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Pigment Production by *Cryptococcus neoformans* from *para*- and *ortho*-Diphenols: Effect of the Nitrogen Source

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Cryptococcus neoformans produced pigments when *p*-diphenols were substrates in a glucose-amino acid-salts medium. The best substrates were 2.5-dihydroxybenzoic acid and 2,5-dihydroxybenzenesulfonic acid. In contrast to the cellular pigment production from *o*-diphenols (hydroxyl groups in the 2,3- or 3,4-position of phenyl ring), the *p*-diphenols (1,4- or 2,5-positions for the hydroxyl groups) produced large amounts of soluble pigments that diffused into the medium. When an optimal source of nitrogen (glutamine, glycine, and asparagine) was used, 89% of the *C. neoformans* strains produced pigments from *p*-diphenols. In contrast, 0 to 67% of the strains produced pigments when a suboptimal nitrogen source (proline, ammonium sulfate, ornithine, and methionine) was used. When glutamine-glycine-asparagine was the nitrogen source, 100% of the *C. neoformans* strains produced pigments from *o*-diphenols, whereas 77 to 100% of the strains produced pigment when proline-ammonium sulfate-ornithine-methionine was the nitrogen source. *Cryptococcus* species other than *C. neoformans* and all tested *Candida* species failed to produce pigments from any of the substrates except when hydroquinone was used. A combination of glutamine-glycine-asparagine and 3,4-dihydroxyphenylalanine allowed differentiation of colonies of *C. neoformans* from *C. albicans* in 3 to 6 days. These data showed that pigment production from *o*- and *p*-diphenols served as an excellent biochemical test for the identification of *C. neoformans*.

Pigment production can be an important aid in the isolation, identification, and classification of microorganisms. Staib (6) in 1962 observed that *Cryptococcus neoformans* produced a brown pigment when cultured on agar medium containing bird droppings. The seed of *Guizotia abyssinica*, commonly used in bird feed, was responsible for the brown color (6, 7) which was specifically produced by *C. neoformans*. A medium prepared with an extract of potatoes and carrots produced an identical pigment (2). The specific compounds that could act as substitutes for pigment production were identified first by Korth and Pulverer (3) as *o*-diphenols. Their results were expanded by Shaw and Kapica (4) and Strachan et al. (9), who concluded that coloration was dependent upon the hydroxyl groups in the 3- and 4-positions of the phenyl ring. The pigments produced were retained by the yeast cells (intracellular) and could not be extracted by common solvents (9). The purpose of the present study is to show that hydroxyl groups in the positions *para* to each other on the phenyl ring (the 1,4- or 2,5-positions) can also be substrates for pigment production by *C. neoformans*. In addition, the effect of various nitrogen sources on pigment formation was tested. The optimum nitrogen sources were incorporated into a medium designed to be clinically useful for the confirmation of cryptococcal infection.

MATERIALS AND METHODS

Yeast cultures. Thirty strains of *Cryptococcus* species including *C. neoformans* (nine strains), *C. luteolus* (two strains), *C. terreus* (five strains), *C. albidus* (five strains), *C. diffluens* (three strains), and *C. laurentii* (six strains) were used in the study. The *Cryptococcus* cultures were supplied by William Kaplan and Virginia Shorall, Mycology Section, Center for Disease Control, Atlanta, Ga., Mark Camblin, St. Mary's Memorial Hospital, Knoxville, Tenn., Smith Shadomy, Medical College of Virginia, Department of Medicine, Richmond, Va., and George N. Little, Division of Laboratories and Research, New York State Department of Health, Albany, N.Y. In addition, *C. neoformans* (ATCC 13690), *C. albidus* ATTC 10666, and one strain each of *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, *C. stellatoidea*, *C. krusei*, *C. pseudotropicalis*, and *C. guilliermondii* (described previously [1]) were used.

Media. A chemically defined medium containing 4 g of KH_2PO_4, 2.5 g of $MgSO_4 \cdot 7H_2O$, 10 mg of thiamine hydrochloride, 20 μg of biotin, 200 mg of

3,4-dihydroxyphenylalanine (DL-DOPA), and 5 g of glucose per liter of distilled water was used. The pH of the medium was adjusted to 5.5 with either 1 M KH_2PO_4 or 1 M K_2HPO_4. Seventeen common nitrogen sources (1 g/liter) including either amino acids, ammonium sulfate, Casamino Acids, yeast extract, or tryptone (Table 1) were tested for their ability to allow *C. neoformans* to form pigment from DL-DOPA. Media ingredients were prepared at ×2 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. Both solutions were adjusted to 55 C, mixed, and poured immediately.

Two more complex nitrogen sources containing at least three amino acids were tested for their ability to allow *C. neoformans* to produce pigment from different diphenols. GGA medium contained glutamine, glycine, and asparagine each at a concentration of 1 g/liter. PAOM medium contained proline, ammonium sulfate, ornithine, and methionine also at a concentration of 1 g/liter for each compound. Eight diphenols were tested separately with GGA or PAOM as nitrogen sources. DL-DOPA was tested at 200 mg/liter. This level was selected since autooxidation was not a factor. Six diphenols (3,4-dihydroxybenzoic acid, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 2,5-dihydroxy-*p*-benzenediacetic acid, hydroquinone, and 2,5-dihydroxybenzenesulfonic acid, potassium salt) were tested at 1 g/liter. Finally, 2,5-dihydroxybenzaldehyde was tested at 20 mg/liter. Chemicals were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Preliminary tests were performed to ascertain the potential of diphenols in a chemically defined medium to isolate *C. neoformans* from clinical sources. The GGA-DL-DOPA agar medium was inoculated with approximately equal numbers of *C. neoformans* and *C. albicans* so that 30 to 300 colonies were obtained. The plates were incubated at 25 C, and the time required for the differentiation of *C. neoformans* from *C. albicans* was recorded. Experiments were performed with nine different strains of *C. neoformans*.

Extraction of pigments. Water-soluble pigments produced by *C. neoformans* were extracted by carefully mincing the agar medium and adding 75 ml of distilled water (three separate 25-ml volumes) to the contents of a petri plate. After 0.5 h, the mixture was centrifuged at 5,000 rpm for 10 min to remove yeast cells, and the supernatant was then membrane filtered (Millipore Corp.). Attempts to extract the intracellular pigments were made with ethanol, ether, acetone, methylethylketone, chloroform, pyridine, dimethylformamide, and dimethylsulfoxide.

Thin-layer chromatography and ultraviolet spectrums. The soluble pigments were subjected to thin-layer chromatography on Eastman Chromagram 6060 silica gel sheets with fluorescent indicator (Eastman Kodak Co., Rochester, N.Y.). Various pigments and standards were applied in 10- to 50-μl volumes. Solvent systems of butanol-ethanol-water (4:1:1), butanol-acetic acid-water (65:10:25), acetic acid-water (20:80), or methanol-chloroform-ammonium hydroxide (MCA) (54:41:5) were used. The ultraviolet spectra of the pigments were obtained with a recording spectrophotometer after the pigments were scraped from the silica gel chromatogram and eluted with water. A control ultraviolet spectrum was also obtained by using the washings of the silica gel.

Molecular weight approximations of soluble pigments. Membrane ultrafilters (Amicon Corp., Lexington, Mass.) UM2 and PM10 were employed to estimate the molecular weight of the pigments derived from hydroquinone, 2,5-dihydroxybenzenesulfonic acid, and 2,5-dihydroxybenzoic acid. The UM2 membrane is said to retain compounds with a molecular weight greater than 1,000; PM10 retains those above 10,000 (Amicon Corp.). A 3-ml volume of solution containing the pigment or the original chemical (1 g/liter) was diluted to 10 ml, which was then filtered under 50 lb/in^2 pressure until 1 ml remained. A 9-ml volume of distilled water was added and the procedure was repeated. The percentage of the pigment or the original chemical retained by the filter was either estimated visually or determined by comparing the intensity of thin-layer chromatograms on the portion passing through the filter to that which did not pass. In addition, dialysis studies were performed on the three pigments and their precursors. A 3-ml volume of pigment or the starting chemical was placed in dialyzed tubing (Arthur H. Thomas Co., Philadelphia, Pa.) and dialyzed against 2 liters of distilled water for 4 days. The distilled water was changed twice daily, and the percentage of the pigment remaining after 4 days was estimated visually.

RESULTS

Table 1 shows pigment production by four strains of *C. neoformans* from DL-DOPA with single nitrogen sources. The three best nitrogen sources (glycine, glutamine, and asparagine) and the four least-desirable nitrogen sources (proline, ammonium sulfate, ornithine, and methionine) were pooled for further testing. *Cryptococcus* and *Candida* species grew well on the GGA or PAOM medium, with the former medium giving slightly better growth. Pigmentation did not occur unless diphenols were added. Table 2 shows pigment production by *C. neoformans* from diphenols with GGA or PAOM as the nitrogen source. A higher number of *C. neoformans* strains produced pigment when GGA was the nitrogen source. Generally, less time was required and the amount of pigment produced was greater if GGA was the nitrogen source. When *o*-diphenols were the substrate, more strains produced pigments than when the substrate was a *p*-diphenol (Table 2). Pigments classified as intracellular could not be extracted from the yeast cells with any common solvent. In contrast, pigments classified as extracellular were soluble and diffused into the surrounding medium. Generally, soluble products were dom-

inant if the substrate was a *p*-diphenol, whereas the *o*-diphenols produced only intracellular pigments, except when the substrate was 3,4-dihydroxybenzoic acid, in which case both soluble and intracellular pigments were produced. With the following exceptions, cultures of *Candida* and *Cryptococcus* other than *C. neoformans* failed to produce pigments from diphenols. One strain of *C. terreus* produced a brown pigment from only 2,3-dihydroxybenzoic acid, one strain of *C. diffluens* produced a brown pigment from only 2,5-dihydroxybenzoic acid, and most *Cryptococcus* species (Table 3) produced reddish-brown pigments from hydroquinone. Thus, pigment formation from hydroquinone did not permit the differentiation of *C. neoformans* from other *Cryptococcus* species. Furthermore, some of the *Candida* species also produced an extracellular reddish-brown pigment, and prolonged incubation (>2 weeks) also resulted in the production of an intracellular pigment. The most striking phenomenon in pigment production from hydroquinone was the difference between the GGA and PAOM media, since the former allowed large amounts of pigments to be produced and the latter yielded no pigments. Hydroquinone might have value in a diagnostic test since *C. luteolus* was the only *Cryptococcus* species inhibited by the compound. Additional strains of *C. luteolus* will have to be tested to confirm this observation.

TABLE 1. *Pigment production by four strains of C. neoformans from* DL-*DOPA with different nitrogen sources*

Nitrogen source	Growth	Pigment production	
		Days	Color
Glutamine	Excellent	1-3	Black
Asparagine	Excellent	1-3	Black
Glycine	Good-fair	1-3	Dark brown
Aspartic acid	Good-fair	2-5	Dark brown
Glutamic acid	Good-fair	2-5	Dark brown
Alanine	Good-fair	2-5	Dark brown
β-Alanine	Good-fair	2-5	Dark brown
Arginine	Fair	2-4	Dark brown
Serine	Fair	2-5	Dark brown
Valine	Fair	2-5	Dark brown
Lysine	Fair	2-5	Dark brown
Proline[a]	Fair	2-7	Brown
Ammonium sulfate	Good	2-7	Light brown
Ornithine[a]	Fair	2-7	Light brown
Methionine	Fair-poor	2-6	Light brown
Creatinine[b]	Poor	2-5	Dark brown
Casamino Acids	Good	2-4	Brown
Yeast extract	Good	2-5	Brown
Tryptone	Good	3-6	Light brown

[a] One strain of *C. neoformans* did not grow.
[b] Three strains of *C. neoformans* did not grow.

Colonies of *C. neoformans* and *C. albicans* were easily differentiated in 3 to 6 days on a DL-DOPA medium that contained GGA as the nitrogen source. The first appearance of pigment in the *C. neoformans* colonies was brown, but after 1 additional day of incubation the colonies turned black. The results (Fig. 1) were representative of the mixed-colony studies.

Soluble pigments remained at the origin of the thin-layer chromatograms when either acidic, basic, or neutral solvent systems were used, but some migration of the soluble pigments was obtained with the strongly acidic system (acetic acid-water, 20:80). However, the pigments tailed badly and distinct R_f values could not be obtained. Since the substrates migrated, the pigment(s) remaining at the origin was eluted

TABLE 2. *Pigment production by C. neoformans from diphenols with GGA or PAOM as the nitrogen source*

Diphenol	Fraction of strains producing pigment(s) with:		Days required for pigment(s) production with:		Color of pigment	Location of pigment
	GGA	PAOM	GGA	PAOM		
3,4-Dihydroxyphenylalanine	9/9	8/9	1-3	1-6	Brown-black	Intracellular
3,4-Dihydroxybenzoic acid	9/9	9/9	1-4	1-6	Brown	Intracellular and extracellular
2,3-Dihydroxybenzoic acid	9/9	7/9	1-4	1-6	Green-black	Intracellular
2,5-Dihydroxybenzoic acid	8/9	6/9	1-4	1-4	Brown	Intracellular and extracellular
2,5-Dihydroxy-*p*-benzene-diacetic acid	8/9	6/9	1-4	1-4	Buff-brown	Intracellular and extracellular
2,5-Dihydroxybenzene-sulfonic acid, potassium salt	8/9	6/9	1-4	1-4	Orange-red	Extracellular
2,5-Dihydroxybenzaldehyde	8/9	0/9	2-7	—	Buff	Intracellular
Hydroquinone	9/9	1/9	2-6	6-10	Reddish-brown	Intracellular and extracellular

TABLE 3. *Pigment production by Cryptococcus and Candida species from hydroquinone (GGA medium) after 2 to 10 days of incubation*

Yeast	No. of strains	Pigment		Inhibition of growth
		Color	Location	
Cryptococcus neoformans	7	Dark reddish-brown	Intracellular and extracellular	None
C. neoformans	2	Light reddish-brown[a]	Intracellular and extracellular	None
C. albidus	5	Dark reddish-brown	Intracellular and extracellular	None
C. laurentii	5	Dark reddish-brown	Intracellular and extracellular	None
C. laurentii	1	Light reddish-brown	Intracellular and extracellular	None
C. diffluens	3	Light reddish-brown	Intracellular and extracellular	None
C. terreus	5	Light brown	Intracellular	None
C. luteolus	2			Yes[b]
Candida albicans	1	None		None
C. tropicalis	1	None		None
C. parapsilosis	1	None		None
C. krusei	1	Light reddish-brown	Extracellular	None
C. guilliermondii	1	Light reddish-brown	Extracellular	None
C. pseudotropicalis	1	Light reddish-brown	Extracellular	None
C. stellatoidea	1	Light reddish-brown	Extracellular	None

[a] The pigment became dark reddish-brown after 10 days of incubation.
[b] Some resistant brown colonies (one strain) developed after 6 days of incubation.

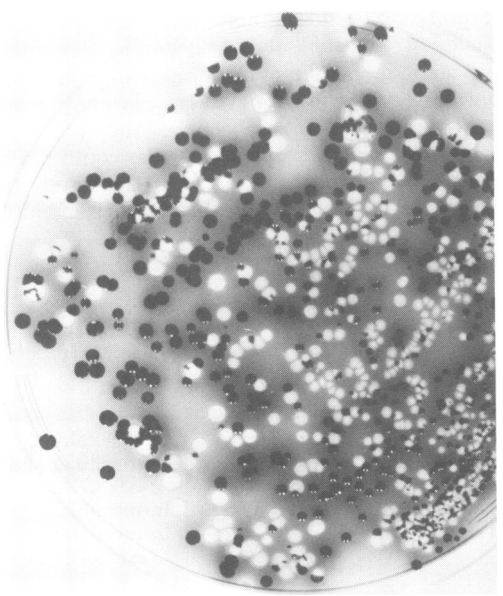

FIG. 1. *Excellent differentiation after 5 days of incubation of colonies of C. neoformans (black) and C. albicans (white) on GGA-DOPA medium. Colonies containing mixtures (black and white) of both C. neoformans and C. albicans can be distinguished. Colonies of this strain of C. neoformans and C. albicans were about the same size (2 to 3 mm in diameter).*

and the ultraviolet spectrum was obtained. The pigments derived from 2,5-dihydroxybenzenesulfonic acid and 2,5-dihydroxybenzoic acid had an ultraviolet spectrum that was distinctly different from the original diphenol. The former pigment had a maximum absorbance at 214 nm and another peak at 325 nm, whereas 2,5-dihydroxybenzenesulfonic acid had a maximum absorbance at 208 nm and had peaks at 225 and 305 nm. The latter pigment had a maximum absorbance at 210 nm and another small peak at 335 nm, whereas 2,5-dihydroxybenzoic acid had maximum absorbance at 220 and 233 nm and another peak at 324 nm. Since the pigments did not migrate from the origin, it is possible that there could be more than one pigment. Finally, it is possible (although improbable, since three solvent systems were used) that other nonpigmented products were also present at the origin.

Table 4 shows the molecular weight approximations of the soluble pigments. The dialysis experiments showed that only part of the pigments derived from hydroquinone and 2,5-dihydroxybenzoic acid could pass through the tubing. This fact and also the complete retention of the pigments by the UM2 filter and partial retention by the PM10 filter indicated that the pigments had a molecular weight between 1,000 and 10,000. This also suggested that the pig-

ments could be small polymers of an altered form of the original diphenol.

DISCUSSION

Previous studies have shown conclusively that *C. neoformans* is the only yeast that produces intracellular pigments from *o*-diphenols. Strachan et al. (9) suggested that the pigments were polymeric compounds, whereas Shaw and Kapica (4), using electron microscopy, showed that the pigment was located in the cell wall. They also showed (4) that *C. neoformans* phenoloxidase (an enzyme that catalyzes the oxidation of *o*-diphenols to melanin) has a wide substrate specificity and is, thus, more similar to the phenoloxidase from mushrooms and *Mycobacterium leprae* than to the mammalian systems, which oxidize only tyrosine and DOPA. The present study indicates that the production of melanin or melanin-like pigments by *C. neoformans* has an even broader spectrum, since the hydroxyl groups may be in the 1,4- or 2,5-positions of the phenyl ring.

The present study showed that a major difference between *para* and 3,4- or 2,3- (*ortho*) positions is that soluble pigments are usually produced when the arrangement is *para* but are rarely produced when the position is *ortho*. Studies suggest that the molecular weight of the soluble pigments may be greater than 1,000 but less than 10,000. It is possible that the soluble pigments are small polymers of an altered diphenol. After 1 to 4 days of incubation, *C. neoformans* formed an orange-red soluble pigment from the substrate 2,5-dihydroxybenzenesulfonic aicd (hydroquinonesulfonic acid). After 5 to 8 days of incubation, small amounts of a light-brown pigment were produced that could not be extracted from the yeast cells. Gentisic acid (2,5-dihydroxybenzoic acid) and hydroquinone also produced large amounts of a brown and reddish-brown soluble pigment as well as large amounts of a brown intracellular pigment. These substrates should prove to be advantageous for studying the pathway leading to pigmentation since the chemical structure of the soluble pigments should be easy to identify in contrast to those that cannot be extracted. Both 2,5-dihydroxy-*p*-benzenediacetic acid and 2,5-dihydroxybenzaldehyde appear to be poor choices for further study since both produced relatively small amounts of pigment and the latter was toxic when added in a concentration above 20 mg/liter.

The importance of the nitrogen source for pigment production from *o*- and *p*-diphenols was demonstrated in the present study. Three amino acids (glycine, glutamine, and asparagine) were the most effective in allowing *C. neoformans* to convert DOPA to melanin. These nitrogen sources allowed excellent growth, and the yeast cells accumulated a black pigment. Other nitrogen sources allowed only brown pigments to be formed. When different substrates were used, the medium containing GGA consistently allowed a higher number of *C. neoformans* strains to produce pigments than did the PAOM medium. Also, more strains of *C. neoformans* were able to produce pigments when the substrate contained hydroxyl groups in the *ortho* rather than *para* positions. Strachan et al. (9) reported previously that 2,5-dihydroxybenzoic acid could not act as a substrate for pigment production. Examination of the glucose-creatinine medium used in their study shows that the three amino acids found to be optimum in the present study were not part of their medium. The differences reported in the two studies emphasize the importance of the nitrogen source. Shaw and Kapica (4), using a potato-carrot-glucose agar medium, obtained pigmentation of *C. neoformans* in a shorter time than when a chemically defined medium containing *o*-diphenols was used. The chemically defined medium they used contained large amounts of ammonium sulfate and asparagine. Our results showed that ammonium sulfate was the least desirable nitrogen source for melanin production by *C. neoformans*. It seems probable that the slower production of melanin by the chemically defined medium occurred because of the presence of ammonium sulfate. Shaw and Kapica (4) reported that single colonies of *C. neoformans* could not be differentiated from *C. albicans* in mixed cultures on their DOPA medium until day 9 or 10, whereas our GGA-DOPA medium allowed differentiation between days 3 and 6. These data suggest that the

TABLE 4. *Molecular weight approximations of soluble pigments*

Compound	% Retained		
	Diaflow ultrafilters		Dialyzer tubing
	UM2	PM10	
2,5-Dihydroxybenzene-sulfonic acid	30	0	0
Pigment derived from 2,5-dihydroxybenzene-sulfonic acid	100	20	0
Hydroquinone	25	0	0
Pigment derived from hydroquinone	100	30	30[a]
2,5-Dihydroxybenzoic acid	10	0	0
Pigment derived from 2,5-dihydroxybenzoic acid	100	20	20[a]

[a] Amount remaining after 4 days of dialysis.

GGA-DOPA medium used in this study might be good for isolating *C. neoformans* from throat cultures, bronchial aspirates, and spinal fluid. The only alteration needed would be the addition of diphenyl and chloramphenicol (5) to inhibit growth of bacteria and fast-growing fungi. Chemically undefined media using *Guizotia abyssinica* seed (5, 6, 8) or potato-carrot extract (2) have already been used as isolation media.

Kapica and Shaw (2) reported that one strain of *C. laurentii* was able to form pigment when grown on a potato-carrot-glucose medium. Individual diphenols were not tested. We encountered one strain of *C. terreus* that produced a brown pigment from only 2,3-dihydroxybenzoic acid and one strain of *C. diffluens* that produced a brown pigment from only 2,5-dihydroxybenzoic acid. Our results suggest that false positive reactions can be minimized by employing at least two different substrates in dual tests. The best diphenols found in our study were DOPA, 3,4-dihydroxybenzoic acid, and 2,5-dihydroxybenzene-sulfonic acid. These diphenols are currently recommended as biochemical tests to identify *C. neoformans*. Finally, an important physiological question is whether or not phenoloxidase can convert 1,4- or 2,5-diphenols (*para*) to melanin. Is there an *ortho* and a *para* enzyme or are they the same?

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