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Genetic Study of Oxygen Resistance and Melanization in *Cryptococcus neoformans*

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Genetic analysis of oxygen-sensitive mutants of *Cryptococcus neoformans* revealed two loci (*oxy1* and *oxy2*) linking hyperoxia sensitivity to production of melanin, a known virulence factor. Hyperoxia-sensitive strain 562 (*oxy1 oxy2*) is albino and avirulent. *oxy2*-defective strains lacking the *oxy1* defect are melanin deficient but show normal hyperoxia resistance. Mutants defective at three additional mapped melanin loci fail to show hyperoxia sensitivity in the *oxy1* background. Revertants of strain 562, which regain the ability to synthesize melanin by mutation at suppressor sites unlinked to *oxy2*, retain the oxygen sensitivity conferred by their *oxy1* and *oxy2* defects. These data identify the melanin gene *oxy2* as unique in its association of hyperoxia resistance and melanization.

Leukocytes reduce molecular oxygen to deploy free radicals and other oxidants against microbes (1, 8, 10, 14). Possible defenses available to pathogens include a variety of antioxidants which protect cells during aerobiosis (13). While correlation studies of superoxide dismutase support its role in pathogenesis (3), the ability of pathogens to resist oxidant fluxes is poorly understood.

Cryptococcus neoformans is a yeast which causes opportunistic central nervous system infections. Studies with mutants suggest that cryptococcal virulence factors include the polysaccharide capsule (4, 5, 21, 22) and melanization (24, 27). However, the absence of mapping data relating the small number of published Mel$^-$ mutants to each other leaves it unproved that all melanin genes are virulence factors. Although cell-mediated immunity (6, 11, 26) is important in host resistance, killing of *C. neoformans* by macrophages often appears inefficient (9), either because of the relatively weak oxidative attack of this leukocyte (2, 9, 31) or because of fungal resistance to oxidants. To study the role of fungal antioxidants in pathogenesis, we have isolated a novel class of cryptococcal mutants sensitive to hyperbaric oxygen, which generates oxygen free radicals (20, 30, 32). We describe the isolation, genetic study, and virulence testing of oxygen-sensitive and albino mutants.

Fresh replica plates of colonies derived from UV-irradiated cells (15) of wild-type strain B-3501 (20) were subjected to pressurized oxygen (4 to 6 h, 25 atm [2, 532.25 kPa], 35°C) (12). The plates were removed and incubated overnight at 36°C to allow wild-type colonies to grow. From 15,000 screened colonies, a sensitive mutant (strain 557; phenotype termed Oxy$^-$) was isolated. This mutant grew normally in air and was not sensitive when hyperbaric nitrogen was substituted for oxygen (Fig. 1). To remove other UV-induced mutations, three consecutive purifying crosses of the mutant to wild-type (Oxy$^+$) strains B-3501 and B-3502 were performed (23). When random spores collected by micromanipulation were analyzed, the ratio of Oxy$^+$ to Oxy$^-$ colonies (15 Oxy$^+$, 14 Oxy$^-$) fit poorly the 3:1 ratio expected if the Oxy$^-$ phenotype depended on two independently segregating *oxy* genes ($P < 0.01$, chi-square test) but fit well that expected if it depended on the meiotic segregation of two alleles of a single chromosomal *oxy* locus ($P > 0.8$), which we have named *oxy1*.

To obtain strains of greater sensitivity, we repeated the UV-induced mutagenesis, this time in the *oxy1* background of strain 557. Of 30,000 colonies screened, several showed greater sensitivity (phenotype termed Oxy$^=$) (Fig. 1 and Table 1). Following three consecutive purification crosses to strain 557, each Oxy$^=$ strain was tested for known virulence factors by using the India ink stain for capsular production and *Guizotia* agar (29) for melanization. Although all strains appeared encapsulated in India ink smears, and mutants 557 and 564 synthesized melanin, Oxy$^=$ mutants 554, 555, and 562 were albino (Mel$^-$) (17).

Random spore analysis of loop-collected spores was performed with crosses of each Oxy$^=$ mutant to strain 557 so that the segregation of the new *oxy* mutations might be observed in a homogeneous *oxy1* background. None of the mutants 554, 555, 562, and 564 showed a ratio of Oxy$^-$ to Oxy$^=$ consistent with 3:1 (totals for four crosses, 79:77, 85:66, 96:55, and 85:75, respectively), suggesting that each bears one *oxy* mutation in addition to the *oxy1* lesion. The poor fit of mutant 562's segregation to the 1:1 ratio in these experiments ($P < 0.001$) was reinvestigated by analysis of micromanipulated spores, which segregated at 13 Oxy$^-$ and 20 Oxy$^=$, confirming single-gene 1:1 ratio segregation ($P > 0.2$). The second mutations of the Oxy$^=$ strains have been mapped by meiotic analysis to two loci distinct from *oxy1*, Mel$^-$ strains 554, 555, and 562 defining *oxy2* and strain 564 defining *oxy3* (17).

To determine the degree to which the slowed recovery of mutant colonies reflected killing, mutant and wild-type colonies growing on brain heart infusion containing glucose at 36°C were treated with oxygen for 9 h and suspended, plated, and allowed to grow into colonies. The plates were observed at regular intervals until survivors had grown into microcolonies of at least 30 cells. Colonies and dead cells were then scored microscopically. In each of three experiments, the viabilities of wild-type strain B-3501, Oxy$^-$ mutant 557, and Oxy$^=$ Mel$^-$ strain 562 paralleled the colony Oxy phenotypes, with the

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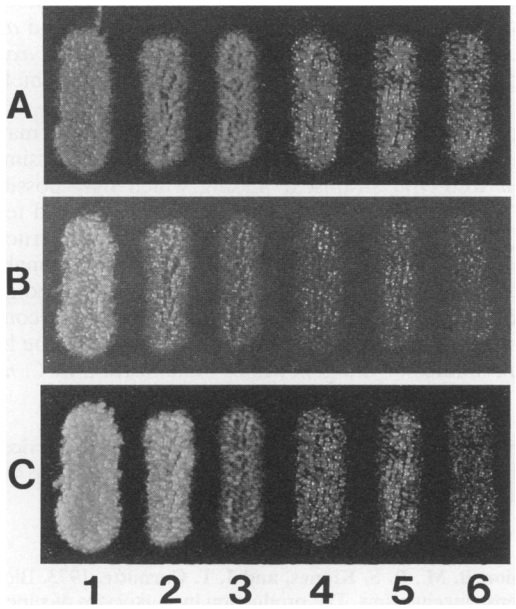

FIG. 1. Phenotypes of oxygen-sensitive mutants. Plates of identical colonies received the following treatments before incubation at 36°C: treatment with hyperbaric nitrogen (A); treatment with hyperbaric oxygen for 4 h, followed by a 16-h incubation at 36°C (B); and same treatment as described for panel B but with 12 additional h of incubation at 36°C (same plate as used for panel B) (C). Strains: 1, B-3501 (wild type); 2, 557 (Oxy^-); 3, 554 ($Oxy^=$); 4, 555 ($Oxy^=$); 5, 562 ($Oxy^=$); 6, 564 ($Oxy^=$).

following average percent survival values (± standard deviations), respectively: 62% ± 26.5%, 40% ± 26.6%, and 10.3% ± 6.13%.

To assess the relationship of the $Oxy^=$ phenotype to virulence, the virulences of strains 562 (*oxy1 oxy2*) and 564 (*oxy1 oxy3*) (17) were compared with that of the wild type (B-3502). For each strain, two groups of five mice (8 weeks old, general purpose Swiss females; National Cancer Institute) were inoculated in the lateral tail vein with either 10^6 or 10^7 cells per mouse. All strains were of mating type **a**. All mice infected with the wild type died by the third week, the average days of death at the 10^6 and 10^7 inocula being days 18 and 14, respectively.

TABLE 1. Genetic properties of cryptococcal strains used in this study

Strain	Phenotype	Genotype
B-3501[a]	Oxy^+ Mel^+	Wild type
B-3502[a]	Oxy^+ Mel^+	Wild type
609	Oxy^+ Mel^+	Wild type
557	Oxy^- Mel^+	*oxy1*
554	$Oxy^=$ Mel^-	*oxy1 oxy2*
555	$Oxy^=$ Mel^-	*oxy1 oxy2*
562	$Oxy^=$ Mel^-	*oxy1 oxy2*
562/r4	Oxy^- Mel^+	*oxy1 oxy2 oxy2*^{rev4}
564	$Oxy^=$ Mel^+	*oxy1 oxy3*
569	Oxy^+ Mel^-	*oxy2*
610	Mel^-	*mel1*
611	Mel^-	*mel1*
612	Mel^-	*mel2*
613	Mel^-	*mel3*

[a] National Institutes of Health strain.

Similarly, $Oxy^=$ strain 564 killed all mice (average days of death at the two inocula, days 11 and 6, respectively). However, $Oxy^=$ Mel^- strain 562 killed no mice by the end of the experiment (42 days). Thus, some, but not all, $Oxy^=$ strains exhibited decreased virulence; the mutant with impaired melanization exhibited defective virulence.

Genetic analyses were undertaken to clarify the relationship between melanin production and resistance to hyperoxia and to test the hypothesis that melanin deficiency per se confers hyperoxia sensitivity upon *C. neoformans*. In its simplest form, this model predicts a correlation between melanin defects and hyperoxia sensitivity, regardless of the particular genes affected. For example, according to this hypothesis, the hyperoxia sensitivity of strain 562 (*oxy1 oxy2*), with two mutations which appear to confer melanin pathway defects (17) and greater sensitivity than that shown by strain 557 (*oxy1*), would be explained as resulting from a further reduction in melanin production. To determine the phenotypes associated with the isolated *oxy2* defect, double mutant 562 was crossed to the wild type and an *oxy2* progeny strain (569) was identified by its ability to reconstitute the phenotypes of strain 562 ($Oxy^=$ Mel^-) when crossed to strain 557 (*oxy1*). However, quite unlike strain 557, which is phenotypically Mel^+ Oxy^-, strain 569 is Mel^- Oxy^+. The lack of correlation between melanin production and Oxy phenotype suggested that the degree of melanin production per se was an imperfect predictor of hyperoxia resistance. The lack of an Oxy phenotype associated with the isolated *oxy2* mutation suggested further that synergism with *oxy1* might be required to express $Oxy^=$ phenotypes associated with melanin defects at other loci.

To test these hypotheses, we obtained additional UV-induced Mel^- mutants of B-3501, isolated by plating UV-treated cells on *Guizotia* agar (29). If melanin production controls hyperoxia sensitivity, it might be expected that single mutant strains showing very low levels of melanin production would show resulting Oxy^- or $Oxy^=$ phenotypes. However, consistent with our finding for melanin mutant 569 (*oxy2*), none of the Mel^- mutants showed sensitivity to hyperoxia.

To identify melanin loci represented by the mutants, we undertook recombinational mapping of their melanin mutations. However, we found interfering allelic differences between the melanin genes of the commonly used but noninbred wild-type strains B-3501 (mating-type α) and B-3502 (mating type **a**). For example, a large proportion of the progeny of crosses between the two wild types is Mel^-. To prevent this phenomenon from confusing mapping analyses, we constructed a mating-type **a** wild type inbred to B-3501, beginning with a Mel^+ mating-type **a** strain from a cross between B-3501 and B-3502. This strain was then backcrossed to B-3501 to allow isolation of an **a** strain genetically closer to B-3501. Backcrosses were continued until an **a** strain (609) sufficiently isogenic with B-3501 was identified, as judged by uniformly Mel^+ progeny produced by its crosses to B-3501. Each melanin mutant was then crossed to strain 609, and Mel^- progeny representing mutant pairs were crossed for recombinational mapping. Nonallelic mutations were identified as those producing large numbers of Mel^+ progeny. This analysis identified three melanin genes (termed *mel1*, *mel2*, and *mel3*) affected in the four mutants tested (Table 2).

To explore further the analogy between the *mel* loci and *oxy2*, the ability of each *mel* mutation to confer the $Oxy^=$ phenotype in the presence of an *oxy1* mutation was compared with that of *oxy2*. When mutants representing each of the *mel* genes were crossed to strain 557 (*oxy1*), none produced $Oxy^=$ progeny. This suggests that the *oxy2* mutation is unique among

TABLE 2. Mapping of melanin mutations

Cross	No. of colonies observed	Mel^+ recombinants (%)	Genetic interpretation
610 × 611	100	1	$mel1^{610} \times mel1^{611}$
610 × 612	80	11	$mel1^{610} \times mel2^{612}$
610 × 613	100	29	$mel1^{610} \times mel3^{613}$
611 × 613	100	11	$mel1^{611} \times mel3^{613}$
612 × 613	100	20	$mel2^{612} \times mel3^{613}$

the tested melanin mutations in its ability to confer the $Oxy^=$ phenotype in an *oxy1* background.

The uniqueness of *oxy2* was confirmed through analyses of spontaneous Mel^+ revertants of strain 562 (*oxy1 oxy2*). If the $Oxy^=$ phenotype of strain 562 results from effects of the *oxy2* mutation on something other than melanin production per se, then Mel^+ revertants of strain 562 due to suppressor mutations at genes other than *oxy2* might retain their $Oxy^=$ phenotype. Of four revertants isolated, three remained $Oxy^=$ despite their regained abilities to synthesize melanin (562/r1, 562/r2, and 562/r3), consistent with earlier experiments suggesting a lack of correlation between melanin production and hyperoxia sensitivity. One strain (562/r4) coreverted the Oxy phenotype, becoming Mel^+ and Oxy^-. If *oxy2* were unique, among the melanin loci capable of reverting the melanin phenotype of 562, in its ability to confer sensitivity to hyperoxia, only reversion at the *oxy2* locus would be expected to corevert both phenotypes. To test this prediction, each Mel^+ revertant was crossed to strain 557 (*oxy1*) to map the reversion site relative to *oxy2*. Random spore analysis was then performed on at least 40 spore colonies collected by scraping with a loop. Mel^+ reversion at a gene other than *oxy2* was inferred by the finding of significant numbers of Mel^- progeny resulting from segregation of the *oxy2* and suppressor mutations, which is only expected if they are unlinked. Consistent with the hypothesis, only the reversion site in 562/r4 mapped to *oxy2*. As was found in the melanin mutant study, manipulating melanin production through mutation at sites other than *oxy1* and *oxy2* does not appear to affect sensitivity to hyperoxia.

Oxy mutants are of interest for several reasons. Initially chosen as a novel and convenient gaseous selection for oxidant-sensitive mutants, hyperoxia has allowed identification and study of a new gene, *oxy2*, implicated in fungal aerobiosis. Moreover, the pleiotropic phenotypes associated with *oxy2* mutations suggest that oxidant resistance is important in pathogenesis and that melanization, long a poorly understood virulence factor of *C. neoformans*, may serve as an antioxidant (17).

While the Mel^- and $Oxy^=$ traits are tightly linked to *oxy2* mutations in the *oxy1* genetic background (17), thus far the association between the traits appears unique to *oxy2* among the defective melanin genes tested and requires the *oxy1* lesion. Notwithstanding such qualifications, the implication of melanin in resistance to oxidants remains strong. Assays of the classical oxidant scavenger enzymes superoxide dismutase and catalase in Oxy mutants have indicated normal activities (18), while assays of melanizing enzymes indicated deficiencies along the melanin pathway (17). Secondly, direct evidence indicates a protective antioxidant role for endogenous or exogenous melanin against the exogenous anionic oxidants hypochlorite and permanganate but not against the neutral oxidant hydrogen peroxide (19). The implied direct antioxidant role of melanin in defending cells from exogenous oxidants contrasts with our present findings, which suggest that the mechanism of defense against endogenous oxidants associated with hyperoxia stress relies most heavily on *oxy1* and *oxy2*. It will thus be of interest to study further the roles of *oxy1* and *oxy2* and the specific mechanisms linking each with melanization.

In attempting to classify melanin genes by meiotic mapping, our early results were confounded by genetic dissimilarity between wild-type strains, a finding which may possibly be related to the regulation of melanization at elevated temperature (16). We approached this problem by constructing a mating-type **a** strain (609) inbred to B-3501 (National Institutes of Health). Using this inbred pair as a corrected genetic background, we resolved three melanin loci by recombinational mapping. These isogenic constructions should be helpful in future studies of the genetics of melanization in *C. neoformans*.

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