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1 **Pyomelanin is Produced by *Shewanella algae* BrY and Effected by Exogenous Iron**

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Abstract

Melanin production by *S. algae* BrY occurred during late/post-exponential growth in lactate-basal-salts liquid medium supplemented with tyrosine or phenylalanine. The antioxidant ascorbate inhibited melanin production, but not production of the melanin precursor, homogentisic acid. In the absence of ascorbate, melanin production was inhibited by the 4-hydroxyphenylpyruvate dioxygenase inhibitor, sulcotrione and Fe(II) (>0.2mM). These data support the hypothesis that pigment production by *S. algae* BrY was a result the conversion of tyrosine or phenylalanine to homogentisic acid which was excreted, auto-oxidized and self-polymerized to form pyomelanin. The inverse relationship between Fe(II) concentration and pyomelanin production has implications that pyomelanin may play a role in iron assimilation under Fe(II) limiting conditions.

Key Words: pyomelanin, *Shewanella*, 4-hydroxyphenylpyruvate dioxygenase, homogentisic acid, sulcotrione

1 The facultative, dissimilatory metal reducer, *Shewanella algae* BrY inhabits the
2 oxic/anoxic zone of marine sediment and produces a dark, extracellular, quinoid pigment
3 characterized as a type of melanin (Caccavo et al. 1992; Turick et al. 2002). *S. algae* BrY
4 exploits the redox cycling properties of melanin through use as a terminal electron acceptor and,
5 in turn, as a soluble electron shuttle capable of reducing insoluble Fe (III)-oxides (Turick et al.
6 2002; Turick et al. 2003). Because melanin also has iron chelation properties, it also plays a role
7 in iron assimilation. For example, Fe(II) assimilation by the yeast *Cryptococcus neoformans* is
8 achieved through the production of eumelanin, a type of melanin (Nyhus et al. 1997). This
9 mechanism incorporates the reductive capacity and metal chelation properties of cell-surface-
10 associated melanin to first reduce exogenous Fe(III) and then assimilate the resulting Fe(II).
11 *Legionella pneumophila* produces pyomelanin, another type of melanin, and was postulated to
12 also use it for extracellular Fe(III) accumulation (Cianciotto et al. 2002). Hence, melanin
13 production may also play an important role in electron transfer to iron oxides related to growth
14 and Fe(II) assimilation of the dissimilatory metal reducing, facultative anaerobe *S. algae* BrY. In
15 order to better understand the role of melanin in the physiological ecology of *S. algae* BrY, it is
16 important to clearly define the type of melanin produced.

17 Melanin is an imprecise term that describes a general category of high-molecular-weight
18 dark pigments of biological origin (Bell and Wheeler 1986). Based on biochemical
19 characteristics, melanin is further differentiated into several types including eumelanin,
20 pheomelanin, allomelanin and pyomelanin. Eumelanin production occurs by the Mason-Raper
21 pathway in which tyrosine is converted to dihydroxyphenylalanine (DOPA) and dopachrome by
22 tyrosinase and oxygen (Bell and Wheeler 1986; Swan 1974; Protá 1992). Phaeomelanins are
23 produced by the conversion of tyrosine to dopachrome which reacts with cysteine (Swan 1974;

1 Protá 1992; Coon et al. 1994). Allomelanins are produced from non-nitrogenous phenols and
2 result in a wide range of diverse phenolic products (Swan 1974; Protá 1992). Tyrosinase plays a
3 significant role in the production of these types of melanin [6-8] and its activity is correlated to
4 exogenous Cu (II) concentrations (Swan 1974; Protá 1992; Ikeda et al. 1996). Pyomelanin
5 (alkaptan) is defined by the conversion of tyrosine or phenylalanine to homogentisic acid (HGA),
6 which is then excreted from the cell (Yabuuchi and Omyama 1972) to form a reddish-brown
7 pigment after autoxidation and self-polymerization. Bacterial production of pyomelanin was first
8 described for *Pseudomonas aeruginosa* (Yabuuchi and Omyama 1972) and has been identified in
9 several bacterial species including *S.colwelliana*, *Vibrio cholera*, and a *Hyphomonas* strain
10 (Ruzafa et al. 1995; Weiner et al. 1985; Kotob et al. 1995) as well as *Alcaligenes eutrophus*
11 (David et al. 1996). With gram-negative bacteria, pyomelanin production occurs through the
12 phenylalanine-tyrosine pathway (David et al. 1996; Lehninger 1975) where phenylalanine is
13 converted to tyrosine, which is then transaminated to 4-hydroxyphenylpyruvate. The
14 metalloenzyme, 4-hydroxyphenylpyruvate dioxygenase (EC1.13.11.27) (4-HPPD), converts 4-
15 hydroxyphenylpyruvate to HGA. In contrast to tyrosinase, 4-HPPD has a non-heme iron
16 complex (Lindbald et al. 1977; Lindstedt et al. 1977). Thus, Fe (II) affects 4-HPPD activity
17 while Cu (II) does not (Lindbald et al. 1977; Lindstedt et al. 1977). HGA is ultimately converted
18 to fumaric acid and acetoacetic acid, but pyomelanin producers are either unable to oxidize HGA
19 or possess a rate of HGA oxidation which is lower than that of 4-HPPD activity. The overall
20 effect results in an increase in HGA and its excretion from the cell (Yabuuchi and Omyama
21 1972; David et al. 1996; Lindstedt et al. 1977; Sanches-Amat et al. 1998).

22 A reddish-brown form of melanin, tentatively identified as pyomelanin (Turick et al.
23 2002) is produced by *S. algae* BrY during late/post exponential growth in the presence of

1 tyrosine. Because this organism uses soluble and insoluble forms of Fe(III) as a terminal electron
2 acceptor and Fe(II) is a component of 4-HPPD, the role of Fe(II) is expected to play a significant
3 role in melanin pigment production as *S. algae* (BrY) transitions back and forth between oxic
4 and anoxic conditions. The type of melanin and factors related to its production were the focus of
5 this study in order to begin to understand the role of this pigment in the physiological ecology of
6 *S. algae* BrY.

7 Bacterial growth and melanin production were as described previously (Turick et al 2002)
8 and used either Tryptic Soy broth (TSB) (McCuen, 1988) or a lactate basal salts medium
9 (LBSM) (Lovley et al. 1996) supplemented with tyrosine or phenylalanine concentrations of 2 g
10 l⁻¹. Except where indicated, iron was omitted from LBSM to reduce its effect on melanin
11 production or behavior. Cell numbers were measured by staining the cells with acridine orange
12 and then visualizing them with an epifluorescence microscope (Hobbie et al. 1977). Chemical
13 determination of melanin was performed as previously described (Ellis and Griffiths 1974;
14 Turick et al. 2002). Melanin content of spent, cell-free culture medium was determined
15 spectrophotometrically at 400 nm (Ruzafa et al 1995) and zeroed against controls (cultures
16 grown without tyrosine). For rate determination, samples were assayed at 400 nm for melanin
17 production at different time intervals throughout the growth cycle. Rates were calculated from
18 the data prior to a plateau in melanin production, by linear regression of log-transformed data.

19 Cell-free spent growth media of *S. algae* BrY were assayed for melanin precursors by high-
20 pressure capillary electrophoresis (HPCE) with a Celect H150 C-8 bonded phase capillary column.
21 Standards (DOPA and HGA) were dissolved in 4 mM ascorbate to a final concentration of 4 mM
22 each. HGA and DOPA were also determined by colorimetric methods. DOPA analysis consisted of

1 the DOPA nitration method (Waite and Benedict 1984). HGA content was determined based on its
2 reaction with cysteine to form 1, 4-thiazine, according to the methods of Fellman et al. (1972).

3 For this study, all experiments were conducted in duplicate or triplicate and performed at
4 least twice. Statistical analysis was conducted with Student's t test.

5 **Extracellular melanin polymerization**

6 Melanin was produced by aerobically grown cultures in all media supplemented with
7 tyrosine or phenylalanine, but was not detected in these media under anoxic conditions. On solid
8 media, melanin production occurred in the regions of the highest colony density on the plates.
9 Pigment was first observed surrounding individual colonies. After several days, the darkness of
10 the colonies, themselves, increased. Colonies growing on T-soy agar (TSA) without
11 supplemental tyrosine showed only slight pigmentation. The onset of melanogenesis occurred
12 24 h earlier on solid media incubated at 37°C than at 28°C. Pigment produced on complex
13 medium (TSA plates) was determined to be melanin and related to the tyrosine and
14 phenylalanine content (300 mg l⁻¹ and 600 mg l⁻¹ respectively) of the medium. Similar results
15 were reported for pyomelanin production by *P. aeruginosa* (Yabuuci and Omyama 1972), *V.*
16 *cholera* (Sanchez-Amat et al. 1998), *S. colwelliana* (Ruzafa et al. 1995; Weiner et al. 1985) and
17 *L. pneumophila* (Ciacciotto et al. (2002).

18 When melanin production was measured by the absorbance at 400 nm (Ruzafa et al.
19 1995), melanin production was 7-fold greater in cell-free, spent LBSM media supplemented with
20 tyrosine (2 g l⁻¹) than phenylalanine (2 g l⁻¹). No melanin was produced in media without either
21 supplement. Melanin production by *S. algae* BrY in LBSM supplemented with phenylalanine
22 occurred 24 h later than in LBSM supplemented with tyrosine (data not shown). After 72 h of
23 incubation, melanin production leveled off in these cultures and resulted in an OD (A₄₀₀) of

1 3.274 for the tyrosine supplemented culture (controls subtracted) and a significantly different
2 ($P < 0.05$) value of 0.459 for the phenylalanine supplemented culture.

3 **Metabolic precursors of melanin**

4 Extracellular melanin is produced by the auto-oxidation and self-polymerization of
5 metabolic precursors such as HGA or DOPA (Yabuuchi and Omyama 1972; Weiner et al. 1985;
6 Kotob et al. 1995; Ruzafa et al. 1994). To determine if extracellular melanin occurred through
7 auto-oxidation, the antioxidant ascorbate was added to the cultures. Cultures of *S. algae* BrY
8 grown for 72 h at 28°C in LBSM supplemented with 12 mM of both the antioxidant ascorbate
9 and tyrosine did not produce melanin. The absorbance (A_{400}) of that cell-free culture supernatant
10 was 1.3, while cell-free culture supernatant from the culture with tyrosine only was 3.5.

11 *S. algae* BrY cultures were grown at 28°C in LBSM with 12 mM tyrosine, (with and
12 without 12 mM ascorbate) and analyzed after 18 and 48 h of growth for the presence of melanin
13 precursors (Figs. 1). For cultures without ascorbate, two peaks were detected after 18 h (Fig.
14 1A), but only one peak remained after 48 h (Fig. 1B). Cultures incubated with ascorbate revealed
15 only one peak, which corresponded to HGA (Fig. 1C and 1D). The peaks with retention times of
16 2.68 min. co-migrated with HGA (Fig. 1D) and were confirmed to be HGA by colorimetric
17 analysis. The HGA peak was not detected in growth medium without tyrosine. The first peak,
18 from the 18 h sample without ascorbate, migrated later than the DOPA peak (Fig. 1A and 1D).
19 Colorimetric analysis of the sample confirmed that the peak was not DOPA. These results
20 support our hypothesis that *S. algae* BrY produces extracellular pyomelanin by the auto-
21 oxidation of excreted HGA but not DOPA.

22 Several other pyomelanin-producing bacteria have been shown to produce pyomelanin by
23 this process of HGA auto-oxidization and self-polymerization (Yabuuchi and Omyama 1972;

1 Ruzafa et al. 1995; Weiner et al. 1985; Ruzafa 1994). Although HGA was consistently identified
2 in culture liquid, the presence of another melanin precursor, DOPA, was not detected in any of
3 the cultures. Only one peak appeared near the DOPA retention time during these studies and
4 was not present in the presence of ascorbate. The absence of this peak in the presence of
5 ascorbate suggests that this compound may be an autooxidation product of another metabolite.
6 With *S. colwelliana*, DOPA is produced, but it is not linked to pigment production (Kotob et al
7 1994). In this study, HGA was detected in cultures prior to detectable melanin production, which
8 has been observed previously (Fuqua and Weiner 1993; Ruzafa et al. 1994). The gene
9 responsible for HGA production by *S. colwelliana* is constitutive (Kotob et al. 1995) and may
10 also be constitutive for *S. algae* BrY.

11 **Enzyme inhibition experiments**

12 Sulcotrione [2-(2-chloro-4-methanesulfonylbenzoyl)-1,3-cyclohexanedione] (Zeneca
13 Ag. Products, Richmond, CA) is a potent inhibitor of 4-HPPD (Lee et al 1997; Schulz et al.
14 1993; Secor 1988). *S. algae* BrY was grown for 48 h in tyrosine-supplemented LBSM with
15 sulcotrione (0, 0.25, 2.5, and 10 μM final concentration) to test its effects on melanin production.
16 Melanin production by *S. algae* BrY grown was completely inhibited with 10 μM sulcotrione
17 (Fig. 2) with an inhibition constant (K_i) of 0.04 μM . The inhibition constant (K_i) for sulcotrione
18 was determined as previously described (Turick and Apel 1997). Cell density and growth were
19 not affected by the sulcotrione concentrations tested in Fig. 2 (data not shown).

20 The enzyme tyrosinase (EC 1.14.18.1) will convert tyrosine to DOPA, which is quickly
21 oxidized to dopachrome (Prota 1992; Yasunobu et al. 1959). The effect of sulcotrione on the
22 activity of commercially-obtained mushroom tyrosinase (Sigma Chem.) was tested to confirm
23 the specificity of the inhibitor. Tyrosinase (EC 1.14.18.1) activity was measured as previously

1 described (Yasunobu et al. 1959). The addition of 8 mM sulcotrione did not inhibit the activity
2 of 1336 units commercially-obtained mushroom tyrosinase. Thus, sulcotrione inhibition of
3 melanin production by *S. algae* BrY supports the hypothesis that the enzyme 4-HPPH and not
4 tyrosinase was responsible for melanin production by *S. algae* BrY.

5 **Effect of Fe (II) on melanin production**

6 Exogenously added Cu(II) enhances tyrosinase activity and related melanin production
7 (Bell and Wheeler 1986; Swan 1974; Ikeda et al. 1996), but does not affect 4-HPPD activity
8 (Linbald et al; 1997; Lindstedt et al 1977). Conversely, exogenous Fe(II) controls 4-HPPD
9 activity but does not affect that of tyrosinase. The effects of these metals on melanin production
10 by *S. algae* BrY was tested in LBSM supplemented with tyrosine (Fig. 3).

11 The growth of *S. algae* BrY was not adversely affected by the Fe (II) concentrations used
12 in this study (Fig. 3A). However, exogenous Fe(II) affected melanin production by *S. algae* BrY
13 (Fig. 3B). The highest rate of melanin production was achieved with 0.18 mM Fe(II), while
14 higher Fe(II) concentrations decreased the rate of melanogenesis. Melanin production in control
15 cultures receiving 3.76 mM Na₂SO₄ was greater than those cultures receiving 3.76 mM FeSO₄,
16 demonstrating that effect on melanin production was specific to iron (Fig. 3B). The addition of
17 20µM Cu(II) did not affect melanin production in *S. algae* BrY indicating a copper-dependent
18 enzyme such as tyrosinase was not involved melanin production.

19 To determine if this decrease in melanin production was caused by Fe(II)-melanin
20 precipitation, 4.0 mM of Fe(II) (as ferrous sulfate) was added to cell-free melanin. The addition
21 of Fe(II) did not alter the spectral scan of the melanin, which indicates that exogenously added
22 Fe(II) did not cause the precipitation of melanin (data not shown). Although iron will precipitate

1 melanin (Turick et al 2002; Swan 1974; Ellis and Griffiths 1974), the concentrations used in this
2 study were insufficient for melanin precipitation.

3 The effect of exogenous Fe(II) was complex. Low levels of Fe(II) resulted in greater
4 melanin production than Fe(II) in excess of 0.376 mM, which had an inhibitory effect (Fig. 3C).
5 Exogenous Fe(II) concentrations greater than 10^{-5} M inhibit 4-HPPD activity in *Pseudomonas* sp.
6 P.J. 874 (Lindstedt et al. 1977). It is possible that an inhibitory effect from elevated Fe(II)
7 concentrations may be the result of oxygen radicals that were generated during growth with
8 Fe(II). Oxygen radicals have been implicated in the inhibition of 4-HPPD activity (Lindbald et
9 al. 1977; Fellman et al. 1972). When grown aerobically, *S. putrefaciens* 200P produces 30 μ M
10 of extracellular H₂O₂ which reacts with Fe(II) and produces oxygen radicals (McKinzi and
11 DiChristina 1999). It is possible that culture conditions in the present study may have resulted in
12 oxygen radical concentrations high enough to inhibit 4-HPPD activity. Melanin production
13 occurred sooner at 37°C than at 28°C even though the optimum growth temperature of *S. algae*
14 is 25-35°C (Caccavo et al 1992). This difference would suggest that the early development of
15 melanin at 37°C was not the result of an increased growth rate but may be related to increased 4-
16 HPPH activity as a function of lower oxygen solubility at the higher temperature.

17 For this study, the pH of the medium was also evaluated during growth to determine its
18 contribution to melanin production. Extracellular melanin production is related to an increase in
19 the pH of the growth medium (Fuqua et al. 1993; Ruzafa et al 1994). In all cultures in this study,
20 the pH remained near 7 for the first 24 h of growth, until the onset of melanin production (Fig.
21 3C). Elevated pH values were recorded after 40h in cultures with higher FeSO₄ concentrations
22 (Fig. 3C). However, these cultures produced the least melanin suggesting that culture pH did not
23 play a role in accelerating melanogenesis.

1 The appearance of melanin coincided with a pH increase to 7.6 – 7.8, except for the 0.376
2 mM and 3.76 mM Fe(II) supplemented cultures which demonstrated a pH of 8.6 (Fig. 3B and
3 3C). Hence, the added iron did not result in decreased pH but rather an increase. Consequently,
4 the decrease in melanin production at the higher iron concentrations was not a function of pH.
5 Since there were higher cell densities in cultures with more iron, low cell density was not a factor
6 in decreased melanin production.

7 **Conclusions**

8 Several lines of evidence demonstrate that pyomelanin was produced by *S. algae* BrY.
9 First, the MW of the melanin produced by *S. algae* BrY was within the range of 12,000 –14,000
10 (Turick et al. 2002). Second, the antioxidant ascorbate, which inhibits auto-oxidation in solution,
11 inhibited melanin production but not extracellular HGA production. Third, tyrosine was
12 converted to extracellular HGA but not DOPA. Fourth, sulcotrione, a specific inhibitor of 4-
13 HPPH activity, inhibited melanin production. Fifth, iron affected melanin production by *S. algae*
14 BrY, but copper had no effect. Lastly, FTIR analysis of the melanin is consistent with
15 pyomelanin (Truick et al. 2002). These results are consistent with pyomelanin production in the γ
16 Proteobacteria indicating that *S. algae* BrY produced pyomelanin.

17 The ability of *S. algae* BrY to produce pyomelanin and exploit its electrochemical properties
18 for growth and iron mineral reduction may provide this organism a significant survival advantage.
19 *S. algae* BrY is a facultative anaerobe that inhabits the oxic/anoxic zone of sediments (Caccavo et
20 al. 1992; Venkateswaran et al.1999). Pyomelanin production may be important during the
21 transition from oxic to anoxic conditions and therefore offer a means for this organism to adapt to
22 this transition. This would be especially important for *S. algae* BrY because it is capable of using
23 pyomelanin to accelerate the rate of dissimilatory iron mineral reduction. Excessive production of

1 pyomelanin may not be necessary since only femtogram quantities per cell are required to
2 significantly increase iron mineral reduction rates (Turick et al. 2002; 2003). In addition, the redox
3 cycling nature of this pigment allows it to be reused as an electron shuttle during anaerobic
4 respiration with iron minerals (Turick et al. 2002; 2003). However, other pyomelanin producing
5 members of the γ Proteobacteria are not known to be dissimilatory metal reducers. Alternatively,
6 melanin is used to reduce Fe(III) to Fe(II) for assimilation (Nyhus et al. 1997; Cianciotto et al.
7 2002). Because pyomelanin production by *S. algae* BrY is enhanced by decreasing concentrations
8 of Fe(II), its production by *S. algae* BrY and other bacteria may also be related to Fe(III) reduction
9 for Fe(II) assimilation under Fe limiting concentrations.

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1 **Figure Legends**

2

3 Figure 1. HPCE analysis for melanin precursors. After 18 h and 48 h *S. BrY* spent culture
4 supernatant liquids were analyzed as described in the Methods. Panels: (A) 18 h *S. algae* BrY
5 (B) 48 h *S. algae* BrY (C) 18 h *S. algae* BrY in ascorbate supplemented medium.(D) HGA and
6 DOPA standards

7

8 Figure 2. Inhibitory effects of sulcotrione on melanin production by *S. algae* BrY. Melanin
9 production as measured A_{400} . Cells were grown in lactate basal medium supplement with
10 tyrosine.

11

12 Figure 3. The effect of Fe(II) concentration on growth and melanin production by *S. algae* BrY.
13 (A) cell density over time, (B) Melanin production as measured A_{400} , and (C) medium pH. Cells
14 were grown in tyrosine (2g liter^{-1}) supplemented lactate basal medium with 0.18 mM FeSO_4
15 (■), 0.376 mM FeSO_4 (▲), 3.76 mM FeSO_4 (●), 3.76 mM Na_2SO_4 (X), and lactate basal
16 medium without tyrosine (◆).





