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Characterization of Genetically Distinct Subgroup of *Candida albicans* Strains Isolated from Oral Cavities of Patients Infected with Human Immunodeficiency Virus

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During the course of a study of oral *Candida albicans* strains from 60 human immunodeficiency virus-infected patients over a 2.5-year period, 18 of the 295 *C. albicans* isolates had genomes that failed to hybridize with a *C. albicans*-specific DNA probe (27A). These strains were germ tube positive and chlamydospore positive and were identified as *C. albicans* by the ID 32C test (API Systems, Montlieu, France). These strains were analyzed for the presence of two other *C. albicans*-specific DNA segments by PCR. The first was a *C. albicans* 1,348-bp species-specific sequence, and the second was a 1,059-bp *C. albicans* repetitive element. The probe 27A-hybridizing strains yielded PCR products which differed from those of the nonhybridizing strains. Five of these genetically atypical *C. albicans* strains and 98 of the *C. albicans* strains were then analyzed for purported virulence factors. The genetically atypical *C. albicans* strains, in comparison with typical *C. albicans* strains, produced greater amounts of extracellular proteinase ($P = 0.038$, Student's *t* test), adhered to a greater degree to buccal epithelial cells ($P = 0.018$, Student's *t* test), and were less susceptible to the antifungal drug 5-flucytosine ($P = 0.0003$, Mann-Whitney test). Analysis of these strains with other common antifungal drugs showed no statistically significant variation in susceptibility. The results of this study indicated that these genetically atypical *C. albicans* strains possess increased virulence in comparison with typical *C. albicans* strains.

Patients infected with the human immunodeficiency virus (HIV) may become infected with a wide variety of opportunistic pathogens. The most common infection of the oral cavity is oral candidosis caused by *Candida albicans* (8, 10, 17, 24). Oral candidosis has been postulated to act as a marker for the development of esophageal candidosis (23). When candidosis occurs in association with HIV infection it is diagnostic of category IV of that infection (7). Several investigators have suggested that some strains of *C. albicans* have a greater propensity to cause systemic, nosocomial, and superficial infections than do other strains (1, 10). Recently, several investigators have reported the isolation from HIV patients of oral *Candida* strains which show many phenotypical traits of *C. albicans* but are genetically different (20, 22). A feature of these strains is the weak hybridization signal after probing genomic DNA with a *C. albicans*-specific mid-repeat sequence (22). We have reported previously a study of oral isolates of *C. albicans* from 60 HIV-infected patients over a 2.5-year period in which a number of strains (18 of 295) that showed similar atypical genetic features were isolated (15). In the present study, we analyzed these strains for other genetic differences and for alterations in several in vitro phenotypic markers of virulence factors, namely extracellular proteinase activity, adherence to buccal epithelial cells, and susceptibility to a panel of antifungal agents.

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MATERIALS AND METHODS

In a previous study (15) of 60 HIV-infected patients' oral *C. albicans* isolates, a total of 295 samples (mean of 6.5 samples per patient) were taken at various intervals ranging from 2 to 24 months (mean of 9.3 months). In nine of these patients, 18 of their isolates had genomes which failed to hybridize with a *C. albicans*-specific DNA probe, 27A (19). Prior to the isolation of these strains, each of these patients had at some stage been taking systemic fluconazole. Each of these strains was regrown on three occasions over an 18-month period, and the species identification was repeated. This identification consisted of examining germ tube production after incubation for 2 h in horse serum, chlamydospore production on rice-agar–Tween agar (Biomerieux, Marcy l'Etoile, France), and their carbohydrate and nitrogen source assimilation patterns by using the ID 32C yeast identification system (API Systems, Montlieu, France). The strains were serotyped into group A or B by a slide agglutination test with the Iatron factor 6 rabbit antiserum (RM 302-4; Iatron Laboratories, Tokyo, Japan). All statistical analyses were performed with the Minitab (Minitab Inc., Lebanon, Pa.) statistical computer program.

Genetic analysis. *Candida* DNA was prepared as described previously (18), except that the DNA was extracted five times with phenol-chloroform (1:1) prior to precipitation in 2 volumes of ethanol.

For Southern hybridization with the 27A probe, approximately 3 μg of DNA was digested with 5 U of *Eco*RI (Boehringer, Mannheim, Germany) overnight at 37°C. The DNA fragments were separated through a 0.7% (wt/vol) agarose gel in TBE buffer (89 mM Tris base, 89 mM boric acid, 0.2 mM EDTA) for 18 h at 3 V/cm and stained with ethidium bromide before examination under UV light to ensure complete digestion was obtained. The DNA was transferred under vacuum to a positively charged nylon membrane (Boehringer) which was then incubated overnight at 42°C in a hybridization solution containing 50% (vol/vol) formamide, 0.9 M NaCl, 50 mM sodium phosphate (pH 7.4), 5 mM EDTA, 0.3% (wt/vol) sodium dodecyl sulfate (SDS), and 100 μg of denatured herring DNA per ml with a digoxigenin-labelled species-specific probe. The probe, 27A, is an *Eco*RI fragment of *C. albicans* cloned into the plasmid vector pUC18 and was kindly supplied by S. Scherer, University of Minnesota, Minneapolis. For labelling, the *Eco*RI fragment 27A was subcloned into plasmid pT7T3 (Pharmacia, Uppsala, Sweden) and a digoxigenin-labelled RNA probe was prepared from the *Sal*I-digested plasmid with T7 RNA polymerase with a labelling kit (Boehringer). After hybridization, the nylon membrane was washed twice at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% (wt/vol) SDS and twice at 50°C with 0.1× SSC–0.1% (wt/vol) SDS before detection of the probe with an alkaline phosphatase-conjugated antibody as described by the manufacturer (Boehringer).

Primers for use in the PCR were designed with two published *C. albicans*-specific sequences. The first was a *C. albicans* 1,348-bp species-specific sequence (16), and primers were designed from this sequence to give a 397-bp PCR product. The primers were designated OREN1 and OREN2, and their sequences were 5′-ATT GGT TGG GCT GGG GTC AT-3′ and 5′-GCG GGT TAG GTG GGC GAT TC-3′, respectively. The second sequence was a 1,059-bp *C. albicans* repetitive element (12), and primers were designed to give a 139-bp PCR product. The primers were designated CARE-2A and CARE-2B, and their sequences were 5′-CTC GGT CTC ATA TCC TAA AT-3′ and 5′-GAA GTA TAT TCT ACA TAC CT-3′, respectively. For PCR, approximately 100 ng of genomic DNA was amplified in a buffer supplied by the *Taq* polymerase manufacturer (Promega) in a 20-μl volume containing 1 μM primers, 2.5 mM $MgCl_2$, 1 U of *Taq* polymerase, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, and 200 μM dTTP. The reactions were performed with an automated thermal cycler (Thermal Reactor; Hybaid, Middlesex, United Kingdom). DNA samples were denatured by incubation for 4 min at 94°C before 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for samples with the OREN primers or 35 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min for samples with the CARE-2 primers. Fifteen microliters of the PCR products was then analyzed by electrophoresis through a 3% (wt/vol) agarose gel in TAE buffer (40 mM Tris-acetate, 0.2 mM EDTA) for 2 h at 10 V/cm and visualized by UV transillumination after ethidium bromide staining.

Virulence assays. A randomly selected subgroup of 103 *C. albicans* strains, which included 5 of the genetically atypical *C. albicans* strains, was analyzed by in vitro tests for virulence-associated phenotypes. The five genetically atypical strains that were included in this analysis were isolated from five different patients when the patients had a typical *C. albicans* strain which was also included in this analysis. For each strain, a single colony from a 48-h incubation on Sabouraud's agar was inoculated into 40 ml of yeast nitrogen base (Difco) supplemented with 500 mM galactose and incubated overnight at 37°C and 100 rpm (Orbital Shaker SS70; Chiltern Scientific, Auckland, N.Z.). This was centrifuged at 1,200 × *g* for 10 min, washed twice in sterile deionized water (dH_2O) by repeated centrifugation, and resuspended in dH_2O to give a final volume of 10^7 cells per ml. The concentration of each sample was assessed and standardized by the use of a Coulter counter (model S-Plus V; Coulter Electronics, Inc., Hialeah, Fla.). This standardized yeast cell suspension was used for the extracellular proteinase production assay, the buccal epithelial cell adhesion assay, and the antifungal susceptibility assays.

Extracellular proteinase production assay. For the extracellular proteinase production assay, 9 ml of GAP medium (13) was inoculated with 1 ml of each strain's standardized yeast cell suspension, resulting in an initial concentration of 10^6 yeast cells per ml. This suspension was incubated at room temperature for 7 days at 160 rpm. The tubes were vortexed to resuspend the cells, and 1 ml was filtered through previously weighed 0.8-μm-pore-size membranes (type AA; Millipore Corp., Bedford, Mass.). These membranes were incubated at 37°C for 4 h and reweighed to measure the dry weight of cells per milliliter. The tubes were then centrifuged for 30 min at 5,000 × *g*, the supernatant was passed through a 0.8-μm-pore-size membrane, and the pH was adjusted to 6.5 with 3 M NaOH. For each strain, 2 ml of the filtrate was mixed with 8 ml of bovine serum albumin solution (10 g/liter) in 0.05 M sodium citrate buffer (pH 3.2). This suspension was incubated for 30 min at 37°C before addition of 1 ml of reaction mixture to 0.5 ml of 50-g/liter trichloroacetic acid at 4°C to stop the reaction. These solutions were left at 4°C for at least 15 min to allow for complete precipitation of the undigested albumin. A control for each strain consisted of 0.8 ml of the bovine serum albumin added to the trichloroacetic acid before precipitation, followed by the addition of 0.2 ml of that strain's filtrate after precipitation. The solutions were centrifuged at 14,000 × *g* for 15 min at 4°C, and 0.5 ml of the supernatant was analyzed by a spectrophotometer for A_{280} against a citrate-buffer blank. The difference in absorbance between the test and the control sample was divided by the dry weight of cells per milliliter to give a measurement of the degree of the extracellular proteinase activity. Each strain was tested in triplicate, and the mean of these three tests was used for further analysis.

Buccal epithelial cell adhesion assay. An aliquot of 4 ml of the standardized yeast cell suspension (10^7 cells per ml) was centrifuged at 4,000 × *g* for 10 min and resuspended in 2 ml of the buccal epithelial cell assay solution (0.05 mM KCl, 1 mM phosphate buffer [pH 6.0], 1 mM $CaCl_2$, 0.01 mM $MgSO_4 \cdot 7H_2O$). Buccal epithelial cells were prepared by scraping the cheek mucosae of 40 healthy volunteers and suspending the cells in dH_2O. The number of cells per milliliter was measured in a Neubauer chamber, and the volume was adjusted to give 10^5 cells per ml. Four milliliters of this suspension was centrifuged at 2,000 × *g* for 10 min and resuspended in 2 ml of the buccal epithelial cell assay solution. The solutions containing the buccal epithelial cells and the yeast cells were mixed and incubated at 37°C for 1 h at 100 rpm. This suspension was filtered through a 20-μm-pore-size filter to remove all nonadherent yeast cells. The cells were washed on the filter twice with 5-ml aliquots of the assay medium and resuspended in 1 ml of the assay medium. A drop of the suspension medium was mounted on a glass slide, air dried, heat fixed, and stained for 1 min with cresyl violet. Adherence was determined by the mean number of yeast cells adhering to 100 epithelial cells. Each strain was tested in triplicate, and the mean of the three tests was used for further analysis.

Antifungal susceptibility assays. A volume of the standardized yeast cell suspension was diluted in 5 ml of dH_2O to give a turbidity corresponding to 0.5 on the Macfarlane telemetric scale. This suspension was streaked onto Casitone complex agar to give a lawn culture, and the sensitivity testing was performed with a disk diffusion method with Neosensitabs (Rosco Diagnostica, Taasrup, Denmark). The plates were incubated in the dark at 37°C for 48 h, after which the diameter of the clear zone around the disk was recorded in millimeters. The antifungal agents used and the cutoff values for zone diameter corresponding with resistance (as indicated by the manufacturer [Rosco Diagnostica]) were as follows: 5-flucytosine, 22 mm; natamycin, 10 mm; amphotericin, 10 mm; nystatin, 10 mm; itraconazole, 15 mm; fluconazole, 20 mm; ketoconazole, 11 mm; clotrimoxazole, 11 mm; and miconazole, 11 mm. There is no known correlation between these in vitro disk diffusion zone diameters and the in vivo situation.

RESULTS

The 18 genetically atypical strains were identified to the species level as *C. albicans* on each of three separate occasions by germ tube and chlamydospore production. On each occasion, all 18 strains were also identified as *C. albicans* by their ID 32C profile, even though there was considerable variation of these profiles within this group. A representative sample of the ID 32C profile of these strains, the relative proximity of these strains to the taxa of the database (percentage of identity), and the strains' proximity to the most typical profile in each of the taxa (T index) as given by the manufacturer's analytical profile index (ATB 32C, 1st edition, 1987; API Systems) are given in Table 1. All 18 of the genetically atypical *C. albicans* strains were serotype A with the Iatron factor 6 antiserum (Table 1).

Figures 1, 2, and 3 show results from a Southern blot with the 27A probe, PCR products with the OREN primers, and PCR products with the CARE-2 primers, respectively. Each figure shows 11 clinical isolates and 8 type strains from either the American Type Culture Collection, the United Kingdom's National Collection of Pathogenic Fungi, or type strains of *Candida stellatoidea* (B-4465 and B-4257) provided by K. J. Kwon-Chung, National Institutes of Health, Bethesda, Md., as used in previous publications (11, 25). The strains in each of these figures are shown in the order given in Table 1.

The genomic DNA of the atypical strains extracted on each occasion failed to hybridize with the 27A probe (Fig. 1). With PCR with primers OREN1 and OREN2, all of the typical, probe 27A-hybridizing *C. albicans* strains yielded a single PCR product of approximately 390 bp whereas all of the atypical, nonhybridizing strains produced several products of various molecular weights (Fig. 2). PCR products with the primers CARE-2A and CARE-2B were of variable molecular weights for the typical strains of *C. albicans*, whereas the genetically atypical strains resulted in quite distinct or negligible PCR products (Fig. 3).

Statistical analysis of the results of the proteinase assay showed that, by comparison with the 98 typical *C. albicans* strains, the five genetically distinct *C. albicans* strains had significantly higher levels of extracellular proteinase activity (Student's *t* test, $P = 0.038$, $t = 3.05$, df = 4). Similar comparison showed that these genetically distinct *C. albicans* strains adhered to a greater degree to buccal epithelial cells (Student's *t* test, $P = 0.018$, $t = 3.24$, df = 6) and were less susceptible to the antifungal drug 5-flurocytosine (Mann-Whitney test, $P = 0.0003$, $W = 493.5$) compared with the typical *C. albicans* strains. Analysis of these strains with the other antifungal drugs tested showed no statistically significant variation in susceptibility.

DISCUSSION

The atypical strains of *C. albicans* isolated from HIV-infected patients described here appear similar to strains previ-

TABLE 1. Results of identification and serotyping of a representative selection of clinical and type culture *Candida* strains

No.	Sample no.	Source or species	ID 32[a]	% ID[b]	T index[c]	Species (API)[d]	Serotype	Hybridization[e]
1	ATCC 90029	*C. albicans*	7347 3400	99.9	0.99	*C. albicans*	A	Positive
2	ATCC 90028	*C. albicans*	7347 3400	99.9	0.99	*C. albicans*	B	Positive
3	93-3084	Clinic	71473400	99.5	0.78	*C. albicans*	B	Positive
4	B-4465[a]	*C. stellatoidea*	3142 3000	99.9	0.94	*C. albicans* 2	B	Positive
5	B-4257[a]	*C. stellatoidea*	3142 3000	99.9	0.94	*C. albicans* 2	B	Positive
6	NCPF 3108[f]	*C. stellatoidea*	7143 3400	27.6	0.52	*C. albicans*	A	Negative
7	ATCC 90030	*C. glabrata*[g]	0001 0102	99.9	0.87	*C. glabrata*	A	Negative
8	ATCC 90018	*C. parapsilosis*	5547 3507	99.9	0.99	*C. parapsilosis*	B	Negative
9	NCPF 3234[f]	*C. kyfer*	7220 3000	99.9	0.92	*C. kyfer*	B	Negative
10	1-169	Clinic	7347 3400	99.9	0.99	*C. albicans*	A	Negative
11	1-136	Clinic	7143 3400	27.6	0.52	*C. albicans*	A	Negative
12	4-424	Clinic	71473400	99.5	0.78	*C. albicans*	A	Negative
13	30-166	Clinic	71473400	99.5	0.78	*C. albicans*	A	Negative
14	38-549	Clinic	7143 3400	27.6	0.52	*C. albicans*	A	Negative
15	5-221	Clinic	71473400	99.5	0.78	*C. albicans*	A	Negative
16	4 24	Clinic	7347 3400	99.9	0.99	*C. albicans*	B	Positive
17	93-4480	Clinic	7347 3400	99.9	0.99	*C. albicans*	A	Positive
18	89-10849	Clinic	71473400	99.5	0.78	*C. albicans*	A	Positive
19	39-553	Clinic	7347 3400	99.9	0.99	*C. albicans*	A	Positive

[a] Type strains donated by K. J. Kwon-Chung, National Institutes of Health.
[b] Strain's relative proximity to the different taxa (ATB 32C, 1st edition; API Systems).
[c] Proximity of the strain to the most typical profile in each of the taxa (ATB 32C, 1st edition; API Systems).
[d] Measured by the strain's reactivity with the Iatron factor 6 antiserum.
[e] Hybridization of the strain's DNA to the probe 27A.
[f] NCPF, British National Collection of Pathogenic Fungi.
[g] *C.* (*Torulopsis*) *glabrata*.

ously described by Sullivan et al. (22). The type culture strain NCPF 3108 used in the genetic analysis in the present study is the same as that used by Sullivan et al. (22), and the results of the analysis for this strain are identical to those for the genetically atypical strains isolated in the present study. The strains supplied by K. J. Kwon-Chung (B-4465 and B-4257) are the same as those previously used in earlier studies of *C. stellatoidea* (11, 25). These *C. stellatoidea* strains gave results identical to those for the typical clinical strains of *C. albicans* and the American Type Culture Collection *C. albicans* type culture strains for all genetic analysis used in this study. It would appear that the nonhybridizing strains isolated in the present study represent a genetically distinct subgroup of *C. albicans* strains. The variation in the ID 32C profile of this subgroup would indicate that it is not a heterogeneous group and does not constitute a single strain.

The absence of hybridization with the 27A probe reported here compared with the reduction in hybridization signal previously reported (11, 20, 22) may be due to the variation in techniques used. In the present study, an RNA probe which

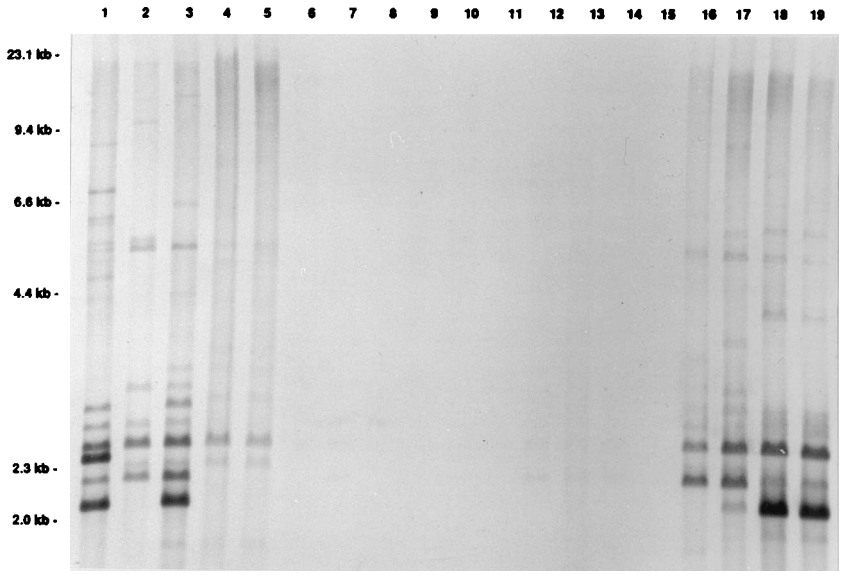

FIG. 1. Southern blot hybridization of *Eco*RI-digested *Candida* DNA with the probe 27A. The order of the isolates is given as listed in Table 1. The migration of DNA molecular size markers is indicated in kilobase pairs.

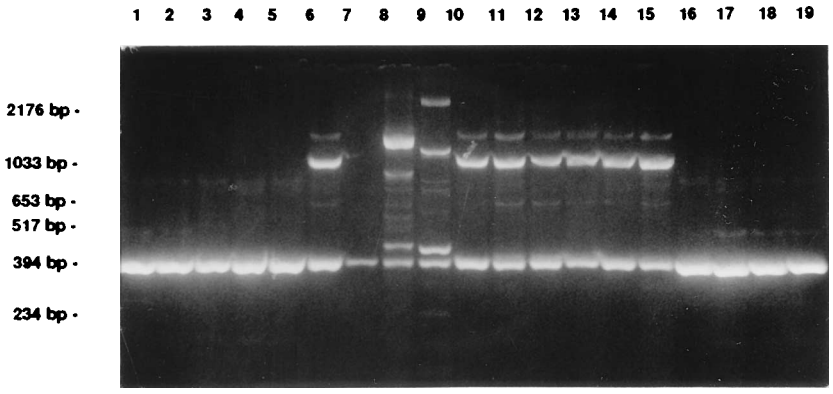

FIG. 2. UV transillumination of ethidium bromide-stained PCR products with the OREN1 and OREN2 primers. The order of the isolates is given as listed in Table 1. The migration of DNA molecular size markers is indicated in base pairs.

was synthesized as a large single strand of nucleic acid was used. In the previous studies, probes prepared by random-primer labelling were used which result in smaller fragments. With a large probe size, regions of little homology between probe and target will affect the hybridization to a greater extent than smaller regions, which have complete homology with the target DNA. This may explain the absence of hybridization seen in the present study compared with decreased hybridization reported in the previous studies. The use of the type strains in the present study shows that this variation is due to differences in technique rather than any inherent differences in the strains.

The variation in the PCR products amplified from the typical and atypical *C. albicans* strains with both sets of primers (OREN1 and OREN2 and CARE-2A and CARE-2b) confirms the genetic differences between these isolates. These primers were designed from two previously published sequences (12, 16). The variation in PCR products and the identical result with the type strain NCPF 3108 but not with other type strains add further evidence to the genetic distinctiveness of this subgroup of *C. albicans* strains.

Several previous investigators have found a correlation between extracellular proteinase activity, adherence to mucosal cells, and pathogenicity (2–6, 9, 21). The results of the present study show that the genetically distinct subgroup of *C. albicans* strains have a significantly higher proteinase activity and a greater adherence to buccal epithelial cells than do typical *C. albicans* strains. Wickes et al. (25) have shown that sucrose-positive type 1 *C. stellatoidea* strains have a 16% increase in their growth rate and a significant increase in in vitro virulence in mice compared with sucrose-negative type 1 *C. stellatoidea* strains. They have postulated that the virulence of the sucrose-positive *C. stellatoidea* strains is midway between the less-virulent sucrose-negative *C. stellatoidea* and *C. albicans* strains. The results presented here of an increase in in vitro virulence with the extracellular proteinase production and buccal epithelial cell adhesion assays indicated that these genetically distinct *C. albicans* strains were more virulent than *C. albicans*. The increase in these in vitro virulence factors may equate with an increase in the ability of these genetically distinct *C. albicans* strains to persist in vivo during conditions of stress. Further prospective research is required to assess the behavior of these strains in vivo.

The decrease in susceptibility of the genetically distinct subgroup of *C. albicans* strains to 5-flucytosine, but not to any of the other antifungal agents tested, was a significant finding, particularly considering that these strains were all serotype A. At the time these genetically atypical strains were isolated, all patients had been treated with fluconazole previously. The unchanged resistance to fluconazole may indicate that these stains are not in direct competition with the predominant oral strain but rather are derived from *C. albicans* during environmental stress, as previously postulated (25). It may be that, in the future, the widespread use of fluconazole will result in the increased isolation of this genetically distinct subgroup of *C.*

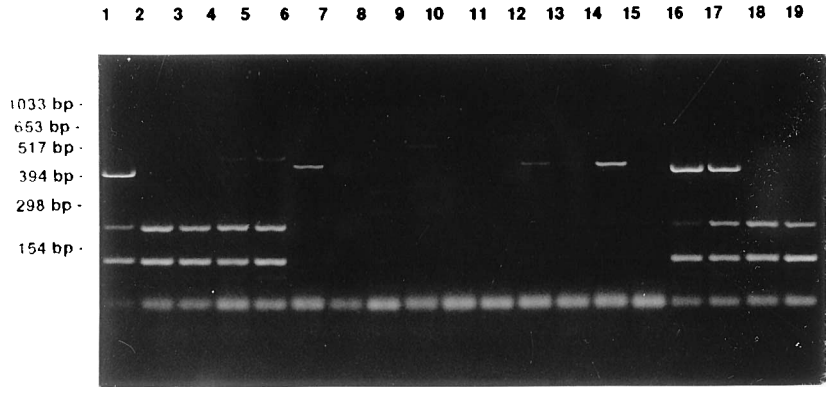

FIG. 3. UV transillumination of ethidium bromide-stained PCR products with the CARE-2A and CARE-2B primers. The order of the isolates is given as listed in Table 1. The migration of DNA molecular size markers is indicated in base pairs.

albicans strains from HIV patients and hence be the causative agent for debilitating esophageal and systemic infections.

The type strain NCPF 3108 used in the present study is unusual in that it is documented as being *C. stellatoidea*, while it is sucrose positive and serotype A. Sullivan et al. (22) have postulated that this strain and their genetically atypical clinical isolates were sucrose-positive variants of type 1 *C. stellatoidea*. On the other hand, Mahrous et al. (14) in a study of the genetic relatedness of *C. albicans* and several isolates of *C. stellatoidea* have concluded that it is not possible to separate these strains and support discarding the name *C. stellatoidea*. Furthermore, the strains of *C. stellatoidea* obtained from K. J. Kwon-Chung used in the present study showed the same genetic features as the type strains and clinical isolates of *C. albicans*. This finding supports the contention that *C. stellatoidea* falls within the range of variability of the species *C. albicans*. The results of the present study show the existence of a genetically distinct subgroup of *C. albicans* which has an increased in vitro virulence compared with the typical *C. albicans* isolates. Whether this subgroup is a different species requires further investigation. The identification of these organisms, however, by routine laboratory procedures is not possible at present because it requires sophisticated and time-consuming genetic analysis.

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