Molecular Probe for Identification of Medically Important *Candida* Species and *Torulopsis glabrata*

MARK M. MASON, BRENT A. LASKER,[†] AND W. STUART RIGGSBY*

Department of Microbiology and Graduate Program in Cellular, Molecular and Developmental Biology, University of Tennessee, Knoxville, Tennessee 37996-0845

Received 11 August 1986/Accepted 21 November 1986

A cloned DNA fragment from *Candida albicans* containing the gene for the protein actin was used to probe the molecular structure of the actin gene of several medically important yeasts (*C. albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida pseudotropicalis*, *Candida krusei*, *Candida parapsilosis*, *Candida guilliermondii*, and *Torulopsis glabrata*). Whole-cell DNA from each species was digested with restriction endonucleases, electrophoresed on agarose gels, and transferred to nitrocellulose. Radioactively labeled *C. albicans* actin gene was hybridized to the DNA fragments on the nitrocellulose. The *C. albicans* probe produced a strong signal with all of the *Candida* DNAs tested, indicating considerable conservation of this gene. In addition, the actin genes of all of the species tested were found to have no internal *EcoRI* or *SalI* restriction sites. With the exception of *C. guilliermondii*, all of the species tested had a single internal *HindIII* recognition site. However, the location of flanking restriction sites was found to be species specific. For all of the enzymes tested, the locations of the flanking restriction sites in *C. albicans* and *C. stellatoidea* were identical; all of the other strains yielded fragments clearly distinct from one another. These differences provide a molecular tool for the differentiation of medically important *Candida* species.

Several species within the genus *Candida* are of recognized medical importance. Clinical isolates of these yeasts are usually differentiated on the basis of a battery of morphological, physiological, and biochemical tests (15, 16), sometimes complemented by serological tests (15). Although it is known that these medically important species may be quite divergent genetically (11), their differentiation on the basis of phenotypic properties is not always simple (15). In this paper, we describe a relatively simple test, involving the analysis of a stable genetic element, which may be valuable in the identification of medically important *Candida* species.

The yeast strains used are listed in Table 1 along with their sources. Species identification of all strains was confirmed by the API 20C system. Strains were maintained as described previously (18). All strains were grown in either YEPL or YEPG (17, 22). These media contained 1% yeast extract (Difco Laboratories, Detroit, Mich.), 2% Bacto-Peptone (Difco), and either 6% DL-sodium lactate (obtained as 60% syrup with equal amounts of D and L stereoisomers; Sigma Chemical Co., St. Louis, Mo.) for YEPL or 2% glycerol (Fisher Scientific Co., Atlanta, Ga.) for YEPG. Cells were grown as 250-ml cultures for 48 h with constant shaking at 30°C; 1 to 4 g (wet weight) of cells could be obtained depending on the strain used. Spheroplasts were prepared as described by Torres-Bauza and Riggsby (18). Whole-cell DNA was purified basically as described previously (14, 19). Spheroplasts were suspended in 0.1 M Tris (pH 8.0)—0.1 M NaCl—0.2 M EDTA supplemented with proteinase K (100 µg/ml) and sodium dodecyl sulfate (final concentration, 1% wt/vol). The lysate was incubated for 1 to 2 h at 37°C, and then it was extracted once with phenol-chloroform-isoamyl alcohol (24:24:1) and twice with chloroform-isoamyl alcohol (24:1); the preparation was then pre-

cipitated with 2 volumes of 95% ethanol. After storage at -20° overnight, the nucleic acids were collected by centrifugation and dissolved in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). The nucleic acid solution was made 0.3 M sodium acetate and 1 mM EDTA (pH 7.0) and precipitated with 0.54 volume of isopropanol. After isopropanol precipitation, the nucleic acids were again dissolved in 0.1× SSC, treated with RNase T₁ (100 U/ml), RNase A (25 µg/ml), α-amylase (100 µg/ml), and β-amylase (100 µg/ml) for 1 to 1.5 h at 37°C. One phenol extraction, two chloroform extractions, and ethanol precipitation as described above were carried out, and the DNA was dissolved in 0.1× SSC. The enzyme treatments, organic extractions, and ethanol precipitation were then repeated. Finally, the DNA was dissolved in 10 mM Tris (pH 7.4)—1 mM EDTA (1× TE buffer) and stored at -20°C.

A 2-kilobase (kb) *SalI* fragment containing the single *Candida albicans* actin gene (identified by homology to the *Saccharomyces cerevisiae* actin gene) was isolated from strain H317 and cloned into the *SalI* site of pBR322 by using *Escherichia coli* DH1 (B. A. Lasker, J. M. Bagash, and W. S. Riggsby, manuscript in preparation). The pBR322 actin DNA was purified by using a standard large-scale plasmid purification method (4, 12), dissolved in 1× TE buffer, and stored at -20°C. The pBR322 actin DNA was labeled by using a nick translation kit supplied by Bethesda Research Laboratories, Inc., Gaithersburg, Md., according to the directions of the manufacturer and [α-³²P]dCTP (New England Nuclear Corp., Boston, Mass.). Restriction endonucleases were purchased from Bethesda Research Laboratories and New England BioLabs, Inc., Beverly, Mass. *SalI* was used in accordance with the instructions of the supplier; for *EcoRI*, *PvuII*, *HindIII*, and *ClaI*, 1× TA buffer (33 mM Tris, pH 8.6, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol) (13) was used. Agarose gel electrophoresis, Southern blotting, and hybridization of the nick-translated DNA probe were all carried out by using common laboratory methods (4, 8), as

* Corresponding author.

[†] Present address: Division of Infectious Diseases, Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110.

TABLE 1. Strains used in this study

Strain designation	Species	Source ^a	Other designation ^b
SR1001	<i>C. albicans</i>	A	ATCC 18804
SR1010	<i>C. albicans</i>	B	H317
SR1102	<i>Torulopsis glabrata</i>	A	ATCC 2001
SR1103	<i>C. tropicalis</i>	A	ATCC 13803
SR1104	<i>C. parapsilosis</i>	A	ATCC 22019
SR1105	<i>C. stellatoidea</i>	A	ATCC 11006
SR1106	<i>C. albicans</i>	A	ATCC 14053
SR1107	<i>C. pseudotropicalis</i>	A	ATCC 2512
SR1110	<i>C. albicans</i>	G	83-57
SR1117	<i>C. albicans</i>	C	CLFMC 260
SR1124	<i>C. albicans</i>	O	PERRY
SR1131	<i>C. krusei</i>	A	ATCC 6258
SR1132	<i>C. krusei</i>	A	ATCC 14243
SR1133	<i>C. albicans</i>	A	ATCC 24433
SR1134	<i>C. stellatoidea</i>	A	ATCC 36232
SR1135	<i>C. tropicalis</i>	A	ATCC 750
SR1136	<i>C. pseudotropicalis</i>	A	ATCC 46764
SR1225	<i>C. albicans</i>	T	25
SR1207	<i>C. guilliermondii</i>	T	7

^a Sources: A, American Type Culture Collection, Rockville, Md.; B, J. M. Becker, University of Tennessee, Knoxville; C, M. Casal Roman, University of Cordoba, Cordoba, Spain; G, D. G. Ahearn, University of Georgia, Athens; O, F. C. Odds, University of Leicester, Leicester, England; T, L. J. Torres-Bauza, University of Puerto Rico, San Juan.

^b Strain designation given by the source from whom the strain was obtained.

previously described (20). Autoradiography was carried out by using Kodak XAR-5 X-ray film placed between two Cronex Lightning-Plus intensifying screens (Du Pont Co., Wilmington, Del.) in a film holder. The film was exposed at -70°C for varying lengths of time to achieve signals from bands at both high intensity and low intensity.

Figure 1 shows restriction digest patterns of various *Candida* species probed with a cloned *C. albicans* actin gene. In Fig. 1A *C. albicans* and *Candida stellatoidea* DNAs exhibit indistinguishable patterns, whereas the patterns produced by the DNAs of all of the other species differ markedly. To determine whether there was variation in *C. albicans* strains from different sources, we performed the same experiment with other *C. albicans* strains. Figure 1B shows the patterns obtained with six additional *C. albicans* strains, all of which produced the same actin-containing fragments. Identical results were obtained with 18 additional *C. albicans* isolates (data not shown).

To determine the reproducibility of the patterns obtained with the other species shown in Fig. 1 and to extend the analysis to other medically important *Candida* species, we carried out analogous experiments with additional strains; representative results are shown in Fig. 2. Patterns identical to that shown for *Candida guilliermondii* in Fig. 2 were also obtained with two additional *C. guilliermondii* strains (data not shown). In all cases, the patterns produced by different strains of the same species were identical.

The sizes of fragments produced by the enzymes *Hind*III and *Eco*RI for each *Candida* species are shown in Table 2. These values were obtained from gels containing DNA size markers. In addition, we digested DNAs of these species with three other restriction endonucleases (*Cla*I, *Sal*I, and *Pvu*II). Southern blots were performed with these digests, and fragment sizes were determined as described above for *Eco*RI and *Hind*III. The fragment sizes obtained with these digests are also shown in Table 2.

Workers in our laboratory have recently cloned the actin

gene of *C. albicans* strain H317 for use as a tool in studying several aspects of the biology of *C. albicans* and other *Candida* species (Lasker et al., in preparation). In a survey of genomic blots of various *Candida* species probed with the cloned *C. albicans* H317 actin gene, we observed that distinctive fragment patterns were produced with different species. These results suggested that the pattern of restriction fragments produced by even a small battery of enzymes may be sufficient to distinguish among these species.

We have now examined eight medically important *Candida* species; we have used at least two isolates of each of these species. With the exception of *C. guilliermondii*, all of the species had the following three features in common: the actin gene of each species was found to have no internal *Eco*RI site, no internal *Sal*I site, and a single internal *Hind*III site. *C. guilliermondii* shares all of these features except that we have not detected an internal *Hind*III recognition site. All species were easily distinguishable except the *C. albicans*-*C. stellatoidea* group, which could not be resolved.

Inability to resolve the *C. albicans*-*C. stellatoidea* group is

FIG. 1. Genomic blots of yeast DNAs cleaved with restriction nucleases and probed with a labeled, cloned actin gene from *C. albicans* H317. Each pair of lanes contains DNA cleaved by *Hind*III (left lane of the pair) and *Eco*RI (right lane). (A) Lane a, *C. albicans* SR1010; lane b, *C. pseudotropicalis* SR1107; lane c, *C. albicans* SR1106; lane d, *C. stellatoidea* SR1105; lane e, *C. parapsilosis* SR1104; lane f, *C. tropicalis* SR1103; lane g, *Torulopsis glabrata* SR1102. (B) *C. albicans* strains. Lane a, strain SR1110; lane b, SR1225; lane c, SR1117; lane d, SR1124; lane e, SR1133; lane f, SR1001.

FIG. 2. Experiments carried out as described in the legend to Fig. 1. Lane a, *C. guilliermondii* SR1207; lane b, *C. krusei* SR1132; lane c, *C. krusei* SR1131; lane d, *C. albicans* SR1010; lane m, markers (*Hind*III fragments of phage lambda).

consistent with our finding (Mason and Riggsby, manuscript in preparation) that these species are nearly identical on the basis of the restriction nuclease digestion patterns of their mitochondrial DNAs. Since mitochondrial DNAs evolve more rapidly than nuclear DNA in a variety of taxa (3), failure to distinguish mitochondrial DNA leads to the prediction that the more conservative actin sequence is also preserved. In addition, gross DNA homology studies (7) have failed to distinguish between *C. albicans* and *C. stellatoidea*, and, in fact, *C. stellatoidea* is now widely regarded as a synonym of *C. albicans* (1, 10).

The restriction patterns for *C. albicans* and *C. stellatoidea* strains are constant over a wide variety of sources with respect to geographical origin and to the clinical condition of the human sources of the strains. The pattern is the same for *C. albicans* and *C. stellatoidea* strains isolated in the continental United States (Fig. 2, lane a), Puerto Rico (Fig. 2, lane b), Spain (Fig. 2, lane c), and the United Kingdom (Fig. 2, lane d). Strains were isolated from patients with oral thrush (strain SR1225), dermatosis (SR1133), systemic infection (SR1106), and acquired immune deficiency syndrome

(SR1110). The same patterns were also obtained with three *C. albicans* strains and two *C. stellatoidea* strains obtained from the American Type Culture Collection, Rockville, Md. These results confirm the utility of restriction patterns for identification of the *C. albicans*-*C. stellatoidea* group and the close genetic relatedness of these two species. It is especially interesting that *Candida tropicalis*, a species which is antigenically very closely related to *C. albicans* (2, 5, 15), has no actin gene restriction fragments in common with *C. albicans*. Thus, restriction fragment analysis appears to provide a more sensitive method for distinguishing these species than immunological methods provide.

It should be noted that none of the other species tested appear to have any fragment in common, with the possible exception of the 6.0-kb *Eco*RI fragment of *C. guilliermondii* and *Candida pseudotropicalis*. This latter similarity is probably coincidental, since the two species have entirely different actin gene restriction patterns for all of the other enzymes tested (Table 2). The same argument can be made for the 4.0-kb *Pvu*II fragments of *C. guilliermondii* and the *C. albicans*-*C. stellatoidea* group. It is unlikely that the 1.27-kb *Cla*I fragment of *C. tropicalis* is the same as the 1.3-kb fragment of *C. albicans* and *C. stellatoidea*, since the *Cla*I site is internal in *C. albicans* and *C. stellatoidea*, whereas *C. tropicalis* appears to have a single *Cla*I fragment, indicating no internal recognition site.

Yarrow and Meyer (21) proposed that members of the genus *Torulopsis* be transferred to the genus *Candida*. Although this recommendation has been accepted by some workers (1, 10, 15, 16), it has been vigorously rejected on nomenclatural and taxonomic grounds by a number of medical mycologists (9). The work reported here does not bear on this question.

For clinical laboratory purposes, two modifications in the details of our methodology would be useful. Our protocols for DNA extraction were originally designed for quantitative extraction of DNA from yeast cells and are therefore much more rigorous than necessary for the purposes proposed here. Any DNA extraction procedure that does not substantially destroy or preferentially exclude the nuclear DNA should suffice. As a probe, we have used ³²P-labeled DNA, whose short half-life makes it unsuitable for a reagent which should preferably have a reasonably long shelf storage life. The substitution of biotinylated, nick-translated probes (6, 7) would give the reagent a long storage life, as well as eliminate the more general safety problems associated with using radioactive reagents.

We thank D. G. Ahearn, J. M. Becker, M. Casal Roman, F. C. Odds, and L. J. Torres-Bauza for gifts of many of the strains used in this study.

This work was supported in part by Public Health Service grant AI 5 T32 07123 from the National Institutes of Health.

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TABLE 2. Sizes of actin-containing DNA fragments produced by digestion of DNAs of various species by various restriction endonucleases

Species	Fragment sizes (kbp) ^a				
	<i>Eco</i> RI	<i>Hind</i> III	<i>Cla</i> I	<i>Pvu</i> II	<i>Sal</i> I
<i>C. albicans</i>	4.0	8.2, 3.9	1.5, 1.3	4.0, 1.1	1.9
<i>C. stellatoidea</i>	4.0	8.2, 3.9	1.5, 1.3	4.0, 1.1	1.9
<i>C. tropicalis</i>	2.8	2.6, 0.7	1.27	17	6.6
<i>C. pseudotropicalis</i>	6.0	3.6, 1.25	1.9, 0.84	16.5	23
<i>C. guilliermondii</i>	6.0	7.9	5.1	4.0, 2.0	5.7
<i>C. parapsilosis</i>	7.0	5.6, 0.8	0.97, 0.5	6.1	23
<i>C. krusei</i>	18	16.5, 5.0	10.5	22	7.2
<i>Torulopsis glabrata</i>	8.0	6.5, 2.75	ND ^b	ND	ND

^a kbp, Kilobase pairs.

^b ND, Not determined.

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