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Dominant Spore Color Mutants of *Aspergillus nidulans* Defective in Germination and Sexual Development

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The ascomycete *Aspergillus nidulans* produces green conidia (asexual spores). Recessive mutants which produce yellow conidia have been previously isolated from haploid strains and have been shown to be deficient in laccase (diphenol oxidase), an enzyme that requires copper for activity. Using a diploid parent strain, we isolated dominant yellow conidial mutants which, in the haploid state, produced even less laccase activity than a recessive mutant. Three isolates of such mutants behaved similarly and define a single complementation group (*yB*) on chromosome VIII distinct from the *yA* locus on chromosome I defined by recessive mutants. Unlike *yA* mutants, whose only discernable phenotype is their conidial color, *yB* mutants are pleiotropic: conidial germination was delayed relative to the wild type, and sexual development was blocked at an early stage. The three phenotypes of *yB* mutants were expressed on yeast extract-glucose medium containing 1.6 μM of added copper. When copper was added to above 5 μM, all three phenotypes were remediated, and near wild-type levels of laccase were produced. We conclude that *yB* mutants have a reduced availability of copper. The dominance of *yB* mutants could result, for example, from an alteration in transport or storage of copper. Using an immunological assay, we detected no laccase antigenic cross-reacting material in *yB* mutants grown on medium of low copper content. We conclude that either the synthesis or the stability of laccase is copper dependent.

During its relatively simple life cycle the ascomycete *Aspergillus nidulans* produces morphologically well-defined and biochemically differentiated structures in a highly synchronized manner. One differentiated cell type is the conidium or asexual spore which, when mature, contains a dark green pigment not present in the vegetative hyphae.

Several classes of mutants with altered spore color have been isolated; one such class produces bright yellow spores (*y* mutants). Clutterbuck (3) has shown that *y* mutants are deficient in the enzyme laccase (*p*-diphenol oxidase), which in the wild type first appears shortly after the initiation of conidiation. The mutants studied by Clutterbuck are recessive to wild type and allelic to one another, defining a single gene *yA* on chromosome I. The fact that a thermosensitive *y* mutant produces a thermosensitive laccase strongly suggests that the *yA* gene codes for at least part of the amino acid sequence of this enzyme (3).

A phenotypically similar class of mutants, yellow-green (*yg*), is also deficient in laccase but is distinct in chromosomal location and other properties from *y* mutants (3, 8). Clutterbuck (3) showed that *yg* mutants, unlike *y* mutants, exhibit laccase activity in vivo if cultures are grown at low pH and in vitro if extracts are supplemented with high levels of copper salts. This latter finding suggests that, like other laccases, the *Aspergillus* laccase requires copper and that the abnormal spore color of *yg* mutants results from a copper-deficient enzyme. Other spore color mutants, *fawn*, *pale*, *chartreuse*, and *white* produce near-normal amounts of laccase, but appear to be altered in the quantity or quality of the pigment precursor (3). *White* mutants are epistatic to all other spore color mutants and thus appear to be blocked at an early step of the pigmentation pathway.

Because the expression of the *yA* gene is temporally regulated and tissue specific, spore color mutants of *A. nidulans* are potentially useful tools with which to study eucaryotic gene regulation. We thus sought new classes of mutants defective in laccase activity with the aim of identifying regulatory loci. The approach we employed takes advantage of the fact that *A. nidulans*, although normally haploid, can exist as rare but stable diploids. In such diploid strains, mutants which can express a yellow-

spore phenotype would be expected to be dominant to the wild type. In this report we describe a new class of semidominant yellow-spore mutants which are deficient in conidial laccase activity and blocked in sexual development. The properties of these mutants suggest that their primary and secondary phenotypes can be explained by a reduction in the availability of copper, a metal which is required by the conidial laccase and by other phenoloxidases that function in sexual development.

MATERIALS AND METHODS

Strain genotypes and gene symbols. Genetic markers carried by the mapping strains of *A. nidulans* have been described by Clutterbuck (5). Strains with the prefix FGSC were obtained from the Fungal Genetics Stock Center. Those used for meiotic recombination analysis are FGSC-270 (*ygA1, biA1*), FGSC-399 (*facB101 riboB2 sE15*), FGSC-484 (*AcrA1; lysB5;sD85 fwA2 facC102*), and FGSC-502 (*sulA1; AcrA1;facA303 lys5*; *sB3*; *chaA1*). Strain FGSC-375 (*suA1adE20, biA1;AcrA1;sA4;pyroA4*; *pA2;lacA1*; *nicB8*; *riboB2*), which has a marker on each of the eight chromosomes, was used to assign mutations to linkage groups by mitotic haploidization. Strains R-21 (*yA2 pabaA1*) and R-153 (*wA3; pyroA4*) were obtained from C. F. Roberts (1). Strain GO-247 (*ygA6; biA1*) was obtained from A. J. Clutterbuck and behaved in all respects like FGSC-270, which carries the *ygA1* allele of the *ygA* locus. Strains with the prefix WIM were constructed in our laboratory.

We used the system of gene symbols suggested by Clutterbuck (4), whereby mutants representing different loci, but having the same primary phenotype, are distinguished by a capital letter following the primary symbol. Thus the yellow-spore mutants described and mapped in this study were designated *yB* to distinguish them from the classical *yA* mutants, which define a different locus.

Genetic methods. General techniques were those of Pontecorvo et al. (21). The yellow-spore strain R-21 and the white-spore strain R-153 were crossed to obtain the green-spore recombinants WIM-69 (*pabaA1*) and WIM-70 (*pyroA4*). These recombinants were used to construct diploid strain WIM-144, from which the dominant yellow-spore mutants (*yB*) were derived. To obtain mutants, suspensions of WIM-144 diploid conidia were exposed to UV light to give 10% survival and plated to give 10^5 survivors per plate. After 2 to 3 days of incubation at 37°C, yellow conidial heads were picked from the green conidial lawn. After purification of the putative mutants by single-colony isolation, haploid yellow-spore strains were obtained by plating the heterozygous diploid conidia on medium containing 0.7 μg of benomyl per ml. Haploid yellow-spore segregants were selected visually by the presence of yellow sectors. The haploid strains so derived were designated WIM-72, WIM-141, and WIM-142, which carry the *yB1*, *yB10*, and *yB35* mutations, respectively. WIM-71 is an alternate haploid *yB1* strain used in certain experiments.

Media. The basic complete growth medium (YG) in most experiments was composed of 0.5% yeast extract (Difco Laboratories, Detroit, Mich.) and 2% glucose. For plates, the medium was solidified with 1.5% agar (Difco). During these studies it was noted that conidial color varied with the particular lot of ingredients used. This variation was eliminated by the addition of trace elements at the micromolar concentrations used by Cove (7): B, 0.4; Cu, 1.6; Fe, 4.3; Mn, 4.3; Mo, 3.2; Zn, 28. Experiments for which this supplemented medium (YGT) was used are indicated in table and figure legends. The defined minimal medium contained 0.01 M sodium nitrate as the nitrogen source and has previously been described (7).

Preparation of cell-free extracts. A 4-ml amount of liquid YG or YGT medium containing 10^5 conidia was layered over 20 ml of YG or YGT agar in a (100 by 15 mm) petri dish. After a 48-h incubation at 37°C the conidiating mycelial mat was peeled from the agar and homogenized in a Ten Broeck tissue grinder with 4.0 ml of extraction buffer (10 mM Tris-hydrochloride [pH 7.4], 1.0 mM EDTA, 10 mM *p*-tosyl-L-arginine methyl ester) containing freshly added phenylmethylsulfonyl fluoride (1 mM). After the removal of particulate material by centrifugation for 20 min at 25,000 × *g*, the extract was used immediately for laccase assays.

Laccase assay. Extracts were assayed for laccase by the colorimetric method of Clutterbuck (3), which employs the chromogenic substrate *N,N*-dimethyl-*p*-phenylenediamine sulfate (DMP). One unit of activity catalyzes the oxidation of 1.0 μmol of DMP per min. For specific activity (units per milligram of protein), protein was determined by the method of Lowry et al. (19) or by the Bio-Rad protein assay. Because absolute activities can vary with different batches of medium, strain comparisons were always performed with the same batch of plates.

Immunological methods. Antibody against purified conidial laccase (manuscript in preparation) was induced in rabbits by intradermal injection with complete Freund adjuvant (12). To remove antibody against possible contaminants in the antigen the serum was preadsorbed with a crude extract prepared from an aconidial mutant that makes no detectable laccase. The gamma globulin fraction was partially purified by precipitation with ammonium sulfate (12). After dialysis against 0.0175 potassium phosphate buffer (pH 6.9), the antibody was used undiluted in Ouchterlony double-diffusion assays (20).

Gel electrophoresis. Discontinuous gel electrophoresis in 7.5% polyacrylamide was performed by the method of Laemmli (13) except that sodium dodecyl sulfate was omitted from the sample and running buffer. Extracts were diluted twofold with sample buffer (0.125 mM Tris-hydrochloride [pH 6.8] containing 20% glycerol) and run at 30 mA until the tracking dye reached the bottom of the gel. Laccase activity was detected by the DMP staining procedure of Clutterbuck (3).

Sexual development. The ability of mutants to develop sexually was assessed by visual observation of cleistothecia and the associated hülle cells under cultural conditions that optimize the sexual development of the wild type. Deep petri plates (100 by 20 mm) containing 50 ml of YGT agar were overlaid with 1 ml

of YGT broth containing 10^5 conidia and incubated at 37°C in the dark. Hülle cells, which appear in grapelike clusters, were scored at 90 h, and cleistothecia were scored after 144 h. The presence of laccase activity associated with hülle cells and cleistothecia (laccase II) was detected by flooding a chloroform-treated mycelial lawn with a chromogenic substrate (1.3 mg of 4-amino-2,6-dibromophenol per ml, 0.3% 3,5-dimethyl aniline in 30% ethanol). Foci with laccase II activity developed a dark blue-green coloration after about 30 min at 37°C.

RESULTS

Isolation of a dominant yellow-spore mutant. Starting with the parental diploid strain WIM-144 (*yA*$^+$ *pabaA1*/*yA*$^+$ *pyroA4*), we isolated dominant yellow-spore mutants by plating large numbers of UV-mutagenized conidia and stabbing those areas on the green lawn which exhibited yellowish conidial heads. The frequency of such variants was about 1 per 10^6 conidia plated, and most isolates had maintained the diploid state as evidenced by the segregation of green sectors on complete medium where the maintenance of diploidy is not required for growth.

One selected diploid yellow-spore variant (WIM-143) was grown in the presence of benomyl to obtain yellow-spore haploid segregants. One such paba-requiring segregant (WIM-72) was grown with a *yA*$^+$ pyridoxine-requiring strain (WIM-69) to reconstruct a heterozygous diploid. This reconstructed diploid produced, as expected, yellow conidia, thus confirming the dominant character of the yellow-spore mutation, which was designated *yB1*. Two additional mutants, *yB10* and *yB35*, were isolated by the same procedure.

To determine whether the yellow conidial color of strains carrying the *yB1* mutation was due to reduced laccase activity or some other defect in pigment formation, crude extracts of various diploid and haploid strains were assayed for laccase activity. The laccase activity of the haploid strain (WIM-72) which carries the *yB1* mutation was less than 5% of its diploid parent (WIM-144) (Table 1). The activity of WIM-72 was, in fact, lower than that of strain R-21, which carries the *yA2* mutation in the structural gene for laccase. In the diploid strains heterozygous for *yB1*, laccase activity was 20 to 30% of the parent diploid level, showing that the dominance was only partial. The partial dominance was, in fact, reflected by the greenish cast of the yellow conidia of the heterozygous diploids. By comparison, the diploid *yA2*/*yA*$^+$ had near wild-type diploid activity, thus confirming the recessive character of *yA* mutations and their difference from *yB* mutations.

That the decreased laccase activity resulting from the *yB1* mutation was not due to a diffusable inhibitor is shown by the experiment of Table 2, in which laccase activity of a wild-type extract was assayed in the presence of various amounts of a *yB1* extract. No significant inhibition was seen even when the *yB1* extract was fourfold greater than the wild-type extract. Furthermore, extensive dialysis of a *yB1* extract against Tris buffer did not significantly increase laccase activity.

Chromosomal location of *yB* mutations. The chromosomal location of *yB1* was determined by analysis of haploid segregants from a diploid strain formed from WIM-72 (which carries *yB1*) and the mapping strain FGSC-375, which has a marker on each of the eight chromosomes. It was found that *yB1* always segregated with the *riboB*$^+$ marker of chromosome VIII and is thus clearly unlinked to the classical *yA* locus on chromosome I (Table 3). The fact that all recombinant classes were recovered also

TABLE 1. *Laccase activity in extracts of conidial color mutants*

Strain	Relevant genotype		Conidial color	Laccase activity (U/mg of protein)
	yA locus	*yB* locus		
WIM-70	+	+	Green	1.91
WIM-144	+/+	+/+	Green	1.55
WIM-143	+/+	*yB1*/+	Yellow-green	0.51
WIM-72/WIM-69	+/+	*yB1*/+	Yellow-green	0.34
WIM-72	+	*yB1*	Yellow	0.05
WIM-71/WIM-72	+/+	*yB1*/*yB1*	Yellow	0.05
R-21	*yA2*	+	Yellow	0.17
R-21/WIM-69	*yA2*/+	+/+	Green	1.10

TABLE 2. *Laccase activity of mixed extracts of the wild type and a yB mutant*

Source of extract	Ratio of extracts	Laccase activity (U/ml)[a]		
		Calculated	Observed	% Inhibition
WT			0.89	
yB1			0.09	
yB1 + WT	1:1	0.53	0.48	9
			0.51	4
yB1 + WT	2:1	0.35	0.35	0
			0.37	0
yB1 + WT	4:1	0.18	0.16	11
			0.16	11

[a] Extracts of strain WIM-70 (wild type) and WIM-72 (*yB1*) were assayed spectrophotometrically before and after mixing. Calculated values for mixed extracts assume simple additivity of the activities of the two extracts.

TABLE 3. *Mitotic segregation of yB1*[a]

Linkage group	Strain of origin	Marker	No. of haploids with genotype:	
			yB1	*yB1*$^+$
I	FGSC-375	+	11	14
	WIM-72	*pabaA1*	15	8
II	FGSC-375	*AcrA1*	3	3
	WIM-72	+	23	19
III	FGSC-375	*sA4*	10	17
	WIM-72	+	16	5
IV	FGSC-375	*pyroA4*	13	13
	WIM-72	+	13	9
V	FGSC-375	*pA2*	NS[b]	13
	WIM-72	+	NS	7
VI	FGSC-375	*lacA1*	16	11
	WIM-72	+	8	11
VII	FGSC-375	*nicB8*	9	13
	WIM-72	+	17	9
VIII	FGSC-375	*riboB2*	0	22
	WIM-72	+	26	0

[a] The haploid strains of the heterozygous diploids were *pabaA1; yB1*. The genotype of the mapping strain is given in the text. Haploid segregants with yellow conidia were considered *yB1*, whereas green haploids were scored as wild type. The *pale* phenotype could not be distinguished in a *yB1*-containing strain. Linked markers assort only into the two parental types, as seen for linkage group VIII.

[b] NS, Not scored.

shows that strain WIM-72 is translocation free relative to FGSC-375.

To further localize the site of the *yB1* mutation, WIM-72 was crossed with various strains carrying chromosome VIII markers. The meiotic recombination frequencies given in Table 4 indicate that *yB1* is closely linked to both *facB* and *riboB*, giving about 10% recombination with each. The other two independently isolated *yB* mutants, *yB10* and *yB35*, gave recombination frequencies of 10% and 7%, respectively, with *facB*. It is therefore likely that all three *yB* mutations are closely linked to one another. Furthermore, diploids of *yB10* and *yB35* with *yB1* produced levels of laccase activity characteristic of *yB1/yB1* diploids rather than the much higher level characteristic of *yB1/yB*$^+$ heterozygous diploids (Table 5). The three dominant yellow-spore mutations thus appear to belong to the same complementation group.

Remediation of *yB* mutants by copper. Copper is an essential component of all known laccases (18), and indeed we have found that purified conidial laccase from *A. nidulans* is a blue protein (unpublished data). To explore the possibility that *yB* mutants have a copper deficiency, *yB1* and other control strains were grown on YG agar containing various amounts of copper sulfate. The conidia produced by *yB1* were observed to undergo a color transition from yellow to green as the copper concentration was increased (Table 6). The other two *yB* mutants behaved identically to *yB1* in this respect. The yellow-green mutant *ygA1* also showed this transition of conidial color but at a considerably higher concentration of copper (10 to 15 μM) than the *yB* mutants (4 to 6 μM). The heterozygous diploid *yB1/yB1*$^+$ produced green conidia at a higher copper concentration than that required by the wild type but at a lower concentration than that of the haploid *yB1*, consistent with the semidominant nature of the *yB1* mutation. By contrast, the *ygA/yg*$^+$ diploid had the same response to copper as the wild-type, showing that the *ygA* mutation is recessive. It is to be noted that a mutant defective in the structural gene for laccase (*yA2* in strain R-21) produces yellow conidia regardless of the copper concentration.

Figure 1 shows the laccase activity as a function of copper concentration in the growth medium for some of the strains discussed above. The response curves generally reflected the transitions in conidial color tabulated in Table 6. Included for comparison is the wild-type homozygous diploid which, at low copper concentrations, was depressed in laccase activity relative to the haploid, but at high concentrations approached haploid activity. This haploid/diploid difference is also seen in the data of Table 1.

TABLE 4. *Meiotic recombination frequencies of yB1 with various markers on linkage group VIII*[a]

Marker	No. of recombinants/total scored	Recombination frequency (%)
sD85	35/79	44
fwA2	41/80	51
facC102	27/63	43
facB101	21/202	10
riboB2	20/202	10
chaA1	112/221	51

[a] Crosses between WIM-72 (*yB1*) and strains carrying markers on linkage group VIII were analyzed for recombination. Several control crosses between strains carrying *facB101* and *riboB2* gave 3 to 6% recombination of these two markers.

TABLE 5. *Laccase activity in yB diploid strains*

Relevant genotype	Laccase activity[a] (U/mg of protein)
yB1/yB1	0.007
yB35/yB1	0.008
yB10/yB1	0.007
yB$^+$*/yB1*	0.073
yB$^+$*/yB*$^+$	0.370

[a] Extracts were prepared from mycelia grown on YGT medium.

TABLE 6. *Effect of copper on conidial color of haploid and diploid strains*[a]

Genotype	Conidial color with copper supplement to YG medium of (μM):											
	0.0	0.2	0.4	1.0	2.0	4.0	6.0	8.0	10	15	20	30
Haploids												
Wild type	yg	g	g	g	g	g	g	g	g	g	g	g
yB1	y	y	y	y	y	yg	yg	g	g	g	g	g
ygA1	y	y	y	y	y	y	y	y	yg	yg	g	g
yA2	y	y	y	y	y	y	y	y	y	y	y	y
Diploids												
+/+	yg	g	g	g	g	g	g	g	g	g	g	g
yB1/+	y	y	yg	g	g	g	g	g	g	g	g	g
ygA1/+	yg	g	g	g	g	g	g	g	g	g	g	g
yA2/+	yg	g	g	g	g	g	g	g	g	g	g	g

[a] Abbreviations: y, yellow; g, green; yg, yellow-green. Copper was added in the form of $CuSO_4 \cdot 5H_2O$.

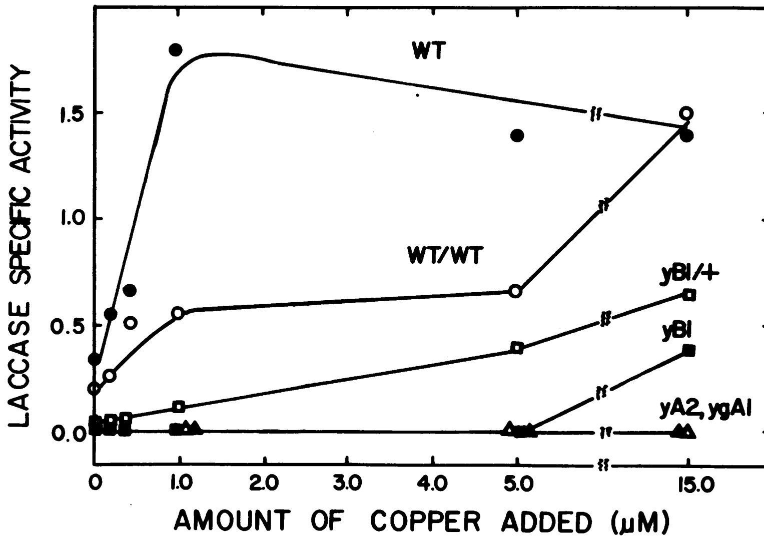

FIG. 1. *Effect of copper in the growth medium on laccase activity. Extracts were prepared from mycelia grown on YG medium supplemented with various concentrations of* $CuSO_4 \cdot 5H_2O$. *Symbols:* ●, *haploid wild type;* ○, *diploid wild type;* ■, *haploid yB1;* □, *heterozygous diploid yB1;* △, *haploid ygA1;* ▲, *haploid yA2.*

The diploid state thus appears to decrease copper availability, though not to an extent that affects conidial color.

The remediation of *yB* mutants was specific to copper. The addition to YG medium of five other commonly supplemented trace elements, Zn, Fe, B, Mo, and Mn, at concentrations of 10 μM or greater did not cause *yB1* to produce green conidia. Remediation did result, however, from the addition of NaCl, but only at concentrations greater than 100 mM, which is some 10^4-fold higher than the reversing concentration of copper. On minimal medium, which has the same copper concentration as YGT, *yB* mutants produced green conidia. Neither the buffering salts nor the copper in this medium was of sufficient concentration alone to reverse the phenotype when added to YG but did so in combination.

Pleiotropy of *yB* mutants. When observed 1 to 3 days after conidial inoculation of YG plates, colonies of haploid strains carrying either of the three *yB* mutations were significantly smaller than their wild-type parents. Measurements of radial growth rate (Fig. 2) showed that the smaller colony size was due to a delay in the initiation of linear growth rather than a reduced growth rate. Microscopic examination of conidia incubated in submerged liquid culture further revealed that the observed delay was due to retarded germination. In crosses, small colony size always segregated with yellow conidial color. Furthermore, several rare green-spore revertants of *yB1* were found to have the colony size of the wild type. Thus the *yB* mutation itself, rather than some extraneous lesion, was responsible for the retardation. This retardation was not exhibited by yB^+/yB diploid conidia. The

lack of apparent dominance of this *yB* phenotype may merely mean that the germination is less sensitive to copper deficiency than is conidial pigment formation. A more interesting alternative explanation will be discussed below.

A second pleiotropic effect of *yB* mutations was a severe impairment of sexual development exhibited by both haploid and heterozygous diploid strains. The rare cleistothecia which were produced appeared much later and were much smaller than the wild type. Both yellow-green mutants *ygA1* and *ygA6* were also blocked in sexual development but were not retarded in growth initiation. Interestingly, the globose hülle cells which developed in association with cleistothecia were produced abundantly by *yB* and *ygA* mutants.

Recent studies in our laboratory, to be reported elsewhere, have identified a laccase associated with hülle cells and the cleistothecial wall. This enzyme, designated laccase II, is electrophoretically distinct from the conidial laccase (laccase I) and can be detected in situ by application of a chromogenic substrate directly to colonies. In contrast to *yA* mutants, which produce laccase II, we found the hülle cells of *yB* and *ygA* mutants to be devoid of this activity.

As indicated in Table 7, which summarizes the pleiotropic phenotypes of the *yB* mutants, copper at a concentration that remediates the spore color phenotype also remediated the delay in growth initiation and sexual impairment.

Immunological assay of laccase in *yB* mutants. The reversal of the phenotype of *yB* mutants by growth on medium containing elevated levels of copper suggests that *yB* mutants may synthesize an inactive form of laccase at low copper concentrations. To test this possibility the presence of laccase antigenic cross-reacting material was assayed by the Ouchterlony double-diffusion method, using antibody prepared against pure laccase. No laccase cross-reacting material was detected in concentrated extracts of *yB1* or *ygA1* when grown on medium containing 1.0 μM copper, but was clearly present in an equivalent wild-type extract even when

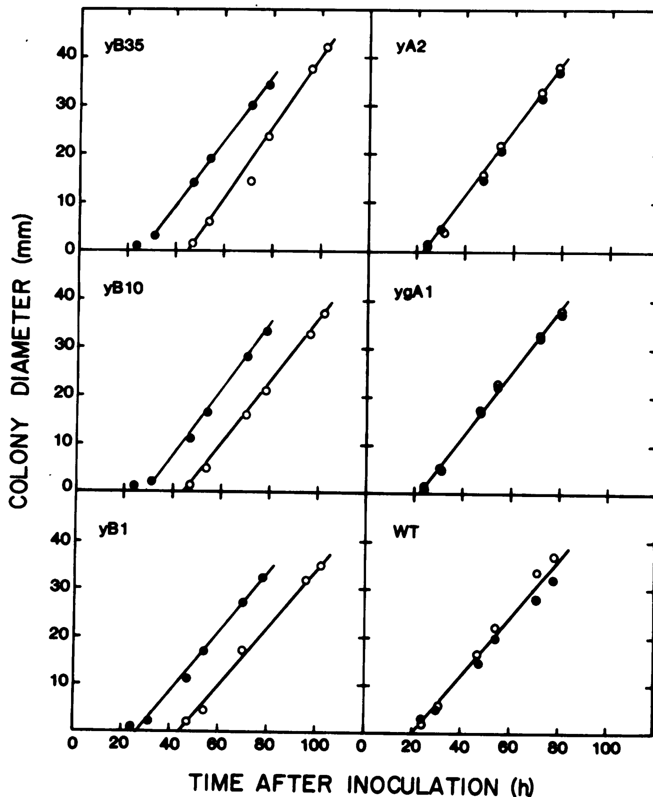

FIG. 2. *Radial growth rates of conidial color mutants. Agar plates were inoculated with less than five conidia and incubated at 37°C. Colony diameters were measured at various times after inoculation. Each measurement is the average of five colonies. Symbols:* ●, *YG medium supplemented with 10* μ*M* $CuSO_4 \cdot 5H_2O$*;* ○, *unsupplemented YG medium.*

diluted 10-fold (Fig. 3). These results suggest that copper deficiency either prevents synthesis of the enzyme, or if synthesized, results in its rapid degradation. This conclusion assumes that the antigenic determinant of the enzyme is not altered by the lack of copper. We have not yet been able to prove that this is the case but have found that neither heat-denaturation nor removal of carbohydrate alters the antigenic reactivity of purified laccase.

Figure 3 also shows that the laccase made by *yB1* when grown on medium containing 15 μM copper was antigenically identical to the wild-type enzyme. It is noteworthy that the standardly used *yA* mutant, *yA2*, showed no evidence of laccase cross-reacting material even at high copper concentrations.

An extragenic suppressor of *yB35*. Revertants of *yB* mutants were sought as a means of identifying additional loci that participate in the control of laccase activity. On a confluent lawn of yellow conidial heads, revertants can be detected as rare greenish patches. The majority of isolates from *yB1* proved to be unstable, giving yellow conidia upon subculture. Stable revertants were most easily obtained from *yB35*, the leakiest of the three *yB* mutants. One of these, *yB35-r9*, was chosen for further characterization.

An electrophoretic comparison of the laccase produced by *yB35-r9* with the wild-type enzyme is shown in Fig. 4. Although there may be a slight difference in mobilities, the activity of the revertant, like that of the wild-type enzyme, is under the control of the *yA* locus as shown by the fact that a *yB35-r9 yA* double mutant totally lacked the activity. The activity of the revertant is thus unlikely to be due to the substitution of some unrelated phenoloxidase. The apparent mobility difference seen in Fig. 4 may be real since studies to be reported elsewhere show that the conidial laccase exhibits a microheterogeneity that depends on the activity of other genes of the conidial pigmentation pathway.

To determine whether *r9* was a second-site mutation at the *yB* locus, a diploid was formed between *yB35-r9;pabaA1* and FGSC-399, a strain which carries a *riboB* marker closely linked to yB^+. Upon haploidization, yellow-spore segregants were obtained, indicating that the *r9* mutation is not on the same chromosome as *yB35* (chromosome VIII). As expected, the *riboB* marker was found only with the fully green conidial progeny. Furthermore, yellow-spore recombinants carrying the *pabaA* allele were obtained, showing that *r9* is not on the same chromosome as *paba* (chromosome I) and is thus not a mutation in the laccase structural gene *yA*.

In a backcross of *yB35-r9* with a wild-type strain, we obtained a class of fully green-spore

TABLE 7. *Pleiotropy of conidial color mutants*

Conidial color mutant	Property expressed at two concn of copper									
	Conidial laccase		Laccase II		Hülle cells		Cleistothecia		Germination	
	1.6 μM	10 μM	1.6 μM	10 μM	1.6 μM	10 μM	1.6 μM	10 μM	1.6 μM	10 μM
Wild type	+	+	+	+	+	+	+	+	Normal	Normal
yB	−	+	−	+	+	+	−	+	Delayed	Normal
ygA	−	−	−	−	+	+	−	−	Normal	Normal
yA	−	−	+	+	+	+	+	+	Normal	Normal

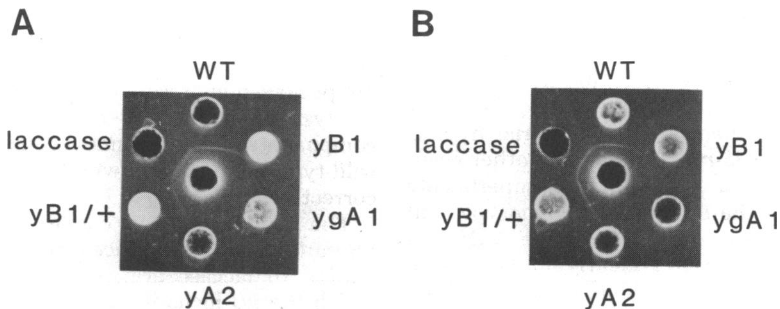

FIG. 3. *Ouchterlony immunoassay of laccase cross-reacting material. Crude extracts prepared as described for the laccase assay were concentrated 10-fold by lyophilization. Each well contained 10 μl of extract except in the case of the wild type where the extract was diluted 10-fold. The antiserum had a titer of at least 1:8 against purified laccase and was used undiluted. (A) Extracts from mycelia grown on YG medium supplemented with 1.0 μM $CuSO_4 \cdot 5H_2O$. (B) Extracts from mycelia grown on YG medium supplemented with 15 μM $CuSO_4 \cdot 5H_2O$. Purified laccase (2.0 μg) was included as a control in each assay.*

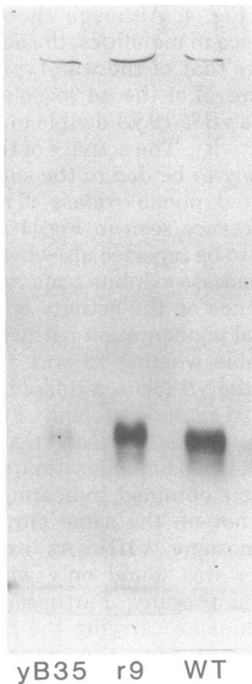

FIG. 4. *Electrophoretic comparison of conidial laccase of the wild type, the pseudorevertant yB35-r9, and yB35. An equal amount of laccase activity was used for the wild type and r9. The amount of total protein added to the gel for yB35 was four times that of the wild type. The gel was stained to visualize laccase activity. Extracts were prepared from mycelia grown on YGT medium.*

recombinants which exhibited a greater profusion of aerial hyphae than did the wild type. The suspicion that this segregant carried the *r9* suppressor was confirmed by a backcross of this segregant to *yB35*. The progeny obtained represented the four predicted classes: *yB35* (yellow), *yB35-r9* (yellow-green), wild type (green), and *r9* (green with aerial hyphae).

Segregants carrying the suppressor *r9* produced very tiny cleistothecia and thus appeared to be almost as defective in sexual development as *yB* mutants themselves. The aerial hyphae produced by *yB35-r9*, *r9* alone, and other pseudorevertants is a characteristic superficially reminiscent of the ve^+ (*velvet*) phenotype controlled by a locus on chromosome VIII. Although the *r9* mutation is clearly not located on chromosome VIII, the possibility that ve^+ alleles suppress *yB* mutations is being explored.

DISCUSSION

The *yB* mutants are a new class of yellow-spore laccase-deficient mutants of *A. nidulans*. The three isolates we studied have semidominant mutations located on chromosome VIII in the *facB-riboB* region and define a single complementation group. These mutants are retarded in conidial germination and are impaired in sexual development.

The remediation of the *yB* mutant phenotype by trace amounts of copper suggests that the laccase deficiency of these mutants results from a reduced availability of copper due, for example, to a defect in transport or storage. In *Neurospora crassa* a low-molecular-weight copper-binding protein (metallothionein) appears to be responsible for the storage of copper (17). Although not yet described in *A. nidulans* such metallothioneins are ubiquitous; the *Neurospora* polypeptide, in fact, has a considerable degree of sequence homology with the Cd-Zn metallothionein from human liver. The reduction or elimination of such a chelator by mutation would likely be a recessive trait. On the other hand, a mutation which caused overproduction of a chelator, or a chelator with greater binding affinity, would decrease copper availability and could be dominant or semidominant.

For several fungi, including *A. nidulans*, it has been shown that conidial germination is dependent on the retention of certain high-affinity iron chelators (siderophores), which are easily lost by the conidia in a medium of high salt concentration (2, 11). Although no specific copper-requiring enzyme has been implicated in the germination process, the fact that the delay in germination of *yB* mutants is overcome by copper suggests the existence of such an activity. The copper required by the conidia may be supplied by a metallothionein or by a non-protein copper chelator analogous to the siderophores. In this context it may be relevant that although *yB* haploids are delayed in germination, yB/yB^+ diploids are not, even though the *yB* mutation is dominant for its other phenotypes. This result could be understood if a *yB* mutation causes the production of a chelator with greater affinity for copper than that of the wild type, but which is not packaged in or is more easily lost by conidia. Analysis of copper content and copper-binding components of *yB* mutants in comparison to the wild type should reveal whether these ideas are correct.

The fact that, at low copper concentrations, *yB* mutants fail to produce protein antigenically related to laccase argues against a model in which the *yB* locus is the structural gene for a second subunit of laccase. Indeed, a recent report (14) suggests that the conidial laccase of *A. nidulans* probably has but one subunit. The absence of laccase cross-reacting material in *yB* mutants is not understood but suggests that either the enzyme is not synthesized under con-

ditions of copper deficiency or that a copper-deficient enzyme is rapidly degraded. In this regard it is noteworthy that mammalian metallothioneins are induced by specific metal ions and that the control is at the level of transcription (6).

The previously described *ygA* mutants (8) are similar in many respects to our *yB* mutants. The two classes differ, however, in dominance, chromosomal location, and copper concentration required for phenotypic remediation. Clutterbuck (3) has reported that dialysis of an extract of a *ygA* mutant against copper salts restores laccase activity. This would appear to be at variance with our finding that *ygA* mutants have no laccase cross-reacting material when the yellow-spore phenotype is expressed. However, we have never been able to demonstrate restoration of laccase activity by the addition of copper to *ygA* extracts that lack cross-reacting material. Growth conditions may well exist that result in an inactive but copper-restorable laccase.

An additional interesting property of *yB* mutants is their inability to complete sexual development. Hülle cells are produced in abundance, but laccase II, which in the wild type first appears in hülle cells, and cleistothecia are absent. The remediation of both of these defects by copper suggests that laccase II or some other unidentified copper enzyme or both is required for cleistothecial morphogenesis. The role of laccase II in sexual development is currently under study. In a screen for mutants showing aberrations in sexual development, many acleistothecial mutants have been found that still produce laccase II. However, all mutants that failed to produce laccase II were also acleistothecial.

A similar correlation between sexual morphogenesis and phenoloxidase activity has been shown for the ascomycete *Podospora anserina* (9, 10) and the basidiomycete *Schizophyllum commune* (15, 16). In *Podospora*, of 14 nonallelic mutants defective in melanin production, 8 are also defective in the formation of perithecia, and all 8 have an altered phenoloxidase spectrum. The sterile mutants *zonata* and *flexuosa*, for example, have two low-molecular-weight laccases instead of the high-molecular-weight laccase of the wild type. Studies with various developmental mutants of *Schizophyllum* also suggest that phenoloxidases are essential for basidiocarp formation. Phenoloxidase inhibitors have been shown to block basidiocarp development at very early stages without affecting vegetative growth (16).

A further examination of extragenic suppressors of *yB* mutations may provide additional insights on the control of laccase synthesis. If, in fact, copper availability is reduced in *yB* mutants, suppression could occur by reducing competition for copper. The *r9* suppressor of *yB35* may indeed be a representative of this class since this mutation by itself reduces cleistothecial production and may thereby eliminate competing copper-requiring reactions of the sexual cycle. If reversion of *yB* mutants is generally due to second-site mutations with phenotypes similar to *r9*, such revertants should provide a rich source of mutants blocked in sexual development. Several additional suppressors of *yB* mutants do, in fact, show sexual abnormalities.

Our initial impetus for isolating dominant spore color mutants was the possibility that regulatory mutants analogous to the rare super-repressor mutants of procaryotes might be obtained. Although the relatively frequent *yB* mutants are probably not of this class, the finding that these mutants are remediated by copper provides a way to screen for rarer dominant classes with the same phenotype.

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