INFECTION AND IMMUNITY, Feb. 1989, p. 527–532
0019-9567/89/020527-06$02.00/0

Vol. 57, No. 2

Genetic Differences between Type I and Type II *Candida stellatoidea*

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Received 25 July 1988/Accepted 2 November 1988

Genetic similarities and differences between type I and type II *Candida stellatoidea* were studied. The electrophoretic karyotype, mitochondrial DNA (mtDNA) restriction patterns, and midrepeat sequence of nuclear DNA in type I *C. stellatoidea* were clearly distinguishable from those of a reference culture of *Candida albicans*. The karyotype and the major bands of the midrepeat sequence of type II *C. stellatoidea* were indistinguishable from those of the reference *C. albicans*. The mtDNA restriction patterns of four type I isolates were homogeneous regardless of the endonucleases and probes used. The mtDNA restriction patterns of type II *C. stellatoidea* varied from strain to strain. Some of them were identical to that of *C. albicans*, while others were the same as that of type I *C. stellatoidea*. Immunofluorescence with *C. albicans* serotype A-specific monoclonal antibody indicated that the four isolates of type I *C. stellatoidea* were serotype B (non-A), whereas all three type II isolates studied were serotype A. Taken together, these results support the hypothesis that the isolates of *C. stellatoidea* type II studied are sucrose-negative mutants of serotype A *C. albicans*. Since *C. stellatoidea* type I differs from *C. albicans* in several major genetic characteristics, it cannot be viewed as a simple mutant derived from *C. albicans*. Hybrids produced by protoplast fusion of type I and type II cells were capable of assimilating sucrose, indicating that the sucrose-negative phenotypes of the parents are due to different mutations.

In clinical laboratories, *Candida stellatoidea* is differentiated from *Candida albicans* by its lack of sucrose assimilation. When the high DNA homology between the two yeasts was discovered, such a phenotypic difference was no longer considered to be sufficiently significant to warrant classifying these yeasts as separate species (11, 12). Kwon-Chung et al. (8) recently found two distinct types among the isolates of *C. stellatoidea*. The two types are distinguishable on the bases of their electrophoretic karyotypes, virulence for mice, resistance to UV irradiation, growth rate in vitro, and proteinase activity on bovine serum albumin agar at pH 3.8.

In this paper, we report further genetic and antigenic differences between the two types of *C. stellatoidea* and discuss their relationship to *C. albicans*.

MATERIALS AND METHODS

Isolates. Four isolates of type I *C. stellatoidea* (B-4252 [ATCC 11006], B-4257 [ATCC 36232], B-4404 [SR 1145], and B-4406 [SR 1146]) and three isolates of type II *C. stellatoidea* (B-4365 [ATCC 20408], B-4403 [SR 1114], and B-4405 [SR 1144]) were either from the American Type Culture Collection, Rockville, Md., or from the collection of W. S. Riggsby. Isolate CA329 was a clonal culture derived from ATCC 36232 which was received from Glenn Roberts at the Mayo Clinic. The isolate of *C. albicans*, B-4201, previously designated C9 (7), was used as a reference strain for two reasons: the growth characteristics and pathogenicity were well characterized, and the orthogonal-field alteration gel electrophoresis (OFAGE) karyotype was identical to that of B-311, a widely used reference culture (10), as well as to those of 18 other *C. albicans* isolates tested in our laboratory, including nine isolates each of serotypes A and B.

OFAGE. An OFAGE apparatus with the specifications of Carle and Olson (2) was used. Chromosome-sized DNA was prepared by the embedded-agarose procedure of Schwartz and Cantor as modified by Merz et al. (10). OFAGE was performed as described by Carle and Olson (2). The circulating buffer was 0.5× TBE (44 mM Tris–44 mM boric acid–2 mM EDTA [pH 8.3]) maintained at 10°C, and electrophoresis was performed with 1.0% agarose in 0.5× TBE with 5-min alternating pulses of 120 V for 42 h. For visualization of DNA, gels were stained with ethidium bromide.

Southern hybridization. DNA was isolated from cells of mid- to late-exponential growth phase by the methods of Overhauser and Radic (14) or Cryer et al. (3). DNA from each isolate was cut with restriction endonucleases purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., or from New England BioLabs, Inc., Beverly, Mass., according to the instructions of the manufacturer.

For the study of mitochondrial DNA (mtDNA) restriction patterns, digested DNA was electrophoresed in 1% agarose gels at 25 V for 18 to 20 h. DNA was transferred from the gels to nitrocellulose paper, and the Southern blots were hybridized and washed as described by Maniatis et al. (9). Template DNAs for the nick-translated probes were *Eco*RI fragments E1 to E6 of mtDNA of *C. albicans* (21).

To study the hybridization pattern with a cloned midrepeat sequence of *C. albicans*, endonuclease-digested DNA was electrophoresed in 0.7% agarose gels. The DNA fragments were transferred to a GeneScreen Plus hybridization membrane (New England Nuclear Corp., Boston, Mass.). Southern blots were hybridized and washed as described by

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Soll et al. (17). The midrepeat sequence of *C. albicans* used as a probe was λCa3, a recombinant bacteriophage containing four contiguous *Eco*RI fragments obtained from *C. albicans* 3153A (J. B. Hicks, M. J. McEachern, E. Bulgac, J. Schmid, and D. R. Soll, submitted for publication).

Complementation. Two isolates of type I (CA329 and B-4252) and one of type II (B-4365) were subjected to mild UV mutagenesis (480 erg/mm^2) to obtain auxotrophic parent strains for hybridization. The auxotrophic strains obtained were CA329*pro*, B-4252*met*, and B-4365*ade*. Crosses were performed according to established methodology (18). Hybrids were obtained by selecting for growth on minimal medium (yeast nitrogen base without amino acids) with 2.0% glucose. After incubation for 7 to 11 days at 25°C, prototrophic colonies were isolated and the cells were plated on glucose minimal medium to purify them by clonal isolation. Prototrophic colonies composed of cells with approximately twice the volume of the parental cells were considered hybrids. The hybrid cells were tested for their ability to utilize sucrose by being plated on minimal medium with sucrose as the sole carbon source. Incubation was at 30°C for 3 days. Only hybrids that formed colonies within 3 days and similar in size to those grown on glucose minimal medium were considered sucrose positive. These were readily distinguished from sucrose-negative hybrids, which formed full-sized (>2 mm) colonies on glucose medium by 3 days but formed only pinpoint-sized colonies on sucrose medium.

Serotyping. All seven isolates of *C. stellatoidea* and *C. albicans* B-4201 were tested with two monoclonal antibodies by an immunofluorescence assay. Murine monoclonal antibody CB6 is an immunoglobulin M that reacts with *C. albicans* serotype A and with *C. tropicalis* but not with *C. albicans* serotype B (15). Monoclonal antibody AC3 is an immunoglobulin M that reacts with all of the cultures described above. Monoclonal antibody CB6 was conjugated to fluorescein isothiocyanate and was used in a direct immunofluorescence assay. The reaction of the cultures with AC3 was judged by an indirect immunofluorescence assay with a goat anti-mouse immunoglobulin M-fluorescein conjugate (Organon Teknika, Malvern, Pa.) (6). Immunofluorescence was obtained with a microscope fitted with a blue excluding BG38 filter, a CP490 exciter filter, and a blau-abs ocular filter and equipped with an HBO 200-W mercury vapor lamp. The cultures which gave bright fluorescence reactions with CB6 and AC3 were judged to be serotype A. The cultures which reacted negatively with CB6 but positively with AC3 were considered to be serotype B. As the positive controls for *C. albicans* serotypes A and B, isolates 65 (A) and Lecocq (B), respectively, were used. *C. parapsilosis* B-390 was used as the negative control.

RESULTS

Electrophoretic karyotype. Figure 1 shows DNA banding patterns of seven isolates of *C. stellatoidea* and one isolate of *C. albicans*. Clear differences between type I and type II isolates were seen in the sizes of the bands. Except for one isolate of type I (lane 4, B-4406), all isolates showed six to seven bands. Isolate B-4406 showed eight bands. Of the six to seven bands, only one large band and two to three small bands were of similar sizes in type I and type II isolates.

mtDNA restriction patterns. The DNAs of type I and type II isolates digested with various restriction enzymes and probed with mtDNA fragments (E1 to E6) of *C. albicans* were significantly different. All four isolates of type I showed

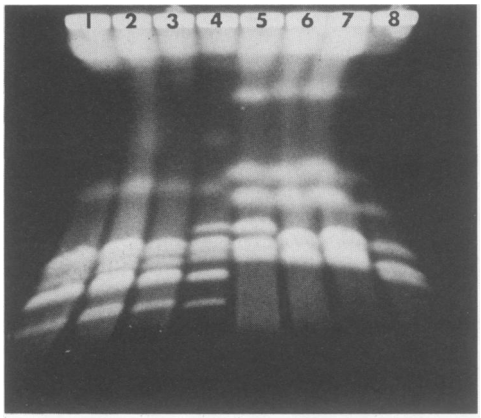

FIG. 1. Chromosome-sized DNA banding patterns of seven isolates of *C. stellatoidea* and *C. albicans* on OFAGE. Lanes: 1 to 4, *C. stellatoidea* type I (B-4252, B-4257, B-4404, and B-4406, respectively); 5 to 7, *C. stellatoidea* type II (B-4365, B-4403, and B-4405, respectively); 8, *C. albicans* B-4201.

the same restriction patterns with every combination of enzyme and probe that was used, and these patterns were always clearly different from the typical patterns found in *C. albicans* (Fig. 2). The restriction patterns in the isolates of type II, however, were heterogeneous. The DNA digested with *Alu*I and probed with the E1 fragment showed that all three isolates of type II had mtDNA patterns identical to that of *C. albicans* B-4201 but different from that of type I (Fig. 2A). The hybridization results when DNA was cut with *Msp*I and probed with E4, however, showed two patterns among the three type II isolates. The fragment patterns of B-4405 were identical to that of *C. albicans*, but the fragment patterns from isolates B-4365 and B-4403 were identical to that of the type I isolates (Fig. 2B). The DNA digested with

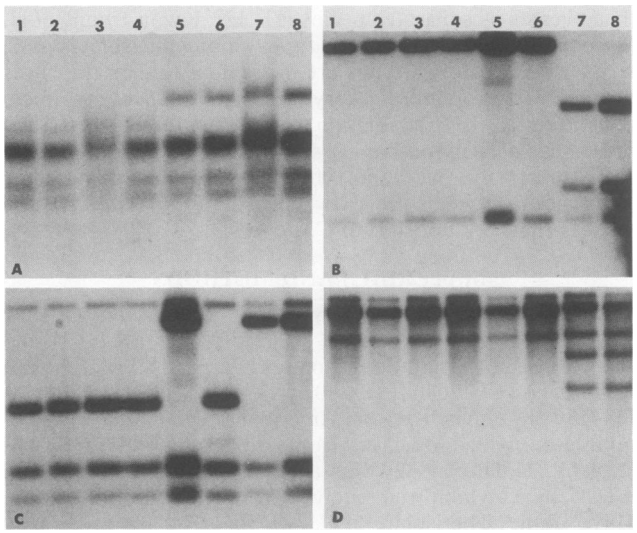

FIG. 2. mtDNA restriction patterns of *C. stellatoidea* type I and type II. Total DNA was cleaved with restriction nucleases and was probed with labeled mtDNA fragments of *C. albicans* (see Materials and Methods) as follows: *Alu*I-E1 (A), *Msp*I-E4 (B), *Msp*I-E5 (C), and *Kpn*I-E1 to -E6 (D). Lanes: 1 to 4, *C. stellatoidea* type I (B-4252, B-4257, B-4404, and B-4406, respectively); 5 to 7, *C. stellatoidea* type II (B-4365, B-4403, and B-4405, respectively); 8, *C. albicans* B-4201.

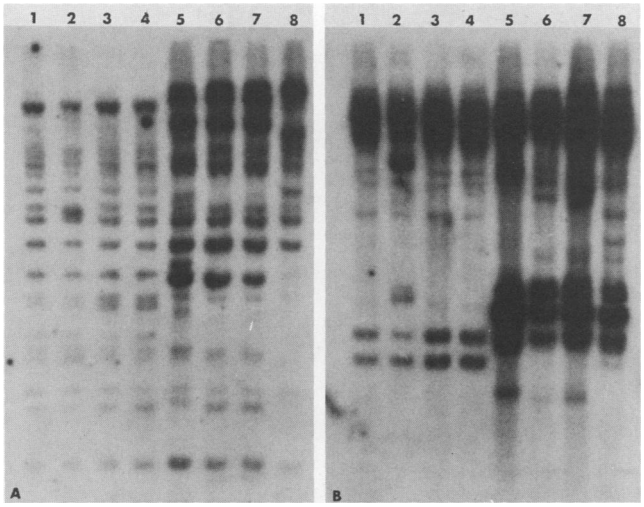

FIG. 3. Hybridization of λCa3 to *C. stellatoidea* types I and II and *C. albicans*. Filters A and B have *Eco*RI and *Cla*I digests, respectively, of *C. stellatoidea* type I (lanes 1 to 4), *C. stellatoidea* type II (lanes 5 to 7), and *C. albicans* B-4201 (lane 8). The sequence of the isolates is identical to that described in the legend to Fig. 2.

*Msp*I and probed with E5 showed that the mtDNA restriction patterns of B-4365 and B-4405 were identical to that of *C. albicans*, while the mtDNA restriction pattern of B-4403 was the same as that of type I *C. stellatoidea* (Fig. 2C). The DNA digested with *Kpn*I and probed simultaneously with all six fragments, E1 to E6, showed that the patterns of B-4365 and B-4403 were identical to that of type I isolates, while isolate B-4405 gave the same pattern as did *C. albicans* (Fig. 2D). In addition to the patterns shown in Fig. 2, mtDNA fragment patterns of type I isolates were identical when tested with enzyme-probe combinations of *Msp*I-E1, *Hae*III-E2, and *Ara*I-E1 to -E6.

Midrepeat sequence. To further evaluate the genetic relatedness between *C. albicans* and the two types of *C. stellatoidea*, total DNA of the eight cultures was digested with *Eco*RI or *Cla*I, separated on agarose gels, Southern blotted, and probed with λCa3. λCa3 is a recombinant λ bacteriophage containing a midrepeat DNA sequence, Ca3, isolated from *C. albicans* 3153A (Hicks et al., submitted). This sequence hybridizes to multiple restriction fragments of *C. albicans* but only weakly to DNA fragments of other yeasts such as *C. tropicalis*, *C. krusei*, *C. glabrata*, *C. guilliermondii*, or *Saccharomyces cerevisiae*.

Figure 3A shows hybridization of λCa3 to the *Eco*RI digests. DNA from all isolates showed multiple bands. Remarkable similarity within each type of *C. stellatoidea* was observed, and the patterns of type I isolates were clearly distinguishable from those of type II isolates. Type I showed much less hybridization, although the amounts of DNA in the lanes were the same. The band pattern of *C. albicans* B-4201 was different from that of *C. stellatoidea* in detail, but the intensity of the bands was similar to that of the bands of type II isolates.

Figure 3B shows hybridization of λCa3 to the *Cla*I digests. The band patterns of type I *C. stellatoidea* were clearly different from those of type II. The major band patterns of type II, however, were similar to that of *C. albicans*.

Complementation. The hybrids formed by crossing two type I isolates (CA329*pro* and B-4252*met*) with a type II isolate (B-4365*ade*) were cultured on sucrose minimal medium. Fifteen randomly picked hybrid clones resulted from CA329*pro* × B-4365*ade*, and 10 of 11 hybrids randomly isolated from the cross B-4252*met* × B-4365*ade* grew well on sucrose minimal medium. These results were taken to mean that the recessive sucrose-negative phenotype of the type I isolates was determined by a mutation different from that responsible for the recessive sucrose-negative phenotype of the type II isolate.

Thirteen randomly isolated hybrids from the cross of two type I isolates (B-4252*met* × CA329*pro*) did not show efficient growth on sucrose minimal medium, although they grew well on glucose minimal medium. This result indicated that the type I isolates studied were mutated in the same gene required for efficient sucrose utilization. Independent evidence for hybridization was sought by comparing putative hybrids with parents in terms of cell size in stationary-phase cultures (see Materials and Methods). All hybrids tabulated were confirmed by showing that stationary-phase cells were distributed over a wide range of sizes that was considerably larger than the range of sizes of parents (Fig. 4). Four of the hybrids (CA329*pro* × B-4365*ade*) were randomly selected and tested for their karyotypes. The bands seen in these hybrids were the sum of the bands seen in the parent isolates (Fig. 5).

When proteinase activity was assessed on bovine serum albumin agar (pH 3.8) by an established method (13), it was found that the hybrid formed by fusing type I isolate B-4252*met* with type II isolate B-4365*ade* produced a strong proteinase reaction similar to that exhibited by type I isolates in a previous study (8).

Serotyping. Table 1 shows the results of serotyping. All of the type II isolates and *C. albicans* serotype A control isolate CDC 65 reacted with both monoclonal antibodies CB6 and AC3. Type I isolates, *C. albicans* B-4201, and *C. albicans* serotype B control isolate Lecocq reacted with AC3 but not with CB6. *C. parapsilosis*, the negative control, reacted with neither CB6 nor AC3. These results indicated that type I isolates and B-4201 are serotype B (non-serotype A), while those of type II are serotype A.

DISCUSSION

The enzyme of *C. albicans* responsible for the hydrolysis of sucrose is different from the invertase of *S. cerevisiae*, which also hydrolyzes the trisaccharide raffinose. *C. albicans*, which can utilize sucrose as a carbon source for growth, does not grow on raffinose, and it is assumed that the enzyme which hydrolyzes sucrose does not hydrolyze raffinose in *C. albicans*. Although studies were not done on the sucrose-hydrolyzing enzyme(s) of *C. albicans*, some authors considered *C. stellatoidea* an α-glucosidase mutant of *C. albicans* (1). We consider that the term α-glucosidase is not sufficiently specific because it does not distinguish hydrolysis of sucrose from hydrolysis of other α-glucosides, for example, maltose.

In recent studies, Kwon-Chung et al. (8) demonstrated that there are two distinct types of sucrose-negative *C. albicans* identified as *C. stellatoidea*. The type culture of *C. stellatoidea* was found to belong to type I. The isolates of type I were found to have OFAGE karyotypes different from those of type II, which are similar to that of *C. albicans*. The type I isolates have longer doubling times (in vitro) than do the type II isolates and fail to cause fatal infections in mice injected with 5×10^5 cells intravenously. The isolates of type II are as virulent as *C. albicans* and produce fatal infections within 20 days in 80 to 90% of mice injected with 5×10^5 cells intravenously. The isolates of type I are more sensitive to UV irradiation than are the type II isolates (8).

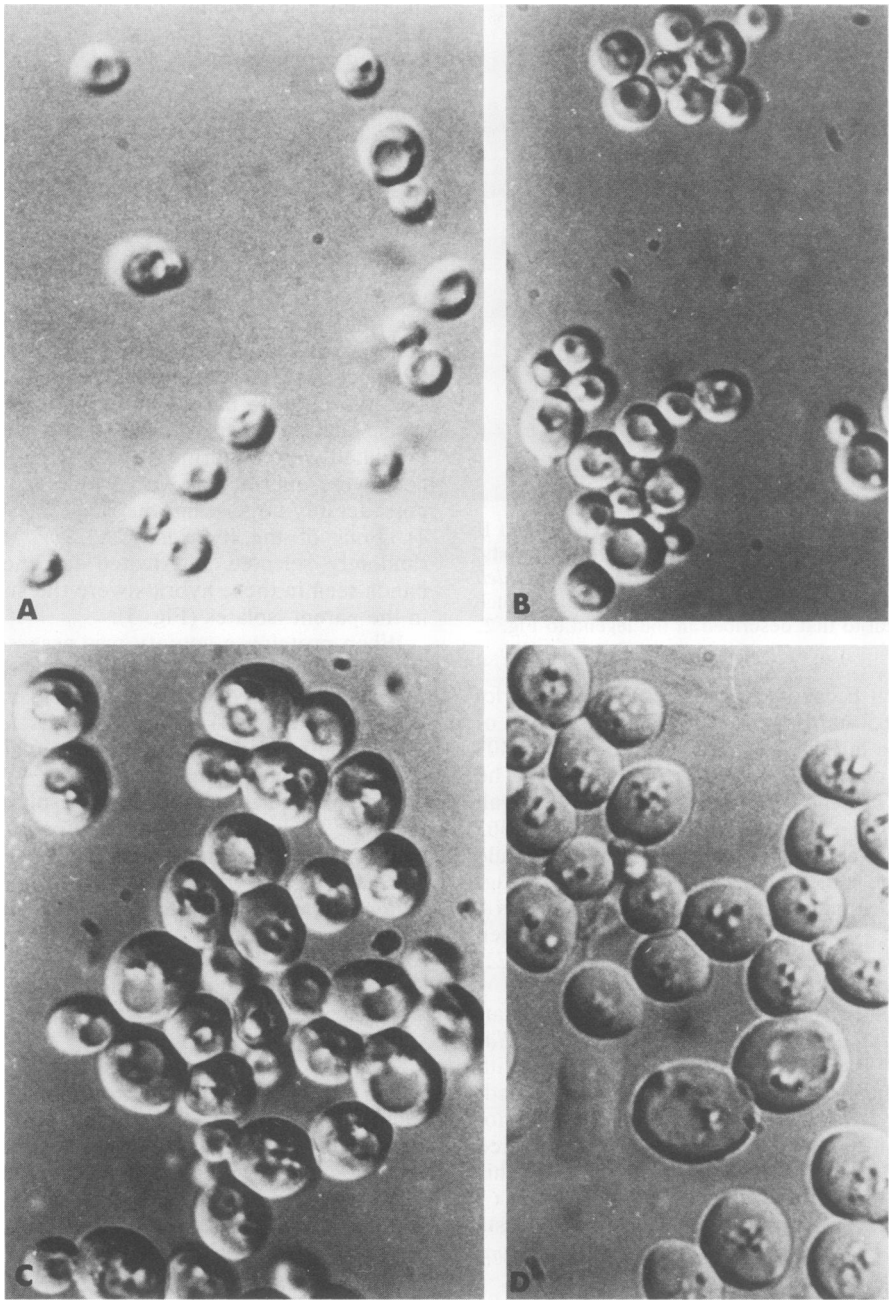

FIG. 4. Stationary-phase cells (grown in yeast extract-glucose-peptone broth) of B-4365*ade* (A), B-4252*met* (B), and two hybrids (C and D) produced by protoplast fusion of the two isolates. Magnification, ×1,000.

The present study revealed further genetic differences between the isolates of the two types and showed similarities between type II *C. stellatoidea* and *C. albicans* B-4201. mtDNA restriction patterns of four type I isolates were identical, regardless of the restriction enzyme or probe used. These patterns were always clearly distinguishable from those of *C. albicans* B-4201. In contrast, the mtDNA restriction patterns of type II isolates were either homogeneous or heterogenous, depending on the enzyme-probe combinations. The DNA digested with *Alu*I and hybridized with the E1 probe showed that the type II mtDNA restriction pattern is identical to that of *C. albicans* B-4201 while clearly different from those of type I. With other combinations of restriction enzymes and probes, however, mtDNAs of type II isolates were either identical to those of type I or identical to that of *C. albicans* B-4201.

Authentic *C. albicans* isolates do not all have identical mtDNA restriction fragment patterns (21). By using the combination *Msp*I-E1, for example, at least 15 patterns have been differentiated among *C. albicans* isolates. Among the 150 *C. albicans* isolates examined, not one had the same mtDNA restriction pattern as type I *C. stellatoidea*, further reinforcing the unique systematic identity of this class of isolates (W. S. Riggsby et al., manuscript in preparation).

The chromosome-sized DNA bands separated by OFAGE showed that the difference between the two types of *C.*

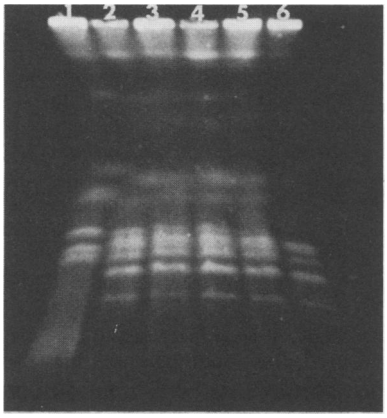

FIG. 5. Karyotypes on OFAGE. Lanes: 1, B-4365*ade*; 2 to 5, hybrids produced by B-4365*ade* × CA329*pro*; 6, CA329*pro*.

stellatoidea lies more in the size than in the number of bands. Our previous OFAGE pictures of *C. stellatoidea* (8) showed different numbers of bands in the two types and numbers different from those seen in this study. The type I isolates appeared to have at least one or two more bands than the type II isolates. These results were obtained by using a different OFAGE apparatus than was used in the previous study (8). The OFAGE protocol used in this study was the same as before (8), except that the duration of electrophoresis was 42 h instead of 48 h. The large- and medium-sized bands in type II isolates were more clearly separated here than in previous runs, and the total number of bands was seven instead of six for type II isolates. As shown in the previous paper (8), two small-sized bands (less than 1,000 kilobases) were seen in type I isolates but not in type II isolates. The numbers of bands seen in the present study were the same among the isolates of the two types and *C. albicans* B-4201, except for B-4406 (type I), which showed two extra bands.

Complementation tests revealed the genetic differences of sucrose-negative phenotypes in the two types of *C. stellatoidea*. Since the hybrids formed by type I × type II crosses were sucrose positive while those of type I × type I crosses remained sucrose negative, the mutations responsible for the sucrose-negative phenotype in the two types appear to be different. One hybrid obtained from cross B-4252*met* × B-4365*ade* was found to be sucrose negative. It is likely that this hybrid is a sucrose-negative derivative of a sucrose-positive hybrid. *C. albicans* hybrids were shown to be unstable in ploidy in previous studies (16, 19, 20). It was observed in the present study that sucrose-negative hybrids arise among subclones of some sucrose-positive hybrids (data not shown). Biochemical studies are needed to identify the lesions in the two types. Complementation tests revealed that the sucrose-negative phenotype is recessive, while the proteinase phenotype at pH 3.8 is dominant.

TABLE 1. Isolates of *Candida* spp. tested with antimannan monoclonal antibodies by immunofluorescence

Species	Isolate	Reaction with monoclonal antibody[a]	
		CB6-fluorescein	AC3
C. stellatoidea	B-4252	−	+
Type I	B-4257	−	+
	B-4404	−	+
	B-4406	−	+
Type II	B-4365	+	+
	B-4403	+	+
	B-4405	+	+
C. albicans	B-4201	−	+
Serotype A	CDC 65	+	+
Serotype B	Lecocq	−	+
C. parapsilosis	B-390	−	−

[a] CB6, Anti-*C. tropicalis* cell wall mannan monoclonal antibody which reacts specifically with mannans of *C. tropicalis* and *C. albicans* serotype A (15). AC3 is produced in a manner similar to that of CB6, except that it reacts specifically with mannans of *C. tropicalis* and *C. albicans* serotypes A and B (15). +, Reaction; −, no reaction.

The analysis of the midrepeat sequence made by using the λCa3 probe showed multiple band patterns in both type I and type II isolates. The major band patterns, however, were clearly different for the two types. With the same amount of DNA, the signals produced in *C. stellatoidea* type I were generally less intense than those seen in type II. This may indicate either that type I isolates contain fewer copies of the Ca3 sequence or that less homology exists between Ca3 and the DNA of type I isolates than between Ca3 and the DNA of type II isolates. Some of the major bands seen in both *Cla*I and *Eco*RI digests of type II DNA were missing in the type I isolates. In spite of all these differences, *C. stellatoidea* type I may not be considered as a separate species, since λCa3 hybridizes to multiple restriction fragments. In a study by Hicks et al. (submitted), λCa3 was shown to hybridize to multiple restriction fragments of *C. albicans* but to hybridize only weakly or not at all to *C. krusei*, *C. tropicalis*, *C. glabrata*, *C. guilliermondii*, or *S. cerevisiae*. The banding patterns with λCa3 support the high percentage of DNA similarities between *C. albicans* and *C. stellatoidea* type I previously observed by DNA reassociation methods (11).

Hasenclever and Mitchell first reported that *C. stellatoidea* is antigenically indistinguishable from serotype B isolates of *C. albicans* (4). The present study shows that this is true only with type I isolates of *C. stellatoidea*. All three isolates of type II *C. stellatoidea* were serologically identical to serotype A isolates of *C. albicans*. These results, together with the genetic information, strongly suggest that the type II isolates of *C. stellatoidea* studied are sucrose-negative mutants of serotype A *C. albicans*. The minor differences seen in the midrepeat sequence between the reference isolate B-4201 (serotype B) and the three isolates of type II may be associated with their serotypic difference. Studies on more isolates of serotype B *C. albicans* are needed to evaluate such differences. The antigenic similarity between serotype B *C. albicans* and *C. stellatoidea* type I cannot be further analyzed until a monoclonal antibody specifically reacting to serotype B *C. albicans* becomes available.

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