Pigment Production by *Cryptococcus neoformans* and Other *Cryptococcus* Species from Aminophenols and Diaminobenzenes

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Cryptococcus neoformans and other *Cryptococcus* species can produce pigment(s) from many aminophenol and diaminobenzene compounds. Pigment production from these compounds is similar to the conversion of diphenols to melanin by *C. neoformans*. Several pigmentation patterns (resulting in the identification or grouping of *Cryptococcus* species) have been observed by using diaminobenzene and aminophenol compounds as substrates. The most common pigmentation pattern observed was pigment production by both *C. neoformans* and *C. terreus*. In contrast to the diphenols, only two aminophenols (4-hydroxymetanilamide and 3-aminotyrosine) were found to be highly specific as substrates. They allowed only *C. neoformans* to produce pigment. When 4-aminosalicylic acid was the substrate, a unique pattern was observed because only *C. terreus*, *C. diffluens*, and *C. albidus* produced pigment. Finally, a pattern was observed in which *C. neoformans* produced large amounts of pigment from aminophenol and diaminobenzene compounds, whereas the other *Cryptococcus* species produced smaller amounts. A simplified scheme with three substrates resulted in the identification of *C. terreus* and *C. neoformans* as well as two groups of other *Cryptococcus* species, group I (*C. albidus* and *C. diffluens*) and group II (*C. laurentii* and *C. luteolus*).

Cryptococcus neoformans is the only yeast known that consistently produces a melanin or melanin-like pigment from many *o*- or *p*-diphenols (2, 4, 9, 11). Pigment production from diphenols by other members of the genus is very rare. However, pigment production by some *Cryptococcus* species can occur when hydroquinone is the substrate (2). *Cryptococcus* species (9) may also produce small amounts of pigment from diphenols after prolonged incubation (10 days or more). Finally, occasional biochemical variants of some *Cryptococcus* species have been observed to pigment from a specific diphenol (2) or from a seed extract of *Guizotia abyssinica* (3).

Intracellular pigments are generally produced when the substrate is an *o*-diphenol, whereas soluble (extracellular) pigments are often the major pigment when the hydroxyl arrangement is *para*. Pulverer and Korth (7) showed that ferric citrate can stimulate pigment production from *p*-diphenols. However, pigment production from *p*-diphenols will also occur if an optimum

nitrogen source (glutamine, asparagine, and glycine) is incorporated into a chemically defined medium (2). The present study reports on the use of aminophenols and diaminobenzenes as substrates for pigment production by *C. neoformans* and *Cryptococcus* species.

MATERIALS AND METHODS

Yeast cultures. A total of 30 previously described (2) isolates of *Cryptococcus* species including *C. neoformans* (nine isolates), *C. luteolus* (two isolates), *C. terreus* (five isolates), *C. albidus* (five isolates), *C. diffluens* (three isolates), and *C. laurentii* (six isolates) were used in this study. In addition one strain each of *Candida albicans*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *C. pseudotropicalis*, *C. stellatoidea*, and *C. tropicalis* (described previously [1]) were used.

Media. The chemically defined diphenol medium and the procedure of Chaskes and Tyndall (2) were used, except that an aminophenol or a diaminobenzene was substituted for the diphenol. The medium contained 4 g of KH_2PO_4 , 2.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg of thiamine hydrochloride, 20 μg of biotin, 5 g of glucose, 1 g of glycine, 1 g of glutamine, and 1 g of asparagine per liter of distilled water. The pH of the medium was adjusted to 5.5 with either 1 M KH_2PO_4 or 1 M K_2HPO_4 . Ingredients were prepared at double strength and sterilized by filtration through 0.45- μm membrane filters (Millipore Corp., Bedford, Mass.). An equal volume of 3% agar was autoclaved for 15 min.

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Both solutions were adjusted to 55°C, mixed, and poured immediately. Because diaminobenzene and aminophenols (like diphenols) are subject to autooxidation, the media were used immediately or stored in an anaerobe jar at 4°C until used. Although shelf-life varied, all media were stable for at least 1 month, and some were stable for up to 6 months. The concentration used for each chemical depended upon three factors: (i) the toxicity of the chemical for the yeasts, (ii) the tendency of the compound to autooxidize, and (iii) the intensity of pigment that was produced.

A total of 12 aminophenol and 6 diaminobenzene compounds were tested as substrates for pigment production by *Cryptococcus* and *Candida* species. A media concentration of 1 g/liter was selected for 3,5-diaminobenzoic acid, 3,4-diaminobenzoic acid, 4-aminosalicylic acid, 4-hydroxymetanilamide, DL-aminophenylalanine, 5-amino-2-hydroxy-3-sulfobenzoic acid, 2-amino-6-hydroxybenzenesulfonic acid, and 2-amino-5-(*p*-chlorophenoxy)-*c*-benzenesulfonic acid, whereas 0.5 g/liter was selected for *m*-aminophenol, 3-aminotyrosine, 5-aminosalicylic acid, *o*-phenylenediamine, *m*-phenylenediamine, and 2,5-diaminobenzenesulfonic acid (2,5-DBSA). A 0.2-g/liter concentration was chosen for *p*-phenylenediamine and 3-amino-4-hydroxyphenyl sulfone, whereas *p*-aminophenol was tested with a 0.1-g/liter level and *o*-aminophenol was tested at 0.02 g/liter. A basic solution of potassium hydroxide (0.3 g/liter of distilled water) was necessary to dissolve the 3-amino-4-hydroxyphenyl sulfone. Aminophenol and diaminobenzene compounds were purchased from the Aldrich Chemical Co., Milwaukee, Wis.

Extraction of pigments. The following procedure was used to extract and purify the water-soluble pigment *C. neoformans* produced when 2,5-DBSA was the substrate. *C. neoformans* was inoculated on 75% of the surface of a petri plate that contained the substrate. After 2 or 3 days of incubation, the quadrant containing the diffusible pigment and no yeast cells was excised and cut into pieces, and the soluble pigment was extracted with five 25-ml amounts of ethanol. During each extraction the ethanol was allowed to remain in contact with the agar medium for 10 to 20 min. The fractions were checked for purity by thin-layer chromatography and were pooled only if one spot was obtained.

Attempts to extract the intracellular pigments were made with ethanol, ether, acetone, methylethylketone, chloroform, pyridine, dimethylformamide, and dimethylsulfoxide.

Thin-layer chromatography and ultraviolet spectra. The soluble pigment obtained after the growth of *C. neoformans* on 2,5-DBSA was subjected to thin-layer chromatography on Eastman Chromagram 6060 silica gel sheets with fluorescent indicator (Eastman Kodak Co., Rochester, N.Y.). The solvent tray was filled with 190 ml of methanol-chloroform-ammonium hydroxide (54:41:5). The chromatogram was allowed to remain in the solvent tray for precisely 90 min and examined after drying under ultraviolet light (366 nm). The ultraviolet and visible spectra of both 2,5-DBSA and the soluble pigment produced by *C. neoformans* from this substrate were obtained by using a recording spectrophotometer. In addition the spectra of the acidified and alkalinized compounds were

obtained. Sufficient acetic acid was added to lower the pH of the pigment and 2,5-DBSA to 3.5, and sufficient sodium hydroxide was added to raise the pH to about 9.0.

RESULTS

Tables 1 and 2 show the pigment production patterns by *C. neoformans*, other *Cryptococcus* species, and *Candida* species from aminophenol and diaminobenzene compounds. Table 1 also shows that the two aminophenols and none of the diaminobenzene substrates were converted only by *C. neoformans* to a pigmented product. 4-Hydroxymetanilamide gave the best results, because all but one isolate of *C. neoformans* produced large amounts of an orange pigment. Frequently, both *C. neoformans* and *C. terreus* produced pigment(s) from the aminophenol and diaminobenzene compounds. Several of these substrates gave excellent results; for example, 3,4-diaminobenzoic acid was converted by all isolates of both species to a pigmented product. Cultures of *C. neoformans* generally produced pigment after 2 to 3 days of incubation, whereas *C. terreus* isolates produced pigment between 4 and 6 days. However, this time differential was not absolute. A second chemical, 3-amino-4-hydroxyphenyl sulfone, also allowed all of the isolates of the two species to produce a pigment, but one isolate of *C. albidus* also produced pigment. A third chemical, 2,5-DBSA, led to the production of an extremely strong, extracellular brownish-yellow green pigment that fluoresced a bright mustard color. Both this pigment and a red-brown intracellular pigment were produced by all *C. neoformans* isolated and by three of five *C. terreus* cultures. Pigment production by *C. neoformans* and *C. terreus* from *m*-aminophenol gave a slightly different pattern: generally, *C. terreus* produced larger amounts of pigment usually within 2 or 3 days, whereas *C. neoformans* often did not produce pigment until day 4 or 5.

Pigment production from 4-aminosalicylic acid gave a unique pattern since all isolates of *C. albidus*, *C. diffluens*, and *C. terreus* produced pigment, whereas *C. neoformans*, *C. laurentii*, and *C. luteolus* did not. Two compounds, *p*-aminophenol and *p*-phenylenediamine, were nonspecific because nearly all *Cryptococcus* and *Candida* species produced pigment from these substrates. However, *C. neoformans* generally produced the largest quantity of pigment, and a 2- to 10-fold dilution of the substrate concentration increased the specificity for *C. neoformans*.

Table 3 shows a simplified scheme, with two aminophenol and one diaminobenzene compound as substrates, to identify some *Cryptococcus* species. *C. neoformans* and *C. terreus* can

TABLE 1. Pigment production by *C. neoformans* and/or *C. terreus* and *C. neoformans* from aminophenol or diaminobenzene compounds

Substrate	Pigment produced by:					
	<i>C. neoformans</i> only	<i>C. terreus</i> and <i>C. neoformans</i>	<i>C. albidus</i> , <i>C. diffluens</i> , <i>C. luteolus</i> , and <i>C. laurentii</i>	<i>Candida</i> species	Color	Location
4-Hydroxymetanilamide	+ ^a	—	—	—	Orange-brown	Extracellular and intracellular
3-Aminotyrosine	+	—	—	—	Brown	Intracellular
5-Amino-2-hydroxy-3-sulfobenzoic acid	—	+ ^{a,b}	—	—	Red-brown or orange	Extracellular and intracellular
DL-Aminophenylalanine	—	+	—	—	Yellow-green	Intracellular
3,4-Diaminobenzoic acid	—	+	—	—	Brown	Intracellular and extracellular
3-Amino-4-hydroxyphenyl sulfone	—	+	— ^c	—	Orange-yellow or red-brown	Intracellular
2,5-DBSA	—	+ ^b	—	—	Red-brown with green	Intracellular and extracellular ^d
<i>m</i> -Phenylenediamine	—	+ ^b	—	—	Brown	Intracellular
2-Amino-5-(<i>p</i> -chlorophenoxy)- <i>o</i> -benzenesulfonic acid	—	+ ^{a,b}	—	—	Brown	Intracellular
<i>m</i> -Aminophenol	—	+	—	+ ^e	Brown	Extracellular

^a Pigment production for eight of nine *C. neoformans* isolates.^b Pigment production for three of five *C. terreus* isolates.^c One isolate of *C. albidus* produced pigment.^d The extracellular pigment fluoresced a brilliant mustard color under ultraviolet light.^e *Candida* species produced small amounts of pigment after 7 days of incubation.TABLE 2. Pigment production by several *Cryptococcus* and/or *Candida* species from aminophenol or diaminobenzene compounds

Substrate	Pigment produced by:					
	<i>C. diffluens</i> , <i>C. albidus</i> , and <i>C. terreus</i>	<i>C. neoformans</i> (major pigment), other <i>Cryptococcus</i> species producing some pigment	<i>Candida</i> species	Pigment not produced	Color	Location
4-Aminosalicylic acid	+	—	—	—	Brown	Intracellular and extracellular
5-Aminosalicylic acid	—	+ ^a	—	—	Brown-black	Intracellular
<i>p</i> -Aminophenol	—	+	+	—	Brown	Intracellular
<i>p</i> -Phenylenediamine	—	+	+	—	Brown	Intracellular
3,5-Diaminobenzoic acid	—	+	—	—	Brown	Intracellular
<i>o</i> -Aminophenol	—	—	—	+	—	—
2-Amino-6-hydroxybenzenesulfonic acid	—	—	—	+	—	—
<i>o</i> -Phenylenediamine	—	—	—	+	—	—

^a 5-Aminosalicylic acid fluoresces a bright green color under ultraviolet light. Fluorescence may disappear after growth of *Cryptococcus* species, especially *C. neoformans*.

be identified by a distinct pigmentation pattern. The scheme allows four other species to be divided into two groups, with the first group (*C. albidus* and *C. diffluens*) producing pigment from 4-aminosalicylic acid and the second group (*C. luteolus* and *C. laurentii*) not producing pigment.

Intracellular pigment extraction from *C. neoformans* and other *Cryptococcus* species after growth on four different substrates, (3,4-diaminobenzoic acid, *p*-phenylenediamine, 5-aminosalicylic acid and 4-aminosalicylic acid) was attempted with a variety of solvents. Difficulty was encountered, and only a small portion of the

intracellular pigment (estimated visually to be 20% or less) could be extracted. Dimethyl sulfoxide extracted the most pigment, and pyridine was the second best solvent. Yeast cells grown on *p*-phenylenediamine were the easiest to extract, and pigment could be removed from every *Cryptococcus* species. Dimethylformamide and ethyl alcohol also removed small amounts of pigment when the substrate was *p*-phenylenediamine. Intracellular pigment was extractable with dimethylsulfoxide and occasionally pyridine when 4- or 5-aminosalicylic acid was the substrate. In contrast, pigment could only be extracted by dimethyl sulfoxide from *C. terreus* but not *C. neoformans* when 3,4-diaminobenzoic acid was the substrate.

The soluble pigment produced from 2,5-DBSA by *C. neoformans* and some *C. terreus* strains was purified by alcohol extraction; the pigment is alcohol soluble, and the substrate is only slightly soluble. The pigment was judged to be pure on the basis of a single spot on thin-layer chromatography (R_f of 0.70 in comparison to 0.52 for 2,5-DBSA) and by its visible and ultraviolet spectra, which contained different maximum absorbance than the substrate (Fig. 1 and 2).

A study to partially characterize the pigment produced from 2,5-DBSA was made by titrating it and the substrate against acetic acid and so-

dium hydroxide. Alcohol extractions of the substrate from 2,5-DBSA plates (not inoculated), titrated against acid and base, showed no color change. However, when 2,5-DBSA was dissolved in distilled water (0.5 g/liter), we observed a light pink color that became a light orange-brown above pH 9. However, raising the pH to 9 did not result in an orange-brown color if acid was added first (pH lowered to 2). When we observed the substrate under a Woods lamp (366 nm) at different pH readings, fluorescence was always a pale blue. In contrast, the pigment, whether in alcohol or water, exhibited reversible color and fluorescence changes. At pH 5.5 and above, the pigment was a light yellow-brown color. Starting at pH 5.3, a pink-red color appeared, and it intensified until pH 4.5. Lowering the pH to 4 resulted in a dark purple-red (burgundy) color. Lowering the pH further did not alter the color. Examination under a Woods lamp revealed a brilliant mustard color fluorescence. The fluorescence increased in intensity as the pH was raised (maximum about pH 9). The fluorescence decreased as the pH was lowered, but substantial fluorescence remained at pH 4. At about pH 3.5 to 3.3 the fluorescence became very weak (now a light-orange fluorescence). Below pH 3 fluorescence was not observed.

Figure 1 shows the ultraviolet spectrum of the pigment (derived from 2,5-DBSA) that has ab-

TABLE 3. Scheme using pigment production from two aminophenol and one diaminobenzene compounds that allows identification or grouping of the major *Cryptococcus* species

Substrate	Pigment produced by:					
	<i>C. neoformans</i>	<i>C. terreus</i>	<i>C. albidus</i>	<i>C. diffluens</i>	<i>C. luteolus</i>	<i>C. laurentii</i>
4-Hydroxymetanilamide	+	—	—	—	—	—
3,4-Diaminobenzoic acid	+	+	—	—	—	—
4-Aminosalicylic acid	—	+	+	+	—	—

FIG. 1. Ultraviolet spectrum (A) of the pigment (derived from 2,5-DBSA) shows absorbance peaks at 218 nm and 287 nm (maximum). B is the spectrum after the pigment is treated with base (pH 9), and C is the pigment spectrum at a pH of 3.

FIG. 2. Visible spectrum (A) of the pigment at pH 5.3 shows absorbance peaks at 445 nm and 580 nm. B shows enhancement at 580 nm when the pH is lowered below 5.3, in this case pH 4.0. The pigment is a burgundy color at pH 4.0. C shows the pigment at pH 9; note that there is no absorbance at 580 nm.

sorbance peaks at 218 nm and 287 nm (maximum). The peak at 287 nm was enhanced by the addition of base and disappeared when the pH was lowered below 3. These results indicate that the peak at 287 nm may be responsible for the brilliant mustard-color fluorescence observed under the Woods lamp. In contrast, the ultraviolet spectrum of 2,5-DBSA had a maximum absorbance of 213 nm, with other peaks at 247 nm and 310 nm. Figure 2 shows the visible spectrum of the pigment at three different pH values. A maximum at about 580 to 585 nm occurred when the pH was below 5.3. Note that at a pH of 5.3 (line A), there is the beginning of a peak, whereas at pH 4 (line B), a distinct absorbance maximum occurs.

The addition of some but not all aminophenol and diaminobenzene compounds to media resulted in a partial inhibition of growth for the *Cryptococcus* and *Candida* species. For example, poor growth of the *Candida* but not the *Cryptococcus* species occurred when 2,5-DBSA was the substrate. Generally, the *Candida* species grew poorly even if the incubation period was 5 to 10 days. In contrast, when 4-aminosalicylic acid was used, some *Cryptococcus* species (including the pigment producers, *C. albidus*, *C. diffluentis*, and *C. terreus*) grew poorly at first (1 to 3 days) but overcame the inhibition by day 4, and heavy growth was noted between days 4 and 6. The inhibition of the *Cryptococcus* species by most aminophenol and diaminobenzene compounds was relatively mild. However, strong inhibition was noted for some compounds that had an *ortho* configuration for the hydroxyl and amino groups. The most toxic compound was *o*-aminophenol; in concentrations above 30 mg/liter, it completely inhibited the growth of the *Cryptococcus* but not the *Candida* species.

DISCUSSION

Our data suggest that the conversion of diphenol, aminophenol, and diaminobenzene compounds by *Cryptococcus* species to a pigment are closely related. First, there are the obvious structural similarities of the substrates. Second, we observed that the intracellular pigment was difficult to extract regardless of the substrate class (aminophenol, diaminobenzene, or diphenol). This observation has also been made for many plant and animal systems (6). A difference that we have noticed in reference to the three substrate classes is that a small amount of pigment can occasionally be extracted when an aminophenol or diaminobenzene compound served as a substrate. A close relationship between the three substrate classes is also suggested because *C. neoformans* generally is the only yeast to produce pigment or the yeast that produced the most pigment. However, exceptions exist in this area. Finally, all three substrate classes yielded enhanced pigmentation when specific amino acids (glutamine, asparagine, and glycine) were the nitrogen source. Enzymatic testing is needed to clarify the exact pathway(s) for production of yeast melanin.

Shaw and Kapica (9) suggested that the phenyloxidase (which converts diphenols to melanin or a melanin-like pigment) of *C. neoformans* resembles the enzymes of mushrooms and *Mycobacterium leprae* because it utilizes many different substrates. In contrast, the mammalian enzyme tyrosinase (phenyloxidase) can utilize only tyrosine and dihydroxyphenylalanine as substrates. The fact that many aminophenol and diaminobenzene compounds can be converted to a melanin-like pigment suggests that the yeast pathway may be even less specific than previ-

ously reported (9). Hydroquinone, 3-aminotyrosine, and *p*-phenylenediamine are inhibitors of melanin formation in the mammalian system. The enzymatic conversion of tyrosine to 3,4-dihydroxyphenylalanine in the mammalian system is inhibited by hydroquinone, whereas in the *Cryptococcus* pathways this step does not exist (9). Thus, conversion of hydroquinone by *Cryptococcus* species (2) to a pigment(s) is consistent with the known pathway. In the mammalian system 3,4-dihydroxyphenylalanine is oxidized to 3,4-dihydroxyphenylalanine quinone, which is further oxidized. The compounds 3-aminotyrosine and *p*-phenylenediamine can combine with *o*-quinones to inhibit melanin formation (5, 8). Although the exact pathway used by *Cryptococcus* species is not known, both of these mammalian inhibitors (5, 8) of melanin formation can act as substrates in the yeast system. In the mammalian system *o*-phenylenediamine also combines with *o*-quinones and inhibits the formation of melanin; in the yeast system this substrate is toxic to the cells.

Several pigmentation patterns were observed with diaminobenzene and aminophenol compounds as substrates. The most common pattern observed was pigment production by both *C. neoformans* and *C. terreus*. This pattern occurred with both diaminobenzene and aminophenol compounds with the reactive groups being 3,4-(*ortho*), 2,5-(*para*) and 1,3-(*meta*) (Table 1). In contrast, *C. terreus* did not produce pigment from diphenols (except for a rare biochemical variant) (2); and *C. neoformans* did not convert *meta* diphenols to melanin (4, 11). Pigmentation of only *C. neoformans* (Table 1) was rare and occurred with just two aminophenols (3,4 position for amino and hydroxyl groups). In contrast, *C. neoformans* was the only yeast that produced a melanin-like pigment from many different diphenols. When 4-aminosalicylic acid was the substrate (reactive groups in the *meta* position), a unique pigmentation pattern was observed: only *C. terreus*, *C. diffluens*, and *C. albidus* produced pigment. Finally, a pattern was observed in which *C. neoformans* produced large amounts of pigment from aminophenol and diaminobenzene compounds, whereas the other *Cryptococcus* species were not consistent in their ability to produce pigment. Compounds that produced this pattern had reactive groups with either a *para* or *meta* configuration.

Just as with diphenols, some aminophenols and diaminobenzene compounds (Table 3) did not elicit the production of pigment by the *Cryptococcus* and *Candida* species. All diphenols, aminophenols, and diaminobenzenes in the *para*

position, without an additional radical on the benzene ring, were converted to a pigment by a large percentage of *Cryptococcus* and *Candida* species. For example, hydroquinone (2) was the only diphenol converted to pigment by the non-*C. neoformans* species. Similarly *p*-aminophenol and *p*-phenylenediamine allowed nearly all *Cryptococcus* species to produce pigment (Table 2). In addition the *Candida* species were also capable of producing pigment from these compounds.

Table 3 shows a scheme to identify some *Cryptococcus* species by using two aminophenol and one diaminobenzene compound. The advantage of this protocol is that both *C. neoformans* and *C. terreus* can be rapidly identified; in addition, two groups of *Cryptococcus* species can be differentiated. Group I consists of *C. albidus* and *C. diffluens*, and group II includes *C. laurentii* and *C. luteolus*. The two groups can be identified as to species if several sugar assimilation tests are performed.

This three-substrate scheme may be useful to the laboratory that wants biochemical identification of *C. neoformans*, but may not wish a complete identification of other species of *Cryptococcus*. When employing the scheme, it is necessary to perform a urease test (10) to be certain that the yeast belongs to the *Cryptococcus* genus. In addition, utilization of 2,5-DBSA, although not entirely specific for *C. neoformans*, should be considered because of the unique mustard-colored fluorescent pigment produced.

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