Pyomelanin Production in a Novel *Pseudomonas* Species is Cell Density-Dependent

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ABSTRACT

Pseudomonas sp. UC17F4 produces pyomelanin (PM) in tyrosine-rich media. We previously reported that PM synthesis in this organism is regulated by visible light, but we also observed that UC17F4 produces increasing amounts of PM with higher cell density, suggesting that quorum sensing also regulates PM production. In this study, we seek further evidence of quorum signaling (QS) in PM regulation in UC17F4 and in PM-deficient mutants. Cellular PM content was analyzed in cultures inoculated at varying densities in TSYE broth. Cultures were incubated under constant illumination for 24 hours. Cell pellets were harvested and lysed, then PM concentration in lysates was expressed as A_{335}/g wet cell weight. Density-dependence of cellular and secreted PM in minimal medium + tyrosine (MMT) was also examined in cell lysates and media supernatants at 335 nm. PM content in cells and supernatants increased, as did the inoculum density. A time-course study was performed to determine cellular PM production prior to stationary phase; a six-fold increase in PM content was observed during the growth period. Varying volumes of conditioned supernatants from a previous study were added to cultures to determine if melanogenesis would be induced due to the presence of QS molecules. Dilute cultures exhibited increased PM production with the addition of up to 50% of conditioned media. Cultures in MMT were incubated with known *Pseudomonas* QS compounds, PQS and C4-HSL, at varying concentrations. PQS inhibited cellular PM production and C4-HSL had no effect. PM-deficient mutants of UC17F4 were isolated by transposon-mediated mutagenesis with mini-Tn5 plasmid pRL27. On TSYE plates, two isolates were unpigmented, one exhibited reduced pigmentation, and one isolate produced unpigmented cells, but overproduced extracellular PM. In TSYE broth, the mutants also exhibited the density-dependent effect on PM production with slightly higher PM content present in the densest cultures. These studies confirm that PM production increases due to quorum sensing in both the WT and mutant strains. Further studies will be performed to identify the QS molecules that regulate PM production in UC17F4, and disrupted genes from the mutant strains will be cloned and sequenced.

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 two bacterial strains, UC179D3 and UC17F4, from the cutaneous microbial flora of female red-backed salamanders. These bacterial strains have the ability to produce potent antifungal compounds. Biochemical and DNA sequence analysis aided the team in discovering that the strains are species of *Pseudomonas*. They were able to identify a number of genes in UC179D3 matching the homology of *P. fluorescens*. However, identification of UC17F4 has 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).

One of the distinctive characteristics of UC17F4 is its reddish-brown pigmentation, which we have determined to be the result of pyomelanin production. Pyomelanin is not uncommon in Pseudomonads; Ogunnariwo and Hamilton-Miller (1974) were able to isolate three strains of *Pseudomonas aeruginosa* that produce the brown pigment. Recent studies in our lab have shown that the organism turns on pyomelanin production when exposed to light; cultures incubated in the dark have reduced pigmentation (Kracke and Aaronson, 2011). Pyomelanin production is also sensitive to the intensity of light and the duration of exposure (Benzing et al., 2012). These observations suggest that pyomelanin production in *Pseudomonas sp.* UC17F4 is photoregulated.

In the present study, we investigate the effects of cell density on the production of pyomelanin in *Pseudomonas* sp. UC17F4 and its mutants in liquid and on solid media.

METHODS

Cultures of *Pseudomonas* sp. UC17F4 were maintained by weekly transfer on tryptic soy – yeast extract (TSYE) or Luria-Bertani (LB) agar. UC17F4 mutants were maintained for treatments in TSYE+Km (50 µg/mL) broth or agar at 37°C. Seed cultures for experiments were prepared by inoculation of bacteria into Nutrient Broth (which minimized pyomelanin production), and incubation overnight at 30ºC in a water bath shaker.

For experimental cultures, seed cultures were diluted, stained with crystal violet solution and counted on a hemocytometer. Cultures were diluted into fresh media at concentrations of 10^7 and 10^4 cells/ml, and 3 ml aliquots of cell suspensions were transferred to 3.0 cm Petri dishes. Plates were exposed to room light (50 lux) from fluorescent light sources. Experiments were conducted in a temperature-controlled room at 30ºC. At the conclusion of light exposure, cells were isolated from liquid broth cultures by transferring 1 mL aliquots of culture to sterile microcentrifuge tubes. The tubes were centrifuged for 3 min at 14,000xG. The resulting supernatants were discarded and microcentrifuge tubes containing the cell pellets were weighed to determine the mass of the pellet. 1 mL of 1% sodium dodecyl sulfate (SDS) was then added to each tube to resuspend each pellet. Tubes were vortexed for approximately 3 seconds to ensure distribution of the pellet, then the lysates were transferred to glass calibrated test tubes, and the volume was brought to 3 mL with 1% SDS. The samples were analyzed for absorbance at 335 nm in a Spectronic 21D UV/VIS spectrophotometer, using 1% SDS as a blank. Pyomelanin content was expressed as absorbance at 335 nm per gram of wet cell pellet weight.

To study further the density-dependent effect on pyomelanin production, mutants deficient in the production pathway were produced by mini-Tn5 transposon mediated mutagenesis of UC17F4**.** Electroporation was performed: in an 0.2-cm gap electroporation chamber, 100 µl of UC17F4 were mixed with 2 µl pRL27 (Larson et al, 2002). The cells were immediately resuspended in 1 mL SOC medium and transferred to a sterile culture tube. The culture was incubated for 1 hour at 37°C with shaking. 9 mL of chilled PBS were added to the cells and were then diluted 1:100 in PBS. 100 µL aliquots of 1:10 and 1:100 dilutions were spread on TSYE+Km (50 µg/mL) plates and the plates were incubated for 22 hours at 37°C. The cultures were allowed to incubate at ambient temperature in constant light, and individual colonies picked for deficient melanin production and restreaked on TSYE+Km (50 µg/ml) media. Cells were screened for deficiency in pigment production.

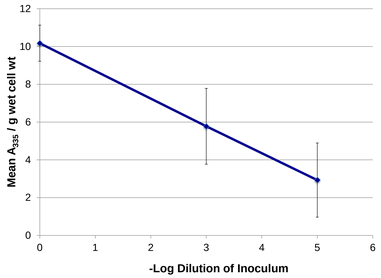

Fig. 1. Pyomelanin production by cells grown on solid media exhibits densityy dependence. Data for "time 0" points from three separate experiments where cultures were grown on TSYE agar plates for 24 hrs at 30ºC under 50 lux illumination were averaged and expressed as means ± standard error of the mean. These data show that under the same conditions of illumination, pyomelanin content in UC17F4 varies with respect to culture cell density. This suggests that quorum sensing pathways may also contribute to the stimulation of pyomelanin synthesis in *Pseudomonas* sp. UC17F4.

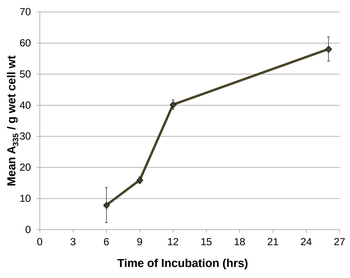

Fig. 2. Pyomelanin production in UC17F4 is growth-phase dependent. 3 mL TSYE broth were inoculated with UC17F4 at 10^7 cells/ml. Samples were collected after 6, 9, 12, and 24 hours of incubation under 50 lux illumination at 30ºC. Cell pellets were obtained by centrifugation and cell lysates were isolated by boiling cell pellets in 1% SDS. These data show that cellular pyomelanin concentration increases 7-fold in a 20 hr period, suggesting that the culture exhibits quorum signaling effects.

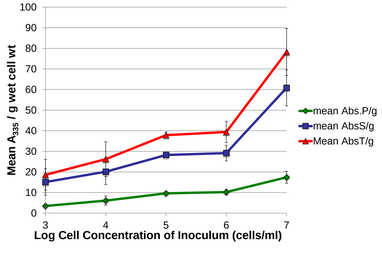

Fig 3. Cell-associated and secreted pyomelanin increase in broth cultures as a function of the concentration of the inoculum. UC17F4 was cultured in 3 mL MMT broth and incubated for 24 hours under 50 lux illumination at 30°C. Pyomelanin was extracted from UC17F4 lysates by boiling cell pellets in 1% SDS and quantified by UV spectrophotometry at 335 nm. The cell culture supernatant was analyzed at 335 nm for extracellular pyomelanin. The data also represent total pyomelanin present in the cells and supernatant. Results indicate that intra- and extracellular pyomelanin increases as inoculum density increases.

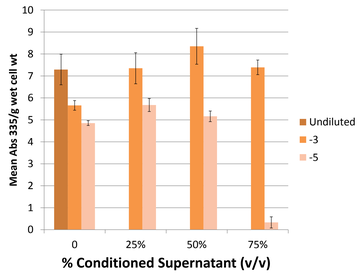

Fig. 4. UC17F4 conditioned media stimulates pyomelanin production. UC17F4 was cultured in 3 mL TSYE broth with varied concentrations of conditioned TSYE broth. Cultures were incubated for 24 hours under 50 lux illumination at 30°C. Pyomelanin was extracted from UC17F4 lysates by boiling cell pellets in 1% SDS and quantified by UV spectrophotometry at 335 nm. The data shows that melanogensis was stimulated in both cultures, suggesting that signaling molecules are secreted from the bacteria and act in the quorum sensing pathway.

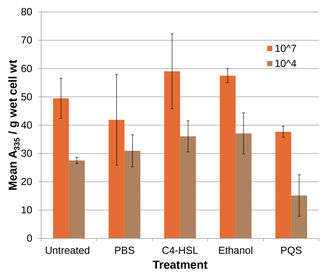

Fig. 5. PQS inhibits UC17F4 pyomelanin production. *Pseudomonas* quorum signaling compounds C4-HSL and PQS were added to UC17F4 cultures with inoculum densities of 10^4 and 10^7 cells/ml and were incubated in 50 lux illumination for 24 hours. C4-HSL showed no effect, while, PQS suppressed pyomelanin production, suggesting that PQS plays a role in pyomelanin regulation under high cell density conditions.

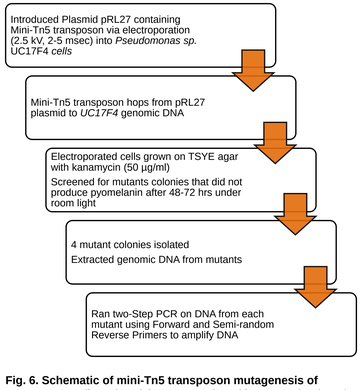

Fig. 6. Schematic of mini-Tn5 transposon mutagenesis of UC17F4. *E. coli* pRL27-mini Tn5 was cultured in LB+Km broth and the plasmid was isolated. UC17F4 cells were electroporated with pRL27. Following a cell-recovery incubation in SOC media, the transformed cells were isolated on TSYE+Km media.

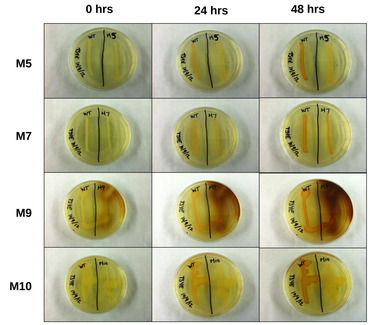

Fig. 7. Melanin-deficient mutants of *Pseudomonas* sp. UC17F4. Mini-Tn5 transposon mutagenesis was performed and mutant isolates were cultured on TSYE+Km media and incubated in the dark at 37ºC for 24 hrs. Plates were then exposed to light for up to 72 hrs. Mutants M5, M7, and M10 exhibit decreased pyomelanin production. M9 exhibits hypersecretion of pyomelanin. These results suggest that the transposon insertion disrupted genes involved in pyomelanin production.

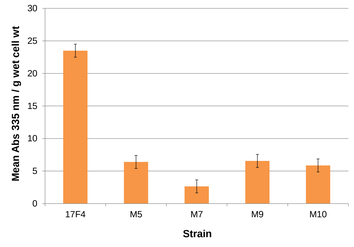

Fig. 8. UC17F4 mini-Tn5 mutants exhibit density-dependent pyomelanin production. UC17F4 and mutants were cultured in 3 mL TSYE broth at 10^7 cells/ml and incubated for 20 hours under 50 lux illumination at 30°C. Pyomelanin was extracted from UC17F4 lysates by boiling cell pellets in 1% SDS and quantified by UV spectrophotometry at 335 nm. All mutants exhibited decreased pyomelanin quantities in at diluted inoculum densities.

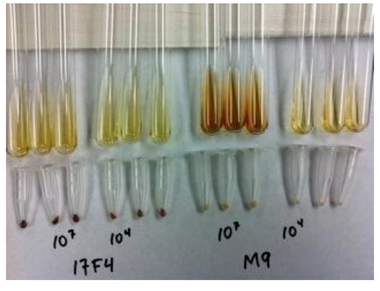

Fig. 9. UC17F4 mutant M9 exhibits reduced intracellular pyomelanin and increased excreted pyomelanin concentrations. The photograph depicts 10^4 and 10^7 cells/ml inoculum densities of UC17F4 and UC17F4-M9 isolated cell pellets and media supernatants after 24 hours of incubation under 50 lux illumination at 30°C. UC17F4-M9 cellular pyomelanin production does not exhibit the density-dependent effect as wild-type. UC17F4 does. However, UC17F4-M9 exhibits a 10-fold increase of secreted pyomelanin over UC17F4.

CONCLUSION

Based on the results of these studies, we are able to conclude that pyomelanin production in *Pseudomonas* sp. UC17F4 is cell density-dependent. In comparison of the pigment production between wild-type and mutant strains of UC17F4, it has been demonstrated that 10x more pigment is exhibited in the wild-type than in the mutant strains. Evidence of quorum sensing in these studies leads us to a greater understanding of the magnitude differences in pyomelanin between different dilutions. Future studies will continue to focus on the elucidation of the signaling compounds and regulatory pathways that govern pyomelanin production in these bacteria. Furthermore, we will increase our gene knock-out library to identify the genes involved in photoregulation and cell density-dependent control of pyomelanin production in *Pseudomonas* sp. UC17F4.

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.*

Gomelsky, M., and Hoff, W.D. 2011. Light helps bacteria make important lifestyle decisions. *Trends Microbiol.* 19: 441-448.

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

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.

Soriano, M., B. Roibas, A. B. Garcia, and M. Espinosa-Urgel. 2010. Evidence of circadian rhythms in non-photosynthetic bacteria? *J. Circadian Rhythms* 8: 1-4.

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.

Wang, Q.W., Mueller, A.P., Leong, C.R., Matsumoto, K., Taguchi, S., and Nomura, C.T. 2010. Quick and efficient method for genetic transformation of biopolymer-producing bacteria. *J. Chem. Technol. Biotechnol.* 85: 775-778.

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