed that they are extremely similar and suggest that they might exhibit similar pathogenic potentials.

Some other clinically important yeast species are discriminated largely by using molecular genetic procedures; thus, *Candida dubliniensis* resembles *C. albicans* (36). Neither *C. dubliniensis* and *C. albicans* nor *C. fermentati* and *C. guilliermondii* are likely to be routinely discriminated from each other in most clinical laboratories. Because of this, the taxa *C. guilliermondii* and *C. albicans* may best be considered species complexes rather than discrete species. The development of convenient procedures in which genetic characteristics are used for primary identification of isolates would be of great value for discriminating phenotypically similar organisms that differ genetically.

ACKNOWLEDGMENTS

This research was supported by a grant from the American Heart Association, Ohio Affiliate.

We thank F.-Y. Bai for providing a copy of his manuscript prior to publication.

REFERENCES

1. Bai, F.-Y. 1996. Separation of *Candida fermentati* comb. nov. from *Candida guilliermondii* by DNA base composition and electrophoretic karyotyping. *Syst. Appl. Microbiol.* **19**:178-181.
2. Barnett, J. A., R. W. Payne, and D. Yarrow. 1990. Yeasts: characteristics and identification, 2nd ed. Cambridge University Press, Cambridge, England.
3. Batista, A. C., J. S. Silveira, and G. L. Silveira. 1959. Um novo *Trichosporon* isolado do apêndice cecal humano. *Rev. Assoc. Med. Bras.* **5**:351-352.
4. Billon-Grand, G. 1989. A new ascosporeogenous yeast genus: *Yamadazyma* gen. nov. *Mycotaxon* **35**:201-204.
5. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-254.
6. Branchini, M. L., M. A. Pfaller, J. Rhine-Chalberg, T. Frempong, and H. D. Isenberg. 1994. Genotypic variation and slime production among blood and catheter isolates of *Candida parapsilosis*. *J. Clin. Microbiol.* **32**:452-456.
7. Buesching, W. J., K. Kurek, and G. D. Roberts. 1979. Evaluation of the modified API 20C system for identification of clinically important yeasts. *J. Clin. Microbiol.* **9**:565-569.
8. Castellani, A. 1912. Observations on the fungi found in tropical bronchomycosis. *Lancet* **i**:13-15.
9. Castellani, A. 1912. Note on the importance of hyphomycetes and other fungi in tropical pathology. *Br. Med. J.* **ii**:1208-1212.

10. Castellani, A. 1920. The etiology of thrush. *J. Trop. Med. Hyg.* **23**:17-22.
11. Christensen, G. D., W. A. Simpson, A. L. Bisno, and E. H. Beachy. 1982. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infect. Immun.* **37**:318-326.
12. Davenport, D. S., R. M. Massanari, M. A. Pfaller, M. J. Bale, S. A. Streed, and W. J. Hierholzer. 1986. Usefulness of a test for slime production as a marker for clinically significant infections with coagulase-negative staphylococci. *J. Infect. Dis.* **153**:332-339.
13. Diddens, H. A., and J. Lodder. 1942. Die Hefesammlung des "Centraalbureau voor Schimmelcultures." Beiträge zu einer Monographie der Hefearten. II. Die anaskosporogenen Hefen, part 2, p. 246-265. N. V. Noord-Hollandsche Uitgevers Maatschappij, Amsterdam, The Netherlands.
14. Fromtling, R. A., J. N. Galgiani, M. A. Pfaller, A. Espinel-Ingroff, K. F. Bartizal, M. S. Bartlett, B. A. Body, C. Frey, G. Hall, G. D. Roberts, F. B. Nolte, F. C. Odds, M. G. Rinaldi, A. M. Sugar, and K. Villareal. 1993. Multicenter evaluation of a broth microdilution antifungal susceptibility test for yeast. *Antimicrob. Agents Chemother.* **37**:39-45.
15. Kemker, B. J., P. F. Lehmann, J. W. Lee, and T. J. Walsh. 1991. Distinction of deep versus superficial clinical and nonclinical isolates of *Trichosporon beigelii* by isoenzymes and restriction fragment length polymorphisms of rDNA generated by polymerase chain reaction. *J. Clin. Microbiol.* **29**:1677-1683.
16. Kimura, K., K. Takayama, and T. Nakanishi. April 1974. Process for producing citric acid by fermentation. U.S. patent 3,806,414.
17. Kurtzman, C. P. 1992. DNA relatedness among phenotypically similar species of *Pichia*. *Mycologia* **84**:72-76.
18. Langeron, M., and P. Guerra. 1938. Nouvelles recherches de zymologie médicale. *Ann. Parasitol. Hum. Comp.* **16**:36-179, 429-476, 481-525.
19. Lehmann, P. F., C.-B. Hsiao, and I. F. Salkin. 1989. Protein and enzyme electrophoresis profiles of selected *Candida* species. *J. Clin. Microbiol.* **27**:400-404.
20. Lehmann, P. F., B. J. Kemker, C.-B. Hsiao, and S. Dev. 1989. Isoenzyme biotypes of *Candida* species. *J. Clin. Microbiol.* **27**:2514-2521.
21. Lehmann, P. F., D. Lin, and B. A. Lasker. 1992. Genotypic identification and characterization of species and strains within the genus *Candida* by using random amplified polymorphic DNA. *J. Clin. Microbiol.* **30**:3249-3254.
22. Lehmann, P. F., and L.-C. Wu. Unpublished data.
23. Lehmann, P. F., L.-C. Wu, W. R. Pruitt, S. A. Meyer, and D. G. Ahearn. 1993. Unrelatedness of groups of yeasts within the *Candida haemulonii* complex. *J. Clin. Microbiol.* **31**:1683-1687.
24. Lin, D., L.-C. Wu, M. A. Rinaldi, and P. F. Lehmann. 1995. Three distinct genotypes within *Candida parapsilosis* from clinical sources. *J. Clin. Microbiol.* **33**:1815-1821.
25. Lodder, J., and N. J. W. Kreger-van Rij. 1952. The yeasts: a taxonomic study, p. 518-524. North-Holland Publishing Co., Amsterdam, The Netherlands.
26. Meyer, S. A., D. G. Ahearn, and D. Yarrow. 1984. Genus 4. *Candida* Berkhout, p. 585-844. In N. J. W. Kreger-van Rij (ed.), The yeasts: a taxonomic study, 3rd ed. Elsevier Science Publishers B. V., Amsterdam, The Netherlands.
27. Meyer, S. A., and H. J. Phaff. 1972. DNA base composition and DNA-DNA homology studies as tools in yeast systematics, p. 375-386. In A. Kocková-Kratochvilová and E. Minárik (ed.), Yeasts; models in science and technics. Proceedings of the First Specialized International Symposium on Yeasts, Smolenice, June 1-4, 1971. Publishing House of the Slovak Academy of Sciences, Bratislava, Slovakia.
28. Negróni, P., and I. Fischer. 1941. Contribución al conocimiento de la flora micológica (microfitos) del delta de Paraná. *Rev. Inst. Bacteriol. Dep. Hig.* **10**:334-342.
29. Odds, F. C., and R. Bernaerts. 1994. CHROMagar *Candida*, a new differential isolation medium for presumptive identification of clinically important *Candida* species. *J. Clin. Microbiol.* **32**:1923-1929.
30. Polonelli, L., C. Archibisacci, M. Sestito, and G. Morace. 1983. Killer system: a simple method for differentiating *Candida albicans* strains. *J. Clin. Microbiol.* **17**:774-780.
31. Rüchel, R., R. Tegeler, and M. Trost. 1982. A comparison of secretory proteinases from different strains of *Candida albicans*. *Sabouraudia* **20**:233-244.
32. Rudek, W. 1978. Esterase activity in *Candida* species. *J. Clin. Microbiol.* **6**:756-758.
33. Saito, K. 1922. Untersuchungen über die atmosphärischen Pilzkeime. *Jpn. J. Bot.* **1**:1-54.
34. San Millán, R., I. F. Salkin, and P. F. Lehmann. 1996. Genetic heterogeneity detected within *Candida guilliermondii*, abstr. F-6, p. 74. In Abstracts of the 96th General Meeting of the American Society for Microbiology 1996. American Society for Microbiology, Washington, D.C.
35. Scarr, M. P., and D. Rose. 1966. Study of osmophilic yeasts producing invertase. *J. Gen. Microbiol.* **45**:9-16.
36. Sullivan, D. J., T. J. Westerneng, K. A. Haynes, D. E. Bennett, and D. C. Coleman. 1995. *Candida dubliniensis* sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. *Microbiology* **141**:1507-1521.
37. Tirunarayanan, M. O., and H. Lundbeck. 1968. Investigations on the enzymes and toxins of staphylococci: assay of lipase using Tween as the substrate. *Acta Pathol. Microbiol. Scand.* **72**:263-276.
38. van der Walt, J. P., and D. Yarrow. 1984. Methods for the isolation, maintenance, classification and identification of yeast. IIC. Physiological and biochemical characteristics, p. 76-97. In N. J. W. Kreger-van Rij (ed.), The yeasts: a taxonomic study, 3rd ed. Elsevier Science Publishers B. V., Amsterdam, The Netherlands.
39. van Uden, N., and H. Buckley. 1971. Genus 2. *Candida* Berkhout, p. 893-1087. In J. Lodder (ed.), The yeasts: a taxonomic study, 2nd ed. North-Holland Publishing Co., Amsterdam, The Netherlands.
40. Wickerham, L. J. 1966. Validation of the species *Pichia guilliermondii*. *J. Bacteriol.* **92**:1269.
41. Wickerham, L. J., and K. A. Burton. 1954. A clarification of the relationship of *Candida guilliermondii* to other yeasts by a study of their mating types. *J. Bacteriol.* **68**:594-597.
42. Zeng, S., L.-C. Wu, and P. F. Lehmann. 1996. Random amplified polymorphic DNA analysis of culture collection strains of *Candida* species. *J. Med. Vet. Mycol.* **34**:293-297.