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Electrophoretic Analysis of *Histoplasma capsulatum* Chromosomal DNA

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Seven chromosome-sized DNA molecules in the Downs strain of *Histoplasma capsulatum* were resolved by using chromosome-specific DNA probes in blot hybridizations of contour-clamped homogeneous electric field (CHEF) and field-inversion gel electrophoresis (FIGE) agarose gels. The sizes of the chromosomal DNA bands extended from that of the largest *Saccharomyces cerevisiae* chromosome to beyond that of the *Schizosaccharomyces pombe* chromosomes. Under our experimental conditions, the order of the five largest DNA bands was inverted in the FIGE gel relative to the CHEF gel, demonstrating a characteristic of FIGE whereby large DNA molecules may have greater rather than lesser mobility with increasing size. Comparison of the Downs strain with other *H. capsulatum* strains by CHEF and FIGE analysis revealed considerable variability in band mobility. The resolution of seven chromosome-sized DNA molecules in the Downs strain provides a minimum estimate of the chromosome number.

Chromosomal mapping studies of medically important lower eucaryotes such as *Trypanosoma brucei* (20), *Plasmodium falciparum* (11), *Leishmania major* (18), and *Candida albicans* (17) have become feasible without the benefit of mating experiments, in part through the use of large-DNA electrophoresis. We have applied these agarose gel techniques to the study of *Histoplasma capsulatum* to provide an initial examination of the genome organization of this dimorphic fungus that is pathogenic for humans.

We detected seven chromosomal DNAs in the Downs strain of *H. capsulatum* by using cloned chromosome-specific DNA probes in blot hybridizations of gels run with two techniques: contour-clamped homogeneous electric field (CHEF) gel electrophoresis (6) and field-inversion gel electrophoresis (FIGE) (3). The CHEF and FIGE techniques provided complementary information because of an apparent difference in the basis for their separation of large DNA molecules in the range of several megabases (1 megabase = 1,000 kilobases). Comparison of different strains of *H. capsulatum* by both techniques revealed considerable polymorphism in band mobility.

MATERIALS AND METHODS

Strains. The Downs strain of *H. capsulatum* has been maintained in our laboratory after isolation from a patient with disseminated histoplasmosis (9). The G184B, G186B, G217B, and G222B strains, also derived from clinical isolates, were obtained from the American Type Culture Collection, Rockville, Md. (ATCC 26028, 26030, 26032, and 26034, respectively). *Saccharomyces cerevisiae* AB972 has been used in previous electrophoretic karyotype studies (3–5). *Schizosaccharomyces pombe* AB4660 was obtained from Jack Szostak, Massachusetts General Hospital, Boston. *Bacillus circulans* WL-12 (19) was obtained from a culture collection in the Department of Food Science and Technology, University of California-Davis.

Sample preparation. For *H. capsulatum*, the embedded-lysis protocol of Schwartz and Cantor (15) was modified as follows. Yeast-phase cells were grown in 2% dextrose–1% yeast extract (Difco Laboratories, Detroit, Mich.) at 37°C. The EDTA for washing, embedding, and overlaying the cell sample was pH 6.5. For cell wall degradation, Zymolyase was replaced by 0.1 ml of an enzyme complex derived from *B. circulans* WL-12 (see below), and 2-mercaptoethanol was omitted. The sample was rotated at 32°C for 3 h. The overlay was then replaced with 5 ml of 0.45 M EDTA (pH 9.0)–1% sarcosyl, 250 μg of proteinase K (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) per ml–10 mM Tris (pH 8.0) and rotated at 42°C for at least 4 h or up to overnight; the agarose visibly cleared to a variable extent during this step but did not become completely transparent.

The enzyme complex was prepared from the supernatant of *B. circulans* WL-12 cultures. The growth medium for *B. circulans* consisted of 0.2% *H. capsulatum* cell walls, prepared as described previously (1), with yeast nitrogen base (Difco) in a sodium phosphate-buffered medium (pH 6.5) (10). After 48 h of growth in a 30°C shaker, the supernatant of the culture was collected and treated for 15 min with polyethyleneimine (pH 8.6) at a final concentration of 0.06%; bacterial nucleic acid was pelleted at 10,000 × *g* for 10 min at 4°C. The enzyme complex was extracted from the supernatant as an ammonium sulfate precipitate (2); briefly, ammonium sulfate was added slowly with gentle stirring at 4°C until 85% saturation was reached. The pH was periodically adjusted to 7.0 with NH_4OH during the addition. The preparation was allowed to stand overnight at 4°C and then was centrifuged at 10,000 × *g* at 4°C. The pellet was suspended in 0.2 M K_2HPO_4 (pH 6.5) with 0.5 mM $CaCl_2$ at 1/25 the volume of the original medium supernatant, dialyzed against the same buffer, and stored at −80°C.

For lysis of *S. cerevisiae*, the Schwartz and Cantor protocol (15) was followed, but with Zymolyase 20T (Miles Laboratories, Inc., Elkhart, Ind.) and with only 250 μg of proteinase K per ml in the lysis step. For lysis of *Schizosac-*

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charomyces pombe, the procedure of Smith et al. (16) was modified by embedding cells previously treated for 2 h with Zymolase 20T in liquid with an additional 5 mg of Zymolyase 20T and proceeding with the usual embedded-lysis protocol (15).

Gels. CHEF gel electrophoresis was performed as described previously (6) with 0.7% agarose. FIGE was carried out with a horizontal apparatus (model H4; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) with 0.4% agarose. Switching was performed with a personal computer linked via the printer port to relay switches (3). Field strengths were measured with a Fluke 8024B multimeter (Scherer Instruments Inc., St. Louis, Mo.). Gels were stained in 0.2 μg of ethidium bromide per ml for 17 min, followed by destaining in water for 1 to 48 h (CHEF) or 16 to 48 (FIGE) before photography. Southern transfers included a 20-min 0.25 M HCl gel pretreatment (4).

DNA probes. *H. capsulatum* DNA clones of alpha- and beta-tubulin and of rDNA were obtained from Grace Harris and Brent Lasker, respectively. Random-sequence chromosome-specific probes were identified by individually probing FIGE blots of Downs strain DNA with 85 anonymous plasmid recombinants constructed by inserting size-selected (4- to 11-kilobase) *Mbo*I restriction fragments of *H. capsulatum* G222B genomic DNA into the *Bam*HI site of pUC18. *Schizosaccharomyces pombe* were derived from plasmid recombinants supplied by John Carbon and consisted of restriction fragments containing the *lys1* (chromosome I), *tps13* (chromosome II), and *ade6* (chromosome III) genes.

H. capsulatum probe DNAs were oligolabeled (7) either as purified restriction fragments (alpha- or beta-tubulin) or as intact recombinants with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). *Schizosaccharomyces pombe* probe DNAs were oligolabeled directly from low-gelling agarose (8). Hybridizations were performed in 3× SSC–5× Denhardt solution (14)–0.1% sodium dodecyl sulfate with 50 μg of denatured calf thymus DNA per ml at 60°C (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate). After 14 to 18 h of hybridization, three 15-min washes in 0.1× SSC–0.1% sodium dodecyl sulfate and three to five 10-min washes in 3× SSC were carried out at 60°C.

RESULTS AND DISCUSSION

Sample preparation. Preservation of intact chromosomal DNA of *H. capsulatum* was achieved with the embedded-lysis method of Schwartz and Cantor (15) with one major modification. Zymolyase, which we found ineffective in degrading the *H. capsulatum* cell wall, was replaced by a mycolytic enzyme complex derived from a culture of *B. circulans* WL-12 grown in medium with *H. capsulatum* cell walls as the sole carbon source. The processing of this enzyme complex was analogous to that described by Phaff and co-workers, who have used it to degrade cell walls of *S. cerevisiae* and other fungi (10, 19). Additional modifications associated with the use of this enzyme complex included the omission of 2-mercaptoethanol, which inhibited lysis, and reduction of the incubation time to 3 h to minimize DNA degradation.

CHEF gel electrophoresis. Six chromosomal DNA bands in the Downs strain of *H. capsulatum* were resolved by CHEF gel electrophoresis (Fig. 1). Only three bands were resolved in G217B (Fig. 1), and only four bands were resolved in G186B (data not shown). Size estimates were made by loading samples of *S. cerevisiae* and *Schizosaccharomyces pombe* chromosomal DNA on the same gel (Fig. 1). The mobilities of the bands in the different strains of *H. capsulatum* extended from that of the largest *S. cerevisiae* chromosome (approximately 2 to 3 megabases) (Fig. 1) to less than that of the three *Schizosaccharomyces pombe* chromosomes (Fig. 1). The latter are designated I, II, and III in decreasing order of size as determined by linkage analysis (12) and, in one strain, have been estimated to be 5.7, 4.6, and 3.5 megabases, respectively (Mitsuhiro Yanagida, personal communication). Hybridization of the *Schizosaccharomyces pombe* chromosome-specific probes confirmed the finding of Vollrath and Davis (22) that under these CHEF conditions, the three chromosomes are separated by size in the conventional order (the largest, chromosome I, migrated the least and the smallest, chromosome III, migrated the furthest; data not shown). According to our analysis, therefore, the *H. capsulatum* chromosomal DNAs extended from about 2 to 3 megabases up to more than 5.7 megabases. An exception to this size range was found in strain G186B, in which a small band, at 0.5 megabase, was present.

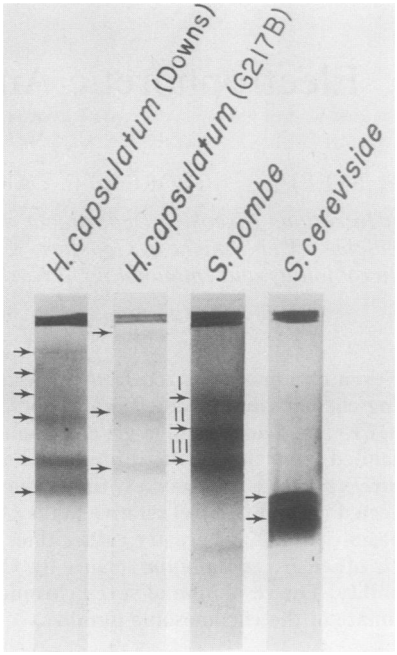

FIG. 1. Negative of an ethidium bromide-stained CHEF gel separation showing six bands in the Downs strain and three bands in the G217B strain (arrows). The three chromosomes of *Schizosaccharomyces pombe* are separated (arrows) and labeled I through III. The largest of the *S. cerevisiae* chromosomes are slightly separated from the remainder of the chromosomes; on further destaining, two bands can be seen in the position of the top arrow. The sample block is seen at the top of each lane. The gel was 0.7% agarose, run for 120 h at 0.5 V/cm (40 V) with a 60-min switching interval.

A relatively low field strength (0.5 V/cm) and long switching interval (60 min) were used to separate these very large DNAs in *H. capsulatum*. Low field strength and long switching intervals have also been used with pulsed-field or CHEF gel electrophoresis to separate the large chromosomes of *Schizosaccharomyces pombe* and *Neurospora crassa* (14, 16, 22).

Considerable variability in band mobility existed among different strains of *H. capsulatum*, as demonstrated for Downs and G217B in Fig. 1. Although some of these

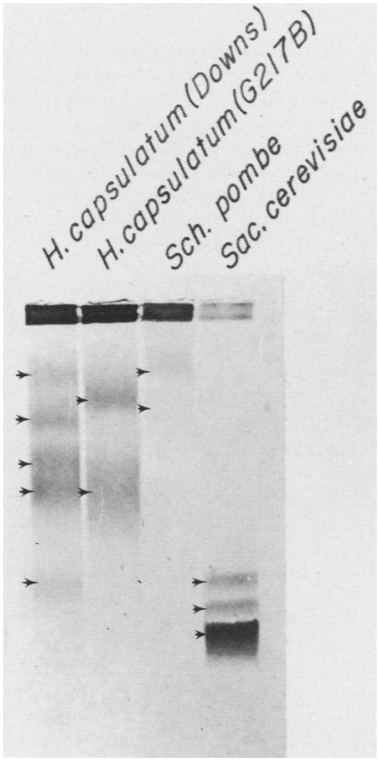

FIG. 2. Negative of an ethidium bromide-stained FIGE separation showing five bands in the Downs strain and two bands in the G217B strain (arrows). Two bands are separated in *Schizosaccharomyces pombe* (arrows); the top band is resolved into two bands in another gel run under these conditions (see Fig. 3B). Two *S. cerevisiae* bands are separated from the remainder of the chromosomal DNA (arrows). The gel is 0.4% agarose, run for 64 h at 0.5 V/cm (20 V) with a switching protocol of 60 min forward, 15 min reverse, followed by 75 h at 1.0 V/cm (40 V) with a switching protocol of 20 min forward, 5 min reverse.

differences could have been due to length polymorphism of the type observed for *S. cerevisiae* (5), it is also possible that the overall karyotypes of those isolates are quite different. For example, the occurrence of a 0.5-megabase chromosome in strain G186B, which is less than 25% of the size of any other molecule in any other strain of *H. capsulatum* we have examined, suggests major strain-to-strain differences in genome structure.

FIGE. At least five chromosomal DNA bands in the Downs strain could be resolved by FIGE gels (Fig. 2). As found with CHEF analysis, there was marked variation in band mobility among strains. Only two bands were resolved in strain G217B (Fig. 2), and three were resolved in G186B (data not shown). For comparison, *S. cerevisiae* and *Schizosaccharomyces pombe* chromosomal DNAs were run on the same gel (Fig. 2); however, a direct size determination of *H. capsulatum* chromosomal DNAs by comparison of FIGE mobility with standards such as *S. cerevisiae* or *Schizosaccharomyces pombe* chromosomes is not always straightforward under fixed switching conditions. In FIGE, switching conditions may be selected in which mobility of the largest DNA molecules can increase rather than decrease with increasing DNA size (3). A consequence of this property is that small and large DNA molecules may comigrate in one region of the gel, thus confounding size determinations by mobility comparisons with standards. The apparent disadvantage of this phenomenon regarding sizing is compensated for by the resolution increase observed in that region of the gel.

With the FIGE gels, we used an initial field strength of 0.5 V/cm to ensure entry of all chromosomes into the gel. (All of the random DNA probes hybridized to at least one of the FIGE bands, which implied that no additional chromosomal DNA molecules were trapped in the sample well.) A second set of slightly higher field strength conditions (1.0 V/cm) was subsequently used to speed separation of bands (see figure legends for conditions). As with CHEF gels, long switching intervals (up to 1 h) with long gel runs (more than 5 days) were required to separate the bands.

Comparison of CHEF and FIGE. We compared CHEF with FIGE separations of *H. capsulatum* chromosomal DNA by using cloned, band-specific probes to determine, to

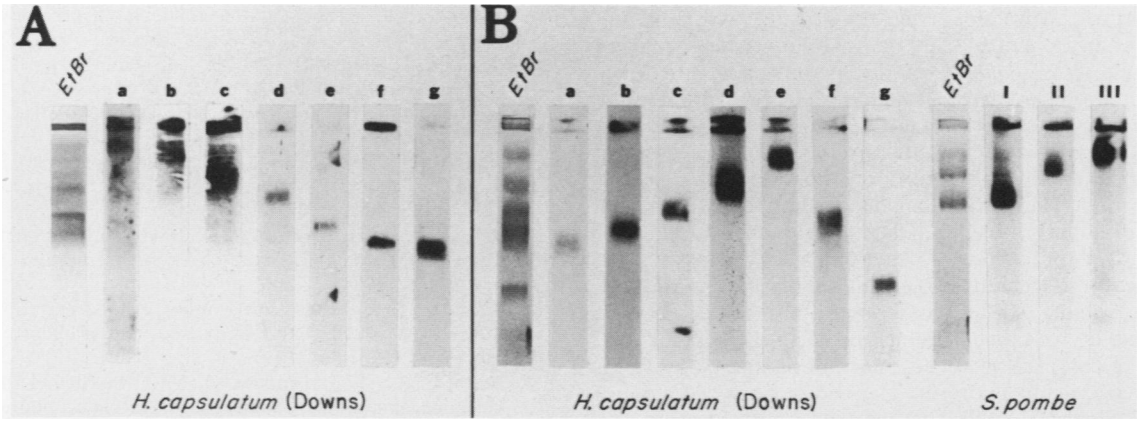

FIG. 3. Hybridizations to chromosome-specific probes. (A) CHEF gel showing six bands in the ethidium-bromide-stained (EtBr) lane of the Downs strain of *H. capsulatum* (left) and hybridizations (a through g) to lanes from the CHEF gel illustrated in Fig. 1 and one other CHEF gel. Lanes: a, c, d, and e, anonymous DNA probes; b, beta-tubulin; f, ribosomal DNA; g, alpha-tubulin. Some hybridization occurred within the sample block at the top of the lanes. (B) FIGE gel, run as in Fig. 2, showing five bands in the Downs strain of *H. capsulatum* (left) and hybridizations to the same seven probes shown in panel A. Three *Schizosaccharomyces pombe* bands were separated on the same gel; hybridization results are shown for each of the three chromosomes (right).

the extent possible, the band mobilities and the maximum number of chromosomal DNAs present. Using blots of Downs strain chromosomal DNA, we were able to achieve hybridization of cloned probes (a through f) to each of the bands which had been separated by CHEF (Fig. 3A). A seventh probe, g, identified a band which comigrated with the f band in this CHEF gel; probes f and g corresponded to different chromosomal DNAs, as shown in the FIGE blot of Downs chromosomal DNA (Fig. 3B). In the FIGE separation, two doublets identified by probes a and b and by probes c and f occurred (Fig. 3B); in both cases, the corresponding bands were separated by CHEF electrophoresis (Fig. 3A).

Comparison of the CHEF and FIGE blot hybridizations of the Downs strain (Fig. 3A and B, respectively) revealed an inverse order of mobility for the first five bands (probes a through e). Since probes a through e identified the five largest DNA molecules in the Downs strain, in decreasing order of size (on the basis of CHEF mobility; Fig. 3A), their patterns of separation by FIGE (Fig. 3B) indicated that these five DNAs exhibited greater mobility with increasing size. The other two chromosomal DNAs (identified by probes f and g) appeared to migrate on the conventional limb of the FIGE separation curve (greater mobility with decreasing size) (Fig. 3B). In this FIGE separation, the inverse mobility of DNA molecules in the size range greater than that of the f and g bands of the Downs strain was confirmed by hybridizing chromosome-specific probes to the *Schizosaccharomyces pombe* bands separated on the same FIGE gel (Fig. 3B). The largest *Schizosaccharomyces pombe* DNA molecule, chromosome I, displayed the greatest mobility, and the smallest molecule, chromosome III, showed the least (Fig. 3B).

Number of chromosomes. The CHEF and FIGE techniques provided complementary information about the chromosome number in the Downs strain of *H. capsulatum* (doublets resolved with one technique were resolved with the other technique; Fig. 3). None of the random-probe hybridizations revealed any inconsistency in this determination of seven bands. In the absence of genetic linkage data, the number of chromosomes cannot be determined with certainty by these electrophoretic techniques, but our results imply that there are at least seven chromosomal DNAs in the Downs strain. Since the ploidy of the organism is unknown, the use of cloned probes was important in the determination of this number, as noncomigrating chromosomal homologs (if present) might produce misleading results with ethidium bromide staining alone. The marked differences in band mobility among *H. capsulatum* strains indicate that Downs may not be representative. Although we have encountered a recent clinical isolate with a FIGE banding pattern nearly identical to that of Downs (unpublished data), it is also true that some other clinical isolates more closely resemble strain G217B in their FIGE band patterns (unpublished data) or mitochondrial and ribosomal DNA restriction patterns (21).

This study represents a starting point for further investigations into the genome organization of *H. capsulatum*. Mapping of genes will be possible with blot hybridizations. The availability of cloned probes for each of the bands will be useful in comparisons of *H. capsulatum* strains by FIGE or CHEF gel electrophoresis. The use of cloned probes to track individual bands will also permit an investigation into the effects of switching-interval or field strength manipulations on band migration and resolution. An improved understanding of the effects of these parameters, particularly in the megabase range, should prove useful for a variety of electrophoretic karyotyping applications.

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