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Identification of *Candida lusitaniae* as an Opportunistic Yeast in Humans

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Four yeast strains, causally associated with infection in a patient with acute myelogenous leukemia, were identified by standard methods currently used in yeast taxonomy as representatives of *Candida lusitaniae* van Uden et do Carmo-Sousa. Because this species has not been recognized previously as an opportunistic yeast in humans, molecular taxonomic methods were applied to confirm its identity. The nuclear deoxyribonucleic acid (DNA) base composition of two clinical isolates was shown to be 45.1 mol% guanine plus cytosine as compared to 44.7 mol% guanine plus cytosine for the type strain of this species. DNA/DNA reassociation experiments revealed more than 95% complementarity between the DNAs from the clinical isolates and that of the type strain of *C. lusitaniae*, thus confirming their classification by conventional taxonomy. A key is provided to differentiate *C. lusitaniae* from two phenotypically similar *Candida* species.

Yeasts implicated in human infections are generally identified in medical laboratories using a limited number of phenotypic characters. Identification of commonly isolated yeasts such as *Candida albicans* is easily accomplished on the basis of germ tube production and chlamydospore formation (1). Less commonly recovered *Candida* species, particularly those fitting the characteristics of the *C. parapsilosis* group sensu Fell and Meyer (9), may be more difficult to distinguish because of interspecific similarities and intraspecific variability (3, 17). Identification of such yeasts in clinical laboratories may be equivocal but could be made more decisive by increasing the number of phenotypic characterizations employed.

We examined four yeast strains, typical of the *C. parapsilosis* group sensu Fell and Meyer, which were cultured from blood and tissue samples taken during the course of infection and postmortem from a patient receiving chemotherapy with daunamycin, 1-β-D-arabinofuranosylcytosine, and 6-thioguanine for acute myelogenous leukemia (14). With the appearance of yeast in blood cultures, intravenous administration of amphotericin B was begun. These yeasts were initially identified as *C. parapsilosis* in a clinical laboratory. Additional phenotypic characterization (17) in our laboratory revealed that the isolates were strains of *C. lusitaniae*, a species that has not been reported as an opportunistic yeast in humans. Genome comparisons of these yeasts with that of the type strain of *C. lusitaniae* and those of other *Candida* species from which they cannot readily be separated in a clinical laboratory confirmed their identity.

MATERIALS AND METHODS

The strains identified here as *C. lusitaniae* were obtained from D. Pappagianis, University of California Medical School, Davis. Strain 45090 (University of California-Food Science and Technology [UCD-FS&T] 78-8) was an isolate from blood early during the illness; 49597 (UCD-FS&T 78-9) was a blood culture obtained later during the illness; 50061 (UCD-FS&T 78-10) was isolated from a lung antemortem; and 50206 (UCD-FS&T 78-11) was isolated from lung tissue postmortem. The other strains (Table 1) were obtained from the yeast collection of the Department of Food Science and Technology, University of California, Davis. Identification of yeast isolates was based on phenotypic criteria as described by Van der Walt (16). The cultures designated UCD-78-8 and UCD-78-11 were included in the deoxyribonucleic acid (DNA)/DNA complementarity studies.

Cells used for nuclear DNA extraction were grown at room temperature in yeast autolysate (Difco, 0.5%) and 5% glucose on a rotary shaker and harvested at stationary phase by centrifugation. Extraction and purification of DNA were done by combination of the protocols of Marmur (11) and Bernardi et al. (4), as described in detail by Price et al. (15).To determine base composition (moles percent guanine plus cytosine [G+C]), each DNA sample was centrifuged at least three times in a CsCl gradient using a Spinco model E analytical centrifuge equipped with photographic optics. For the reassociation experiments, purified DNA was concentrated by electrophoresis in an ISCO concentrator and subsequently adjusted to a concentration of ~700 μg/ml prior to shearing. Reference DNA

TABLE 1. *Sources and DNA base composition of certain species of Candida*

UCD designation	Original designation[a]	Organism	Mol% G+C ± SD[b]
78-47	CBS 2162[c]	*C. freyschussii* Buckley et van Uden	43.82 ± 0.25
78-50	NRRL Y-7957	*C. freyschussii*	43.85 ± 0.15
61-4	CBS 4413[c]	*C. lusitaniae* van Uden et do Carmo-Sousa	44.71 ± 0.09
68-36	CBS 5901	*C. lusitaniae*	45.15 ± 0.18
78-8	45090	*C. lusitaniae*, clinical isolate susceptible to amphotericin B	45.15 ± 0.19
78-11	50206	*C. lusitaniae*, clinical isolate resistant to amphotericin B	45.08 ± 0.25
67-30	IGC 2704[c]	*C. obtusa* (Dietrichson) van Uden et do Carmo-Sousa ex van Uden et Buckley	45.02 ± 0.17
60-17	IGC	*C. obtusa*	45.26 ± 0.16
68-34	CBS 5837[c]	*C. obtusa* (Dietrichson) van Uden et do Carmo-Sousa ex van Uden et Buckley var. *arabinosa* Montrochet	47.26 ± 0.20
61-27	CBS 604[c]	*C. parapsilosis* (Ashford) Langeron et Talice	39.54 ± 0.10

[a] CBS, Centraalbureau voor Schimmelcultures, Yeast Division, Delft, Holland; NRRL, Northern Regional Research Laboratories, United States Department of Agriculture, Peoria, Ill.; IGC, Instituto Gulbenkian de Ciência, Oeiras, Portugal.

[b] Average of at least three buoyant density determinations. SD, Standard deviation.

[c] Type strain.

to be labeled with ^{125}I was further purified by ultracentrifugation (Beckman model L) to eliminate residual protein and ribonucleic acid prior to iodination. Peak fractions as determined by absorbance at 260 nm were pooled and dialyzed against 0.113 M NH_4-acetate–0.053 M acetic acid buffer at pH 5 for 48 h. Labeling with ^{125}I was done after denaturing the DNA solution in a glycol bath at 110°C, according to the method of Commerford (8), using approximately 200 μCi per 200 μg of DNA. The activity of the labeled DNA, after subsequent shearing and removal of rapidly renaturing sequences (15), was approximately 5 × 10^5 cpm/μg of DNA.

Unlabeled DNA was sheared to an average mass of approximately 300,000 daltons by two passages through a miniature French press at 13,500 lb/in^2. Reference DNA at a concentration of approximately 150 μg/ml was sheared after iodination by the same method. All DNA was filtered after shearing through Metricel filters, 0.45- or 1.2-μm pore size, to remove debris. Rapidly renaturing sequences were removed from the labeled DNA by the method of Price et al. (15).

The final concentration of unlabeled and labeled DNA was determined by the diphenylamine test (7). Subsequently, unlabeled DNA was diluted to 500 μg/ml, and labeled DNA was made to 2 μg/ml for renaturation experiments.

Renaturation kinetics of the reference DNA was determined by C_0t analysis (6). Reannealing reactions were done by following the protocol of Price et al. (15). Separation of single- and double-stranded DNA was done by the hydroxylapatite batch method (5), as modified by Price et al. (15). Resultant samples were transferred to counting tubes and counted in a Nuclear Chicago 1185 gamma ray counter (90% efficiency). Calculation of sequence complementarity was done according to Price et al. (15).

RESULTS

The clinical strains supplied to us were first subjected to a thorough analysis of their phenotypic properties (16). Identification of the strains by means of the key to the species of the genus *Candida* (17) indicated that the four strains were representatives of *C. lusitaniae* van Uden et do Carmo-Sousa. This species shows some resemblance to *C. freyschussii* Buckley et van Uden and to *C. parapsilosis* (Ashford) Langeron et Talice. All three species grow well at 39°C or higher and have many assimilative and fermentative properties in common.

To confirm the identification of the four clinical strains as *C. lusitaniae* and to differentiate them with certainty from phenotypically similar *Candida* species, a number of representative strains were investigated by molecular taxonomy.

Nuclear DNA base composition values for the cultures examined are presented in Table 1. The values were consistently somewhat lower than those reported in the literature (12), possibly because of different degrees of DNA purity and/or different methods for G+C determination.

Because of their significantly different DNA base compositions (Table 1), *C. parapsilosis*, *C. obtusa* var. *arabinosa*, and *C. freyschussii* do not appear to be closely related to the clinical isolates or to each other. Thus far, in our laboratory, yeasts with base compositions differing by more than 1 to 2 mol% G+C (by the buoyant density equilibrium centrifugation technique) were not found to be closely related (15).

The congruity of base composition values of the clinical isolates with those of *C. lusitaniae* and its synonym *C. obtusa* (13) supports but does not provide conclusive evidence for the phenotypic identification of the clinical isolates as *C. lusitaniae*. Nuclear genome comparisons were, therefore, made as described under Materials and Methods.

The DNA/DNA reassociation data are presented in Table 2. The DNAs from the clinical isolates 78-8 and 78-11 showed greater than 95% complementarity with that of the type strain of *C. lusitaniae*, thus confirming their identification as *C. lusitaniae*. *C. obtusa* also demonstrated a high degree of relatedness (>95%) with *C. lusitaniae*, confirming an earlier report on the synonymy of this species with *C. lusitaniae* (13). *C. obtusa* var. *arabinosa* showed very little DNA sequence complementarity with the reference DNA and must be considered a species separate from *C. lusitaniae*. This result is not surprising in view of the significant difference in DNA base composition between the two designated varieties of *C. obtusa*.

The two strains of *C. freyschussii* are not closely related to *C. lusitaniae*, because they exhibited only insignificant DNA complementarity with the reference strain. *C. parapsilosis*, used in the homology work as a negative yeast control on the basis of its G+C content (Table 1), also showed very low complementarity with the reference DNA and is not considered to be closely related to *C. lusitaniae*.

TABLE 2. *Renaturation reactions using ^{125}I-labeled DNA from C. lusitaniae 61-4 (at 65°C)*

Source of unlabeled DNA			
Organism	Strain designation	% Actual binding ± SD[a]	% Relative binding[b]
C. lusitaniae	61-4[c]	78.10 ± 0.26	100.00
Clinical isolate	78-8	75.45 ± 1.28	96.62
Clinical isolate	78-11	74.77 ± 0.67	95.74
C. obtusa	67-30[c]	75.63 ± 0.48	96.84
C. obtusa var. *arabinosa*	68-34[c]	3.77 ± 1.08	4.83
C. parapsilosis	61-27[c]	4.55 ± 2.00[d]	5.70
C. freyschussii	78-47[c]	3.58 ± 1.26	4.58
C. freyschussii	78-50	3.84 ± 1.08	4.92
Calf		4.77 ± 1.44	6.11

[a] Average of triplicate samples, corrected for zero-time binding (15). SD, Standard deviation.

[b] Corrected for self-reassociation of labeled DNA (15).

[c] Type culture.

[d] Average of duplicate samples, corrected for zero-time binding.

DISCUSSION

The ability of the yeasts in question to assimilate cellobiose should have precluded their identification in a clinical laboratory as *C. parapsilosis*. The separation of *C. lusitaniae* from the superficially similar *C. parapsilosis*, *C. freyschussii*, and *C. obtusa* var. *arabinosa* can be made by the use of the following key:

1a. Cellobiose not assimilated or fermented ..... *C. parapsilosis*
b. Cellobiose assimilated or fermented ..... (2)
2a. Growth in media containing 8% NaCl positive ..... *C. lusitaniae*
b. Growth in media containing 8% NaCl negative ..... (3)
3a. L-Arabinose not assimilated ..... *C. freyschussii*
b. L-Arabinose strongly assimilated ..... *C. obtusa* var. *arabinosa*

Another difference is that ribitol is assimilated (sometimes latently) by *C. lusitaniae* and by *C. parapsilosis*, but not by *C. freyschussii* or by *C. obtusa* var. *arabinosa*. More fundamentally, the data in Tables 1 and 2 show that these four yeasts differ in nuclear DNA base composition and have no significant DNA base sequences in common. The differentiation of these four species in most clinical laboratories is difficult, however, because the necessary criteria as described above are not routinely included by all laboratories in the identification regimen. An additional complication is encountered if the growth characteristics of yeast isolated from clinical sources vary from the norm.

Some growth characteristics of the *C. lusitaniae* strains isolated during the course of the disease (14) were not identical. Strains FS&T 78-10 and 78-11, which had been isolated during the terminal stage of the disease and postmortem, had developed resistance to amphotericin B (14), and concomitantly there was a discernible difference in gross colony morphology and an increasing degree of latency in the assimilation of several carbon compounds. This led initially to a presumptive identification of these strains as *C. freyschussii* in our laboratory until it became apparent that the isolates were capable of assimilating ribitol latently and grew in the presence of 10% sodium chloride. These altered growth characteristics that we noted may be due to changes in the sterol composition in the plasma membrane, which has been considered as a possible mechanism in the development of resistance to amphotericin B (14).

The maximum growth temperature of the clinical isolates is 42°C. The other species of *Candida* examined are also capable of growth at

temperatures exeeding 37°C (17), making it possible for these yeasts to survive in association with humans and possibly to develop as opportunistic pathogens. However, the ability of a microorganism to grow at 37°C or above does not necessarily impart to it the capability to invade or to survive in the tissues of warm-blooded animals.

The ecological specificity of the yeasts studied by us may provide insight into their capacity to produce human disease. *C. parapsilosis* is commonly found in association with warm-blooded animals; in humans it has been associated with endocarditis, transient fungemia, and systemic mycoses. *C. lusitaniae* is also found in association with warm-blooded animals (17) and now must be regarded also as opportunistically associated with humans. *C. freyschussii* and *C. obtusa* var. *arabinosa* have not been reported in association with warm-blooded animals, nor are they considered to be opportunistic yeasts in medical mycology literature.

Yeast infections are increasingly recognized as complications of various life-prolonging procedures currently used in medicine, i.e., immunosuppressive therapies, extensive use of bacterial antibiotics, hyperalimentation, and others (2). Generally, the yeasts considered responsible for such infections are species of the imperfect genera *Candida, Cryptococcus,* and *Torulopsis,* although members of *Rhodotorula* and *Trichosporon* are being recognized from time to time (2). Caution should be exercised in designating a yeast as the causative agent of infections because the mere presence of yeast does not necessarily denote tissue invasion. Under normal conditions, yeasts can be eliminated by the natural defense mechanisms of the host. It is also difficult to demonstrate the invasive ability of yeasts using animal models (10). Therefore, a detailed case history demonstrating repeated recovery and tissue invasion over the course of infection should be requisite for reporting novel yeast infections. Pappagianis and co-workers (14) believe that *C. lusitaniae* was involved in the deterioration of their patient, but its contribution to death of the patient remains uncertain.

The expanding spectrum of adventitious yeasts necessitates the reevaluation of criteria used for yeast characterization in clinical laboratories. The identification scheme now generally employed cannot differentiate the various cellobiose-positive yeasts examined in this study. The correct identification of yeasts associated with systemic mycoses, endocarditis, or other clinical syndromes is of obvious importance. Because many of these yeasts are members of the *C. parapsilosis* group (9), a more extensive characterization regimen is necessary to correctly identify isolates belonging to this group, as we have shown here for *C. lusitaniae.*

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