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Extensive Allelic Variation in *Cryptococcus neoformans*

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The orotidine monophosphate pyrophosphorylase (OMPPase) gene locus of the DNA of 13 *Cryptococcus neoformans* var. *neoformans* strains, including 10 recent clinical isolates, was studied by using restriction fragment length polymorphisms and nucleotide sequence analysis. The OMPPase locus (*URA5*) is highly polymorphic, and at least six alleles were identified. The nucleotide sequences of some alleles differed by up to 5%. The majority of the nucleotide polymorphisms in the protein-coding region occurred at the third codon position and were silent. The low frequency of replacement nucleotide substitutions relative to silent nucleotide substitutions implied that there is strong selection against amino acid changes in OMPPase. The allelic variation suggested that there is extensive genomic diversity among *C. neoformans* clinical isolates from one geographic area. The various alleles are potentially useful markers in the study of the population structure, epidemiology, and pathogenesis of *C. neoformans* strains.

Approximately 5 to 10% of patients with AIDS suffer life-threatening infections with *Cryptococcus neoformans*, a basidiomycetous yeast which has been divided into two varieties known as *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* (1, 5, 13–15, 26). In the United States, cryptococcal infections are caused primarily by *C. neoformans* var. *neoformans* strains (6, 19), but *C. neoformans* var. *gattii* strains are more common in other countries (8, 9).

Despite the medical importance of *C. neoformans*, relatively little is known about the genetic variation of clinical isolates. Restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA from 20 strains of *C. neoformans* showed that there is extensive heterogeneity among isolates (22). However, RFLP analysis of polymerase chain reaction (PCR)-amplified segments of the rRNA gene locus from 19 strains revealed no differences (23).

The aim of this work was to study the genetic variation among clinical strains and to develop molecular tools for classifying *C. neoformans* strains. *C. neoformans* is a fungus that reproduces sexually, and genetic exchange can occur in its natural habitat. This information is critical for understanding the population structure of clinical isolates. In addition, genetic markers are necessary for addressing such clinically important issues as whether relapse of cryptococcal meningitis is the result of new infection versus recrudescence of the original infection or whether patients are infected with more than one strain. The orotidine monophosphate pyrophosphorylase (OMPPase) (*URA5*) gene was chosen for study because it is present in a single copy and its sequence was available (7). Results of our study indicated that there is a surprising degree of allelic variation among 10 clinical strains from New York City.

MATERIALS AND METHODS

***C. neoformans* strains.** The strains from the American Type Culture Collection (ATCC; Rockville, Md.) used in this study were ATCC 24064 (serotype A) and ATCC 24067 and ATCC 52817 (serotype D). Strain GH was isolated from a patient with AIDS at the Bronx Municipal Hospital Center (Bronx, N.Y.) in 1989 (4). Strain R was isolated at the Rikers Island Prison in New York City and was the kind gift of Eran Bellin. Strain W was isolated from a patient with AIDS at the Weiler Hospital of the Albert Einstein College of Medicine. Strains J1, J2, J3, J4, J5, J6, and J7 were sequential isolates of *C. neoformans* from the Bronx Municipal Hospital Center obtained in February, March, and April of 1991. Each isolate came from a different patient. With the exception of the GH strain, which was streaked once to obtain a single colony, all clinical strains were streaked twice to obtain single colonies. Clinical isolates were identified as *C. neoformans* by the hospital microbiology laboratories by using standard criteria. Strains were maintained in Sabouraud agar at 4°C. All clinical isolates belonged to the *C. neoformans* var. *neoformans* group on the basis of their susceptibilities to L-canavanine (17).

DNA isolation. *C. neoformans* genomic DNA was made by using a modification of existing protocols (18, 22). Briefly, stationary-phase *C. neoformans* was washed two times in CPE (40 mM citric acid, 120 mM sodium phosphate, 50 mM EDTA [pH 6.0]) buffer and was then resuspended in 1.2 M sorbitol in CPE buffer. NovoZym 234 (Novo Biolabs, Bagsvaerd, Denmark) was added to a concentration of 10 mg/ml, and the suspension was incubated at 30°C for 1 h. The protoplasts were harvested, washed once with 1.2 M sorbitol in CPE buffer, resuspended in 0.1 M sodium citrate–10 mM EDTA (pH 5.8), and then lysed by adding sodium dodecyl sulfate to a concentration of 2% (wt/vol). After 1 h at 37°C, the lysate was heated to 65°C for 10 min. After pelleting the cell debris, the lysate was extracted three times with a chloroform-isoamyl alcohol (24:1) solution. DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethyl alcohol. The DNA was then resuspended in 0.01 M Tris-hydrochloride–0.001 M EDTA (pH 8.1), and the DNA was further purified by using a size exclusion column packed with Ultrogel AcA 22 (Pharmacia LKB, Piscataway, N.J.).

PCR. The OMPPase gene was amplified by using a thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) by using as

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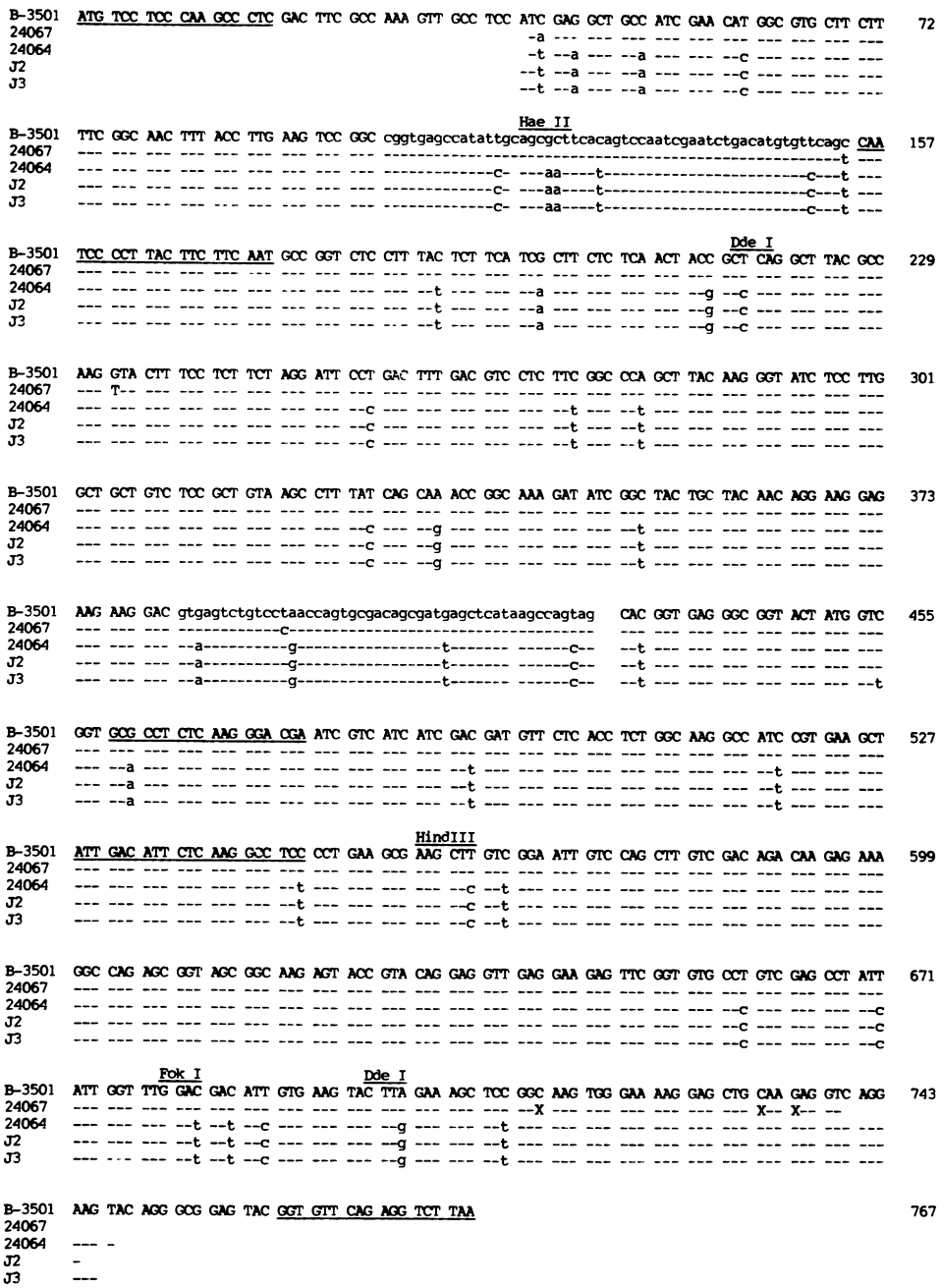

FIG. 1. Nucleotide sequence of the OMPPase gene from strains B-3501 (7), ATCC 24067, ATCC 24064, J2, and J3. A hyphen indicates that the base is identical to that in B-3501. A space implies that the base was not present in that allele. An X indicates an uncertain base. Only one replacement base change was found (position 233 in the sequence of strain ATCC 24067) and is indicated by a capital letter. Silent base changes are shown as lowercase letters. The sequences corresponding to the oligonucleotides primers used in this study are underlined. Sequences near the two amplification primers were not readable when these primers were used for sequencing. The restriction sites for restriction enzymes *Dde*I, *Hae*II, and *Hin*dIII in strains B-3501 and ATCC 24067 are shown. These sites were not present in the ATCC 24064, J2, and J3 gene sequences because of base changes. The *Fok*I site was present in the ATCC 24067, J2, and J3 gene sequences but not in that of B-3501 or ATCC 24067.

primers the oligonucleotides TTAAGACCTCTGAACACC and ATGTCCTCCCAAGCCCTC, which were designed from the published sequence (7). Oligonucleotides were made in the DNA synthesis facility of the Albert Einstein Cancer Center. Standard conditions for PCR were 92°C for 1 min, 42°C for 1 min, and 72°C for 1.5 min; this was performed for 35 cycles. PCR products were analyzed by agarose gel electrophoresis. The identities of the amplified bands were confirmed by blotting the gels (see below) and probing these blots with a [γ-^{32}P]dATP-labeled oligonucleotide (TCGTCCCTTGAGTGGCGC) complementary to an internal OMPPase sequence. Low stringency (35°C) conditions were used to detect PCR products containing sequence polymorphisms.

Southern blots. Restriction enzyme fragments were separated in 0.8 to 1.0% agarose gels and transferred to modified

nylon membranes by using 10× SSC (1× SSC is a 0.15 M NaCl plus 0.015 M sodium citrate solution). Blots were hybridized in a solution of denatured salmon sperm DNA (0.5 to 1.0 mg/ml) in 5× SSC–7% sodium dodecyl sulfate–10× Denhardt's reagent–10% dextran sulfate in 20 mM phosphate buffer (pH 7.2). Blots were probed with [γ-^{32}P]dATP-labeled oligonucleotides. The genomic DNA blot was probed with the gene from the strain ATCC 24064 PCR fragment in the pCR 1000 vector (see below) labeled with [^{32}P]dCTP by using random primers. Restriction enzymes, the nylon membranes, and the random primer kit were obtained from Boehringer Mannheim (Indianapolis, Ind.).

DNA sequencing. PCR-amplified OMPPase DNAs from strains ATCC 24064 and ATCC 24067 and clinical isolates J2 and J3 were sequenced after the PCR product was cloned into the pCR 1000 vector by using the TA cloning system (Invitrogen, San Diego, Calif.). DNA was sequenced by the dideoxynucleotide-chain termination method by using the Sequenase version 2.0 kit (United States Biochemical, Cleveland, Ohio).

RESULTS

The OMPPase genes of 10 clinical isolates (J1 to J7, GH, W, R) and three ATCC strains (ATCC 24064, ATCC 24067, and ATCC 52817) were studied. All strains were *C. neoformans* var. *neoformans*. The OMPPase gene is required to synthesize uracil, and cryptococci defective in this gene fail to grow on minimal salts (SD) medium lacking uracil (7). All 10 clinical isolates grew on SD medium lacking uracil, and therefore, all clinical isolates made a functional enzyme. For all the strains, PCR amplification of the OMPPase gene yielded a single band with the expected size of about 0.8 kb.

Figure 1 shows sequence data for the OMPPase genes from strains ATCC 24064, ATCC 24067, J2, J3, and B-3501 (7). The data revealed the existence of at least three alleles for this gene corresponding to those in B-3501 (7), ATCC 24064 (J2 and J3), and ATCC 24067. The gene from strain ATCC 24067 differed by four bases from that from strain B-3501 (7), of which only one resulted in a replacement substitution at amino acid 60 (valine to leucine). However, the genes from strains ATCC 24064, J2, and J3 had many base differences from that of B-3501 (and ATCC 24067), amounting to 5% of the determined sequence. The strain J2 sequence was identical to that of strain ATCC 24064 but differed by one silent base change from that of strain J3. All the base differences between strains B-3501 and ATCC 24064 in the protein-coding region occurred in the third position of the codon, and all were silent substitutions. Therefore, the primary OMPPase structures deduced from the B-3501, ATCC 24064, J2, and J3 DNA sequences are identical, indicating conservation of the enzyme structure, despite DNA sequence divergence.

Analysis of these five OMPPase gene sequences indicated restriction enzyme site differences. Specifically, there were two *Dde*I, one *Hae*II, and one *Hin*dIII site present in the B-3501 and ATCC 24067 genes; those sites were absent from the ATCC 24064, J2, and J3 genes (Fig. 1); in addition, the ATCC 24064, J2, and J3 genes had a *Fok*I site which was not present in the B-3501 and ATCC 24067 genes (Fig. 1). The presence of these predicted restriction sites was confirmed by digestions of PCR products. The OMPPase PCR products from the 10 clinical isolates (J1 to J7, GH, R, W) were subjected to RFLP analysis with the enzymes *Dde*I, *Hae*II, *Hin*dIII, and *Fok*I. Restriction digests of the PCR products

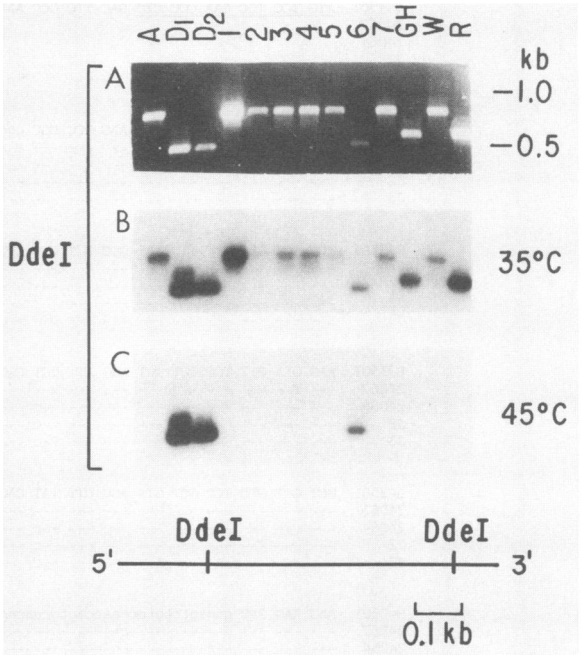

FIG. 2. *Dde*I analysis of OMPPases from strains ATCC 24064 (A), ATCC 24067 (D_1), ATCC 52817 (D_2), and 10 clinical isolates. The restriction map of B-3501 (7) and ATCC 24067, as predicted by their sequences. (A) A 1.2% agarose gel containing the *Dde*I digest of PCR products from the various strains after staining with ethidium bromide. Only the OMPPases from strains ATCC 24067 (D_1), ATCC 52817 (D_2), J6, GH, and R were cut by *Dde*I. The fragments from strains ATCC 24067 (D_1), ATCC 52817 (D_2), and J6 are consistent with the presence of both restriction sites. The OMPPases from strains GH and R lack the 3′ site. As predicted by their DNA sequences (Fig. 1), the OMPPases from strains ATCC 24064 (A), J2, and J3 were not cut by *Dde*I. The OMPPases from strains J1, J4, J5, J7 and W also lack *Dde*I sites. Panels B (35°C) and C (45°C) show blots of the gels probed at different temperatures with the oligonucleotide TCGTCCCTTGAGTGGCGC, which is complementary to an internal sequence of the strain ATCC 24067 gene but which has a 1-bp difference (G to A at position 460) from that of the ATCC 24064, J2, and J3 genes (see Fig. 1). This base difference allows binding to strains ATCC 24064, J2, and J3 at low stringency (35°C) but not at higher stringency (45°C). At 45°C, only ATCC 24067 (D_1), ATCC 52817 (D_2), and J6 bound the oligonucleotide, indicating sequence polymorphisms in this segment in strains J1 to J5, J7, GH, R, and W.

were probed with three internal oligonucleotides (Fig. 1) to ascertain the correct assignment of restriction sites. Figure 2 shows the data for the *Dde*I analysis (data for *Hae*I, *Hin*dIII, and *Fok*I analyses are not shown). As predicted from the DNA sequences, *Dde*I did not cut the PCR product from ATCC 24064, since that strain lacks *Dde*I sites; in contrast, the PCR products of both strains ATCC 24067 and ATCC 52817 were cut into fragments consistent with the presence of two *Dde*I sites. Of the 10 clinical strains, strains J1 to J5, J7, and W were not cut by *Dde*I; strain J6 contained two *Dde*I sites; and strains GH and R each had one *Dde*I site. To confirm the sequence polymorphisms for codon 119 (G to A change at nucleotide 460) in the B-3501 and ATCC 24064 sequences (Fig. 1) and to investigate the presence of polymorphisms in the other genes, a blot containing the *Dde*I restriction fragments of the 10 isolates was probed with an oligonucleotide complementary to that region in strain ATCC 24067 (Fig. 2). At 45°C, the oligonucleotide did not

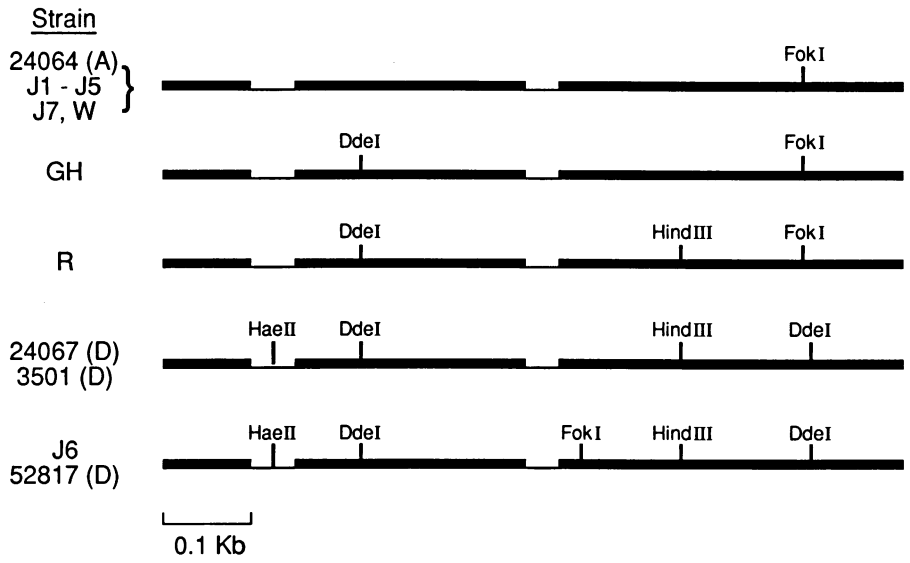

FIG. 3. Restriction map of enzymes *Dde*I, *Hae*II, *Hin*dIII, and *Fok*I for the various strains. The map was constructed by using agarose gel, DNA sequence, and oligonucleotide hybridization data. The heavy lines indicate the protein-coding regions, and the thin lines correspond to the introns. Part of the data for the *Dde*I analysis are shown in Fig. 2. The data for *Hae*II, *Hin*dIII, and *Fok*I are not shown. The ATCC 52817 allele may have a second *Fok*I site near the 3′ end of the gene. The map for strain B-3501 was deduced from its published sequence (7).

hybridize to the PCR products of strains ATCC 24064, J1 to J5, J7, GH, W, or R, confirming sequence polymorphisms for these strains relative to those for ATCC 24067, ATCC 52817, and J6 in this region.

Figure 3 shows the restriction map for the enzymes *Dde*I, *Hae*II, *Hin*dIII, and *Fok*I of the OMPPase genes analyzed in this study. Five different restriction patterns are evident, indicating that there are at least five distinct alleles. Strains assigned to a given group on the basis of RFLPs do not necessarily share the same allele. For example, the nucleotide sequences of the B-3501 (7) and ATCC 24067 genes differed by four bases, yet their restriction enzyme sites for these four enzymes were identical. The nucleotide sequence differences for strain B-3501 relative to the sequence of strain ATCC 24067 indicated a sixth allele for the OMPPase locus (Fig. 1). The significance of the single-base difference between strains J2 and J3 is uncertain. It could represent another allele of this gene, but the possibility of a *Taq* polymerase error cannot be excluded (see below).

Southern blot analysis of *Hin*dIII genomic DNA digests revealed additional sequence polymorphisms in the OMPPase gene-flanking sequences. For all strains except strain R, a single band was observed for the OMPPase gene. The size of the band was as follows: for strains ATCC 24067, ATCC 52817, and J6, 5.5 kb; for strains ATCC 24064, J1, J2, J4, J5, and GH, 5.0 kb; for strains J3, J7, and W, 4.0 kb; and for strain R, two bands of 2.7 and 2.0 kb. For strain R, the second band could be a second allele (as would occur in rare diploid strains [25]) or a *Hin*dIII polymorphism. Given that some strains have an intragenic *Hin*dIII site, one might expect the occurrence of two hybridizing bands; however, the presence of a *Hin*dIII site immediately 3′ of the gene (7) results in a small restriction fragment which was not present in our blots. For all strains except strain R, the occurrence of a single band strongly suggests that there is one type of OMPPase allele per strain. This is to be expected since the predominant *C. neoformans* yeast form arises from haploid basidiospores (14) and the overwhelming majority of clinical isolates are yeast forms of the α mating type (16).

DISCUSSION

The 10 *C. neoformans* clinical isolates were *C. neoformans* var. *neoformans*. This is consistent with reports that *C. neoformans* var. *neoformans* strains are responsible for the majority of infections in the United States (6, 19). Allelic variation at the OMPPase locus was detected by analysis of the nucleotide sequence and RFLP analysis of the PCR-amplified gene product and genomic DNA. Since the sequence data were obtained from PCR-amplified DNA, the possibility of sequence variation being an artifact of a *Taq* polymerase error must be a concern. However, four lines of evidence lead us to conclude that most, if not all, base differences are indeed genuine. First, in all cases where nucleotide differences were predicted to result in RFLPs for the enzymes used, the RFLPs were subsequently confirmed. Second, the nucleotide sequence variation found between the B-3501 (7), ATCC 24064, and ATCC 24067 genes was much greater than that expected from a *Taq* polymerase error, which is approximately 10^{-4} per nucleotide (10). Third, all except one of the base changes in the exons occurred at the third position in the codon and were silent mutations, and hence, they were unlikely to be random. Fourth, most of the base changes were shared by more than one gene (since each allele was amplified and cloned independently, the likelihood of a shared *Taq* polymerase error was remote). Although we cannot rule out the possibility that some base changes result from a *Taq* polymerase error, the conclusion that the OMPPase locus is highly polymorphic can be made on the basis of RFLP analysis of PCR-amplified genes alone.

We identified at least six alleles for the OMPPase-coding region on the basis of nucleotide differences. Flanking genomic DNA OMPPase RFLPs revealed additional differences between strains that were grouped together on the basis of their intragenic restriction patterns (such as strains J1 and J2). The OMPPase locus polymorphism contrasts with the absence of RFLPs reported for the ribosomal DNA genes (which were not sequenced) (23). This difference may

be explained either by the fact that there is a high degree of conservation in the ribosomal DNA loci or by the use of restriction enzymes, which did not reveal differences in those loci. Indeed, we were able to choose enzymes which had a high likelihood of revealing allelic differences only by comparison of the nucleotide sequences of several distinct alleles (Fig. 1). The intragenic sequence diversity for some alleles amounted to 5%. This degree of sequence variation was similar to that observed for alleles in gram-negative bacterial (2, 3) and *Drosophila* (12) populations. Despite significant nucleotide diversity between alleles, only one base difference which would result in an amino acid substitution (valine to leucine) was identified. The low frequency of replacement substitutions relative to the frequency of silent substitutions implies that there is strong selection against amino acid changes in the OMPPase protein.

C. neoformans can reproduce sexually (11, 13, 24). The issue of sexual versus clonal reproduction of pathogens is of medical interest because sexual reproduction has the potential for generating rapid genetic variation as a result of segregation and recombination. Virtually all environmental and clinical *C. neoformans* isolates are capable of mating (16). Recently, it has been suggested that *C. neoformans* may have a clonal population structure (21) on the basis of isoenzyme electrophoresis data (20). However, electrophoretic motility has limited sensitivity for detecting protein differences (3). Our data indicate that the OMPPase locus is highly polymorphic at the DNA level. Since we analyzed only one locus, we cannot make conclusions regarding the population structure of *C. neoformans* strains. However, the polymorphism of the OMPPase locus and the earlier mitochondrial DNA RFLP analysis provide evidence in favor of great diversity among *C. neoformans* strains.

The extensive allelic variation of the OMPPase locus provides another tool for studying the epidemiology of *C. neoformans* infections. One could use the variation of this locus to investigate whether patients are infected with more than one strain, to determine whether clinical relapses are a result of relapse of infection with the original strain or a new infection, and to investigate the relationship between environmental and clinical isolates.

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REFERENCES

1. **Aulakh, H. S., S. E. Strauss, and K. J. Kwon-Chung.** 1981. Genetic relatedness of *Filobasidiella neoformans* (*Cryptococcus neoformans*) and *Filobasidiella bacillispora* (*Cryptococcus bacillisporus*) as determined by deoxyribonucleic acid composition and sequence homology studies. Int. J. Syst. Bacteriol. **31:**97–103.
2. **Barcak, G. T., and R. E. Wolf, Jr.** 1988. Comparative nucleotide sequence analysis of growth-rate-regulated *gnd* alleles from natural isolates of *Escherichia coli* and from *Salmonella typhimurium* 1T-2. J. Bacteriol. **170:**372–379.
3. **Bisercic, M., J. Y. Feutrier, and P. R. Reeves.** 1991. Nucleotide sequences of the *gnd* genes from nine natural isolates of *Escherichia coli*: evidence of intragenic recombination as a contributing factor in the evolution of the polymorphic *gnd* locus. J. Bacteriol **173:**3894–3900.
4. **Casadevall, A., and M. D. Scharff.** 1991. The mouse antibody response to infection with *Cryptococcus neoformans*: V_H and V_L usage in polysaccharide binding antibodies. J. Exp. Med. **174:**1483–1491.
5. **Chuck, S. L., and M. A. Sande.** 1990. Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. N. Engl. J. Med. **321:**794–799.
6. **Diamond, R. D.** 1985. *Cryptococcus neoformans*, p. 1460–1468. *In* G. L. Mandell, R. G. Gordon, and J. E. Bennett (eds.), Principles and practice of infectious diseases. John Wiley & Sons, Inc., New York.
7. **Edman, J. C., and K. J. Kwon-Chung.** 1990. Isolation of the *URA5* gene from *Cryptococcus neoformans* var. *neoformans* and its use as a selective marker for transformation. Mol. Cell. Biol. **10:**4538–4544.
8. **Ellis, D. H.** 1987. *Cryptococcus neoformans* var. *gattii* in Australia. J. Clin. Microbiol. **25:**430–431.
9. **Ellis, D. H., and T. J. Pfeiffer.** 1990. Ecology, life cycle, and infectious propagule of *Cryptococcus neoformans*. Lancet **386:** 923–924.
10. **Erlich, H. A., D. Gelfand, and J. J. Sninsky.** 1991. Recent advances in the polymerase chain reaction. Science **252:**1643–1651.
11. **Jacobson, E. S., D. I. Ayers, A. C. Harrell, and C. C. Nicholas.** 1982. Genetic and phenotypic characterization of capsule mutants of *Cryptococcus neoformans*. J. Bacteriol. **150:**1292.
12. **Kreitman, M.** 1983. Nucleotide polymorphism at the alcohol dehydrogenase locus of *Drosophila melanogaster*. Nature (London) **304:**412–417.
13. **Kwon-Chung, K. J.** 1975. A new genus, *Filobasidiella*, the perfect state of *Cryptococcus neoformans*. Mycologia **67:**1197–1200.
14. **Kwon-Chung, K. J.** 1976. Morphogenesis of *Filobasidiella neoformans*, the sexual state of *Cryptococcus neoformans*. Mycologia **68:**821–833.
15. **Kwon-Chung, K. J.** 1976. A new species of *Filobasidiella*, the sexual state of *Cryptococcus neoformans* B and C serotypes. Mycologia **68:**942–946.
16. **Kwon-Chung, K. J., and J. E. Bennett.** 1978. Distribution of a and α mating types of *Cryptococcus neoformans* among natural and clinical isolates. Am. J. Epidemiol. **108:**337–340.
17. **Kwon-Chung, K. J., I. Polacheck, and J. E. Bennett.** 1982. An improved diagnostic medium for the separation of *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus neoformans* var. *gattii*. J. Clin. Microbiol. **15:**535–537.
18. **Restrepo, B. I., and A. Barbour.** 1989. Cloning of 18S and 25S rDNAs from the pathogenic fungus *Cryptococcus neoformans*. J. Bacteriol. **171:**5596–5600.
19. **Rinaldi, M. G., D. J. Drutz, A. Howell, M. A. Sande, C. B. Wofsy, and W. K. Hadley.** 1986. Serotypes of *Cryptococcus neoformans* in patients with AIDS. J. Infect. Dis. **153:**642.
20. **Safrin, R. E., L. A. Lancaster, L. E. Davis, and A. I. Braude.** 1986. Differentiation of *Cryptococcus neoformans* serotypes by isoenzyme electrophoresis. Am. J. Clin. Pathol. **86:**204–208.
21. **Tibayrenc, M., F. Kjellberg, J. Arnaund, B. Oury, S. F. Breniere, M.-L. Darde, and F. Ayala.** 1991. Are eukaryotic microorganisms clonal or sexual? A population genetics vantage. Proc. Natl. Acad. Sci. USA **81:**5129–5133.
22. **Varma, A., and K. J. Kwon-Chung.** 1989. Restriction fragment polymorphism in mitochondrial DNA of *Cryptococcus neoformans*. J. Gen. Microbiol. **135:**3353–3362.
23. **Vilgalys, R., and M. Hester.** 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J. Bacteriol. **172:**4238–4246.
24. **Whelan, W. L.** 1987. The genetics of medically important fungi. Crit. Rev. Microbiol. **14:**99–170.
25. **White, C. W., and E. S. Jacobson.** 1985. Occurrence of diploid strains of *Cryptococcus neoformans*. J. Bacteriol. **161:**1231–1232.
26. **Zuger, A. E. Louie, R. S. Holtzman, M. M. Simberkoff, and J. J. Rahal.** 1986. Cryptococcal disease in patients with the acquired immunodeficiency syndrome. Ann. Intern. Med. **104:**234–240.