

## Evaluation of Phenotypic Markers for Selection and Identification of *Candida dubliniensis*

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Received 16 June 1999/Returned for modification 2 October 1999/Accepted 27 December 1999

*Candida dubliniensis* is often associated with *C. albicans* in cultures. Easy-to-perform selective isolation procedures for these closely related species do not exist. Therefore, we evaluated previously described discriminatory phenotypic markers for *C. dubliniensis*. A total of 150 oral rinses from human immunodeficiency virus (HIV)-infected patients were cultured on CHROMagar Candida. Dark green colonies described as being indicative of *C. dubliniensis* and other green colonies, 170 in total, were isolated. Chlamyospore formation, intracellular  $\beta$ -D-glucosidase activity, ability to grow at 42°C, carbohydrate assimilation pattern obtained by the API ID 32C, and Fourier transform infrared (FT-IR) spectroscopy were used for phenotypic characterization. Sequencing of the 5' end of the nuclear large-subunit (26S) ribosomal DNA gene was used for definitive species identification for *C. dubliniensis*. *C. dubliniensis* was found in 34% of yeast-colonized HIV-infected patients. The color of the colonies on CHROMagar Candida proved to be insufficient for selecting *C. dubliniensis*, since only 30 of 53 proven *C. dubliniensis* isolates showed a dark green color in primary cultures. The described typical chlamyospore formation can give only some indication of *C. dubliniensis*. The assimilation pattern proved to be insufficient to discriminate *C. dubliniensis* from *C. albicans*. All *C. dubliniensis* strains showed no or highly restricted growth at 42°C and a lack of  $\beta$ -D-glucosidase activity. Unfortunately, atypical *C. albicans* strains can also exhibit these phenotypic traits. FT-IR spectroscopy combined with hierarchical clustering proved to be as reliable as genotyping for discriminating the two species.

Various reports of the recently described yeast species *Candida dubliniensis* (32) indicate a worldwide occurrence (11, 26, 30, 31) of this fungus, which is phylogenetically closely related to *C. albicans*. The pathogenic potential of *C. dubliniensis*, so far recovered mainly from the oral cavity of human immunodeficiency virus (HIV)-infected patients (2, 23) and rarely from cases of candidemia in HIV-negative patients (16), is virtually unknown. While some *C. dubliniensis* isolates showed a higher level of adherence to human buccal epithelial cells when grown in glucose-rich media, the in vivo virulence of *C. dubliniensis* in a mouse model was found to be lower than that of *C. albicans* (4). Evidence for the inducibility of stable fluconazole resistance in vitro in *C. dubliniensis* strains (17, 18) may indicate an emerging pathogen for immunocompromised patients receiving long-term fluconazole prophylaxis.

Since *C. dubliniensis* is normally found in mixed cultures with *C. albicans*, several methods for the identification of *C. dubliniensis* and discrimination from *C. albicans* have been reported; these include the formation of dark green colonies on CHROMagar Candida (29), no or strictly reduced growth at 42°C (26), and lack of intracellular  $\beta$ -D-glucosidase activity (29). *C. dubliniensis* often shows characteristic chlamyospore formation (32) and may differ from *C. albicans* in its distinctive carbohydrate assimilation pattern (27). Furthermore, genetic differences between *C. albicans* and *C. dubliniensis* have been described (32). For a limited number of strains it has already been shown that Fourier transform infrared (FT-IR) spectroscopy

can successfully discriminate *C. albicans* from *C. dubliniensis* (35).

The detection and identification of microorganisms strongly depend on the availability of easy-to-perform screening methods. In planning an epidemiological study, we were confronted with the problem of selective isolation of *C. dubliniensis*. Primary experiments clearly showed that the sole use of green color on CHROMagar Candida was insufficient. Therefore, we evaluated systematically the discriminatory power of various phenotypic markers, i.e., colony color on CHROMagar Candida, growth at 42°C, intracellular  $\beta$ -D-glucosidase activity, kind of chlamyospore formation, assimilation pattern, and FT-IR spectroscopy. For reference, definitive species identification was achieved by sequence analysis of the 5' end of the nuclear large-subunit (26S) ribosomal DNA (rDNA) gene for suspected *C. dubliniensis* isolates or atypical *C. albicans* isolates (14).

### MATERIALS AND METHODS

**Clinical specimens.** Oral rinses from 150 consecutive HIV-infected patients attending an outpatient clinic for infectious diseases at Humboldt University in Berlin, Germany, between September and November 1997 were studied. HIV-infected patients (30 female and 120 male patients; 24 to 70 years old) with different stages of HIV infection (57 with AIDS-related complex and 66 with AIDS) were consequently receiving different treatments. T-cell (CD4<sup>+</sup>) counts were more than 500/ $\mu$ l in 13 patients, 200 to 500/ $\mu$ l in 78 patients, 100 to 200/ $\mu$ l in 42 patients, and less than 100/ $\mu$ l in 17 patients. Samples were taken regardless of clinical symptoms.

In addition to the patient isolates, the following reference strains were studied: *C. dubliniensis* CBS 7987<sup>T</sup> and CBS 7988 and *C. albicans* ATCC 10261, ATCC 48867, ATCC 76615, ATCC 90028, CBS 1905 (formerly the type strain of "*C. stellatoidea*"), A 1618, A 1626, and A 1634. The latter three, originating from vaginal swabs taken from women in Angola, had been described as morphologically and biochemically "atypical" *C. albicans* strains not capable of forming chlamyospores and of assimilating glucosamine and/or *N*-acetylglucosamine

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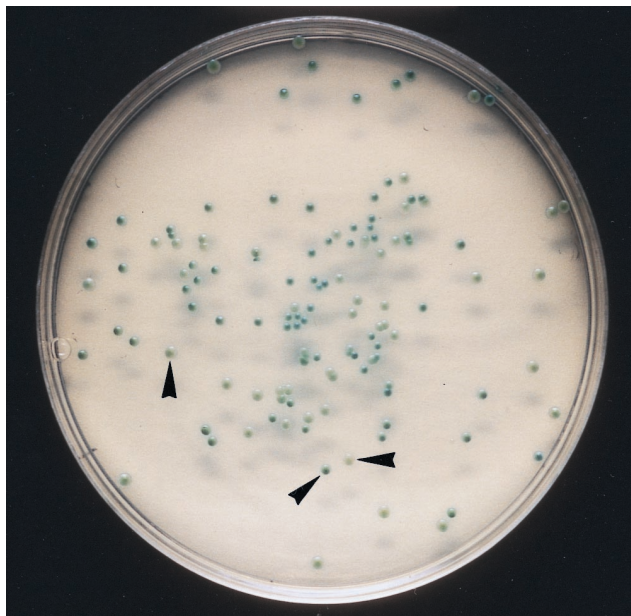


FIG. 1. Primary culture of an oral rinse on CHROMagar Candida after 48 h at 37°C showing colonies with three different shades of green (arrowheads), all finally identified as *C. dubliniensis*.

(34). Stock cultures of yeast isolates were kept on ceramic beads (Microbank; Mast Diagnostica, Reinfeld, Germany) at  $-70^{\circ}\text{C}$ .

**Media.** CHROMagar Candida (lot 332-c; CHROMagar Company, Paris, France; distributed by Mast Diagnostica), brain heart infusion (BHI) agar (Difco, Detroit, Mich.), and Sabouraud dextrose (SD) agar containing 2% glucose (E. Merck AG, Darmstadt, Germany) were prepared according to the manufacturers' instructions. Potato dextrose agar (PDA) was prepared using a filtrate of 100 g of boiled new potatoes, 20 g of dextrose, and 20 g of agar/liter, adjusted to pH 5.6. Rice-Tween 80 agar (RTagar) was prepared as described by Taschdjian (33).

**Sample processing.** Fresh oral rinse (10 ml) was centrifuged at  $3,000 \times g$  for 10 min, and the resulting sediment was resuspended in 0.5 ml of sterile saline. This suspension was microscopically examined and, if the yeast cell count was  $>10^4 \text{ ml}^{-1}$ , diluted to about  $10^3 \text{ cells ml}^{-1}$  with sterile saline. A 100- $\mu\text{l}$  quantity of this suspension was plated onto CHROMagar Candida and cultivated for 48 h at 37°C in ambient air. The occurrence of colonies in various shades of green (Fig. 1) was determined, and one colony of each green shade was randomly isolated and further characterized phenotypically.

**Chlamydospore formation.** According to the description given by Sullivan et al. (32), chlamydospore formation on RTagar was studied after prior subculturing on PDA for 48 to 72 h at 37°C. Chlamydospore formation was scored on days 2, 3, and 7 of incubation at 26°C as (i) suspected *C. dubliniensis*, i.e., abundant chlamydospores, often occurring in clusters or contiguous pairs on short branching pseudomycelia, or (ii) suspected *C. albicans*, i.e., chlamydospores occurring mostly singly on elongated pseudomycelia. Scoring was done by inspection of 20 fields (magnification,  $\times 200$ ) independently by two trained persons.

**Intracellular  $\beta$ -D-glucosidase activity.** Intracellular  $\beta$ -D-glucosidase activity was assessed twice as described elsewhere in detail (1, 29). Isolates grown on BHI agar were subcultured overnight in BHI broth in Erlenmeyer flasks on an orbital shaker at 120 rpm and 35°C. The following steps were performed at room temperature. One milliliter of the broth was centrifuged at  $15,000 \times g$  for 2 min. Pellets were resuspended in 1 ml of sodium acetate buffer (pH 5.5) containing 1 mg of 4-methylumbelliferyl- $\beta$ -D-glucoside (Sigma, Deisenhofen, Germany) per ml. Cells were disrupted by vigorous vortexing four times for 30 s each time after the addition of 0.4 g of glass beads (0.5-mm diameter). After centrifugation at  $15,000 \times g$  for 2 min, 0.1 ml of supernatant was transferred into a well of a microtiter plate. The occurrence of bright fluorescence indicative of intracellular  $\beta$ -D-glucosidase activity was judged visually after 15, 30, and 60 min using a UV transilluminator (Spectroline TR 302; Spectronics Corp., Westbury, N.Y.) with an excitation of 302 nm.

**Growth at 42°C.** Each isolate was subcultured on PDA at 37 and 42°C (30) for 48 h using a multipoint inoculator (Denley Corp., Sussex, United Kingdom). The growth of isolates at 42°C was scored as (i) none or poor and (ii) not reduced in comparison to that at 37°C.

**Extended phenotypic characterization.** Yeast isolates suspected of being *C. dubliniensis*, i.e., having at least two of the aforementioned phenotypic markers indicative of this species (e.g., abundant chlamydospore formation, lack of intracellular  $\beta$ -D-glucosidase activity, and/or significant growth reduction at 42°C; color on CHROMagar Candida not included), were further characterized by the assimilation pattern and FT-IR spectroscopy.

**Carbohydrate assimilation.** The assimilation pattern was studied using the API ID 32C (bioMérieux, Nürtingen, Germany). Strains were precultured on SD agar at 37°C for 48 h. Reactions were examined after 48 h of incubation at 30°C. Reading was done visually in strict accordance with the manufacturer's instructions; i.e., any cupule more turbid than the control was judged positive. Evaluation of the assimilation pattern was done with the ID 32C database (A-Software 2.6.8, version 2.0, 1999).

**FT-IR spectroscopy.** For FT-IR spectroscopy, yeast cells were subcultured on SD agar for 24 h at 30°C. Cells were harvested and prepared for FT-IR measurements as already described for bacteria (9, 10, 20). Briefly, 25  $\mu\text{l}$  of a yeast cell suspension in distilled water was transferred to a ZnSe optical plate and dried to a transparent film under mild vacuum (2.5 to 7.5 kPa). All spectra between 500 and 4,000  $\text{cm}^{-1}$  were recorded on an IFS 28/B spectrometer (Bruker Analytik, Karlsruhe, Germany) equipped with a deuterated triglycerine sulfate detector. Nominal physical resolution was set to 6  $\text{cm}^{-1}$ , Blackman-Harris apodization was used for Fourier transformation, and a zero filling factor of 4 was applied to yield an encoding interval of approximately one datum point per wave number. Spectral data were collected and evaluated with OPUS 3.0 software. Data processing included calculation of second derivatives to minimize baseline problems and to enhance apparent resolution. For hierarchical cluster analysis, an unsupervised classification technique (10), Ward's algorithm, was used to construct dendrograms. As a distance measure, Pearson's product-moment correlation coefficient was used as defined previously (9, 10).

For artificial neural network (ANN) analysis, the Stuttgart Neuronal Net Simulator was used. Analysis was performed as described previously (28). Automatic feature selection was based on variance analysis (28). This strategy generally improved the quality of the classifier and reduced the complexity and dimensionality of the classification model. Prior to feature selection, data reduction was performed by averaging three datum points, resulting in 1,206 datum points out of 3,620. The spectral range used for ANN analysis was 750 to 1,500  $\text{cm}^{-1}$ . Only vector-normalized spectra were used. A three-layer feed-forward network with 150 input neurons, 75 hidden layers, and 2 output units allowing shortcut connections within the net was established. As the learning function, resilient back propagation was used. A total of 150 training cycles were performed, with a relative training error (i.e., error divided by the variance of the outputs) of 0%.

TABLE 1. Characterization of 170 isolates derived from green colonies on CHROMagar Candida upon primary culturing

Group (no. of isolates)	Unrestricted growth at 42°C <sup>a</sup>	Type of chlamydospore formation	$\beta$ -D-Glucosidase activity	Presumed species
I (76)	+	Mostly single on elongated hyphae	+	<i>C. albicans</i>
II (23)	+	Abundant, in clusters or contiguous pairs	+	<i>C. albicans</i>
III (3)	+	None	+	<i>C. albicans</i> <sup>b,c</sup>
IV (9)	+	Mostly single on elongated hyphae	–	<i>C. albicans</i> <sup>b,c</sup>
V <sup>d</sup> (6)	+	Abundant, in clusters or contiguous pairs	–	<i>C. dubliniensis</i> or <i>C. albicans</i> <sup>b,c</sup>
VI <sup>d</sup> (46)	–	Abundant, in clusters or contiguous pairs	–	<i>C. dubliniensis</i> <sup>b</sup>
VII <sup>d</sup> (7)	–	Mostly single on elongated hyphae	–	<i>C. dubliniensis</i> <sup>b</sup>

<sup>a</sup> In comparison to growth at 37°C. +, positive; –, negative.

<sup>b</sup> Further characterized by sequencing.

<sup>c</sup> All confirmed as *C. albicans*.

<sup>d</sup> Isolates were further characterized by the assimilation pattern determined with the API ID 32C gallery and FT-IR spectroscopy.

TABLE 2. Phenotypic characteristics of 170 isolates derived from green colonies on CHROMagar Candida

Characteristic	Result <sup>a</sup> for:	
	<i>C. albicans</i> ( <i>n</i> = 117)	<i>C. dubliniensis</i> ( <i>n</i> = 53)
Colony color on CHROMagar Candida		
Light green	34 (29)	15 (28)
Green	47 (40)	8 (15)
Dark green	36 (31)	30 (57)
Growth at 42°C <sup>b</sup>		
Unrestricted	117 (100)	—
None or highly restricted	—	53 (100)
Chlamyospore formation in clusters or contiguous pairs	29 (25)	46 (87)
MIC <sub>80</sub> of fluconazole	ND	
≤0.5 µg/ml		50 (94)
2 to 8 µg/ml		3 (6)
Intracellular β-D-glucosidase activity	102 (87)	—
Assimilation (including weak) of <sup>c</sup> :		
D-Xylose	5/6 <sup>d</sup>	31 (58)
DL-Lactate	6/6 <sup>d</sup>	46 (87)
MDG	6/6 <sup>d</sup>	29 (55)

<sup>a</sup> Reported as number (percent) of strains, unless indicated otherwise. ND, not done; —, not found.

<sup>b</sup> In comparison to that at 37°C.

<sup>c</sup> API ID 32C results were read after 48 h.

<sup>d</sup> For isolates belonging to group V (Table 1).

**Sequencing for genetic characterization.** Partial sequence analysis of the nuclear rDNA was performed for all isolates presumed to be *C. dubliniensis* (groups VI and VII in Table 1; *n* = 53) and for doubtful *C. albicans* isolates (groups III, IV, and V in Table 1; *n* = 18).

Yeast cells were harvested after 2 days of incubation at 30°C on SD agar and suspended in sterile saline. DNA was prepared according to the procedure described by Goodwin and Lee (7). With primers NS7 and ITS2 (39), the 3' end of the nuclear 18S rRNA gene, along with the adjacent internal transcribed spacer 1 (ITS1) region, was amplified by PCR, whereas the 5' end of the nuclear large-subunit (26S) rDNA gene was amplified by use of a procedure described recently (13).

Direct sequencing of the resulting products was done using a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit and a 373 A DNA Sequencer (both from Applied Biosystems, Weiterstadt, Germany) as well as amplification primers for sequencing. Details of this procedure have been given recently (8). Concatenation of the resulting sequences and alignment using the respective sequences of *C. albicans* (EMBL accession no. AB013586 and AB032172) as a reference were done with the help of the DCSE software package (3). The ITS1 sequences of *C. dubliniensis* CBS 7987<sup>T</sup> and of *C. albicans* CBS 1905 have been deposited under EMBL accession no. AJ010332 and AJ010333, respectively.

**Fluconazole susceptibility testing.** A microdilution assay was performed in duplicate according to National Committee for Clinical Laboratory Standards performance standard M 27A (18a), with the modification that a final glucose concentration of 2% was used in RPMI medium. Final fluconazole concentrations ranged from 0.1 to 128 µg/ml. After 24 h of incubation at 35°C, each isolate showed good growth in the control well. Growth was then determined spectrophotometrically, and MICs were calculated as the MICs at which 80% of isolates were inhibited.

## RESULTS

Yeasts could be cultured from 117 (78%) of 150 oral rinses.

**Colony color on CHROMagar Candida and characterization of yeast isolates.** All samples showing growth of yeasts on CHROMagar Candida also yielded green colonies. As one specimen provided colonies with up to three different shades of green, 170 different colonies in total (light green, 49; green, 55; dark green, 66) were isolated randomly and further character-

ized phenotypically (Table 2). FT-IR spectroscopic measurements were obtained for 59 isolates after judgment of their phenotypic characteristics, i.e., abundant chlamyospore formation, no or highly restricted growth at 42°C, and/or no intracellular β-D-glucosidase activity (Table 1, groups V, VI, and VII). Analysis of the sequence of the 5' end of the nuclear large-subunit (26S) rDNA gene allowed unequivocal species assignment with the homolog signature nucleotides A (278), G (288), T (492), C (494), and G (496) for *C. albicans* (positions from GenBank sequence X70659) and G (176), A (186), A (392), T (394), and A (396) for *C. dubliniensis* (positions from GenBank sequence U57685). Therefore, all isolates of groups III, IV, and V (Table 1) proved to be *C. albicans*, whereas isolates of groups VI and VII proved to be *C. dubliniensis*. Together with definitive phenotypic identification for groups I and II, all green colonies were finally identified as either *C. albicans* (*n* = 117) or *C. dubliniensis* (*n* = 53).

Only 30 of the 53 *C. dubliniensis* isolates showed the dark green pigmentation described as typical of this species in primary cultures. Fifteen isolates were clearly light green, a color regarded as indicative of *C. albicans*. Eight of the *C. dubliniensis* isolates showed an intermediate intensity of green. Furthermore, *C. albicans* colonies also displayed every shade of green noted on CHROMagar Candida (Table 2).

**Chlamyospore formation.** All but 3 of 170 isolates tested produced chlamyospores. ITS1 sequencing revealed that the three non-chlamyospore-forming isolates were *C. albicans*. "Typical" chlamyospore formation of *C. dubliniensis*, i.e., abundant chlamyospores frequently arranged in triplets or contiguous pairs on short branching pseudomycelia (30, 32), was found in 46 isolates. Of 117 *C. albicans* strains, 85 showed single terminal chlamyospores, regarded as typical of *C. albicans*, whereas 29 showed chlamyospores typical of *C. dubliniensis*.

In contrast to the *C. albicans* American Type Culture Collection reference strains, which produced single terminal chlamyospores, strains A 1618, A 1626, and A 1634 produced no chlamyospores (34). Incubation for more than 72 h did not alter these results.

**Intracellular β-D-glucosidase activity.** Reproducible determination of β-D-glucosidase activity was achievable only after 30 min of incubation with the substrate. For all 53 proven *C. dubliniensis* isolates, no fluorescence could be detected. Bright fluorescence was seen for 102 (87%) of the 117 confirmed *C. albicans* isolates as well as the reference strains. Fifteen (13%) of 117 *C. albicans* isolates and strains A 1618, A 1626, and A 1634 showed no fluorescence, indicating a lack of intracellular β-D-glucosidase activity.

**Growth at 42°C.** No or highly restricted growth at 42°C was found for all *C. dubliniensis* isolates (Table 2), whereas all studied *C. albicans* strains, except for strains CBS 1905, A 1618, A 1626, and A 1634, grew well at this temperature.

**Carbohydrate assimilation.** No unique assimilation pattern for *C. dubliniensis* isolates was obtained with α-methyl-D-glucoside (MDG), DL-lactate, and D-xylose. Seven of 53 *C. dubliniensis* isolates failed to assimilate DL-lactate, and 24 were unable to assimilate MDG after 48 h. Twenty-two of 53 isolates failed to assimilate D-xylose. Only one of the 31 isolates able to assimilate D-xylose showed a strong reaction. Of note, the two *C. dubliniensis* reference strains also showed very weak assimilation of the above-mentioned three carbohydrates, but after a further 5 days of incubation, the assimilation reactions were clear-cut.

When the 53 *C. dubliniensis* isolates were identified using the assimilation pattern and the API 32C database, only for 2 isolates was *C. dubliniensis* quoted first (percentage of identi-



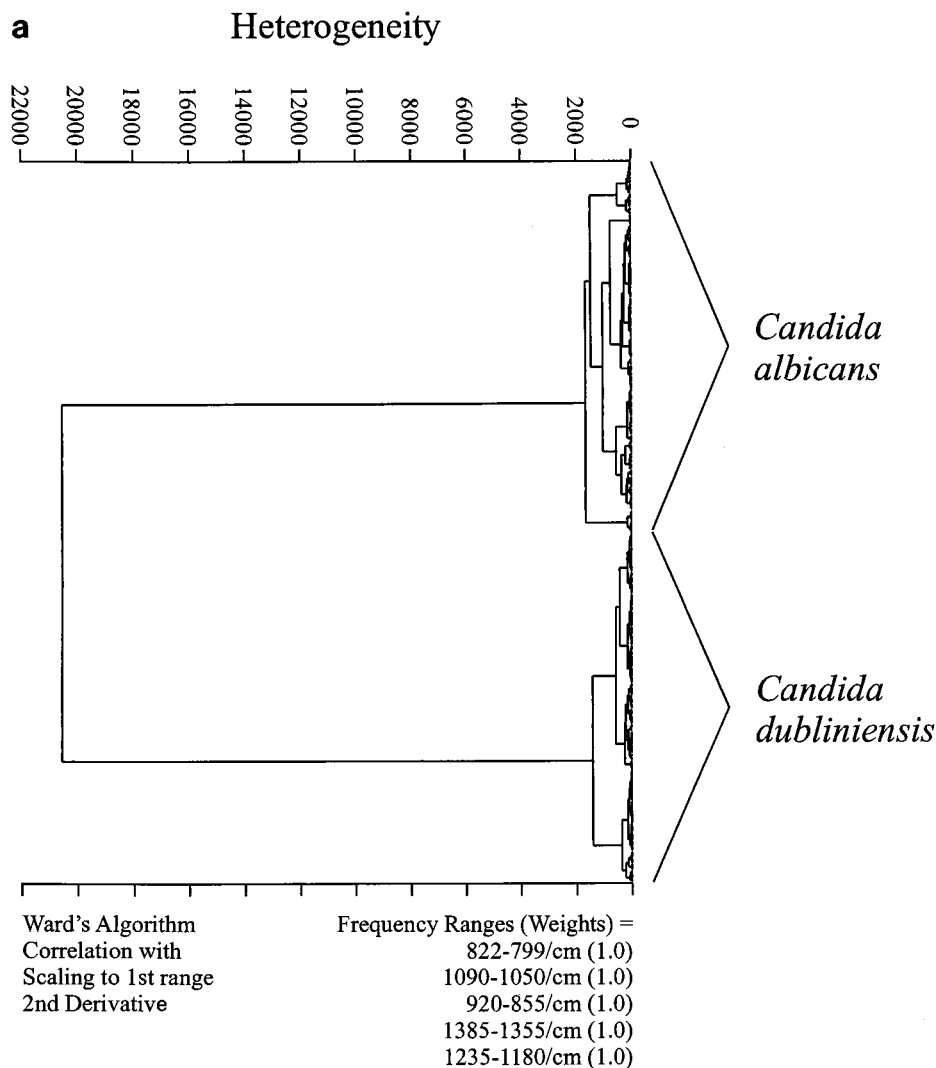


FIG. 2. (a) Dendrogram of a hierarchical cluster analysis performed on 421 spectra measured from 58 strains of *C. albicans* and 53 strains of *C. dubliniensis*. The discriminating information used for clustering is based on five spectral ranges: 822 to 799, 920 to 855, 1,090 to 1,050, 1,235 to 1,180, and 1,385 to 1,355  $\text{cm}^{-1}$ . (b) Representative second-derivative spectra of *C. albicans* and *C. dubliniensis* in the four most discriminatory spectral windows.

fication [%id], 81.9; T = 0.50). For the other isolates, an identification of "*C. albicans*" was given (%id, 68.0 to 99.9; T, 0.40 to 1.00). These results were due to the frequencies of assimilation of D-xylose, MDG, and DL-lactate being given as 0, 0, and 10%, respectively, in the database. Interestingly, one *C. dubliniensis* isolate was unable to assimilate N-acetylglucosamine.

**FT-IR spectroscopy. (i) Hierarchical cluster analysis.** Grouping of the spectra into distinct clusters was achieved by varying the number and combination of the spectral ranges contributing maximally to the desired classification (9). By use of the information encoded in five spectral ranges (822 to 799, 920 to 855, 1,090 to 1,050, 1,235 to 1,180, and 1,385 to 1,355  $\text{cm}^{-1}$ ) (equally weighted) as input data for the calculation of spectral distances and cluster analysis, a dendrogram was obtained (Fig. 2a). This dendrogram, comprising more than 350 spectra, exhibited two main clusters clearly separating the *C. albicans* and *C. dubliniensis* strains into two groups. All replicate measurements on the strains gave very similar distances, yielding extremely dense subgroupings within the two main clusters. Atypical *C. albicans* strains A 1618, A 1626, A 1634,

and CBS 1905 clustered as a monophyletic group together with the other *C. albicans* strains studied.

Figure 2b shows typical center-of-class spectra extracted from the *C. albicans* and *C. dubliniensis* clusters. The spectra are plotted for the spectral ranges used to calculate spectral distances. Figure 2b evidences a number of phenotypic differences responsible for the separation of the two *Candida* species into two spectral classes.

**(ii) ANN analysis.** A second approach, ANN analysis, a supervised classification method known to be superior to hierarchical clustering (5, 6, 35), was used to discriminate between *C. albicans* and *C. dubliniensis* and to establish a reference database suitable for the rapid identification of the two *Candida* species. The whole set of 270 FT-IR spectra collected from 40 strains of *C. albicans* and 41 isolates of *C. dubliniensis* was divided into two subsets, comprising 135 spectra of *C. albicans* and 135 spectra of *C. dubliniensis* for training and validation of the ANN. Prior to training and validation, the data set was subjected to an automatic wavelength selection process to extract class-specific spectral traits from the spectra

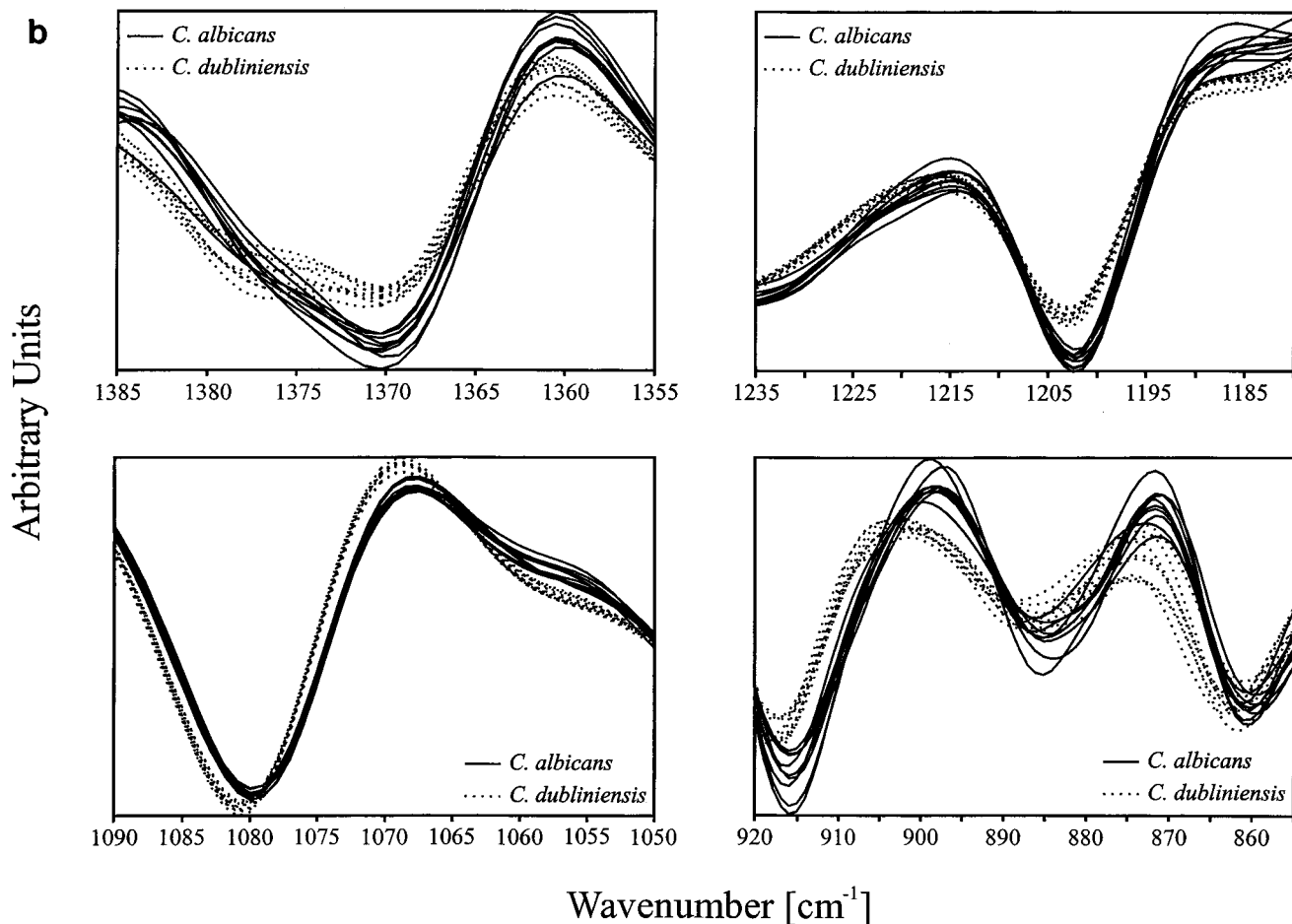


Fig. 2—Continued.

and to obtain data reduction. The selected wavelengths (data not shown) were used as input data for ANN analysis. All spectra (100%) of the training and validation data sets were correctly assigned to their respective classes, with the activation value of the output neurons being either 0 or 1. In a last step of analysis, the ANN was challenged with an independent test set of 87 FT-IR spectra obtained from 13 *C. albicans* strains and 17 *C. dubliniensis* strains not included in the training and validation. Spectra of this test data set were assigned correctly to their respective classes, indicating that the ANN is a stable reference for identifying unknown isolates of *C. albicans* and *C. dubliniensis*.

**ITS1 sequencing.** Analyses of the alignment of all ITS1 sequences obtained revealed two clusters (Fig. 3). All sequenced *C. albicans* strains (groups III, IV, and V in Table 1;  $n = 18$ ) showed a sequence identical to that of CBS 1905. The type strain of *C. dubliniensis* and the other *C. dubliniensis* isolates showed identical sequences, thus representing a second cluster. The alignment (Fig. 3) showed that one region (positions 482 to 491) is very distinctive for both species and thus is capable of being used as a signature sequence.

Additional sequencing and alignment of the 3' end of the nuclear small-subunit (18S) rDNA for the three reference strains of *C. dubliniensis* (CBS 7987<sup>T</sup>, CBS 7988, and NCPF 3108) showed no difference from the results for *C. albicans* (Fig. 3), a result which is in contrast to the deposited sequence of the type strain of *C. dubliniensis* (X99399).

**Fluconazole susceptibility.** For all 53 *C. dubliniensis* isolates, the MIC was  $\leq 8 \mu\text{g/ml}$ , indicating fluconazole susceptibility for all isolates, according to recent interpretation guidelines (25). Fifty isolates were highly susceptible (MIC,  $\leq 0.5 \mu\text{g/ml}$ ). Three isolates for which the MIC was between 2 and 8  $\mu\text{g/ml}$  were isolated from a single sample.

## DISCUSSION

Twenty-seven percent of HIV-infected patients studied were colonized with *C. dubliniensis*, a rate comparable to the rate reported from Ireland (2), whereas colonization is much lower in HIV-negative individuals (2, 23). The presence of *C. dubliniensis* may be underestimated when phenotypic markers used for selective isolation prove to be insufficient. Colonization with *C. dubliniensis* was not found to be correlated with the stage of HIV infection. Induction of fluconazole resistance in *C. dubliniensis* strains due to treatment with this drug, as seen in *in vitro* studies (18), seemed not to have occurred in our patients.

*C. dubliniensis* as the sole identified yeast species cultivated in 11 oral rinses cannot be definitively interpreted because isolation of different green colonies was done randomly, with possible *C. albicans* not being recognized.

The main obstacle to clarification of the epidemiological role of *C. dubliniensis* is the lack of an easy-to-perform screening method capable of selective identification of this yeast,



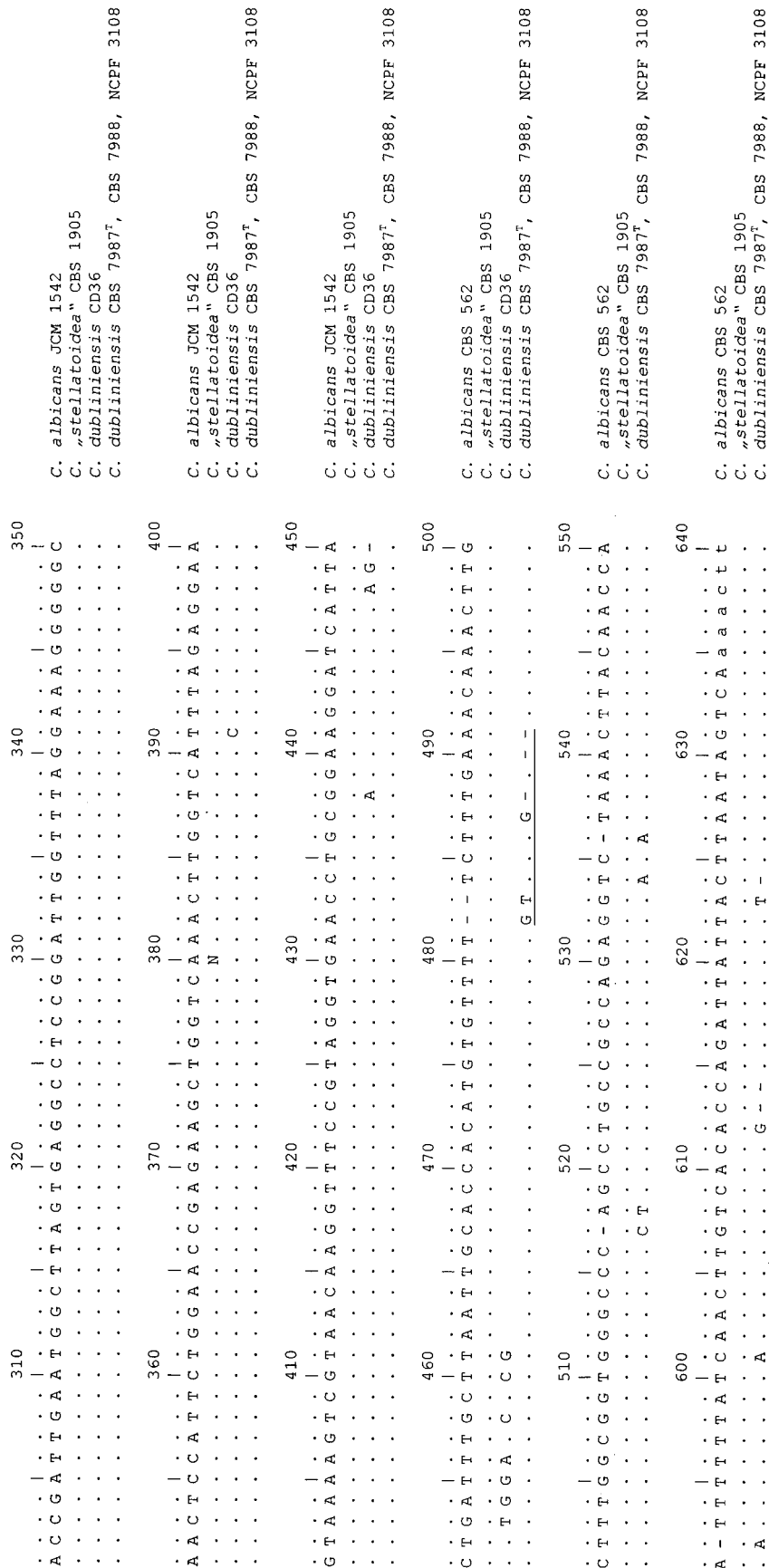


FIG. 3. Alignment of the 3' end of the small-subunit (18S) rDNA sequences of *C. albicans* JCM 1542 (EMBL accession no. AB013586), *C. dubliniensis* CD36 (GenBank accession no. X99399), and *C. dubliniensis* CBS 7987<sup>T</sup>, CBS 7988, and NCPF 3108 and of ITS1 sequences of *C. albicans* CBS 562 (EMBL accession no. AB032172), "*C. stellatoidea*", CBS 1905, and *C. dubliniensis* CBS 7987<sup>T</sup>, CBS 7988, and NCPF 3108. Identical nucleotides are indicated by dots, and gaps are indicated by dashes. The ITS1 region begins at position 451 and ends at position 634. Underlining indicates the species-specific signature sequence that could be used to assign a strain unequivocally to *C. albicans* or *C. dubliniensis*.

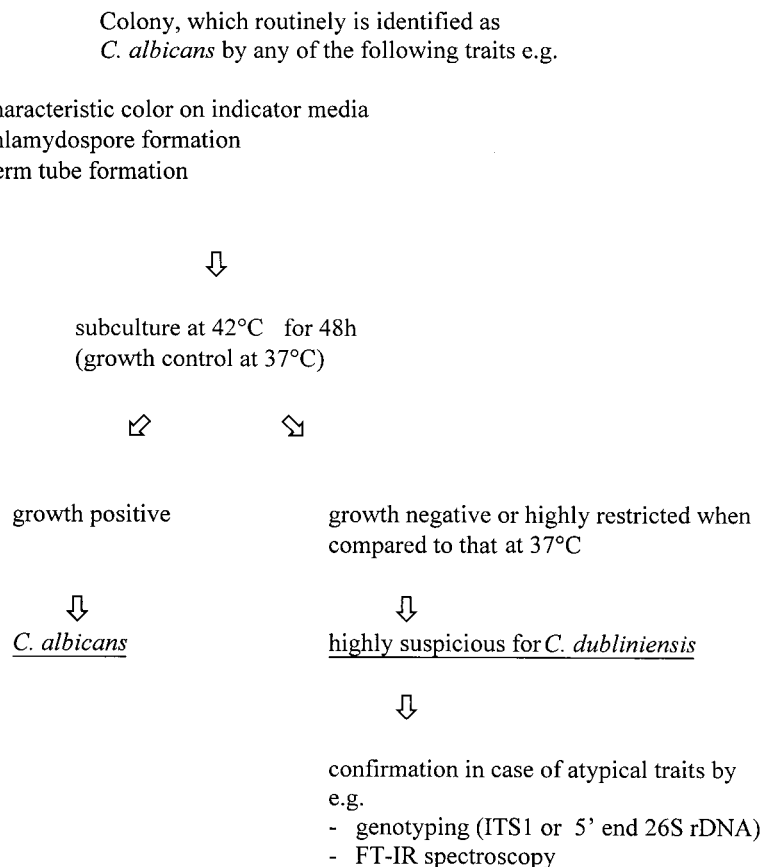


FIG. 4. Proposed scheme for identification of *C. dubliniensis*. Some authors claim that a clearer distinction between *C. albicans* and *C. dubliniensis* is obtained by subculturing at 45°C (24).

which occurs mostly in mixed cultures. Such a “positive” phenotypic marker is still missing. CHROMagar Candida is a medium with chromogenic substances allowing presumptive identification of several clinically important *Candida* species (22). A dark green color on CHROMagar Candida was described as a potential phenotypic marker for *C. dubliniensis* in primary cultures (2, 29). Since this phenomenon was found not to be reproducible after subculturing and storage of isolates (29, 30), use of this marker may be limited to primary cultures. In a large-scale retrospective study by Odds et al. (23), the shade of green proved to be unreliable for discrimination between *C. albicans* and *C. dubliniensis*. The results obtained in our study are in accordance with this observation. Therefore, an approach that uses color on CHROMagar Candida in primary or secondary cultures (11) as a sole selective criterion may underestimate the frequency of *C. dubliniensis*.

The use of reduction of 2,3,5-triphenyltetrazolium chloride (TTC) by *C. dubliniensis* and not by *C. albicans* as a positive screening parameter (38) is also of limited value, since TTC reduction is also seen regularly in *C. tropicalis*, a species frequently encountered in human specimens.

The type of chlamyospore formation was to be variable in both *C. dubliniensis* and *C. albicans* strains and therefore is also not reliable for differentiation of these two species (29, 30). Since 57% of *C. dubliniensis* isolates (compared to 15% of *C. albicans* isolates) showed abundant chlamyospores frequently arranged in clusters of contiguous pairs, the predictive value of this characteristic is low.

No or highly restricted growth at 42°C proved to be an easily

obtainable and reliable phenotypic marker for the presence of *C. dubliniensis*; this trait is rarely encountered in atypical *C. albicans* strains. Use of an incubation temperature of 45°C, as recently proposed (24), in order to obtain more clear-cut discrimination seems not to be mandatory, since the highly restricted growth of some *C. dubliniensis* isolates at 42°C did not negatively affect the discriminatory power, because of unrestricted growth of *C. albicans* isolates. This difference could best be seen after incubation for 48 h, since the growth of *C. albicans* was less evident after 24 h at 42°C (data not shown). Of note, the maximum growth temperature of *C. albicans* varies from 43 to 46°C (37). Nevertheless, one should be aware that rare atypical *C. albicans* strains, e.g., the former “*C. stellatoidea*” type I and strains A 1618, A 1626, and A 1634 (34), are not able to grow at 42°C.

When the recommended procedure was used (1), none of the *C. dubliniensis* isolates showed intracellular  $\beta$ -D-glucosidase activity. Nevertheless, the predictive value of this trait is lower than that of the inability to grow at 42°C, since 13% of *C. albicans* isolates showed no  $\beta$ -D-glucosidase activity. This result is in accordance with the results reported by Odds et al. (23), who found nearly the same percentage of *C. albicans* isolates without  $\beta$ -D-glucosidase activity.

According to Schoofs et al. (29) but contrary to the reported uniformly negative assimilation of DL-lactate, D-xylose, and MDG in *C. dubliniensis* strains (27, 30–32), we found considerable variability in the assimilation of DL-lactate, D-xylose, and MDG when using the API ID 32C gallery and reading the test



visually. This result may have been due to very weak reactions after 48 h of incubation.

FT-IR spectroscopy monitors the composition and particular structures of all cell compounds within an intact cell environment. Thus, the spectral signatures observed are the complex superposition of the infrared active vibrational spectroscopic features of all cell components present in a cell (9, 10, 19, 21). To extract the relevant discriminatory information from large spectral data sets, data reduction and application of pattern recognition techniques are mandatory.

FT-IR spectroscopy is undoubtedly capable of discriminating between clinical isolates of *C. albicans* and *C. dubliniensis*. This discrimination is, however, achievable only when multivariate statistical techniques are applied to the analysis of complex spectral data. These spectral differences cannot yet be interpreted in terms of biochemical and/or chemical structures, however. The two classification models elaborated in this study prove that the information contained in the microbial FT-IR spectra is sufficient to differentiate phenotypically between the two closely related species of *Candida*. The potency of FT-IR spectroscopy for differentiating these two *Candida* species has already been shown by Timmins et al. (35). The present study, however, is the first example of a successful application of FT-IR spectroscopy to previously unidentified clinical isolates of *C. albicans* and *C. dubliniensis*. Subgrouping within the two species evidences the presence of strain-specific substructures within the classification scheme and demonstrates the clear ability to use the FT-IR spectroscopy technique as a fingerprinting-like method for further studies. The ANN approach elaborated in this paper proved to be extremely efficient, since once a validated ANN had been generated, unknown isolates could be identified within seconds without again performing the whole elaboration procedure. Thus, FT-IR spectroscopy can be used as a very rapid and versatile technique for screening large numbers of isolates in the context of epidemiology and outbreaks.

Species assignment based on analysis of the 5' end of the nuclear large-subunit (26S) rDNA proved to be a reliable procedure, especially for yeasts (12, 13), including medically important species (14). The variability found in the ITS1 region of the rDNA gene is also often used for the discrimination of phylogenetically closely related fungi (36). Upon comparison, both regions of the nuclear rRNA gene showed identical clustering. The ITS1 sequence contained one species-specific signature sequence that could be used to assign a strain unequivocally to *C. albicans* or *C. dubliniensis*. The same was true for phenotypically atypical strains of *C. albicans*, such as the former type strain of "*C. stellatoidea*" or *C. albicans* strains not producing chlamydo spores. Therefore, this signature sequence may be used for the design of a species-specific gene probe, allowing a colony blot to be performed in order to recognize *C. dubliniensis* in mixed cultures. Since there are several different adjacent nucleotides, the design and application of a gene probe might be easier than the use of single different nucleotides, as in the case of the 5' end of the nuclear large-subunit (26S) rDNA (15). Such an approach might help to overcome the difficulties encountered when phenotypic markers are used for this purpose, as revealed in our study. However, sequence differences in the region of the 3' end of the nuclear 18S rRNA gene in one strain of *C. dubliniensis* relative to *C. albicans* (32) could not be revealed in the reference strains (CBS 7987<sup>T</sup>, CBS 7988, and NCPF 3108) studied by us. This finding may provide an explanation for the unsuccessful application of a gene probe (data not shown) designed on the basis of the sequence differences in positions 114 to 124 in the alignment (Fig. 3) when we started our study.

In conclusion, the main problem in the selection and identification of *C. dubliniensis*, which is present in more than one-third of yeast-colonized HIV-infected patients, is still the lack of a reliable discriminative phenotypic marker. Use of dark green colonies on CHROMagar *Candida* proved to be insufficient. One indication of the presence of *C. dubliniensis* in chlamydo spore-forming isolates is the combination of no or highly restricted growth at 42°C and the absence of intracellular  $\beta$ -D-glucosidase activity. Both ITS1 sequencing and FT-IR spectroscopy with multivariate data analysis allow reliable identification of *C. dubliniensis*.

The flow scheme proposed in Fig. 4 is based on the results obtained in our study and might help in identifying *C. dubliniensis*.

#### ACKNOWLEDGMENTS

We thank Mareike Kurz and Beate Melzer-Krick for excellent work and H.-J. Tietz for providing us with three atypical *C. albicans* strains. The help and suggestions offered by J. Schmitt in applying ANN analysis to the FT-IR spectroscopy data are particularly acknowledged.

Part of this work was supported by EC Biomed II project BMH4-97-2054.

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