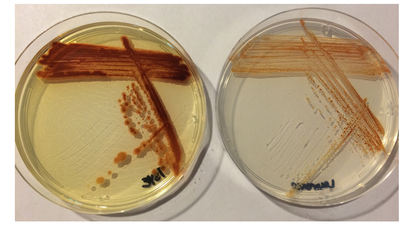
Melanogenesis in a Novel *Pseudomonas* Species: Evidence of Two Pathways

Pamela L. Lawrence*, Viktoria Yudchits and Lawrence R. Aaronson
Biology Department, Utica College, Utica, NY



ABSTRACT

Pseudomonas sp. UC17F4 is a novel species isolated from the skin of red-backed salamanders for its potent antifungal activity. A hallmark of UC17F4 is its rich brown color on nutrient-rich media. The pigment, which is tyrosine-dependent has been identified as melanin. Melanogenesis is also induced by visible light. Under certain conditions, UC17F4 produces only high MW intracellular melanin, while under other conditions it also secretes low MW melanin. We hypothesize that the intracellular form is eumelanin, which is produced through a tyrosinase- dependent pathway, while the secreted form is pyomelanin, which is not tyrosinase dependent. When UC17F4 was grown in minimal media with various carbon sources, only succinate and citrate allowed the bacteria to grow. Citrate titration studies showed that both pigment production and growth were optimal with 1% citrate. Titration of L-tyrosine and Fe3+ showed that optimal melanin production occurred at Fe3+ concentration > 100 µM and tyrosine concentrations of 0.1%. To ensure that exogenous tyrosine did not produce an artificial response in melanin production, cells were grown with 0%, 0.05%, and 0.1% tyrosine. Cultures grown with exogenous tyrosine produced more pyomelanin than those cultured without, but no difference in eumelanin production was seen. Cultures of the bacterium were grown in standing cultures to promote biofilm formation and in shaking planktonic culture conditions. Eumelanin content was 80% lower in planktonic conditions and pyomelanin was about 60% less in comparison to standing cultures, suggesting that bacteria in biofilms are more melanogenic. Density dependence was also seen when cells were grown with inocula ranging from 107 cells/ml down to 102 cells/ml. Cultures from lower inoculum concentrations produced less pyomelanin and eumelanin, affirming that quorum signaling may regulate melanogenesis. To determine if intracellular melanin is produced by a tyrosinase, cultures were grown with p-coumaric acid (pCA) and oxyresveratrol (Oxy), known inhibitors of tyrosinase. Oxy inhibited melanogenesis, while pCA showed only slight inhibition. UC17F4 DNA was probed for homologues of a tyrosinase gene using PCR with primers derived from sequences of tyrosinase, tyrosinase melC2, laccase and hmgA. Laccase and hmgA primers generated amplicons, tyrosinase primers produced a weak signal. This suggests that a novel tyrosinase may be involved in melanogenesis in *Pseudomonas* sp. UC17F4.

BACKGROUND

In 2002, two students in our microbiology lab at Utica College isolated a novel bacterial strain from red-backed salamanders (*Plethodon cinereus*) based on its potent antifungal activity. The novel bacterium is a nitrate reducer and a facultative anaerobe (11). The organism does not utilize glucose or other carbohydrates as a carbon source, but can grow efficiently using citrate or succinate. It is capable of peptonizing proteins as a carbon source, secreting proteases and alkalinizing the medium. This bacterium is also β-hemolytic. Thorough biochemical analysis of the organism failed to yield an identification, but 16s rDNA sequence analysis suggested it to be a species of *Pseudomonas,* with the closest phylogenetic matches being *Pseudomonas putida, P. asplenii, and P. fuscovaginae* anaerobe (11). Using PCR primers to amplify the gyrB and rpoD genes (commonly used in *Pseudomonas* phylogeny), sequence analysis of the amplicons indicated only an 85%-88% sequence identity to homologous sequences from *P. putida and P. fluorescens* (3). This suggested that there was a strong possibility that *Pseudomonas* sp. UC17F4 was a previously undescribed species of *Pseudomonas.* We have unofficially named this putative species, *Pseudomonas uticensis.* One of the most interesting characteristics of *UC17F4* is its ability to produce the brown pigment, melanin (2, 5, 9, 11). Originally we suspected that our bacterium produced just pyomelanin, a low molecular weight derivative of tyrosine, that is oxidized through the homogentisic acid pathway. Further investigation revealed that the bacterium produced two different forms of melanin: low molecular weight (<4 kDa) extracellular pyomelanin and high molecular weight (>30 kDa) intracellular eumelanin as well. Eumelanin is the main type of melanin in mammalian skin, and few bacterial species are known to have intracellular melanin (8). The few species that are able to produce eumelanin, use tyrosinase or a tyrosinase-like enzyme, that is secreted to make eumelanin. We further discovered that tyrosine is a requisite substrate for for the production of either form of melanin. Eumelanin synthesis appears to be regulated by light and cell density, suggesting a complex regulatory mechanism in the bacterium (9, 11). In the present study, we further explore the biosynthesis of intracellular melanin in *Pseudomonas* sp. UC17F4 and provide evidence that the pigment is produced through a tyrosinase-dependent pathway.

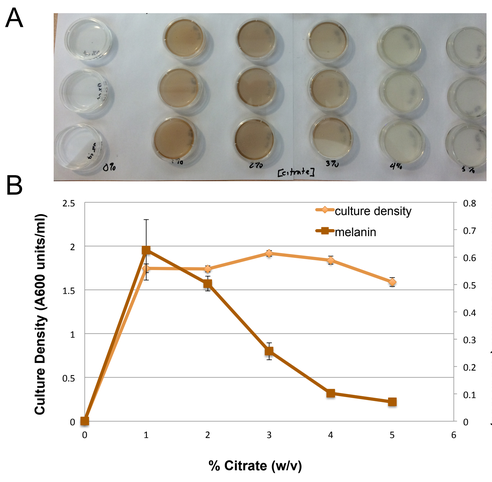


Figure 1. A citrate concentration of 1% is needed for optimal growth and melanin production. Cells were grown in liquid culture with a defined medium, using 10µM $FeCl_3$ and increasing amounts of citrate. Cultures were inoculated at 1x10^{7}cells/mL, grown at 30°C in an illuminated incubator, at 450 lux, for 20hrs. Cell density was measured spectrophotometrically at 600 nm. Cell pellets were then lysed with 1% SDS, and melanin content was determined by UV absorbance at 335nm. **A**. Photograph of cultures after 20 hr incubation. **B**. Concentration dependence of cell density and cellular melanin content.

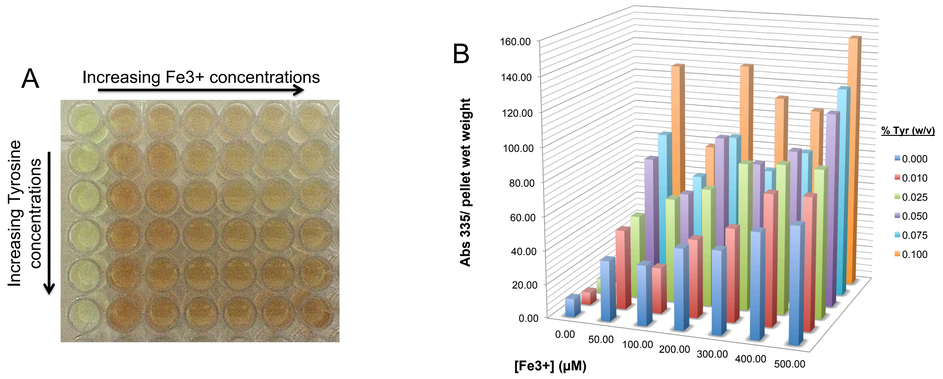


Figure 2A and 2B. Tyrosine- and Fe^{3+}- dependence of melanin production in UC17F4. Cells were grown in liquid culture with a defined medium, using 1% sodium citrate, with increasing concentrations of tyrosine and $FeCl_3$. Incubation and cell lysis were performed as previously described. Optimal concentrations for melanin production were 1 mM tyrosine and 100 µM Fe^{3+}. Based on these findings, Lawrence Minimal Media was developed for future use. Lawrence minimal media is a modified Vogel's minimal medium, containing Fe-free trace elements, 1% sodium citrate, and 100µM $FeCl_3$. Iron concentration is consistent with the iron content of the soil where bacterium were isolated.

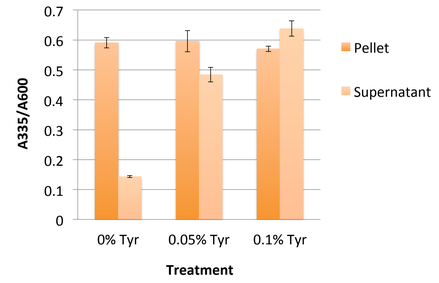


Figure 3. Higher tyrosine concentrations increase extracellular pyomelanin production, but do not effect intracellular melanin production. Cells were grown in Lawrence minimal media with increasing amounts of tyrosine. Cells were incubated, lysed and analyzed for melanin content as previously described.

Pyomelanin and….

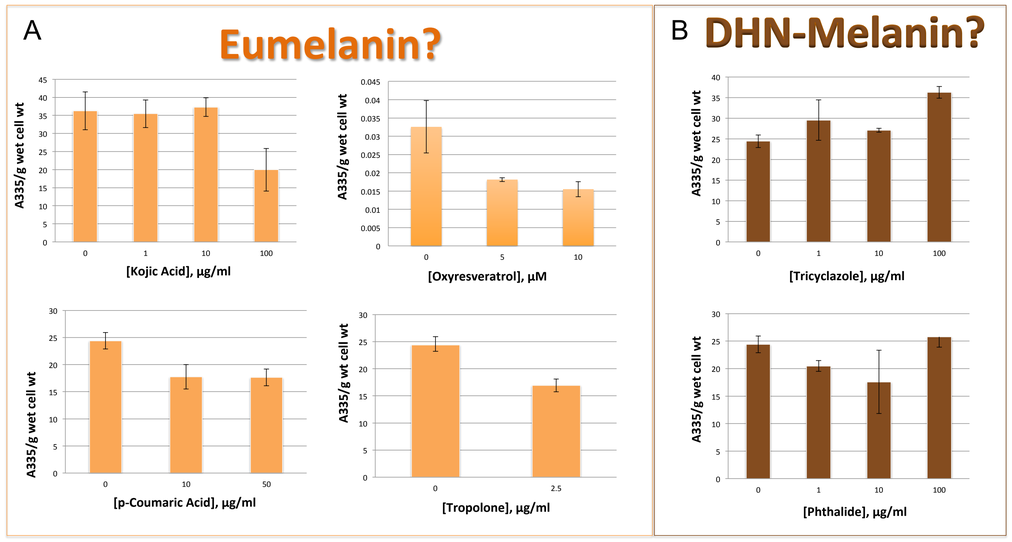


Figure 4A. Increasing concentrations of eumelanin synthesis inhibitors produced a decrease in intracellular melanin production. Cells were grown in Lawrence minimal media with tyrosinase inhibitors kojic acid (7), oxresveratriol (10) in DMSO, *p*-coumaric acid (1) in ethanol, and tropolone (7) in ethanol. Controls were made with each vehicle used in this experiment. Concentrations of each did not exceed 0.1% (v/v) in final solution. Cells were incubated, lysed and analyzed for melanin content as previously described. Results indicate that all of the tyrosinase inhibitors result in decreased intracellular melanin content, suggesting that the pigment is eumelanin.

Figure 4B. Increasing concentrations of DHN-melanin synthesis inhibitors produced no decrease in intracellular melanin production. Cells were grown in Lawrence minimal media with DHN-melanin synthesis inhibitors tricyclazole and phthalide (7) in ethanol. Controls were made with each vehicle used in this experiment. Concentrations of each did not exceed 0.1% in final solution. . Cells were incubated, lysed and analyzed for melanin content as previously described. Results show that neither inhibitor produced significant reductions in intracellular melanin, suggesting that this pigment is not DHN-melanin.

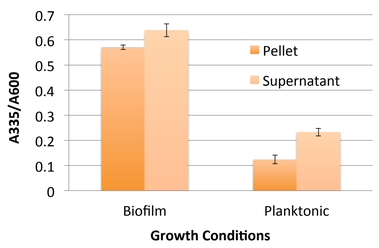


Figure 5. Biofilm heavy cultures, grown in standing plates, have five-times higher intracellular melanin content than those grown planktonic culture. Cells were grown in Lawrence minimal media. Standing cultures were incubated as previously described. Planktonic cells were grown in 30°C shaker bath, at 225 rpm with illumination over night. Cells were incubated, lysed and analyzed for melanin content as previously described. Cells in biofilms produced higher levels of both intracellular eumelanin and extracellular pyomelanin.

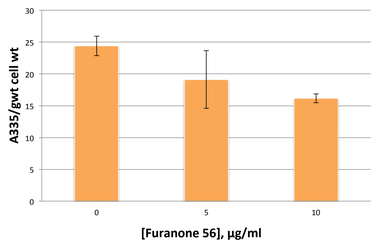


Figure 6. UC17F4 showed a decrease in production of intracellular melanin when treated with Furanone 56, a known biofilm / *las* inhibitor in *P. aueruginosa* (4). Cells were grown in Lawrence minimal media, with increasing concentrations of Furanone 56 in ethanol final concentrations of [0-10µg/mL], with the vehicle not exceeding 0.1% of final volume. Cells were incubated, lysed and analyzed for melanin content as previously described. Increasing concentrations of the inhibitor results in lower melanin content.

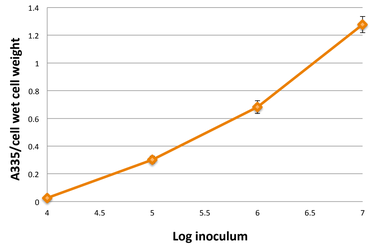
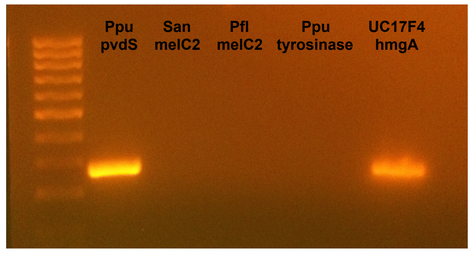


Figure 7. Inoculation density affects melanin production in UC17F4. Cells were inoculated in Lawrence minimal media at 1x10^4-1x10^7 cells/mL inoculation density. Incubation, lysis and analysis for melanin content were preformed as previously described. Denser cultures produce higher levels of intracellular melanin, suggesting a quorum-signaling phenomenon.



Gene	Primer sequences
Pseudomonas putida pvdS	F: ATGGCGGAACAACTATCCAC R: ATGGCGGAACAACTATCCAC
Streptomyces antibioticus melC2	F: CGTCGAACTGCATGTGATGA R: GCTCGAACTCCAGCGAAAT
Pseudomonas fluorescens melC2	F: GGCATCGTGAAATGTTGTTG R: CGAGTTGACCAAACGTGAGA
Pseudomonas putida tyrosinase	F: CCACCTGTTGCTGGAAAATC R: TTCCCT A TTGGGACTGGA TG
Pseudomonas sp. UC17F4 hmgA	F: AGTTCGGCATACAGGCCATA R: TGACGGTCTTCACCAATCAA

Figure 8. PCR analysis of UC17F4 DNA with primers for bacterial tyrosinase genes reveals no homologues. PCR was performed using primer sets listed above. PvdS (pyoverdin production) and hmgA (homogentisate-1,2-diooxygenase) were used as positive controls. PCR products were run on a 1.2% agarose gel at 120V. No bands were seen for all three possible tyrosinase/melanin genes. This data leaves the possibility of a new non-homolgous gene in our previously undescribed bacterial species.

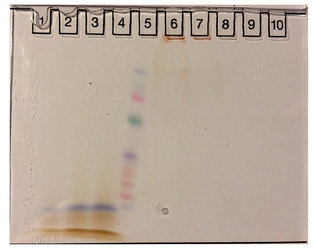


Figure 9. Evidence of low MW pyomelanin and high MW eumelanin produced by UC17F4. Extracts of supernatants (lanes 2-4) and cell pellets (lanes 6-8) run on a 4-15% SDS-PAGE gel. Low MW pyomelanin is evident only in culture supernatants, while high MW eumelanin is evident in cell pellets.

Conclusions

- Higher concentrations of tyrosine increases pyomelanin production; eumelanin can be synthesized from endogenous sources in a minimal medium
- Inhibitors of eumelanin synthesis reduce the melanin content in cells
- DHN-melanin inhibitors produce no significant effect on intracellular melanin production.
- Cells grown in biofilm-inducing cultures produce more intracellular and extracellular melanin.
- Furanone 56-treated cells show a decrease in production of intracellular melanin, suggesting possible quorum signaling regulation.
- Increasing cell density results in higher cellular melanin content
- No genes homologous to known bacterial tyrosinase genes have been identified, suggesting the possibility of a novel tyrosinase.

LITERATURE CITED

1. An, S.M., J.-S. Koh and Y.C. Boo. 2010. *p*-Coumaric acid not only inhibits human tyrosinase activity *in vitro* but also melanogenesis in cells exposed to UVB. *Phytother. Res.* 24: 1175–1180.

2. 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.*

3. 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.*

4. Hentzer. M., K. Riedel, T.B. Rasmussen, A. Heydorn, J.B. Andersen, M.R. Parsek, S.A. Rice, L. Eberl, S. Molin, N. Høiby, S. Kjelleberg and M. Givskov. 2002. Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology* 148: 87–102.

5. 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.*

6. 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.

7. Pal, A.K., D.U. Gajjar and A.R. Vasavada. 2014. DOPA and DHN pathways orchestrate melanin synthesis in *Aspergillus* species. *Med. Mycol.* 52:10-18.

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

9 Seifert S,L,, K. Shikula.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.*

10. Shin, N.-H., S.Y. Ryu, E.J. Choi, S.-H. Kang, I.-M. Chang, K.R. Min, and Y. Kim. 1998. Oxyresveratrol as the potent inhibitor on dopa oxidase activity of mushroom tyrosinase. *Biochem. Biophys. Res. Comm.* 243: 801–803

11. 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. 435.3.*

ACKNOWLEDGEMENTS

This work was supported in part by the Harold T. Clark, Jr. Endowed Professorship and a grant from Donald and Sally Majka to L.R.A. Presentation of this poster was supported by a Utica College Student Travel Grant to P.L.L. and V.Y.

View this poster at www.aaronsonmicrobiology.com