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Catecholamine Uptake, Melanization, and Oxygen Toxicity in *Cryptococcus neoformans*

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Oxygen sensitivity mutations of *Cryptococcus neoformans* were mapped to three genetic loci. Three oxygen-sensitive mutants had mutations that appeared allelic and exhibited albinism tightly linked to oxygen sensitivity; these three and a fourth exhibited defects in catechol uptake and catechol oxidation to melanin. Catecholamine metabolism appears to protect *C. neoformans* from oxidants.

Melanin is widely distributed among the fungi (26). In *Cryptococcus neoformans* (15, 22) and *Wangiella dermatitidis* (7), it has been related to virulence. However, its role in infection has been unclear.

Leukocytes produce antimicrobial oxidants (1, 14). However, many microorganisms have evolved defenses against oxidants, since oxidants are unavoidably associated with aerobiosis (3, 12); moreover, superoxide dismutases in *Nocardia asteroides* and *Histoplasma capulatum* may participate in the pathogenesis of infection (2, 13). We recently isolated hyperbaric-oxygen-sensitive chromosomal mutants of *C. neoformans*, a yeast which causes meningitis. Strain 557, which is moderately oxygen sensitive (Oxy^-), bears a single mutation, while the more sensitive ($Oxy^=$) strains 554, 555, 562, and 564 each bear second mutations in the background of strain 557. The Oxy phenotypes of these mutants are expressed as a lesser (Oxy^-) or greater ($Oxy^=$) delay in the growth of colonies after oxygen exposure. At least one Oxy mutant (an albino) is hypovirulent for mice. The isolation and preliminary characterization of these mutants will be reported separately (8). Here we link susceptibility to oxygen toxicity with defects in melanization.

Strains (Table 1) were stored at −70°C on brain heart agar. Random spore analysis allowed meiotic mapping (24). Oxygen traits of multiple colonies were scored on brain heart agar by replica stamping 24-h colonies, treating the fresh imprints with 25 atm (ca. 2,500 kPa) of oxygen in steel chambers (11) for 4 h at 35°C, and observing growth in air for 24 h. Melanin production in vivo was assessed on *Guizotia abyssinica* agar (16). Cultures for biochemical assays were grown at 37°C with agitation in asparagine medium (19). Phenoloxidase activity was assayed in toluene-ethanol-permeabilized cells (21), and catecholamine uptake was assayed in 0.05 mM L-dihydroxyphenylalanine (DOPA) containing 0.3 μCi of [^{14}C]DOPA (Research Products International Corp.) at 37°C (19). Biochemical values were normalized to dry weight. Statistical analysis was done by Student's one-tailed *t* test.

Crosses of double mutants to the wild type determined linkage between the first and second Oxy mutations. Crosses between double mutants were made to assess linkage between their respective second mutations. In each case, Oxy^- progeny (Fig. 1) indicated recombination. All progeny could be classified as parental or recombinant Oxy^-; no unpredicted types were seen. Reversion and partial complementation in diploids (27) were excluded as alternative explanations for Oxy^- colonies. The results (Table 2) revealed at least three *oxy* loci. All crosses between the double mutant ($Oxy^=$) strains 554, 555, and 562 produced infrequent (2%) recombinants, and all three were albino (Mel^-), suggesting that the three second mutations define a single locus, designated *oxy2*. When crossed with the wild type, all three yielded frequent (16%) Oxy^- recombinants, showing the *oxy2* locus to be well separated from *oxy1*, the locus of the single mutation which each double mutant shares with 557. The crosses involving double mutant 564 map the second mutation of this strain to a third locus, *oxy3*.

The linkage of the $Oxy^=$ and Mel^- traits was studied. The $Oxy^=$ albino strains 554, 555, and 562 were crossed to the Oxy^- pigmented strain 557, and 200 random progeny from each of the three crosses were scored for oxygen sensitivity and pigmentation. All 600 progeny exhibited the parental combinations of the Oxy and melanin traits (data not shown), suggesting that the two traits are pleiotropic effects of a single gene.

When tested for melanization, strains 557 and 564 exhibited normal pigmentation, while strains 554, 555, and 562 (the closely linked triplet) were albino (Fig. 2). DOPA uptake was markedly reduced in strain 557 and in the three albino double mutants (Table 1). Phenoloxidase activity was reduced by 60% in strain 557 and by 75 to 80% in the double mutants, 554, 555, and 562 ($P < 0.01$).

Current oxygen toxicology theory emphasizes toxic oxygen free radicals and protective free radical-scavenging enzymes (6, 10, 11, 23, 25). Notwithstanding, two of the three *oxy* loci in *C. neoformans* control melanization. Recalling the original characterization of melanin as a potentially protective, stable free radical (5), the free radical hypothesis of oxygen toxicity supports a protective role for melanin. The high concentration of polyphenolic rings in melanin has been proposed to be the locus of free radicals of the semiquinone type, greatly stabilized by the possibility of resonance throughout the highly conjugated polymer (17). The present report provides genetic support for the model, linking melanization with resistance to oxygen toxicity.

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TABLE 1. Origins and properties of yeast strains

Strain	Phenotype	Genotype	Source or reference	Phenoloxidase (U/mg [dry wt])[a]	Catechol uptake (nmol/min/mg [dry wt])
B-3501	Oxy$^+$ Mel$^+$	Wild type	NIH[b] B-3501	31.7 ± 13.5	0.18 ± 0.08
B-3502	Oxy$^+$ Mel$^+$	Wild type	NIH B-3502	26.7 ± 9.4	0.22 ± 0.11
557	Oxy$^-$ Mel$^+$	*oxy1-219 oxy2*$^+$	8	12.6 ± 5.7	0.04 ± 0.03
554	Oxy$^=$ Mel$^-$	*oxy1-219 oxy2-85*	8	6.6 ± 4.4	0.02 ± 0.03
555	Oxy$^=$ Mel$^-$	*oxy1-219 oxy2-227*	8	4.3 ± 2.2	0.03 ± 0.02
562	Oxy$^=$ Mel$^-$	*oxy1-219 oxy2-233*	8	7.3 ± 1.7	0.01 ± 0.04
564	Oxy$^=$ Mel$^+$	*oxy1-219 oxy3-261-1*	8	ND[c]	ND

[a] Values are reported as mean ± standard deviation.
[b] NIH, National Institutes of Health.
[c] ND, Not done.

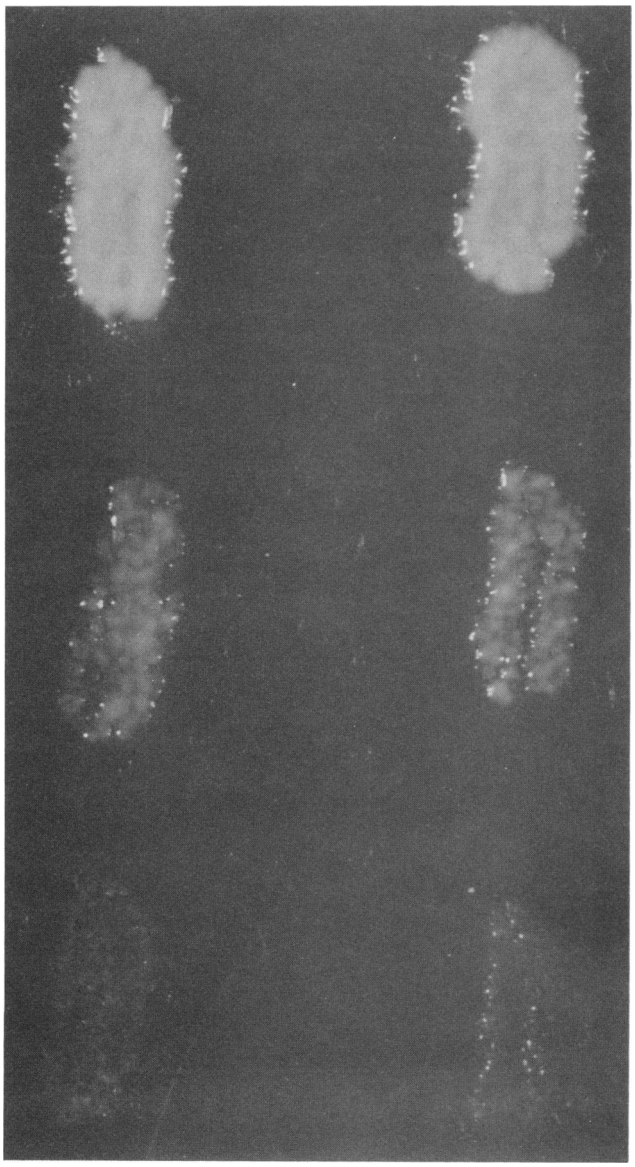

FIG. 1. Oxygen phenotypes of progeny of sexual cross of strain 562 with strain B-3501 16 h after exposure of fresh replica plate to hyperbaric oxygen (in duplicate). Top row, Oxy$^+$ parentals; middle row, Oxy$^-$ recombinants; bottom row, Oxy$^=$ parentals. Conditions are described in text. All colonies grew at equal rates on a control plate incubated continuously in air (not shown).

Since albinism and oxygen sensitivity map within 0.17 centimorgans (a typical genic width [9]), they are most probably pleiotropic effects of a single gene. Thus, mutations selected for oxygen sensitivity resulted in albinism. The converse argument is also made, namely, that cryptococcal mutations selected for albinism confer oxidant susceptibility (20). The evidence for a relationship between melanization and oxidant resistance thus seems very strong.

Melanization in *C. neoformans* requires exogenous catecholamines; the pathway comprises only the steps of uptake and initial oxidation (19). Both activities were reduced in most oxygen-sensitive mutants. The Oxy$^-$ strain 557 exhibited an 80 to 90% decrease in L-DOPA uptake and a 60% decrease in phenoloxidase activity, leading to the inference that the genetic locus *oxy1* may be concerned with both functions. The Oxy$^=$ double mutants, strains 554, 555, and 562, exhibited a further 50% decrease in phenoloxidase activity, implying that *oxy2* contributes to expression of the phenoloxidase. The degree of oxygen resistance in any particular strain appears proportional to the phenoloxidase activity.

Oxygen sensitivity in the single mutant, strain 557, may not be adequately explained by 60%-reduced phenoloxidase activity. However, catecholamines react with oxygen radicals (18) and norepinephrine can function as an intracellular antioxidant (4), so the fivefold decrease in catecholamine uptake in Oxy strains may contribute to oxygen sensitivity. Thus, the genetic association of oxidant resistance, catecholamine uptake, melanization, and decreased virulence supports the hypothesis that catecholamine metabolism allows *C. neoformans* to resist leukocytic oxidants.

TABLE 2. Mapping of oxygen sensitivity mutations

Cross	No. of colonies observed	Oxy$^-$ recombinants (%)
wt[a] × 564	100	19
wt × 554	92	16
wt × 555	40	17
wt × 562	100	16
554 × 555	200	3
554 × 562	200	2
555 × 562	200	2
564 × 554	100	30
564 × 555	100	27
564 × 562	100	35

[a] wt, Wild type.

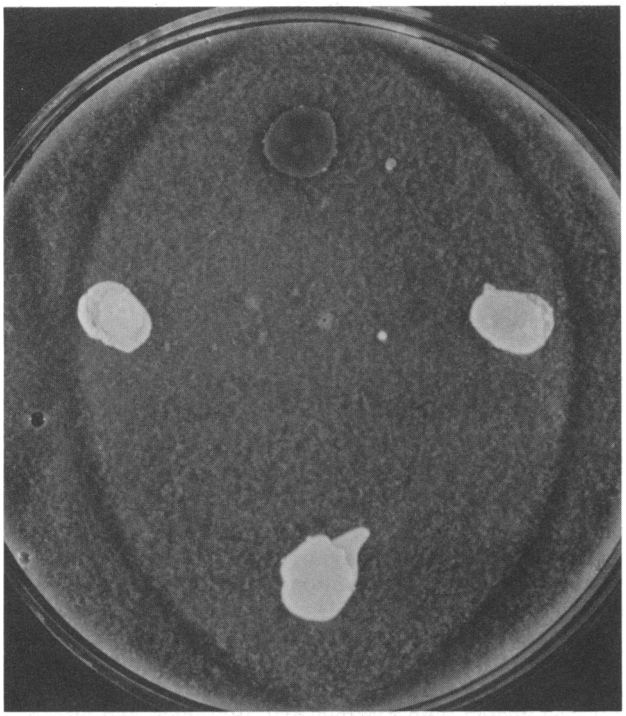

FIG. 2. Pigment formation in oxygen-sensitive mutants after 6 days at 37°C. Strains tested (clockwise from top): B-3501 (wild type), 554 ($Oxy^{=}$), 555 ($Oxy^{=}$), and 562 ($Oxy^{=}$).

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