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Unrelatedness of Groups of Yeasts within the *Candida haemulonii* Complex

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Isoenzyme and protein profiles of clinical isolates initially identified as *Candida haemulonii* demonstrated the presence of two distinct groups. DNA relatedness studies with representative cultures confirmed the presence of two species. Physiological features that can be used to separate two groups within *C. haemulonii* are reported.

Recently, six isolates of *Candida haemulonii* (van Uden et Kolipinski) Meyer et Yarrow, a species that has been associated with thickened toenails, ulcers of the feet or legs, and occasionally with fungemia, all in Europe (5, 8, 10, 14, 18), have been reported to be found in clinical specimens obtained in the United States (6). The isolates were distinguished by their negative-to-weak assimilation of both cellobiose and raffinose from the phenotypically similar species *Candida famata* (Harrison) Meyer et Yarrow (synonym, *Torulopsis candida* Saito; teleomorph, *Debaryomyces hansenii* (Zopf) Kreger-van Rij) and *Candida guilliermondii* (Castellani) Langeron et Guerra (teleomorph, *Pichia guilliermondii* Wickerham). The API 20C (Analytab Products, Plainview, N.Y.) profiles of the six isolates of *C. haemulonii*, five of which were obtained from feet and one from blood, differed from each other, and some assimilations determined with the API 20C kit deviated from those obtained by using Wickerham broth assimilation tests (6).

During the later stages of the study, five additional clinical isolates were obtained from the United States; again, these were mostly from feet, and the isolates were grouped with *C. haemulonii* on the basis of their phenotype. As with the first group, this second group of isolates had varied API 20C profiles, but they could be distinguished from the first group by their positive utilization of ribitol. This feature was distinct only with the API 20C system and not with broth assimilations. Most of the second group also showed a more rapid utilization of raffinose and a tendency for delayed formation of sparse, mycotorula-type pseudohyphae. At first, these findings were not considered significant, especially since representative isolates of the second group had the same guanine-plus-cytosine content in the DNA as authentic *C. haemulonii* isolates. However, isoenzymes and DNA-DNA homology analysis showed the presence of two genetically distinct groups within the species complex. Further investigations have led to the discovery of physiological differences that may be used to distinguish between the groups. Here we describe features distinguishing the two groups.

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MATERIALS AND METHODS

Fungi. Isolates are listed in Table 1. No clinical history was available for the isolates obtained from David H. Pincus, Analytab Products (isolates designated MR). With the exception of these isolates and the *C. haemulonii* type strain, CBS 5149, the isolates were clinical cultures that had been received at the Centers for Disease Control. Of the latter, the isolates that are related to *C. haemulonii* have been described previously (6), and the sources of the five clinical isolates, which were found to be unrelated to *C. haemulonii*, are as follows. CDC 89-015867 was isolated from the toenail of a patient in Alabama, and isolates CDC 90-003593, CDC 90-006053, CDC 90-019484, and CDC 90-019652 were derived from ulcers on the feet of patients residing in Alabama, Georgia, Tennessee, and Virginia.

Physiologic profiles. The profiles were based on growth in liquid broth (19) or in the API 20C system (4).

Fungal extracts. The procedures used to obtain a soluble extract for studies on enzymes and proteins were based on methods and materials which have been published in detail previously (11). The method is given briefly below, including the modifications used for this study. The yeasts were grown in 100 ml of 0.10 M sucrose–0.03 M mannitol–yeast nitrogen base (without amino acids) broth in 250-ml Erlenmeyer flasks placed on a gyratory shaker (6 days at 28°C; 150 rpm); then crude extracts were obtained by disruption of the yeast cells with glass beads. After centrifugation of the extracts (13,000 × *g* for 5 min), the supernatants were collected, assayed for protein content by the method of Bradford (3), and loaded onto discontinuous native polyacrylamide gel slabs for electrophoresis. Following electrophoresis, the slabs were washed twice in buffer and then stained for enzyme activity, as described previously (7, 11).

DNA relatedness. The methods used were described previously (6). The thermal denaturation method of Marmur and Doty (15) was employed for determination of DNA base composition, and the optical renaturation method of Seidler and Mandel, as modified by Kurtzman et al. (9), was used for determining the percent DNA relatedness.

Computer analysis of phenotypic characteristics. The Yeast

TABLE 1. Carbohydrate assimilation in liquid by *C. haemulonii* groups 1 and 2 and API 20C codes

Group (*n*) and isolate	Day of first observation of assimilation[a]										API 20C code[b]
	αMG	Gal (4)	Glt (6)	Ara (5)	Sor (5)	Rib	Rbt	Raf (4)	Rha (6)	Mlz (3)[c]	
Group 1 (12)											
CBS 5149	−	8	9	−	−	−	−	11	+	5	6102170
86-032926[d]	−	8	12	12	−	−	8	11	+	11	2102170
86-041135[d]	−	9	12	12	−	−	8	11	+	12	6102170
89-019312[d]	−	−	−	−	−	−	−	11	+	−	2102170
90-001121[d]	−	−	−	−	−	−	−	11	+	−	6102170
90-006399[d]	−	−	−	11	−	−	−	9	+	9	6102170
90-006551[d]	−	11	−	−	−	−	−	10	+	−	6102170
87-MR68	−	−	−	−	−	−	−	+[e]	+	−	2102170
86-MR10	−	−	−	−	−	−	−	6	11	−	6102172
87-MR97	−	12	−	−	−	−	11	6	+	13	6102172
89-MR23	−	7	−	11	−	−	10	8	+	6	6102170
87-MR53	−	−	−	8	−	−	9	5	−	4	6102170
Group 2 (14)											
89-015867[d]	8	+	+	+	+	12	−	+	11	+	6142173
90-003593[d]	10	+	+	+	+	−	−	+	12	+	6152073
90-006053[d]	7	+	+	+	+	11	6	+	−	+	6142171
90-019484[d]	7	+	+	+	+	11	7	+	11	+	6172173
90-019652[d]	7	+	+	+	7	−	−	+	12	+	6142173
82-MR35	8	+	+	+	+	−	−	+	−	+	6152171
81-MR292	10	+	+	+	+	−	8	+	11	+	6152173
82-MR90	8	+	+	+	+	11	5	+	−	+	2152173
86-MR22	7	+	+	+	9	11	−	+	11	+	6142171
87-MR32	10	+	+	+	+	−	11	+	11	+	6152173
88-MR80	7	+	+	+	+	11	10	+	11	+	6146173
90-MR72	8	+	+	+	+	11	10	+	11	+	6152171
86-MR104	10	+	+	+	+	−	4	+	11	+	6152173
86-MR21	7	+	+	+	+	−	3	+	11	+	6156173

[a] Abbreviations: αMG, methyl-α-D-glucoside; Gal, D-galactose; Glt, galactitol; Ara, L-arabinose; Sor, sorbose; Rib, D-ribose; Rbt, D-ribitol; Raf, raffinose; Rha, rhamnose; Mlz, melezitose. Each cutoff day (in parentheses) is the latest day after inoculation that assimilation was considered rapid for each carbohydrate. A cutoff day for rapid assimilation was not scored for methyl-α-D-glucoside, D-ribose, or D-ribitol. +, rapid assimilation; −, no assimilation seen by 14 days.

[b] Code derived from the API 20C kit (4).

[c] For melezitose assimilation, all group 2 strains showed assimilation of melezitose within 24 h of showing assimilation of glucose. Group 1 strains showed assimilation of melezitose no earlier than 3 days after assimilation of glucose was first noted.

[d] Centers for Disease Control identifier.

[e] Assimilation seen at 4 days.

Identification PC program, version 2 (purchased from J. A. Barnett, Norwich, United Kingdom) (2) run on an IBM PC-compatible personal computer (International Business Machines Inc.) with an MS-DOS operating system (Microsoft Corporation) was used in the comparative analyses of phenotypic characteristics of different yeasts.

RESULTS

Isoenzyme profiles. The isoenzyme and protein profiles separated the clinical isolates into two distinct groups (Table 1), and representative protein and isoenzyme profiles of these are shown with those of *Candida lusitaniae* CBS 6936 in Fig. 1. Though there appeared to be some heterogeneity associated with the more slowly migrating β-glucosidase band in *C. haemulonii* strains, this was not seen in two replicate experiments. Only for α-mannosidase, superoxide dismutase, and malate dehydrogenase were the enzyme profiles of the two *C. haemulonii* groups considered indistinguishable.

The groups could still be differentiated by isoenzymes when representative yeasts were grown for shorter periods at a higher temperature (48 h at 35°C; 200 rpm). Alternative methods used to prepare the extracts, which included extraction in the presence of 2 mM phenylmethylsulfonyl fluoride (a proteinase inhibitor [17]) and precipitation with ammonium sulfate and renaturation by centrifugation though minicolumns containing Sephadex G-25 (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) (the spun column technique [13]), gave the same results as obtained with the crude soluble extracts (data not shown).

The isoenzyme profiles of the two groups were unlike those of *C. famata* (*T. candida*), *C. guilliermondii*, or *Candida maltosa*; a selection of the enzyme patterns is shown in Fig. 2. In addition, the profiles did not resemble those of *C. lusitaniae* (Fig. 1), *Candida albicans* MCO50 (11), or the type strain of *Candida parapsilosis*, ATCC 22019 (data not shown).

Phenotypic characteristics. The assimilation and fermentation reactions of the five clinical isolates composing the new group were quite similar to those reported for *C. haemulonii* by Gargeya et al. (6) and led to their initial placement within this species. All strains failed to ferment raffinose and did not assimilate cellobiose or salicin rapidly; however, delayed reactions with at least one of these two compounds were observed for a few isolates of each species. This made it unlikely that they represented clinically derived strains of *C. famata* (*T. candida*) or *C. guilliermondii*, which usually, in our experience, assimilate both compounds. Additionally, both Barnett et al. (1) and Meyer et al. (16) reported that fermentation of raffinose was typical for *C. guilliermondii*

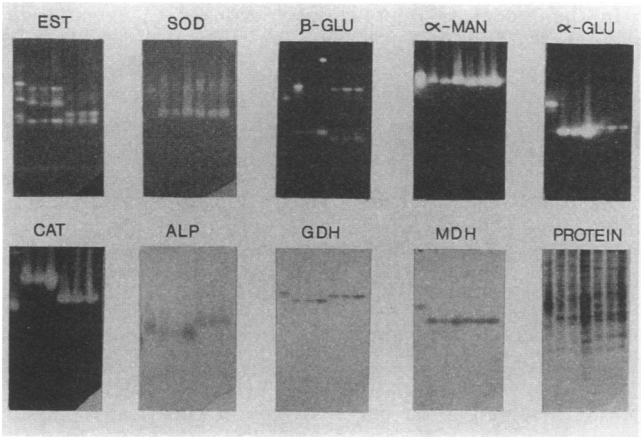

FIG. 1. Enzyme and protein profiles of *C. haemulonii* groups 1 and 2. From left to right, lanes contain extracts of *C. lusitaniae* CBS 6936, three *C. haemulonii* group 1 strains (CBS 5149 [type strain], CDC 89-019312, and CDC 90-006399), and three *C. haemulonii* group 2 strains (CDC 90-003593, CDC 90-019484, and CDC 90-019652). Abbreviations: EST, esterases developed with 4-methylumbelliferyl acetate; SOD, superoxide dismutases; β-GLU, β-glucosidases; α-MAN, α-mannosidases; α-GLU, α-glucosidases; CAT, catalases; ALP, alkaline phosphatases; GDH, glucose 6-phosphate dehydrogenases; MDH, malate dehydrogenases.

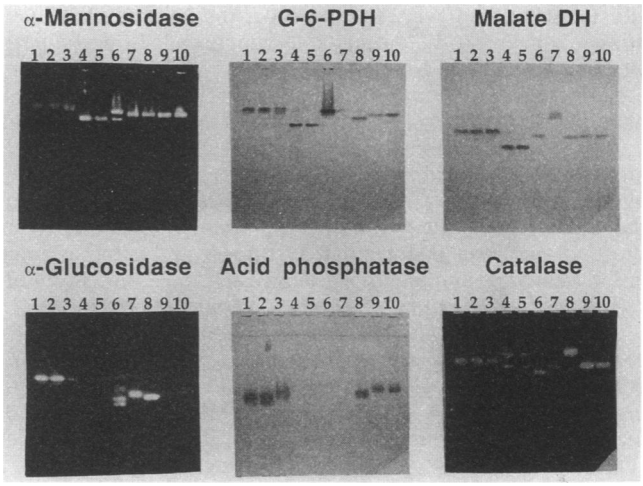

FIG. 2. Differentiation of *C. haemulonii* groups 1 and 2 from *C. famata*, *C. guilliermondii*, and *C. maltosa* by isoenzyme patterns. Lanes 1 to 3, *C. guilliermondii* CD-26, G-26, and GD-17, respectively; lanes 4 and 5, *C. famata* (*T. candida*) 115 and 117, respectively; lane 6, *C. maltosa* R-42; lane 7, *Candida* sp. strain TN90-001049; lane 8, *C. haemulonii* type CBS 5149 (group 1); lanes 9 and 10, *C. haemulonii* CDC 89-015867 and CDC 90-003593 (group 2), respectively. All strains are held in the culture collection of D. G. Ahearn at Georgia State University. Malate DH, malate dehydrogenase; G-6-PDH, glucose 6-phosphate dehydrogenase.

and could be found in some strains of *C. famata* (*T. candida*).

There were several discrepancies between the results obtained with the Wickerham assimilation tests and the API 20C system (4). For example, discrepancies may be noticed for the assimilation of D-galactose, ribitol (adonitol), L-arabinose, methyl-α-D-glucoside, and melezitose (Table 1). With the exception of L-arabinose assimilation, the majority of the inconsistencies were associated with a rather slow assimilation of the compounds when the broth method was used.

Physiological characteristics that allowed differentiation of the two groups of *C. haemulonii* were discovered. With broth assimilation tests, there was no assimilation of methyl-α-D-glucoside or sorbose by group 1 isolates, but these sugars were assimilated by group 2 isolates. Rapid assimilation of arabinose, galactose, galactitol, and raffinose characterized the group 2 isolates; in contrast, with the single exception of 87-MR68, which assimilated raffinose rapidly, group 1 isolates showed either negligible or greatly delayed assimilation of these carbohydrates. No group 1 isolate assimilated ribose, but ribose assimilation was detected within 14 days for many of the group 2 isolates. Rapid assimilation of melezitose was noted for group 2 isolates, while group 1 isolates showed either no assimilation or slower assimilation of this sugar. Rhamnose assimilation by *C. haemulonii* group 2 isolates was delayed or absent, but it was characteristically rapid for *C. haemulonii* group 1, with the exception of two isolates, 86-MR10 and 87-MR53 (Table 1).

The API 20C codes showed that isolates positive for galactose assimilation (shown by scores of 4 to 7 in the third position), ribitol assimilation (a score of 5 or 7 in the third position), methyl-α-D-glucoside assimilation (a score of 6 in the fourth position), or melezitose assimilation (a score of 1 or 3 in the seventh position) were typically *C. haemulonii* group 2. With the single exception of the group 2 isolate CDC 90-006053, which failed to assimilate melezitose in the API 20C test, the differential assimilation of galactose and melezitose separated *C. haemulonii* group 1 from group 2.

DNA relatedness of the two *C. haemulonii* groups. The DNA base composition and DNA relatedness of representative cultures are presented in Table 2. Representatives of the two groups had similar moles percent G+C in their DNAs, but when tested against each other, they showed an insignificant DNA homology.

DISCUSSION

Isolates of *C. haemulonii* group 2 have morphological and biochemical phenotypes that resemble those of group 1, which includes the type strain, and the two groups share a clinical association with feet and nails (6). Filamentous forms have been observed for several group 2 strains cultured on cornmeal agar, and this is also the case for some isolates of *C. haemulonii* group 1. Indeed, in spite of the original designation as *Torulopsis haemulonii* (20) and the lack of filaments observed for the type strain and other

TABLE 2. DNA base composition and DNA relatedness of *C. haemulonii* groups 1 and 2

Strain	Group	Mol% G+C[a]	Group 1 strain compared	% DNA relatedness
CBS 5149[b,c]	1	46.3 ± 0.0	CDC 90-001121	89
CDC 86-041135[b]	1	45.4 ± 0.5	CBS 5149[c]	92
CDC 90-001121[b]	1	47.9 ± 0.3	CDC 86-041135	100
CDC 90-003593	2	48.0 ± 0.0	CBS 5149	<28
CDC 90-019484	2	47.3 ± 0.0	CBS 5149	<28

[a] Values are means ± standard deviations (n = 2).
[b] Data for *C. haemulonii* group 1 strains are from the work of Gargeya et al. (6).
[c] Type strain.

isolates when they were first isolated, the failure to produce hyphae appears to be an unstable phenotype. In particular, we have noticed that some isolates from both groups develop a capacity to produce limited hyphal growth when cultures have been passaged in the laboratory.

Whether *C. haemulonii* group 2 represents an anamorph of an already described species is under investigation. The *C. haemulonii* complex has phenotypic properties similar to those described for *C. famata* (*T. candida*), particularly for those of the latter isolates that test negative for the assimilation of lactose and melibiose. However, the assimilation of cellobiose by *C. famata* (*T. candida*) separates it from the *C. haemulonii* complex. Therefore, isolates of *C. famata* (*T. candida*) are distinguishable from those in the *C. haemulonii* complex by their typical assimilation of cellobiose, by having a DNA with a lower moles percent G+C content (39 to 40 mol%), and by producing a very different isoenzyme profile (Fig. 2).

In the "minimal key" for phenotypic characteristics developed by Barnett et al. (1), the only difference between *D. hansenii* (teleomorph of *C. famata* [*T. candida*]) and *C. haemulonii* is the formation of ascospores by the former. Using the Yeast Identification PC program (2), we have analyzed other species that were found to differ by one characteristic from *C. haemulonii* on the basis of the following minimal key characters: fermentation of sucrose; assimilation of sucrose, cellobiose, erythritol, or citrate; growth at 37°C; and growth in the presence of 0.1% cycloheximide or 60% glucose. Fourteen species were found, most of which differed from *C. haemulonii* in the ability to grow in the presence of either 0.1% cycloheximide or 60% glucose. The majority of these species, which included *C. albicans*, had morphology obviously very different from that of isolates within the *C. haemulonii* complex. Thirteen species had significantly different G+C contents, with the moles percent G+C varying >4% from either group of *C. haemulonii*. Only *Candida mogii* Vidal-Leiria had a similar G+C content of 46%; however, this species, which has also been reported to be associated with human toenails (5), shows some phenotypic features that separate it from both groups of *C. haemulonii*. Differential characteristics for *C. mogii* include both its failure to assimilate melezitose or L-rhamnose and its production of a well-developed pseudomycelium on cornmeal agar; further, isoenzyme profiles show that *C. mogii* ATCC 18364 is genetically distinct from both groups of *C. haemulonii*. In contrast to the characteristic features associated with *C. mogii*, only 3 of 14 isolates of *C. haemulonii* group 2 tested negative for L-rhamnose assimilation; the remainder showed weak but definite assimilation, and all isolates showed a very rapid assimilation of melezitose (Table 1).

When the assimilation results for galactose, raffinose, xylose, and xylitol are added to the minimal key characters listed by Barnett et al. (1), the key indicates *C. haemulonii* but still fails to differentiate between the two groups. While molecular genetic techniques, including DNA-DNA homology or isoenzyme analysis, can then be employed to discriminate between them, the groups may also be determined from their differential assimilation of L-sorbose and from differences in their rates of assimilation of methyl-α-D-glucoside, D-galactose, galactitol, L-arabinose, raffinose, rhamnose, and melezitose (Table 1). With the API 20C system, and taking into account previous data (6), ribitol (adonitol), methyl-α-D-glucoside, and melezitose generally differentiated these two groups, though assimilation of the sugars is not necessarily found for all isolates within group 2.

In the recent taxonomic description of *C. haemulonii* given by Barnett et al. (1), there are variable results for the assimilation of a number of the compounds that we have found useful in discriminating between the two groups of *C. haemulonii*. Therefore, it is possible that the five strains examined therein (1) included isolates representing both of these groups.

Isoenzyme analysis has proved to be a rapid, inexpensive, and relatively simple procedure for screening large groups of phenotypically similar fungi for genetic similarity. As we have reported here, profound differences in isoenzyme profiles are associated with different species, while similar profiles characterize a single species (11). The use of isoenzyme analysis and similar approaches, such as employing random amplified polymorphic DNA (12), is likely to be very helpful in defining the major groups of genetically related yeasts that are to be found within the *C. haemulonii-C. famata* (*T. candida*) complex.

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