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The physiological role and characterization of melanin produced by Shewanella algae BrY

Charles Edwin Turick
University of New Hampshire, Durham

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THE PHYSIOLOGICAL ROLE AND CHARACTERIZATION OF MELANIN PRODUCED BY *SHEWANELLA ALGAE* BRY

BY

Charles Edwin Turick

B.S., California University of Pennsylvania, 1976

M.S., West Virginia University, 1986

DISSERTATION

Submitted to the University of New Hampshire

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In

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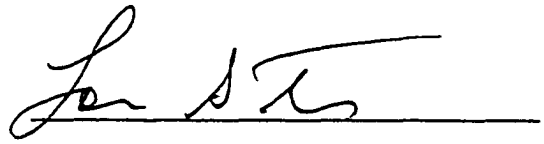

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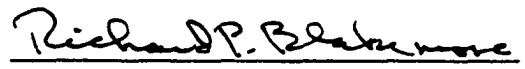

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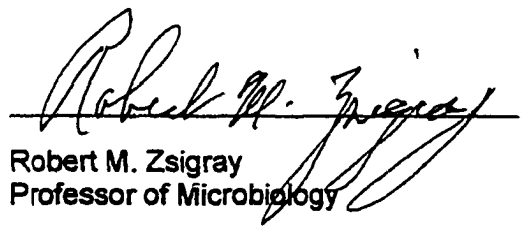
Professor of Microbiology



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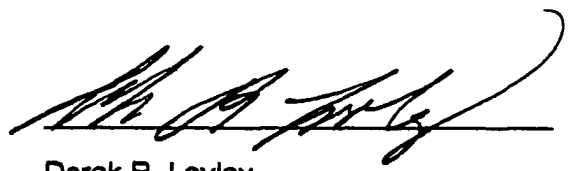

Derek R. Lovley
Distinguished Professor of Microbiology
University of Massachusetts

1/22/01

Date

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ABSTRACT

THE PHYSIOLOGICAL ROLE AND CHARACTERIZATION OF MELANIN PRODUCED BY *SHEWANELLA ALGAE* BRY

by

Charles E. Turick

University of New Hampshire, May, 2001

Shewanella algae BrY is a Gram negative, facultative anaerobe isolated from the Great Bay Estuary of New Hampshire. This member of the γProteobacteria demonstrates extensive versatility in the variety of terminal electron acceptors it uses for anaerobic respiration. Since the natural habitat of this non-fermenting organism is the oxic/anoxic interface of sediments, the ability to utilize various terminal electron acceptors contributes to its survival and growth. Among the terminal electron acceptors used by *S. algae* BrY are insoluble iron minerals. The transfer of electrons from a bacterial cell to an insoluble compound presents a strategic challenge for this organism. This dissertation focuses on understanding the mechanism of electron transfer from *S. algae* BrY to insoluble iron minerals and characterizing the component used in bacterial-mineral electron transfer. Here I show that *S. algae* BrY produces the quinone rich heteropolymer, melanin, when grown with tyrosine. The redox-cycling capabilities of melanin provide it with the ability to serve as a terminal electron acceptor and soluble electron shuttle for iron mineral reduction. When melanin is associated with the cell surface, it accelerates the rate of electron transfer from the cell membrane to iron minerals.

INTRODUCTION

Microorganisms play significant roles in fundamental geological processes and mineral cycling through formation and transformation of minerals (Ehrlich 1996). One role is the use of oxidized metals and metaloids as terminal electron acceptors during anaerobic respiration. This dissimilatory metabolic process occurs in anaerobic environments and can employ either soluble or insoluble mineral oxides. Dissimilatory metal reducing bacteria (DMRB) oxidize organic matter which results in the transfer of electrons to a terminal electron acceptor with consequential energy coupling for growth (Ehrlich 1996).

Biotechnology development is predicated upon a fundamental understanding of microbial physiology. For example, development of bioremediation technologies for treatment of toxic metals have included bacterial dissimilatory reduction of soluble oxides of U(VI) and Cr(VI) resulting in their precipitation and recovery in bioreactor systems (Gorby and Lovley1992; Turick, *et al.* 1997; Turick and Apel 1997; Rege, *et al.* 1997). The ubiquity of anaerobic Cr(VI)-reducing bacteria (Turick, *et al.* 1996), has lead to the development of *in-situ* bioreduction technologies for detoxification and immobilization of this carcinogenic soil contaminant (Turick *et al.* 1998; Schmieman, et al.1997; Schmieman, *et al.* 1998.). Fe(III)-reducing bacteria also offer tremendous potential for the *in-situ,* direct oxidation of organic contaminants as well as indirect chemical transformation of halogenated and substituted aromatic compounds (Fredrickson and Gorby 1996)

One of the most important chemical changes occuring in the development of anaerobic soils and sediments is the reduction of Fe(III) (Ponnamperuma 1972).

Phylogenetically and physiologically diverse DMRB use iron as a terminal electron acceptor. This, in turn, results in the complete oxidization even of complex organic compounds to carbon dioxide (Lovley 1993). A significant portion of the Fe(III) reduction measured in anaerobic soils and sediments is enzymatically catalyzed by DMRB (Lovley 1987; Lovley 1991; Lovley1993; Lovley and Phillips1988). Therefore, DMRB play an important role in the geochemical cycling of iron as well as other elements including carbon, sulfur, phosphorous and trace metals. Because of their physiological versatility, DMRB offer considerable potential for bioremediation in environments contaminated with organic or halogenated compounds as well as metals (Fredrickson and Gorby 1996). An understanding of the physiology and mechanism(s) of iron mineral reduction will provide the opportunity to control and manipulate the physiological activities of DMRB in various anaerobic environments for bioremediation.

The oxidation of organic matter coupled to Fe(III) reduction is an exergonic process (Lovley and Phillips 1988). The standard free energy at pH 7 that is available from acetate oxidation coupled to Fe(III) reduction (-814 kJ/reaction) is similar to the amount available with oxygen as a terminal electron acceptor (-849 kJ/reaction). This value is much greater than that associated with a comparable reaction with sulfate as the electron acceptor (-52 kJ/reaction) (Lovley and Phillips 1988). Numerous bacteria gain energy for growth by catalyzing Fe(III) reduction with organic electron donors (Lovley 1997). Although the concentrations of Fe(III) range from the micromolar to millimolar (Nealson and Saffarini 1994), the predominant forms of Fe(III) are highly insoluble minerals. These minerals range in their degree of crystallinity, particle size, surface area, and reactivity (Schwertmann and Talylor 1977) and include, amorphous Fe(III) oxyhydroxide, goethite and hematite (Chukhrov et al. 1973).

DMRB must overcome the problem of accessing abundant, but insoluble minerals as electron acceptors. The bioavailability of Fe(III) in natural environments

limits the rates and extent of dissimilatory Fe(III) reduction (Lovley 1987). Although the metabolism of DMRB is operationally similar to that of oxygen respirers, nitrate reducers, sulfate reducers or methanogens, the minimal bioavailability of Fe(III) as an electron acceptor may be an important constraint on the ability of DMRB to compete with these other organisms for organic carbon. The mechanism(s) by which DMRB utilize insoluble Fe(III) minerals as substrates for growth in natural environments is essentially unknown. Furthermore, the transfer of electrons from the cell membrane to an insoluble mineral oxide presents an interesting mechanistic dilemma. Several models for this have been presented: first, surface-associated carriers have been proposed for the transport of electrons to minerals (Ehrlich 1993; Lovley, 1987). Electron transfer from bacterial membranes to minerals has been hypothesized to occur via metals associated with the cell surface (Ehrlich 1993). These surface-associated metals would act as an electron conduit for the cytochromes to reduce insoluble minerals. This first model was based on increased reduction rates of Mn(IV) relative to the manganese content of the cell surface (Ehrlich 1993). Second, cytochromes of *Shewanella putrifaciens* MR-1 are located near the cell surface and are postulated to be another mechanism of direct electron transfer to insoluble minerals (Lovley 1987). However, it is not known whether the cytochromes transfer electrons directly to the mineral surface or if an electron carrier is required (Lovley 1987; and Myers and Meyers 1992). A third proposed mechanism for mineral reduction is the excretion of soluble electron shuttles by DMRB. Seeliger et al. (1998) proposed that *Geobacter sulfurreducens* excretes soluble cytochromes as a mechanism for iron mineral reduction. This was based on the isolation of c type cytochromes in growth medium that were capable of iron oxide reduction (Seeliger et al. 1998). Based on thermodynamic considerations, Lloyd et al. (1999) challenged this model and showed that the addition of purified cytochromes to washed cell suspensions did not enhance iron mineral reduction of *G. sulfurreducens*. Environmental humic compounds serve as

terminal electron acceptors and electron shuttles for iron reduction by *S. algae* BrY and *G. metallireducens* (Lovley et al.1996). Subsequent studies have demonstrated that this phenomenon is widespread among DMRB (Coates et al. 1998). Soluble humic compounds transfer electrons to iron minerals due to the redox cycling capabilities of the quinone moities that are associated with these heteropolymers (Scott et al. 1998). The humic analogue 2,6-anthraquinone disulfonate also acts as a terminal electron acceptor for DMRB and has provided strong evidence that quinone moieties act as electron acceptors and electron shuttles to Fe(III) (Lovley et al. 1996).

Excreted quinones play a role in extracellular electron transfer by the DMRB *S. oneidensis* [formally *putrefaciens*)(Venkateswarn et al.1999)] MR-1(Newman and Kolter 2000). These quinones reduce the humic acid analogue, anthraquinone-2-6-disulphonate and have also been proposed to act as electron shuttles for DMRB (Newman and Kolter 2000).

The capability of DMRB to utilize quinoid compounds, especially humics as terminal electron acceptors and as electron shuttles for iron mineral reduction (Lovley et al. 1996) offers some insight on a biological mechanism for iron mineral reduction by DMRB. Humic acids are heterogeneous polymers of partially decomposed organic compounds containing polyaromatic structures with quinones and hydroquinones as well as carboxyl and hydroxyl groups (Clapp 1990). The quinone moieties are speculated to be involved in the mechanism by which humics achieve electron transfer to Fe(III) (Lovley et al. 1996 ; Scott et al 1998). Although humic acids originate from the partial decomposition of organic molecules such as tannins, they are also produced from microbial activity via extracellular melanin production. Thus, microbial melanins are a small, but significant portion of the humic acid pool in many soils (Clapp 1990). DMRB reduce Fe(III) minerals without addition of humics, albeit at slower rates (Lovley and Phillips 1988; Lovley et al.1996). This suggests that these DMRB either produce

another component to assist in electron transfer to Fe(III) minerals or they actually produce a humic-like compound in small quantities to facilitate Fe(III) mineral reduction. A bacterial metabolite structurally similar to humic acids could, hypothetically, serve as a biological mechanism for electron transfer to insoluble Fe(III) minerals by DMRB.

Melanin is a microbial metabolite that is structurally similar to humic acids with similar redox cycling properties. (Menter and Willis 1997; Clapp 1990). Several properties and functions have been ascribed to melanin: (1) protection from; UV and ã radiation, desiccation, temperature extremes, free radicals, and hydrolytic enzymes (Bell and Wheeler 1996); (2) microbial virulence (Bell and Wheeler 1996; Coyne and Al-Harthi 1992); (3) settlement and attachment of oyster larvae (Weiner et al. 1985); and (4) cation exchange and metal sequestration (White 1958; Nyhus et al 1997). For instance, melanized cell cultures of the fungus *Aureobasidium pullulans* show increased adsorption to numerous metals and tributyltin chloride relative to non-melanized cells (Gadd et al 1990). Uranium accumulation has also been reported in the melanin-containing lichen *Trapelia involuta* when grown on uranium minerals (McLean et al 1990).

Electrochemical properties of melanin include the ability to act as an amorphous semiconductor, a threshold switch, and an electron donor and electron acceptor (McGinness 1972; McGinness et al. 1974; Menter and Willis 1997; Pullman and Pullman 1961). The electron transfer properties of melanins have been demonstrated through the coupling of hydroxybenzene depigmenting-compound oxidation to ferric cyanide reduction (Menter and Willis 1997). The polymeric nature of melanin, which confers its semiconductor properties allows melanin to function as a threshold switch by transferring stored electrons to a metallic surface (McGinness et al. 1974). These metal adsorption and redox cycling properties of melanin-like polymers may also constitute a mechanism

of electron transfer to Fe(III) (Ellis and Griffiths 1974).

Melanin is a generic term describing high molecular weight dark pigments of biological origin (Bell and Wheeler 1986) which are classified into several groups based on their biochemical characteristics. Eumelanins are produced from the conversion of tyrosine to dihydroxyphenylalanine (DOPA) and subsequently to dopaquinone by the Mason-Raper pathway (Swan 1974). Phaeomelanins are produced from the conversion of tyrosine to dopachrome followed by the reaction with cysteine or glutamine (Coon et al. 1994; Prota 1996; Swan 1974). Allomelanins are produced from non-nitrogenous phenols and result in a wide range of diverse phenolic products (Coon et al. 1994; Prota 1996; Swan 1974). Melanin production is a function of polyphenol oxidases including tyrosinase (Bell and Wheeler 1986), a copper (Cu) containing enzyme that has been shown to play a significant role in the production of many other types of melanin (Bell and Wheeler 1986; Prota 1996; Swan 1974). Tyrosinase activity has been shown to be related to exogenous copper concentrations (Prota 1996; Swan 1974) with activity increasing with exogenous Cu (Swan 1974).

Pyomelanins (alkaptans) are produced by the initial conversion of tyrosine to *p*-hydroxyphenylpyruvate which is then converted to homogentisic acid (HGA) by the enzyme *p*-hydroxyphenylpyruvate hydroxylase (pHPPH) (also known as *p*-hydroxyphenylpyruvate dioxygenase) (Katob et al. 1995; Coon et al 1994; Ruzafa et al. 1994). The resulting homogentisic acid is excreted by bacterial cells and undergoes autooxidation and self polymerization, extracellularly, to form melanin (Yabuuchi and Omyama 1972; Ruzafa et al. 1995). Pyomelanin has a molecular weight ranging from 12,000 to 120,000 (Ruzafa et al. 1994; Weiner et al.1985). It is smaller than DOPA melanins which have molecular weights over 1,000,000 (Bridelli 1998). In contrast to tyrosinase, the metaloenzyme pHPPH contains a non-heme iron complex. Its activity is

controlled by exogenous Fe while supplemental Cu has no effect on pHPPH activity (Lindstedt et al.1977; Lindbald et al. 1977).

Bacterial production of pyomelanin was first described for *Pseudomonas aeruginosa* (Yabuuchi and Omyama 1972) and has been identified in several bacterial species including *S. colwelliana* D, *Vibrio cholerae* 14035, and a *Hyphomonas* strain (Ruzafa et al. 1995, Weiner et al. 1985; Katob et al. 1995). Pyomelanin is found both in the cell-free culture medium and associated with the cells (J, Ruzafa et al. 1995). The *melA* gene of *S. colwelliana* D which encodes for pHPPH has been cloned and sequenced (Coon et al 1994; Ruzafa et al. 1994). *P. aeruginosa* produces pyomelanin from either tyrosine or phenylalanine, but this bacterium is unable to use either amino acid as a sole carbon source (Yabuuchi and Omyama 1972). Sanchez-Amat et al.(1998) showed that melanin production in *V. cholera* 14035 was related to the carbon source-to-tyrosine ratio, where increased glutamate concentrations decreased melanin production. Sanchez-Amat et al.(1998) proposed that melanin production is a stress response to low nutrient conditions such as carbon limitation or decreased oxygen or trace metal availability. *V. cholera* ATCC 14035, was able to use a small percentage of tyrosine as a carbon source but did not use it as a sole source of carbon (Sanchez-Amat et al. 1998). This strain produced pyomelanin by the incomplete breakdown of tyrosine to HGA and its subsequent autooxidation and polymerization (Sanchez-Amat et al. 1998). The non-melanogenic strain, *V. cholera* CETC 557 (Sanchez-Amat et al. 1998), has seven times more HGA oxidase activity than the melanin-producing *V. cholera* ATCC 14035 and is able to use tyrosine as a sole source of carbon. Thus, because pHPPH activity is higher than HGA oxidase activity (Sanchez-Amat et al. 1998) melanin production by *V. cholera* ATCC 14035 occurs as a result of HGA accumulation in the medium. With *V. cholera* HTX-3, *S. colwelliana* D and a *Hyphomonas* sp. the mechanism of melanin production involves the control of the constitutive pHPPH by

environmental factors (Katob et al. 1995), resulting in overproduction and excretion of HGA.

S. colwelliana D is one of the most characterized pyomelanin-producing microorganisms (Coon et al 1994; Fuqua et al. 1993; Ruzafa et al.1994). The genus *Shewanella* is part of the gamma Proteobacteria, in the family Vibrionaceae (Venkateswarn et al.1999) Dissimilatory Fe(III) oxide reduction has also been well characterized for this genus with 9 out of the 11 accepted species capable of this form of anaerobic respiration (Venkateswarn et al.1999). In particular, *S. algae* BrY and *S. oneidensis* MR-1 demonstrate remarkable versatility in the number of terminal electron acceptors they can use for anaerobic respiration which include manganese, iron, chromate, cobalt, uranium and humic compounds (Venkateswarn et al.1999). Both species have clinical significance because they are opportunistic pathogens (Khashe and Janda 1998, Dhawan et. al 1998). For example, *S. algae* was involved in disease outbreaks in Denmark (Vogel et al. 2000; Gram et. al 2000). Several clinical strians have been reported to produce pigments when grown on phenolic compounds (Khashe and Janda 1998). Since melanin production has been associated with virulence in microorganisms, including *V. cholera* (Bell and Wheeler 1984; Coyne and Al-Harthi 1992; Kwon-Chung et al 1992), and melanin-like compounds (humic acids) serve as electron shuttles for iron-oxide reduction, a further understanding of melanin production in these bacteria may also have significance in biogeochemistry, geomicrobiology and public health.

The overall goal of this study is to elucidate the mechanism of insoluble iron oxide reduction by DMRB. My hypothesis is that a soluble electron shuttle is involved in the electron transfer process. Since melanin is a humic-like heteropolymer consisting of quinones, it has the properties of an electron acceptor and an electron donor (McGinness 1972; Menter and Willis. 1997). The aims of this study were to determine

whether: 1) melanin is produced by *S. algae* BrY; 2) melanin accepts electrons from *S. algae* BrY; 3) as a terminal electron acceptor, melanin supports the growth of *S. algae* BrY; 4) soluble extracellular melanin is an electron shuttle and reduces insoluble iron oxides; 5) melanin can be associated with the cell surface and serve as an electron conduit from the cytochromes to insoluble iron oxides; and 6) to characterize melanogenesis by *S. algae* BrY.

CHAPTER ONE

Melanin Production and Use as a Terminal Electron Acceptor and a Soluble Electron Shuttle for Fe(III) Oxide Reduction by *Shewanella algae* BrY

ABSTRACT

Although dissimilatory metal reducing bacteria (DMRB) play a vital role in biogeochemical cycling, a paucity of information exists on the biochemical mechanism(s) for electron transfer to insoluble minerals by these organisms (Lovley 1995; Fredrickson and Gorby 1996). DMRB also use environmental humic compounds as terminal electron acceptors and electron shuttles to iron minerals under anaerobic conditions (Lovley et al. 1996; Scott et al. 1998). The quinone moities associated with humics are responsible for their redox cycling capabilities (Scott et al. 1998). In addition, DMRB excrete quinones that may mediate extracellular electron transfer to humics (Newman and Kolter 2000). I therefore investigated the hypothesis that a quinone-containing, humic-like compound, melanin, is produced by DMRB and could serve as a terminal electron acceptor as well as an electron shuttle to insoluble iron minerals under anaerobic conditions. Here I report that melanin is produced by two species of DMRB in the genus *Shewanella*. *S. algae* BrY-produced melanin serves as a terminal electron acceptor and supports growth of cells during anaerobic respiration. Growth of *S. algae* BrY occurred in minimal medium supplemented with extracted melanin from previously grown cultures of *S. algae* BrY. Melanin served as a sole terminal electron acceptor. The reduced, soluble melanin, in turn, reduced Fe(III) minerals in the absence of bacteria, demonstrating that *S. algae* BrY produces a

soluble Fe(III) - reducing compound. In the presence of bacteria, melanin acted as a potential electron conduit to Fe(III) minerals and increased Fe(III) mineral reduction rates. Based on kinetic studies, approximately 35 femtograms of supplemented melanin per cell were required to achieve half the maximum rate of iron mineral reduction. Melanin produced by *S. algae* BrY imparts increased versatility to this organism as a terminal electron acceptor, soluble Fe(III) reductant and a an electron conduit for iron mineral reduction. These findings show that melanin production by facultative anaerobic DMRB offers an extraordinary survival strategy for these organisms. Melanin production by DMRB could have a significant role in biogeochemical cycling of Fe.

INTRODUCTION

DMRB display remarkable versatility in the number of terminal electron acceptors they use for anaerobic respiration, including iron minerals (Venkateswaran *et al.* 1999). Although electron transfer by bacteria to insoluble minerals is known, the molecular mechanism(s) of electron transfer from DMRB to the minerals has yet to be elucidated (Fredrickson and Gorby 1996). One model suggests that close physical contact to minerals is required for reduction. For example, the extent of hydrous ferric oxide (HFO) reduction by *S. algae* BrY is directly related to the degree of attachment (Das and Caccavo 2000). Another model postulates the production of soluble electron shuttles by DMRB. Soluble extracellular cytochromes may play a role in mineral reduction (Seeliger et al. 1998), but the feasibility of this process has been questioned (Lloyd et al. 1999). With *S. oneidensis* MR-1 [formally *S. putrefaciens* MR-1(Venkateswaran *et al.*1999)] a relation between electron transfer to humics has been linked to excreted quinones (Newman and Kolter 2000). The electron transport properties of humic compounds are directly related to redox cycling capabilities of their quinone moities (Scott et al. 1998). Since soluble environmental humic compounds mediate electron transfer to iron minerals (Lovley et al. 1996), one plausible biological mechanism by which DMRB can shuttle electrons to minerals is through the production of a polyquinoid humic-like metabolite. The basidiomycete *Cryptococcus neoformans* uses melanin, a humic-like compound to reduce soluble Fe(III) to Fe(II) for assimilation (Nyhus et al. 1997). Melanin has electrochemical properties which include the ability to act as an amorphous semiconductor, a threshold switch and an electron donor and electron acceptor (McGinness 1972; McGinness et al. 1974; Menter and Willis 1997;

Pullman and Pullman 1961). These properties, along with its chemical and functional similarities to humic compounds (Scott and Martin 1990) led me to hypothesize that melanin is produced by facultatively anaerobic DMRB in the genus *Shewanella.* Coon et al. (1994) and Ruzafa et al. (1994) have shown that *S. colwelliana* produces melanin. The type of melanin produced is pyomelanin and is made by converting tyrosine to homogentisic acid with the enzyme p-hydroxyphenylpyruvate dioxygenase (pHPPD) Homogentisic acid is excreted, autooxidized and self-assembled into the quinone-containing polymer, pyomelanin (Ruzafa et al. 1994; L, Coon et al. 1994). I hypothesized that melanin is produced by facultatively anaerobic DMRB and could serve as a terminal electron acceptor as well as a soluble electron shuttle to iron minerals, thereby offering a significant growth and energetic advantage to these DMRB.

MATERIALS AND METHODS

Melanin production. *S. algae* BrY (Caccavo et al. 1992; formally *S. alga* BrY; Truper and de'Clari 1997) and *S. oneidensis* MR-1 were used in this study. Cultures were maintained on Tryptic Soy agar (TSA; DIFCO Labs, Detroit, MI). For bacterial growth and melanin production, a basal medium (Lovley et al. 1996) contained (in g liter^{-1} dH_2O): NH_4Cl (1.5), KCl (0.1), KH_2PO_4 (0.5), PIPES [Piperazine-N,N'-bis(2 ethanesulfonic acid)] (7.57), NaCl (10.0), 60% Na lactate (9 ml liter^{-1}), vitamin solution (10 ml liter^{-1}), mineral solution (10ml liter^{-1}) with tyrosine concentrations of 2 g liter^{-1} in the growth study and 0, 0.25, 0.5, 1.0, and 2.0 g liter^{-1} in the pigment production study. Aerobic 50 ml duplicate cultures were incubated at 28°C with shaking at 150 rpm. Inocula (10%, v/v) were from a 24 hr culture grown in basal medium (above), but without tyrosine. Cell density was measured with acridine orange direct counts using an epifluorescence microscope (Hobbie et al. 1997). Melanin production in the spent, cell free culture medium (background subtracted) was determined spectrophotometrically at a wavelength of 400 nm (Ruzufa et al. 1995).

Melanin preparation. A 5 liter culture of *S. algae* BrY was grown in lactate minimal medium supplemented with 2 g liter^{-1} of tyrosine as above, for 12 days, until pigment production stabilized. The cell-free supernatant fluid was acidified with 6N HCl to a pH < 2.0 and it was allowed to precipitate for 4 hrs at 20°C. The concentrated melanin was transferred to 8 KDa dialysis tubing and dialyzed for 24 hours in dH_2O with 4 changes of water during that time. The dialyzed melanin was then dried at 60°C and powdered prior to storage under nitrogen gas in glass vials which served as the melanin stock. HGA-melanin was produced *in-vitro* by autooxidation of homogentisic acid (Sigma Chem Co., St.Louis, MO.) which was constantly stirred for 7 days in dH_2O with the pH

adjusted to 10 with 10N NaOH as described previously (Ruzufa et al. 1995). The HGA-melanin was concentrated and dried as described above. For use in experiments, dried melanin was dissolved in dH_2O with its pH adjusted to 12 with 10 N NaOH. After melanin was in solution, the pH was adjusted to 7.0 by the addition of 1N HCl and diluted to a volume of 100 ml. Aliquots of this concentrated stock solution and were diluted as needed.

Molecular weight analysis. Concentrated melanin and HGA-melanin (final concentration, 2 g $liter^{-1}$) were dissolved in dH_2O by the addition of 1N NaOH to a pH of 12 and this solution was neutralized with 1N HCl to a final pH of 7.0. The molecular weight (MW) of the solutions were determined by high speed sedimentation (Yphantis 1960). Experiments were conducted at 20° C in a Beckman XL-I Ultracentrifuge at 35,000 rpms using absorbance detection. For each sample, three loading concentrations were examined ranging from 0.5-1.50 OD in PIPES buffer and 100 mM NaCl. Solvent density (1.0017 g ml^{-1}) was calculated using the Sednterp program (Laue et al. 1992) and an assumed partial specific volume of 0.71 ml g^{-1} were used in MW calculations. Data were fit using NONLIN (Johnson et al. 1981).

Quinone assay. The presence of quinones in the cell-free supernatant fluid was determined by the nitroblue tetrazolium/glycinate assay (Paz et al. 1991). The reagents, which consisted of 300 ì l of 2 M potassium glycinate (pH 10), and 1 ml of 0.24mM nitroblue tetrazolium, dissolved in 2 M potassium glycinate (pH 10) were added to 100 ì l of cell free culture supernatant. Reagents were stored on ice until use. The mixture was incubated at 28°C in the dark for 1 hr and absorbance was read at 530 nm. In addition, concentrated, dried pigment (see below) was dissolved in dilute NaOH (pH10)

and aliquoted into dH_2O to concentrations of 0.1-1.0 mg liter^{-1}. The response with this assay was linear with r^2=0.9937 with a slope = 0.729 and was consistent with that obtained with other quinoid compounds (Paz et al. 1991).

Terminal electron acceptor experiments. Aerobic cultures of *S. algae* BrY were grown in tryptic soy broth (30 g liter^{-1}) for 15 hrs., at 28°C with shaking (150 rpm). The cells were harvested by centrifugation (8000 x g, 4°C, 20 min), and washed twice with anaerobic 30mM sodium bicarbonate buffer under a nitrogen atmosphere. To cell suspensions, bacterial melanin was added (2 g liter $^{-1}$ final concentration) in 30 mM sodium bicarbonate buffer (pH 7.2) and made anaerobic by boiling and cooling under a constant stream of 80% N_2, 20% CO_2. The mixture was transferred anaerobically to serum vials and kept under the same atmosphere, after which they were autoclaved. Washed cells were then added to the above mixture to a final density of 10^9 ml^{-1}. Hydrogen gas (10 kPa) served as electron donor and was added aseptically to vials with a syringe fitted with a sterile 0.22 ìm filter. Vials were incubated with agitation (150 rpm) in the dark at 28°C. At different time intervals, samples were removed for analysis.

Melanin reduction assay. To quantify melanin reduction, 1 ml of cell free supernatant fluid was added to 50 µl of 10 mM soluble ferric citrate and the mixture was incubated anaerobically for at least 15 min., as described previously for humic compounds (Lovley et al. 1996). In this procedure, reduced melanin reduced Fe(III), resulting in Fe(II), which was quantified spectrophotometrically with ferrozine (Lovley et al. 1996). The ability of reduced melanin to reduce 1mM of Fe(III) to Fe(II) is defined as 1 milliequivalent of reduced melanin.

Growth assay. Melanin prepared as described above was added to an anaerobic minimal lactate medium (as above) at a final concentration of 6 g liter^{-1}. The inoculum consisted of anaerobic cells of *S. algae* BrY that were grown for 48 hrs at 28°C in the lactate basal medium with fumarate (30 mM) as terminal electron acceptor and lactate as carbon and energy source. Cells were harvested by centrifugation (8000 x g, 4°C, 20 min), and washed twice with anaerobic, 30mM bicarbonate buffer under a nitrogen atmosphere, and inoculated into lactate basal medium with melanin. Melanin reduction was determined as described above. Cell numbers were determined by direct counting with acridine orange staining and an epifluorescence microscope (Hobbie et al. 1997).

Melanin reduction of insoluble Fe(III) oxide. Melanin which was previously reduced during the terminal electron acceptor study was centrifuged anaerobically (8000 x g, 20°C, 20 min.), to remove cells. One ml of cell-free melanin was added to 200µl of 6mM insoluble hydrous ferric oxide (HFO) and it was incubated at 20° C under anaerobic conditions for 90 min. The amount of Fe(II) produced from the reduction of HFO was determined as described above.

HFO reduction rate as a function of melanin concentration. HFO reduction over time, by cell suspensions of *S. algae* BrY and H_2 (prepared as described above) was determined with various concentrations of melanin. HFO reduction to Fe(II) was measured as described above. HFO reduction rates were calculated with the equation

$$a=a_i(1-e^{-kt}) \qquad (1)$$

a_i = maximum HFO reduction [as mM Fe(II) produced]; k = HFO reduction rate hr^{-1}. Nonlinear curves were determined with Sigma Plot curve-fitting software (version 7.1). Fe(II) concentrations were normalized by subtracting Fe(II) values of the control (no

added melanin). The normalized Fe(II) values were then fit to the Michaelis-Menton equation,

$$k = k_{max} (S/K_s+S) \quad (2)$$

where: k= HFO reduction rate (hr^{-1}); k_{max} = maximum HFO reduction rate (hr^{-1}); S= melanin concentration (g $liter^{-1}$) and K_s = half saturation constant for melanin (g $liter^{-1}$). Nonlinear curves were determined with Sigma Plot (Jandel Co., Chicago, IL) curve fitting software (version 7.1).

RESULTS

When grown aerobically with tyrosine, *S. algae* BrY and *S. oneidensis* MR-1 produced a dark extracellular pigment (Fig. 1.1). The concentrated pigment reduced nitroblue tetrazolium to formazan in the presence of oxygen and excess glycine at pH 10, indicating the existence of quinoid compounds (Paz et al. 1991). At pH $\leq$2, the soluble, cell-free pigment precipitated rapidly, further indicating melanin and humic-like qualities (Ellis and Griffiths 1974; Scott and Martin 1990). Because of these results, I tentatively identified the pigment as melanin (Ellis and Griffiths 1974). Its use as a terminal electron acceptor for *S. algae* BrY was evaluated by monitoring hydrogen-dependent melanin reduction by anaerobic cell suspensions of *S. algae* BrY (Fig. 1.2). Melanin was reduced in the presence of *S. algae* BrY plus hydrogen, but was not reduced by heat-killed, washed cells with hydrogen or by hydrogen alone (Fig. 1.2). The amount of melanin reduced by *S. algae* BrY with hydrogen was significantly greater than the amount reduced in the presence of *S. algae* BrY without hydrogen ($P < 0.01$). These results are consistent with the conclusion that *S. algae* BrY uses melanin as a sole terminal electron acceptor.

The ability of cultures of *S. algae* BrY to use melanin as a sole terminal electron acceptor (to support cell growth) was examined (Fig. 1.3). Melanin reduction coincided with more than a 100 fold increase in cell numbers (Fig. 1.3) and the reduced cell-free melanin was capable of reducing 1.2 mM of soluble Fe(III) to Fe(II). In a parallel experiment, an initial melanin concentration of 3 g liter^{-1}, with lactate as electron donor, produced a 50 fold increase in bacterial density and an Fe(III) reduction potential of 0.6 mM from the reduced melanin (data not shown). No growth occurred with melanin in the absence of an electron donor. Melanin was also produced *in-vitro* by the autooxidation of commercially available homogentisic acid (HGA-melanin). *S. algae*

Figure 1.1. Melanin was produced by *S. algae* BrY and *S. oneidensis* MR-1 (results for *S. algae* BrY shown here) when grown in tyrosine-supplemented, lactate basal medium (left). Without tyrosine, no melanin production was observed (right).

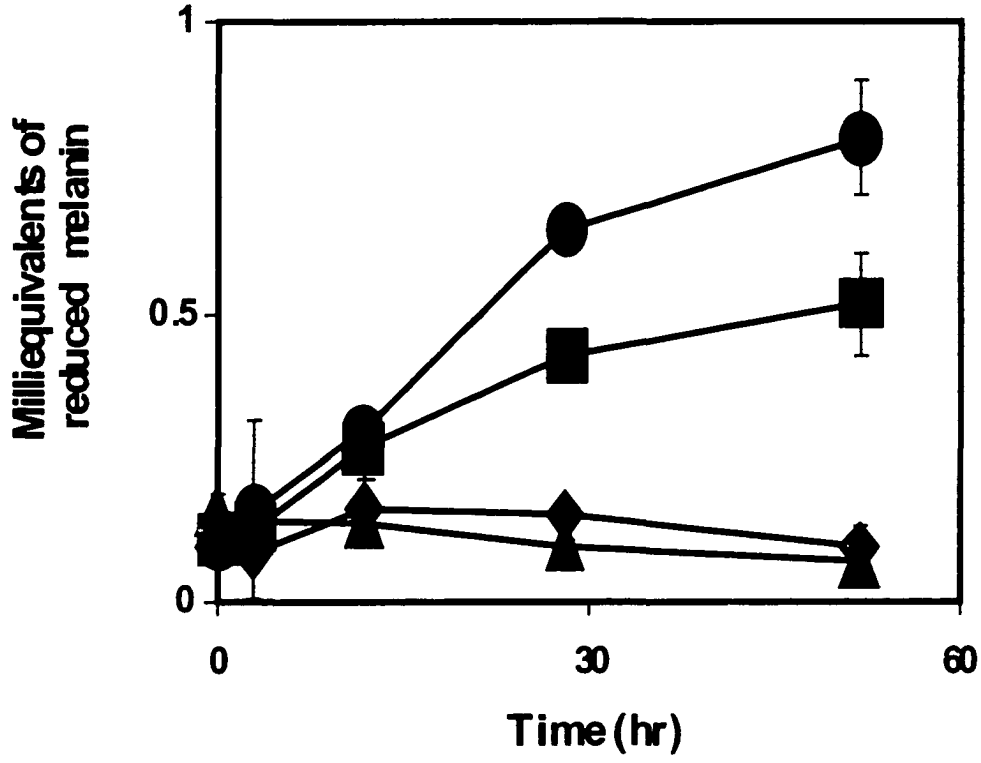


Figure 1.2. *S. algae* BrY cell suspensions coupled H_2 oxidation to melanin reduction under anaerobic conditions. Melanin reduction in anaerobic 30mM sodium bicarbonate buffer by suspensions of *S. algae* BrY cells (10^9 ml^{-1}) with H_2 (●), *S. algae* BrY cells without H_2 (■), H_2 alone (▲) and heat-killed cells with H_2 (◆).

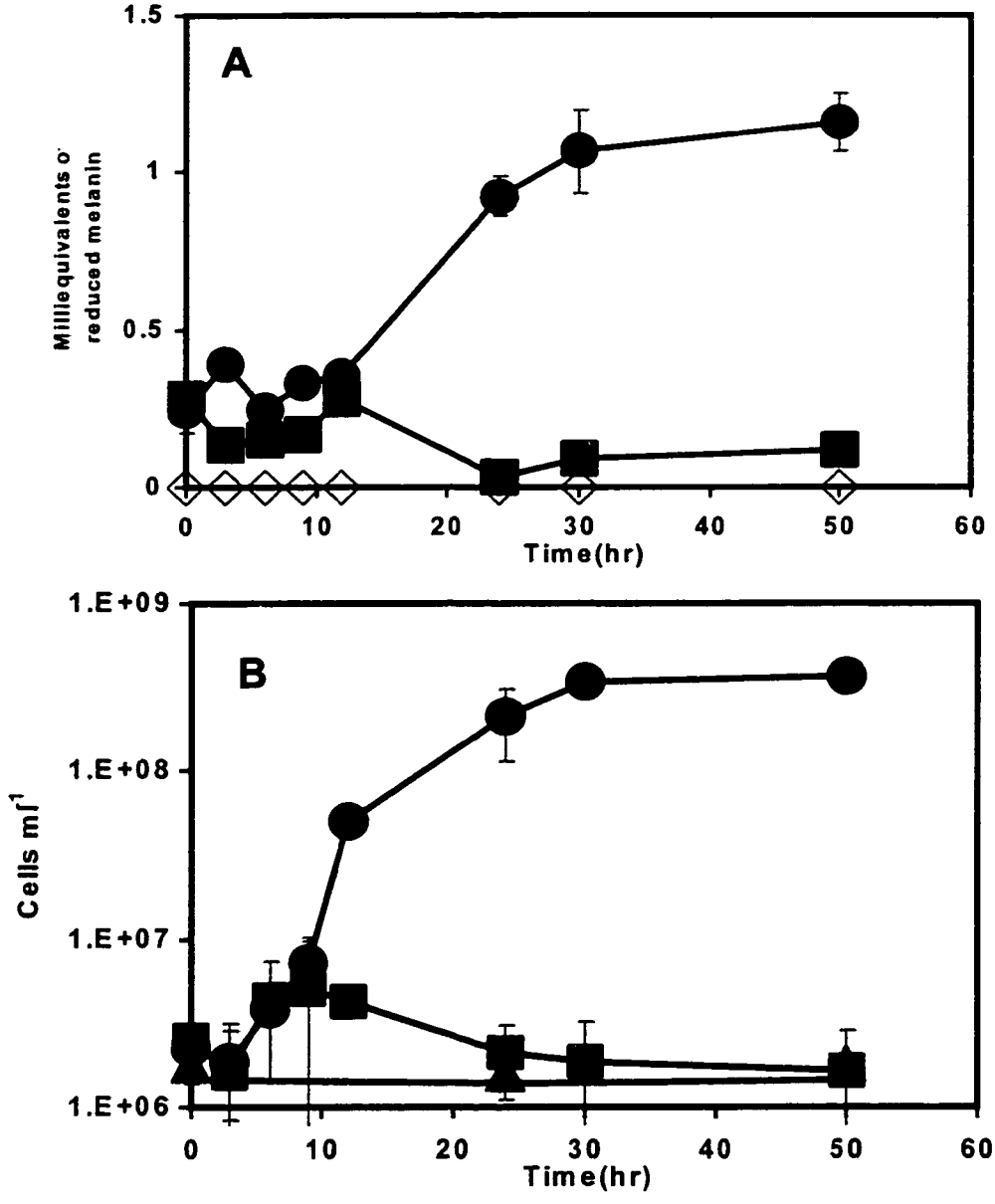
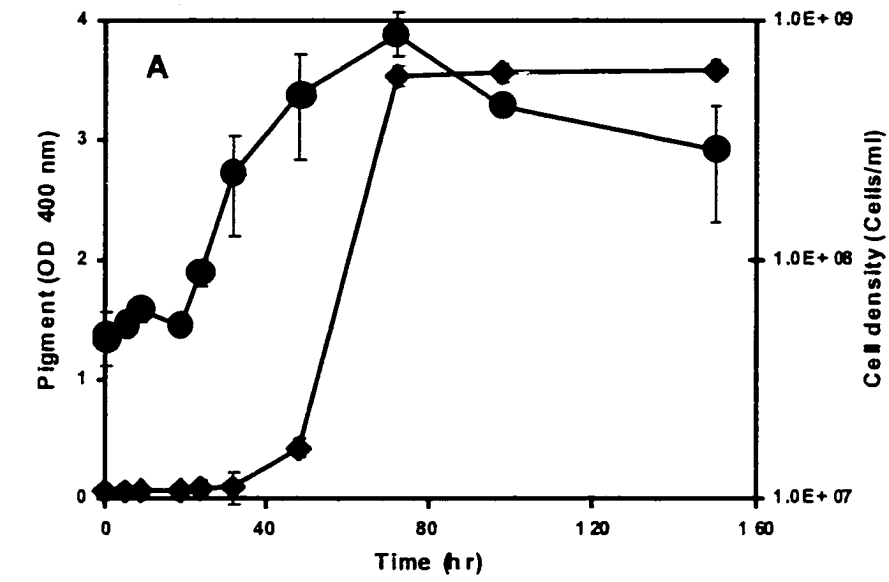


Figure 1.3. Melanin (6 g liter^{-1}) as sole electron acceptor supported growth of *S. algae* BrY in sodium lactate basal medium. (A) Melanin reduction during growth of*S. algae* BrY cells with melanin and sodium lactate basal medium (●), *S. algae* BrY cells without melanin (■), medium with melanin but without cells (◇). (B) Cell growth measurements of *S. algae* BrY with melanin as an electron acceptor, (●) *S. algae* BrY cells without melanin (■) and *S. algae* BrY cells with melanin but without carbon and energy source (▲).

BrY grew anaerobically with 6 g liter^{-1} HGA-melanin as a terminal electron acceptor and had a >50 fold increase in cell numbers after 48 hrs. of incubation (data not shown).

Extracellular pigment production by *S. oneidensis* MR-1 (Fig. 1.4A) and *S. algae* BrY (data shown only) occurred during the late exponential/early stationary growth phase in tyrosine-supplemented minimal medium. The amount of soluble pigment produced was directly proportional to the initial tyrosine concentration in the medium (Fig. 1.4B). The pigment had an absorbance spectrum (300 - 800 nm) similar to that of commercial humic acid (Aldrich Chem. Co.). In addition, the slope of the log absorbance vs. wavelength (400-600 nm) of the spectrum was equal to -0.0035, indicative of melanin (Ellis and Griffiths 1974). Following precipitation, the washed, dialyzed, dried melanin resulted in a black powder with the following properties indicative of melanin (Ellis and Griffiths 1974): (a) insoluble in organic solvents (ethanol, chloroform and acetone); (b) soluble in NaOH solutions $\geq$ pH 10; (c) decolorized in H_2O_2; (e) precipitated by $FeCl_3$ and (f) retained by a 8kDa dialysis membrane. HGA melanin and concentrated extracellular melanin had MW's ranging from 11,000-17,000 and 12,000-14,000, respectively.

The *melA* gene of *S. colwelliana*, which encodes for pHPPD has been identified and sequenced (Ruzafa et al. 1994; Fuqua et al. 1991). The genome of *S. oneidensis* MR-1 has been almost completely sequenced (TIGR database) and this database was analyzed by the BLAST program (TIGR database) to identify *melA* homologues. An ORF was identified whose product has a 92.5% amino acid similarity and 82% identity to the *melA* gene product. Melanin is also produced by some organisms by converting tyrosine to dihydroxyphenylalanine with the enzyme tyrosinase (Mercado-Blanco et al. 1993). No regions of similarity or identity were identified by a BLAST search of the *S. oneidensis* MR-1 database. These results are consistent with the melanin produced by *S. oneidensis* MR-1 is a result of homogentisic acid production from the enzyme



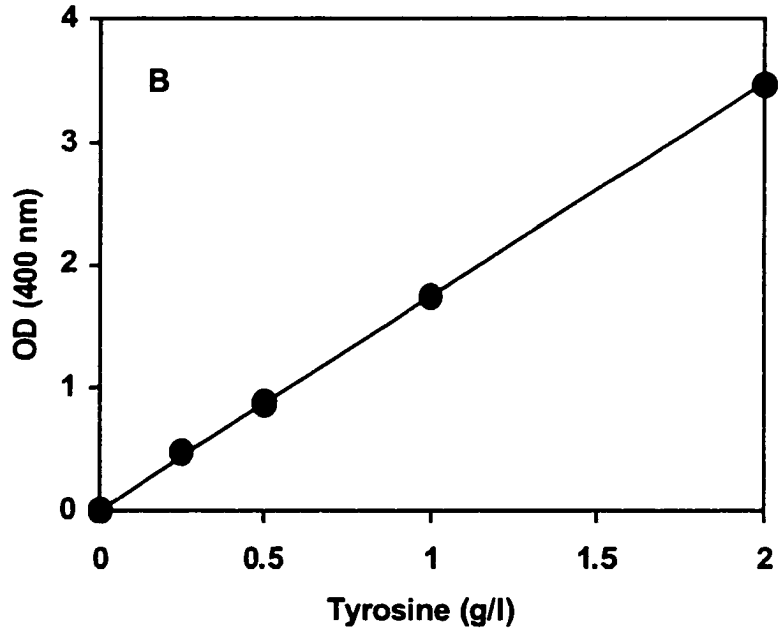


Figure 1.4. Melanin production by *S. algae* BrY in lactate basal medium supplemented with tyrosine. (A) Melanin production by (◆) and growth of (●) an aerobic culture of *S. algae* BrY with 2 g liter^{-1} tyrosine. (B) Melanin production (●) by aerobic *S. algae* BrY cultures was tyrosine-dependent.

pHPPD and is most probably pyomelanin. This particular type of melanin has also been identified in other members of the gamma Proteobacteria including , *Vibrio cholerae,* a *Hyphomonas* strain (Ruzafa et al. 1994; Fuqua et al 1991; Katob et al. 1995) and *Pseudomonas aeruginosa* (Yabuuchi and Omyama 1972). Our results suggest that *S. algae* BrY produces pyomelanin.

The addition of cell-free, reduced, bacterial melanin to anaerobic hydrous ferric oxide (HFO) resulted in HFO reduction to Fe(II) (Fig. 1.5A). The amount of HFO reduced by reduced melanin was significantly greater than the amount reduced by oxidized melanin ($P < 0.01$). This is the first demonstration of a soluble DMRB metabolite that is capable of Fe(III) oxide reduction.

Melanin also accelerated the rate of bacterial HFO reduction during anaerobic respiration. The rates of Fe(III) oxide reduction by cell suspensions of *S. algae* BrY increased as a function of oxidized melanin concentration (Fig. 1.5B). Fe(II) adsorption to Fe(III) oxides limits the rate and extent of bacterial Fe(III) oxide reduction (Roden and Urrutia 1999). Since melanin complexes with soluble iron (Nyhus et al. 1997), one possible explanation for my results is that, melanin increased Fe(III) reduction rates by complexing with Fe(II) which would normally have bound to the oxide surface. However, an analysis of the kinetics of iron reduction shows that the data did not fit a Langmuir isotherm (Veith and Sposito 1977) ($r^2 = 0.54$) (data not shown), but were more precisely described by a Michaelis-Menton model ($r^2=0.98$) (Fig. 1.5C). This demonstrates that melanin acted directly to increase HFO reduction rates rather than just to impede Fe(II) sorption to HFO. The Michaelis-Menton kinetic analysis yielded a half saturation constant of 32 mg liter^{-1} melanin and an optimum concentration of approximately 200 mg liter^{-1} of melanin. These results demonstrate that melanin increased the rate of bacterial Fe(III) oxide reduction. One possible mechanism for this effect is by electron shuttling through quinone moieties as previously described for

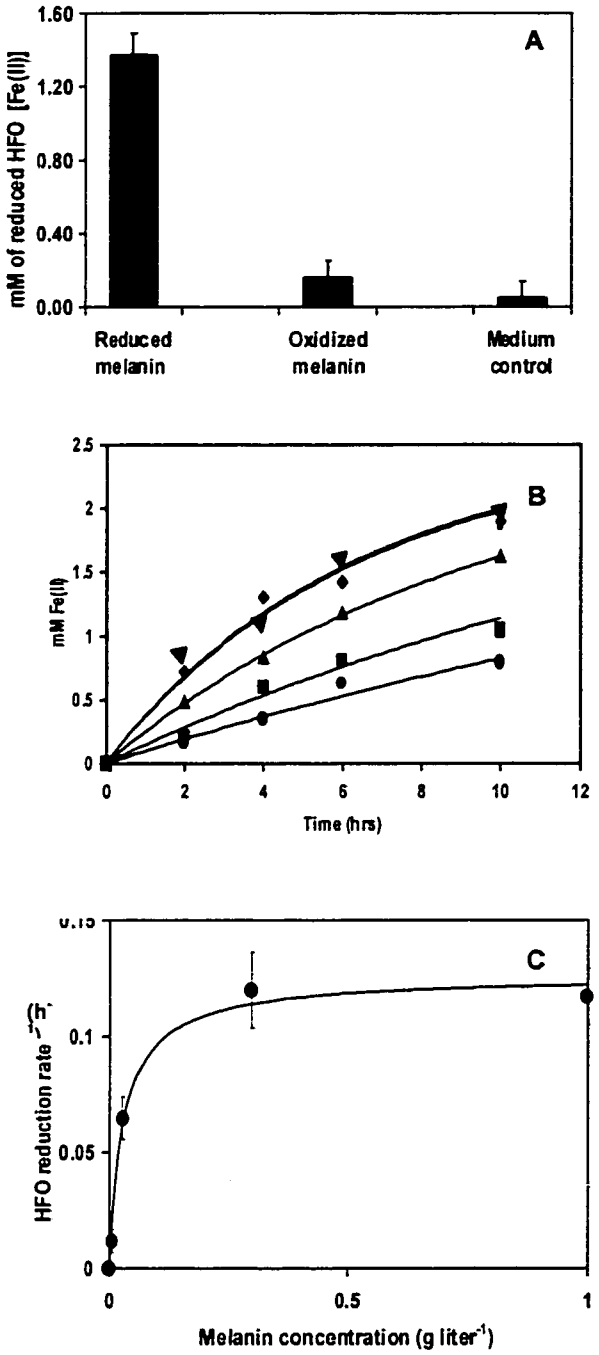


Figure 1.5. Reduced melanin reduces HFO. (A) Melanin, reduced by *S. algae* BrY, reduced HFO in the absence of cells. Oxidized melanin and spent culture liquid without melanin demonstrated minimal HFO reduction. (B) The rate of HFO reduction by cell suspensions of *S. algae* BrY and H_2, supplemented with melanin concentrations of 0 mg liter^{-1} (●), 6 mg liter^{-1} (■), 30 mg liter^{-1} (▲), 300 mg liter^{-1} (▼), and 1000 mg liter^{-1} (◆). (C) Michaelis-Menton kinetics (r^2=0.98) demonstrating HFO reduction rates by *S. algae* BrY (from Fig. 1.5 B) as a function of melanin concentration. Maximum HFO reduction rate (k_{max}) = 0.12 hr^{-1} and Ks = 0.032 g liter^{-1}.

humic compounds (Scott et al. 1998). Results of my kinetic analyses, based on the cell density in these experiments, indicates that *S. algae* BrY requires only 32 femtograms of melanin per cell to achieve half of the maximum HFO reduction rate.

DISCUSSION

These results provide the first example of a compound produced and used as a terminal electron acceptor for anaerobic respiration by the same organism. This is thermodynamically feasible for melanin. Hydroxylated melanin precursors are excreted from the cells and then undergo autooxidation to quinones, followed by self assembly into melanin polymers (Ruzufa et al. 1994; Fuqua et al. 1991). Although melanin production by microorganisms is well documented, the physiological significance of microbial melanogenesis is not completely understood.

The results of this study indicate that melanogenic, facultatively anaerobic bacteria utilize their own extracellular melanin for energy and growth and for increased rates of insoluble iron mineral reduction. Due to the small quantities of melanin required for significant increases in Fe(III) reduction, and the redox cycling behavior of melanin, bacterial cells may only require trace amounts of tyrosine and oxygen for melanin production and subsequent Fe(III) mineral reduction in the environment. Other quinoid compounds also significantly enhance Fe(III) reduction, even in quantities as low as 50ìg Kg^{-1} of sediment (Nevin and Lovley 2000). Reduced melanin can also serve as a soluble metabolite capable of mineral reduction in the absence of bacteria. This physiological trait may play an important role in biogeochemical cycling, especially where small pore size of sediments may limit direct bacterial contact with iron minerals. Bacterial melanogenesis by DMRB has the potential to exert a significant influence on bacterial survival as well as the cycling of carbon and metals in oxic/anoxic environments.

CHAPTER TWO

Electron Transfer From *Shewanella algae* BrY to Hydrous Ferric Oxide is Mediated by Cell-Associated Melanin

ABSTRACT

For dissimilatory metal reducing bacteria (DMRB), the use of insoluble mineral oxides as terminal electron acceptors requires the transfer of electrons from their outer membrane to the mineral surface. The mechanism of electron transfer from cell surface to mineral surface has not been elucidated. I tested the hypothesis that the electrochemical properties of melanin would allow it to serve as an electron conduit for bacterial-mineral reduction. The DMRB, *Shewanella algae* BrY, produces extracellular melanin from supplemental tyrosine. Extracellular melanin then becomes associated with the cell surface, as determined by Fourier transform infrared spectroscopy (FT-IR), and cell surface hydrophobicity tests. With H_2 as electron donor, washed cell suspensions of melanin-coated *S. algae* BrY reduced hydrous ferric oxide (HFO) 10 times faster than cells without melanin. *S. algae* BrY grown without tyrosine did not produce extracellular melanin. However, the addition of H_2 and melanin (20 ìg/ml) to these washed cell suspensions doubled HFO reduction rates. Furthermore, H_2-reduced cytochromes were oxidized by anaerobic melanin as evidenced from spectrophotometric scans of whole cells. This is the first demonstration of dissimilatory reduction where a surface-associated metabolite mediates electron transfer from bacterial cytochromes to an insoluble mineral.

INTRODUCTION

Dissimilatory metal reducing bacteria (DMRB) are capable of utilizing insoluble minerals as terminal electron acceptors, however the mechanism(s) of electron transfer remains unclear. There are several reports of the use of soluble electron shuttles by DMRB for iron mineral reduction (Seeliger et al. 1998; Lovley et al. 1996). *Geobacter sulfurreducens* has been reported to excrete a soluble c-type cytochrome as a mechanism for iron mineral reduction (Seeliger et al. 1998). However, this model is based on indirect evidence and has been challenged, primarily because the addition of the purified cytochrome to washed cell suspensions did not enhance iron mineral reduction by *G. sulfurreducens* (Lloyd et al. 1999). Environmental humic compounds serve as terminal electron acceptors and electron shuttles for iron reduction in *S. algae* BrY and *Geobacter metallireducens* (Lovley et al. 1996) along with other DMRB (Coates et al. 1998). Soluble humic compounds transfer electrons to iron minerals through the redox cycling capabilities of the quinone moities associated with these heteropolymers (Scott et al. 1998). With the DMRB *S. oneidensis* [formally *putrefaciens* MR-1(Venkateswaran et al 1999)], excreted quinones also play a role in extracellular electron transfer (Newman and Kolter 2000). These non-proteinaceous, uncharacterized quinones restore the ability of menaquinone-minus mutants to reduce the humic acid analogue, anthraquinone-2-6-disulphonate (Newman and Kolter 2000) and have been proposed to act as electron shuttles for anaerobic respiration of minerals.

The extracellular heteropolymer, melanin, is produced by the DMRB *S. algae* BrY and *S. oneidensis* MR-1 (Chapter One). The polyquinoid nature of melanin provides it with the electrochemical properties of an amorphous semiconductor capable of redox cycling (McGinness 1972; McGinness et al. 1974; Menter and Willis 1997). Due to their redox cycling ability, only small quantities of quinoid compounds such as melanin and

humics, are required to enhance Fe(III) reduction (Nevin and Lovley 2000; Chapter One). Because of these properties, melanin has been proposed to be an excellent electron acceptor (Menter and Willis 1997; Pullman and Pullman 1961).

I have recently shown that melanin will act as a terminal electron acceptor for *S. algae* BrY and as a soluble electron shuttle for iron mineral reduction in the absence of bacteria (Chapter One). In the presence of *S. algae* BrY and iron minerals, kinetic studies revealed that only femtogram quantities per cell of supplemental melanin were required to double the rate of iron mineral reduction (Chapter One). Due to the redox cycling properties of quinones, these molecules can be cycled back and forth from the reduced hydroquinone state to the oxidized quinone conformation (Clapp 1990; Scott et al. 1990). In this case, only minute quantities of melanin are required per cell to effectively transfer electrons repeatedly to iron minerals.

In the absence of soluble electron shuttles, close physical contact to minerals by DMRB is required for reduction to occur (Lovley 1994). For example, hydrous ferric oxide reduction by *S. algae* BrY requires attachment (Das and Caccavo 2000). However, the mechanism(s) of electron transfer from the cell surface to minerals during physical contact has not yet been demonstrated, but has been postulated to be dependent on a surface associated cellular component (Fredrickson and Gorby 1996; Lovley 1994; Lovley 1997). With the yeast *Cryptococcus neoformans,* surface-associated melanin in the hydroquinonic state reduces soluble Fe(III) and is a means of acquiring Fe(II) by this organism (Nyhus et al. 1997). Since melanin is coupled to electron transport (Capter One) its association with the cell surface would provide a significant bioenergetic advantage. Surface melanin has the potential to serve as an electron conduit from the bacterial surface to a mineral surface during dissimilatory mineral reduction.

Melanin production in several species of the gamma proteobacteria, including *S. colwelliana* has been reported to occur outside the cell by way of autooxidation and self-

assembly of melanin precursors. For instance, tyrosine is ultimately converted to homogentisic acid which is then excreted from the cell (Coon et al 1994; Ruzafa et al 1994; Ruzafa et al 1995; Yabuuchi and Omyama 1972). Melanin formation then occurs abiotically, outside the cells of these organisms to form polymers of MW ranging from 12,000-120,000 (Ruzafa et al 1994; Weiner et al. 1985). Melanin produced by *S. colwelliana* has been reportedly found both in the cell-free culture medium and associated with the cells (Fuqua et al. 1991; Ruzafa et al 1995). Similarly, extracellular melanin formed in shake cultures has been shown to adhere to the outer surfaces of some microorganisms (Bell and Wheeler 1986). If melanin is associated with the cell surface of *S. algae* BrY, it would then have to be incorporated onto the cell after it is formed. Here I demonstrate that extracellular melanin becomes associated with the cell surface of *S. algae* BrY and significantly increases the rate of anaerobic iron mineral reduction.

MATERIALS AND METHODS

Culture conditions and cell preparation. *S. algae* BrY (Caccavo et al. 1992),used throughout this study was maintained on Tryptic Soy agar (TSA) (DIFCO Labs, Detroit, MI). For bacterial growth and melanin production, the lactate basal medium (Lovley et al. 1996) contained (in g liter^{-1} dH_2O): NH_4CL (1.5), KCl (0.1), KH_2PO_4 (0.5), PIPES [Piperazine-N,N'-bis(2 ethanesulfonic acid)] (7.57), NaCl (10.0), 60% Na lactate (9 ml liter^{-1}), vitamin solution (10 ml liter^{-1}), mineral solution (10ml liter^{-1}) with tyrosine concentrations of 2g liter^{-1}. Aerobic 50 ml, duplicate cultures, were incubated at 28°C and 150 rpm for 48-200 hr. Inocula for all studies (10%, v/v) were from a 24 hr culture grown as above but without tyrosine. Cells from melanin-producing cultures (tyrosine supplemented) were termed "melanized", while those grown under conditions that did not produce melanin (e.g. not supplemented with tyrosine) were termed "non-melanized".

Determination of cell-associated melanin. *S. algae* BrY was grown in 1l of tyrosine-supplemented minimal medium as described above, for 12 days, until melanin production stabilized. Cultures were centrifuged (6,000 x g, 10 min., 4°C) and washed in sodium bicarbonate buffer (30 mM, pH 6.8) 3 times to remove any soluble melanin. The cells were acidified and subsequently lysed with 10 ml, 6N HCl to a pH of 1.5. The precipitate was removed by centrifugation (6,000 x g, 10 min., 4°C), then10 N NaOH was added to a final pH of 12 and the solution was centrifuged as above. The resulting dark solution was acidified to pH 1.5 with 6N HCl. The resulting precipitate was then centrifuged (as above), washed with pH 5 deionized water and centrifuged again. The precipitate was dried at 60°C. The presence of melanin was determined as previously described (Ellis and Griffiths 1974).

To determine if the degree of cell-associated melanin corresponds with soluble melanin production in culture liquid, cultures were grown in lactate basal medium (60% Na lactate 9 ml liter^{-1}) with the following concentrations of tyrosine (0.1, 0.25, 0.5, 1.0, 2.0 g liter^{-1}). Cultures were sampled 48 hr and 144 hr after the onset of melanin production. Melanin content from liquid cultures was determined spectrophotometrically at a wavelength of 400 nm with the spent, cell-free culture medium (Ruzafa et al 1994). Bacterial density was determined with acrodine orange staining and an epifluorescence microscope (Hobbie et al. 1977). Melanin association with the cells was analyzed by determining the quinone content of the washed cells (see below).

Quinone assay. Because melanin is a quinone-containing heteropolymer, the presence of cell-associated quinones was used to determine melanin concentrations with the nitroblue tetrazolium/glycinate (NBT) assay as described previously (Paz et al. 1991). An increase in the quinone content of the melanized cells relative to non-melanized cells was therefore indicative of melanin. This assay is specific for quinones which reduce nitroblue tetrazolium to formazan in the presence of oxygen and excess glycine at alkaline pH (Paz et al. 1991). Three hundred ì l of 2 M potassium glycinate (pH 10), combined with 1 ml of 0.24mM nitroblue tetrazolium, dissolved in 2M potassium glycinate (pH 10) were added to 100 ì l of a washed (20 mM phosphate buffer) cell suspension (OD_{600} = 0.5). The chemical solutions were kept on ice until use. The mixture was incubated at 28°C in the dark for 1 hr, and the absorbance was read at 530 nm. A standard curve for melanin was created with dried, concentrated melanin dissolved in dH_2O adjusted to pH 10 with 1N NaOH to insure dissolution. The solution was then adjusted to pH 7 with 1N HCl and diluted to final concentrations of 0.1-1.0 mg liter^{-1}. The response was linear (Paz et al 1991) with r^2=0.9937 with a slope = 0.729 .

Values for melanized cells were corrected for background activity by subtracting the values obtained for non-melanized controls.

Fourier Transform Infrared Spectroscopy (FT-IR). Melanized and non-melanized cultures were grown as described above, pelleted, washed three times in sterile dH_2O (pH 7.2) and lyophilized for 24 hr. Lyophilized cells (1-3 mg) were combined with dry KBr powder and pelleted as described previously (Abu et al. 1991). The resulting pellet was measured on a Nicolet 520 FT-IR spectrophotometer (Nicolet Instruments, Madison, WI) with an Mercury-Cadmium-Telluride (MCT) detector, against a reference of a non-absorbing KBr-matrix.

Hydrophobicity assay. Melanized and non-melanized cells were harvested by centrifugation, and washed three times in 20 mM potassium phosphate buffer (pH 6.8). The cells were resuspended to a final OD_{600} of 0.3 (Spec. 21 spectrometer, Unicam, Rochester, NY). Hydrophobicity was determined by the Bacterial Adhesion to Hydrocarbon Test (BATH) as described previously (Rosenberg et al. 1980). Samples of dilute bacterial suspensions were added (3 mls each) to 10ml glass cuvettes. After 25 ìl of pure hexadecane (Sigma Chem.) were added to each suspension, they were mixed by vortexing at low speed for 1 min. as previously described (Rosenberg et al. 1980). A second study was conducted with 5 ìl of hexadecane and 2 min. of agitation. Optical densities at 600 nm of the suspensions were read prior to and 30 min. after agitation with hexadecane. Bacteria that adhered to the hexadecane droplets rose to the top of the liquid and resulted in a decrease in absorbance. The degree of hydrophobicity was calculated as the Log_{10} ([initial OD_{600} {A} minus the final OD_{600} $\{A_1\}$] x 100) (Rosenberg et al. 1980).

Hydrous ferric oxide reduction. Aerobic cultures of melanized and non-melanized *S. algae* BrY were grown as described above. To determine whether increased rates of HFO reduction were due to tyrosine conversion to melanin or due to another aspect of amino acid catabolism, one treatment consisted of cells grown with lactate and phenylalanine (2g liter^{-1}) instead of tyrosine. Phenylalanine is a tyrosine analogue. Melanin was not produced with lactate and phenylalanine (2g liter^{-1}) in minimal medium within 48 hr. The cells were harvested at 48 hr of growth by centrifugation (8000 x g, 4°C, 20 min), and washed twice with anaerobic bicarbonate buffer (30 mM) under a N_2 atmosphere. Washed cells (final density of 10^9 ml^{-1}) were added to sterile anaerobic vials containing 50ìl of 6mM insoluble hydrous ferric oxide (HFO) in 10 ml pH 7.4 bicarbonate buffer (30 mM) with a head space of N_2 (80%), CO_2 (20%). To one set of duplicate tubes containing non-melanized cells,100 ìl of a 2g liter^{-1} melanin solution (Chapter One) were added (final concentration of 20 ìg ml^{-1}). Two abiotic controls (without cells), one with HFO and the other with HFO and 20 ìg/ml melanin were included. The electron donor was H_2, and was added (10 kPa) aseptically to all vials prior to incubation. Vials were incubated with agitation (150 rpm) in the dark at 28°C. At different time intervals, samples were removed for analysis of Fe(II) as previously described (Lovley et al. 1996). HFO reduction rates were calculated as previously described (Chapter One).

Cytochrome assay. *S. algae* BrY grown aerobically in minimal medium with lactate as the carbon source were washed (30mM sodium bicarbonate buffer) and concentrated under N_2 (approx. 10^9 cells/ml). A reduced cytochrome scan was generated as previously described (Gorby and Lovley 1991) by placing an air oxidized cell suspension in the reference cell of a duel beam spectrophotometer (Shimadzu UV-2401 PC) with a dithionite reduced cell suspension in the sample cell. For determination

of the effect of melanin on the cytochromes, 100ìl of an anaerobic oxidized melanin solution (2g liter^{-1}) was added anaerobically to the H_2 reduced cell suspension and read against a H_2 reduced cell suspension.

RESULTS

Cell-associated melanin. The washed centrifuged pellets of melanin producing cells were darker than those of non-melanin-producing cultures, suggesting melanin association with the cells (Fig. 2.1). After acid treatment and lysis, followed by adjustment to pH 12, melanin-producing cells revealed the presence of a dark soluble pigment with the same chemical characteristics as previously described for melanin (Ellis and Griffiths 1974; Prota 1986). The pigment had an absorbance spectrum (300 - 800 nm) similar to that of commercial humic acid (Aldrich Chem. Co.) (data not shown). In addition, the slope of log absorbance vs. wavelength (400-600 nm) scan of the pigment's spectrum was equal to -0.0035, which is indicative of melanin (Ellis and Griffiths 1974). Following precipitation, the washed, dialyzed, dried pigment was a black powder with the following properties indicative of melanin (Ellis and Griffiths 1974): (1) insoluble in organic solvents (ethanol, chloroform and acetone); (2) soluble in NaOH solutions $\geq$ pH 10; (3) decolorized in H_2O_2; (4) precipitated by $FeCl_3$ and (5) retained by an 8kDa dialysis membrane. Melanin was not detected in the liquid after the third wash of melanized cells, indicating that melanin was indeed associated with the cells and did not diffuse into the liquid. No pigment with characteristics of melanin was detected with lysed, non-melanized cells.

To further characterize and quantify cell-associated melanin, the NBT assay was used. Since this assay is specific for quinones (Paz et al 1991), it was used to quantify the quinone-rich melanin present with washed cells. Increased quinone content of whole melanized cells was confirmed with the NBT assay using concentrated *S. algae* BrY melanin as a standard (Table 1). Melanin production in the culture supernatant increased with tyrosine concentration. However there was no correlation between the relative amount of cell-associated melanin and initial tyrosine concentrations or extracellular melanin levels at 48 or 144 hr. after pigment production began (Table 1).

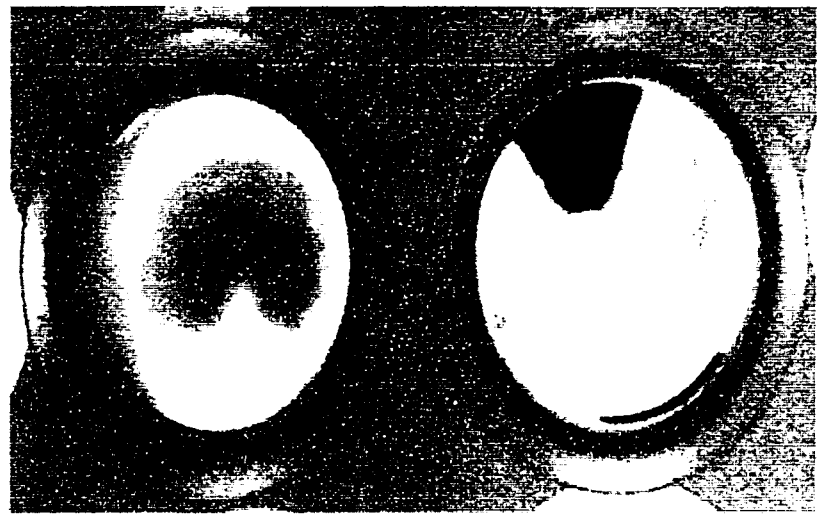


Figure 2.1. Photograph of pelted non-melanized cells (left) and melanized cells (right) of *S. algae* BrY, demonstrating differences in coloration.

Table 2.1. Soluble and cell associated melanin concentrations relative to initial tyrosine concentrations

	48 hr[a]		144 hr	
Initial tyrosine concentration (g/l)	cell-assoc. melanin/cell (femtograms)	soluble melanin A_{400}	cell-assoc. melanin/cell (femtograms)	soluble melanin A_{400}
0	0	0	0	0
0.1	0	0.04 (0.04)	0	0.11 (0.05)
0.25	29 (1.1)[b]	0.34 (0.01)	57 (2.7)	0.36 (0.11)
0.50	53 (3.5)	0.94 (0.20)	113 (10.0)	1.30 (0.07)
1.00	49 (3.2)	2.15 (0.11)	126 (16.0)	2.94 (0.04)
2.00	52 (5.6)	2.62 (0.11)	116 (18.6)	3.09 (0.03)

[a] hr. after onset of pigmentation
[b] standard deviation

Cell-associated melanin concentrations increased with time even after melanin production ceased (Table 1). This suggests a gradual association of extracellular melanin with cells. In addition, by 48 hr., the quantity of melanin associated with cells was not significantly different ($P<0.05$) in the cultures receiving 0.25 – 2.0 g liter^{-1} tyrosine (Table 1). By 144 hr however, cellular melanin content increased by more than 2 times in the cultures receiving 0.5 – 2.0 g liter^{-1} tyrosine (Table 1). Although the melanin concentration increased in the culture supplemented with 0.25 g liter^{-1} tyrosine, it was significantly lower ($P< 0.05$) than with the higher tyrosine concentrations. The culture receiving 0.1 g liter^{-1} tyrosine did not display a detectable increase in melanin concentration during any time of this study (Table 1) and may be due to the detection limits of this assay. By 48 hr after pigmentation, cell densities ranged from 3.6 - 3.9 x 10^9 and did not vary significantly between treatments. The melanin content of the melanized cells was then calculated. Melanin concentrations were not significantly different ($P<0.05$) for cultures with tyrosine concentrations from 0.5 – 2.0 g liter^{-1}, for their respective incubation times. Average melanin concentrations were 51 and 118 femtograms/cell at 48 and 144 hours, respectively. By 144 hr., cells had significantly higher ($P<0.05$) melanin content compared to those at 48 hr. These data demonstrate that melanin is associated with the cells and that it increased with time. However this assay could not determine if melanin was associated with the cell surface.

Fourier Transform Infrared Spectroscopy (FT-IR). The molecular surface composition of lyophilized bacteria was analyzed by FT-IR spectroscopy to determine their physiochemical properties (van der Mei et al. 1989) and detect cell surface-associated melanin. The FT-IR bands showed definite surface differences between melanized and non-melanized cells (Fig. 2.2) and corroborated the quinone data. In particular, those

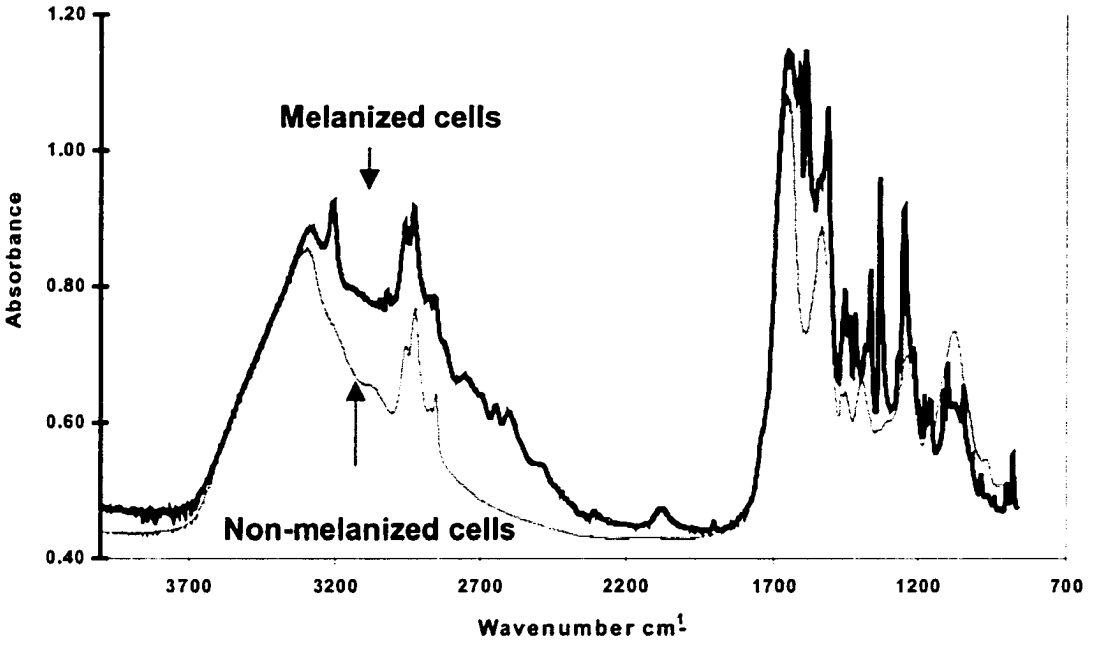


Figure 2.2. FT-IR specta of lypholized whole cells of *S. algae* BrY. Compared to non-melanized cells, melanized cells demonstrate increased response indicative of melanin at wavenumbers 2931, 1660, 1592 and1230 which are indicative of the C-H stretch of aromatics, C=O stretch, C=C stretch of aromatics and C-C stretch of phenolics, respectively.

differences consistent with the presence of melanin (Ellis and Griffiths 1974) include; a broad band at 3333 cm^{-1}, bands at 2860 cm^{-1}, 1650 - 1600 cm^{-1}, 1510 cm^{-1}, and 1410-1310 cm^{-1}, which are indicative of the OH stretch of polymeric structures (Silverman 1991), aliphatic C-H bonds (MacCarthy and Rice 1985), aromatic C=C conjugated with C=O and/or COO^- groups (MacCarthy and Rice 1985), aromatic C=C bonds (MacCarthy and Rice 1985) and OH groups of phenolics (Conley 1966), respectively. Non-melanized cells incubated for 6 days with 2 g $liter^{-1}$ melanin also demonstrated FT-IR bands indicative of melanin (above) when compared to non-melanized cells without contact with melanin (data not shown). These results provide evidence that the surface differences between melanized and non-melanized cells are due to surface-associated melanin.

Hydrophobicity determination. The FT-IR results suggested that melanin was present on the cell surface. Given the hydrophilic nature of melanin (Prota 1992; White 1958), I evaluated the differences in cell surface hydrophobicity between melanized and nonmelanized cells of *S. algae* BrY using the BATH test (Rosenberg et al. 1980). If melanin is associated with the cell surface, the cells would be expected to display a decrease in hydrophobicity. Based on the BATH assay, hydrophobicity values for melanized cells were 1.95 compared 1.92 for non-melanized cells. These values are significantly different ($P<0.01$) and demonstrate that melanized cells were less hydrophobic than non-melanized cells. The decrease in hydrophobicity supports the hypothesis of surface-associated melanin.

Hydrous ferric oxide reduction. Since melanin has redox cycling capabilities, surface-associated melanin should have the capacity for electron transfer from the cell surface to iron minerals. Surface associated melanin should therefore increase the rate of iron mineral reduction. Melanized cells had a significantly ($P<0.01$) higher HFO

reduction rate compared to non-melanized cells (Fig. 2.3). Calculations from non-linear regression analysis of Fe(II) evolution revealed that melanized cells reduced HFO 10 times faster than the non-melanized cells (Fig. 2.3). No HFO reduction occurred in the abiotic controls of HFO or HFO and melanin (data not shown). The addition of melanin to non-melanized cell suspensions accelerated HFO reduction (Fig. 2.3) to a rate of 0.002 h^{-1}, double that of the non-melanized cells without supplemental melanin. These data support the hypothesis that melanin is surface-associated and has the potential to significantly increase the rate of iron mineral reduction by *S. algae* BrY. Furthermore, based on the amount of supplemental melanin used in this experiment, and a cell density of 10^9 cells/ml, approximately 20 femtograms of melanin in solution was required per cell to double the HFO reduction rate of non-melanized cells.

Cells grown with lactate and supplemented with the tyrosine analogue phenylalanine did not produce melanin prior to harvesting and did not reduce HFO at a significantly greater rate ($P < 0.05$) than non-melanized cells grown with lactate as a sole carbon source (data not shown).

Cytochrome assay. For melanin to transfer electrons to HFO, the electrons must first be transferred from the cell to melanin. In order to determine if cytochromes were involved in electron transport to oxidized, soluble melanin, whole cell cytochrome scans were conducted. Dithionite reduced, minus air oxidized scans revealed peaks at 423, 523, and 552nm, indicative of bacterial cytochromes (Obuekwe and Westlake 1982) Whole cell absorbance scans from H_2 reduced, minus H_2 reduced plus anaerobic, oxidized melanin, revealed oxidized cytochromes (Fig. 2.4). These results illustrate that the reduced cytochromes of *S algae* BrY are capable of oxidization by soluble, anaerobic, oxidized melanin.

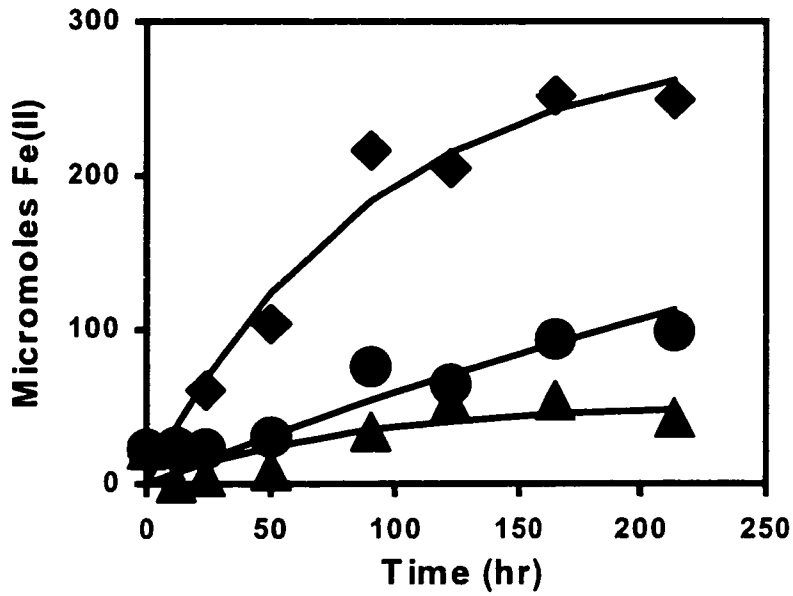


Figure 2.3. HFO reduction by melanized and non-melanized *S. algae* BrY cells. Cell suspensions (approx. 10^9 cells ml^{-1}) in bicarbonate buffer (30 mM) (pH 7.2) with 50 µl of 6mM HFO and N_2/CO_2 (80%/20%) headspace demonstrated HFO reduction with H_2 as electron donor. Melanized cells (◆) reduced HFO at a rate of $0.011h^{-1}$, non-melanized cell suspensions pregrown in lactate basal medium (▲) reduced HFO at a rate of $0.001h^{-1}$. HFO reduction rates increased by two fold ($0.002h^{-1}$) with the addition of 20mg $liter^{-1}$ of melanin to nonmelanized cell suspensions (●). HFO reduction was not detected in controls consisting of HFO only or HFO plus 20mg $liter^{-1}$ of melanin (data not shown). Non-melanized cell suspensions pregrown in lactate basal medium with phenylalanine demonstrated HFO reduction rates similar ($0.001h^{-1}$) to the other non-melanized control (data not shown).

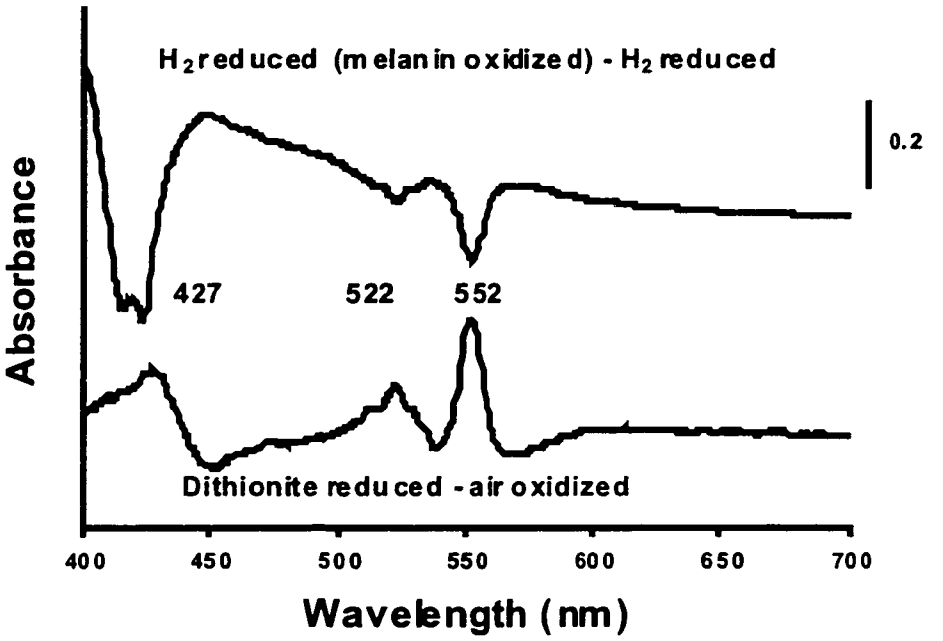


Figure 2.4. Difference spectra for whole cells of *S. algae* BrY. The upper scan is for H_2 - reduced (with oxidized, anaerobic melanin) minus H_2 – reduced cells. The lower scan is for (dithionite) - reduced minus (air) – oxidized cells.

DISCUSSION

In the absence of soluble electron shuttles, DMRB require close physical contact with minerals for reduction to proceed. Yet a mechanism for cell-to-mineral electron transfer has not been reported to date. Electron transport from the membrane to minerals has been theorized to occur by way of metals associated with the cell surface (Ehrlich 1993). In this case, surface-associated metals may act as an electron conduit for the cytochromes to reduce insoluble minerals. In another hypothesis, cytochromes which are proximal to the cell surface, may be involved in the direct transfer of electrons to insoluble minerals as with *S. putrifaciens* MR-1 (Lovley 1994). Whether the cytochromes transfer electrons directly to the mineral surface or whether an electron carrier is required has not been determined (Myers and Myers 1992; Lovley 1994). Here I show that: 1) melanin is associated with the cell surface; 2) melanized cells reduce HFO 10 times faster than non-melanized cells; 3) non-melanized cells, supplemented with melanin reduce HFO 2 times faster than non-melanized cells; 4) reduced cytochromes of whole cells of *S. algae* BrY reduce melanin. Melanin association with the cell surface, along with its ability to accept electrons from the cytochromes and reduce HFO offers a plausible mechanism of cell-to-mineral electron transfer by *S. algae* BrY.

Melanin production has been previously documented in the gamma proteobacteria, including the genus *Shewanella* (Katob et al. 1995; Weiner et al. 1985; Ruzafa et al 1994; Ruzafa et al 1995: Sanchez-Amat et al. 1998; Fuqua and Weiner 1993; Yabuuchi and Omyama 1972). The nature of melanin production in many microorganisms involves extracelluar autooxidation and self assembly of an excreted melanin precursor (Ruzafa et al 1994; Ruzafa et al 1995; Yabuuchi and Omyama 1972). Essentially, extracellular melanin is produced abiotically by specific precursors excreted

by the bacteria. Melanin that is produced extracellularly is also cell-associated (Ruzafa et al 1995). Therefore, for extracellular melanin to become associated with the cell surface, it must be acquired from the external environment. In this study, the increased quinone content of melanized cells denotes melanin association with the cells. The increase of melanin concentrations of the cells appears to be time dependent. Several lines of evidence indicating that melanin is associated with the cell surface are: 1) signature FT-IR absorbance bands for melanin, and; 2) melanized cells are less hydrophobic than non-melanized cells. Since melanin is a type of humic acid, it has the cation exchange properties of humics (Clapp 1990; White 1958). Extracellular melanin may therefore associate with the bacterial surface through a similar adsorption mechanism as reported for humics. Sakai (1986) demonstrated increased adsorption of *Aeromonas salmonicida* to humic acid-covered sand particles that was linked to ion exchange between the cell surface and humic acids.

Since Fe(III) reduction by hydroquinones results in regeneration to the quinone state, their low concentrations and redox cycling capacity can have a tremendous impact on Fe(III) reduction rates (Lloyd et al 1999; Nevin and Lovley 2000). Small quantities of quinoid compounds significantly increase the rate of HFO reduction (Nevin and Lovley 2000; Chapter One). Here I show that melanized cells increase the HFO reduction rate by 10 fold compared to non-melanized cells. Supplemental melanin doubled the rate of HFO reduction by non-melanized cells, further demonstrating that melanin was responsible for increased HFO reduction rates. Furthermore, the supplemental melanin concentration used in this study equates to 20 femtograms of melanin per cell. Melanized cells had about 100 femtograms of melanin per cell, based on the quinone assay (Table 1). That could explain their 5 fold higher HFO reduction rate compared to non-melanized, melanin supplemented cells. Melanin concentration does influence HFO reduction rates (Chapter One) and that may explain the differences evident in this study.

Non-melanized cells reduced HFO, but at much slower rates than melanized cells and non-melanized cells with melanin added. There are several possible explanations for this. There may be another mechanism for HFO reduction that does not include melanin or intracellular tyrosine could be produced de novo and converted to small amounts of melanin not detected during this study.

Previously, iron reduction rates of the DMRB *Pseudomonas* putrefaciens MR-1 were increased when cells were pregrown in basal medium supplemented with yeast extract and beef peptone compared to an unsupplemented basal medium (Obuekwe and Westlake 1982). In that report, the increased rate of iron reduction was attributed to increased cytochrome content ascribed to growth in the supplemental carbon sources (Obuekwe and Westlake 1982). In order to determine if growth in tyrosine may have contributed to increased HFO reduction rates in some other way than melanin production, cells were grown in lactate minimal medium supplemented with the tyrosine analogue phenylalanine. These cells did not produce melanin during incubation and the washed cell suspensions reduced HFO at a similar rate as the non-melanized cells, but at a significantly lower rate than melanized cells. Thus, these data provide further evidence that melanin production from tyrosine was responsible for the increased rate of HFO reduction.

Cell contact to iron minerals is necessary for dissimilatory reduction to occur by DMRB (Lovley 1994). In this study, increased contact through adhesion to HFO brought about by melanin could not have been responsible for the increased HFO reduction rate of the melanized cells. Increased adhesion of *S.algae* BrY to HFO was shown to be a function of increased hydrophobicity (Caccavo et al. 1997). Melanized cells were actually less hydrophobic than the non-melanized cells in this study, consequently the melanized cells would be expected to be somewhat less adherent to HFO. Since the

cultures in this study were shaken during incubation, sufficient surface contact was encountered throughout the experiments.

This work also raises the question of the likelihood of melanin production in the environment by DMRB. Previous reports demonstrated that melanin production occurs during the transition from exponential to post exponential growth, under aerobic conditions (Coyne and Al-Harthi 1992; Katob et al. 1995; Ruzafa et al 1995; Weiner et al. 1985; Chapter One). Melanin production is believed to be a response to changing growth conditions such as anoxia or carbon limitation (Coyne and Al-Harthi 1992). Faced with imminent anoxia, nonfermentative, facultative anaerobes such as *S. algae* could derive a potential bioenergetic benefit from melanin production. Since only low concentration of melanin is required per cell for effective iron oxide reduction (Chapter One), only trace amounts of tyrosine would be required. These might be scavenged from the environment for melanin production to occur.

CHAPTER 3

Characterization of Melanin Production by *Shewanella algae* BrY

ABSTRACT

Shewanella algae BrY produces melanin and uses it as a sole terminal electron acceptor and an electron shuttle for dissimilatory reduction of insoluble minerals. This exploitation of the electro-chemical properties of melanin offers a significant bioenergetic survival advantage for this organism. Therefore, the characterization of melanogenesis is necessary to understand the role of this substance in the physiology and ecology of *S. algae* BrY. Melanin production occurs during exponential/post-exponential growth in tyrosine-supplemented liquid media. Phenylalanine-supplemented medium does not result in the same degree of pigmentation and the onset of cell culture pigmentation is delayed relative to growth with tyrosine. Either tyrosine or phenylalanine serve as sole carbon sources, but the degree of pigmentation decreases under these conditions. The onset of melanogenesis on solid media supplemented with tyrosine occurs sooner at 37°C than at 28°C. The antioxidant ascorbate inhibits melanin production, but does not inhibit production and excretion of homogentisic acid, the melanin precursor. In the absence of ascorbate, melanin production is inhibited by the *p*-hydroxyplenylpyruvate hydroxylase inhibitor, sulcotrione, which is consistent with homogentisic acid production via this enzyme. The rate and degree of melanin production is related to exogenous Fe(II) concentrations. Inhibition of melanin production occurs above 0.2mM Fe(II). Exogenous Cu (20 ìM) has no effect on melanin production. These data support the

hypothesis that melanin production by *S. algae* BrY is a result of tyrosine conversion to homogentisic acid which is autooxidized and self-polymerized to pyomelanin.

INTRODUCTION

Melanin is a generic term that describes high-molecular-weight dark pigments of biological origin (Bell and Wheeler 1986). Numerous protective properties have been ascribed to melanin, including protection from environmental stresses such as, UV and ã radiation, desiccation, temperature extremes, free radicals, and hydrolytic enzymes (Bell and Wheeler 1986). Melanin also plays an important role in microbial virulence (Bell and Wheeler, 1986; Coyne and Al-Harthi, 1992), and iron scavenging (Nyhus et al.1997). The electrochemical properties of melanin allow it to function as an amorphous semiconductor, a threshold switch, and an electron donor and an electron acceptor (McGinness 1972; McGinness et al. 1974; Menter and Willis 1997; Pullman and Pullman 1961).

Based on biochemical characteristics, melanin is further characterized by type. Eumelanin production occurs by the Mason-Raper pathway in which tyrosine is converted to DOPA and dopachrome by tyrosinase and oxygen (Bell and Wheeler 1986; Swan, 1974; Prota 1992). Phaeomelanins are produced by the conversion of tyrosine to dopachrome which reacts with cysteine or glutamine (Coon et al. 1994; Prota 1992; Swan, 1974). Allomelanins are produced from non-nitrogenous phenols and result in a wide range of diverse phenolic products (Coon et al. 1994; Prota 1992; Swan, 1974). Overall, melanin production is a function of polyphenol oxidases such as tyrosinase. Tyrosinase, a copper-containing enzyme, plays a significant role in the production of some types of melanin (Prota 1992; Bell and Wheeler 1986; Swan 1974). Tyrosinase activity is correlated to exogenous copper concentrations (Prota 1992; Swan 1974).

Pyomelanin (alkaptan) is produced by the conversion of tyrosine to *p*-hydroxyphenylpyruvate and subsequently to homogentisic acid (Yabuuchi and Omyama

1972). The enzyme, *p*-hydroxyphenylpyruvate hydroxylase (*p*-HPPH) (also known as *p*-hydroxyphenylpyruvate dioxygenase) is responsible for this conversion (Kotob et al.1995; Coon et al. 1994; Ruzafa et al.1994). In contrast to tyrosinase, *p*-HPPH, also a metaloenzyme, has a non-heme iron complex (Lindstedt 1977; Lindbald et al.1977). Exogenous Fe controls *p*-HPPH activity, but supplemental Cu has no affect on activity (Lindstedt 1977; Lindbald et al.1977). The resulting homogentisic acid, produced by *p*-HPPH, is excreted by the bacteria and it undergoes autooxidation and self-polymerization to form pyomelanin (Yabuuchi and Omyama 1972; Ruzafa et al. 1995). Pyomelanin has a molecular weight ranging from 12,000 to 120,000 (Ruzafa et al.1994; Weiner et al. 1985) and is smaller than eumelanin which has molecular weights over 1,000,000 (Bridelli 1998).

Bacterial production of pyomelanin was first described in *Pseudomonas aeruginosa* (Yabuuchi and Omyama 1972) and has since been identified in several bacterial species including *S. colwelliana, Vibrio cholerae*, and a *Hyphomonas* strain (Ruzafa et al. 1995; Weiner et al. 1985; Kotob et al.1995). The *melA* gene, which encodes for p-HPPH in *S. colwelliana,* has been sequenced and cloned (Coon et al. 1994; Ruzafa et al.1994).

S. algae BrY and *S. oneidensis* MR-1 are facultative anaerobes capable of dissimilatory mineral reduction (Venkateswaran et al. 1999; Caccavo et al. 1992) and also produce extracellular melanin (Chapter One). *S. algae* BrY melanin serves as a terminal electron acceptor and an electron shuttle to insoluble mineral oxides (Chapters One and Two). This contribution to bacterial bioenergetics is a novel use of melanin by a microorganism. Furthermore melanin production may play an important role in the physiological ecology of *S. algae* BrY. A further understanding of melanin production by *S. algae* BrY is necessary in order to determine its role in the growth and survival of this organism.

Melanin production by both *S. algae* BrY and *S. oneidensis* MR-1 occurs during the exponential/post exponential growth phase when grown with tyrosine (Chapters One and Two) and is similar to pyomelamin production by *S. colwelliana* (Coon et al. 1994; Fuqua and Weiner 1993). A BLAST search of the nearly completely sequenced genome of *S. oneidensis* MR-1 identified an ORF with 92% amino acid similarity and 82% identity to the *melA* gene product of *S. colwelliana* (TIGR website), which suggests that *S. oneidensis* MR-1 is also a pyomelanin producer. Due to the physiological and phylogenetic similarities of *S. oneidensis* MR-1 and *S. algae* BrY, *S. oneidensis* MR-1 was included in several key comparative experiments and to further characterize melanin production in the genus *Shewanella*.

MATERIALS AND METHODS

Culture conditions and cell preparation. *S. algae* BrY and *S. oneidensis* MR-1 were used in this study and were maintained on Tryptic Soy agar (TSA) (Difco LBS, Detroit, MI)). For bacterial growth and melanin production, the lactate basal medium (Lovley et al. 1996), which contained (in g liter^{-1} dH_2O): NH_4CL (1.5), KCl (0.1), KH_2PO_4 (0.5), PIPES [Piperazine-N,N'-bis(2 ethanesulfonic acid)] (7.57), NaCl (10.0), 60% Na lactate (9 ml/l), vitamin solution (10 ml/l), mineral solution (10ml/l) with tyrosine or phenylalanine concentrations of 2g liter^{-1} was used. Final pH was 6.8-7.2. Cultures (50 ml) were inoculated with (10% v/v) 18-24 hr. old cells grown in either Tryptic Soy broth (TSB) (Difco) or lactate basal medium and were incubated aerobically (150 rpm) at 28°C.

Anaerobic media consisted of lactate basal medium (above) with 30 mM fumarate as an electron acceptor. Media were made anaerobic by boiling and cooling under a constant stream of an anaerobic gas mixture (80% N_2, 20% CO_2). The media were transferred anaerobically to serum vials, stored under the same atmosphere, and autoclaved.

Bacterial growth studies and melanin production on solid media were conducted with either tyrosine supplemented (2g liter^{-1}) TSA or the lactate basal medium, supplemented with tyrosine (2g liter^{-1}) and 2%agar. Control cultures were grown in the same media, but without tyrosine. Cultures were incubated at 28°C and 37°C.

Sole carbon source studies were conducted aerobically in basal medium broth without lactate as described above with the addition of 0.25 g liter^{-1} tyrosine or 0.25 g liter^{-1} phenylalanine.

Cell density measurements. Cell numbers were measured by acridine orange

staining and an epifluorescence microscope as described by Hobbie et al. (1977).

Determination of extracellular melanin polymerization. To determine if extracellular melanin was produced by self-assembly of excreted metabolic precursors, the antioxidant ascorbate was added in equal molar concentrations to tyrosine supplemented lactate basal medium described above. *S. algae* BrY, pregrown in lactate basal medium, served as inoculum (10% v/v) and was incubated for 72 hr. as described above. Melanin was determined spectrophotomectrically in the cell-free culture supernatant as described below.

Melanin measurement. Melanin content of spent, cell-free culture medium was determined spectrophotometrically (UV-2401 PC recording spectrophotometer, Shimadzu Instruments, Columbia, MD) at 400 nm (Ruzafa et al. 1995) or 800-300 nm (Ellis and Griffiths 1974) and zeroed against controls (cultures grown without tyrosine). For rate determination, melanin production was measured at 400 nm at different time intervals throughout the growth cycle. Melanin production was plotted over time. Rates were then calculated from the data prior to a plateau in melanin production, by linear regression of log transformed data.

Melanin concentration and HGA-melanin production. Melanin was concentrated from batch reactors with tyrosine supplemented basal medium as described above and concentrated as previously described (Chapters One and Two). HGA-melanin was produced by the autooxidation of homogentisic acid (Sigma Chemical, St. Louis, MO) as previously described (Ruzafa et al. 1995).

Metabolic precursor determination. Cell-free liquid culture aliquots of *S. algae* BrY and *S. oneidensis* MR-1 were assayed for melanin precursors by high pressure capillary electrophoresis (HPCE) (Heiger 1992). Cells were grown in lactate basal medium supplemented with tyrosine (2 g liter^{-1}). To prevent autooxidation and to increase melanin precursor concentrations, equal molar concentrations of ascorbate were added to some of the cultures (Kotob et al.1995).

Standards (tyrosine, DOPA and HGA) were dissolved in 4 mM ascorbate at 4 mM each. After samples (1 mL) were removed from the cultures at 18 and 48 hr growth, they were centrifuged (8,000 x g) to remove cells and transferred to teflon autosample tubes for further analysis.

A Celect H150 C-8 bonded phase capillary column (Supelco, Belefonte, PA), 30 cm long with a 50ìm ID, was used with HPCE (Heiger 1992). Operating conditions were: cassette temperature, 25.0°C; positive polarity; voltage, 20.00 kV; injection pressure, 50.00 m bar for 1.00 sec. The injection volume was 1 nL (Heiger 1992). Buffer was 7.14 g liter^{-1} NaB_4O_7 solution in dH_2O with a pH of 9.2. Optical density of samples was detected on-capillary at 200 nm.

HGA and DOPA were also determined by colorimetric methods. DOPA analysis consisted of the DOPA nitration method (Waite and Benedict 1984). HGA content was determined based on its reaction with cysteine to form 1,4 thiazine, according to the methods of Fellman et al. (1972). Reactants were measured with a Shimadzu UV-2401 PC recording spectrophotometer.

Enzyme inhibition assays. Sulcotrione (Zeneca Ag. Products, Richmond, CA) (250ìM) was made in lactate basal medium and filter sterilized (0.22ìm Nucleopore membrane). Sterile sulcotrione was added (0, 0.25, 2.5, and 10 ìM final concentrations) to aerobic lactate basal medium (50 ml) supplemented with 0.25 g liter^{-1} tyrosine. These

media were inoculated (10% v/v) with 24 hr cultures of *S. algae* BrY that were grown in basal medium. Cultures were aerobically incubated with shaking (150 rpm) at 28°C for 48hr. Spent cell free medium was measured spectrophotometrically (400 nm) for melanin, as described above. An inhibition constant (K_i) for sulcotrione was determined as previously described (Turick and Apel 1997).

In some experiments, *S. algae* BrY and *S. oneidensis* MR-1 were grown in lactate basal medium with tyrosine (2 g liter^{-1}) and filter sterilized sulcotrione (30ìM, final concentration). Cultures were aerobically incubated with shaking (150 rpm) at 28°C for 48hr.

Mushroom tyrosinase activity was measured as previously described (Yasunobu et al. 1959). The reaction mixture consisted of 0.2 mg tyrosinase (6680 units/mg) (Sigma Chem.), 0.45 ìg tyrosine, 260ìM phosphate buffer (pH 6.8) and 8 mM sulcotrione per 3 ml reaction vessel. Controls contained no sulcotrione. Samples were incubated at 25°C for 1 hr. and activity was determined spectrophotometrically by optical density measurements (500 to 250 nm) on a Shimadzu UV-2401 PC recording spectrophotometer.

Effects of meta!s on melanogenesis. For melanin production studies, lactate basal medium with tyrosine (2g liter^{-1}) contained the following supplements: $CuSO_4$ (20 ìM), or $FeSO_4$ (0.01, 0.18, 0.38, 3.76 mM), or Na_2SO_4 (3.76 mM). Cultures (50 ml) were inoculated (10% v/v) with 18-24 hr old cells grown in lactate basal medium with tyrosine, Cu and Fe excluded.

RESULTS

Growth studies. Melanin was produced aerobically in all tyrosine supplemented media evaluated, but was not detected under anaerobic conditions. On solid media, melanin production commenced in regions of highest colony density on the plates. The pigment was first observed surrounding individual colonies and after several days was followed by the darkening of the colonies themselves. This observation would suggest that the pigment was excreted by the cells and undergoes autooxidation. Slight pigmentation was also observed on TSA without supplemental tyrosine. This slight degree of pigmentation may have been melanin that was produced from background levels of tyrosine as part of the amino acid complement of the medium. The onset of melanogenesis occurred 24 hr. earlier on solid media at 37°C than at 28°C.

The onset of melanin production in lactate basal medium supplemented with phenylalanine occurred 24 hr. later than pigment production in medium supplemented with tyrosine (data not shown). After 72 hr. of incubation, melanin production leveled off in these cultures and a significant difference ($P<0.05$) in the amount of melanin was determined (Student's t test) (Table 1). Both tyrosine and phenylalanine served as a sole carbon source for *S. algae* BrY (data not shown). Less melanin was produced, however, during growth with only phenylalanine or tyrosine as compared to growth with 0.25 g liter^{-1} lactate and tyrosine (Table 3.1).

Extracellular melanin polymerization. Pigment production on plates suggested that melanin production was the result of autooxidation and self-assembly of melanin precursors into polymers. To test this hypothesis, the antioxidant ascorbate was added to the cultures. Cultures grown with ascorbate and tyrosine did not produce melanin

Table 3.1. Melanin production by *S. algae* BrY with various carbon sources[1]

Carbon sources	A_{400}	standard deviation
Na lactate (5.4 g liter^{-1}) + tyrosine (2.0 g liter^{-1})	3.274	(0.086)
Na lactate (5.4 g liter^{-1}) + phenylalanine (2.0 g liter^{-1})	0.459	(0.049)
Na lactate (5.4 g liter^{-1}) + tyrosine (0.25 g liter^{-1})	0.285	(0.036)
tyrosine (0.25 g liter^{-1})	0.168	(0.025)
phenylalanine (2 g liter^{-1})	0.165	(0.046)

1, Melanin concentration after 72hr. of incubation.

(Fig. 3.1.). Their spectral scans were similar to those of cultures without tyrosine. Cultures with tyrosine produced a spectral scan characteristic of melanin (Fig. 3.1).

Metabolic precursor determination. Extracellular melanin is produced by the autooxidation and self-polymerization of metabolic precursors such as HGA or DOPA (Bell and Wheeler 1986; Ruzafa et al. 1995; Weiner et al. 1985; Kotob et al.1995). The growth media from 18 and 48 hr. cultures of *S. algae* BrY and *S. oneidensis* MR-1 were analyzed for the presence of melanin precursors by the use of HPCE (Figs. 3.2A and B). With *S. algae* BrY, two peaks were detected from the growth medium supplemented with tyrosine (Fig. 3.2A). The second peak co-migrated with HGA and was confirmed with colorimetric analysis (data not shown). The presence of ascorbate caused an increase in the HGA peak. The HGA peak was not detected in growth medium without tyrosine (data not shown). With *S. oneidensis* MR-1, one peak, HGA, was detected under these conditions (Fig. 3.2B). The first peak of the *S. algae* BrY sample migrated just after the DOPA peak (Fig. 3.2A). Colorimetric analysis of the sample indicated that the peak was not DOPA. These results support our hypothesis that extracellular melanin is produced *S. algae* BrY and *S. oneidensis* MR-1 by the autooxidation of excreted HGA.

Enzyme inhibition. Melanin production by *S. algae* BrY was completely inhibited with10 ìM sulcotrione (Fig. 3.3). The inhibition constant (K_i) of sulcotrione for *S. algae* BrY was 0.04 ìM. Melanin production by *S.* oneidensis MR-1 and *S. algae* BrY was also inhibited by 55% and 69% ($P<0.05$) respectively, after 48 hr. growth in basal medium supplemented with 2g liter^{-1} tyrosine and 25 ìM sulcotrione. Sulcotrione had no effect on cell viability (data not shown).

In the presence of the enzyme tyrosinase, tyrosine is converted to DOPA and quickly oxidized to dopachrome (Prota 1992; Yasunobu et al.1959). Sulcotrione did not

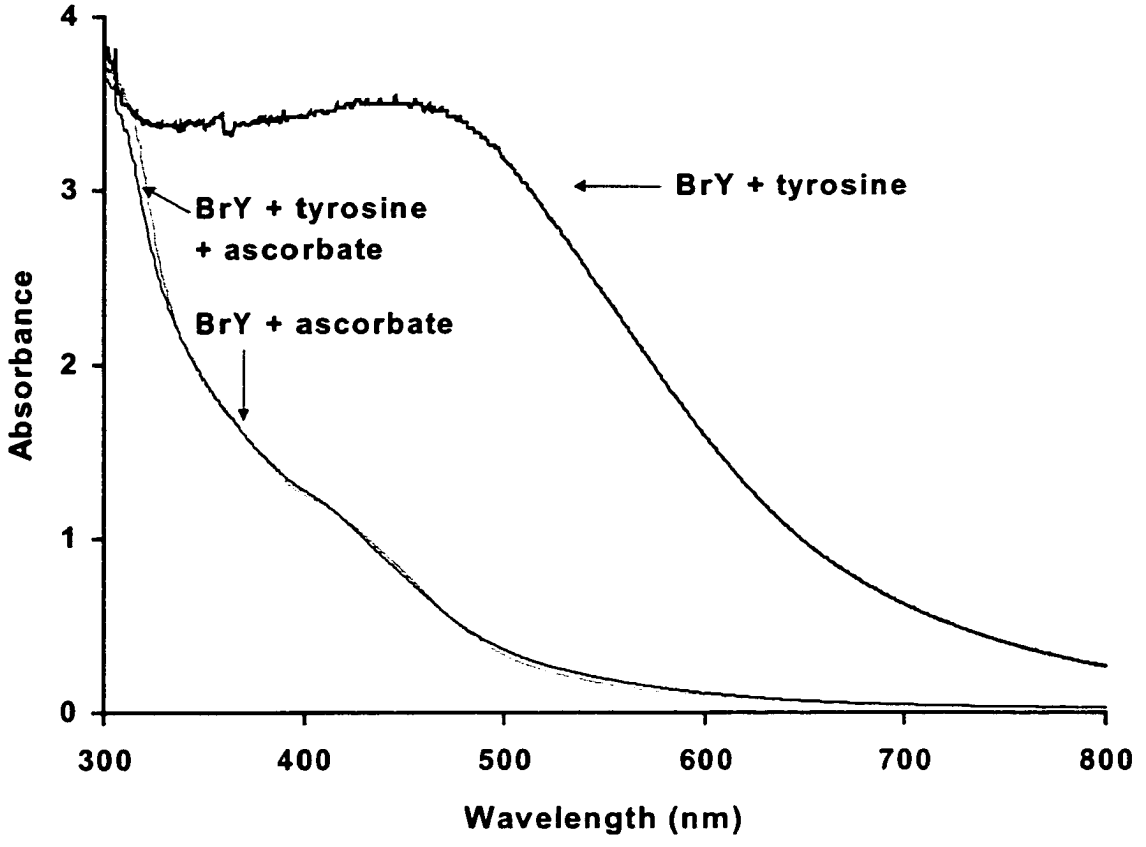


Figure 3.1. Effect of ascorbate (8 mM) on melanin production by *S. algae* BrY grown aerobically in lactate basal medium supplemented with 2g liter^{-1} tyrosine.

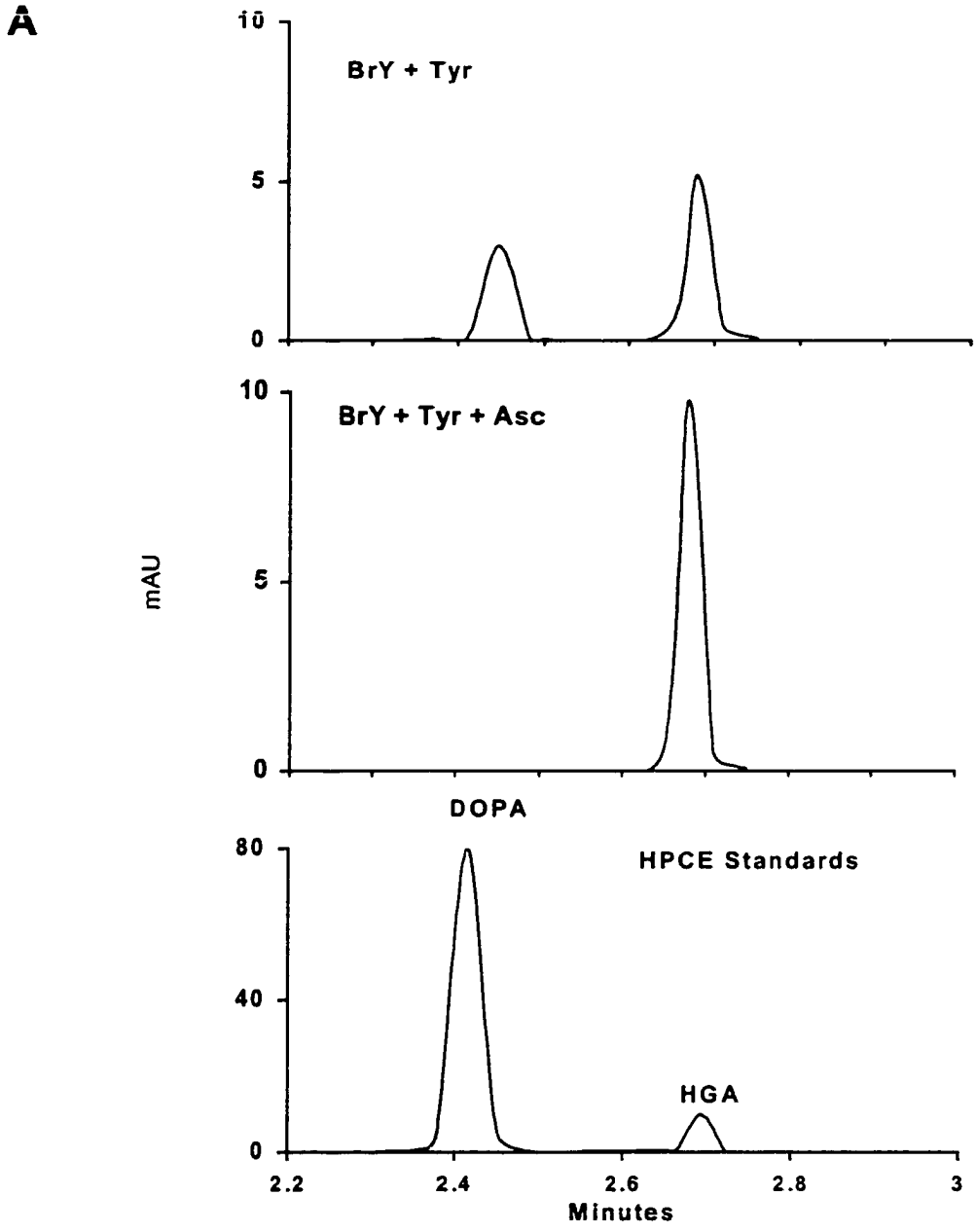


Figure 3.2 A. HPCE analysis of *S. algae* BrY spent culture supernatant liquids.

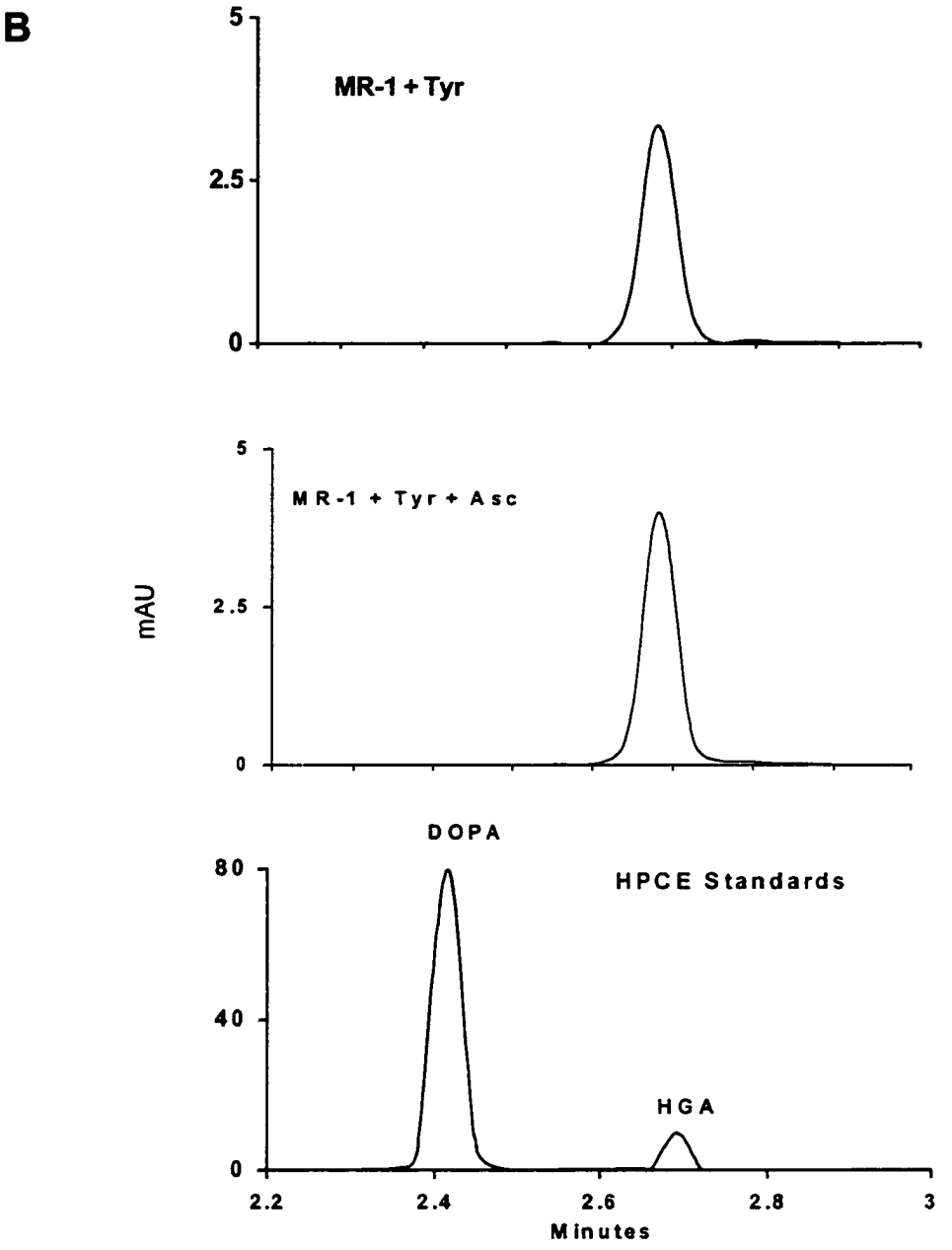


Figure 3.2 B. HPCE analysis *S. oneidensis* MR-1 spent culture supernatant liquids.

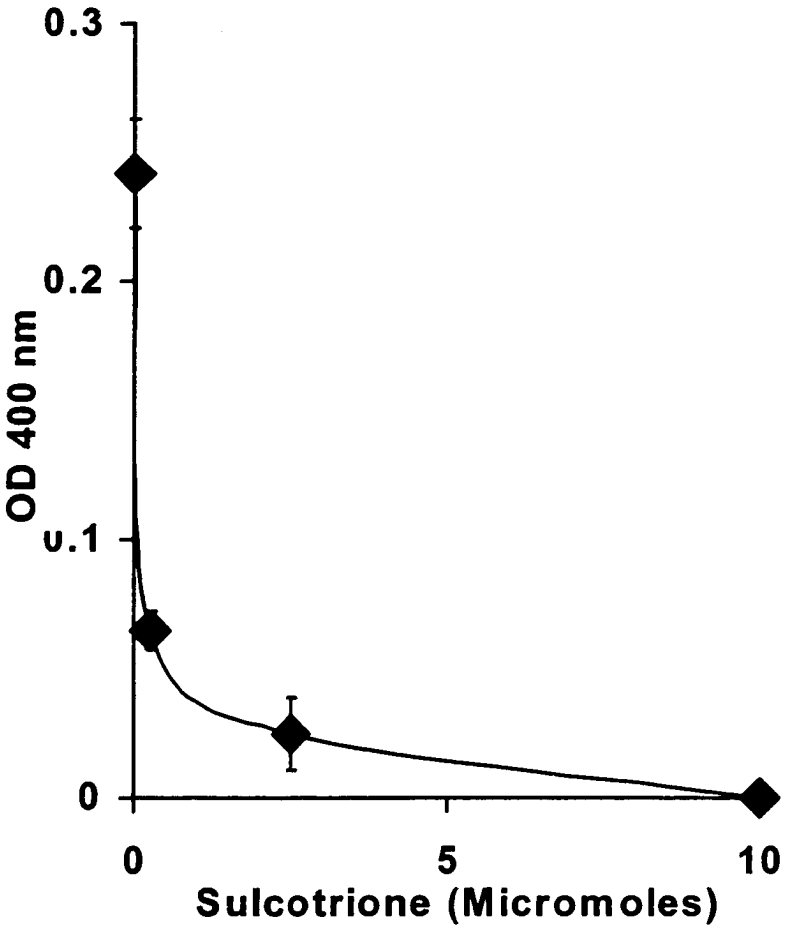


Figure 3.3. Melanin inhibition in cultures of *S. alage* BrY by the *p*HPPD inhibitor sulcotrione.

inhibit the activity of the enzyme, mushroom tyrosinase. In the presence of tyrosine and mushroom tyrosinase, a typical spectral scan of dopachrome (Yasunobu et al.1959) resulted both from sulcotrione treated and untreated samples (Fig. 3.4). These results further support our hypothesis that the enzyme p-HPPH, not tyrosinase, is responsible for melanin production by *S. algae* BrY and *S. oneidensis* MR-1.

Effects of metals on melanin production. The concentration of Fe(II) affected melanin production by *S. algae* BrY (Fig. 3.5A). To determine if the exogenous Fe(II) was contributing to melanin precipitation and consequently the decrease in optical density, 4.0 mM of Fe(II) (as ferrous sulfate) was added to cell-free melanin. The addition of Fe(II) did not affect the spectral scans of melanin (data not shown). Although iron will precipitate melanin, the concentration used in this study was insufficient for melanin precipitation. Melanin production was not affected in cultures receiving 3.8 mM Na_2SO_4, demonstrating that the sulfate in the ferrous sulfate additions did not affect melanin production (Fig. 3.5A). Melanogenesis was also not affected by the exclusion or addition of copper (20ìM) (data not shown).

We also evaluated the pH of the medium during growth to determine if that contributed to the decrease in melanin. Extracellular melanin production is related to an increase in the pH of the growth medium (Ruzafa et al. 1995; Fuqua and Weiner 1993). In all cultures in this study, the pH remained near 7 for the first 24 hr. of growth, until the onset of melanin production (Fig. 3.5B). The appearance of melanin coincided with a pH increase to 7.6 – 7.8, except for the 0.376 mM and 3.76 mM Fe(II) supplemented cultures which demonstrated a pH of 8.6 (Fig. 3.5B). Hence, the added iron did not result in decreased pH but rather an increase. Consequently, the decrease in melanin production at the higher iron concentrations was not a function of pH.

The Fe(II) concentrations used in this study did not have an adverse affect on

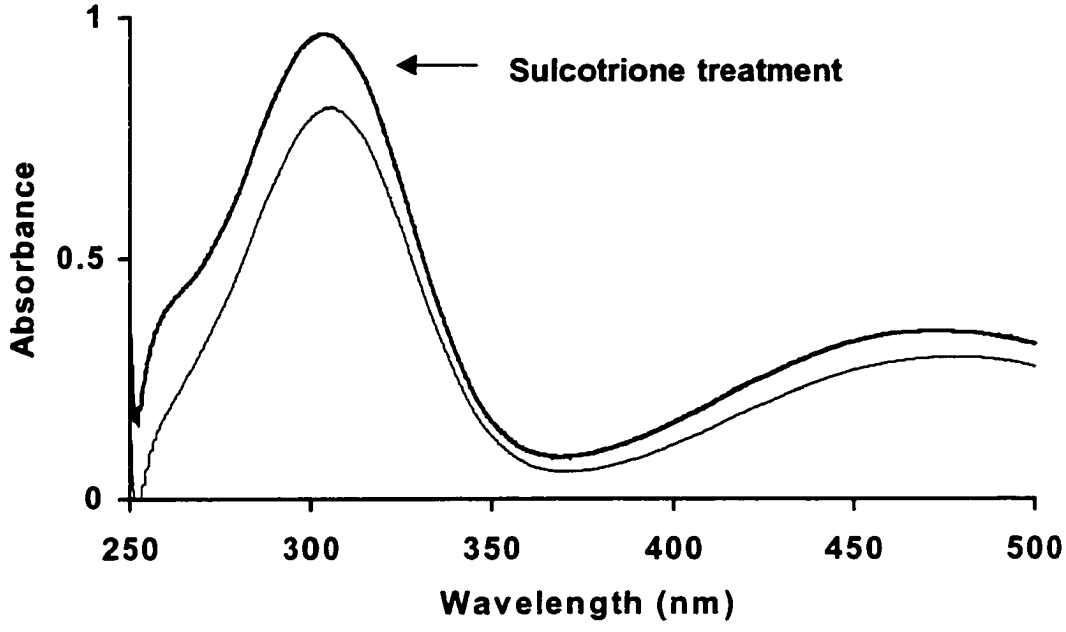


Figure 3.4. Spectral scan of products from purified mushroom tyrosinase incubated with tyrosine, with and without sulcotrione (30 ìM).

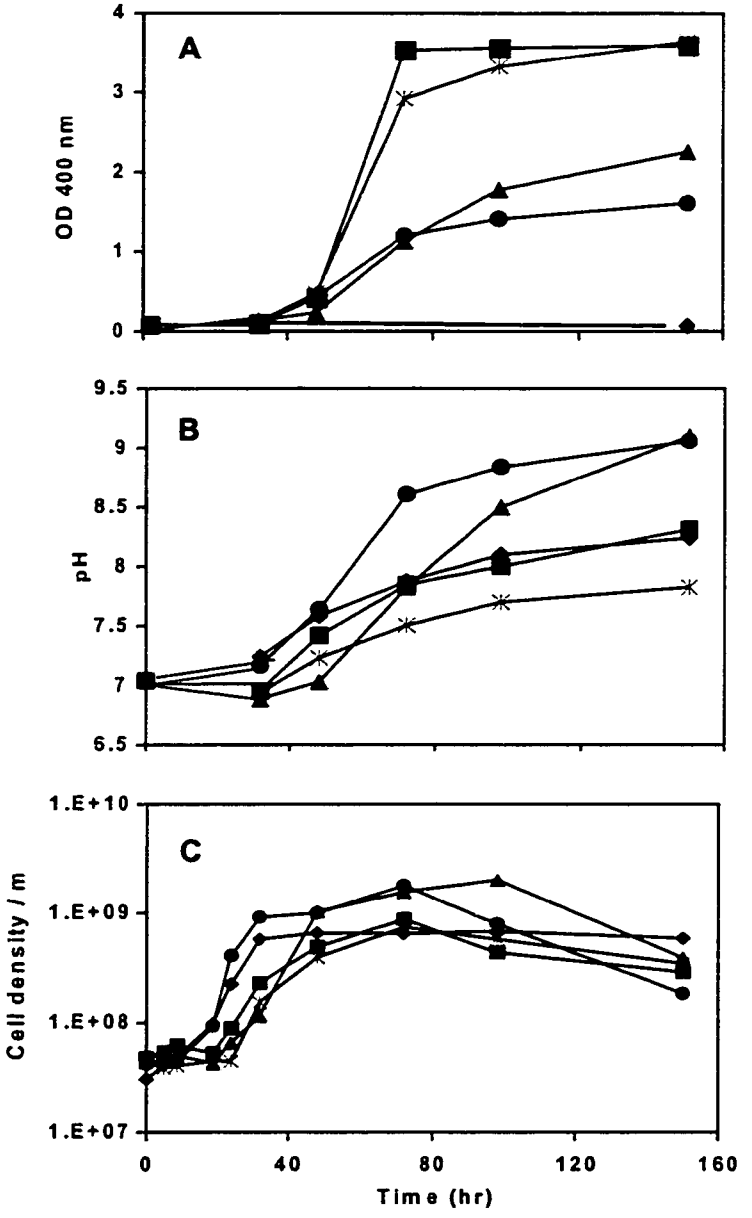


Figure 3.5. Growth and melanin production by *S. algae* BrY. Melanin production (A) medium pH (B) and cell density (C) from tyrosine (2g liter^{-1}) supplemented lactate basal medium with $FeSO_4$ concentrations of; 0.01 (■), 0.376 (▲) and 3.76 mM (●). Controls consisted of tyrosine-supplemented medium with 3.76 mM Na_2SO_4 (∗), and lactate basal medium without tyrosine (◆).

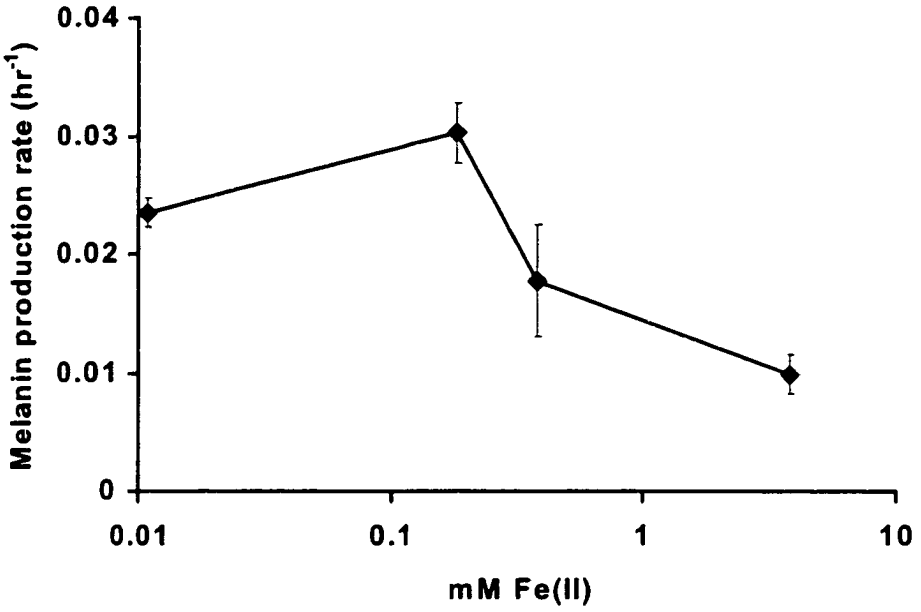


Figure 3.6. Effect of Fe(II) on extracellular melanin production rates by *S. algae* BrY grown in lactate basal medium.

growth (Fig. 3.5C). Cell densities reached 7 - 9 x 10^8 /ml in all but those receiving higher Fe(II) concentrations, which had cell densities of 1.6 -1.8 x 10^9 /ml (Fig. 3.5C). Since there were higher cell densities in cultures with more iron, cell density was not a factor in decreased melanin production.

The highest rate of melanin production was achieved with 0.18 mM Fe(II) (Fig. 3.6). Higher Fe(II) concentrations decreased the rate of melanogenesis (Fig. 3.6). These data suggest that Fe(II) concentrations affect p-HPPH activity in *S. algae* BrY which results in a decreased rate of melanogenesis at Fe(II) concentrations above 0.18mM.

DISCUSSION

S. algae BrY and *S. oneidensis* MR-1 have been shown to produce melanin (Chapters One and Two) and the manner of production was characterized in this study. Melanin production by *S. algae* BrY was by a pathway similar to that of many other species in the ã Proteobacteria (Kotob et al.1995; Ruzafa et al. 1995; Ruzafa et al. 1994; Yabuuchi and Omyama 1972). Specifically, during exponential/post-exponential growth, tyrosine is converted to melanin from the precursor HGA. This is a characteristic of pyomelanin production by bacteria (Kotob et al.1995; Ruzafa et al. 1995; Ruzafa et al. 1994; Yabuuchi and Omyama 1972) and the degree of melanin production is proportional to tyrosine concentration (Fuqua and Weiner 1993; Ruzafa et al. 1995; Chapters One and Two. Evidence that *S. algae* BrY produces pyomelanin includes: (1) extracellular melanin is produced from tyrosine and phenylalanine; (2) both tyrosine and phenylalanine serve as sole carbon sources, but result in more melanin when lactate is the other source of carbon; (3) extracellular melanin production occurs through autooxidation and self polymerization of an excreted metabolic precursor; (4) melanin production in liquid culture coincides with a pH increase of the bulk liquid; (5) melanin production occurs during exponential/post exponential growth; (6) increased growth temperature accelerates the onset of melanin production; (7) melanin produced by *S. algae* BrY has a MW of 13,000-17,000 and is similar to autooxidized HGA-melanin but not eumelanin. In addition, results from studies with both *S. algae* BrY and *S. oneidensis* MR-1 show that: (1) HGA but not DOPA is excreted prior to melanin production; (2) exogenous Cu has no effect on melanin production; (3) melanin production is inhibited by exogenous Fe(II) at concentrations $\geq$ 0.376 mM and by the p-HPPH inhibitor sulcotrione.

S. algae BrY utilized tyrosine and phenylalanine for pigment production as well as sole carbon sources. Previously, *P. aeruginosa* was reported to produce pyomelanin from both tyrosine and phenylalanine, but was unable to use either of them as sole carbon sources (Yabuuchi and Omyama 1972). A melanogenic strain, *V. cholera* ATCC 14035, converts tyrosine, but not phenylalanine to HGA and subsequently to pyomelanin (Ruzafa et al. 1994). However *V. cholera* ATCC 14035 is unable to use either tyrosine or phenylalanine as sole carbon sources (Ruzafa et al. 1994). In contrast, the non-melanogenic strain, *V. cholera* CETC 557 is able to use tyrosine as a sole source of carbon (Ruzafa et al. 1994). Pyomelanin production is thereby due to the incomplete breakdown of tyrosine to HGA followed by excretion from the cell (Ruzafa et al. 1994).

The production of more melanin in the presence of lactate as a carbon source compared to tyrosine or phenylalanine as a sole source of carbon has not been demonstrated previously. Sanchez-Amat et al. (1998) showed that melanin production in *V. cholera* 14035 was related to the carbon source to tyrosine ratio, where increased glutamate concentration decreased melanin production. Melanin production was proposed to be a stress response during low nutrient conditions (Ruzafa et al. 1994). Since *S. algae* BrY can grow and produce melanin with tyrosine as a sole carbon source, melanin production by *S. algae* BrY may not be a result of carbon limitation.

The accelerated onset of melanogenesis at 37°C compared to 28°C in this study may be a result of lower oxygen solubility at elevated temperatures. Since the optimum growth temperature of *S. algae* is 25-35°C (Venkateswaran et al. 1999;), the early development of melanin at 37°C could not be a result of an increased growth rate.

MW analysis demonstrated a similarity between melanin produced by *S. algae* BrY and HGA-melanin produced from autooxidized HGA. These values are also consistent with previous reports of the MW of pyomelanin (Ruzafa et al. 1994; Weiner et

al. 1985), but are several orders of magnitude lower than eumelanin (Bridelli 1998)

Cultures grown with ascorbate produced HGA, but not extracellular melanin, due to the antioxidant properties of ascorbate. This demonstrates that auto-oxidation and self polymerization of HGA into extracellular melanin occurs abiotically with *S. algae* BrY, and is similar to other bacteria that produce pyomelanin (Yabuuchi and Omyama 1972; Kotob et al.1995; Weiner et al. 1985; Ruzafa et al. 1994). Furthermore, HGA was consistently identified in culture liquid, but the presence of DOPA was not verified in any of the cultures. Only one peak appeared near the DOPA retention time during these studies and was not present in the presence of ascorbate. DOPA was detected in similar studies with *S. colwelliana* (Kotob et al.1995). The presence of DOPA indicated that *S. colwelliana* used a second biochemical pathway for melanin production (Kotob et al.1995). However a knock-out of the *melA* gene, which encodes for the enzyme p-HPPH and is responsible for HGA production in *S. colwelliana,* resulted in no pigment production (Fuqua and Weiner 1993). Since no pigmentation was detected in the absence of *melA*, the potential role of DOPA in *S. colwelliana* remains unknown. HGA was detected in cultures in the present study prior to detectable melanin production and is corroborated by previous reports (Fuqua and Weiner 1993; Ruzafa et al. 1995) The genes responsible for melanin production by *S. algae* BrY and *S. oneidensis* MR-1 may therefore be constitutive, such as the *melA* gene of *S. colwelliana* (Kotob et al.1995).

Sulcotrione [2-(2- chloro- 4- methane sulfonylbenzoyl)-1,3-cyclohexanedione)] is a potent competitive inhibitor of the enzyme p-HPPH (Lee et al. 1997; Schulz et al. 1993;, Secor 1994) and has been demonstrated effective in plants (Secor 1994) and mammals (Lindbald et al.1977). Sulcotrione's inhibitory effect is specific to biochemical steps in pigment biosynthesis (Secor 1994) via inhibition of p-HPPH, but is unreactive with other related enzymatic activity (Schulz et al. 1993). Inhibition of melanin production by sulcotrione is further evidence that melanin from *S. algae* BrY arises from the p-

HPPH enzyme. These data denote that a tyrosinase is not involved here in melanin production based on (1) the inability of sulcotrione to inhibit mushroom tyrosinase, (2) the absence of DOPA in liquid culture, and (3) the inability of exogenous Cu to affect melanin production.

Enzymes such as tyrosinase and p-HPPH are controlled by exogenous concentrations of copper and iron, respectively. Therefore, the effects on melanin production by these metals were evaluated to gain a better understanding of the enzyme responsible for melanin production. The rates and degrees of melanin production were inhibited by Fe(II) concentrations in excess of 0.376 mM (Figs. 5 and 6). Iron is incorporated into the p-HPPH enzyme and the addition of Fe(II) concentrations greater than 10^{-5}M inhibited p-HPPH activity in *Pseudomonas* sp. P.J. 874 (Lindstedt et al. 1977). However, in those studies, Fe(II) only inhibited the enzyme in conjunction with ascorbate and catalase, but without ascorbate Fe(II) had no effect (Lindstedt et al. 1977). Our studies regarding metal effects on melanin production did not incorporate ascorbate. A possible scenario of enzyme inhibition in the present study may have resulted from the generation of oxygen radicals during growth with Fe(II). Oxygen radicals have been implicated in the inhibition of p-HPPH activity (Lindbald et al.1977; Fellman et al. 1972). Aerobically respiring bacteria typically produce H_2O_2. For instance, *S. putrefaciens* 200P produces 30 ìM of extracellular H_2O_2 when grown aerobically (McKinzi and DiChristina 1999). When Fe(II) and H_2O_2 react, oxygen radicals result from the reactions (McKinzi and DiChristina 1999). The culture conditions in the present study may have resulted in oxygen radical concentrations high enough to inhibit p-HPPH activity.

Another similarity to p-HPPH involves the pH optima which have been reported to be 7.5 and 7.8 for p-HPPH from *Pseudomonas* sp. P.J. 874 and human liver, respectively (Lindstedt 1977; Lindbald et al.1977). The increase of pH in the medium

during growth coincides with the appearance of melanin and is also consistent with previous reports for pyomelanin production (Ruzafa et al. 1995; Fuqua and Weiner 1993). In addition, HGA production from *V. cholera* 14035 has an optimum near pH 7 (Ruzafa et al. 1995). If the pH optimum for the p-HPPH in *S. algae* BrY and other pyomelanin producers is similar, the sudden increase in melanin production may be due to increased HGA concentration in the medium as culture pH increases. Although HGA polymerization is also pH dependent, the two phenomena could operate simultaneously.

Increased Fe(II) concentrations in the medium resulted in higher cell densities but a decrease in melanin. This may be related to decreased activity of p-HPPH resulting in less HGA excretion, thereby providing more carbon for growth.

Cu (20 ìM) did not affect melanin production in *S. algae* BrY in this study, further indicating that melanin is not produced via an copper-dependent enzyme such as tyrosinase. Previous investigators have found that exogenous Cu had no effect on the activity of the enzyme p-HPPH (Lindstedt 1977; Lindbald et al.1977). However, the production of melanin from DOPA involves the enzyme tyrosinase which is enhanced by Cu concentrations in the medium (Bell and Wheeler 1986; Swan and Griffiths 1974).

These data support the hypothesis that *S. algae* BrY and *S. oneidensis* MR-1 produce pyomelanin from the conversion of tyrosine or phenylalanine to HGA via the enzyme p-HPPH. This corroborates the previous BLAST search of the *S. oneidensis* MR-1 genome (TIGR website), where an ORF very similar to the *melA* gene of *S. colwelliana* was identified. Results from this study are also consistent with the description of pyomelanin production by other bacteria. Melanin provides the bioenergetic utility of a terminal electron acceptor and an electron shuttle to iron minerals. Both *S. algae* BrY and *S. oneidensis* MR-1 use iron oxides as terminal electron acceptors (Venkateswaran et al. 1999), and both of these organisms produce melanin. Iron oxide reduction is a common characteristic in the genus *Shewanella* (Venkateswaran et al. 1999). Pyomelanin production, similar

to that by *S. colwelliana,* may be a common characteristic in this genus and play a significant role in the physiological ecology and survival of members of this genus.

Conclusions

Several members of the in family Vibrionaceae produce melanin, including *Shewanella colwelliana* (Ruzafa et al. 1995; Weiner et al. 1985; Kotob et al.1995).. The work presented here has demonstrated melanin production by *S. algae* BrY and *S. oneidensis* which is characteristically similar to that of other members of the Vibrionaceae. Previous work on melanin production by the Vibrionaceae focused on its contribution to virulence (Bell and Wheeler 1986; Coyne and Al-Harthi 1992) and settlement of oyster larvae (Weiner et al. 1985). My study has concentrated on the electrochemical properties of melanin and its relation to bioenergetics. Because of its polyquinoid nature, melanin is well suited to function as a terminal electron acceptor (Pullman and Pullman 1961; Menter and Willis. 1997). Melanin is a type of humic acid and therefore these heteropolymers are very similar (Clapp 1990). One specific similarity is their abundance of quinone moieties and hence bioenergetic potential (Clapp 1990). Both melanin and humics serve as soluble terminal electron acceptors and electron shuttles to metals (Lovley et al. 1996; McGinness et al. 1974).

The production of melanin by DMRB potentially offers a considerable survival advantage for these organisms, especially in light of their physiological ecology. *S. algae* BrY is a facultative anaerobe with no fermentative ability (Venkateswaran et al. 1999). Its survival ability in the oxic/anoxic interface of marine sediments is due to the variety of compounds it uses as terminal electron acceptors during anaerobic respiration (Venkateswaran et al. 1999; Caccavo et al. 1992). Because potential electron acceptors are often in the form of insoluble minerals, their bioavailability to these bacteria is diminished.

Melanin may offer several survival advantages to *S. algae* BrY in its natural habitat. Fluctuations of oxygen concentration are drastic and continual in esturarine sediments. The ability to produce melanin may allow these organisms to respire in the absence or minimal availability of other electron acceptors. Melanin associated with the cell surface could also provide another survival advantage. In the presence of mineral oxides by melanin may act as an electron conduit from the bacterial surface to the mineral oxide, thereby significantly enhancing the rate of mineral reduction and hence bacterial growth.

Soluble extracellular melanin formation results in an oxidized heteropolymer that is reduced on contact with the cell. Upon reduction, melanin plays a significant role in mineral transformation by reducing metal oxides on contact. This capability may mean that DMRB contribute more to biogeochemical cycling than previously thought possible, especially in marine environments. Previously, mineral reduction by bacteria was thought possible only if bacteria were intimately associated with the minerals (Myers and Meyers 1992). Reports of reduced humics serving as soluble electron shuttles shows that mineral biotransformation does not require physical contact by bacteria (Lovley et al. 1996). Thus, mineral bioreduction may occur in areas where low pore volume makes minerals physically inaccessible to bacteria. Melanin could also function in the same capacity, however, if it is produced as needed, the degree of biogeochemical cycling may be largely a function of bacterial metabolism and ultimately, enzyme activity. Consequently the phenomenon of melanin production by DMRB could contribute significantly to mineral cycling in the environment.

Since melanin is also similar to humics in their degree of recalcitrance (Clapp 1990), melanin production could contribute to carbon sequestration in aquatic environments. However, the importance and production of melanin by *S. algae* BrY in the environment has yet to be determined. Because melanin is similar in its chemical

characteristics to humic acids and only minute quantities may be required per cell, the presence of melanin in the environment may be difficult to ascertain. The production of melanin in-situ could be inferred by in-vitro experiments incorporating marine sediments with and without inhibitors of the enzyme *p*-hydroxyphenyl pyruvate hydroxylase. If these sediments are cycled between aerobic/anaerobic conditions and monitored for Fe(III) reduction, sediments treated with the inhibitor should show a decrease in iron reduction. Prior to these studies however, a thorough understanding of the effects of the inhibitor to the physiology of microorganisms is necessary. Alternatively, radio-labeled tyrosine could be used to determine if melanin is produced in the sediment under in-vito conditions. Recovery of radio labeled melanin can be accomplished by conventional humic acid recovery procedures.

Melanin production by DMRB also offers significant utility in bioremediation. In the event its importance can be demonstrated in-situ, the rates of metal biotransformation can be increased by as much as 10 times. The economics of such an endeavor of course need to be addressed, especially since carbon sources containing tyrosine may be expensive. Considering the minute quantities of melanin (and hence tyrosine) required for a significant increase in iron mineral reduction rates, an initial expense in a relatively costly carbon source may be economically viable.

Ex-situ bioremedial technologies also offer considerable potential. With reduced melanin as an electron shuttle, it can be added to toxic metals for reduction and precipitation. Some industrial and military wastes are present in very harsh environments, such as high radiation. These environments may not be conducive for growth by *S. algae* BrY or similar bacteria. However, the addition of cell free reduced melanin may offer the reduction potential necessary for successful remedial efforts to be undertaken. Considering the metal sequestration properties of melanin, reduction and precipitation of toxic radioactive metals may be even more feasible.

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