Antibiotic Interactions with *Pseudomonas uticensis*: Erythromycin Induces Pyomelanin Synthesis

Emra Klempic, Celia R. DeJohn and Lawrence R. Aaronson
Biology Department, Utica College, Utica, NY

ABSTRACT

Pseudomonas uticensis is a novel bacterial isolate from the cutaneous microbiota of red backed salamanders, identified on the basis of its potent antifungal properties. A distinctive characteristic of *P. uticensis* is the chocolate brown pigmentation of colonies when the bacteria are cultured on nutrient-rich media such as tryptic soy agar (TSA). The brown pigment is a high MW intracellular form of melanin, which is synthesized through a tyrosinase-independent pathway. *P. uticensis* can also synthesize pyomelanin (PM), a low MW, water-soluble pigment derived by catabolism of tyrosine through the homogentisate pathway. Since melanin synthesis is uncommon in bacteria, we have been investigating the biosynthesis and biological role of these pigments in *P. uticensis*, and have isolated several mutant strains of the organism that are altered in melanin production. As part of our efforts to characterize this species, we subjected it to a standard battery of antibiotic sensitivity tests using a Kirby-Bauer disk diffusion assay. Because the bacterium was an environmental isolate and likely resident in soil, we screened bacterial sensitivity to antibiotics not only on standard Mueller-Hinton agar (MHA), but also on TSA and Lawrence Minimal Medium (LMM), a chemically-defined medium optimized for *P. uticensis*. The bacteria exhibit sensitivity to bacitracin, ciprofloxacin, gentamycin and polymixin B, while displaying intermediate sensitivity to tetracycline and neomycin. *P. uticensis* also was sensitive to sulfisoxazole on LMM agar, but not on MHA or TSA. Of particular interest, however, was the fact that on TSA agar, a zone of hypermelanization appeared around the erythromycin disk, while no growth inhibition was evident. Wild-type and melanin biosynthetic mutant strains MM9, CD4 and CD9 were plated on LMM supplemented with tyrosine or *p*-hydroxyphenylacetate (both precursors of melanin synthesis in *P. uticensis*) with erythromycin disks, and the various patterns of melanization indicated that PM production was stimulated in the bacteria. Since other species of *Pseudomonas* may also produce PM under some conditions, cultures of *P. aeruginosa*, *P. fluorescens* and the closely related species *P. vranovensis* were grown on plates of LMM+tyrosine with erythromycin Sensi-Discs; only *P. uticensis* and *P. aeruginosa* produced PM. The mechanism behind the stimulation of PM production only by erythromycin among the antibiotics tested remains unresolved, but we hypothesize that this may represent a distinctive stress response in this species.

BACKGROUND

Pseudomonas uticensis is a novel species isolated from redbacked salamanders (van Kessel et al., 2003). 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 form of melanin. *P. uticensis* can produce two distinct forms of melanin: an insoluble, high molecular weight polymer that is retained in cells, and pyomelanin, which is derived through the homogentisate pathway of tyrosine catabolism (Lawrence et al.,2015).

In an effort to describe the novel species *P. uticensis*, we subjected it to a standard battery of antibiotic sensitivity tests using the Kirby-Bauer disc diffusion assay method. We chose to conduct these assays not only on Mueller-Hinton agar (MHA), but also on tryptic soy agar (TSA) and Lawrence Minimal Medium (LMM; Lawrence et al. 2017). The pattern of antibiotic sensitivity was generally unremarkable for a pseudomonad. However, exposure to erythromycin resulted in no inhibition of growth, but did produce a zone of increased melanization around the disc. In the present study, we demonstrate that erythromycin induces the production of pyomelanin in *P. uticensis*.

Antibiotic	MHA	TSA	LMM
Ampicillin 10µg	N	N	N
Bacitracin 10U	S	S	S
Cefazolin 30µg	N	N	N
Chloramphenicol 30µg	N	N	N
Ciprofloxacin 5µg	S	S	S
Doxycycline 30µg	N	N	N
Erythromycin 15µg	N	N	N
Gentamycin 120µg	S	S	S
Kanamycin 30µg	N	N	N
Naladixic Acid 30µg	N	N	N
Neomycin 30µg	I	I	I
Novobiocin 30µg	N	N	N
Penicillin 10U	N	N	N
Polymixin B 300U	S	S	S
Streptomycin 300µg	N	N	N
Sulfisoxazole 250µg	N	N	S
Tetracycline 30µg	N	N	N
Vancomycin 30µg	N	N	N

Table I. Antibiotic sensitivity of *P. uticensis* varies with growth medium. *P. uticensis* was spread on plates of MHA, TSA and LMM. BD BBL Sensi-Discs containing various antibiotics were placed on plates, which were incubated for 24 h at 30ºC. Antibiotic sensitivity was established using a standard Kirby-Bauer assay.

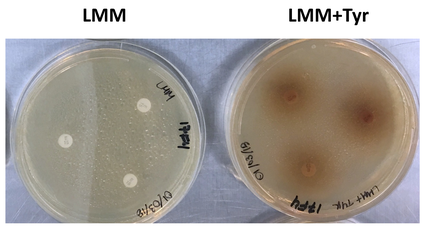

Fig. 1. Erythromycin induces a zone of hyper-melanization in *P. uticensis* around the Sensi-Disc. Lawns of *P. uticensis* were grown on LMM agar and LMM + 0.1% tyrosine agar plates and incubated with erythromycin Sensi-Discs for 24 h at 30ºC. A distinct zone of diffuse brown pigment was observed around the discs on tyrosine-supplemented media, suggesting the stimulation of a form of melanin.

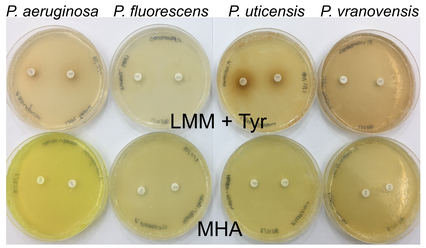

Fig. 2. Erythromycin induces pyomelanin production in *P. uticensis* predominantly among select *Pseudomonas* species. Cultures of *P. aeruginosa*, *P. fluorescens*, *P. uticensis* and *P. vranovensis* were spread on plates of LMM agar containing 0.1% tyrosine (top row) and Mueller Hinton agar (bottom row), and incubated for 24 h at 30ºC with Erythromycin Sensi-Discs. All bacterial strains except *P. fluorescens* exhibited pyomelanin production on LMM+Tyr medium, but *P. uticensis* and *P. aeruginosa* to a lesser extent display hypermelanization around the Sensi-Discs.

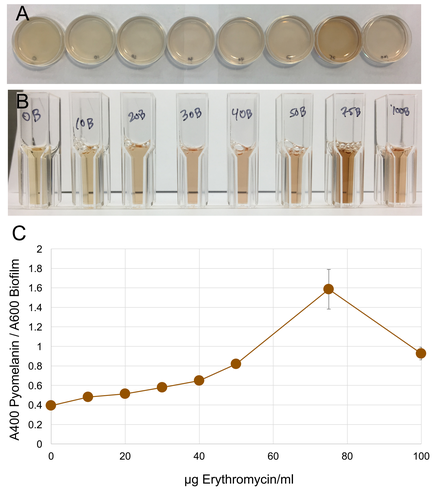

Fig 3. Erythromycin-induced pyomelanin production is concentration-dependent in broth cultures. *P. uticensis* was grown in LMM broth containing 0.1% tyrosine (w/v) with varying concentrations of erythromycin for 24 h at 30ºC in 35 mm Petri plates. Biofilms were suspended, and OD_{600} was measured to establish cell density. Cell suspensions were then centrifuged at 13,000xg for 3 min, and supernatants were collected and analyzed spectrophotometrically at 400 nm to measure pyomelanin. A: plate cultures after 24 h. B: supernatants from cell cultures. C: pyomelanin production expressed as a ratio of A_{400}/OD_{600}. Maximum pyomelanin production occurs with a concentration of 75 µg/ml erythromycin in broth cultures.

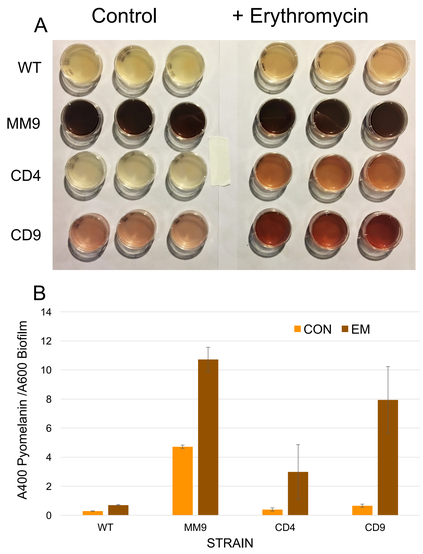

Fig. 4. Erythromycin-induced pyomelanin production is concentration-dependent in agar plate cultures. A. *P. uticensis* WT and mutant strains altered in melanin production were grown in LMM + Tyr broth with 75 µg/ml erythromycin in 35 mm plates in standing culture at 30ºC. Photographs were taken of plates After 24 h. B. Relative pyomelanin levels in culture supernatants were measured spectrophotometrically as previously described. Results show that erythromycin stimulates pyomelanin production more than 2-fold in WT and MM9 strains, and more than 6-fold in CD4 and CD9 strains.

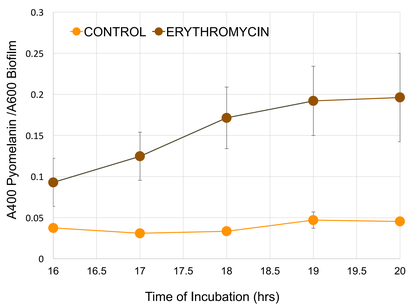

Fig. 5: Pyomelanin production increases over time in broth cultures. P. uticensis was grown in LMM + Tyr broth in 35mm plates with or without 75 µg/ml erythromycin, as described above, for up to 20 h. Cultures were sampled at 2 h intervals and OD_{600} of the suspended biofilms, and then the A_{400} of pyomelanin in the bacterial supernatants were determined spectrophotometrically. Results indicate that pyomelanin increases with time of incubation and increase in cell mass, suggesting that hypermelanization by erythromycin may involve quorum signaling.

CONCLUSION

The aminoglycoside antibiotic, erythromycin does not exhibit inhibition of growth and reproduction of the Gram-negative bacterium, *Pseudomonas uticensis*. Exposure to erythromycin does, however, induce the production of extracellular pyomelanin in WT and mutant strains of the bacterium in broth and agar plate cultures. Induction of pyomelanin is concentration-dependent and appears to correlate with increased cell density. Intracellular melanin synthesis on P. uticensis is known to be density dependent (Lawrence and Aaronson, 2016), so both processes may be associated with quorum sensing responses. Pyomelanin is not, however, synthesized by the same metabolic process as intracellular melanin (Nicolette DeJohn and Aaronson, 2017). Pyomelanin production protects *P. uticensis* from oxidative stress (Lawrence and Aaronson, 2015), and has protective properties in other bacterial species as well (Plonka and Grabacka, 2006). Consequently, pyomelanin synthesis in response to erythromycin exposure may represent a novel stress response.

LITERATURE CITED

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

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., the Harold T. Clark, Jr. Summer Research Fellowship to C.R.D. 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 Anthony DiMambro and Tashiry Maldonado to this project.

View this poster at www.aaronsonmicrobiology.com