Characterization of Pyomelanin-Overproducing Mutants of *Pseudomonas uticensis*, a Novel Melanogenic Bacterial Species

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ABSTRACT

Pseudomonas uticensis is a novel bacterial species, isolated from the skin of red-backed salamanders due to its potent antifungal properties. Among the characteristics of this bacterium is the production of pyomelanin (PM), which confers a brown pigment to cells grown on enriched medium. We used transposon-mediated mutagenesis to isolate strains altered in PM synthesis. Among these were strains mutated in the gene for homogentisate 1,2-dioxygenase (HDO), which overproduce and secrete PM into their environment. In the present study, we compare the biology of mutants MM7, MM8 and MM9 to wild type *P. uticensis*. Growth curves were conducted on the mutant and the wild type (WT) strains. The WT had the fastest rate of growth and reached stationary phase faster than the mutants. The mutants are characterized by abundant secretion of PM on both liquid and solid growth media, and produced 4-8 times more PM in the supernatant of TSYE broth cultures than did WT. When grown on nutrient agar (NA), *P. uticensis* did not produce PM as when grown on TSYE agar. The HDO mutants, however, secrete abundant PM on NA. Tyrosine is a precursor in the synthesis of both PM and the green fluorescent siderophore, pyoverdin (PV). Because these mutants have a defect in PM production, we wanted to see if this mutation also affected PV synthesis. PV is not produced in the mutants, unlike WT, when incubated with the quorum signal compound, PQS. In other microbes, melanin has antioxidant properties. When challenged with H2O2 on TSYE agar, 17F4 can withstand H2O2 better than when grown on NA, suggesting that intracellular PM has a protective antioxidant effect. However, on NA the mutants survive better against H2O2 than WT, indicating that extracellular PM also protects bacteria from oxidative stress. *P. uticensis* is β-hemolytic, and mutants also exhibit normal hemolytic activity, though MM8 was slightly more hemolytic than the other strains. The bacterium also has antifungal properties, so we challenged *Candida albicans* with the WT and mutant secretions. *P. uticensis* has strong antifungal activity, with slightly reduced activity in the mutants. These data show that HDO is a critical enzyme in the catabolism of tyrosine to produce intracellular PM and PV in *P. uticensis*, and that both intracellular and extracellular PM affect the survival of this organism.

BACKGROUND

Pseudomonas uticensis is a novel melanogenic species isolated from red backed salamanders for its antifungal properties (van Kessel et al.2003). *P. uticensis* produces two different forms of melanin; high molecular weight eumelanin, and low molecular weight pyomelanin (McHarris et al., 2015). In attempt to better understand our microorganism, we performed transposon-mediated mutagenesis to isolate mutant strains of *P. uticensis*, that were altered in melanin production (Morreale et al., 2014). Three of the isolates, MM7, MM8, MM9, that were found overproduced melanin and was secreting it into the medium. After molecular analysis we were able to determine that is was secreted pyomelanin. RT-PCR was preformed and it was determined that the three isolates had a disruption in the hmgA gene, encoding the production of homogentisate 1,2-dioxygenase (McHarris et al., 2015). These mutants have other phenotypically interesting characteristics, including a defect in the transport of tyrosine, which is very important building block to melanin synthesis. Present studies have also shown the other characteristic differences between the wild type, and these three mutants.

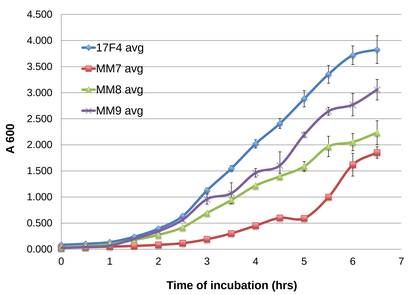


Fig 1. *Pseudomonas uticensis* mutants grow at a slower rate than wild-type. Cells were grown in TSYE broth at 30° C in a rotary shaker bath at 225 rpm. Samples were monitored every half hour using a spectrophotometer, measuring culture density at 600 nm.

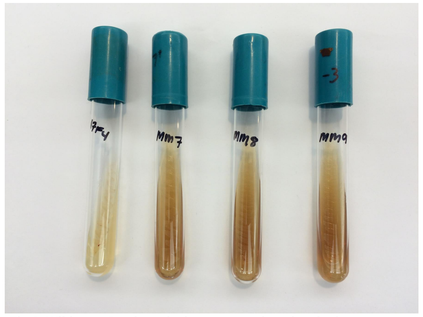


Fig 2. Wild-type *P. uticensis* does not produced melanin on nutrient agar while mutant strains secrete an abundance of pyomelanin. Bacteria were cultured on nutrient agar slants incubated for 24 hrs at 30°C in a dark incubator, then left at room temperature for another 24 hrs, and observed for growth and PM production. Most of the PM from the mutant strains diffused into the agar.

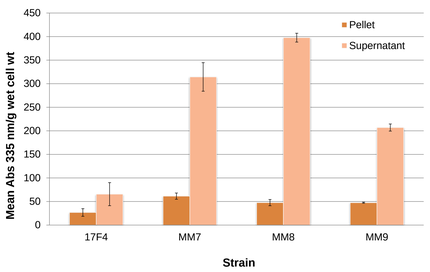


Fig 3. Wild-type and mutant strains show differences in extracellular and intracellular melanin production in nutrient broth cultures. Small petri plates were filled with nutrient broth cultures of the bacterial strains at approx. 1x10^7 cells/ml and incubated at 30°C overnight in an illuminated incubator. Samples were taken from plates and spun down in pre-weighed microcentrifuge tubes. Supernatants were then place in cuvettes for spectral analysis at 335 nm. Pellet weights were obtained, and then pellets were taken up in 1.5mL of 1% SDS and boiled for five minutes. Lysates were then placed in cuvettes as well and were analyzed at 335 nm. Absorbance values were normalized to wet cell mass (Benzing et al., 2012)

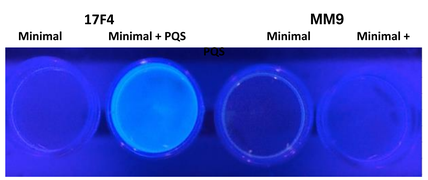
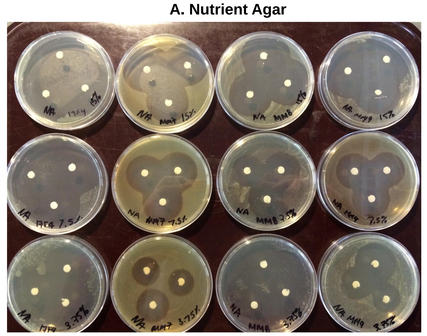
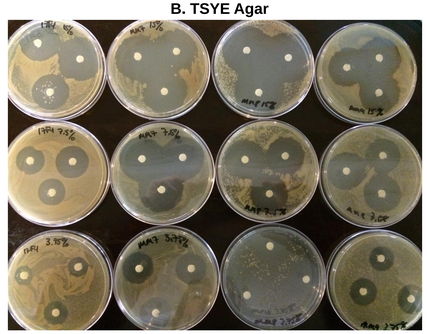


Fig 4. Pyoverdin is produced in wild-type *P. uticensis* when treated with quorum signal PQS but not in mutant strains. Strains were grown in minimal media with and without 12.5 µM PQS, in an illuminated 30°C incubator overnight (Shikula et al, 2013). Plates were then viewed on a UV light box. Results show that only the WT produces PV when incubated with PQS.

A. Nutrient Agar



B. TSYE Agar



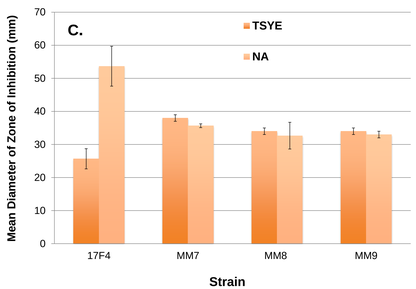


Fig 5. Wild-type *P. uticensis* shows more H_2O_2 resistance on TSYE agar than on nutrient agar; mutant strains show no significant differences. *P. uticensis* was inoculated into temperate TSYE or nutrient agar at 1x10^6 cells/ml and poured into plates. Blotting paper disks were saturated with different concentrations of H_2O_2 and incubated at 30°C in a dark incubator overnight. Zones of inhibition were then measured for analysis.
A. NA cultures with different concentrations of H_2O_2. Top row 15%, middle row 7.5%, bottom row 3.75%.
B. TSYE cultures with different concentrations of H_2O_2. Concentrations in same order as above.
C. Comparison of the mean diameter of zones of inhibition. When grown on TSYE, the WT is produces intracellular melanin, and is more resistant to oxidative stress than when grown on NA, where WT produces little intracellular melanin.

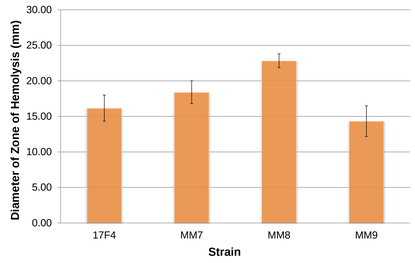


Fig 6. *P. uticensis* shows β-hemolysis on blood agar plates, with mutant strain MM8 showing a slightly larger zone of hemolysis. Strains were placed on blotting paper discs to saturation, then placed on 5% blood agar plates, incubated at 30°C in dark incubator overnight. Zones were measured the next day, showing that the mutant phenotype does not significantly affect hemolytic activity.

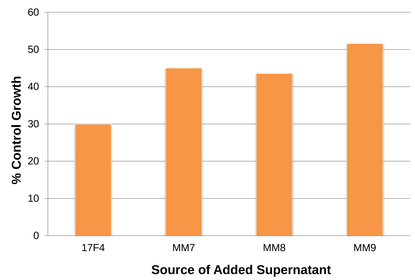


Fig 7. Mutant strains exhibit decreased antifungal activity compared to wild-type. Overnight cultures of *C. albicans* were treated with supernatants of broth cultures from each strain, and incubated for 4 hrs at 30°C in shaker at 225 rpm. Control cultures were incubated with an equal volume of TSYE broth. After incubation, cells were collected by centrifugation. Calcoflour White was added to cultures and incubated at room temperature for 30 minutes. Biomass was determined using a fluorescent plate reader (Stagoj et al., 2004). Supernatants from mutants inhibited fungal growth less than WT.

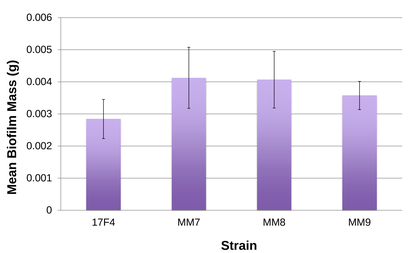


Fig 8. Mutant strains of *P. uticensis* have slightly increased biofilm production compared to wild-type. Preweighed sterile glass slides were placed in tubes with 10 mL of bacterial cultures (approx. 1x10^7 cells/ml) and placed in 30°C shaker at 120 rpm overnight. Slides were carefully removed and placed in oven to dry for 1 hr at 70°C. Dry biofilm slides were weighed to determine biofilm mass. Mutant dry biofilm masses were greater than that of WT.

CONCLUSION

Pseudomonas uticensis has a number of differences from its three mutant strains discussed here. WT cells seem to be more protected from exposure to different concentrations of hydrogen peroxide, and also seem to be able to produce the fluorescent iron siderophore, pyoverdin in the presences of PQS, unlike the mutants. The mutant strains seem to have diminished antifungal properties as well and decreased growth rates compared to the WT. Biofilm production is seen to increase, and in one strain, MM8, even an increase in hemolysin production can be seen. The most interesting characteristic differences seen were the differences in the ratio of intracellular melanin (eumelanin), to the extracellular melanin (pyomelanin), and how different media and nutrient availablity can effect the synthesis of the two different types of melanin present.

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 112th Gen. Mtg. of the Am. Soc. for Microbiol.*

Shikula, K., Seifert, S.L., and Aaronson, L.R. 2013. The Quorum Signaling Compound PQS Induces Pyoverdin Production in a Novel *Pseudomonas* Species. *Abstracts of the 113th Gen. Mtg. of the Am. Soc. for Microbiol*

Stagoj, M.N., Komel, R., and Comino, R. 2004. Microtiter Plate Assay of Yeast Cell Number Using The Fluorescent Dye Clacofluor White M2R. *Biotechniques* 36: 380-382.

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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