The Role and Regulation of Melanin Production by *Shewanella oneidensis* MR-1 in Relation to Metal and Radionuclide Reduction and Immobilization

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Abstract

Bacteria in the genus *Shewanella* grow by transferring electrons to soluble and insoluble metals for energy production, thereby decreasing metal toxicity and mobility. Humic compounds are known to accelerate the process by which microorganisms transfer electrons to metals. The pigment melanin is a particularly important humic compound in this process, and is produced by *Shewanella oneidensis* MR-1. In the presence of melanin *S. oneidensis* MR-1 reduces the insoluble mineral hydrous ferric oxide (HFO) at a greater rate than without melanin. This is accomplished because, under anaerobic conditions, melanin serves as a terminal electron acceptor and soluble electron shuttle to iron minerals.

The overall hypothesis of this work is: Melanin production in the genus *Shewanella* plays a significant role as a mechanism of metal and radionuclide reduction and immobilization, and its production can be manipulated with the addition of proper nutrients. By understanding the role and regulation of melanin production in microorganisms, remediation of metal and radionuclide-contaminated environments may be accelerated. We are concentrating our studies on *S. oneidensis* MR-1, the type organism of this genus.

To date we have demonstrated the inhibition of melanin production by *S. oneidensis* MR-1 with the enzyme inhibitor sulcotrione ((2-(2- chloro- 4- methane sulfonylbenzoyl)-1,3-cyclohexanedione). Sulcotrione is a competitive inhibitor of the enzyme 4-hydroxyphenylpyruvate dioxygenase (4HPPD). This enzyme is responsible for production of homogentisic acid, the precursor to melanin production in *S. oneidensis* MR-1. Using the suicide vector system (pDS3.1) developed in our lab we generated a 700-bp deletion in the gene *melA* which encodes for 4PHPPD. The *melA* minus mutant was unable to produce melanin in the presence of tyrosine. Preliminary studies indicate that HFO reduction was not completely halted in the absence of *melA*, however the rate HFO reduction was decreased. Studies are ongoing related to the role of melanin production on iron reduction.

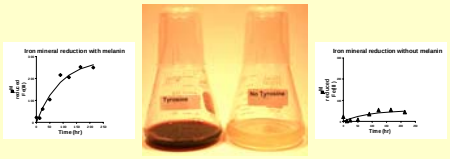

Figure 1. Melanin production in *Shewanella* cultures occurs as a function of tyrosine supplementation to a lactate basal salts medium (Turick et al. 2002. *Appl. Environ. Microbiol*. 68:2436–2444). Hydrous ferric oxide reduction rates are increased when cells contain surface associated melanin (Turick et al. 2003. *FEMS Microbiology Letters* 220:99-104).

Objectives

Determine the impact of Fe(III) oxide reduction by melanin produced by *S. oneidensis* MR-1.

- Prevent melanin production with enzyme inhibition.
- Develop a mutant deficient in melanin production by deletion of the *melA* gene.
- Develop a melanin over producer by deletion of the *HmgA* gene.
- Determine the rate and degree Fe(III) oxide reduction when melanin production is prevented and when it is over produced.
- Relate the results to previous, similar studies using minimal or nutrient rich growth media.

Conclusions

Melanin production is prevented by inhibition of 4-hydroxyphenyl pyruvate dioxygenase activity and by deletion of the *MelA* gene, which encodes for 4-hydroxyphenyl pyruvate dioxygenase.

Melanin is over produced by deletion of the *HmgA* gene, which encodes for homogentisate 1,2-dioxygenase.

Melanin production contributes to metal reduction when cells are grown in rich nutrient media.

In the absence of melanin, metal reduction s still possible, indicating the presence of other mechanisms of metal reduction.

Melanin production occurs as a result of tyrosine concentrations in nutrient rich media such as tryptic soy broth.

In previous Fe(III) oxide reduction studies with *Shewanella* that incorporated either rich nutrient media (such as tryptic soy broth) or minimal media with amino acid supplements (i.e. yeast extract or caseamino acids), melanin may have been responsible for a portion of metal reduction reported.

Inhibition Studies

Fig. 2. Tyrosine degradation pathway in *S. oneidensis* MR-1 (from the KEGG database). Melanin is produced from the partial breakdown of tyrosine to homogentisic acid. Excretion of excess homogentisic acid from the cell results in autooxidaiton and self-polymerization to melanin (specifically pyomelanin).

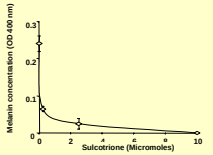

Figure 3. Inhibition of melanin production in *Shewanella* with the pHPPD enzyme inhibitor Sulcotrione.

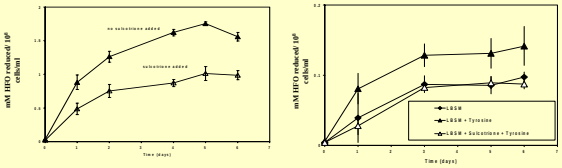

Figure 4. HFO reduction in resting cell studies. Sulcotrione inhibited the enzyme 4 hydroxylphenylpyruvate dioxegenase, resulting in no melanin production by *S. oneidensis* MR-1 when grown in tryptic soy broth (left) or lactate basal salts medium supplemented with 0.25mg/l tyrosine (right). The inhibition of melanin production decreased the rate and extent of hydrous ferric oxide reduction in resting cell studies in both cases.

Mutagenesis studies

Gene organization of the melA locus on the S. oneidensis MR-1 chromosome.

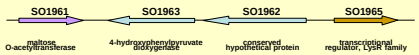

Figure 5 . Analysis of the whole-genome sequence of *S. oneidensis* MR-1 indicated that the putative *melA* gene (SO1963) is located 120 base pairs downstream of an ORF encoding a conserved hypothetical protein (SO1962) (Figure 2). Although no potential *rho* -independent transcriptional terminator sequences are found downstream of SO1962, an operon organization of SO1962 and SO1963 is still to be determined.

Mutagenesis studies (cont.)

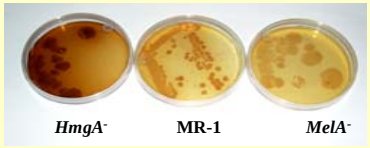

Figure 6. Deletion mutagenesis . Following deletion mutagenesis studies, the *MelA* ⁻ phenotype was deficient of melanin production (right) while the *HmgA* phenotype over produces melanin (left), compared to MR-1 (middle). Cultures shown here were grown on lactate basal salts medium with 2g/l tyrosine.

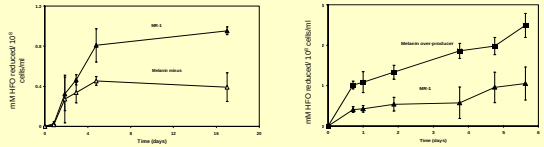

Figure 7. HFO reduction by resting cells. Resting cell studies of cells pregrown in TSB (24h) demonstrated decreased HFO reduction efficiency by the melanin minus strain (*melA* ⁻) relative to MR -1 (left). An increased rate of HFO reduction occurred with the melanin over producing strain (*hmgA* ⁻) (right).

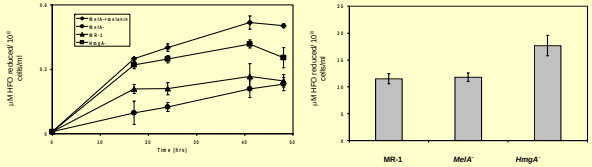

Figure 8. The role of melanin on HFO reduction. Resting cell studies (left) with cells pregrown for 48 hrs. in LBSM+250mg/l tyrosine demonstrated increased HFO reduction efficiency as a function of melanin production or melanin supplementation. Cells pregrown for 10 days in LBSM with no supplemental tyrosine (right). The melanin overproducing mutant (*HmgA*⁻) reduced HFO to a greater extent than the wild type and the melanin minus mutant (*MelA*⁻) after 24 hrs. This suggests that tyrosine is scavenged proceeding cell death during stationary phase growth.

Supplemental data

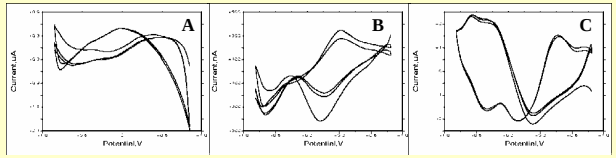

Figure 9. Cyclic voltammetry of (A) the melanin minus mutant (*MelA*⁻), (B) wild-type (MR-1) and (C) the melanin overproducing mutant (*HmgA*⁻). Voltammograms of 5 day old biofilms grown on glassy carbon working electrodes demonstrated enhanced redox activity on the bacterial surface as a function of melanin content.