Classification of *Histoplasma capsulatum* Isolates by Restriction Fragment Polymorphisms

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Received 9 September 1985/Accepted 11 November 1985

Twenty isolates of the dimorphic, pathogenic fungus *Histoplasma capsulatum* were divided into three classes based on comparisons of restriction enzyme digests of their mitochondrial DNA and rDNA. The majority of isolates, including most North American strains and the African *H. capsulatum var. duboisi* variants, belong to class 2. Isolates from Central America and South America make up class 3. The attenuated Downs strain is the only member of class 1.

The development of a reliable classification of different isolates of the dimorphic, pathogenic fungus *Histoplasma capsulatum* and a precise determination of their interrelatedness has been hampered by the lack of suitable genetic markers and simple biochemical or serologic tests (1, 3–5, 20, 24, 25, 27, 29, 32, 39). Comparisons of restriction fragment patterns of mitochondrial DNA (mtDNA) have been especially useful in determining taxonomic and evolutionary relationships among fungi, protozoans, and animals (2, 6–12, 14, 16–18, 21, 22, 30, 31, 36). Restriction fragment polymorphisms of rDNA have also been used for such comparisons (18, 38, 41).

In this work, we compared restriction digest patterns of mtDNAs and rRNAs of *H. capsulatum* isolates from human and animal sources of diverse geographical origin. We identified three distinct classes of *H. capsulatum* based on such comparisons.

MATERIALS AND METHODS

**Strains of *H. capsulatum***. Table 1 lists the isolates used in this study and their sources.

**Growth of cultures.** Yeast- or mycelial-phase cultures of *H. capsulatum* were grown to the late-log or early stationary phase in liquid GYE medium (2% [wt/vol] glucose, 1% [wt/vol] yeast extract) at 37 or 25°C, respectively (28). Purity of the culture was determined by microscopic examination and subculture.

**Antigenic studies.** The exoantigen test of Standard and Kaufman (39) was used to demonstrate antigenic identity of all of the isolates of *H. capsulatum* used in this study. Briefly, the mycelia and the GYE agar medium of 14-day-old cultures grown at room temperature were homogenized in saline containing 10% Formalin solution. The extract was recovered by filtration and dialyzed against four changes of distilled water. The extract was concentrated to 1/10th its original volume and assayed by immunodiffusion against antisera containing h and m antibodies specific for *H. capsulatum* (lot 10AH6; Meridian Diagnostics, Inc., Cincinnati, Ohio) and compared with a standard histoplasmin (HKC-43; obtained from Coy D. Smith, University of Kentucky, Lexington).

**Isolation of DNA species.** mtDNA of *H. capsulatum* was isolated from a partially purified mitochondrial preparation by a modification of procedures described for the isolation of *Saccharomyces cerevisiae* mtDNA (23, 40). Cells were harvested by centrifugation at 1,800 × g at 4°C (yeast) or by filtration (mycelia). The cells were washed with 100 mM EDTA (pH 6.5) and incubated in 100 mM EDTA (pH 6.5; 2 ml/g [wet weight] of cells) plus 1/40 volume of 2-mercaptoethanol (0.3 M final concentration) for 15 to 30 min at 37°C. They were then incubated in 1 M sorbitol–100 mM EDTA, pH 8.5 (2 ml/g [wet weight] of cells) plus Zymolyase 5000 (Seikagaku Co., Ltd., obtained from Miles Laboratories, Inc., Elkhart, Ind.) at a concentration of 2 to 3 mg/g (wet weight) of cells for 1 to 2 h at 37°C. Although Zymolyase 5000 was not effective in removing *H. capsulatum* yeast-phase cell walls, it facilitated breakage. Treated cells (5 to 15 g [wet weight]) were pelleted at 10,000 × g and suspended in 20 to 30 ml of breakage buffer (0.9 M sorbitol, 1 to 10 mM EDTA, 0.1% bovine serum albumin, 2 to 5 mM Tris hydrochloride, pH 7.2) plus an equal volume of glass beads. After gentle agitation for 2 to 5 min (or until 90% of the organisms were cellular ghosts), the homogenate was decanted and combined with washings of the beads.

Cell debris and unbroken cells were pelleted at 1,500 × g (4°C), and mitochondria were pelleted from the supernatant at 30,000 × g (4°C). The crude mitochondrial pellet was suspended in 0.9 M sorbitol–10 mM EDTA–2 mM Tris hydrochloride (pH 7.5) and washed once at 30,000 × g. The washed mitochondrial pellet (contaminated with traces of other cellular fractions) was suspended in 1 to 3 ml of lysis buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris hydrochloride, pH 8.1), made 2% (wt/vol) in sodium dodecyl sulfate (SDS), and allowed to lyse at room temperature for 1 to 3 h. After the addition of a saturated CsCl solution (2 to 3 ml/ml of lysate), the mixture was partially deproteinized by centrifugation at 40,000 × g. Bisbenzimide (HOECHST 33258) solution (7.5 μg/ml) was added (1/50 volume), and the refractive index was adjusted to 1.397. The preparation was then centrifuged to equilibrium at 150,000 to 200,000 × g for 37 to 42 h at 20°C in a Beckman Ti50 rotor.

The gradients generally showed two or three major DNA bands, the uppermost and lowermost of which were separated by about 1 cm. The mtDNA (upper) band was collected by either gradient fractionation, side puncture, or pipetting; rDNA (the lower part of the bottom band) could only be
isolated by gradient fractionation. After removal of the dye by extraction with isopropanol and removal of CsCl by dialysis, the gradient fractions were analyzed by restriction endonuclease digestion and gel electrophoresis.

**Micrococcal nuclease treatment of mitochondria.** Washed mitochondrial pellets were suspended in incubation buffer (0.9 M sorbitol, 25 mM KCl, 5 mM CaCl₂, 2 mM dithiothreitol, 1 mM EDTA, 100 mM Tris hydrochloride, pH 8.2) and incubated for 3 min at 37°C. Micrococcal nuclease (10 U Sigma Chemical Co., St. Louis, Mo.) was added, and the incubation was continued for 5 to 10 min. An equal volume of 100 mM NaCl–10 mM EDTA–10 mM Tris hydrochloride (pH 8.1) plus proteinase K (1 mg/ml) was added, and the mitochondria were lysed by the addition of SDS to a final concentration of 2%. The samples were then processed as above.

**Restriction endonuclease digestion and gel electrophoresis.** Restriction endonucleases were obtained from New England BioLabs, Inc. (Beverly, Mass.) or Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and were used according to the directions provided by the suppliers. Restriction fragments were separated by electrophoresis through horizontal gels containing 0.7 to 2% agarose in Tris acetate buffer (pH 8.05) at 1 to 2 V/cm. Gels were stained with 0.5 μg of ethidium bromide per ml and photographed with transillumination at 302 nm. Fragment mobilities were measured from a projected image (approximately 10× magnification), and molecular weights were calculated by using *HindIII* digests of bacteriophage lambda DNA as molecular weight standards.

**Blotting and hybridization.** DNA gel patterns were transferred to nitrocellulose (BA85; Schleicher & Schuell, Inc., Keene, N.H.) by the method of Southern (37). Blots were prehybridized in 50% formamide–5× SSC (0.75 M NaCl plus 75 mM sodium citrate)–50 mM NaPO₄ (pH 6.5)–150 μg of tRNA per ml–0.2% SDS (9 to 100 μl/cm² of nitrocellulose). Blots were probed with *H. capsulatum* (Downs strain) large rRNA that had been partially hydrolyzed and end labeled with [γ-³²P]ATP (Amersham Corp., Arlington Heights, Ill.) and T4 polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Approximately 2 × 10⁷ cpm of probe were hybridized to the blot for 24 h at 43°C in 48% formamide–10% dextran sulfate–4× SSC–40 mM NaPO₄ (pH 6.5)–120 μg of tRNA per ml–0.16% SDS. The blots were washed in 2× SSC–0.1% SDS at room temperature and then with 0.1× SSC–0.1% SDS at 50°C. Kodak XAR-5 film was exposed to the dried nitrocellulose with intensifying screens (Du Pont, Wilmington, Del.) for 16 h at −70°C.

**Electron microscopy.** Electron microscopy of mtDNA was carried out by the formamide spreading technique of Davis et al. (13). The DNA-containing hyperphase (0.01 M EDTA, 0.1 M Tris hydrochloride [pH 8.5], 40 to 50% formamide, 100 μg of cytochrome c per ml) was spread on a hypophase of 0.001 M EDTA–0.01 M Tris hydrochloride (pH 8.5)–10 to 30% formamide. DNA picked up on Parlodion-coated grids was stained with 0.005 M uranyl acetate in 90% ethanol and rotary shadowed with platinum-palladium.

### RESULTS AND DISCUSSION

Each of the 20 fungal isolates used in this study (Table 1) was identified as *H. capsulatum* by morphological criteria. On Sabouraud dextrose agar at 25°C, the isolates grew as white- to buff-colored mycelia which on microscopic examination possessed characteristic round-to-yrifrom tuberculuate macroaleuriospores, 8 to 12 μm in diameter. Numerous microaleuriospores, 2 to 4 μm in diameter, were also present. All isolates were converted to the budding yeast morphology on blood agar at 37°C. To confirm their taxonomic identities, the isolates were evaluated by the exoantigen test of Standard and Kaufman (39). Extracts prepared from all of the isolates showed lines of identity when reacted against commercial antiserum to *H. capsulatum* (data not shown).

The Downs strain has lost its ability for sexual reproduction and the capacity to sporulate asexually. It was the only representative of class 1 (see below), it seemed necessary to carefully document its identity as an *H. capsulatum* isolate. Figure 1 illustrates the antigenic identity of the Downs strain to standard commercial histoplasmin HKC-43. There was no reactivity of the standard antiserum to blastomyces (lot 10CB3; Immuno-Mycologies, Inc.) nor to a concentrated extract of *Septonium* sp., a fungus whose morphology is similar to that of *H. capsulatum* (15). Our identification of the Downs isolate as *H. capsulatum* was confirmed by Leo Kaufman, Mycology Unit, Centers for Disease Control, Atlanta, Ga., using the exoantigen procedure (39). The Downs strain was previously identified as mating type (−) by K. J. Kwon-Chung of the National Institute of Allergy and Infectious Diseases, Bethesda, Md. (27).

Figure 2 shows an experiment in which DNAs from the Downs strain of *H. capsulatum* were separated in a CsCl- bisbenzimide gradient. The gradient showed two visible bands corresponding to fractions 7 and 8 (upper band) and 3 to 5 (lower band). DNAs in individual gradient fractions

<table>
<thead>
<tr>
<th>Class</th>
<th>Isolate</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>1 Downs (human)</td>
<td>North America (19)</td>
<td></td>
</tr>
<tr>
<td>2 2310 (human)</td>
<td>North America (A. Body, Lexington, Ky.)</td>
<td></td>
</tr>
<tr>
<td>2 6617 (human)</td>
<td>North America (33)</td>
<td></td>
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<tr>
<td>2 6622 (cat)</td>
<td>North America (33)</td>
<td></td>
</tr>
<tr>
<td>2 6623 (opossum)</td>
<td>North America (33)</td>
<td></td>
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<tr>
<td>2 6628 (human)</td>
<td>North America (33)</td>
<td></td>
</tr>
<tr>
<td>2 6624 (human)</td>
<td>North America (33)</td>
<td></td>
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<tr>
<td>2 UCLA 505 (human)</td>
<td>North America (D. H. Howard, University of California–Los Angeles)</td>
<td></td>
</tr>
<tr>
<td>2 1073 (human)</td>
<td>North America (33)</td>
<td></td>
</tr>
<tr>
<td>2 District (human)</td>
<td>North America (L. Pine, Centers for Disease Control, Atlanta, Ga.)</td>
<td></td>
</tr>
<tr>
<td>2 Grand Island (human)</td>
<td>North America (L. Pine, Centers for Disease Control, Atlanta, Ga.)</td>
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<tr>
<td>2 28 (human)</td>
<td>North America (33)</td>
<td></td>
</tr>
<tr>
<td>2 G217A (human)</td>
<td>North America (4)</td>
<td></td>
</tr>
<tr>
<td>2 G217B (human)</td>
<td>North America (4)</td>
<td></td>
</tr>
<tr>
<td>2 G222B (human)</td>
<td>North America (4)</td>
<td></td>
</tr>
<tr>
<td>2 Duboisii 2100 (human)</td>
<td>Africa (L. Ajello, Centers for Disease Control, Atlanta, Ga.)</td>
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</tr>
<tr>
<td>2 Duboissi 2591 (human)</td>
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</tr>
<tr>
<td>3 G184A (human)</td>
<td>Central America (3)</td>
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</tr>
<tr>
<td>3 G184B (human)</td>
<td>Central America (3)</td>
<td></td>
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<td>3 G186A (human)</td>
<td>Central America (3)</td>
<td></td>
</tr>
<tr>
<td>3 G186B (human)</td>
<td>Central America (3)</td>
<td></td>
</tr>
<tr>
<td>3 H₂ (human)</td>
<td>South America (A. Restrepo, Medellin, Colombia)</td>
<td></td>
</tr>
<tr>
<td>3 H₃ (human)</td>
<td>South America (A. Restrepo, Medellin, Colombia)</td>
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were digested with EcoRI and analyzed by gel electrophoresis (Fig. 2).

The upper gradient band was identified as mtDNA by its resistance to micrococcal nuclease digestion of the mitochondrial preparation before lysis and centrifugation in CsCl-bisbenzimide gradients. Electron microscopic examination of DNA from these fractions showed that they included circular DNA molecules whose contour lengths corresponded to the sum of the molecular weights of the restriction fragments (appeared to be full-length mtDNAs).

The lower gradient band was composed of micrococcal nuclease-sensitive (extramitochondrial) nuclear DNA contaminating the mitochondrial preparation (the smear of restriction fragments in Fig. 2, lanes 4 and 5). The highest-density fraction contained a DNA species producing discrete bands in restriction digests (lane 3). This DNA was identified as rDNA by hybridization to 25S rRNA of the yeast-phase Downs strain. The rDNA could be isolated relatively free of other nuclear DNA and mtDNA only in the bottom fractions (Fig. 2, lane 3), although it was also present in other gradient fractions. Large restriction fragments in the nuclear DNA smear also hybridized to the rRNA probe, probably owing to incomplete digestion of the DNA.

DNAs from other strains of H. capsulatum gave similar gradient profiles, but class 2 strains (Table 1) also contained a third unidentified band between the nuclear and mtDNA. That band did not hybridize with rDNA and disappeared after treatment of mitochondrial preparations with micrococcal nuclease. Additional unidentified minor gradient bands were also present in some preparations.

Restriction digest patterns of mtDNA from 21 strains of H. capsulatum and 2 strains of H. capsulatum var. duboisii (Table 1) could be divided into three distinct classes (Fig. 3). Each restriction endonuclease (17 enzymes with 6-base-pair recognition sequences were tested) produced an identical and characteristic pattern on the mtDNA isolated from each member of a class; each of the three classes had a unique pattern.

A comparison of restriction patterns for representative strains of each class (class 1, Downs; class 2, G217B; class 3, G184B) for each of four restriction enzymes is shown in Fig. 3A. KpnI, HhaI, and XbaI digests show relatively few bands in common among the different classes. However, BglII digests (Fig. 3A, lanes 13 and 14) show at least six fragments in common between classes 2 and 3. The BglII digests of mtDNA isolated from members of all three classes are displayed in Fig. 3B. They show the common pattern shared by members of the same class and the close similarity of the class 2 and class 3 patterns (compare lanes 3 to 7 and 8 to 11). In contrast, the class 1 (lane 2) pattern is markedly different from the other two.

Molecular weights of the mtDNAs of the strains in each class were calculated as the sum of the molecular weights of the restriction fragments. Class 1 mtDNA has an apparent molecular weight of 47 kilobases; class 2 mtDNA, 38 kilobases; class 3 mtDNA, 33 kilobases. The estimated molecular weights were approximately equal to the contour lengths of the circular molecules observed by electron microscopy.

A comparison of EcoRI digests of rDNA from representative strains of the three classes of H. capsulatum (Table 1; Fig. 4) showed that their restriction fragment patterns also fell into the same three classes as those determined by comparisons of mtDNA polymorphisms. The rDNA repeating units of classes 1, 2, and 3 were 6.9, 7.3, and 8.4 kilobases, respectively. We also monitored rDNA by South-
by mtDNA and rDNA restriction patterns. Included in this group are strains which span the apparent spectrum of diversity noted above, including as extreme examples the *H. capsulatum var. duboisii* variants. All members of the class share common mtDNA and rDNA restriction patterns when enzymes with 6-base-pair recognition sequences are used. Class 3 is composed of strains with a limited geographical distribution (Central America and South America). The size and restriction pattern differences between mtDNAs of class 2 and class 3 *H. capsulatum* isolates were roughly similar to those observed for other filamentous fungi (10, 16, 34, 35).

mtDNA restriction patterns of *H. capsulatum var. duboisii*, a variant with unique morphology and a relatively distinct clinical presentation of infection (23), clearly identify it as a member of class 2, which contains most of the strains we studied. Therefore, the phenotypic differences between *H. capsulatum var. duboisii* and other organisms in class 2 may represent a relatively small number of differences in nuclear genotype (18, 26).

Thus far, the Downs strain is the only member of class 1. The mtDNA of the Downs strain is the largest of those found in the three classes, and restriction patterns of its mtDNA and rDNA appear to be markedly different from those of classes 2 and 3. Downs has antigenic identity with standard strains of *H. capsulatum* and has been successfully mated with mating type (+) strains (27). In addition, its phase transitions to yeasts or mycelia are qualitatively the same as the other strains of *H. capsulatum*. These observations support its classification as *H. capsulatum*. However, in

FIG. 3. (A) *KpnI*, *HhaI*, *XbaI*, and *BglIII* digests of mtDNA from representative strains of the three classes of *H. capsulatum*. mtDNAs from strains representing class 1 (Downs), class 2 (G217B), and class 3 (G184B) of *H. capsulatum* were digested with each of four restriction endonucleases and electrophoresed through a 0.7% agarose gel. The mtDNAs are ordered from left to right (class 1-class 2-class 3) for each enzyme: *KpnI* (lanes 2 to 4), *HhaI* (lanes 5 to 7), *XbaI* (lanes 9 to 11), *BglIII* (lanes 12 to 14). *HindIII* digests of phage lambda DNA (lanes 1, 8, and 15) are molecular weight standards. (B) *BglIII* digests of mtDNA from strains representing the three classes of *H. capsulatum*. mtDNAs from representative strains of each of the three classes of *H. capsulatum* were digested with *BglIII* and electrophoresed through a 0.7% agarose gel. All class 2 strains (lanes 3 to 7: G217A, G217B yeast phase, G217B mycelial phase, G222 B, District, respectively) have the same electrophoretic pattern. All class 3 strains (lanes 8 to 11: G184A, G184B, G186A, G186B, respectively) share a different but related pattern. The class 1 Downs strain (lane 2) has a unique pattern. *HindIII* digests of phage lambda (lanes 1 and 12) are molecular weight standards.

FIG. 4. Strain-specific variation of *EcoRI* digests of rDNAs from strains of *H. capsulatum*. rDNAs from strains representing the three classes of *H. capsulatum* (class 1, Downs; class 2, G217B; and class 3, G184B) were digested with *EcoRI* and electrophoresed through a gel containing 0.7% agarose. The *EcoRI* patterns of rDNA are also class specific: lane 3 (class 1, Downs), lane 4 (class 2, G217B), lane 5 (class 3, G184B). Lanes 1 and 6 contain *HindIII* digests of phage lambda DNA, used as molecular weight standards. Lane 2 is a *HindIII* digest of *S. cerevisiae* (ID41-6161) rDNA as a reference.

The isolates of *H. capsulatum* examined above are a very diverse group of strains. They include isolates from both human and animal sources from widely separated geographical regions, morphological subtypes (mycelial A and B forms, *H. capsulatum var. duboisii*), and strains of varying virulence. We also examined yeasts and mycelial phases of most strains and both fresh clinical isolates and laboratory-adapted strains from several sources.

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addition to its unique restriction fragment pattern of mtDNA and rDNA, the Downs strain has other unusual characteristics. It is a vaginal isolate from a patient with an unusual clinical presentation (19). It is relatively avirulent, as determined by mouse infection and is more sensitive to elevated temperature than the other more virulent strains of \textit{H. capsulatum} (28; unpublished data). Perhaps all of these divergent characteristics indicate that the Downs strain evolved in a special environmental niche isolated from those of other \textit{H. capsulatum} strains that we have studied.

**ACKNOWLEDGMENTS**

This work was supported by Public Health Service grant AI 16228 and training grants AI07172 and AI07015 from the National Institutes of Health. R.D.V. was the recipient of National Research Service Award AI 07060. We wish to thank Leo Pine and Libero Ajello of the Centers for Disease Control, Atlanta, Ga., and Angela Restrepo-M. of Medellin, Colombia, South America, for some of the isolates of \textit{H. capsulatum} used in this study. We are also indebted to Cletus P. Kurtzman for reviewing this manuscript and for helpful discussions. We thank Patricia Yuckenberg for the gift of highly purified \textit{H. capsulatum} RNA. Thomas Rosinsky and Barbara Armbruster provided assistance in electron microscopic analysis of DNAs.

**LITERATURE CITED**


