Electrophoretic Karyotype of the Pathogenic Yeast *Cryptococcus neoformans*

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The electrokaryotype of the pathogenic yeast *Cryptococcus neoformans* is described for the first time. Three different patterns were seen: (a) serotypes B and C (variety *gattii*) are similar and consist of nine chromosome mobility groups of > 580 kb; (b) serotype A (variety *neoformans*) revealed eight chromosome-like groups > 700 kb; (c) serotype D (the second serotype of variety *neoformans*) not only differs from those described above, but each D isolate tested showed a different distribution of bands. The discrepancy, and the importance of electrokaryotyping as a taxonomic tool, are discussed.

INTRODUCTION

Cryptococcus neoformans is a pathogenic yeast that causes cryptococcosis, growing preferentially in the central nervous system. This disease has a high mortality rate (Butler *et al.*, 1964) and is now occurring with increasing frequency among AIDS patients. According to a recent survey in the USA, 8.6% of AIDS victims developed cryptococcosis (Zuger *et al.*, 1986) and in African AIDS cases it is one of the most commonly observed opportunistic infections (Quinn *et al.*, 1986).

C. neoformans is an encapsulated basidiomycete with a known sexual stage and life cycle (Kwon-Chung, 1976*a, b*). There are two described taxonomic varieties of *C. neoformans*: var. *neoformans*, designated as either serotype A or D; and var. *gattii*, designated as serotype B or C (Kwon-Chung *et al.*, 1982*a*). Although extensive virulence studies on this yeast have been reported (Kwon-Chung *et al.*, 1982*b*; Kwon-Chung & Rhodes, 1986; Polacheck *et al.*, 1982; Polacheck & Kwon-Chung, 1988; Rhodes *et al.*, 1982), our knowledge of its genetic organization is currently limited to mutant isolation (White & Jacobson, 1985) and some genetic complementation tests (Whelan & Kwon-Chung, 1986).

By contrast, the genetics of the ascomycete *Saccharomyces cerevisiae* has been extensively studied. Chromosome mapping and genetic analysis indicate that the genome consists of 17 linkage groups. That 16 of these groups actually represent distinct chromosomes was confirmed by orthogonal field alternation gel electrophoresis (OFAGE) (Carle & Olson, 1985).

We have modified and adapted the OFAGE technique described for *Schizosaccharomyces pombe* (Smith *et al.*, 1987) to separate the chromosomal mobility groups of the four serotypes of *C. neoformans*. Determining the number of chromosomes in this important pathogen would have taxonomic value and would also permit the mapping of chromosome-specific probes for genetic analysis.

METHODS

Strains. All the strains used in this work were kindly provided by Dr I. F. Salkin (Wadsworth Center for Laboratory Research, New York State Department of Health, Albany, New York, USA) and by Dr K. J. Kwon-Chung (LCI, NIAID, NIH, Bethesda, Maryland, USA) (Table 1). The isolates were taken from diverse sources,

Abbreviation: OFAGE, Orthogonal field alternation gel electrophoresis.

Table 1. *Yeast strains used in this study*

Strain	Serotype	Source*	ATCC no.†
<i>Saccharomyces cerevisiae</i>			
2012	—	—	—
<i>Cryptococcus neoformans</i>			
var. <i>gattii</i>			
191	C	CSF; NIH	32608
CP110	C	CSF; Oklahoma	—
298	C	CSF; California	—
444	B	Sputum; NIH	32609
997	B	CSF; Brazil	—
365	B	CSF; Thailand	—
<i>Cryptococcus neoformans</i>			
var. <i>neoformans</i>			
372	A	Pigeon droppings; NIH	34870
38-1	A	CSF; NIH	34869
162	A	CSF; NYS	—
160	A	Blood; NYS	—
174A	A	CSF; NIH	—
12	D	Human bone lesion; NIH	28957
430	D	Pigeon droppings; Denmark	28958
B-3501	D	SB from cross 12 × 430	34873
433	D	Pigeon nest; NIH	34875
161	D	Sputum; NYS	—
157	D	CSF; NYS	—

* CSF, cerebrospinal fluid. SB, single basidiospore; NIH, National Institutes of Health; NYS, New York State Department of Health.

† ATCC, American Type Culture Collection.

and were identified using conventional procedures (Kwon-Chung & Fell, 1984). They all grew at 37 °C, were urea- and inositol-positive and nitrate-negative, and produced a brown pigment when grown on Niger seed agar. Furthermore, all were identified with the API 20C test and showed similarity in the assimilation pattern of at least 19 carbohydrates. Some of them showed variation, in assimilation of L-arabinose. The isolates were serotyped both with specific antibody reagents and with canavanine-glycine-bromthymol blue agar. All isolates were maintained on Sabouraud dextrose agar until used.

DNA preparation. Chromosomal DNAs were prepared from *Saccharomyces cerevisiae* by the method of Smith *et al.* (1987) and from *C. neoformans* as follows. An inoculum from an overnight culture was transferred into 100 ml yeast peptone dextrose (YPD) medium (10 g yeast extract, 20 g Bacto-peptone and 20 g glucose l⁻¹) to yield a density of 10⁴ yeasts ml⁻¹.

The yeast cells were grown overnight at 30 °C on a shaker up to 2 × 10⁷ yeasts ml⁻¹ (early exponential phase). Approximately 10 ml of cells was then washed twice in 50 mM-EDTA pH 7.5 and resuspended in 1 ml 20 mM-citrate/phosphate buffer pH 5.6, 50 mM-EDTA and 0.9 M-sorbitol, containing 2–8 mg NovoZym 234 ml⁻¹ (Novo Industrias, Bagsvaerd, Denmark). This suspension was incubated in a water bath at 30 °C. A 10 µl sample was removed every 15 min and mixed with an equal volume of 0.25 M-EDTA, 1% (w/v) SDS and checked for lysis under a phase-contrast microscope. When total lysis had occurred it was assumed that there was 90–100% protoplast formation; this usually occurred within 45–60 min. Alternatively, the protoplasts were stained with 0.1% methylene blue in 50 mM-phosphate buffer pH 7.0.

A 1 ml volume of protoplasts was mixed with 1 ml 1% low-melting-point agarose (BRL) made up in 0.125 M-EDTA pH 7.5. Then 1 ml of this mixture was placed in each of two wells of a 24-well tissue culture cluster (well diameter 16 mm) and allowed to set. Each block was cut into smaller pieces and these were mixed in a sterile tube with 5 ml 0.25 M-EDTA pH 7.5, 1% (w/v) SDS and incubated at 60 °C for 2 h. After incubation, the gel pieces were transferred to 3–5 ml 0.5 M-EDTA pH 9, 1% (w/v) sarkosyl and 1 mg proteinase K ml⁻¹ (Sigma) and incubated for 24 h at 50 °C. This step was repeated with a fresh solution and samples were stored at 4 °C in 0.5 M-EDTA pH 9. The modified method gave no significant background of partially degraded DNA molecules when used in OFAGE analysis.

OFAGE analysis. All the separations were carried out on 24 cm 1.5%, 100 ml agarose slab gels (Pharmacia agarose NA) with wells measuring 5 × 3 × 7 mm. The apparatus used was constructed in our institution according to the specifications of Carle & Olson (1984), with some modifications to the cooling system. Gels were

cast and run in $0.5 \times$ TBE buffer ($10 \times$ TBE is 0.89 M-Tris base, 0.89 M-boric acid, 25 mM-EDTA, pH 8). This buffer was maintained at 12–15 °C by circulating cooled water through glass spirals positioned in the tank on either side of the cooled gel platform. In addition the electrophoresis buffer was constantly circulated through the electrophoresis chamber with the aid of an external peristaltic pump.

Gels were run at 300 V (constant voltage) with a pulse time of 55 s for a period of 25 h. They were then removed and stained in 0.5 mg ethidium bromide ml⁻¹ for 45 min and after this destained for up to 2 d in numerous changes of $0.5 \times$ TBE buffer. Photographs were taken with Polaroid type 667 film using an ultraviolet transilluminator (Camag Reprostar, Switzerland).

RESULTS

The electrokaryotypes of 17 isolates of *C. neoformans* (Table 1) representing both varieties *gattii* and *neoformans*, each with their two respective serotypes, were studied. All the isolates were from diverse sources, having been obtained from different hosts, niches or geographical localities. In order to check the validity of the technique, at least one isolate of each serotype from the American Type Culture Collection was included.

The electrokaryotypes differed among the isolates of *C. neoformans* studied. The main differences were found between the two varieties. Fig. 1 shows the pattern of the chromosome mobility groups of variety *gattii* (lanes 1 and 2) compared with those of variety *neoformans* (lanes 3–5).

There was great similarity among the isolates of the variety *gattii*. The same band pattern was obtained when using isolates from either serotype C or B of this variety (Fig. 1, lanes 1 and 2). Each lane has nine bands, the smallest being around 580 kb and the largest >1.6 Mb as estimated from the *Saccharomyces cerevisiae* marker in lane 6 (Carle & Olson, 1984; Chu *et al.*, 1986; Mortimer & Schild, 1985). Isolates 191 and 444 were tested a number of times and the

Fig. 1. An OFAGE gel of *C. neoformans* chromosomal DNA. Lanes 1, 2, 3, 4 and 5 represent isolates 191 (serotype C), 444 (serotype B), 372 (serotype A), B-3501 (serotype D) and 12 (serotype D). Lane 6 represents *Saccharomyces cerevisiae* 2012, as a marker; the positions and sizes of its 13 chromosome bands are shown on the right. Sizes ≤ 0.7 Mb are as determined by OFAGE (Carle & Olson, 1985); sizes > 0.7 Mb are current physical estimates (Mortimer & Schild, 1985).

Fig. 2. (a) Electrokaryotype of *C. neoformans* var. *gattii*. Lanes 1, 2 and 3 represent isolates of serotype C: 191, CP110 and 298, respectively. Lanes 4, 5 and 6 represent isolates of serotype B: 444, 997 and 365, respectively. (b) Electrokaryotype of serotype A of *C. neoformans* var. *neoformans*. Lanes 2–6 represent isolates 38-1, 160, 372, 174A and 162, respectively. Lane 1 represents *Saccharomyces cerevisiae* 2012.

Fig. 3. Electrokaryotype of serotype D of *C. neoformans* var. *neoformans*. Lanes 2–7 represent isolates B-3501, 430, 433, 12, 161 and 157, respectively. Lane 1 represents *Saccharomyces cerevisiae* 2012.

results were consistent with those shown in Fig. 1. In addition we ran gels of a number of different isolates of serotypes B and C (listed in Table 1): all showed similar patterns with respect to the size and distribution of the chromosomal DNA elements (Fig. 1, lanes 1 and 2; Fig. 2a).

The variety *neoformans* showed a different and less consistent pattern. There was apparent

similarity among isolates of serotype A (Fig. 2*b*), while each of the six isolates of serotype D showed a different electrophoretic karyotype (Fig. 1, lanes 4 and 5; Fig. 3). Comparison of the running of the marker lanes in Figs 2 and 3 shows that the gels are comparable. With serotype A (Fig. 1, lane 3), there appear to be eight bands (the lower band is a doublet, as confirmed on other gels) the smallest being approximately 700 kb and the largest > 1.6 Mb. Lanes 4 and 5 of Fig. 1 show isolates of serotype D. These also appear to have eight bands in a range of 580 to > 1600 kb; however, the distribution of the bands is not the same as that seen in serotype A. To pursue this point further we ran gels of a number of isolates of both serotypes (listed in Table 1); Fig. 2(*b*) (lanes 2–6) shows the chromosomal distribution of five isolates of serotype A; they all appear quite similar. In contrast, each of six isolates of serotype D (Fig. 3, lanes 2–7) demonstrates a unique banding pattern. We have found no two isolates of serotype D that give the same band distribution. The good separation of the marker bands in Fig. 3 (lane 1) indicates that the differences found in band distribution are not a result of gel distortion.

In addition to the data presented in Figs 1–3, four more isolates of each serotype from diverse sources were tested for the electrophoretic karyotype (data not presented). All of them showed patterns similar to those described above.

DISCUSSION

C. neoformans is an encapsulated yeast belonging to the class Basidiomycetes, and has a different cell wall from other yeasts such as *Saccharomyces cerevisiae*, *Candida albicans* and *Schizosaccharomyces pombe*. We thus modified the published methods of DNA extraction (Carle & Olson, 1985; Smith *et al.*, 1987) by introducing a novel protoplast formation step. NovoZym was the only cell-wall-hydrolysing enzyme tested that produced satisfactory results. This method allowed chromosomes of *C. neoformans* to be visualized for the first time. Furthermore, the lysis steps outlined in the procedure of Smith *et al.* (1987) gave higher yields of DNA with this yeast than did the methods of Carle & Olson (1985) that we had tried previously.

A diversity of isolates was chosen for this study, rather than a group with similar origins and physiology. However, all the isolates show similarities and represent the range of characteristics within the current concept of the species complex (Kwon-Chung & Fell, 1984). The serotyping of *C. neoformans* is based mainly on the polysaccharide structure of the capsule (Bhattacharjee *et al.*, 1984). It is believed that there are discrepancies among the serotyping results from different laboratories. However, we found no major differences between the isolates obtained from the mycological laboratory in NIH and those obtained from the reference laboratory in New York State.

Our results demonstrate that *C. neoformans* possesses its own unique distribution of chromosome mobility groups when compared to those of the various species of yeast already published. *Schizosaccharomyces pombe* is reported to have only three extremely large chromosomes estimated at 3 Mb, 5 Mb and 7 Mb (Vollrath & Davis, 1987). The diploid yeast *Candida albicans* is reported to have a somewhat variable number of bands, between five and nine (Lott *et al.*, 1987; Magee & Magee, 1987; Snell & Wilkins, 1986). *Saccharomyces cerevisiae* chromosomal DNA has been resolved to 12–15 distinct bands of known sizes ranging from 260 kb (chromosome I) to approximately 2.2 Mb (chromosome XII) (Carle & Olson, 1984; Chu *et al.*, 1986; Mortimer & Schild, 1985). The *Saccharomyces cerevisiae* strain used as a marker in this study gave 13 bands.

Our study of the *C. neoformans* electrokaryotype revealed nine bands in the variety *gattii* and eight bands in the variety *neoformans*. These bands were well distributed over the size range of most of the *Saccharomyces cerevisiae* markers. This is in marked contrast to the band distribution seen in *Schizosaccharomyces pombe* and the majority of *Candida* species, where the chromosomes appear to be very large (Magee & Magee, 1987; Smith *et al.*, 1987). The definitive chromosome numbers for *C. neoformans* are not clear. Our gel system does not resolve chromosome XII of *Saccharomyces cerevisiae* (2.2–2.5 Mb) (Chu *et al.*, 1986; Vollrath & Davis, 1987). In addition, all the OFAGE gels appeared to have significant fluorescence at a position above the largest size marker (chromosome IV of *Saccharomyces cerevisiae*), indicating that large bands of *C. neoformans* may still be concealed at the top of the gel.

Our results show that isolates of different varieties of *C. neoformans* have different chromosome patterns, a feature unique to this species. In other yeasts such as *Candida* species the electrophoretic karyotype is species-specific (Lott *et al.*, 1987). The electrophoretic karyotype should perhaps be included as a taxonomic character for yeasts in the same manner as the G + C content, since it represents a major biological property. Our results would thus suggest that taxonomists should reconsider the status of the two varieties of *C. neoformans*. Although there is a significant DNA sequence homology between the two varieties (Kwon-Chung & Fell, 1984), they may represent two different species.

According to the literature, a cross between serotypes A and D always results in D phenotypes among the progeny (Bhattacharjee *et al.*, 1984). This may indicate dominance of the D phenotype; however, in contrast to this phenotypic dominance, serotype D is rarely found in clinical specimens compared to serotype A, which accounts for 75% of cryptococcosis in the USA (Kwon-Chung & Bennett, 1984). In general, when strains of opposite mating types of the different *C. neoformans* varieties are crossed no fertile progeny are found, with one exception – a cross between serotypes B and D gives about 30% viable progeny, all of which are of the D phenotype (Kwon-Chung *et al.*, 1982a; Kwon-Chung & Fell, 1984). The above data may explain why each isolate of serotype D tested gave a different chromosome band pattern. Some of the serotype D strains are perhaps the result of a cross between the two mating types of serotype D, while others may be the progeny of crosses between serotypes A and D or B and D.

The data presented here give an overall view of the physical structure of the cryptococcal genome. The use of isolates from diverse sources, including isolates from an international culture collection, and the similar pattern of the running of the *Saccharomyces cerevisiae* marker in each gel, indicates the reproducibility and validity of the OFAGE technique. We believe that the electrophoretic karyotype of pathogenic fungi will become an important taxonomic tool.

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