Isolation and Characterization of hmgA Mutants of *Pseudomonas uticensis*: Evidence of a Tyrosine Transport Deficiency

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ABSTRACT

Pseudomonas uticensis is a novel bacterium originally isolated in our laboratory from the cutaneous microbial flora of the red-backed salamander, *Plethodon cinereus*. *P. uticensis* was isolated on the basis of its ability to produce and secrete potent antifungal compounds, which have been shown to inhibit hyphal growth of *Neurospora crassa*, as well as growth, hyphal development and biofilm formation in *Candida albicans*. One of the novel characteristics of *P. uticensis* is the brown pigmentation of bacterial cells when grown on nitrogen-enriched media, such as tryptic soy- yeast extract (TSYE) agar. The brown pigment has been determined to be pyomelanin (PM), and is only produced when tyrosine is abundant in the media. Furthermore, PM production in *P. uticensis* increases with exposure to visible light and under conditions of higher cell density. Despite the evidence that PM synthesis is regulated, the biological role of the pigment in this bacterial isolate remains unclear. We used transposon-mediated mutagenesis to isolate 3 PM-overproducing mutants of *P. uticensis*. The 3 mutant isolates are designated as MM7, MM8, and MM9. Semi- random two-step PCR was used to amplify regions of genomic DNA flanking the transposon within these 3 mutant isolates. Genetic analysis indicated that all 3 mutants have homology to the 5` end of the hmgA gene, encoding homogentisate 1,2-dioxygenase (HDO). HDO is responsible for the third enzymatic step in tyrosine catabolism leading to PM synthesis. Using SDS-PAGE analysis, secreted PM in mutants has been estimated to have a molecular weight averaging 45 kDa compared to intracellular PM with an average of 60 kDa. In preparation for pulse-chase studies with [14C]-tyrosine, tyrosine uptake in the mutant and wild-type strains was examined. The hmgA mutants have reduced rates of tyrosine uptake, exhibiting greater than 70% inhibition. Kinetic analysis of MM9 reveals an increase in Km and a decrease in Vmax compared to the wild-type. Our results indicate that a transport defect appears to be tyrosine- specific due to the fact that no inhibition was evident in N-acetylglucosamine uptake. PM-overproducing mutants have interesting biological properties, and will be useful for further study of regulation of PM synthesis in *P. uticensis*.

BACKGROUND

Microorganisms can be found in a variety of environments – in soil, in the guts of animals, on inanimate objects, or on mountaintops. They have evolved to produce unique survival mechanisms in the event of environmental change. The survival mechanism of interest in this study is the production of melanin. Melanin production and color differ among organisms, but in general, it is considered to be "[a substance of dark color], insoluble in aqueous or organic fluids, resistant to concentrated acid and susceptible to bleaching by oxidizing agents" (Nosanchuk and Casadevall, 2003). There are two types of melanin that are produced by bacteria – pyorubrin, which consists of a red-brown hue, and pyomelanin, which features more of a light-brown hue. *Pseudomonas* species produce pyomelanin in order to protect the cell against damage from ultra-violet (UV) light (Ogunnariwo and Hamilton-Miller, 1975; Nosanchuk and Casadevall, 2003).

In 2003, Van Kessel, Scanlon and Aaronson isolated the bacterial strain UC17F4 from the cutaneous microbial flora of female red-backed salamanders. This bacterial strain has the ability to produce potent antifungal compounds. Biochemical and DNA sequence analysis aided in discovering that UC17F4 is a species of *Pseudomonas*, but identification of UC17F4 had not been successful since sequence analysis of two signature sequences, rpoD and gyrB showed no more than 88% sequence identity with its closest neighbors in the GenBank database (Butler and Aaronson, 2006). Subsequent analysis of additional genes in this strain confirm that *Pseudomonas* sp. UC17F4 is most likely a novel species, which we have tentatively named *Pseudomonas uticensis*.

One of the distinctive characteristics of *P. uticensis* is the chocolate brown pigmentation of cells when grown in tyrosine-enriched media, which we hypothesize to be eumelanin. Pyomelanin, which is secreted from bacteria, is not uncommon in Pseudomonads; Ogunnariwo and Hamilton-Miller (1974) were able to isolate three strains of *Pseudomonas aeruginosa* that produce the brown pigment. Intracellular eumelanin, however, is rare in bacteria. Studies in our lab have shown that the organism turns on melanin production when exposed to light; cultures incubated in the dark have reduced pigmentation (Kracke and Aaronson, 2011). Melanin production is also sensitive to the intensity of light and the duration of exposure (Benzing et al., 2012). These observations suggest that melanin production in *P. uticensis* is photoregulated. We have also observed density-dependent production of melanin in this organism (Seifert et al., 2013).

In order to study the regulation and biochemistry of melanin production in *P. uticensis*, we used transposon-mediated mutagenesis (Larson et al., 2002) to isolate mutant strains of the bacterium that are altered in melanin production. In the present study, we report the isolation and initial characterization of three mutant strains of *P. uticensis* that over-produce the secreted pigment, pyomelanin. These mutant strains all have transposon insertions in the hmgA gene, which encodes homogentisate-1,2-diooxygenase (HDO), resulting in the the accumulation and secretion of homogentisic acid which oxidizes and polymerizes to form the low moleculer weight pyomelanin pigment. One of the interesting phenotypes of these mutants is a defect in tyrosine transport.

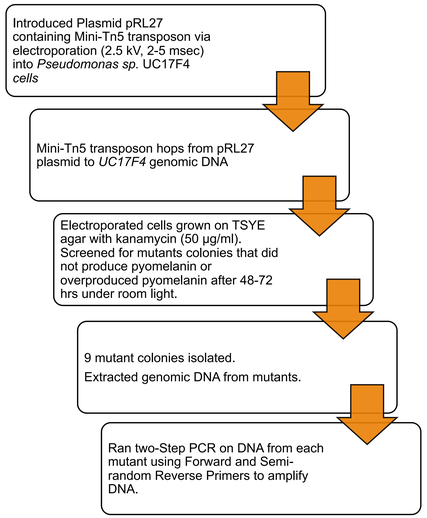


Fig. 1. Schematic of transposon mutagenesis and isolation of pyomelanin-defective and pyomelanin-overproducing mutants of *Pseudomonas* sp. UC17F4.

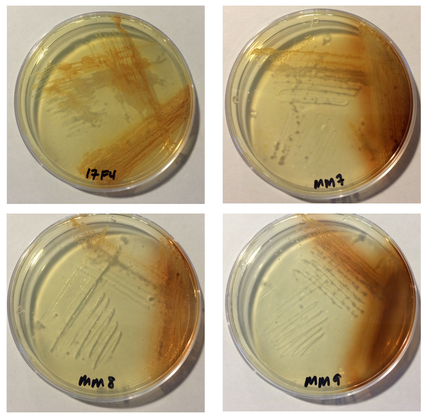


Fig. 2. Production of melanins in wild-type and mutant strains of *Pseudomonas* sp. UC17F4. Bacteria were streaked on plates of TSYE agar, and incubated under constant illumination at 30ºC for 48 hours. Melanin in the wild-type strain is contained exclusively in the bacterial cells; pyomelanin secretion is evident by the diffuse brown pigmentation of the agar.

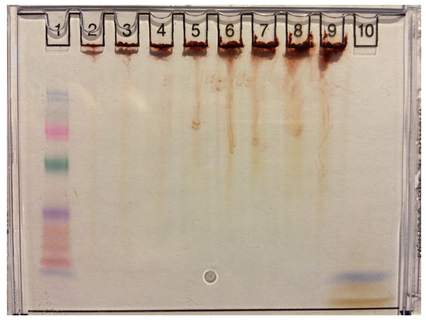
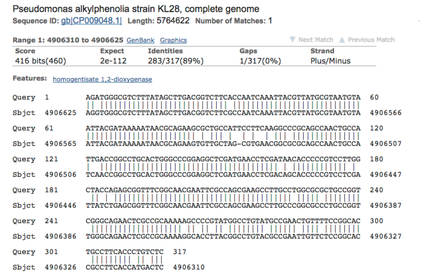


Fig 3. *P. uticensis* produces two different forms of melanin. Bacterial biofilms were scraped from TSYE plates into TE buffer. Cells were washed and resuspended in a small volume of 15% glycerol, and different volumes of the packed cells were loaded into wells of a 4-15% SDS-PAGE gel. Lanes 2-9 were loaded with 2, 4, 6, 8, 10, 12, 14 and 16 µl of cell suspension, respectively. Lane 10 was loaded with 20 µl of supernatant from MM mutants. Cell associated melanin was high molecular weight, consistent with eumelanin, while secreted pyomelanin had a molecular mass of 4-6 kDa.


Pseudomonas alkylphenolia strain KL28, complete genome
Sequence ID: gb|CP009048.1| Length: 5764622 Number of Matches: 1

Range 1: 4906310 to 4906625 GenBank Graphics

Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
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Features: homogentisate 1,2-dioxygenase

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Fig. 4. Pyomelanin over-producing mutants have a transposon insertion in the hmgA gene, encoding homogentisate-1,2-dioxygenase. Semi-random two-step PCR was performed to amplify sequences flanking the transposon using nested primers (ref.). BLAST analysis shows homology to the hmgA gene sequence of numerous *Pseudomonas* species, although 89% sequence identity is the best we obtained.

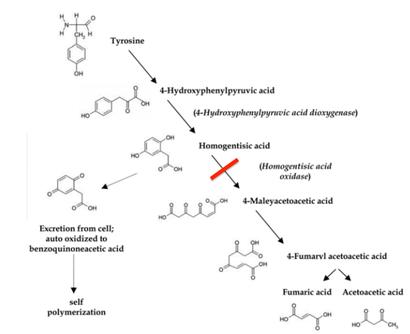


Fig 5. Schematic of pyomelanin synthesis. Pyomelanin is derived by enzymatic oxidation of tyrosine to homogentisic acid, which accumulates due to the defect in HDO (red bar).

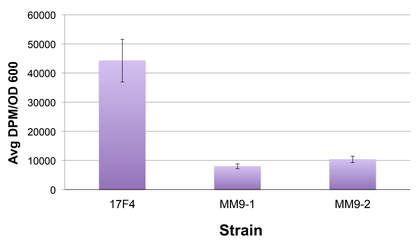
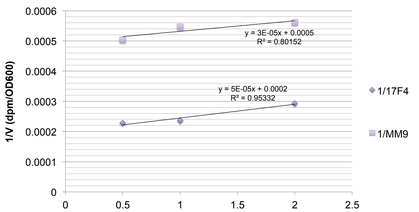


Fig 6. Pyomelanin overproducing strains exhibit a defect in tyrosine transport. Wild-type *P. uticensis* and two different cultures of the MM9 mutant were incubated with [^{14}C]-L-tyrosine in minimal medium for 5 min. Cells were harvested and washed by filtration of 0.45 µm nitrocellulose filters, and then analyzed for tyrosine uptake by liquid scintillation analysis. Mutant strains repeatedly exhibit a 70% reduction in the rate of tyrosine transport compared to mutant strains.



Strain	Vmax (dpm/ OD600)	Km (µM)
17F4	50000	0.25
MM9	20000	0.06

Fig. 7. Kinetic analysis of tyrosine transport revelas alterations in both K_M and V_{max} . Cultures of MM9 and *P. uticensis* were incubated for 5 min. with varying concentrations of [14C]-tyrosine. Cells were harvested, washed and analyzed as described above. MM9 exhibits a decrease in both K_M and V_{max} as compared to the wild-type strain, and appears to be a case of uncompetitive inhibition.

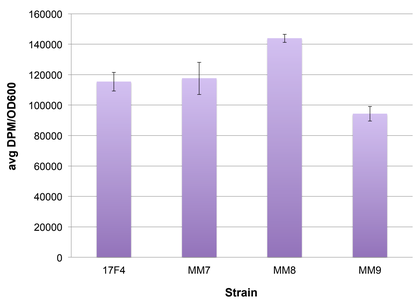


Fig. 8. The transport defect in pyomelanin-overproducing mutants appears to be tyrosine-specific. Analysis of N-acetyl-[3H]-glucosamine uptake was performed with wild-type and mutant strains of *P. uticensis*. No significant difference in transport rates was observed among the strains, suggesting that the tyrosine transport deficiency is not due to a generalized membrane transport defect.

CONCLUSION

Pseudomonas uticensis is a novel melanogenic bacterial species. Wild-type cells produce high molecular weight eumelanin, but may also secrete a much lower molecular weight form of pyomelanin. Pyomelanin overproducing mutant strains have been isolated by transposon mediated mutagenesis, and are defective in the hmgA gene, which encodes the enzyme homogentisate-1,2-dioxygenase. Mutant strains have several interesting phenotypic characteristics, including a defect in the rate of tyrosine transport. Other phenotypic characteristics are characterized in our companion poster (Lawrence and Aaronson, 2015)

LITERATURE CITED

Benzing, S.L., Cotrupe, C.C.,and Aaronson, L.R. 2012. Light effects on pyomelanin production in a novel Pseudomonas species. *Abstracts of the 113th Gen. Mtg. of the Am. Soc. for Microbiol.*

Butler, A.M. and Aaronson, L.R. 2006. Identification of two unknown antifungal *Pseudomonas* species and their effect on *Neurospora crassa* hyphal morphology. *Abstracts of the 106th Gen. Mtg. Of the Am. Soc. for Microbiol.*

Das, S., Noe, J.C., Paik, S. and Kitten, T. 2005. An improved arbitrary primed PCR method for rapid characterization of transposon insertion sites. *J. Microbiol. Meth.* 63: 89–94

Kracke, K.M., and Aaronson, L. R.. 2011. Photoinduction of pyomelanin production in a novel *Pseudomonas* species. *Abstracts of the 111th Gen. Mtg. of the Am. Soc. for Microbiol.*

Larson, R.A., Wilson, M.M, .Guss, A.M, and Metcalf, W.W. 2002. Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria *Arch. Microbiol.* 178 :193–201.

Lawrence, P.L. and Aaronson, L.R. 2015. Characterization of Pyomelanin-Overproducing Mutants of *Pseudomonas uticensis*, a Novel Melanogenic Bacterial Species. . *Abstracts of the 115th Gen. Mtg. of the Am. Soc. For Microbiol.*

Liu, G.Y. and V. Nizet. 2009. Color me bad: microbial pigments as virulence factors. *Trends in Microbiol.* 17: 406-413.

Nosanchuk, J. D., and A. Casadevall. 2003. The contribution of melanin to microbial pathogenesis. *Cell. Microbiol.* 5: 203-223.

Ogunnariwo, J., and J. M. T. Hamilton-Miller. 1975. Brown- and red-pigmented *Pseudomonas aeruginosa*: Differentiation between melanin and pyorubrin. *J. Med. Microbiol.* 8: 199-203.

Plonka, P.M. and M. Grabacka. 2006. Melanin synthesis in microorganisms — biotechnological and medical aspects. *Acta Biochim. Pol.* 53: 429–443

Seifert S,L,, K.and L.R. Aaronson. 2013. Pyomelanin production in a novel *Pseudomonas* species is cell density-dependent. *Abstracts of the 113th Gen. Mtg. of the Am. Soc. for Microbiol..*

Van Kessel, J.C., Scanlon, T.L., and Aaronson, L.R. 2003. Identification of the cutaneous antifungal microbial flora of the red-backed salamander, *Plethodon cinereus*. *Abstracts of the 103rd Gen. Mtg. Of the Am. Soc. for Microbiol. pg. 435.*

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