Casamino Acids Suppress Pyoverdin Production in a Novel *Pseudomonas* Species

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Abstract

Pseudomonas species UC17F4, originally isolated from the cutaneous flora of red-backed salamanders for its antifungal properties, produces brown-pigmented colonies when grown on tyrosine-enriched media. The brown pigmentation is the result of pyomelanin (PM) production and accumulation in the bacteria, which we have previously shown to be light- and cell density-dependent. Addition of *Pseudomonas* quinolone signal (PQS), a quorum-signaling molecule, to cultures grown in minimal medium (MM) supplemented with 0.1% tyrosine (MT) results in a switch from PM synthesis to the production of the green fluorescent siderophore, pyoverdin (PV). Delivery of PQS dissolved in ethanol or NP-40 into MT induces PV production, but not when a dried residue of PQS is rehydrated in culture tubes with MT. However, when PQS is added to MT supplemented with 1.5% casamino acids (CAA), PV production is suppressed and PM is produced. We found that only in CAA concentrations of 0.125% or less is PV produced by UC17F4, indicating a threshold level for suppression. We hypothesized that one or more amino acids in CAA may suppress synthesis of PV, so we grew cells in MM supplemented with PQS and Tyr in binary combinations with the 17 other amino acids constituting CAA. No suppression of PV synthesis was observed in any of these combinations. Furthermore, when we added the 17 amino acids in the same molar proportions found in CAA to MT with PQS, PV was produced. Growth of UC17F4 in MT and NH_4NO_3 at concentrations as high as 0.1% resulted in no suppression of PV production, suggesting that the suppression effect is not a generalized response to elevated nitrogen levels in the media and that more complex components of CAA cause this effect. To better understand this regulatory problem, transposon mutagenesis was employed to isolate mutants of UC17F4 that are altered in PV synthesis. Several strains were isolated that are defective in PV production on MT with PQS. One mutant strain, PV22, which does not produce PV, is mutated in a gene encoding a presumptive ABC transporter protein. Another mutant, C11, produces 9 times more PV than the wild type when grown in 0.25% CAA. Future research will involve analysis of the disrupted genetic sequence in C11 involved in regulation of the switch from PV to PM production, and of the target of suppression by CAA.

Introduction

Isolated for its antifungal properties by Van Kessel, Scanlon, and Aaronson (2003), UC17F4 was identified as a novel *Pseudomonas* species by analysis of signature genes (Butler and Aaronson, 2006). UC17F4 produces a reddish-brown pigment, which was characterized as pyomelanin (Kracke and Aaronson, 2011); it is not uncommon for pyomelanin synthesis to occur in *Pseudomonas* species (Nikodinovic-Runic *et al*., 2009; Ogunnariwo and Hamilton-Miller, 1975). Pyomelanin is characterized by a light brown, reddish-brown, or dark brown color (Ogunnariwo and Hamilton-Miller, 1975) and is synthesized via a tyrosine degradation pathway (Turick *et al*., 2010). Previous research has shown that pyomelanin production in UC17F4 is light-dependent (Benzing *et al*., 2012); more pyomelanin is produced in the light than in the darkness (Benzing *et al*., 2012; Kracke and Aaronson, 2011). Under high-intensity light, photobleaching of pyomelanin occurs (Benzing *et al*., 2012). Moreover, a greater degree of photobleaching is observed at lower cell densities. Benzing *et al.* (2012) proposed that quorum sensing may influence pyomelanin production in UC17F4, and this supposition was supported in later research. Seifert *et al.* (2013) found that cells inoculated at higher cell densities produced more pyomelanin than cells inoculated at lower densities, suggesting a quorum sensing response. When cell-free extracts of UC17F4 grown for 24 h added were added to cell cultures at the time of inoculation, pyomelanin production increased (Seifert *et al*., 2013).

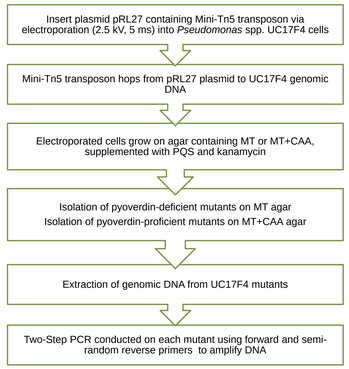
Addition of *Pseudomonas* quinolone signal (PQS), a quorum signaling molecule, decreased pyomelanin content of UC17F4 (Seifert *et al*., 2013). Diggle *et al.* (2003) propose that PQS is produced at the end of exponential phase, and may stimulate cells to enter stationary phase. When growing UC17F4 in Vogel's Minimal Medium *N* (Vogel, 1956), supplemented with 0.1% (w/v) L-tyrosine (MT) and 12.5 µM PQS, we observed that UC17F4 produces a green fluorescent pigment instead of pyomelanin (Shikula *et al*., 2013). We identified this pigment as the iron siderophore pyoverdin by comparing the known spectrum of pyoverdin to the absorbance spectrum of the isolated pigment (Shikula *et al.,* 2013). In addition, the production of green fluorescent pigment in MT with PQS decreased as ferric iron concentration increased (Shikula *et al*., 2013). Cox and Adams (1985) previously found that *P. aeruginosa* produces pyoverdin in response to iron deprivation, further supporting that the green fluorescent pigment is pyoverdin.

In the present study, we investigated the genetic basis for the switch from pyomelanin to pyoverdin production in UC17F4. The effects of casamino acids, an acid hydrolyzed form of casein, on pigment production were also investigated.

Materials and Methods

To generate UC17F4 mutants, we electroporated plasmid pRL27 Larson et al., 1997) into UC17F4. This plasmid has a transposon, mini-Tn5, with a kanamycin-resistant (Km^r) gene and an origin of replication. After shocking cells at approximately 2.5 kV for 5 ms, cells recovered 1 h in SOC media, allowing transposition and expression of Km^r. We then plated electroporated cells on MT agar (1.5%, w/v) plates with 12.5 µm PQS and 50 µm kanamycin to isolate pyoverdin deficient mutants. To identify pyoverdin proficient mutants, which produce pyoverdin in the presence of casamino acids, we grew cells on MT agar plates containing 0.5% (w/v) casamino acids with 12.5 µm PQS and 50 µm kanamycin. To screen for pyoverdin deficient mutants, we placed petri plates on a UV box after 24 h of growth at 30˚C and screened for colonies without a green fluorescent halo. Screening for pyoverdin proficient mutants, we isolated colonies with a green fluorescent halo when grown with casamino acids. To isolate genomic DNA, we grew overnight cultures of UC17F4 mutants, washed cells with Tris EDTA buffer, and autoclaved cultures for 1 min to disrupt cells. Semi-random PCR amplification of the sequences flanking the transposon was performed as previously described by Stolyar *et al.* (2007). Once DNA was extracted, a Qubit Assay (Life Technologies, Inc.) was used to determine the concentration of DNA. Ten ng/µL of PCR products were sent to GENEWIZ, Inc. for sequencing. A BLASTN search was conducted to establish sequence identity.

For growth experiments, overnight seed cultures of UC17F4 were inoculated in Nutrient Broth and placed in a water bath shaker at 30˚ C. The next day cells were diluted, stained with Crystal Violet, and counted using a hemocytometer to establish the titer of the seed culture. We transferred 3 mL of UC17F4 at final concentrations of 10^7 cells/mL in MT supplemented with casamino acids (at varying concentrations) with or without 12.5 µm PQS, into 35 mm petri plates, with three replicates per treatment group. Cultures were grown at 100 lux for 24 h at 30˚ C. We collected 1.5 mL from each plate into pre-weighed tubes and centrifuged samples at 14,000 rpm for 3 min. Absorbance of cell-free extract at 400 nm was measured, and cell pellets were weighed to determine the wet cell weight of each sample. Pyoverdin content in UC17F4 was expressed as $A_{400\ nm}$ of culture supernatants per g of wet cell weight.



First Step PCR Primers	Second Step PCR Primers
tpnRL17-1 5-AACAAGCCAGGGATGTAACG-3	tpnRL17-2 5-AGCCCTTAGAGCCTCTCAAAGCAA-3
CEKG2A 5-GGCCACGCGTCGACTAGTACN10AGAG-3	CEKG4 5-GGCCACGCGTCGACTAGTAC-3
CEKG2B 5-GGCCACGCGTCGACTAGTACN10ACGCC-3	
CEKG2C 5-GGCCACGCGTCGACTAGTACN10GATAT-3	

Figure 1. Schematic of transposon mutagenesis and table of primers used in Two-Step PCR procedures. pRL27-mini Tn5 was electroporated into UC17F4 cells. Mutants were isolated on MT or MT+CAA agar, supplemented with PQS and kanamycin.

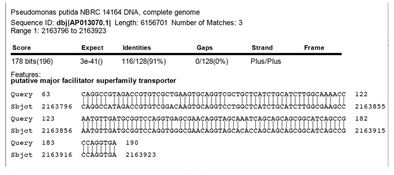


Figure 2. Sequence identity of UC17F4 pyoverdin-deficient mutant PV22. By conducting a BLASTN search, we found that PV22, a mutant that does not produce pyoverdin on MT with PQS, exhibits a 91% sequence identity to a presumptive ABC transporter protein in *Pseudomonas putida.*

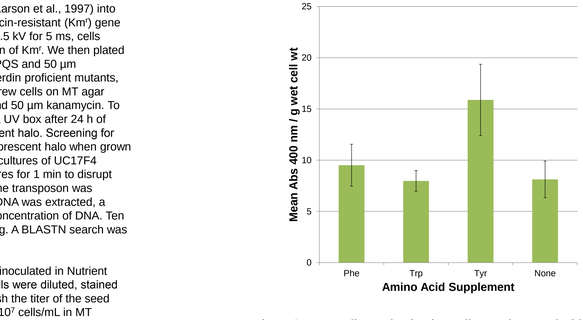


Figure 3. Pyoverdin production in media supplemented with aromatic amino acids. Wild-type UC17F4 grown in Vogel's Minimal Medium *N* supplemented with 5.52 mM phenylalanine, tryptophan, or tyrosine. UC17F4 produced more pyoverdin in media supplemented with tyrosine than without amino acids or with phenylalanine or tryptophan.

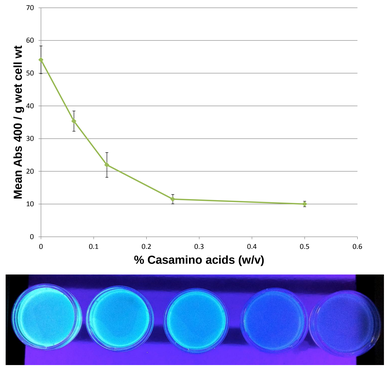


Figure 4. Casamino acids concentrations greater than 0.125 % (w/v) suppress PQS-induced pyoverdin production. In wild-type UC17F4, we found a threshold of 0.125% (w/v) CAA in media below which we observe pyoverdin production, and above which we observe pyomelanin production.

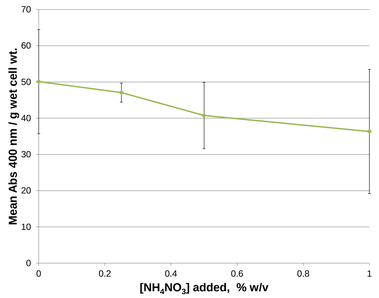


Figure 5. The casamino acids-induced suppression of pyoverdin production is not a generalized response to increased nitrogen levels. We grew wild-type UC17F4 in MT with 12.5 µM PQS and increasing concentrations of ammonium nitrate, which did not result in a switch to pyomelanin production. This suggests that the CAA-induced suppression of pyoverdin is not a result of an increase in nitrogen levels in the media.

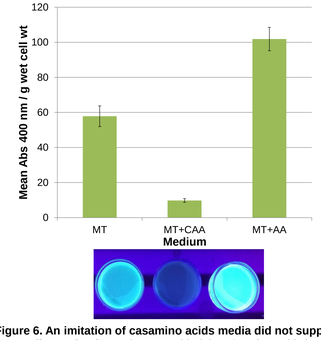


Figure 6. An imitation of casamino acids media did not suppress pyoverdin production. When we added the 18 amino acids in the same molar proportions found in 1.5% (w/v) CAA to minimal media with PQS, pyoverdin was produced, indicating that more complex elements of CAA result in pyoverdin suppression by CAA.

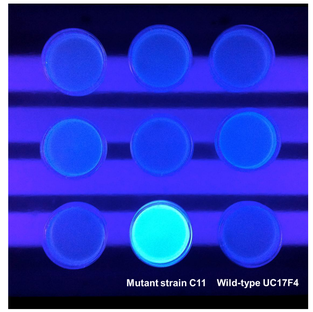


Figure 7. Pyoverdin over-producing mutant C11 exhibits fluorescence even when grown in MT with PQS and 0.25 % (w/v) CAA. Transposon mutagenesis was used to generate mutants that were resistant to kanamycin. C11 produced pyoverdin even in 0.25% (w/v) CAA, a condition in which wild-type UC17F4 produces pyomelanin.

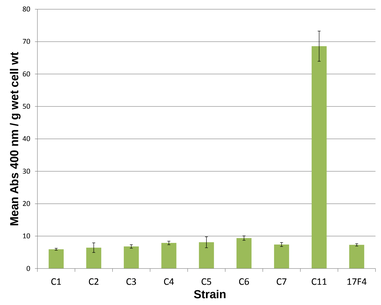


Figure 8. Mutant C11 produces 9 times more pyoverdin in MT with 0.25% (w/v) CAA and 12.5 µM PQS than wild type UC17F4. We quantified the pyoverdin produced by the isolated UC17F4 mutants using spectrophotometric analysis.

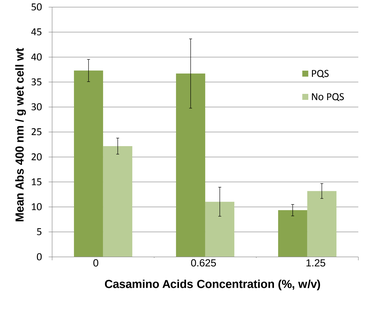


Figure 9. Mutant C11 produces pyoverdin even in the absence of PQS. We grew C11 in MT with increasing concentrations of CAA with and without PQS. Greatest pyoverdin production was observed when PQS was added to MT with 0 or 0.625% (w/v) CAA.

Conclusions

In the present work, we found that pyoverdin is produced as a result of the specific addition of tyrosine to Vogel's Minimum Medium *N* with PQS, not from addition of any aromatic amino acid. The aromatic amino acids phenylalanine and tryptophan induced less pyoverdin production than tyrosine. By conducting a BLASTN search, we found that a pyoverdin-deficient mutant of UC17F4, PV22, exhibited sequence identity to a *P. putida* transporter gene.

In addition, we identified a threshold level above which pyoverdin suppression by casamino acids occurs. We found that pyoverdin production is suppressed at concentrations of casamino acids higher than 0.125% (w/v). We thought that perhaps this suppression occurred as a result of a generalized response to elevated nitrogen levels, as casamino acids is composed of 18 amino acids digested by acid. However, pyoverdin production was not suppressed as nitrogen levels in the media increased. Finally, we thought that the interactions among the 18 amino acids in the casamino acids may suppress pyoverdin production above 0.125% (w/v) CAA in minimal media. Yet amino acid addition in the molar proportions found in casamino acids resulted in pyoverdin, not pyomelanin, production. In casamino acids, some small peptide fragments of the original casein proteins may remain, suggesting that more complex components of the casamino acids media suppress pyoverdin production.

We isolated and cloned a mutant, C11, that over-produces pyoverdin in casamino acids media. We aim to isolate more pyoverdin over-producing mutants of UC17F4 in casamino acids by transposon mutagenesis in an effort to identify genes that are involved in the regulation of pyoverdin production. We then aim to identify the gene products involved in signal transduction or regulation of gene expression in the process of pigment production in UC17F4.

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