# The Effects of Cell Density and Biofilm Formation on Melanin Production in a Novel *Pseudomonas* Species

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## ABSTRACT

*Pseudomonas* sp. UC17F4 is a novel bacterial species that was isolated from the cutaneous flora of red-backed salamanders on the basis of its antifungal activity. One of the most interesting characteristics of this species is its ability to produce two different forms of the brown pigment, melanin. Eumelanin is a high MW molecule that is contained within the cytoplasm of the bacterial cell, while pyomelanin is a low MW secreted form. Studies in our lab have shown that melanin production in this bacterium is regulated by light exposure, and by the availability of iron and suitable carbon sources. We have recently observed that melanogenesis is dependent on high cell density and the development of biofilms. Cultures of UC17F4 were grown in either tryptic soy-yeast extract (TSYE) broth or in citrate-enriched Lawrence Minimal Medium (LMM). Eumelanin content was assayed by spectrophotometric analysis of cell lysates in 1% SDS. As culture densities increased, so did cellular melanin content, increasing 6-fold from the onset of stationary phase. Cultures inoculated at higher cell density also exhibited higher melanin content. Biofilm-forming cultures produced 5-fold higher levels of eumelanin than did planktonic broth cultures. Biofilm cultures were grown in 35 mm plates in both TSYE and LMM broth, and exhibited density-dependent melanin content. Similar results were observed in biofilms formed on nitrocellulose filters placed on LMM and TSYE agar plates. To further study the dependence of UC17F4 biofilm formation of melanogenesis, NaCl was added to cultures to inhibit the development of biofilms. Bacterial eumelanin content in the biofilm mass declined by 90% as NaCl concentration increased to 1% (w/v) compared to untreated controls. Treatment of cultures with Furanone 56, an inhibitor of quorum signaling in Pseudomonads, resulted in a 30% reduction in eumelanin content. The quorum signaling compound C12-homoserine lactone stimulates only a modest increase in melanogenesis. Supplementation of early exponential-phase cultures with cleared supernatants from 48 hr biofilm cultures resulted in up to a 2-fold increase in cellular melanin content. These data strongly suggest that melanogenesis in *Pseudomonas* sp. UC17F4 is under the control of one or more signaling compounds. Ongoing studies are being conducted to isolate and characterize melanogenic signal compounds, and identify signaling and regulatory pathways in the bacterium.


## BACKGROUND

*Pseudomonas* sp. UC17F4 is a novel melanogenic species isolated from red backed salamanders for its antifungal properties (van Kessel *et al.*2003). Biochemical and DNA sequence analysis strongly suggests that this isolate is a type culture of a previously undescribed species, for which the name *Psuedomonas uticensis* has been proposed (Lawrence *et al.*, manuscript in preparation). *P. uticensis* produces two different forms of melanin; high molecular weight eumelanin, and low molecular weight pyomelanin (McHarris *et al.*, 2015). Recent studies in our laboratory have revealed that melanin production in this organism is dependent on culture density and biofilm production. This suggests that pigment production may be controlled by quorum signaling pathways. In the present study, we provide preliminary evidence that at least one quorum signaling pathway may be involved in regulation of melanogenesis.

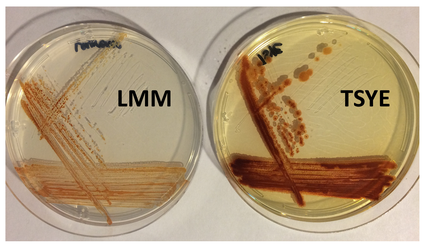


**Fig 1. Pigment production in *Pseudomonas uticensis* grown on minimal and enriched media.** Bacteria were streaked on agar plates containing Lawrence Minimal Medium (LMM, left) or TSYE (right) and at 30° C for 48 hrs. LMM is a modified form of Vogel's medium N (Vogel, 1956) containing 1% citrate (w/v) as the sole carbon source and 100 µM $FeCl_3$.

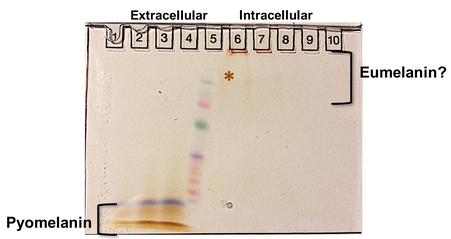


**Fig 2. Two forms of melanin produced by *P. uticensis*.** Supernatants from TSYE broth cultures were collected, and bacterial cell pellets were precipitated with HCl, neutralized with NaOH, washed with ethanol and resuspended in $dH_2O$. Supernatants and precipitates were loaded on a 4-20% polyacrylamide gel and electrophoresed using a Tris/glycine/SDS running buffer. Low MW melanin (<3000 Da) was observed in supernatants, while high MW melanin (>100,000 Da) was observed in cell pellets.

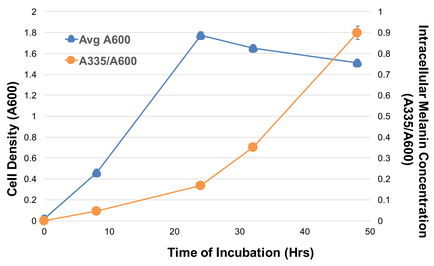


**Fig 3. Intracellular melanin content increases with culture density over time.** *P. uticensis* was inoculated into TSYE broth at a starting density of 0.02 $OD_{600}$U/ml. Samples were collected at various intervals. Cell density was measured spectrophometrically at 600 nm. Cell pellets were lysed in 1% SDS at 100°C for 5 min, and melanin content was measured at 335 nm. Intracellular melanin concentration was expressed as A335 nm/A600 nm. Intracellular melanin content increases dramatically upon the culture reaching stationary phase.

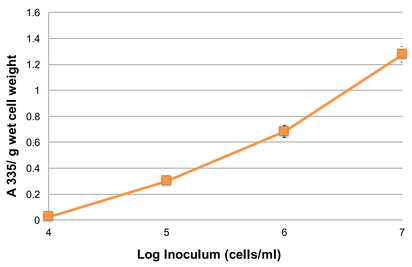


**Fig 4. Melanin content is increased in cultures with a higher starting inoculum.** *P. uticensis* was inoculated into TSYE broth at varying concentrations, and incubated at 30°C for 24 hrs. Cells were pelleted by centrifugation in preweighed microcentrifuge tubes; supernatants were removed and wet cell pellet masses were determined. Pellets were lysed in SDS and lysates were analyzed at 335 nm, as described above. Melanin content increased proportionately with increasing inoculum density.

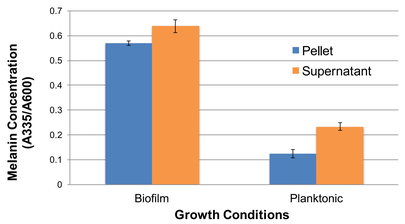


**Fig 5. *Pseudomonas uticensis* produces higher concentrations of melanin when grown as biofilms than in planktonic cultures.** Bacteria were inoculated into TSYE broth, and cultivated for 24 hrs at 30°C in 35mm petri plates or in culture tubes in a water bath shaking at 200 rpm. Cell density was measured at 600 nm, and then cultures were centrifuged. Melanin content in supernatants and in cell lysates was measured at 335 nm. Intracellular melanin content is approx. 5-fold higher in biofilm cultures than in planktonic cells. Extracellular pyomelanin is approx. 3 times greater under biofilm growth conditions.

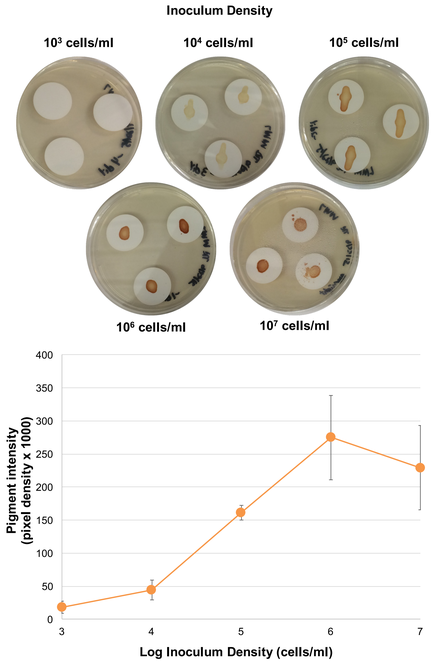


**Fig 6. Melanin content is increased in biofilms with a higher starting inoculum.** An overnight culture of *P. uticensis* was diluted to a concentration of 1 x $10^7$ cells/ml, and then 10-fold serial dilutions were made. 50 µl aliquots of the dilutions were inoculated onto nitrocellulose filters with a 0.45 µm pore size on TSYE agar plates, and incubated at 30°C for 48 hrs. Plates were photographed under identical lighting conditions, converted to grayscale, inverted in color, and pigment intensity was determined using ImageJ software. Biofilm melanin content increased proportionately with increasing inoculum density.

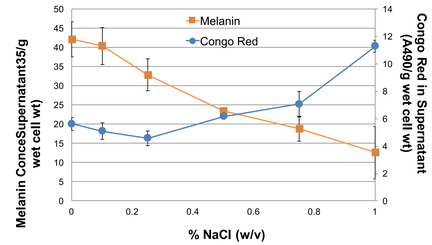


**Fig 7. Inhibition of *P. uticensis* biofilm formation results in decreased melanin content.** *P. uticensis* was inoculated into TSYE broth containing increasing concentrations of NaCl, and incubated at 30°C for 24 hrs. Cells were pelleted by centrifugation in preweighed microcentrifuge tubes; supernatants were removed and wet cell pellet masses were determined. Pellets were lysed in SDS and lysates were analyzed at 335 nm, as described above. In parallel cultures, the Congo Red assay for biofilm extracellular polysaccharide development (Ghafoor *et al.*, 2011) was employed. Melanin content decreased as biofilm extracellular matrix levels declined.

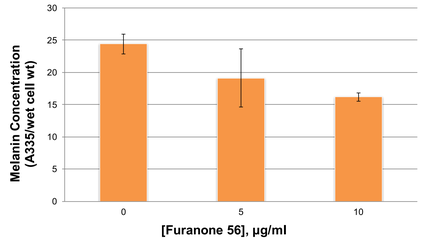


**Fig 8. Biofilm development inhibitor Furanone 56 reduces melanin content in *P. uticensis*.** *P. uticensis* was inoculated into TSYE broth containing increasing concentrations of Furanone 56 (Hentzer *et al.*, 2002), and incubated at 30°C for 24 hrs. Cells were pelleted by centrifugation in preweighed microcentrifuge tubes; supernatants were removed and wet cell pellet masses were determined. Pellets were lysed in SDS and lysates were analyzed at 335 nm, as described above.

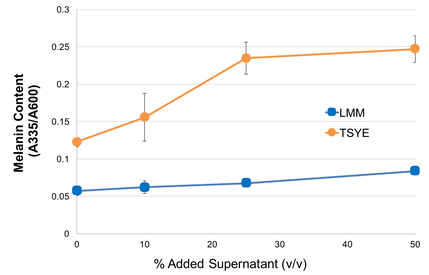


**Fig 9. Addition of conditioned supernatant stimulates melanin production in *P. uticensis*.** *P. uticensis* was inoculated at a density of 0.02 $OD_{600}$U/ml into LMM or TSYE broth containing increasing concentrations of cleared, sterile-filtered conditioned supernatants from 48 hr cultures in the respective media, and incubated at 30°C for 6 hrs. Cell density was measured spectrophometrically at 600 nm. Cell pellets were lysed in 1% SDS at 100°C for 5 min, and melanin content was measured at 335 nm. Intracellular melanin concentration was expressed as A335 nm/A600 nm. Melanin levels increased 50% compared to controls in LMM broth, but doubled in TSYE broth, suggesting the presence of a melanizing signal in the conditioned supernatants.

## CONCLUSION

*Pseudomonas uticensis* is a novel melanogenic bacterial species, producing two distinct forms of the brown pigment. The extracellular form, pyomelanin, is a low MW polymer, while the intracellular form, hypothesized to be eumelanin, is a much larger polymer. Accumulation of the intracellular pigment increases with the culture density, peaking during stationary phase. Increased melanogenesis also appears to be associated with biofilm formation, as melanin content is much lower in cells grown in planktonic culture. Inhibitors of P. uticensis biofilm formation, such as NaCl and Furanone 56 result in lower melanin content in cells. Addition of conditioned supernatants from dense cultures stimulates melanin production, suggesting the role of a diffusible melanizing or quorum signaling compound in the regulation of melanin synthesis in *P. uticensis*. Efforts are currently underway in our laboratory to isolate and characterize such signal compounds.

## LITERATURE CITED


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