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Distinction of Deep versus Superficial Clinical and Nonclinical Isolates of *Trichosporon beigelii* by Isoenzymes and Restriction Fragment Length Polymorphisms of rDNA Generated by Polymerase Chain Reaction

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Fifteen clinical and environmental strains of *Trichosporon beigelii* were analyzed for similarities by using morphological features, biochemical profiles based on carbon compound assimilation and uric acid utilization, isoenzyme electrophoresis, and restriction fragment length polymorphisms of a segment of genes coding for rRNA expanded with the polymerase chain reaction. The findings suggest that strains that cause invasive disease are distinct from the superficial and the nonclinical isolates and that isolates from the skin and mucosae represent a number of different organisms, including some environmental forms. The study shows that *T. beigelii* is a complex of genetically distinct organisms and that more than one type is found in clinical samples.

Trichosporon beigelii (Kuchenmeister et Rabenhorst) Vuillemin is an emerging pathogen in immunocompromised patients. Although long known as the cause of white piedra in immunologically normal hosts, increasingly this basidiomycetous, arthroconidial yeast is being reported as an opportunistic pathogen causing deep-seated and disseminated infections in immunocompromised patients (9, 24–26). Current taxonomical treatment in *The Yeasts* (12) places *T. beigelii* in synonymity with *T. cutaneum* (De Beurmann, Gougerot et Vaucher) Ota; however, this taxon contains organisms that differ from each other in a number of morphological, physiological, and genetic characteristics (6, 7, 13, 29). Lee et al. demonstrated that deeply invasive isolates of *T. beigelii* were distinguishable from superficial and environmental isolates by colonial and microscopic morphology (13). Homology studies, using DNA-DNA reassociation, of the type strain of *T. cutaneum* and some of the strains that have been placed in synonymity with *T. cutaneum* in *The Yeasts* have shown that *T. cutaneum* is unrelated to *T. cutaneum* var. *antarcticum*, *T. infestans*, *T. loubieri*, *T. lutetiae*, *Geotrichum vanriji*, and *T. dulcitum* (7). There has been a report that *T. beigelii* has a variable ubiquinone type (29), and Guého, Kurtzman, and Peterson communicated the unpublished results of Billon-Grand that the ubiquinone type of a strain of *T. beigelii* from white piedra in a monkey differed from that of the type strain of *T. cutaneum* (6). The 18S and 25S RNA sequences support the placing of these two strains into distinct, though clearly related species (6). However, there appears to have been no detailed study on the genetic relationships of clinical isolates from humans.

That invasive clinical isolates of *T. beigelii* (previously known as *T. cutaneum*) may be genetically distinct from superficial clinical isolates has been suggested earlier (5). Here we report on a series of different clinical isolates, including ones causing deep-seated infections that were isolated from blood and ones obtained from mucosal and cutaneous sites. Also studied are some nonclinical or environmental strains. The characteristics of the organisms have been compared for morphological features, carbon substrate assimilation profiles, utilization of uric acid, isoenzyme profiles, and restriction fragment length polymorphisms (RFLPs) in a segment of the gene coding for rRNA (rDNA) which had been expanded with the polymerase chain reaction. The rDNA, studied herein, was composed largely of the two internally transcribed spacer (ITS) regions and the 5.8S rDNA that lie between the nuclear small rDNA and nuclear large rDNA sequences in the genome (Fig. 1). This area was chosen because ITS regions appear to undergo more rapid evolutionary change than is found for the associated rDNA sequences and may show heterogeneity when strains of closely related species are compared (28). In contrast, the nuclear small, nuclear large, and 5.8S rDNA sequences appear to evolve more slowly and have been useful in placing organisms into different groups on the basis of their phylogeny (23).

MATERIALS AND METHODS

Fungi. Fifteen isolates were obtained for study as listed in Table 1. Ten of these were derived from clinical sources, and five were from the American Type Culture Collection, Rockville, Md. One isolate, TSAS-87, showed four different stable morphotypes, and these were analyzed separately. Isolates were stored on potato dextrose agar (Remel, Lenexa, Kans.) slants at −70°C and then were subcultured to Sabouraud glucose agar (D-glucose, 20 g; neopeptone [catalog 0119; Difco, Detroit, Mich.], 10 g; agar, 15 g/liter of water). The morphological features of these strains have been studied in detail (13) and are listed in Table 1. All strains were tested by the diazonium blue B test (27) by using freshly made reagent that was applied to 3-week-old colonies that had been grown on a modified Sabouraud agar (neopeptone, 1 g; D-glucose, 4 g; yeast extract [catalog 0127; Difco], 0.5 g; agar, 1.5 g/100 ml).

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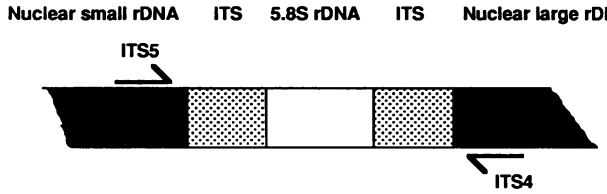

FIG. 1. Map of DNA sequence amplified in the polymerase chain reaction. ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TC CTCCGCTTATTGATATGC) are oligomer primers that bind to conserved sequences on opposite DNA strands just within an end of the nuclear small and nuclear large rDNA genes (28). The amplified DNA is composed primarily of two ITS regions and the 5.8S rDNA.

Physiological studies. All cultures were incubated at 30°C. For carbon compound assimilation profiles, the API 20C kit (Analytab, Plainview, N.Y.) was employed. This tested the abilities of the strains to produce visible growth after 3 days in an agar medium containing one of a panel of different compounds as the sole source of carbon. Details of the compounds are given in Table 1 with the results. Utilization of uric acid was determined after the fungi were inoculated as a streak on medium containing a suspension of uric acid (19), buffered at pH 5.0 using 20 g of KH_2PO_4 per liter (18). The production of a clear zone in the agar after 14 days of growth was considered indicative of uric acid utilization.

Isoenzyme preparation. Multiple colonies of 3-day-old growth on Sabouraud glucose agar were used to inoculate 50 ml of YNB-mannitol-sucrose broth (yeast-nitrogen base without amino acids, 0.03 M sucrose, 0.1 M mannitol) in 250-ml Erlenmeyer flasks (15). The flasks were incubated (66 h, 27°C, 250 rpm on a gyratory shaker), and then the fungus was harvested and proteins were extracted for isoenzyme analysis as described previously (14). Briefly, the cells were washed in 100 mM Tris-hydrochloride buffer, pH 8.0, and then broken by vortexing with 0.45-mm-diameter glass beads. The homogenate was centrifuged (13,000 × *g*, 5 min), and then the supernatant was assayed for protein content by using the Bradford method (3). The extract was then applied, with 250 μg of protein per lane, to native discontinuous polyacrylamide gels (either 5.0 or 7.5% [wt/vol] acrylamide content, as shown in Table 2) and subjected to electrophoresis (16 h, 5 V/cm). Following electrophoresis, the gels were removed and washed (15 to 30 min, 60 rpm) twice in 100 ml of buffer appropriate for detecting enzyme activity. Enzyme activity was detected by using reagents and buffers listed in Table 2, and bands of enzyme activity were recorded by photography.

Cluster analysis. As in a previous study (15), the unweighted pair mean group average clustering and centroid clustering algorithms were used.

DNA extraction. The miniprep method, based on that described for *Candida albicans* (22), was used. A small inoculum was placed into 50 ml of YNB-mannitol-sucrose broth in 250-ml Erlenmeyer flasks. After incubation for 66 h at 27°C in a 250-rpm gyratory shaker, 10 ml of cells were transferred to a 15-ml polypropylene centrifuge tube and

TABLE 1. Morphology and physiological features of *T. beigelii*

Strain	Site	Source[a]	Morphology[b]	Utilization[c]						
				Uri	Gly	Ino	Sor	Tre	Mlz	Raf
Clinical[d]										
Deep seated										
TSAS-87P	Blood	UT	Powdery	−	−	−	−	+	−	−
TSAS-87R	Blood	UT	Rugose	−	−	−	−	+	−	−
TSAS-87PG	Blood	UT	Powdery-grey	−	−	−	−	+	+	−
TSAS-87RG	Blood	UT	Rugose-grey	−	−	−	−	+	−	−
TCM-86	Blood	NCI	Powdery-grey	+	−	−	−	+	−	−
1135-88	Blood	NYSDH	Powdery	+	−	−	−	−	+	−
1181-82	Blood	NYSDH	Powdery/powdery-grey	+	−	−	−	+	+	−
UMSMT-1	Blood	UMSMT	Powdery	−	−	−	−	+	−	−
UMSMT-2	Blood	UMSMT	Rugose	−	−	−	−	+	−	−
UMSMT-3	Blood	UMSMT	Rugose	−	−	−	−	+	−	−
Mucosa associated										
297-87	Stool	NYSDH	Powdery/powdery-grey	+	−	−	−	+	−	−
958-85	Sputum	NYSDH	Powdery	+	−	−	+	+	+	−
Superficial										
342-85	Toenail	NYSDH	Creamy	+	+	+	−	+	+	−
38300	Skin (leg)	ATCC	Creamy	−	−	+	+	+	+	+
Nonclinical[d]										
10266	Soil	ATCC	Grey	+	−	−	−	−	−	−
11115	Fat synthesis	ATCC	Grey	+	−	−	−	+	+	−
14905	Patent strain	ATCC	Grey	+	−	−	−	+	+	−
28574	Water	ATCC	Grey	+	−	−	−	+	−	−

[a] UT, University of Texas Health Science Center at San Antonio; NCI, National Cancer Institute, Bethesda, Md.; NYSDH, New York State Department of Health, Albany; UMSMT, University of Maryland School of Medical Technology, Baltimore; ATCC, American Type Culture Collection, Rockville, Md.

[b] Colony morphologies are described in detail elsewhere (13).

[c] Uri, uric acid; Gly, glycerol; Ino, inositol; Sor, sorbitol (glucitol); Tre, trehalose; Mlz, melezitose; Raf, raffinose. All stains utilized D-glucose, 2-keto-D-gluconate, L-arabinose, D-xylose, D-galactose, α-methyl-D-glucoside, *N*-acetyl-D-glucosamine, cellobiose, lactose, maltose, and sucrose. No strain utilized ribitol or xylitol. Utilization of all compounds except uric acid was determined with the API 20C kit.

[d] All strains were positive in the diazonium blue B test.

TABLE 2. Enzyme stains[a]

Enzyme[b]	Buffer	Substrate[c]	Visualization[d]
α-Glucosidase (7.5)	0.1 M Na acetate, pH 5.5	4-Methylumbelliferyl α-D-glucoside, 3 mg	Fluorescent bands[e] (10 min, 25°C)
β-Glucosidase (7.5)	0.1 M Na acetate, pH 5.5	4-Methylumbelliferyl β-D-glucoside, 3 mg	Fluorescent bands[e] (30 min, 25°C)
Esterase (7.5)	0.1 M Na phosphate, pH 7.0	4-Methylumbelliferyl acetate, 20 mg	Fluorescent bands[e] (15 min, 25°C)
Esterase (7.5)	0.1 M Na phosphate, pH 7.0	α-Naphthyl acetate, 20 mg; Fast Blue RR salt, 40 mg	Brown bands (120 min, 37°C)
Glucose-6-phosphate dehydrogenase (7.5)	0.1 M Tris-hydrochloride, pH 8.0	D-Glucose-6-phosphate, 30 mg; NADP, 10 mg; PMS+MTT+buffer[f]	Purple bands (5 min, 37°C)
Malate dehydrogenase (7.5)	0.1 M Tris-hydrochloride, pH 8.0	L(−)-Malic acid, 300 mg; NAD, 10 mg; PMS+MTT+buffer[f]	Purple bands (45 min, 37°C)
Superoxide dismutase (7.5)	Water	Nitroblue tetrazolium, then riboflavin + TEMED[g]	Clear bands[h]
Catalase (5)	Water	Hydrogen peroxide, then $FeCl_3$ + $K_3Fe(CN)_6$[i]	Clear bands[a] (15 min, 25°C)

[a] Preparation of most substrates and buffers was based on formulae described by Harris and Hopkinson (8).
[b] The amount of protein loaded was 250 μg per lane, and the percent concentration of polyacrylamide in the separating gel (g/100 ml) is listed in parentheses.
[c] Chemicals, except inorganic salts, were obtained from Sigma Chemical Co., St. Louis, Mo. Amounts are given for 100 ml of staining solution except for detection of dehydrogenases.
[d] Typical times are given.
[e] The gel was placed on a UV light box having a midrange filter and photographed through a dark-green filter (no. 58; Tiffen, Hauppauge, N.Y.) with Polaroid 667 film (Polaroid, Cambridge, Mass.).
[f] PMS, phenazine methosulfate, 5 mg; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg; buffer, 0.1 M Tris-hydrochloride, pH 8.0, 20 ml.
[g] TEMED, *N,N,N′, N′*-tetramethylethylenediamine.
[h] Method of Beauchamp and Fridovich (2). Clear bands developed after exposing gel to light.
[i] Hydrogen peroxide was prepared by adding 10 μl of a 30% solution into 100 ml of water. After washing, the gel was developed by using a mixture prepared from equal parts of aqueous solutions of ferric chloride (2 g/100 ml) and potassium ferricyanide (2 g/100 ml).

centrifuged (5 min, 1,500 × *g*). The pellet was resuspended in 1.0 ml of a 1.0 M sorbitol solution, and spheroplasts were prepared by adding both 100 μl of a 300-μg/ml solution of Zymolyase 20T (20,000 U/g; Miles Laboratories, Elkhart, Ind.) in 1.0 M sorbitol and 100 μl of 2-mercaptoethanol (Eastman Kodak, Rochester, N.Y.). After gentle mixing, the tubes were incubated on a reciprocal shaker (60 min, 37°C, 75 rpm). The tubes were then centrifuged (1,500 × *g*), and the supernatant was removed. The cells were resuspended in 500 μl of TE buffer (10 mM Tris-hydrochloride, pH 8.0, containing 1 mM sodium EDTA); 100 μl of aqueous sodium dodecyl sulfate (SDS) solution (10 g/100 ml) was then added to each tube. After mixing, by inverting the tubes several times, the tubes were incubated (30 min, 65°C). The suspension was transferred to 1.5-ml microcentrifuge tubes, and the DNA was extracted twice with an equal volume of phenol-chloroform; this was followed by an extraction with chloroform-isoamyl alcohol (21). The DNA was precipitated following the addition of 2.5 volumes of absolute alcohol and 10 μl of 5.0 M NaCl and the placing of tubes at −20°C overnight. After centrifugation (13,000 × *g*, 15 min), the pellet was rinsed by adding 1.0 ml of 70% (vol/vol) ethanol and leaving this in the tube for 5 min at 4°C. The ethanol was removed, and the pellet was dried in vacuo. The pellet was resuspended in 125 μl of TE buffer, and 20 μl of boiled RNase A in TE buffer was added. Following incubation (37°C, 60 min), the DNA was reprecipitated with ethanol and NaCl and then washed and dried as described above. After being suspended in 125 μl of TE buffer, the concentration and purity of the DNA preparations were determined by measuring the optical density at 260 and 280 nm (21).

Polymerase chain reaction. The polymerase chain reaction was used as a method to expand a segment of rDNA covering two ITSs and the 5.8S rDNA (Fig. 1). The ITS5 and ITS4 primers, described by White et al. (28), were obtained from Genosys Biotechnologies, Inc., The Woodlands, Tex. These primers have been found to bind conserved sites in rDNA from numerous fungal groups (28). The procedure for polymerase chain reaction utilized the protocol described by White et al. Briefly, the reaction mixture containing nucleotide triphosphates, buffer, and *Taq* polymerase (Ampligen kit; Perkin Elmer Cetus, Norwalk, Conn.), ITS5 and ITS4 primers (20 μM each), and *Trichosporon* DNA (5 to 10 ng) was overlaid with mineral oil and placed on a thermal cycler (Perkin Elmer Cetus). The amplification conditions were the following. There was an initial denaturation for 2.5 min at 95°C, and then 35 cycles of annealing for 0.5 min at 55°C, followed by extension for 1.5 min at 72°C, and denaturation for 0.5 min at 95°C. After these cycles, the final extension was allowed to run for 10.0 min at 72°C. Following amplification, the expanded fragments were analyzed by agarose gel electrophoresis (21) using 2% (wt/vol) SeaKem GTG agarose (FMC BioProducts, Rockland, Maine; 40 mM Tris-acetate buffer containing 1 mM EDTA, pH 8.0). Size markers were derived from ΦX174 cut with *Hae*III (Bethesda Research Laboratories Technologies, Inc., Gaithersburg, Md.).

Restriction enzyme digestion of polymerase chain reaction product. The polymerase chain reaction fragment was digested with 4-base recognition restriction enzymes *Sau*3A, *Alu*I, and *Hae*III under the conditions recommended and the buffers provided by the supplier (Bethesda Research Laboratories). The fragments sizes were then analyzed by using agarose gel electrophoresis as for the polymerase chain reaction product.

TABLE 3. Matrix showing enzyme electrophoresis data for *T. beigelii* strains

Strain	Enzyme activity in position[a]:																																													
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r
TSAS-87P	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	1
TSAS-87R	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	1
TSAS-87PG	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	1
TSAS-87RG	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	1
297-87	1	0	1	1	0	0	0	0	1	1	0	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0
342-85	0	0	1	1	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	0	1	0	0	1	1	0	1	1	0	0	1	0	1	1	0	0	0	0	0	1	1	1
958-85	0	0	0	1	0	0	0	0	1	1	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	1	1	0	1	0	0	0	1
1135-88	0	0	0	1	0	0	0	0	1	1	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	1	1	0	1	0	0	0	1
1181-82	1	0	1	1	0	0	0	0	1	1	0	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0
UMSMT-1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	1
UMSMT-2	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	1
UMSMT-3	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	1
TCM-86	0	1	0	1	1	0	0	0	1	1	0	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	1	0	0	1	0
10266	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	1	0	0	1	0	0	1	0	1	1	0	0	0
11115	0	0	1	1	0	0	0	0	0	0	0	1	0	1	0	1	0	0	1	0	1	0	1	0	0	0	0	0	1	1	0	1	0	0	0	1	0	0	1	1	0	0	0	0
14905	0	1	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	1	0	0	0	0
28574	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	0	1	1	0	0
38300	0	0	0	1	0	0	1	1	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	1	1	0	0	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0

[a] Absence (0) and presence (1) of band of activity for α-glucosidase (columns A to C), β-glucosidase (columns D to F), catalase (columns G to L), superoxide dismutase (columns M to Q), glucose-6-phosphate dehydrogenase (columns R to T), malate dehydrogenase (columns U to Y), esterases based on α-naphthyl acetate (columns Z to i, not included in cluster analyses [see text]), esterases based on 4-methylumbelliferyl acetate (columns j to r).

RESULTS

Physiological tests. The strains of *T. beigelii* showed some heterogeneity in their abilities to assimilate uric acid, glycine, inositol, sorbitol, trehalose, melezitose, and raffinose (Table 1). However, there was no clear pattern that characterized organisms as belonging to one of the clinical groups or as being a nonclinical isolate. Three of the four morphological variants of TSAS-87 behaved identically in their abilities to utilize different carbon compounds; however, TSAS-87PG differed from the others in being able to assimilate melezitose. The positive diazonium blue B test for all strains was in agreement with the described basidiomycetous affinity of *T. beigelii*.

Isoenzyme profiles. Table 3 shows the pattern of enzyme bands seen for the different strains of *T. beigelii*. Heterogeneity was detected in the patterns of bands of enzyme activity that developed on the polyacrylamide gels. Examples of the heterogeneity found are shown in Fig. 2. Glucose-6-phosphate dehydrogenase, α-glucosidase, and β-glucosidase activities were observed in three different positions each, catalase was found in six different positions, superoxide dismutase activity and malate dehydrogenase were found in five different positions, and esterases, detected with 4-methylumbelliferyl acetate, were present in nine positions. In addition to these enzyme stains, stains for mannitol dehydrogenase and acid phosphatase were performed, but there was poor resolution of the bands of activity. No bands of activity were observable in gels stained for alkaline phosphatase or for sorbitol dehydrogenase.

A valuable feature of the isoenzyme assay was its ability to detect unrelated organisms. For example, another strain that showed very different isoenzyme profiles turned out to be a contaminant; it was diazonium blue B negative.

Cluster analyses. Cluster analysis was applied to the data, and strains were clustered for similarity on the basis of the enzyme patterns alone or the enzyme patterns with the carbon compound utilization data. Because very similar esterase profiles were obtained with 4-methylumbelliferyl acetate and α-naphthyl acetate as substrates, the results

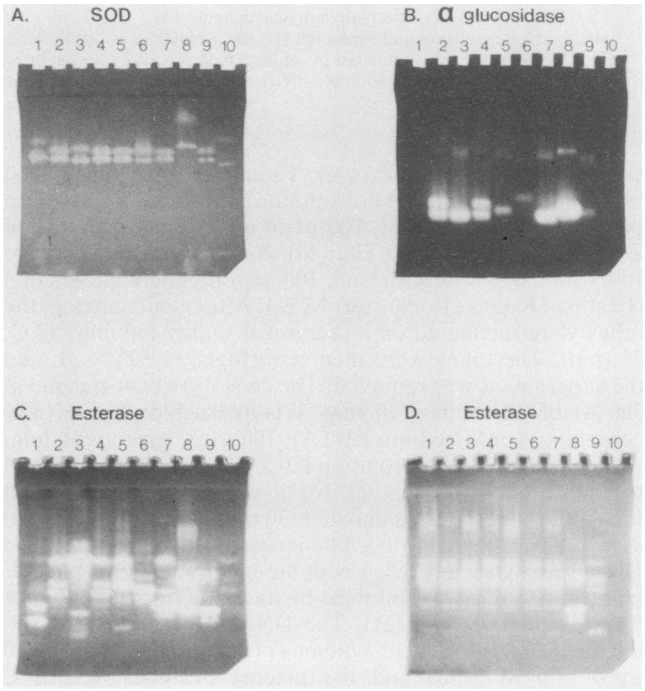

FIG. 2. Isoenzyme variation in *T. beigelii*. Superoxide dismutases (SOD) (A), α-glucosidases (B), and esterases (C) stained with 4-methylumbelliferyl acetate of (lanes 1 to 10, respectively) MCO585, 297-87, 342-85, 1181-82, TCM-86, 10266, 11115, 14905, 28574, and 38300. (D) Esterases of seven strains obtained from blood and of two unrelated strains. Lanes 1 to 9, TSAS-87P, TSAS-87R, TSAS-87PG, TSAS-87RG, UMSMT-1, UMSMT-2, UMSMT-3, 28574, and 38300 (lane 10 was left empty). MCO585 was a contaminant unlike *T. beigelii*; almost no activity was observed for it or for 38300 in the gel stained for α-glucosidase (B). Although extra bands of esterase activity may be seen in panel D when compared with panel C, for example, in strain 38300, replicate gels did not regularly show these bands, so they have not been included in Table 3 or in the cluster analyses.

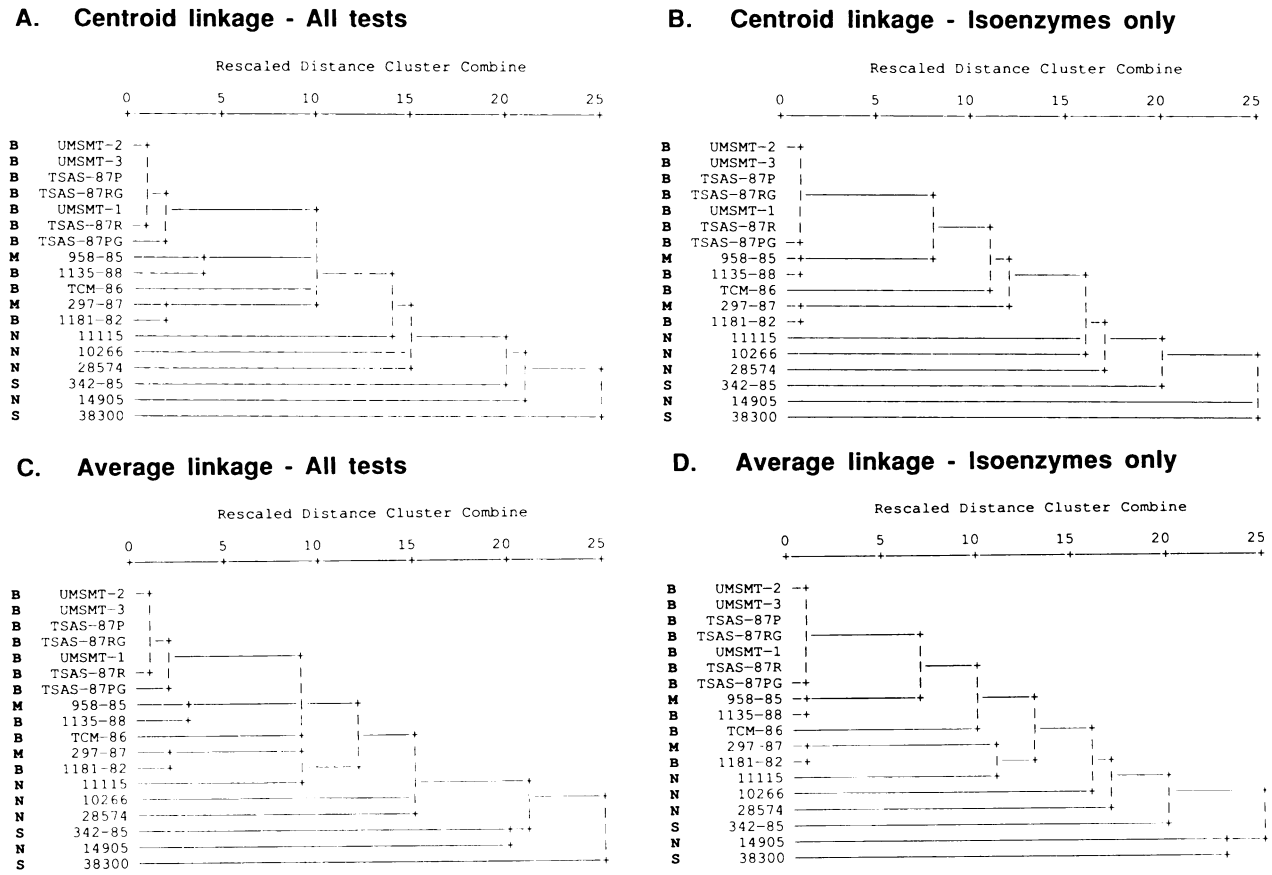

FIG. 3. Dendrograms showing results of cluster analyses applied to isoenzyme patterns and the physiological characteristics of *T. beigelii* strains listed in Table 1. (A) Analysis using centroid linkage of both isoenzyme patterns and physiological characteristics. (B) Analysis using centroid linkage of the isoenzyme patterns alone. (C) Analysis using average linkage between groups of both the isoenzyme patterns and physiological characteristics. (D) Analysis using average linkage between groups of the isoenzyme patterns alone. Strain identifiers are shown on the left of dendrograms. B, isolate from blood; M, isolate from mucosal site; S, isolate from superficial site; N, nonclinical isolate.

derived from the latter reagent were not included in the data set used for the cluster analyses.

The results are shown in Fig. 3. The strains isolated from blood clustered closely together, while there were substantial differences between the strains that were from nonclinical and superficial sources. Indeed, in no case was there evidence for a close relationship between any of the four nonclinical strains and the two strains from superficial sources. Clustering patterns depended in part on the algorithm used, but regardless of the method used, the majority of blood and mucosa-associated isolates clustered separately from the majority of nonclinical strains and superficial isolates. The addition of physiological test results to the isoenzyme data did not change the form of the clusters substantially. When the centroid clustering method was applied to the isoenzyme data, it separated all the blood and mucosa-associated strains from the nonclinical strains and strains from superficial sites (Fig. 3C), while the average linkage clustering method placed two strains, 1181-82 from blood and 297-87 from feces, into a different group. In all analyses, two blood strains appeared somewhat different from the other blood strains; these were 1181-82 and TCM-86.

RFLPs of rDNA fragments. The differences seen by isoenzyme analysis were supported by studies of RFLPs generated by *Sau*3A and *Alu*I digestion of the rDNA expanded with the polymerase chain reaction. Figure 4 shows the variation in DNA sizes found. Variation was not obvious with the other enzymes used. In all cases, the *Sau*3A digest gave rise to a 180-bp fragment, but analysis of the other fragment sizes placed the strains into four groups (Table 4). The *Alu*I digests gave rise to two groups. With both restriction enzymes, the two clinical isolates from superficial sites appeared quite different from the remaining strains. The *Sau*3A-derived RFLPs placed the nonclinical strains into a separate group from that of the blood and mucosa-associated isolates (Table 4); however, overall, there was not as much heterogeneity in the combined RFLP patterns as could be obtained with all the isoenzyme patterns combined.

DISCUSSION

T. beigelii is described in standard reference works as having a wide range of colony forms and characteristics as seen by microscopy (1, 4, 5, 10–12, 17, 20). It is characterized by the presence of arthroconidia, blastoconidia, pseudohyphae, and hyphae; however, the selection of strains examined here indicates that some of these features, especially those of the colony texture, may be restricted to organisms of particular genetic backgrounds. On the basis of the large variety of isoenzyme patterns present, there appear to be several different organisms grouped under the name *T. beigelii* (= *T. cutaneum*) according to current criteria (12).

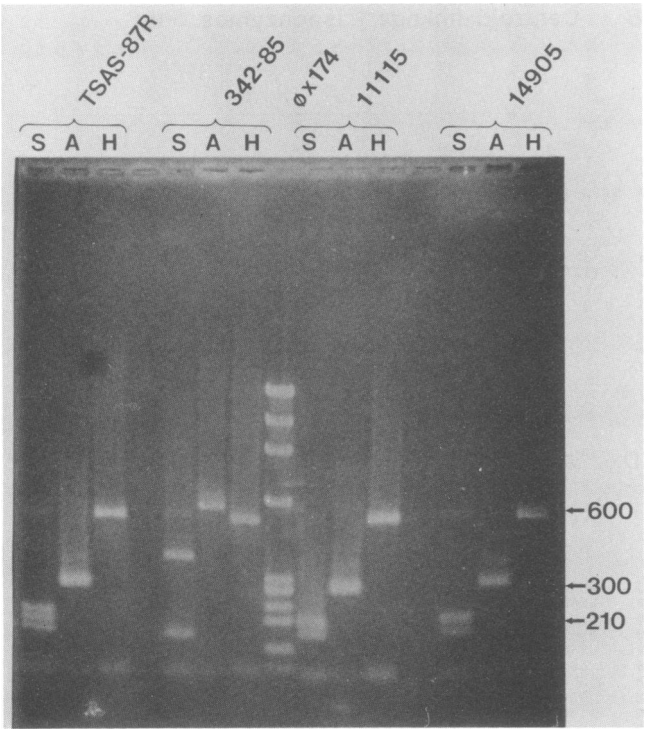

FIG. 4. Ethidium bromide-stained DNA showing RFLPs of polymerase chain reaction product from four *T. beigelii* strains after electrophoresis in a 2% (wt/vol) agarose gel. Strain identifiers are shown at the top. S, *Sau*3A; A, *Alu*I; H, *Hae*III. Lanes 4 and 12 were left empty, and lane 8 is a *Hae*III digest of ΦX174. Arrows on the right show lengths of DNA in bases.

It was notable that, in the series studied here, all the strains with a colony having a powdery or rugose texture were derived from blood or mucosal sites; these same strains formed a subgroup of the isolates when their isoenzyme patterns, with or without their physiological tests, were subjected to cluster analysis. This was seen most clearly when centroid linkage was used in the analysis (Fig. 3A and B), for the average linkage clustering algorithm did not separate all blood and mucosal isolates from two nonclinical strains (Fig. 3C and D).

TABLE 4. RFLP groups of *T. beigelii* determined with *Sau*3A and *Alu*I

Group and strain	DNA fragments (bp)[a]	
	*Sau*3A	*Alu*I
Group 1 (blood isolates) TSAS-87P, TSAS-87PG, TSAS-87R, TSAS-87RG, 297-87, 1181-82, UMSMT-1, UMSMT-2, UMSMT-3	220, 200, 180	310, 290
Group 2[a] (superficial site), 342-85	420, 180	600
Group 3 (nonclinical) 10266, 11115, 14905, 28574	210, 210, 180	310, 290
Group 4[a,b] (superficial site), 38300	240, 180, 180	600

[a] Fragment sizes were estimated from comparison with *Hae*III-digested ΦX174 fragments. The size of the uncut fragment was 600 bp.

[b] The *Sau*3A digest of strain 38300 had two clear bands that were best interpreted with the sizes given.

Neither of the clinical strains derived from superficial sites (342-85 and 38300) showed evidence for any close genetic relationship to the strains derived from blood specimens or to the mucosal isolates, nor were the two strains at all similar in isoenzyme patterns. Furthermore, they differed in the fragment sizes derived from *Sau*3A-digested rDNA and showed differences in their abilities to grow on glycerol, sorbitol, raffinose, and uric acid. However, both strains had characteristics, including a creamy colony texture, that would agree with previous descriptions given to *T. cutaneum* obtained from cases of white piedra. Thus, isoenzyme analysis supports the contention of Emmons et al. (5) that the superficial forms are different species from those that cause invasive infection. Clearly, further studies are mandated to determine the characteristics of other isolates obtained from superficial sites and from cases of white piedra.

Solely on the basis of the differences in isoenzyme patterns within the group of strains studied here, it appears that there may be several different fungal species grouped under the name *T. beigelii*. However, the isolates from blood and mucosal sites may represent only a single species. This is of interest, because it is another example of a situation in which the invasive pathogenic fungi form a genetically restricted subset of a variety of fungi that appear extremely similar morphologically. Such a restriction has been noticed for fungi resembling *Sporothrix schenckii* which had been obtained from a single batch of contaminated sphagnum moss. In that instance, there seemed to be numerous species that were nonpathogenic, while only one group of strains was pathogenic, and this had isoenzyme profiles that were characteristic of authentic pathogenic *S. schenckii* strains (16).

Clinical microbiology laboratories are often called upon to interpret the clinical significance of a fungal isolate. A common dilemma is to determine whether a fungal isolate from a normally sterile site represents contamination or infection. Similarly, it is important to know the pathogenic potential of a fungal isolate when it has been recovered from a surveillance culture. The molecular genetic studies, reported herein, would be applicable to these problems. For example, in surveillance cultures, the detection of isolates which have the characteristics of an invasive strain could prompt early and aggressive initiation of antifungal therapy in febrile, neutropenic patients. While we have not yet examined a very large number of invasive and noninvasive strains of *T. beigelii*, the current association of the invasive isolates with a variety of powdery and rugose colonial morphologies suggests that these phenotypes may be useful for detecting strains with invasive potential. However, molecular genetic characterizations should be continued to be applied, whether isoenzyme profiles or methods involving the characteristics of the fungal DNA are used, as these procedures should help determine the usefulness of phenotypes chosen to identify the different groups of *T. beigelii*.

The relationships of the *Trichosporon* isolates studied here require further study by techniques that quantitate their degrees of divergence one from the other. The measurement of DNA homology and the comparison of nucleic acid sequences would be valuable. Though the isoenzyme procedure does not allow precise quantitative differences between strains to be recorded, it has proven very valuable as a simple, relatively rapid method for placing fungi into groups that are likely to be found to be closely related genetically. It has provided evidence that the name *T. beigelii* must now be considered as representing several distinct entities that are

likely to differ in their clinical significance. In their development of diagnostic procedures and therapies for invasive trichosporonosis, researchers should be sure that they evaluate isolates of *T. beigelii* from invasive disease rather than using possibly unrelated strains obtained from culture collections.

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