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# Catechol Formation and Melanization by $Na^+$-Dependent *Azotobacter chroococcum*: a Protective Mechanism for Aeroadaptation?

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**Aeroadaptive microaerophilic *Azotobacter chroococcum* 184 produced a cell-associated black pigment when grown at high aeration rates under nitrogen-fixing conditions. This pigment was shown to be a catechol melanin. Polyphenol oxidase activity was detected in cell extracts of cells grown for 72 h. Melanin formation was optimal in the later stages of growth, and there was no correlation between nitrogenase activity and melanization. Nitrogenase activity in strain 184 was optimal at 10% $O_2$, and melanin formation was suppressed by $O_2$ limitation. In the presence of charcoal, an adsorbent of toxic oxygen intermediates, and benzoic acid, a scavenger of hydroxyl radicals, melanization was inhibited. However, in the presence of copper, the intensity of pigment color increased and melanization was accelerated. Copper also eliminated catalase and peroxidase activities of the organism but still permitted aerobic growth. In the presence of low levels of iron, melanization was accelerated under high aeration rates, and under low rates of aeration, melanization was observed only at higher levels of iron. Hydroxamate-siderophore production was detectable in the presence of soluble iron under high rates of aeration but was repressed by the same levels of iron under low aeration rates. Unlike melanization and hydroxamate formation, catechol formation was observed under both low and high rates of aeration under nitrogen-fixing conditions. Catechol formation and melanization were repressed by 14 mM $NH_4^+$, at which level nitrogenase activity was also repressed. Copper reversed the repressive effect of $NH_4^+$. A role for catechol formation and melanization in aeroadaptation is proposed.**

Organisms of the genus *Azotobacter* are free-living nitrogen-fixing obligate aerobes (6). Among species of this genus, *A. chroococcum* has been most commonly isolated from the soils worldwide. Recently, strains of *A. chroococcum* absolutely dependent on $Na^+$ for growth have been isolated from the soils of Alberta, Canada (31). These organisms have an iron-dependent growth phenotype (32), and the iron dependence has been linked to the activity of catalase, peroxidase, and superoxide dismutase (33). The activities of the former two enzymes are very low in the $Na^+$-dependent strains, and these bacteria share many traits with microaerophilic bacteria. However, despite the low activity of these enzymes, these bacteria can grow and fix nitrogen aerobically, unlike other microaerophilic bacteria (31, 33).

We have become interested in the intense brown-black pigment formed by the $Na^+$-dependent strains. This pigment has been described but has not been proven to be a melanin in *A. chroococcum* (20, 43). Melanins are formed by the oxidative polymerization of phenolic compounds such as catechol and 1,8-dihydroxynaphthalene (allomelanins) (1), dopa (eumelanins), and cysteinyl-dopa (phaeomelanins) (42). The eumelanins and phaeomelanins are found mainly in animal species, whereas the allomelanins are found in plants and microorganisms (30). Among microorganisms, melanin formation has been observed in species of the genera *Micrococcus* (28), *Bacillus* (3), *Streptomyces* (2), *Vibrio* (19), *Mycobacterium* (36), *Rhizobium* (8), and recently in an *Azospirillum* species (40), to name a few. Melanins are not considered essential for growth and development of cells but are required in order to enhance the ability of the producing species to compete and survive under certain environmental conditions, such as in the presence of UV radiation (7). Sakina et al. (41) describe antioxidant properties of melanin in cases in which lipid peroxidation is inhibited due to the ferrous ion-binding and antiradical activities of the pigment.

All aerobic organisms form the superoxide radical and hydrogen peroxide (11). Both $O_2^-$ and $H_2O_2$ can do direct damage to cellular targets (12), but on the whole their reactivities are limited. However, in the presence of iron catalysts, $O_2^-$ and $H_2O_2$ interact to form the highly reactive hydroxyl radical (OH ·) via the Fenton reaction (15):

$$O_2^- + H_2O_2 \xrightarrow{\text{Fe catalyst}} OH \cdot + OH^- + O_2.$$

The enzymes catalase, peroxidase, and superoxide dismutase are involved in protecting aerobic organisms by preventing the formation of these radicals (21). Melanins can act as cation exchange polymers which can protect tissues against oxidizing and reducing conditions and trap free radicals (26). Goodchild et al. (13) have demonstrated the superoxide-scavenging ability of melanin, and Bell and Wheeler (7) describe melanin as a sponge for free radicals which result from environmental stress. Korytowski et al. (22) describe a pseudo-superoxide dismutase activity of a dopa-melanin, indicating a further role in $O_2$ protection. The present study concerns the characterization of the melanin produced by strain 184, the influence of environmental conditions on melanin formation, and the possible role of melanin in allowing this microaerophilic organism to adapt to aerobic growth conditions.

## MATERIALS AND METHODS

**Organism, media, and growth conditions.** The $Na^+$-dependent strain of *A. chroococcum* 184 (31) isolated from Alberta soils was used in this investigation. It was maintained on slants of Burk nitrogen-free medium containing 0.85 mM

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NaCl. Liquid medium, pH 7.2, contained 4.6 mM $K_2HPO_4$, 1.46 mM $KH_2PO_4$, 0.8 mM $MgSO_4 \cdot 7H_2O$, 1 μM $Na_2MoO_4 \cdot 2H_2O$, 13.2 mM $CH_3COONa \cdot 3H_2O$, and 0.453 mM $CaCO_3$. The nitrogen source was either 14 mM ammonium acetate or $N_2$, and 2% (wt/vol) sucrose was used as the carbon and energy source. The iron content of the medium was adjusted using ferric citrate. To study the effect of light on melanin formation, slants were illuminated by placing them circa 60 cm below a fluorescent lamp (15-W cool white) in a water bath at 30°C for 7 days.

Cultures (15 ml of medium per 50-ml Erlenmeyer flask) were incubated under aerobic conditions at 30°C in a gyratory water bath shaker (model G-76; New Brunswick Scientific Co., Inc.) at a shaking speed of 250 rpm in all the studies. Rates of aeration of nitrogen-fixing strain 184 were varied by using 5- to 30-ml volumes in 50-ml Erlenmeyer flasks. Cultures were inoculated by using a 1% (vol/vol) inoculum as described previously (32). Growth was monitored by estimating culture protein content as described by Bradford (9), with bovine serum albumin (Sigma Chemical Co.) as the standard. Variability of up to 10% was observed in results obtained between replicate experiments.

**Identification of catechol.** The catechol produced by strain 184 was extracted from acidified (with HCl) (pH 2) growth supernatant fluid with ethyl acetate. The ethyl acetate extract was reduced to dryness by rotary evaporation at 30 to 34°C. The residue was then dissolved in methanol-chloroform (5:95 [vol/vol]), and further purification was carried out by preparative thin-layer chromatography on Silica Gel G plates by using the same solvent mixture. The spray reagents used were ferric chloride: 2,2′-dipyridyl (23) and 0.1 M $FeCl_3$ in 0.1 N HCl. Pure catechol, 2,3-dihydroxybenzoic acid, and 3,4-dihydroxybenzoic acid were used as standards. The silica gel containing catechol was scraped from the plates and placed in a Pasteur pipette, and the catechol was eluted with chloroform. The catechol purified from the culture supernatant fluids had the same $R_f$ value as authentic catechol in all of the following solvent systems: methanol-chloroform (5:95, 3:0.3, 3:1, 3:2, 1:1, 0.1:1, and 0.2:1 [vol/vol]), benzene-acetic acid water (2:2:1, 2:3:1, and 2:4:1 [vol/vol]), butanol-acetic acid-water (4:1:5 [vol/vol]), and chloroform-acetic acid-water (140:8:52 [vol/vol]). Pure chloroform, benzene, methanol, and ether were also used as solvents.

UV-visible wavelength scan of the purified catechol was carried out with a Perkin-Elmer Lambda 3 spectrophotometer. The compound was conclusively identified as catechol by gas chromatography (on a 30-m DB-1 capillary column with helium as the carrier gas at a flow rate of 45 cm $s^{-1}$ and a holding temperature of 90°C) coupled to mass spectroscopic analysis.

Quantitative estimations of catechol in culture supernatant fluids was carried out by the Arnow procedure as modified by Barnum (5). Variability in results between replicate experiments was up to a maximum of 15%.

**Melanin estimation.** The black pigment produced by the organism was identified as a melanin on the basis of the properties described by White (44). Melanin was purified from the cells by the method of Whittaker (45), in which the cell pellet was extracted three times with 5% trichloroacetic acid, twice with ether-alcohol (1:1 [vol/vol]), and once with absolute ether to remove impurities. The residual material was then dissolved in 0.05 M sodium carbonate by treatment in a 100°C water bath for 10 min. After centrifugation to remove insoluble material, the solution was transferred to aluminum foil cups and dried overnight in an oven at 160°C. The dry residue was redissolved in warm 0.85 N KOH to obtain a 1-mg $ml^{-1}$ solution of melanin. Melanin concentration was estimated by relating $A_{400}$ values to known concentrations of the purified melanin. Variability in results was up to a maximum of 50% between replicate experiments and up to 20% between duplicates in the same experiment. The absorption spectrum of the purified melanin was obtained by scanning with a Perkin-Elmer Lambda 3 spectrophotometer. Standard dopa-melanin (Sigma), autooxidized catechol, and autooxidized indole-melanin were used as authentic standards in this analysis.

**Enzyme activity analysis.** Cell extracts were obtained after sonication of the cells suspended in 0.5 M phosphate buffer, pH 6.8, with a Branson Sonic Power Sonifier model 350. The cells were disrupted by four 20-s bursts of full power at 1-min intervals. Cell debris was removed by centrifugation, and the protein content of the cell extract was determined.

Phenol oxidase (EC 1.10.3.1) activity was determined as described in the Worthington Enzyme Manual (46). Enzyme activity was estimated by monitoring the oxidation of catechol at $A_{390}$ (10) or L-tyrosine at $A_{280}$ at 1-min intervals.

Nitrogen-fixing activity was determined by using 3 ml of cell suspension in a 10-ml Erlenmeyer flask which was sealed with a serum stopper and had 10% of the atmosphere replaced by acetylene. To determine the effect of oxygen on nitrogenase activity, cells were grown in 48 ml of medium in 160-ml serum bottles. The bottles were sparged with air for 1 min, and volumes of air were replaced with nitrogen to obtain various oxygen concentrations (11.2 ml for 10% $O_2$ and 16.8 ml for 5% $O_2$). For estimating nitrogenase activity, the bottles were sparged with helium for 2 min and 10% of the atmosphere was replaced with acetylene. The flasks and bottles were incubated under the same conditions used for growth for 1 h, and ethylene produced was estimated by injecting 0.5 ml of the gas phase into a Hewlett-Packard gas chromatograph (model 5890A) fitted with a Poropak R column. The specific activity of nitrogenase was expressed as micromoles of ethylene produced milligram of cell $protein^{-1}$ $hour^{-1}$. There was up to 20% variability in results between replicate experiments.

Catalase and peroxidase activities were assayed as described previously (33). Hydroxamate production was estimated as described previously (32). Variability of up to 15% was observed in results obtained in replicate experiments.

## RESULTS

**Pigmentation of cells.** *A. chroococcum* 184 cells became increasingly pigmented during growth in Burk nitrogen-free medium containing 10 μM ferric citrate (Table 1). The color of the cells changed from colorless to light brown after 72 h and to black at 96 h, after which time there was no further detectable change.

The cell-associated pigment was extracted from the cells as described in Materials and Methods and was identified as a melanin on the basis of the following characteristics: solubility in hot 0.5 M NaOH and in 1 M $Na_2CO_3$ and insolubility in cold water, hot water, ethanol, chloroform, acetone, and cold 0.5 M NaOH. Upon addition of 0.1 M $FeCl_3$, a flocculent brown precipitate formed; the pigment was bleached by $H_2O_2$ (15% [wt/vol]), and there was a decrease in intensity of the color upon addition of glutathione (5% [wt/vol]). The absorption spectrum of the purified pigment was characteristic of a melanin (Fig. 1).

**Effect of amino acids, charcoal, benzoic acid, and light on melanin formation.** Strain 184 pigmentation appeared normal

TABLE 1. Melanin production and nitrogen-fixing activity over a period of 5 days[a]

| Incubation period (h) | Protein ($\mu g\ ml^{-1}$) | Melanin (mg mg of protein$^{-1}$) | Catechol ($\mu$mol mg of protein$^{-1}$) | Nitrogen fixation ($\mu$mol of ethylene produced mg of protein$^{-1}$ $h^{-1}$) |
|---|---|---|---|---|
| 24 | 200 | 0.63 | 0.04 | 4.60 |
| 48 | 310 | 1.31 | 0.41 | 1.33 |
| 72 | 295 | 4.86 | 1.00 | 1.00 |
| 96 | 300 | 48.70 | 0.54 | 0.76 |
| 120 | 293 | 38.10 | ND[b] | 0.00 |

[a] All values are means of at least two duplicate determinations. Samples were withdrawn from the cultures (150 ml per 500-ml Erlenmeyer flask) at the appropriate time intervals.
[b] ND, Not determined.

when the cells were grown on nitrogen-free agar slants containing L-tyrosine, L-cysteine, or L-tyrosine plus L-cysteine (Table 2). However, these amino acids did not promote melanin formation when ammonium acetate was the nitrogen source. Melanization was also unaffected by incubation of slants in the light or in the dark (data not shown).

Quantitative studies using liquid growth medium indicated that all the amino acids depressed melanin formation (Table 2). Melanization was also suppressed by the presence of charcoal or benzoic acid in the medium. Significant stimulation of melanin formation (in the presence of $NH_4^+$) or intensification of melanin color (in the presence of $N_2$) was caused by copper.

**Presence of catechol in culture supernatant fluids.** After 72 h of incubation, the culture supernatant fluid of nitrogen-fixing strain 184 contained a compound which absorbed strongly at 272 nm and was reactive in the Barnum assay for

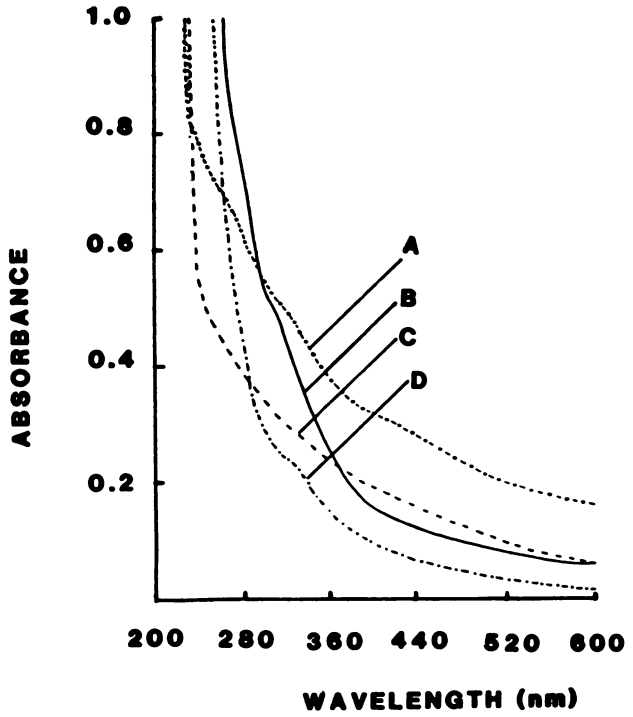


FIG. 1. Absorption spectra of autooxidized catechol-melanin (A), autooxidized indole-melanin (B), standard dopa-melanin (Sigma) (C), and purified strain 184 melanin (D).

TABLE 2. Effect of amino acids, copper, and $NH_4^+$ on melanin formation

| Addition(s) to growth medium | Melanization in: | |
|---|---|---|
| | Solid medium[a] | Liquid medium[b] |
| $-NH_4^+$ | | |
| None | + | 34 |
| CuCl (2.5 $\mu$M) | +++ | 38 |
| L-Tyrosine (0.1%) | + | 16 |
| L-Tyrosine + L-cysteine (0.1%) | + | 18 |
| L-Cysteine (0.1%) | + | 18 |
| Charcoal (0.2%)[c] | ND[d] | 7 |
| Benzoic acid (1 mM) | ND | 4 |
| $+NH_4^+$ (14 mM) | | |
| None | − | 0 |
| CuCl (2.5 $\mu$M) | +++ | 17 |
| L-Tyrosine (0.1%) | − | 0 |
| L-Tyrosine + L-cysteine (0.1%) | − | 0 |
| L-Cysteine (0.1%) | − | 0 |

[a] After 7 days of incubation. +++, Dark brown to black; +, brown; −, no brown color. All values are means of at least two duplicate determinations.
[b] After 4 days of incubation. Values are expressed as milligrams of melanin milligram of protein$^{-1}$ and are means of at least two duplicate determinations.
[c] 14/60-mesh charcoal (Sigma).
[d] ND, Not determined.

*o*-dihydric phenols. Its migration pattern on silica gel with the solvent systems described in Materials and Methods was identical to that of authentic catechol. The red color developed in the Barnum assay also showed an identical absorption maximum to that of standard catechol. Gas chromatographic-mass spectrometric analysis of the purified compound clearly identified the compound as catechol. Resorcinol (1,3-benzenediol) and hydroquinone (*p*-dihydroxybenzene), which had the same molecular weight as catechol, did not react in the Barnum assay.

Catechol was produced under a range of aeration conditions imposed by shaking different volumes of medium at 250 rpm (Table 3). Catechol released into the culture supernatant fluid were inversely related to melanization and increased aeration. Furthermore, it was observed that as the cells melanized, the catechol disappeared from the culture supernatant fluids (Table 1). These data, combined with the spectral data (Fig. 1), suggested that the strain 184 melanin was a catechol melanin.

TABLE 3. Effect of aeration on hydroxamate, catechol, and melanin formation by nitrogen-fixing cells

| Culture volume (% of flask volume) | Production of[a]: | | | |
|---|---|---|---|---|
| | Protein ($\mu g\ ml^{-1}$)[b] | Hydroxamate ($\mu$g of hydroxamate-nitrogen mg of protein$^{-1}$)[b] | Catechol ($\mu$mol mg of protein$^{-1}$)[c] | Melanin (mg mg of protein$^{-1}$)[d] |
| 10 | 321 | 3.00 | 0.22 | 10.30 |
| 20 | 301 | 1.07 | 0.82 | 18.10 |
| 30 | 274 | 0.15 | 0.99 | 32.00 |
| 40 | 224 | —[e] | 3.40 | 4.50 |
| 50 | 221 | — | 3.77 | 1.76 |
| 60 | 177 | — | ND[f] | 1.65 |

[a] All values are means of at least two duplicate determinations.
[b] After 40 h of growth.
[c] After 72 h of growth.
[d] After 96 h growth.
[e] —, Not detectable.
[f] ND, Not determined.

Melanization was absent when the cells were grown under static, very-low-aeration, or microaerophilic growth conditions. Melanization at low-aeration conditions (shaking at 150 rpm) was enhanced by the presence of iron in the medium. With 10 μM ferric citrate, there was no melanization after 96 h, but with 50 and 100 μM ferric citrate the cells melanized to a light brown color. Vigorously aerated cultures, on the other hand, melanized by 72 h at all iron levels.

**Oxygen effect on nitrogenase activity and hydroxamate production.** Strain 184 appeared to have optimal nitrogenase activity at oxygen concentrations lower than atmospheric concentrations. A 10% $O_2$ concentration supported the highest nitrogenase activity. The activities at 20% $O_2$ and 5% $O_2$ were 66 and 76%, respectively, of the activity at 10% $O_2$ after 18 h of growth.

Furthermore, when strain 184 was grown under microaerophilic or static conditions, it did not produce the hydroxamate siderophore reported earlier (32). Similarly, when grown at various aeration rates, hydroxamate production was not detectable at lower aeration rates (Table 3). The iron content of the cells varied from 1.27 to 1.41 μg of $Fe^{3+}$ mg of protein$^{-1}$ at the higher aeration rates to 0.77 to 0.98 μg of $Fe^{3+}$ mg of protein$^{-1}$ at the lower aeration rates. Cells from cultures grown at higher aeration rates contained 70 to 100% of the added iron after 40 h, whereas cells grown at the lower aeration rates had 30 to 50% of the added iron. Hydroxamate production was probably repressed at low aeration rates because of the presence of repressive levels of iron which remained in the growth medium because of low iron utilization. These levels of iron were much higher than the iron-limited levels of 0.36 to 0.59 μg of $Fe^{3+}$ mg of protein$^{-1}$ reported previously (32), although cultures at higher aeration rates were apparently becoming externally iron limited.

**$NH_4^+$ effect on melanization and catechol formation.** $NH_4^+$ appeared to repress melanization (Table 2). Levels of $NH_4^+$ that completely inhibited nitrogenase activity (14 mM) also prevented melanization and catechol production (Table 4). Lower levels of $NH_4^+$ which partially inhibited nitrogenase activity also delayed melanization and depressed catechol production. Levels of $NH_4^+$ which did not depress nitrogenase activity also were without effect on catechol formation and melanization (Table 4). When $NH_4^+$ (14 mM) was added at various stages of growth under nitrogen-fixing conditions, it was observed that catechol production was suppressed when ammonium was added during the lag phase (0 to 10 h) and during the exponential phase of growth (10 to 28 h). However, when $NH_4^+$ was added during the stationary phase (after 28 h), catechol had already been produced and melanization occurred, although to a lesser extent (data not shown).

The culture fluids from $NH_4^+$-grown cells after periods of incubation from 1 to 3 days did not contain any inhibitory compound which would inhibit melanin formation when added to nitrogen-fixing cells. This suggested that $NH_4^+$ repression occurred as a result of an internal change and not through some excreted product.

Polyphenol oxidase activity was observed in cell extracts prepared from 72-h nitrogen-fixing cells but not from $NH_4^+$-grown cells. $NH_4^+$ (up to 10 mM) added to cell extracts did not inhibit the activity of this enzyme in vitro, and mixing the cell extracts from $NH_4^+$-grown and $N_2$-grown cells did not decrease the enzyme activity more than was expected from the resulting dilution. Enzyme activity was not observed in the cell extracts prepared from 24- and 48-h $N_2$-fixing cultures. However, when approximately 1 μmol of catechol mg of protein$^{-1}$ was observed in the culture supernatant fluid, polyphenol oxidase activity was detectable in the cells (0.0052 $\Delta A_{390}$ min$^{-1}$ mg of protein$^{-1}$), which suggested that catechol was involved in the induction of this enzyme.

TABLE 4. Effect of $NH_4^+$ on catechol, melanin, and nitrogenase activity[a]

| Incubation conditions and time (h) | Catechol production (μmol mg of protein$^{-1}$) | Nitrogenase activity (μmol of ethylene mg of protein$^{-1}$ h$^{-1}$) | Time of initial melanization (final intensity)[b] |
|---|---|---|---|
| Nitrogen fixing | | | |
| 24 | 0.04 | 2.67 | 96 h (+++) |
| 48 | 0.97 | 0.96 | |
| 72 | 1.00 | —[d] | |
| 2 mM $NH_4^+$[c] | | | |
| 24 | 0.04 | 2.87 | 96 h (+++) |
| 48 | 0.70 | 0.99 | |
| 72 | 1.18 | — | |
| 4 mM $NH_4^+$[c] | | | |
| 24 | 0.04 | 0.86 | 96 h (++) |
| 48 | 0.42 | 0.86 | |
| 72 | 0.85 | — | |
| 8 mM $NH_4^+$[c] | | | |
| 24 | 0.03 | 0.90 | 7 days (++) |
| 48 | 0.09 | 0.95 | |
| 72 | 0.18 | — | |
| 14 mM $NH_4^+$[c] | | | |
| 24 | 0.02 | — | No melanization |
| 48 | 0.04 | — | |
| 72 | 0.02 | — | |

[a] All values are means of at least two duplicate determinations.
[b] +++, Dark brown to black; ++, brown.
[c] $NH_4^+$ was added as ammonium acetate.
[d] —, Not detectable.

**Melanin formation and nitrogenase activity.** Melanin formation and nitrogenase activity were monitored over a period of 5 days (Table 1). It was observed that maximum melanization occurred after 4 days of growth, whereas nitrogenase activity was at its peak in the early stages of growth and was very low after 3 days (Table 1 and 4). Thus, there was no correlation between nitrogenase activity and melanin formation, which suggested that melanin per se was not playing a protective role for nitrogenase activity.

**Effect of copper on melanization.** When strain 184 was grown in medium containing copper ($Cu^{2+}$ or $Cu^{1+}$), melanization was accelerated so that peak production in liquid medium occurred after 48 h. Copper also stimulated melanization of cells grown in the presence of $NH_4^+$ (Table 2), and therefore appeared to partially reverse the inhibitory effects of $NH_4^+$. Cells grown in the presence of copper and $NH_4^+$ also contained polyphenol oxidase activity (0.01 $\Delta A_{390}$ min$^{-1}$ mg of protein$^{-1}$). In an attempt to determine whether cells grown with $NH_4^+$ contained an inactive polyphenol oxidase, it was observed that the addition of copper to this cell extract resulted in a rapid change of $A_{390}$ without the addition of catechol substrate. This background reaction resulted in a constant value of 0.02 $\Delta A_{390}$ min$^{-1}$ mg of protein$^{-1}$. By comparison, when copper was added to cell extracts prepared from $N_2$-fixing minus copper, $N_2$-fixing plus copper, and $NH_4^+$ plus copper growth conditions, the substrate-dependent enzyme specific activity values were 0.138, 0.208, and 0.074 $\Delta A_{390}$ min$^{-1}$ mg of protein$^{-1}$, respectively.

**Effect of copper on catalase and peroxidase levels in strain 184.** In the presence of copper, strain 184 did not contain detectable catalase or peroxidase activities. However, copper did not inhibit aerobic growth under these conditions, as indicated by similar protein values. The addition of 15 μM copper did not affect the in vitro activity of active catalase or peroxidase in cell extracts. Thus, it would appear that in the presence of copper, strain 184 can still grow substantially even in the absence of the enzymes that normally would be required for aerobic growth and would normally protect the cell from toxic oxygen products.

## DISCUSSION

When *A. chroococcum* 184 is grown under nitrogen-fixing conditions and relatively high aeration, it produces a cell-associated melanin in the later stages of its growth cycle. Catechol which is produced by the organism disappears upon melanization, suggesting that the melanin is derived from catechol and that the catechol may be both an inducer and a substrate for melanization. Catechol melanins have been observed previously in plant seeds and in the spores of *Ustilago maydis* (29, 34). The amino acids L-tyrosine and L-cysteine did not enhance melanin formation in strain 184, unlike the melanization of *Vibrio cholerae* (19) and *Azospirillum brasilense* (40), but in fact decreased it to a certain extent. On the basis of these results, the $Na^+$-dependent strain of *A. chroococcum* appears to produce an allomelanin rather than a eumelanin or phaeomelanin.

Maximum pigmentation of strain 184 occurred at relatively high aeration, and pigment formation was suppressed by $O_2$ limitation. Melanin formation was not observed in cells grown under static growth conditions or in cells grown microaerophilically. Thus, melanin formation appears to be necessary for or a consequence of high aeration rates. Ivins and Holmes (19) similarly report melanin production by *V. cholerae* Htx-3 when grown under higher aeration and not when grown under static or anaerobic growth conditions. These results could be simply due to the presence or absence of $O_2$, which participates directly in the enzymatic oxidation of polyphenols (25).

In the presence of charcoal, a free-radical trap and an adsorbent of toxic $O_2$ intermediates (18), strain 184 cells do not melanize. Hoffman et al. (18) used charcoal in laboratory media to increase the aerotolerance of microaerophilic organisms such as *Legionella* spp. Similarly, benzoic acid, which can act as a scavenger of hydroxyl radicals (15), also had a suppressive effect on melanization. These results suggest that there may be an inverse relationship between melanization and the presence of toxic oxygen species in strain 184.

Copper accelerated melanin formation and also increased the intensity of the pigment color in strain 184. This ion is an integral part of polyphenol oxidase and is a known stimulator of enzyme activity (25). However, copper eliminated the catalase and peroxidase activities of strain 184 but did not prevent aerobic growth of the cells. These results indicate that an alternative mode of protection from toxic oxygen species must exist in these cells.

Azotobacters normally display a high respiratory rate under nitrogen-fixing conditions to scavenge $O_2$ and create a suitable reducing environment for nitrogenase (39). The $Na^+$-dependent *A. chroococcum* also appear to use respiratory protection of their nitrogenases when grown aerobically (33), and nitrogen-fixing cells would therefore be expected to produce greater amounts of toxic $O_2$ species than $NH_4^+$-grown cells. However, strain 184 does not increase catalase activity in response to increased aeration (33). Strain 184 possesses an active superoxide dismutase (33), but the end product of this enzymatic reaction would be $H_2O_2$. Whatever the oxygen protection system of strain 184 is, it must be relatively inefficient compared with the enzymatic protection present in other azotobacters, because the addition of small amounts of $H_2O_2$ is very toxic to the $Na^+$-dependent strain (33).

The $Na^+$-dependent strains of *A. chroococcum* possibly have developed an oxygen protection system, based on catechol formation and melanization, which is especially important for aerobic growth under nitrogen-fixing conditions. Catechol formation may also occur in $NH_4^+$-grown cells, although it is not excreted. This is suggested by the presence of phenol oxidase activity when $Cu^+$ is added to cell extracts without added substrate.

The superoxide-dependent formation of hydroxyl radicals requires the presence of transition metal ions, and it has been found that iron and copper are the most effective (16). Both ions accelerated melanization in strain 184. Molecules which bind iron can prevent it from participating in the Fenton reaction and thereby protect a cell from the damaging effects of the hydroxyl radical. Catechol is such a molecule and could itself be a target for the hydroxyl radical. In a model chemical system, the catechol Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid) is aerobically oxidized to the *o*-semiquinone radical by superoxides (27). Semiquinone radicals are also intermediates in the formation of catechol melanin (7). Catechols, such as Tiron, can react directly with reduced flavin-$O_2$ compounds, resulting in the oxidation of the catechol, and reaction of metalloflavoproteins with oxygen also result in the oxidation of Tiron (27). Hydroxylation of aromatic compounds has been used as an in vitro assay for the production of the hydroxyl radical from superoxides (38). Higher levels of iron result in higher levels of hydroxylation (16). Salicylic acid in *Mycobacterium* species and dihydroxybenzoic acids in *Enterobacter aerogenes* have been reported to accumulate in culture media of iron-deficient organisms (37). Salicylic acid and phenol were hydroxylated to diphenols by the hydroxyl radical in in vitro hydroxylation studies (16, 38). Thus, catechol formation could be mediated by hydroxy radicals, and the catechol so formed could be oxidized to further protect the cell from superoxide-mediated oxidative damage.

Due to the ability of melanins to bind cations such as iron, melanins may protect cell membranes from damage by hydroxyl radicals generated via the Fenton reaction. Pilas et al. (35) reported a decrease in hydroxyl radical formation when ferrous ion is bound to melanin at low iron concentrations. Conversely, Aver'yanov et al. (4) reported an increased sensitivity of the mutant spores of *Pyricularia oryzae*, which lacked melanin, to the hydroxyl radical and the superoxide radical. Melanins also have the ability to oxidize 80% of the $O_2^-$ to $O_2$ and reduce 20% of supplied $O_2^-$ to $H_2O_2$, thus demonstrating a pseudo-superoxide dismutase-peroxidase activity (22). Since strain 184 lacks catalase and has only low peroxidase activity, the ability of melanin to remove harmful oxygen species without the formation of significant levels of $H_2O_2$ could be advantageous to the cell.

The hydroxamate deferrioxamine inhibits hydroxyl radical formation by the iron-catalyzed Fenton reaction by chelating iron and rendering it less reactive (14). Strain 184 produces a hydroxamate-type siderophore which appears to function in iron assimilation, but its regulation is unusual (31). This

hydroxamate is formed in the presence of iron concentrations that would normally repress the formation of a siderophore (32). As shown in this study, the hydroxamate also appears to be more important to aerobically grown cells than to microaerophically grown cells, although both must grow by respiratory metabolism. It is possible that the iron-binding ability of the siderophore and its resulting ability to diminish the reactivity of free iron in the Fenton reaction could further protect the cell from oxidative damage.

As noted previously, catechol formation and melanization appear to be most important to nitrogen-fixing cells, possibly as a consequence of respiratory protection of nitrogenase and the increased number of iron-containing redox enzymes and potential sites for oxygen inactivation (39). When nitrogenase is repressed by $NH_4^+$, the production of catechol and melanization are also repressed. Since *nifA* regulates melanin formation in *Rhizobium leguminosarum* biovar phaseoli (17), melanization must be subject to $NH_4^+$ repression in this species. The *Rhizobium* melanization is not necessary for symbiotic nitrogen fixation (24), but *Rhizobium* spp. only fix $N_2$ microaerophilically. Catechol formation and melanization in strain 184 may be an example of a chemical protection system which has been developed for aerobic nitrogen fixation and is an alternative to the more traditional enzymatic oxygen protection systems found in obligate aerobes.

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