The Quorum Signaling Compound PQS Induces Pyoverdin Production in a Novel *Pseudomonas* Species

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Abstract

Pseudomonas species produce a variety of quorum sensing molecules, which relay signals regarding population dynamics of their surroundings. *Pseudomonas* sp. UC17F4, isolated in our lab in 2003 for its production of potent antifungal compounds, produces brown-pigmented colonies on nutrient-enriched media due to the production of pyomelanin. The synthesis of pyomelanin in this organism appears to be cell density dependent, suggesting that quorum sensing may regulate production. To study whether the addition of *Pseudomonas* quinolone signal (PQS) affects pyomelanin production, we added PQS in ethanolic solution to UC17F4 growing in minimal medium supplemented with 0.1% (w/v) L-tyrosine. Normally UC17F4 produces pyomelanin in this medium, but upon addition of PQS secretion of a green fluorescent pigment was observed. We hypothesized that this pigment is the fluorescent iron siderophore, pyoverdin, which was confirmed by comparing the absorbance spectrum of the isolated pigment to the known spectrum of pyoverdin. To further investigate the mechanism of this pigment switch in UC17F4, we grew the bacteria in the aforementioned medium under constant illumination (300 lux) at 30˚ C for 24 h in all experiments, adding PQS at the time of inoculation, unless otherwise indicated. Pyoverdin content in cell-free supernatants was expressed as A_{400}/g wet cell mass. We observed concentration-dependent stimulation of pyoverdin production, with a peak at 12.5 µM PQS. The time of addition of PQS to cultures also affected pyoverdin production; pigment levels were 2x greater in cultures receiving PQS at t=0 than in those with PQS introduced at 12 h of incubation. Addition of the quorum-signaling compound C4-HSL to media did not stimulate pyoverdin production when added alone, nor did C4-HSL affect pyoverdin production when added in combination with PQS. Pyoverdin production in minimal medium appears to be tyrosine dependent; the pigment is not produced in the absence of L-tyrosine, and curiously, it is not produced in minimal medium supplemented with casamino acids, nor in enriched media such as TSYE broth. PQS induction of pyoverdin is also repressed by Fe^{3+} concentrations in excess of 0.1 µM. These data reveal an interesting regulatory switch in pigment production in *Pseudomonas* sp. UC17F4 that is worthy of further study.

Introduction

UC17F4, a novel *Pseudomonas* species isolated by Van Kessel, Scanlon, and Aaronson (2003) for its antifungal properties, produces a reddish-brown pigment, previously characterized as pyomelanin (Kracke and Aaronson, 2011). It is not uncommon for *Pseudomonas* species to produce pyomelanin (Nikodinovic-Runic *et al*., 2009; Ogunnariwo and Hamilton-Miller, 1975). This pigment is visually characterized by a light brown, reddish-brown, or dark brown color (Ogunnariwo and Hamilton-Miller, 1975).

Quorum sensing, the cell signaling in microorganisms which relays information on population dynamics, may influence pyomelanin production in UC17F4. Recent work showed that cells inoculated at higher cell densities produce more melanin than cells inoculated at lower densities when grown in Vogel's Minimal Medium N (MM) (Vogel, 1956), supplemented with 0.1% (w/v) L-tyrosine (MM+Tyr). In addition, pyomelanin production increases during the growth phase of this bacterium. When supernatants from UC17F4 grown for 24 h were added to cell cultures at time of inoculation, pyomelanin production increased (Seifert *et al.*, 2013).

Certain quorum signaling molecules have been identified in *P. aeruginosa*. Addition of 2-heptyl-3-hydroxyl-4(1H)-quinolone (*Pseudomonas* quinolone signal, or PQS) increased the production of pyocyanin and elastase in wild-type *P. aeruginosa* (Diggle *et al*., 2003). PQS is produced at the end of exponential phase and may stimulate cells to enter stationary phase, propose Diggle *et al.* (2003). Pesci *et al.* (1999) found that *P. aeruginosa* utilizes PQS to regulate expression of the quorum sensing *lasB* gene in this bacterium, which encodes an important virulence factor (Pearson *et al.*, 1994). Prior to this, it was identified that *N*-(3-oxododecanoyl)-l-homoserine lactone and *N*-butyryl-l-homoserine lactone (C4-HSL) controlled *lasB* expression (Pearson *et al.*, 1994; Pearson *et al.*, 1995).

In the following experiments, we tested the hypothesis that quorum sensing regulates pigment production in UC17F4. We grew bacteria in the presence of varying concentrations of exogenous PQS or C4-HSL to determine whether these quorum signaling molecules altered pyomelanin production. Although we expected that pyomelanin production would increase with PQS as well as C4-HSL addition, a fluorescent green pigment was produced when PQS was added. The research presented here involved further analysis of the observed PQS-stimulated switch in production of a brown to a green pigment in UC17F4.

Materials and Methods

Stock cultures of wild-type UC17F4 were maintained by weekly transfer of bacteria to fresh tryptic soy-yeast extract (TSYE) agar plates. Seed cultures of UC17F4 were inoculated in Nutrient Broth and placed in a water bath shaker at 30˚ C overnight. The next day cells were diluted, stained with Crystal Violet, and counted using a hemocytometer to determine the titer of the seed culture. We transferred 3 mL of UC17F4 at final concentrations of 10^7 and 10^4 cells/mL in MM+Tyr, with three replicates for each treatment. In our experiments, these treatments included adding PQS and C4-HSL at varying combinations and concentrations, using differing concentrations of cells, and adding PQS at varying times during growth. In other experiments, we altered the media when adding PQS: cells were grown either in MM+Tyr or MM supplemented with 1.5% casamino acids (w/v). Further, we altered iron and tyrosine concentrations when adding PQS. All cultures were grown 24 h at 30˚ C, exposed to room light (300 lux).

We collected 1.5 mL from each plate into pre-weighed microcentrifuge tubes, centrifuging at 14,000 rpm for 3 min. Supernatants were transferred to glass tubes, and cell pellets were weighed to determine the wet cell weight of each sample. We added 1 mL of 1% sodium dodecyl sulfate (SDS) to microcentrifuge tubes to resuspend the pellets, when pellets were studied for certain experiments. We transferred resuspended pellets to calibrated tubes, which were then vortexed for 4 s and placed in boiling water for 5 min to lyse the cells. We added 75 µL 20% SDS to the supernatants, for a final concentration of 1% SDS in each sample. Supernatants were placed in boiling water for 5 min, and A_{335} or A_{400} per g of wet cell weight was determined spectrophotometrically, using 1% SDS as a blank. Pyomelanin and pyoverdin (the green fluorescent pigment, as identified by matching absorbance spectra and physical qualities) content in UC17F4 was expressed as A_{335} or A_{400} of culture supernatants, respectively, per g of wet cell weight.

To generate mutants of UC17F4 deficient in pyoverdin production, we electroporated plasmid pRL27 into UC17F4. This plasmid contains a transposon, mini-Tn5, with a kanamycin-resistant (Km^r) gene and an origin of replication. After shocking cells at 2.5 kV for 5 msec, cells recovered 1.5 h in SOC media, allowing transposition and expression of Km^r. We then plated cells on MM+Tyr agar plates (1.5% agar, w/v) containing 12.5 µm PQS and 50 µm kanamycin. Plates were placed under UV light after 48 h of growth at 30˚C and colonies without a green fluorescent halo were streaked on plates. Mutant clones were subcultured, and A_{400} of supernatants was determined for mutants grown in MM+Tyr supplemented with PQS

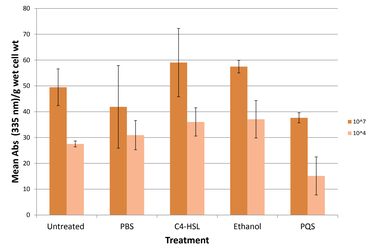

Figure 1. PQS stimulated decreased pyomelanin content in UC17F4 grown in 50% TSYE. Cultures were inoculated at 10^7 and 10^4 cells/mL, grown in 50% TSYE with treatment, and absorbance of cell lysates determined 24 h later. Treatments consisted of media to which we added 2.5 µm PQS dissolved in ethanol, 10 µm C4-HSL dissolved in PBS, ethanol only, PBS only, or untreated. Only PQS appeared to decrease pyomelanin production in UC17F4, as determined by A_{335}/g.

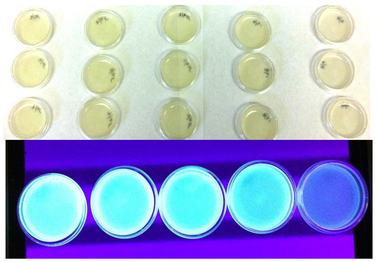

Figure 2. Addition of PQS to MM+Tyr inhibited pyomelanin production. After 24 h of growth, cultures appeared light green and fluoresced under ultraviolet light. This pigment was identified as pyoverdin by comparing the absorbance spectrum of the isolated pigment to the known spectrum of pyoverdin.

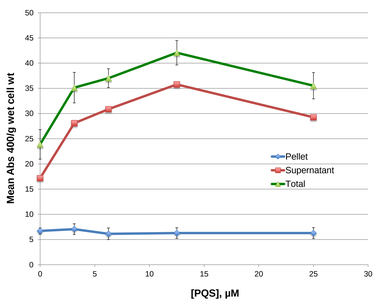

Figure 3. Pyoverdin production was highest at a concentration of 12.5 µM PQS in MM+Tyr. Cultures inoculated at 10^7 cells/mL exhibited an increase in pyoverdin content of the supernatants from 0 to 12.5 µM PQS and a decrease from 12.5 to 25 µM after 24 h of growth. Intracellular pyoverdin was consistently low among the varied concentrations, as determined by spectrophotometric analysis.

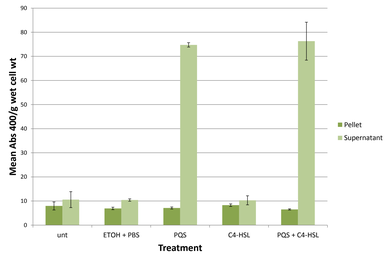

Figure 4. Pyoverdin content is dependent on PQS, not C4-HSL, addition. Cultures inoculated with PQS alone in MM+Tyr exhibited high pyoverdin content; likewise, cultures inoculated with PQS and C4-HSL together exhibited high pyoverdin content. C4-HSL, when added alone, did not stimulate pyoverdin production. The ethanol and PBS group served as a control, since PQS was dissolved in ethanol and C4-HSL in PBS.
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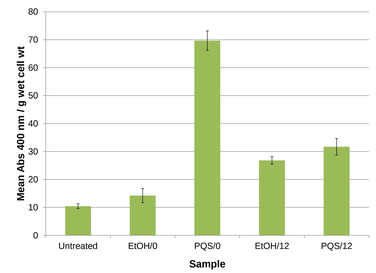
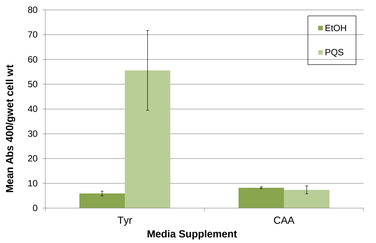


Figure 5. Pyoverdin content was 2x greater in cultures receiving PQS at t=0 than t=12h after incubation. Cultures grown in MM+Tyr were either supplemented with 12.5 µM PQS at the time of inoculation or 12 h following inoculation.



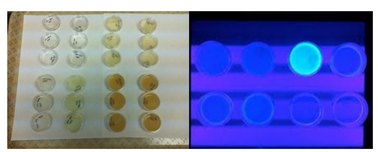


Figure 6. Media with 12.5 µM PQS added to 1.5% casamino acids inhibited production of pyoverdin in minimal media. UC17F4 was grown for 24 h in minimal media supplemented with either 1.5% casamino acids or 0.1% tyrosine; 12.5 µM **PQS** was added to all treatments including the ethanol control. A brown pigment was observed in cultures grown with casamino acids, whereas a green fluorescent pigment was observed in cultures supplemented with tyrosine.

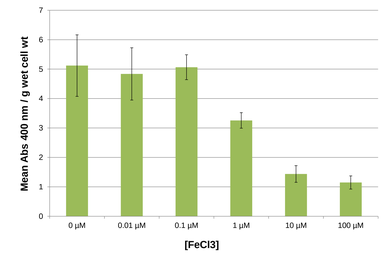


Figure 7. Increasing concentrations of $FeCl_3$ decreased pyoverdin content. $FeCl_3$ at concentrations of 0, 0.01, 0.1, 1.0, 10, and 100 µM was added to UC17F4 grown in MM+Tyr with 12.5 µM PQS. A decrease in pyoverdin production was observed at $FeCl_3$ concentrations greater than 0.1µM.

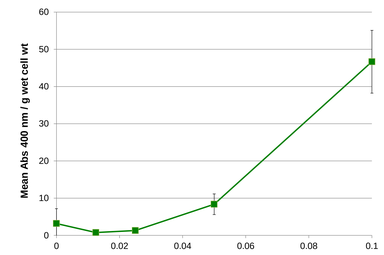


Figure 8. **Increased concentrations of tyrosine stimulated greater pyoverdin production after 24 h of growth.** UC17F4 grown in MM+Tyr supplemented with 0, 0.0125, 0.025, 0.050, or 0.10 % (w/v) tyrosine and 12.5 µM PQS exhibited an increase in pyoverdin production when tyrosine concentrations were above 0.025%.

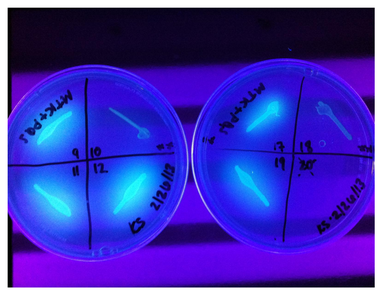


Figure 9. Pyoverdin deficient mutants grown on MM+Tyr agar plates containing 12.5 µM PQS exhibit decreased fluorescence under ultraviolet light. UC17F4 was electroporated with plasmid mini-Tn5 having a kanamycin-resistant (Km^r) gene and an origin of replication. Mutants were selected by examination under UV light, choosing colonies with decreased fluorescence.

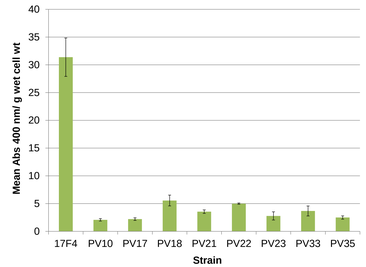


Figure 10. Pyoverdin deficient mutants secrete less pyoverdin than wild-type UC17F4. Wild-type UC17F4 and pyoverdin-deficient were grown in MM+Tyr supplemented with 12.5 µM PQS. Pyoverdin content in culture supernatants was determined as A_{400}/g cell weight.

Conclusions

Although *Pseudomonas* sp. UC17F4 typically secretes pyomelanin when grown on TSYE or MM+Tyr media, introducing the quorum signaling compound PQS alters pigment production in this bacterium. When grown in TSYE, PQS induces a decrease in pyomelanin production. PQS may act by inducing cells to enter stationary phase (Diggle *et al.*, 2003); consequently, a decrease in melanin production may be indicative of a retardation of growth. When grown in MM+Tyr, PQS induces secretion of a green fluorescent pigment, which we identified as the iron siderophore, pyoverdin. Pyoverdin production was repressed by $FeCl_3$ concentrations greater than 0.1µM in MM+Tyr with 12.5 µM PQS. This further confirms that the green fluorescent pigment is pyoverdin. Since pyoverdin levels were 2x greater in cultures receiving PQS at t=0 than t=12h after incubation, we conclude that pyoverdin has a prolonged effect on cells, perhaps inducing transcriptional changes. To further study the PQS mediated switch in pigment production from pyomelanin to pyoverdin in UC17F4, we have isolated mutants defective in pyoverdin production when grown in MM+Tyr with 12.5 µM PQS. Future experiments will involve analyzing the disrupted sequences in these mutants to study the genetic regulation of pigment production in *Pseudomonas* sp. UC17F4.

Literature Cited

Diggle, S. P., K. Winzer, S. R. Chhabra, K. E. Worrall, M. Camara, and P. Williams. 2003. The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates *rhl*-dependent genes at the onset of the stationary phase and can be produced in the absence of *lasR*. *Molecular Microbiol.* **50:** 29-43.

Kracke, K.M., and L. R. Aaronson. 2011. Photoinduction of Pyomelanin Production in a Novel *Pseudomonas* Species. *Abstracts of the 111th Gen. Mtg. of the Am. Soc. for Microbiol.*

Nikodinovic-Runic, J., L. B. Martin, R. Babu, W. Blau, and K. E. O'Connor. 2009. Characterization of melanin-overproducing transposon mutants of *Pseudomonas putida* F6. **298:** 174-183.

Ogunnariwo, J., and J. M. T. Hamilton-Miller. 1975. Brown- and red-pigmented *Pseudomonas aeruginosa*: differentiation between melanin and pyorubin. *J. Med. Microbiol*. **8:** 199-203.

Pearson, J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and E. P. Greenberg. 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc. Natl. Acad. Sci. USA* **91:**197–201.

Pearson, J. P., L. Passador, B. H. Iglewski, and E. P. Greenberg. 1995. A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **92:**1490–1494.

Pesci, E. C., J. B. Milbank, J. P. Pearson, S. McKnight, A. S.Kende, E. P. Greenberg, and B. H. Iglewski. 1999. Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **96**:11229–11234.

Seifert, S.L., K. Shikula, and L.R. Aaronson. 2013. Pyomelanin Production in a Novel *Pseudomonas* Species is Cell Density-Dependent Abstracts of the 114th Gen. Mtg. of the Am. Soc. For Microbiol.

Van Kessel, J.C., T. L. Scanlon, and L. R. Aaronson. 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.*

Vogel, H. J. 1956. A convenient growth medium for *Neurospora* (medium N). *Microbiol. Genet. Bull*. **13:** 42-43.

Acknowledgements

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