Pseudomonas uticensis Synthesizes Pyomelanin Through Two Distinct Pathways

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Poster No. MBP-FRIDAY-613

Abstract No. 4854

UTICA UNIVERSITY

ABSTRACT

Pseudomonas uticensis **is a novel bacterial species isolated from red-backed salamanders which has a distinctive brown pigment in its colonies and biofilms from pyomelanin (PM). Many pseudomonads can produce PM, which is derived from catabolism of tyrosine to homogenisate. *P. uticensis* produces PM in media containing phenylalanine or tyrosine, but can also synthesize the pigment in synthetic media with citrate or succinate as the sole carbon source. Three of the most closely related species, *P. donghuensis*, *P. wadenswilerensis* and *P. vranovensis* are not pigmented on tryptic soy agar or on minimal medium. Our interest is why *P. uticensis* produces PM under culture conditions where other *Pseudomonas* species do not. We hypothesized that it may result from differences in the structure or regulation of two key enzymes in the PM pathway: 4-hydroxyphenylpyruvate dioxygenase (HPPD) and/or homogentisate-1,2-dioxygenase (HGD). We tested this using a comparative genomics approach. Databases of DNA sequences were assembled for the two genes from 40 different *Pseudomonas* species and BLASTn was used to align the sequences and create a phylogenetic tree for relatedness to the *P. uticensis* sequences. DNA sequences were translated into amino acid sequences, which were also aligned using BLASTp and CLUSTAL. There were no major sequence differences between *P. uticensis* and other pseudomonads nor in the upstream regulatory region of the HmgA gene that codes for HGD and in sequences of the HmgR regulatory protein. Sequences of enzymes downstream from HGD that lead to the synthesis of fumarate also show strong similarity. This metabolic connection to fumarate in the citric acid cycle suggests that there may be an alternate pathway from citrate or succinate to the synthesis of PM. 2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione (NTBC), an inhibitor of HPPD was used to test for PM production when the pathway from tyrosine to homogentisate was blocked. PM production was inhibited in NTBC-treated *P. uticensis* cultures containing tyrosine, but no inhibition was observed in cultures in LMM with either carbon source. We conclude that *P. uticensis* employs two different pathways for PM production: one that involves tyrosine catabolism to homogentisate, and one that does not. We are exploring the possibility that abundant citrate and succinate sources may reverse the dynamic equilibrium in the downstream pathway from homogentisate to fumarate causing an accumulation of homogentisate that results in a spillover forming PM. Our studies are ongoing to elucidate the latter pathway.**

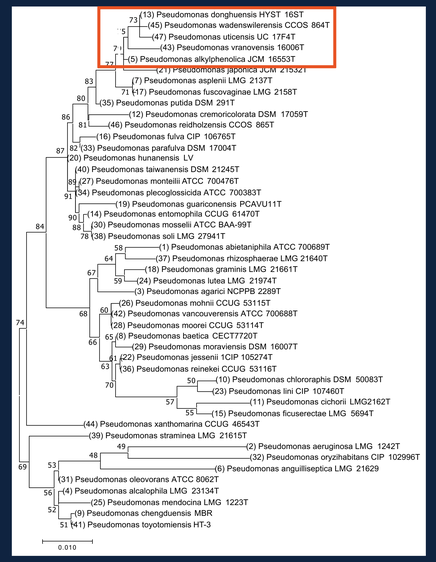

FIG. 1. Neighbor-Joining Tree of *Pseudomonas* species based on 16S rRNA sequences. Group containing *P. uticensis* highlighted in box.

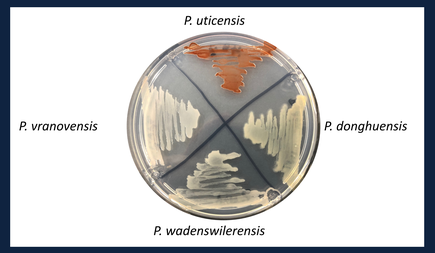

Fig. 2. On Lawrence Minimal Agar (LMM), *P. uticensis* produces a brown pigment, while closely related species do not.

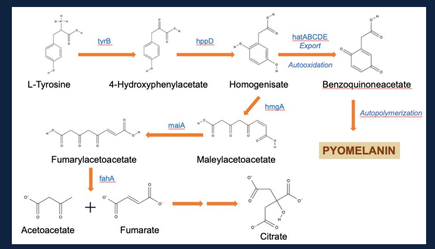

FIG. 3. Pathway of pyomelanin production from L-tyrosine via the homogentisate pathway. Pyomelanin is usually produced when hmgA activity is blocked or reduced in bacteria. (From Turick *et al.,* 2010)

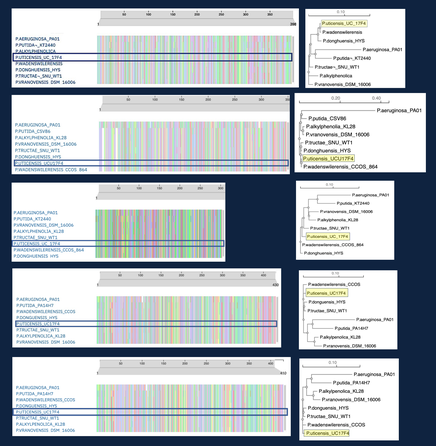

FIG. 4. MUSCLE alignment of amino acid sequences of enzymes of the homogentisate pathway from closely related Pseudomonads. Sequences were obtained from the Pseudomonas Genome Database (pseudomonas.com) and Joint Genomics Institute - Integrated Microbial Genetics database (https://mg.jgi.doe.gov/cgi-bin/mer/main.cgi). Phylogenetic trees were generated by MUSCLE website (https://www.ebi.ac.uk/jdispatcher/msa/muscle). Amino acids are indicated using the color code below:

No significant difference in amino acid sequences are evident among the most closely related species.

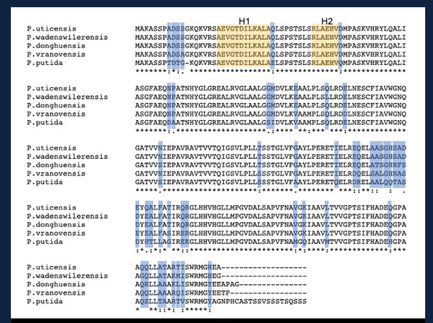

FIG 5. A. Alignment of amino acid sequences of hmgR protein among *Pseudomonas* species closely related to *P. uticensis*. Orange-shaded sequences indicate the two N-terminal helical motifs which comprise the DNA-binding domain. B. Secondary structure ribbon model of hmgR protein generated by Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) and MEGAX software. The structure of the *P. uticensis* protein (orange) is superimposed on the structure of the homologous protein from *P. vranovensis* (aqua).

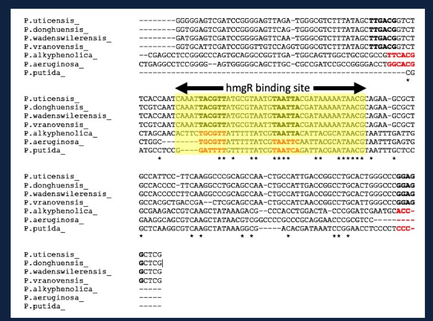

FIG. 6. Nucleotide sequence alignment of the in the upstream region of *hmg*A gene in species closely related *to P. uticensis*. Sequences highlighted in yellow represent the hmgR-binding site in the promoter region (Arias-Barrau *et al*., 2004).

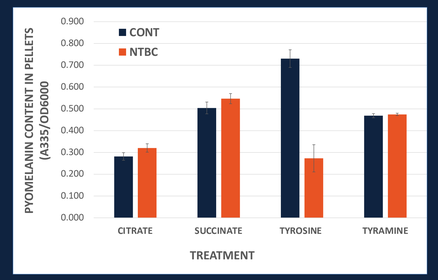

FIG. 7. NTBC, an inhibitor of HPPD (Ketelboeter *et al*., 2014) reduces melanin levels in the pellet fractions of *P. uticensis* only when tyrosine is supplemented in LMM medium. Bacteria were grown in standing cultures in LMM broth in 30 mm plates for 24 hours. Biofilms were collected and OD_{600} of the biofilm suspension was measured. Biofilm suspensions were centrifuged at 10,000xG for 3 min. Supernatants were discarded and pellets were lysed in 1% SDS at 65ºC for 15 min. Pyomelanin content in the lysates measured at 335 nm, and the relative pyomelanin content was expressed as A335/OD600. Pyomelanin in cultures grown with tyramine, succinate and citrate is not produced through the homogentisate pathway.

FIG. 8. *P. uticensis* strain MC15 is a transposon Tn5-induced mutant with a defect in fatty acid synthesis. The mutant grows unpigmented on TSA, but does form pyomelanin when 1% yeast extract is added to the media (inset). MC15 was grown in Tryptic Soy Broth and Tryptic Soy – Yeast Extract Broth +/- NTBC. The inhibitor had no effect on pyomelanin content compared to controls on either medium, suggesting that the pigment is not derived through the homogentisate pathway.

FIG. 9. Schematic diagram of the hypothesized alternate pathway of pyomelanin synthesis through the HGA pathway of tyrosine catabolism. We suggest that high levels of pyomelanin in *P. uticensis* on LMM media may be due to high levels of citrate in the media causing a back-up of HGA (green arrows) causing spillover into pyomelanin synthesis. (Adapted from From Turick *et al.,* 2010)

CONCLUSIONS

- ***Pseudomonas uticensis*** **produces a brown pigment under culture conditions in which closely related *Pseudomonas* species do not.**
- **Pyomelanin is the brown pigment in *Pseudomonas uticensis***
- **Amino acid sequences of the enzymes in the homogentisate pathway from tyrosine to fumarate exhibit strong identity among species closely related to *P. uticensis***
- **Modeling of the hmgA and hmgR protein structures inferred from amino acid sequences does not reveal significant differences in secondary or tertiary structures of these proteins in species closely related *P. uticensis***
- **Alignment of the hmgR binding site in the hmgABC promotor reveals no significant difference among species closely related *P. uticensis***
- **NTBC, an inhibitor of the homogentisate pathway of tyrosine catabolism does not inhibit pyomelanin production on minimal medium containing citrate or succinate as the sole carbon source, suggesting the existence of an alternate pathway of pyomelanin synthesis**

LITERATURE CITED

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ACKNOWLEDGEMENTS

This work was supported by the Harold T. Clark, Jr. Endowed Professorship to L.R.A and grants from Donald and Sally Majka, TheCommunity Foundation of Herkimer and Oneida Counties, and The Alden Trust.

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