Effects of Carbon Source on Alginate Biosynthesis and Biofilm Adhesion in the Melanogenic Bacterium, *Pseudomonas uticensis*

Evan V. Vescio, Jennifer A. Liu, Sarah F. Mirza and Lawrence R. Aaronson
Biology Department, Utica College, Utica, NY

ABSTRACT

Pseudomonas uticensis is a novel bacterial species with potent antifungal properties. It is nonfermenting, nitrogen-reducing, and utilizes citrate and succinate as its preferred carbon sources. A distinctive characteristic of this species is its reddish-brown pigmentation when grown on media with citrate or tyrosine, suggesting that the pigment is a form of melanin. The bacteria can produce two different forms of melanin: pyomelanin, which is a low MW secreted form, and a high MW intracellular melanin. *P. uticensis* also produces robust biofilms, and colonies have a mucoid characteristic, suggesting that it synthesizes abundant extracellular polysaccharide (EPS). The *P. uticensis* genome sequence contains numerous genes encoding enzymes for the biosynthesis of alginate, a major EPS component of the bacterial biofilm matrix. One function of alginate and other matrix components is adhesion of *P. uticensis* biofilms to surfaces. In studies unrelated to biofilm development, we observed that adhesion of lawns of *P. uticensis* on agar media varied depending upon the composition of the media. Bacteria exhibited strong adhesion when grown on agar plates containing citrate as the primary carbon source, but easily washed off agar surfaces in its absence, despite the abundance of tryptones and amino acids as carbon sources. We hypothesized that the form of carbon source altered the synthesis and composition of the EPS, and tested this hypothesis by using transposon-mediated mutagenesis to isolate mutants of *P. uticensis* that did not adhere to agar medium containing citrate. Ten isolates were found that easily washed off of citrate-enriched agar medium. A Congo Red binding assay was used to directly quantify alginate yield in the mutant strains, and 7 out of 10 isolates produced up to 5-fold lower amounts of alginate compared to the wild-type. Another peculiar phenotype of the alginate mutants is the hypersecretion of pyomelanin in tyrosine-enriched media. We hypothesized that this was the result of melanin that is normally trapped in the matrix being released from the alginate-deficient biofilm matrix. To test this idea, biofilms of wild-type cells were treated with alginate lyase, and melanin release was measured spectrophotometrically. Results showed that melanin release from biofilms was 4-17 times higher with alginate lyase treatment than in untreated controls, suggesting that some of the colony- and biofilm-associated pigment is pyomelanin trapped in the EPS.

BACKGROUND

Pseudomonas uticensis is a novel species isolated from redbacked salamanders (van Kessel et al., 2003) local to Central NY. Biochemical and molecular genetic evidence suggest that this isolate is the prototype of a previously undescribed species (Lawrence et al., 2017). One of the characteristic phenotypes of *P. uticensis* is the brown pigmentation of colonies and biofilms when cultivated on nutrient-enriched medium, including minimal medium containing citrate as a carbon source, or tyrosine as a supplement. Biochemical analysis suggests that the brown pigment is a relatively low MW form of melanin known as pyomelanin, which can be biofilm-associated, or released into the extracellular environment (Lawrence et al., 2015). Pyomelanin is derived through the homogentisate pathway of tyrosine catabolism (Turick et al, 2010).

P. uticensis produces robust, mucoid biofilms when grown in nutrient-enriched medium. We observed in a previous study that biofilm adhesion to agar plates varies with the composition of growth medium, and is greatest when bacteria are cultivated in media containing citrate as the primary carbon source. We hypothesized that biofilm matrix composition, which is comprised of alginate, varies with nutrient availability. In order to better understand the role of carbon-source availability in *P. uticensis*, we conducted transposon-mediated mutagenesis to isolate mutants that exhibit an alginate-deficiency phenotype. In the present study we analyzed the effect of media composition on alginate production, adhesion of biofilms to agar surfaces and pyomelanin content.

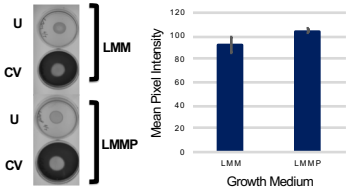
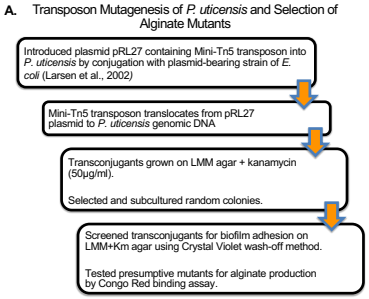


Fig. 1. Differential adhesion of *P. uticensis* biofilms to agar media. Bacterial suspensions were spot-inoculated on Lawrence Minimal Agar (LMM) or LMM+1%peptone (LLMP) and incubated for 24 h at 30ºC. Plates were stained with 0.5% Crystal Violet for 30 min the rinsed gently with water. Plates were photographed and the pixel intensity of washed area was measured with ImageJ. U: unstained plate; CV: plate stained with crystal violet after rinsing with water.



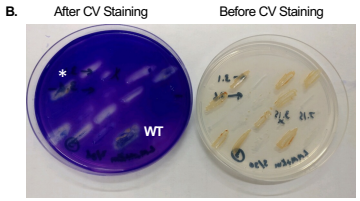


Fig. 2. Isolation of adhesion-defective mutants of by transposon-mediated mutagenesis. A. Flowchart of transposon-mediated mutagenesis and mutant selection and screening in *P. uticensis*. **B.** Agar plate assay for screening of adhesion-defective mutants. Transconjugants were patched on plates containing LMM+Kanamycin agar. After 24 h incubation at 30ºC, plates were stained with 0.5% Crystal Violet (CV) for 30 min. Plates were then rinsed with a gentle stream of water, and non-adherent strains were evident by exposure of unstained agar under bacterial biofilms. Adherent isolates indicated by (•). Wild type strain is identified as WT.

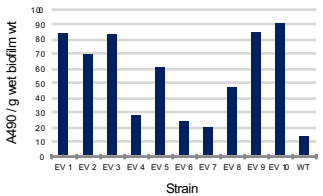


Fig. 3. Mutant strain biofilms of *P. uticensis* produce less alginate than WT in Congo Red binding assay of alginate. *P. uticensis* alginate production was quantified after exposure of biofilms to 40 µg/ml Congo Red in LMM broth. Biofilm pellets were centrifuged and supernatants were measured spectrophotometrically at 490 nm to determine the amount of Congo Red dye not bound to biofilm alginate. Values were normalized to wet biofilm pellet mass. Higher values indicate lower alginate production.

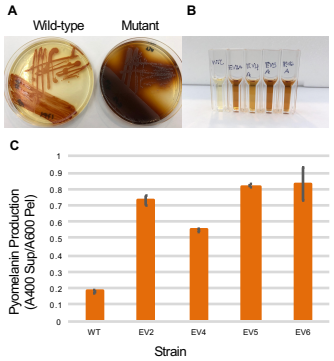


Fig. 4. Alginate mutants hyperproduce pyomelanin. A. Tryptic soy agar plates showing that pyomelanin is contained within the biofilm mass of *P. uticensis* wild-type, but hypersecreted into the agar by mutant strain EV2. **B.** Supernatants of standing broth biofilm cultures of WT and alginate mutant strains of *P. uticensis*. **C.** Quantitative analysis of pyomelanin secretion in supernatants shown in **B.** Pyomelanin content was estimated by spectrophotometric analysis at 400 nm, and values were normalized to cell mass (determined by optical density of dispersed biofilm pellets at 600 nm). Mutant strains secrete up to 4-fold higher levels of pyomelanin than WT.

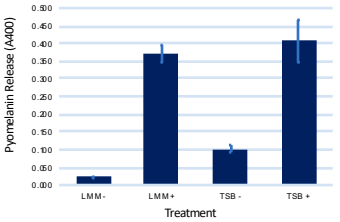


Fig. 5. Alginate lyase digestion of *P. uticensis* biofilms releases pyomelanin. Biofilms were growth in standing liquid cultures in either LMM or tryptic soy broth for 24 h at 30ºC. Biofilm pellets were collected by centrifugation and resuspended in PBS containing alginate lyase, or PBS alone as a control. Mixtures were incubated for 1 h at 30ºC, then centrifuged. Pyomelanin in supernatants was measured as previously described. Results indicate that pyomelanin is released into the supernatant following alginate lyase treatment, suggesting that the pigment is trapped in the extracellular polysaccharide matrix, rather than housed within the bacterial cells.

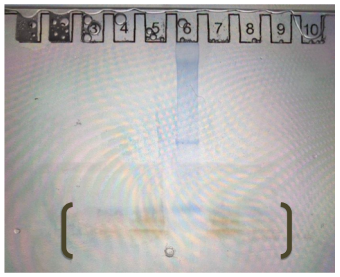


Fig. 6. Polyacrylamide gel to identify pyomelanin in mutant strains. *P. uticensis* wild-type and mutant broth biofilm culture supernatants applied to a 4-15% gradient polyacrylamide gel to identify the molecular weight of the melanin samples produced by the strains. Results shows brown bands of low molecular weights (<2 kDa) in all specimens. In the supernatants from mutant strains EV4, EV5 and EV2 are loaded in columns 4,5, and 7, respectively, with WT in lane 8.

CONCLUSIONS

Transposon mutagenesis and screening assays were carried out successfully, leading to isolation of *P. uticensis* mutants that are significantly deficient in alginate synthesis. Analysis of the alginate lyase wild-type cultures led to the new discovery that pyomelanin is trapped in the EPS matrix, rather than in the cytoplasm of the bacterial cells. The alginate lyase-treated wild-type cultures revealed a similar hypersecretion of pyomelanin. The genome of *P. uticensis* has been completely sequenced (Lawrence et al., 2017), which will allow us to directly compare the mutant genomes to the wild-type. Discovering the specific mutations that cause alginate deficiency in *P. uticensis* mutants will provide the ability to explore the role of alginate production in biofilm formation and the interactions of the bacterial extracellular matrix with pyomelanin molecules.

LITERATURE CITED

Boyd, A., and Chakrabarty,A.M. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 60.7 (1994): 2355-2359.

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.

Lawrence, P.L. and Aaronson, L.R. 2015. Characterization of pyomelanin-overproducing mutants of *Pseudomonas uticensis*, a novel melanogenic bacterial species. *Abstracts of the 115th General Meeting of the American Society for Microbiology*.

Lawrence, P.L. and Aaronson, L.R. 2016. The effects of cell density and biofilm formation on melanin production in a novel *Pseudomonas* species. *Abstracts of ASM Microbe 2016*

Lawrence, P.L, van Kessel, J.C., Butler, A.M, Nicolette DeJohn, C.N. and Aaronson, L.R. 2017. Genomic and biochemical characterization of Pseudomonas uticensis, a novel melanogenic bacterial species. *Abstracts of ASM-Microbe 2017.*

Lawrence, P.L, Yudchits, V. and Aaronson, L.R. 2015. Melanogenesis in a novel *Pseudomonas* species: Evidence of two pathways. *Abstracts of Pseudomonas 2015 International Conference*

Nicolette DeJohn, C.R. and L.R. Aaronson. 2017. Genomic and biochemical analysis of melanin biosynthesis in *Pseudomonas uticensis. Abstracts of ASM Microbe 2017.*

Plonka, P.M. and M. Grabacka. 2006. Melanin synthesis in microorganisms — biotechnological and medical aspects. *Acta Biochim. Pol.* 53: 429–443

C.E. Turick, Knox, A.S., Becnel, J.M., Ekechukwu, A.A. and Milliken, C.E. (2010). Properties and Function of Pyomelanin, Biopolymers, Magdy Elnashar (Ed.), ISBN: 978-953-307-109-1, InTech

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.*

ACKNOWLEDGEMENTS

This work was supported by the Harold T. Clark, Jr. Endowed Professorship to L.R.A and grants from Donald and Sally Majka, The Community Foundation of Herkimer and Oneida Counties, and The Alden Trust. The authors also wish to acknowledge the contributions of Nicole Pickett to this project.

View this poster at www.aaronsonmicrobiology.com