Temporal and Spatial Analysis of Pyomelanin Synthesis in Wild-Type and Mutant Colonies of *Pseudomonas uticensis* Using the Triton Bio Mira Smart Incubator

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ABSTRACT

Pseudomonas uticensis is a novel species of bacteria isolated on the basis of its potent antifungal properties. Bacterial colonies produce pyomelanin (PM) and form small, dome-shaped, brown-pigmented colonies on agar culture medium plates. We also isolated a mutant strain MC13 of the bacterium, that has a transposon insertion in the purF gene, and produces broadly-spreading, lobate colonies with altered pigmentation. Incubation of agar plate cultures in a cabinet incubator makes it difficult to record the temporal dynamics of colonial growth and pigmentation in fixed intervals over a period of several days. However, we recently employed the Mira Smart Incubator from Triton Bio, Inc. for these types of studies. Mira is a programmable cloud-connected incubator with AI capabilities and can photograph Petri plates at user-designated intervals and generate data for up to 6 culture plates at a time. Studies with MIRA have expanded our understanding of wild-type (WT) and mutant colony development and pigmentation on different growth media. We used digital image analysis to measure colony diameter and pigment intensity in intervals from 15-30 min over a period of up to 72 hrs at 30◦C. Results revealed that colonies of the WT and MC13 mutant strain both produce pyomelanin at greater levels when incubated on a chemically defined medium, compared to tryptic soy agar. Peak pigmentation was achieved in both strains around 60 hrs of incubation. ImageJ software enabled creation of pixel intensity surface plots of selected colonies over time, elucidating both the temporal and spatial production of pigment. In previous studies, we observed that the macrolide antibiotic, azithromycin (AZM) inhibits extracellular polysaccharide synthesis and reduces pigmentation in *P. uticensis* biofilms forming in standing broth cultures. In studies of the effects of AZM on colony development and pigmentation in agar plate cultures, a reduction in pyomelanin content at concentrations as low as 12.5 mg/ml was observed in both strains. Differences in the spatial pattern of PM pixel density were also evident. Pigment intensity in WT colonies declines in a smooth distribution, with the greatest intensity in the center of the colony. PM intensity in MC13 colonies is organized in concentric circles, which become more evident and lighter as AZM concentration increases. These results confirm the utility of the Triton Bio Mira Smart Incubator for temporal and spatial analysis of colonial growth, enumeration, morphogenesis and pigmentation in microbiological research.

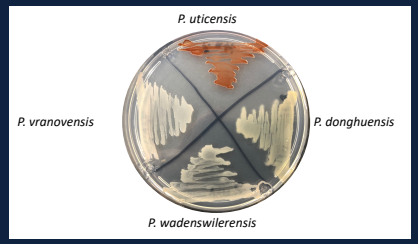


Fig.1. *Pseudomonas uticensis* UC 17F4[T] (ATCC TSD-65, NCTC 14185) and three close phylogenetic relatives grown on Lawrence Minimal Medium (LMM) which contains 1% sodium citrate as the sole carbon source; only *P. uticensis* produces brown pigmentation due to the production of pyomelanin

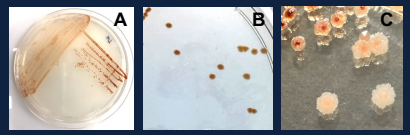


Fig. 2. A. *P. uticensis* growth on LMM agar plate. B. Magnified view of *P. uticensis* colonies on LMM agar. Average colony diameter =1.5 mm). C. Magnified view of colonies of *P. uticensis* transposon-insertion mutant MC13 that has a disruption in the purF gene (encoding amidophosphoribosyltransferase in *de novo* biosynthesis of purines). Average diameter of larger lobate colonies is 4-5 mm.

Fig. 3. Exterior view of Triton Bio Mira Smart incubator used in these studies. The device was programmed to take photos at 20 min intervals during incubation periods of up to 72 hrs at 30◦C. Data were uploaded to cloud storage where they were retrieved for image processing, For more information on the instrument and its functionality, please click on the QR code above or visit tritonbio.com.

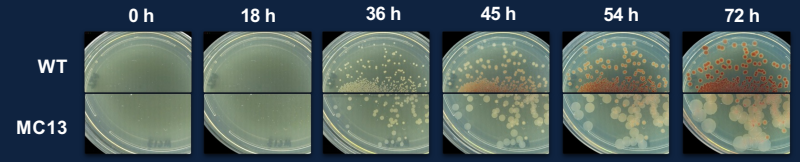


Fig.4. Composite images of *P. uticensis* wild-type (WT) and *P. uticensis* MC13 mutant strain growing on tryptic soy agar, taken by the Mira instrument over a 72 hr growth period at 30◦C. Differences in the temporal development of colony morphogenesis and pigmentation between the strains are evident.

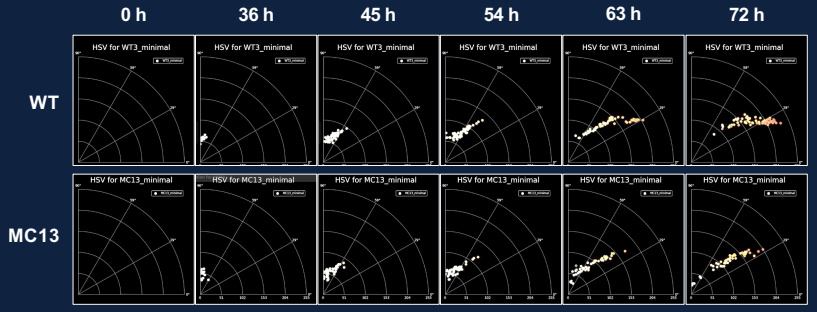


Fig.5. Hue, saturation and value (HSV) analysis of colony pigmentation in *P. uticensis* WT and MC13 strains grown on LMM agar plates. Images collected by Mira were scanned and an AI program tagged individual colonies as they appeared and developed on plates. Each colony was subjected to an HSV analysis, and the data were transformed for expression on a color wheel. In this representation, each dot represents an individual colony, and its placement relative to the center of the wheel indicates the saturation of pigmentation. The radial position of the points indicates the hue (or color) of the colony, with green at the 90◦ and red at 0◦ positions on the wheel. Results indicate more brown pigment saturation in WT beginning around 60 hrs of incubation compared to a slower rate of saturation and brown hue development in MC13.

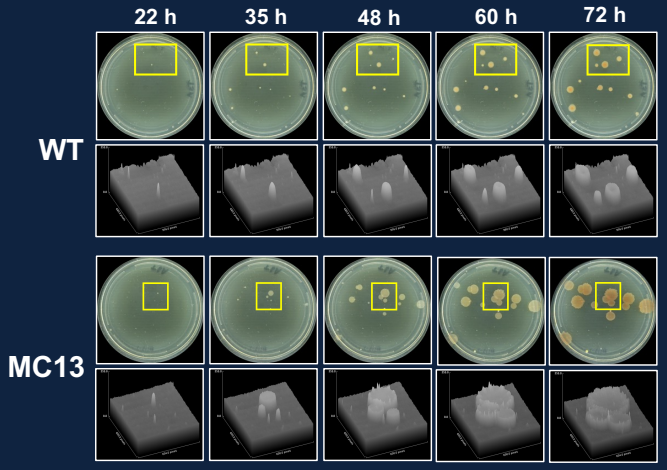
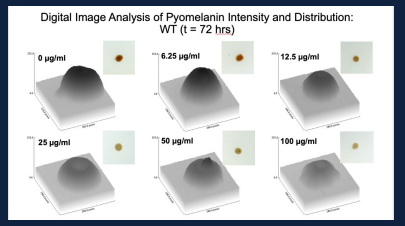


Fig.6. Surface plot analysis of temporal effects of pigment distribution in *P. uticensis* WT and MC13 strains using Fiji image processing software. Bacterial strains were grown on tryptic soy agar for 72 hrs and images were obtained in 20 min intervals. Images were converted to gray scale using Fiji software and colonies in the boxed areas on plates were transformed by surface plot analysis to create the 3-D images shown below the photographs. In these images, white pixel intensity increases vertically. Each peak represents a distinct colony. Initially colonies are lightly pigmented, but as pyomelanin is produced, the peaks collapse downward indicating increased pigmentation. In WT colonies, pigmentation is evident beginning at about 60 hrs of incubation, while onset of pigmentation occurs around 48 hours of growth. Pigmentation is not uniform in colonies of either strain, occurring predominantly in the center of the colonies.



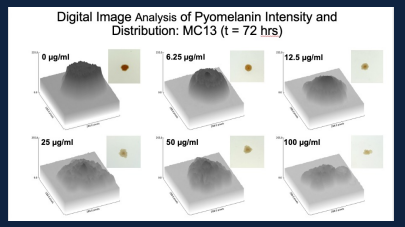


Fig.7. Surface plot analysis of temporal effects of pigment distribution in *P. uticensis* WT and MC13 strains treated with varying concentrations of azithromycin (AZM). The macrolide antibiotics have been shown to inhibit the formation of the extracellular polysaccharide matrix (EPS) of biofilms and colonies in several species of Pseudomonas. We observed that AZM treatment reduces pigmentation of *P. uticensis* biofilms and colonies due to secretion of the pigment into the surrounding medium. In this experiment, *P. uticensis* WT and MC13 strains were spot inoculated onto LMM plates containing AZM at concentrations as high as 100 µg/ml. Images were collected over 72 hrs incubation at 30◦C. Single colonies were analyzed using Fiji software as previously described. In this case, because the background was lighter than the colonies, the surface plots were reversed so that darker pixel intensity increases vertically. Results show a distinct decline in pigment intensity in WT, though colonial morphology appears unaffected. By contrast, pigment intensity and spatial distribution of pyomelanin is dramatically altered in MC13. Surface plots reveal that pyomelanin is organized in concentric rings, which is not obvious from microscopic observation. AZM alters the pigmentation in the mutant strain by up to 75% at 100 µg/ml.

CONCLUSIONS

- The Triton Bio Mira Smart Incubator was effectively used to investigate the temporal and spatial development of pyomelanin production in in the novel bacterium, *Pseudomonas uticensis*.
- Photographic quality produced by the instrument was consistent and enabled the use of digital imaging software to extract vital information from the photographs
- We used Mira to evaluate the temporal kinetics of pyomelanin pigmentation in *Pseudomonas uticensis* wild-type and mutant strains with greater reproducibility than taking plates out of a cabinet incubator and taking photos at regular intervals
- Images of colonies could be tracked by AI software to permit HSV analysis of individual colonies over the incubation period. Differences in pigmentation hue, saturation and color were observed between the WT and MC13 mutant strains.
- Fiji software was used for analysis of spatial distribution of pyomelanin in WT and mutant colonies over time, revealing unusual distribution of the pigment in the MC13 morphological mutant

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