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# Development of Two Species-Specific Fingerprinting Probes for Broad Computer-Assisted Epidemiological Studies of *Candida tropicalis*

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***Candida tropicalis* has emerged as the second most frequent colonizing *Candida* species, and it has been documented in nosocomial infections. To develop an effective fingerprinting system for this species, which is amenable to computer-assisted analyses and which provides information on the genetic relatedness of independent isolates, two DNA fragments, Ct3 (18,000 bp) and Ct14 (20,000 bp), were cloned from a genomic library of *Sau*3AI partial digestion products. Both probes generate complex Southern blot patterns containing 8 to 20 bands, when hybridized to *Eco*RI- or *Eco*RI-*Hae*III-digested DNA of independent *C. tropicalis* isolates. The two probes show no cross-hybridization and are both species specific for *C. tropicalis*. A comparison of the capacity of the two probes to identify the same strain in different isolates, and differentiate unrelated strains, using computer-assisted computation of similarity coefficients and the genesis of dendrograms, suggests that while Ct14 is more effective in grouping independent isolates, Ct3 is more effective in discriminating small differences in the patterns of highly related isolates and is therefore a more effective probe for determining microevolution within a clonal population and substrain shuffling in recurrent infections. Because of their alternative attributes, it is suggested that both probes be used in fingerprinting studies of *C. tropicalis*.**

*Candida tropicalis* is the second most common *Candida* species colonizing humans (9). It is responsible for a significant proportion of vulvovaginitis (10) and systemic (36) infections, and it has emerged as a major infecting agent in bone marrow transplant patients (6, 34). In cancer patients, *C. tropicalis* infections correlate with a higher proportion of deaths than other *Candida* species (7, 43). *C. tropicalis*, therefore, warrants an effective fingerprinting system which can be used to assess the genetic relatedness of nosocomial infections, the microevolution of virulent strains, the relationship between commensal and infecting strains, the specificity of emerging drug-resistant strains, and the specificity of strains to particular diseases. A fingerprinting system will be required which is amenable to computer-assisted analyses, in order to perform broad epidemiological studies and to develop databases for retrospective and comparative studies (27, 31).

Several genetic-based typing systems have been applied to *C. tropicalis*, including isoenzyme profiles (5), pulsed-field gel electrophoresis (5, 39), restriction fragment length polymorphisms (4, 5, 39), PCR-based methods (14, 19, 24, 37), and restriction enzyme-digested DNA probed in Southern blots with a moderately repetitive, species-specific probe (22, 34, 41). The last method was deemed superior to the preceding ones because it provides a pattern (34) which is complex enough to compute similarity coefficients between unrelated strains, but is simple enough to be amenable to computer-assisted analysis. However, the use of the first developed DNA probe, Ct13-8, to measure the genetic distance of independently isolated strains of *C. tropicalis* was never validated by an unrelated fingerprinting system, as has been done for the Ca3 fingerprinting probe of *Candida albicans* (16), and the probe was not adequately maintained for general distribution. Therefore, to develop an effective fingerprinting system for *C. tropicalis*, we screened a genomic library of *C. tropicalis* for two species-specific, moderately repetitive, unrelated sequences and have used them to cross-verify their effectiveness in fingerprinting *C. tropicalis*. It is demonstrated that while the Ct14 probe is more effective in grouping strains into genetically related clusters, the Ct3 probe is more effective in distinguishing microevolution within a strain. Therefore, when used together, they should provide all of the information deemed relevant in broad epidemiological studies of *Candida* spp. (15, 16, 20a, 27, 31).

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## MATERIALS AND METHODS

**Cloning the moderately repetitive sequences Ct3 and Ct14.** The method to clone moderately repetitive DNA sequences was the same one developed by Scherer and Stevens (26) to clone 27A from *C. albicans* and by Soll et al. (22, 33) to clone Ca3 from *C. albicans* and used by Girardin et al. (8) to clone a series of probes from *Aspergillus fumigatus*. In brief, a genomic library of *C. tropicalis* T26 was constructed from *Sau*3AI partial digestion products (9 to 23 kb) in phage λ EMBL3 between *Bam*HI sites according to established protocols (23). The library was amplified and plated at a density of $10^4$ recombinant phage per plate. Duplicate nitrocellulose filters were prepared from each plate and pretreated at 65°C for 20 min with a solution containing 1% bovine serum albumin, 7% sodium dodecyl sulfate (SDS), 0.5 M $NaH_2PO_4$ (pH 7.0), and 1 mM EDTA (3). One filter of each set, designated filter A, was then hybridized overnight with $10^6$ cpm of random primer-labeled ([$^{32}$P]dCTP) *Sau*3AI- and *Taq*I-digested genomic DNA of *C. tropicalis* T26 per ml. The second filter, designated filter B, was hybridized overnight with $10^6$ cpm of random primer-labeled ribosomal DNA of *C. albicans* per ml (35). Filters were then washed with a solution containing 5% SDS, 40 mM $NaH_2PO_4$, and 1 mM EDTA for 20 min and then with a solution containing 1% SDS, 40 mM $NaH_2PO_4$, and 1 mM EDTA for 20 min. The filters were finally autoradiographed. Because the combined conditions of driver DNA concentration (labeled genomic DNA) and incubation time did not result in complete hybridization of radioactive probe with λ DNA immobilized on the membrane, those λ clones harboring repetitive sequences generate a stronger signal than those λ clones harboring unique sequences and could, therefore, be discriminated. Clones on filter A which exhibited intense hybridization and had no correlate hybridization with the ribosomal probe were selected and rescreened by the same protocol described above. Clones which produced strong hybridization signals in filter A and no signal on filter B were plaque purified (18).

TABLE 1. History of *C. tropicalis* isolates used in the characterization of the cloned fingerprinting probes

| Patient | Isolate | Date of collection[a] | Body location (pathology)[b] | Place of collection[c] |
|---|---|---|---|---|
| P1 | T1 | 10/94 | Blood (neutropenic) | Holland |
| P2 | T3 | 10/93 | Anal (gynecology) | Belgium |
| P3 | T5 | 02/94 | Oral ($HIV^+$) | Germany |
| | T6 | 02/94 | Oral | |
| | T8 | 02/94 | Oral | |
| | T9 | 02/94 | Oral | |
| P4 | T12 | 10/93 | Oral ($HIV^+$) | Germany |
| P5 | T14 | 02/93 | Anal (gynecology) | Belgium |
| P6 | T16 | 10/94 | Blood (neutropenic) | Holland |
| P7 | T18 | 02/94 | Anal (gynecology) | Belgium |
| P8 | T19 | 10/94 | Blood (neutropenic) | Holland |
| P9 | T26 | 02/94 | Vagina (healthy patient) | USA |
| P10 | T360 | | | UK |
| P11 | T362 | | | UK |
| P12 | T838 | 09/05/95 | Trachea (intracranial hemorrhage) | Germany |
| | T971 | 10/13/95 | Catheter | |
| | T983 | 10/17/95 | Catheter | |
| P13 | T888 | 08/22/95 | Sputum (psoriasis) | Germany |
| P14 | T890 | 09/25/95 | Urine (urinary catheter) | Germany |
| | T988 | 10/18/95 | Urine | |
| P15 | T921 | 10/04/95 | Urine (cervical spine myelitis) | Germany |
| P16 | T925 | 10/05/95 | Urine (urinary infection) | Germany |
| P17 | T967 | 10/10/95 | Sputum (polyradiculitis) | Germany |
| P18 | T986 | 10/16/95 | Tonsil swab (chronic tonsillitis) | Germany |
| P19 | T995 | 10/17/95 | Sputum (pneumonia) | Germany |
| P20 | T1008 | 10/11/95 | Trachea (fragile endoprosthesis) | Germany |
| P21 | T1038 | 10/20/95 | Throat ($HIV^+$) | Germany |
| P22 | T1081 | 11/02/95 | Bronchial fluid (mesenteriectomy after arterial clotting) | Germany |
| | T1098 | 12/27/95 | Bronchial fluid | |
| P23 | T1087 | 11/08/95 | Throat (non-Hodgkin lymphoma) | Germany |
| | T1102 | 11/28/95 | Throat | |
| P24 | T1090 | 11/21/95 | Bronchial (lung opacity) | Germany |
| | $T31_{smooth\ (Sm)}$ | | ATCC 34139 | USA |
| | $T32_{wrinkle\ (Wr)}$ | | ATCC 34139 | USA |
| | TJ[d] | | | Belgium (Janssen Research Foundation) |
| | *aTJ*[e]: $J_1$(smooth), $J_2$(wrinkle) | | | |

[a] Given as month/year or month/day/year.
[b] $HIV^+$, human immunodeficiency virus positive.
[c] USA, United States of America; UK, United Kingdom.
[d] Strain originally isolated as a clinical sample and routinely used in screening tests at the Janssen Research Foundation, Beerse, Belgium.
[e] Substrain of the TJ isolate which displayed an atypical appearance on CHROMagar *Candida* medium, suggesting a yeast species different from *C. tropicalis*. However, sugar assimilation patterns in ID32C panels conformed perfectly with results expected for *C. tropicalis*.

**Southern blot hybridization and the analysis of fingerprinting gels.** Southern blot hybridization and the computer-assisted analysis of gel patterns were performed according to methods previously described for the probe Ca3 (12, 15, 27, 28, 31). In brief, DNA was isolated from each strain (25) and digested with either *Eco*RI (4 U/μg of DNA) or a combination of *Eco*RI and *Hae*III (2 U each per μg of DNA) for 16 h at 37°C. Digested DNA (3 or 6 μg per lane) was then electrophoresed in a 0.8% agarose gel overnight at 35 V and transferred by capillary blotting to a nylon or a nitrocellulose membrane (18). In each multilane gel, digested DNA of the reference strain T12 was run in the left and right outermost lanes, and the patterns of the outer lanes were used to unwarp gels and assess molecular weights in computer-assisted analyses. For Southern blot hybridization, probes were random primer labeled with [$^{32}$P]dCTP and incubated with membranes at $10^6$ cpm/ml (27). Prehybridization (7 h at 65°C) and hybridization (overnight at 65°C) were performed in a solution of 50 mM $NaH_2PO_4$ (pH 7.5)–50 mM EDTA–0.9 M NaCl–5% dextran sulfate–150 μg of sheared and denatured sperm DNA per ml–0.3% SDS. Membranes were washed at 45°C with a solution containing 0.3 M NaCl, 0.03 M sodium citrate (pH 7.0), and 2% SDS and then exposed to XAR-S film (Eastman Kodak Co., Rochester, N.Y.) with a Cronex Lightning-Plus intensifying screen (DuPont Co., Wilmington, Del.).

To analyze gel patterns, autoradiograms were digitized into the Dendron software package version 2.0 data file (Solltech, Iowa City, Iowa) with a Scanjet IIcx flatbed scanner (Hewlett-Packard, Palo Alto, Calif.) equipped with the transparency option. Distortions in gels were straightened ("unwarping option" of Dendron), and the lanes and bands were automatically identified. The similarity coefficient ($S_{AB}$) computation was based on band positions alone for every pair of patterns (isolates) according to the formula $S_{AB} = 2E/(2E + a + b)$, where $E$ is the number of bands in patterns A and B sharing the same position, $a$ is the number of bands in pattern A with no correlate in pattern B, and $b$ is the number of bands in pattern B with no correlate in pattern A. An $S_{AB}$ of 1.00 represented identical patterns, an $S_{AB}$ of 0.00 represented patterns with no correlate bands, and $S_{AB}$s from 0.01 to 0.99 represented patterns with increasing numbers of bands at the same positions. Dendrograms based on $S_{AB}$ values were automatically generated by the Dendron software program, based on the unweighted pair group method (29). To compare lanes visually from different gels, two or more digitized lane images were windowed, normalized to a global standard, and placed side by side (neighbored) with the appropriate Dendron software program. In the case of one gel (Fig. 6A), one overloaded lane was windowed and selectively deintensified with the processing software of Dendron.

**Strain maintenance.** The *C. tropicalis* isolates used in this study are described in Table 1. Isolates from the United Kingdom were generous gifts from Richard Barton, University of Leeds. Each isolate was maintained on a YPD agar slant (2% agarose, 2% Bacto Peptone, 1% yeast extract, 2% agar) in a capped tube. For experimental purposes, cells were transferred to a YPD agar plate, grown for 2 days at 25°C, and harvested.

In two isolates, ATCC 34139 and aTJ, more than one colony morphology (smooth and wrinkled) was observed on YPD plates. In these cases, cells of the alternative phenotypes were subcloned and individually analyzed. The clone aTJ was isolated as a variant colony of strain TJ on CHROMagar *Candida* medium (CHROMagar, Paris, France), which didn't display the blue-grey color typical of *C. tropicalis*. However, the sugar assimilation pattern of aTJ with the ID32C kit (bioMérieux, Marcy l'Etoile, France) was consistent with *C. tropicalis*.

For experiments in which pattern stability was assessed over many generations,

cells were inoculated into YPD medium in Erlenmeyer flasks at an initial concentration of $5 \times 10^4$ cells per ml and grown to stationary phase (~24 h at 25°C). Stationary-phase cells were then diluted into fresh medium, and the process was repeated. At intervals of 100 generations, cells were removed and stored on YPD slants in capped tubes.

## RESULTS

**Isolation of the moderately repetitive sequences Ct3 and Ct14.** A genomic library was constructed in λEMBL3 from a *Sau*3AI partial digest of DNA from *C. tropicalis* T26. The *Sau*3AI partial digestion products ranged between 9 and 23 kb, and the library contained five genome equivalents. Duplicate membranes prepared from the library and containing approximately 10,000 recombinants were hybridized in parallel with either radiolabeled *C. tropicalis* genomic DNA or a ribosomal DNA probe of *C. albicans*. Because the conditions for DNA hybridization with genomic DNA were performed under nonsaturating conditions, clones containing repeat sequences generated stronger signals than clones containing unique sequences. Hybridization of a duplicate blot with a ribosomal DNA probe identified ribosomal DNA clones for exclusion. Thirty clones which hybridized intensively with genomic DNA but not with the ribosomal probe were selected for further analysis. The screen was repeated to verify that each clone contained a nonribosomal repeat sequence. Each putative repetitive sequence was then labeled and hybridized to a Southern blot of *Eco*RI-digested DNA from three unrelated strains of *C. tropicalis* to assess the effectiveness of the sequence as a fingerprinting probe. The hybridization patterns had to be complex enough (i.e., contain enough bands) to provide a reasonable range of similarity coefficients between unrelated strains, but the bands had to be separated and sharp enough to be amenable to automatic detection by the Dendron program. Of the original 30 clones, 10 displayed complex banding patterns which varied among the three *C. tropicalis* strains. Of these 10 latter clones, two general patterns emerged for seven and three of the clones, respectively, suggesting that two families of repetitive sequences were represented in the collection. One representative clone from each family, Ct3 and Ct14, was selected for further analysis. The estimated sizes of Ct3 and Ct14 were 18,000 and 20,000 bp, respectively. Each generated a hybridization pattern of 8 to 20 bands when used to probe Southern blots of the *Eco*RI-digested DNA of the three *C. tropicalis* test strains.

**Unrelatedness of Ct3 and Ct14.** To demonstrate that Ct3 and Ct14 are unrelated, the respective lambda clones were digested simultaneously with *Eco*RI and *Sal*I, and the Southern blot of digested DNA was hybridized with either the Ct3 or the Ct14 probe (Fig. 1). If they are unrelated, there should be no cross-hybridization. In each case, the probe hybridized to a 9,000- and a 20,000-bp fragment, representing the arms of the lambda vector. The Ct3 probe hybridized to 10 Ct3 fragments ranging in size from 4,500 to below 1,160 bp, but to no fragments of Ct14 (Fig. 1A), and the Ct14 probe hybridized to nine Ct14 fragments ranging in size from 4,380 to below 1,125 bp, but to no fragments of Ct3 (Fig. 1B). These results demonstrate that there is no cross-hybridization between Ct3 and Ct14 and that they, therefore, represent different families of repeat elements.

**Species specificity of Ct3 and Ct14.** To test the species specificity of Ct3 and Ct14, each was used to probe the *Eco*RI-digested DNA of 13 yeast species besides *C. tropicalis*. No hybridization was observed between either probe and any of the other species, including *C. albicans*, demonstrating that both probes are species specific to *C. tropicalis* (Fig. 2).

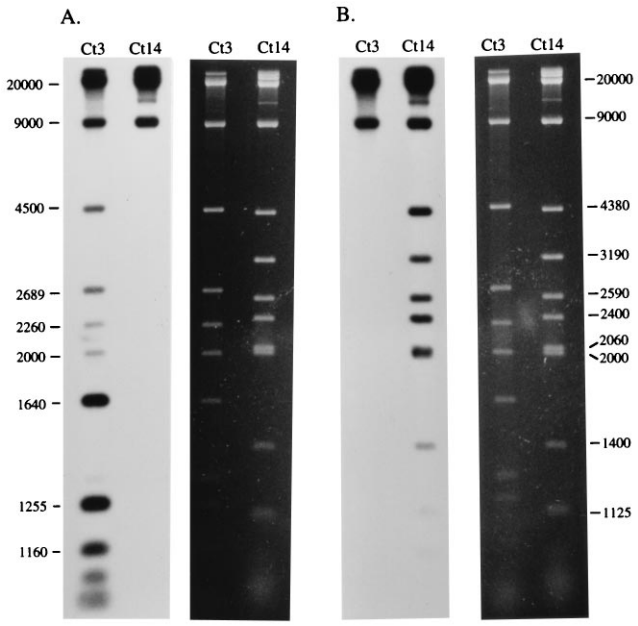


FIG. 1. Ct3 and Ct14 do not cross-hybridize. *Eco*RI-*Sal*I-digested Ct3 and Ct14 were probed with intact Ct3 (A) or Ct14 (B) probe in Southern blots. The first image in panel A or B represents the Southern blot hybridization pattern, and the second image in panel A or B represents the ethidium bromide-stained gel for each blot. The fragments at 20,000 and 9,000 bp represent the arms of the lambda vector. The molecular sizes of the major hybridization bands are presented in base pairs on either side of panels A and B.

**The capacity of Ct3 and Ct14 to discriminate between unrelated strains and to identify the same strain in different isolates.** A primary function of a species-specific probe is to distinguish between completely unrelated strains as well as identify the same strain in different isolates (27, 31). Both probes were, therefore, tested for these capabilities through Southern blot hybridization to a variety of unrelated and related *C. tropicalis* isolates. In Fig. 3A and B, Ct3 and Ct14 were hybridized, respectively, to Southern blots containing the reference strain T12 in lanes 1, 7, and 14; switch phenotypes of the ATCC strain 34139 (T31 and T32) in lanes 2 and 3; and nine additional, unrelated isolates in lanes 4 through 6 and 8 through 13.

The Ct3 probe generated a complex pattern of between 8 and 15 bands of different intensities in the >2.0-kb range (Fig. 3A). The bands were of varying intensity, discrete, and sufficiently separated for automatic computer-assisted analysis. The Ct3 hybridization patterns of the same strain (e.g., T12 in lanes 1, 7, and 14 and T31 and T32 in lanes 2 and 3) were identical, demonstrating the usefulness of the probe in identifying the same strain in different isolates. For the 11 unrelated isolates in the blot, the patterns were significantly different (Fig. 3A). Among the 26 unrelated isolates used in this study (Table 1), 25 different Ct3 Southern blot hybridization patterns were generated. For computer-assisted analysis, it is useful, but not essential, to have in a DNA fingerprinting pattern invariant bands for removing linear and nonlinear distortions and for normalizing to a global standard (31). The 25 different patterns generated by Ct3 in the isolate collection for this study did not contain prominent invariant bands.

The Ct14 probe generated a complex pattern of between 12 and 20 bands in the >2.0-kb range (Fig. 3B). The bands were of varying intensity, discrete, and sufficiently separated for automatic computer-assisted analysis. Just as in the case of Ct3,

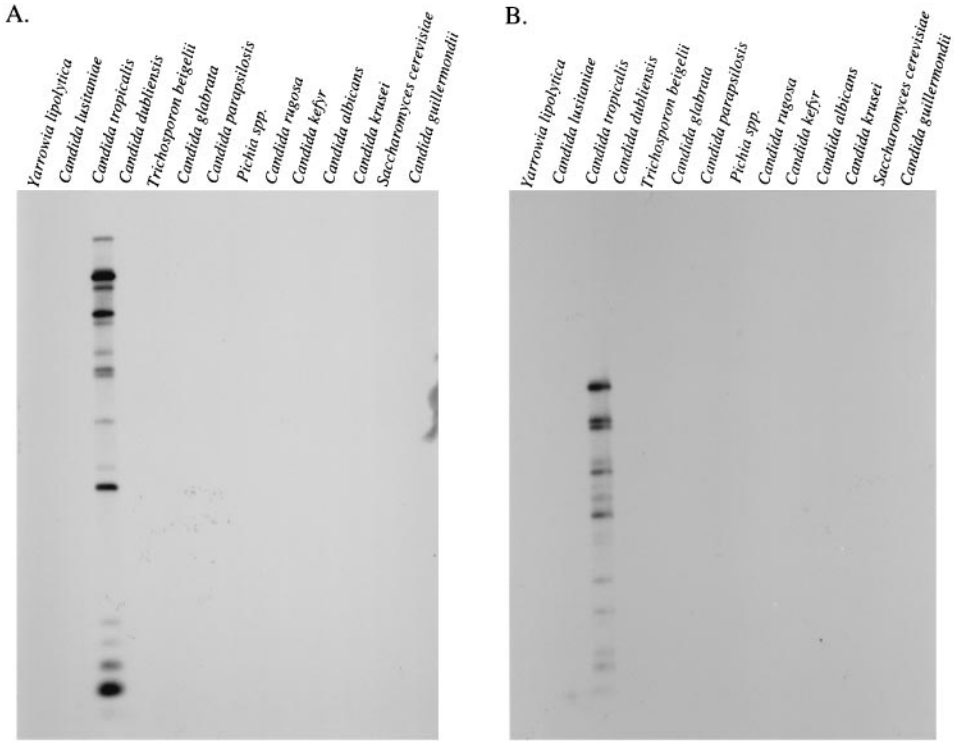


FIG. 2. Ct3 and Ct14 are specific to *C. tropicalis*. *Eco*RI-digested DNA of 14 different yeast species was probed with Ct3 (A) and Ct14 (B). As is evident, only *C. tropicalis* DNA hybridized with either probe.

Ct14 generated identical patterns for the same strain (e.g., lanes 1, 7, and 14 and lanes 2 and 3), demonstrating the usefulness of the probe in identifying the same strain in different isolates. For the 11 unrelated strains in the blot, the patterns were significantly different (Fig. 3B). Among the 26 unrelated isolates used in this study (Table 1), 21 different Ct14 Southern blot hybridization patterns were generated, indicating that the Ct14 probe is less sensitive in revealing variability than the Ct3 probe. In addition, Ct14 generated the same Southern blot hybridization pattern for several independent isolates differentiated by Ct3, demonstrating that it is less sensitive than Ct3 in identifying the same strain. For computer-assisted analysis, however, Ct14 is superior to Ct3 since it generates in its pattern three prominent invariant bands (noted with arrows in Fig. 3B) which facilitate the removal of distortions and normalization to a global standard.

**Further comparison of the strain-discriminating capabilities of Ct3 and Ct14.** It is clear from the patterns generated by Ct3 and Ct14 (Fig. 3) that both probes are capable of identifying the same strain in independent isolates, although Ct3 is more discriminating, and that both probes are capable of differentiating unrelated isolates, although again Ct3 is superior to Ct14. To test whether similarity coefficients ($S_{AB}$s) computed between the patterns generated by either probe were indicators of genetic distance, we tested whether the patterns generated by the two probes grouped nonidentical strains in a similar fashion. Dendrograms were generated from similarity coefficients ($S_{AB}$s) computed between all pairs of fingerprinted isolates, and clustering of strains by the two probes was compared (Fig. 4). In this case, $S_{AB}$s were based upon band positions alone (31). If both probes generated patterns which differed according to genetic distance, then the clustering patterns in the dendrograms of each probe should be similar under the hypothesis that *C. tropicalis* has a clonal population structure (21, 38), as has been demonstrated for *C. albicans* (21). Of the 37 fingerprinted isolates in this study, multiple isolates were derived from five individuals, four from P3, three from P12, two from P23, two from P22, and two from P14. In addition, multiple colony phenotypes were fingerprinted for strain aTJ and strain ATCC 34139.

In four of the five cases of multiple isolates derived from the same patient, and in all cases of multiple colonies from the same isolate, both Ct3 and Ct14 clustered the multiple isolates (Fig. 4A and B). In only one case, isolates T1081 and T1098 from patient P22, which were isolated 2 months apart from the same body location (Table 1), the isolates were found to be unrelated with both probes (Fig. 4A and B). The variability between the Ct3 hybridization patterns of independent isolates was greater than the Ct14 hybridization patterns, and this was evident in the average $S_{AB}$ for each probe. While the average $S_{AB}$ for the entire collection of isolates fingerprinted with the Ct3 probe was $0.60 \pm 0.15$, that with the Ct14 probe was $0.77 \pm 0.11$ (Fig. 4). However, each probe separated a comparable major cluster of isolates, referred to as group I isolates. One hundred percent of the independent isolates (i.e., from different patients) of the Ct3 group I cluster were present in the Ct14 group I cluster, while 76% of the independent isolates of the Ct14 group I cluster were present in the Ct3 group I cluster (Fig. 4). Although both probes clustered similar group I isolates, Ct3 was clearly more sensitive in discriminating between highly related strains. While Ct14 could not discriminate between T838, T983, T971, T1008, T1038, T986, T888, T988, and T890, Ct3 discriminated small differences between most of them.

A second large cluster, group II, was evident in the Ct14 dendrogram. This general cluster had a lower average $S_{AB}$

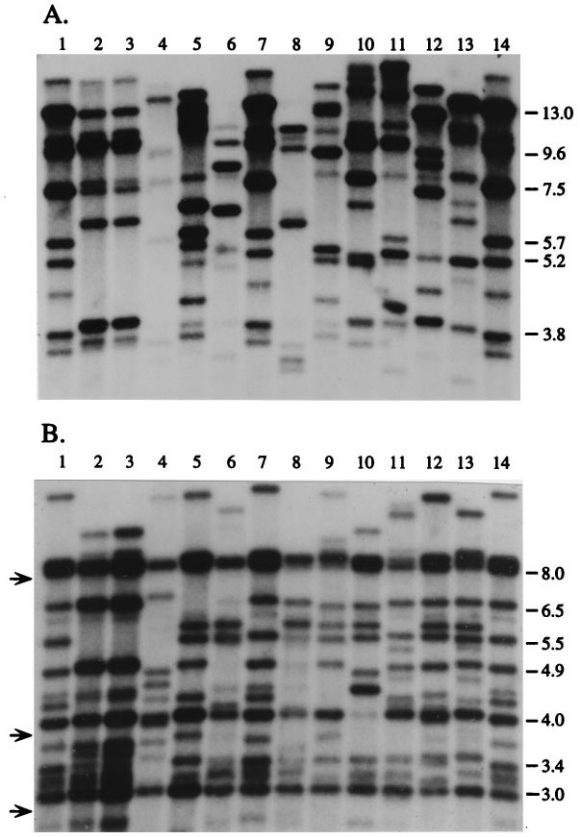


FIG. 3. Southern blot hybridization patterns of *Eco*RI-digested DNA from a variety of *C. tropicalis* isolates and either the Ct3 (A) or Ct14 (B) probe. Lanes 1, 7, and 14, reference strain T12; lane 2, smooth phenotype of ATCC 34139; lane 3, wrinkled phenotype of ATCC 34139; lane 4, T362; lane 5, T360; lane 6, T925; lane 8, T1081; lane 9, T1090; lane 10, T26; lane 11, T1; lane 12, T14; lane 13, T13. The origins of these isolates are reviewed in Table 1. Key molecular sizes are presented in kilobases to the right of each panel. Arrows to the left of panel B represent prominent invariant bands in the Ct14 pattern.

than the group I cluster and included a majority of isolates in two minor clusters in the Ct3 dendrogram, group IIa and group IIb. One hundred percent of individual group IIa isolates and 84% of individual group IIb isolates in the Ct3 dendrogram were present in the Ct14 group II cluster (Fig. 4). These results suggest that both probes are effective in discriminating genetic distance, and that while Ct3 is more sensitive in discriminating between related isolates, Ct14 is more effective in separating strains into general groups.

**Stability of the Ct3 and Ct14 patterns.** For a fingerprinting probe to be effective in identifying the same strain in independent isolates and in discriminating between different strains in independent isolates, the pattern it generates must be stable within a strain over many generations. To test for this stability, T31, T8, and T5 were grown over 600 generations, and isolates were removed and stored after 0, 100, 200, 300, and 600 generations. These isolates were then fingerprinted with the Ct3 (Fig. 5A) and Ct14 (Fig. 5B) probes. In each strain, both the Ct3 and the Ct14 pattern remained invariant over 600 generations, demonstrating that both probes have the necessary stability.

**Ct3 is a better indicator of microevolution within a strain than Ct14.** The capacity to discriminate microevolution within a colonizing strain has proven useful in demonstrating microevolution of *C. albicans* strains at sites of colonization over time (15), substrain specialization of *C. albicans* for anatomical locales (32), and substrain shuffling between recurrent vaginal yeast infections (16). Ct3 appears to be a far more powerful indicator of microevolution than Ct14. This is demonstrated in Fig. 6A, in which *Eco*RI-digested DNA of three isolates from patient P12, two isolates from patient P23, two isolates from patient P14, and four isolates from patient P3 was probed with Ct3. Single band differences were observed in the P12 and P3 sets (noted with dots in Fig. 6A). However, similar variability was not observed when the same isolates were probed with Ct14 (Fig. 6B). The microevolution discriminated by Ct3 within the sets of isolates from individuals P12 and P3 was better resolved when the DNA of isolates from each set was digested with a combination of *Eco*RI and *Hae*III and then probed with Ct3 (Fig. 6C). The number of band differences increased in both sets of isolates. Again, no variability was observed in the sets of isolates from P23 and P14 (Fig. 6C). To test how stable the microevolutionary changes within a colonizing strain were, *Eco*RI-*Hae*III-digested DNA from T5 and T8 (which differed originally by one major band shown in Fig. 6C) was prepared after 0, 100, 300, and 600 generations and probed with the Ct3 probe in Southern blots (Fig. 7). The patterns remained invariant through 600 generations, suggesting that the rate of microevolution of the Ct3 pattern was below one band change per 600 generations.

**Stability of the Ct3 and Ct14 patterns during phenotypic switching.** Most strains of *C. tropicalis* are capable of undergoing high-frequency phenotypic switching (34), in a fashion similar to that of *C. albicans* (30). An example is presented in Fig. 8 of the smooth white and wrinkled phenotypes in the switching repertoire of strain ATCC 34129 of *C. tropicalis*. The Ct3 and Ct14 Southern blot hybridization patterns were compared between a smooth (T31sm) and a wrinkled (T32wr) isolate of strain ATCC 34139, and a smooth (aTJ1sm) and a wrinkled (aTJ2wr) isolate of strain aTJ (Table 1). In the case of two morphologically different isolates from ATCC 34139, the Ct3 patterns and the Ct14 patterns were identical (lanes 2 and 3, Fig. 3; see also Fig. 4). In the case of the two morphologically different isolates of aTJ, the Ct14 and Ct3 patterns were also identical (Fig. 4). However, the patterns of the two switch phenotypes of the aTJ derivative of isolate TJ differed by two bands from that of the parent strain TJ.

## DISCUSSION

*C. tropicalis* is the second most frequent *Candida* species isolated from the oral cavity and the third most frequent species isolated from the anorectal region, vaginal region, and skin (20). It is also the second most frequent *Candida* species isolated from blood cultures (20) and represents a major opportunistic pathogen in immunocompromised patients (6, 34). Single strains have been responsible for documented nosocomial infections (4, 14). It is, therefore, imperative that a fingerprinting system be developed which is effective in broad epidemiological studies involving large numbers of isolates and which, therefore, is amenable to computer-assisted analysis (31). Several genetically based fingerprinting systems have been applied to *C. tropicalis*, including restriction fragment length patterns (4, 39), PCR-based methods (2, 6, 14, 17, 19, 37, 40), pulsed-field gel electrophoresis (5, 6, 39), isoenzyme profiles (5), and Southern blot hybridization with repetitive DNA probes (22, 24, 34, 40). However, in none of these studies was the fingerprinting method verified for reflecting genetic distance, and in no case was it assessed for amenability to automatic computer-assisted analysis, a requisite for broad

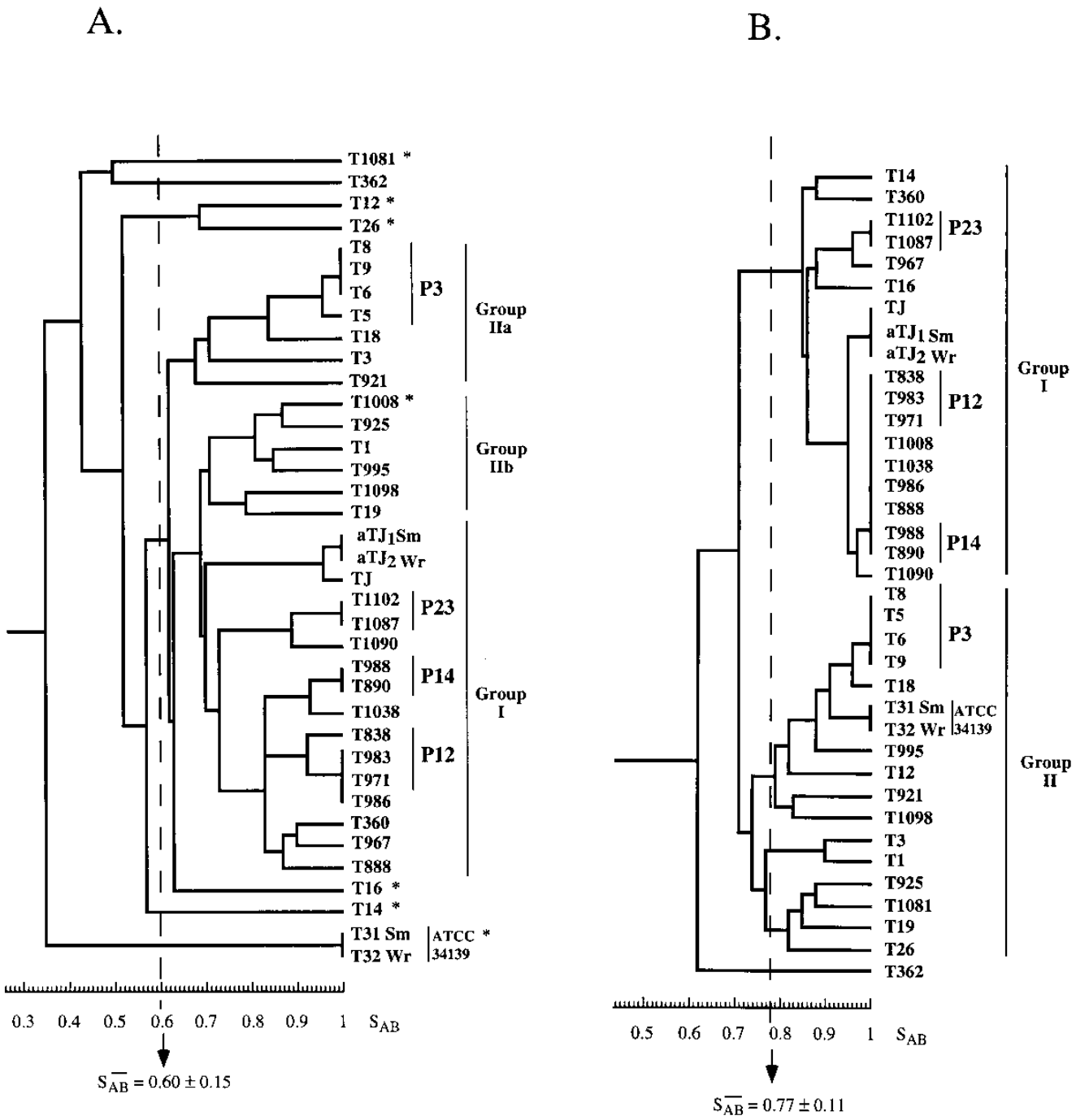


FIG. 4. Dendrograms generated from the similarity coefficients ($S_{AB}$s) computed for all possible pairs of the 37 isolates fingerprinted with the Ct3 (A) and Ct14 (B) probe. In this case, the $S_{AB}$s were computed for band position alone (see Materials and Methods). The main clusters are indicated to the right of each dendrogram (groups I, IIa, and IIb in panel A; groups I and II in panel B). The isolates noted with asterisks in panel A for Ct3 are not clustered in the same groups as in panel B for Ct14. Multiple isolates were analyzed for four patients (four from P3, three from P12, two from P23, and two from P14). These are noted with vertical bars. Isolates T31 and T32 are the smooth and wrinkled switch phenotypes, respectively, of ATCC 34139. $aTJ_1$ and $aTJ_2$ are the smooth and wrinkled switch phenotypes of aTJ, which is a substrain of TJ. See Table 1 for the history of the isolates.

epidemiological studies with the potential for continued retrospective analysis.

Because of the demonstrated reproducibility of fingerprinting patterns generated by Southern blot hybridization with nonribosomal, complex probes containing repetitive elements, and because of the amenability of the fingerprinting patterns generated by this method to computer-assisted analysis (8, 12, 16, 27, 28, 31, 32), we cloned two DNA probes which were unrelated and which fulfilled the prerequisites we have established for probes to be effective in broad epidemiological studies. We cloned two relatively complex probes which showed no cross-hybridization, which represent members of distinct re-

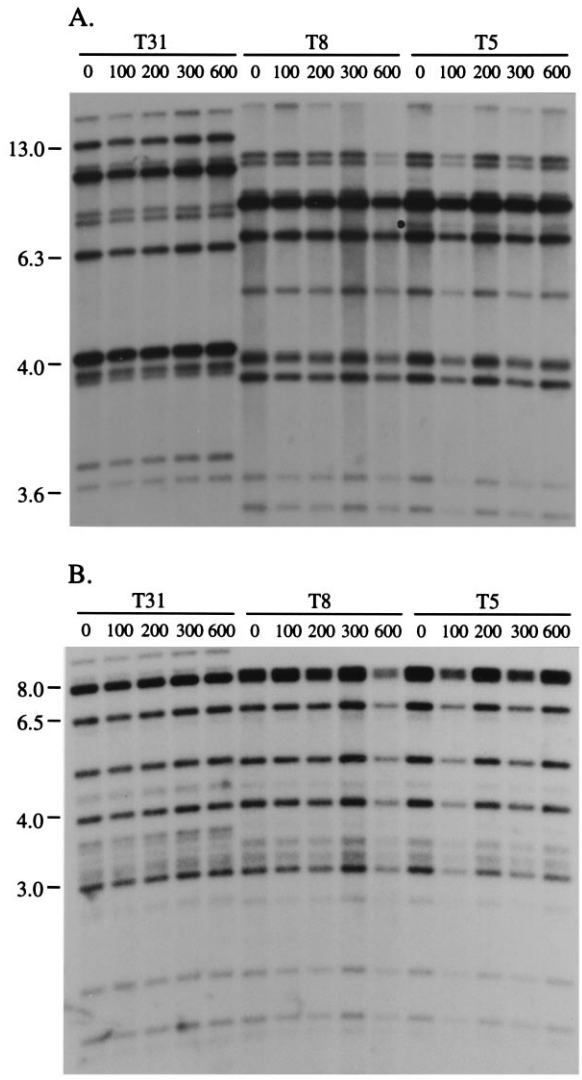


FIG. 5. The Ct3 and Ct14 Southern blot hybridization patterns are stable for 600 generations in three test isolates. *Eco*RI-digested DNA of isolates T31, T8, and T5 after 0, 100, 200, 300, and 600 generations was probed in Southern blots with either Ct3 (A) or Ct14 (B). The single band difference between T8 and T5 is noted with a dot. Note that there is no change in the pattern of each of the test isolates. Key molecular sizes in kilobases are presented at the left of each gel image.

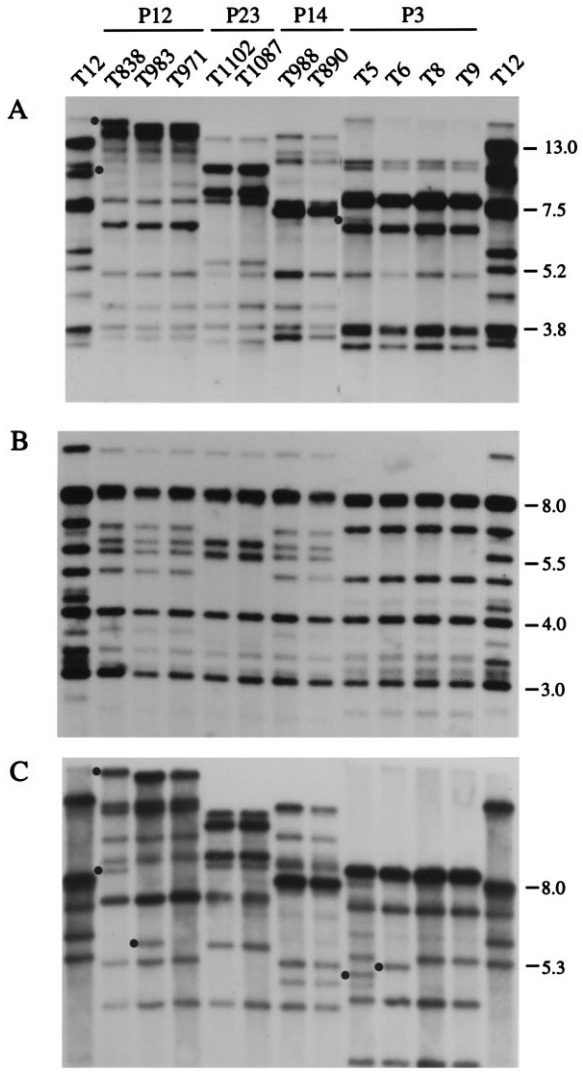


FIG. 6. Ct3, but not Ct14, identified small pattern changes in multiple isolates from the same patient. (A) *Eco*RI-digested DNA from multiple isolates of patient P12, P23, P14, and P3 is probed with Ct3. (B) *Eco*RI-digested DNA from the multiple isolates is probed with Ct14. (C) *Eco*RI-*Hae*III-digested DNA from the multiple isolates is probed with Ct3. Bands differing between isolates from the same patient are noted with dots. Reference strain 12 was run in the two outermost lanes of the gel to assist in computer-assisted unwarping and normalization to a global standard. Because of overloading of lane P3, T5, in panel A, the gel image was digitized and the particular lane was windowed and selectively disintensified with the processing software of Dendron. Key molecular sizes in kilobases are presented at the right.

petitive DNA families, and which are species specific to *C. tropicalis*. Ct3 generated a pattern of 8 and 15 hybridization bands for the set of test isolates in this study, and Ct14 generated a pattern of 12 and 20 hybridization bands. In both cases, the generated patterns differed between most unrelated isolates of *C. tropicalis*. The bands in the patterns were distinct and easily identified in digitized images by the Dendron software system. In addition, each probe generated a hybridization pattern which remained stable through 600 generations in each of three tested isolates. Both probes readily grouped identical or highly similar strains isolated from the same infected individual or known subisolates of a parental cloned isolate. More importantly, both probes grouped the tested collection of isolates into a major cluster, referred to as group I, which represented 38% of all isolates from different hosts in the case of Ct3, and 50% of all isolates in the case of Ct14. One hundred percent of the group I isolates clustered by Ct3 were in the group I cluster defined by Ct14, while 76% of the group I isolates clustered by Ct14 were in the group I cluster of Ct14. Ct14 clustered the majority of the remaining isolates into a second, less-related group, group II, while Ct3 grouped the majority of these strains into two groups, group IIa and group IIb.

Although both probes are effective in identifying and grouping isolates of the same strain, and grouping less-related isolates into similar clusters, Ct3 is more effective in discriminating differences in highly related but nonidentical isolates and in separating unrelated isolates. This is evident in the much lower average $S_{AB}$ of isolates clustered into group I by Ct3 than by Ct14. While Ct3 revealed two patterns in the P3 isolates T5, T6, T8, and T9 and two patterns in the P12 isolates T838, T983,

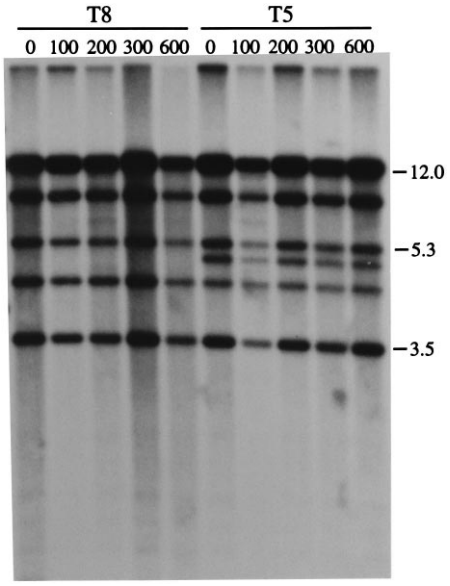


FIG. 7. The variations in bands identified by Ct3 in multiple isolates from the same patients remain stable over 600 generations. *Eco*RI-*Hae*III-digested DNA of isolates T8 and T5 after 0, 100, 200, 300, and 600 generations by one band was stable for 600 generations. Key molecular sizes in kilobases are presented to the right of the gel image.

and T791, Ct14 revealed only one pattern in each set, and while Ct3 revealed differences among the independent isolates T838, T1038, T986, T888, and T988, Ct14 generated identical patterns for the five independent isolates. In addition, while Ct3 did not group T1008 in the group I cluster, Ct14 did and could not differentiate it from T838, T1038, T986, T888, and T988. More interestingly, while Ct14 did not reveal a genetic difference between strain TJ and its CHROMagar variant aTJ, Ct3 did. The strain aTJ was isolated as a colony variant of TJ on CHROMagar and upon plating exhibited phenotypic switching between a smooth and a wrinkled colony morphology. The Ct3 pattern contained two band differences between TJ and aTJ, but no change between the switch variants, further demonstrating the effectiveness of this probe in differentiating microevolution as well as diagnosing the parent and variant as members of the same strain, when the CHROMagar biotyping system misdiagnosed them as different species.

We tentatively conclude that sequences in the genome homologous to Ct3 vary at a greater frequency, or diverge at a greater rate, than sequences homologous to Ct14. This is most evident in isolates from the same patient which exhibit minor Ct3 band differences, but no Ct14 band differences. The difference in resolution between Ct3 and Ct14 is analogous to the difference between the *C. albicans* probes Ca3 (22, 33) and CARE2 (13). While Ca3 can readily discriminate microevolution within a clonal population, CARE2 is far less discriminating for the same clonal population (16). The discriminating ability of the Ca3 probe is due to the repeat sequence in the *Eco*RI C1 subfragment (1), which has been demonstrated to contain a hypervariable repetitive element (1, 11, 15). It is quite possible that Ct3 contains an analogous repeat element. Indeed, more rapid and, in some cases, sporadic reorganization of Ct3 sequences may lead to more rapid divergence of the Ct3 fingerprint than the Ct14 fingerprint of a strain, and this may explain why strain ATCC 34139 was not placed in the group I cluster by Ct3 but was placed in the group I cluster by Ct14. Sequence analysis of the hypervariable region of Ct3 is now in progress and should reveal whether the changes in Ct3 patterns involve high rates of reorganization of hypervariable elements.

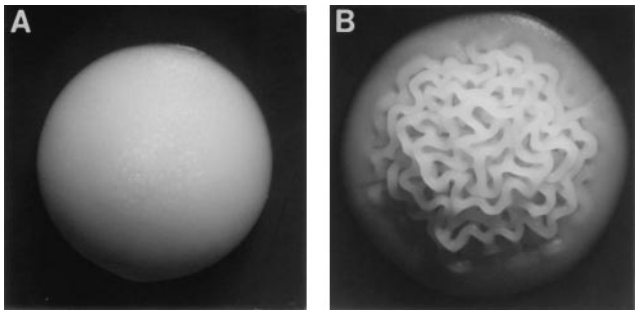


FIG. 8. The smooth (A) and wrinkled (B) colony morphologies displayed by ATCC 34139. Smooth, isolate T31; wrinkled, T32 (Table 1).

Although the discrepancies observed between the dendrograms obtained from the Ct3 and those from Ct14 patterns have been interpreted to be due to a greater rate of Ct3 sequence reorganization, they may also be due to a limited level of sexual recombination. To rule out this latter possibility, it will be necessary to assess the population structure of *C. tropicalis.*

The Ct14 probe is, therefore, more effective in grouping moderately related strains, while the Ct3 probe is more effective in analyzing the microevolution of clonal populations (28) and such differentiating phenomena as substrain shuffling (16) in recurrent or nosocomial infections. In this regard, the term "microevolution" has been used in a relative sense to mean simply rapid genomic changes in a clone with time. The fingerprint pattern generated by Ct14 is more amenable than the pattern generated by Ct3 to automated computer-assisted pattern analysis because it contains invariant bands throughout the pattern, which allow rapid removal of distortions and easy normalization to a global standard for broad epidemiological studies. However, the Ct3 patterns do have some relatively common bands which, in a multilane gel, can be used in the same manner. Alternatively, reference strain DNA could be run in an internal lane as well as the outside lanes of each gel, as we have done in the gel in Fig. 3, to facilitate computer-assisted unwarping protocols. Because of the alternative attributes of the two probes, Ct3 and Ct14, we suggest that both be used in fingerprinting studies of *C. tropicalis* in order to obtain maximum information on strain relatedness.

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