Pyomelanin Enhances Virulence and Antifungal Activity of a Novel *Pseudomonas Species*

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ABSTRACT

Pseudomonas sp. UC17F4 is a novel bacterium originally isolated in our laboratory from the cutaneous microbial flora of the red-backed salamander, *Plethodon cinereus*. The bacterium was isolated on the basis of its ability to secrete potent antifungal compounds, which have been shown to inhibit hyphal growth of *Neurospora crassa*, as well as growth, hyphal development and biofilm formation in *Candida albicans*. One of the novel characteristics of UC17F4 is the brown pigmentation of bacterial cells when grown on nitrogen-enriched media, such as tryptic soy – yeast extract (TSYE) agar. The brown pigment has been determined to be pyomelanin (PM), and is only produced when tyrosine is abundant in the media. Furthermore, PM production in UC17F4 increases with exposure to visible light and under conditions of higher cell density. Despite the evidence that PM synthesis is regulated, the biological role of the pigment in this bacterial isolate remains unclear. We have used transposon-mediated mutagenesis to isolate a library of mutants of UC17F4 that produce different levels of PM, from completely unpigmented strains to PM-overproducing isolates. Because melanin production has been shown to enhance virulence in several species of bacteria and fungi, we tested the virulence of our mutant strains using the *Caenorhabditis elegans* model system. *C. elegans* larvae were transferred to lawns of bacterial strains on Nematode Growth Media (NGM) agar plates supplemented with tyrosine and observed after 24 hours of feeding. Wild-type and melanogenic strains MM8 and MM9 resulted in greater than 90 percent mortality, while unpigmented strains MM2 and PV21 produced no mortality. To determine if PM production is related to the antifungal activity of UC17F4, mutant strains were tested for their ability to inhibit the growth of *C. albicans* and *N. crassa* in agar plate assays. Strains with wild-type levels of PM were strongly inhibitory to both fungal species while PM-deficient strains did not significantly inhibit fungal growth. These results suggest that PM contributes to virulence and antifungal activity in *Pseudomonas* sp. UC17F4. Future studies with wild-type and mutant strains will explore the mechanisms of toxicity and virulence of pyomelanin.

BACKGROUND

Microorganisms can be found in a variety of environments – in soil, in the guts of animals, on inanimate objects, or on mountaintops. They have evolved to produce unique survival mechanisms in the event of environmental change. The survival mechanism of interest in this study is the production of melanin. Melanin production and color differ among organisms, but in general, it is considered to be "[a substance of dark color], insoluble in aqueous or organic fluids, resistant to concentrated acid and susceptible to bleaching by oxidizing agents" (Nosanchuk and Casadevall, 2003). There are two types of melanin that are produced by bacteria – pyorubrin, which consists of a red-brown hue, and pyomelanin, which features more of a light-brown hue. *Pseudomonas* species produce pyomelanin in order to protect the cell against damage from ultra-violet (UV) light (Ogunnariwo and Hamilton-Miller, 1975; Nosanchuk and Casadevall, 2003).

In 2003, Van Kessel, Scanlon and Aaronson isolated the bacterial strain UC17F4 from the cutaneous microbial flora of female red-backed salamanders. This bacterial strain has the ability to produce potent antifungal compounds. Biochemical and DNA sequence analysis aided the team in discovering that UC17F4 is a species of *Pseudomonas*, but identification of UC17F4 has not been successful since sequence analysis of two signature sequences, rpoD and gyrB showed no more than 88% sequence identity with its closest neighbors in the GenBank database (Butler and Aaronson, 2006).

One of the distinctive characteristics of UC17F4 is its reddish-brown pigmentation, which we have determined to be pyomelanin. Pyomelanin is not uncommon in Pseudomonads; Ogunnariwo and Hamilton-Miller (1974) were able to isolate three strains of *Pseudomonas aeruginosa* that produce the brown pigment. Recent studies in our lab have shown that the organism turns on pyomelanin production when exposed to light; cultures incubated in the dark have reduced pigmentation (Kracke and Aaronson, 2011). Pyomelanin production is also sensitive to the intensity of light and the duration of exposure (Benzing et al., 2012). These observations suggest that pyomelanin production in *Pseudomonas sp.* UC17F4 is photoregulated. We have also observed density-dependent production of pyomelanin in this organism (Seifert et al., 2013).

Pyomelanin has been demonstrated to be a virulence factor in many species of bacteria and fungi (Liu and Nizet, 2009; Planka and Grabacka, 2006). In the present study, we investigate the effects of pyomelanin production on the virulence and antifungal properties of *Pseudomonas* sp. UC17F4. Virulence will be assessed using the nematode worm, *Caenorhabditis elegans*. *C. elegans* has been used as a host in numerous studies of bacterial virulence (reviewed by Marsh and May, 2012), and has shown sensitivity to pyomelanin production by *Cryptococcus neoformans* (Mylonokis et al., 2002). We have isolated mutant strains of UC17F4 that are pyomelanin deficient or hypersecrete the pigment, and we can control pyomelanin production in the wild-type strain by limiting tyrosine availability. We show that only melanized strains of the bacteria cause death in *C. elegans*.

METHODS

Culture conditions: Cultures of *Pseudomonas* sp. UC17F4 were maintained by weekly transfer on tryptic soy – yeast extract (TSYE) or Luria-Bertani (LB) agar. UC17F4 mutants were maintained for treatments in TSYE+Km (50 µg/mL) broth or agar at 37°C. Seed cultures for experiments were prepared by inoculation of bacteria into Nutrient Broth (which minimized pyomelanin production), and incubation overnight at 30ºC in a water bath shaker.

Mutant isolation: To study further the biological effects of pyomelanin production, mutants altered in the production pathway were produced by mini-Tn5 transposon mediated mutagenesis of UC17F4. Electroporation was performed: in an 0.2-cm gap electroporation chamber, 100 µl of UC17F4 were mixed with 2 µl pRL27 (Larson et al, 2002). The cells were immediately resuspended in 1 mL SOC medium and transferred to a sterile culture tube. The cultures were incubated for 1 hour at 37°C with shaking. 100 µL aliquots of cultures were spread on TSYE+Km (50 µg/mL) plates and incubated for 24-48 hrs at 30oC under constant illumination. Unpigmented colonies and those secreting pyomelanin into the agar were restreaked on TSYE+Km (50 µg/ml) media. Cells were screened for deficiency in pigment production or for hypersecretion of pyomelanin.

Virulence studies: Broth cultures of UC17F4 were spread on 60 mm plates of Nematode Growth Medium (NGM) agar, with or without 0.1% L-tyrosine. Cholesterol and uracil were omitted from the basic formulation of NGM with no apparent effect on the growth or behavior of the worms. Plates were incubated for 24 hrs at 20ºC under constant illumination. L1 and L2 stage *C. elegans* worms were transferred to bacterial lawns and incubated at 25ºC for up to 24 hrs. Plates were observed by microscopy, and viable worms were those who exhibited motility.

Antifungal studies: TSYE agar plates were prepared containing Candida albicans blastospores (1x105 cells/ml) and kanamycin (50 µg/ml). A loopful of broth cultures of UC17F4 and mutant strains were streaked on plates, which were incubated at 30ºC for 24-48 hrs. Plates were photographed on a light box.

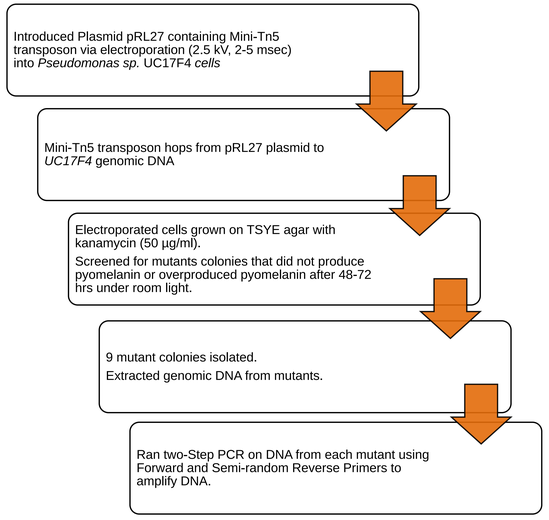

Fig. 1. Schematic of transposon mutagenesis and isolation of pyomelanin-defective and pyomelanin-overproducing mutants of *Pseudomonas* sp. UC17F4.

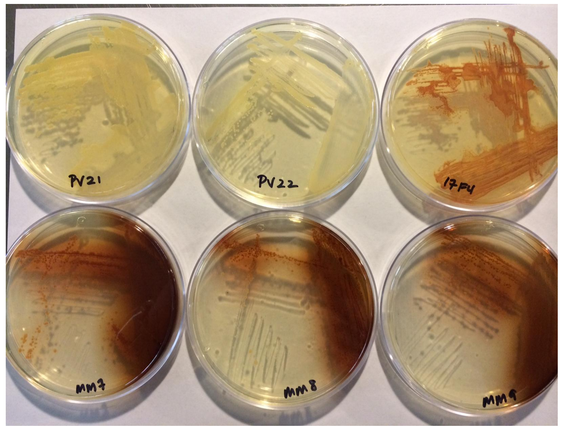

Fig. 1. Pyomelanin production in wild-type and mutant strains of *Pseudomonas* sp. UC17F4. Bacteria were streaked on plates of TSYE agar, and incubated under constant illumination at 30ºC for 48 hours.

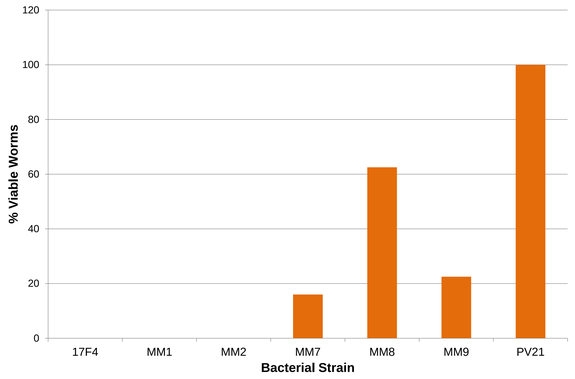

Fig 3. Mutant strains of UC17F4 with high levels of cell-associated pyomelanin have exhibit high virulence in *C. elegans*. L1 and L2 stage worms were transferred to lawns of various mutant strains, and analyzed for viability after 24 hrs of incubation. Data are expressed as percentage of total worms inoculated onto plates that were viable after 24 hours.

Time (hours)	Trial 1 (+tyr)	Trial 2 (+tyr)	Trial 3 (+tyr)	Trial 3 (-tyr)	Trial 4 (+tyr)	Trial 4 (-tyr)	Trial 5 (+tyr)	Trial 5 (-tyr)
0	100	100	100	100	100	100	100	100
2.5	100							
5.5	97							
6.5	93							
9	88							
10			91	100				
10.5		0						
11			81	100				
13.5			55	100				
14					0	100	0	90
15	75		0	92				

Fig. 4. Pyomelanin production in *Pseudomonas* sp. UC17F4 results in loss of viability in *C. elegans*. Lawns of bacteria were grown on NGM with or without 0.1% tyrosine. Only bacteria grown on tyrosine produced pyomelanin. L2/L3-stage worms were transferred to bacterial lawns, and analyzed for viability at various time intervals. Data are expressed as percentage of total worms inoculated onto plates that remained viable at the various time intervals. Loss of viability was only observed with melanized bacterial cultures.

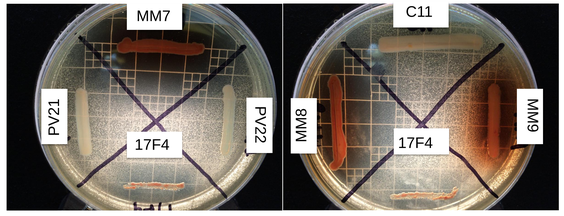

Fig. 5. Antifungal activity of UC17F4 is associated with pyomelanin production. TSYE+Km agar plates were prepared containing *C. albicans* blastospores. Bacterial cultures were streaked on plates and incubated under constant illumination at 30ºC. Zones of inhibition were evident around melanized bacterial streaks, but not around unpigmented strains.

CONCLUSION

Based on the results of these studies, we are able to conclude that pyomelanin production in *Pseudomonas* sp. UC17F4 is associated with virulence of the organism in *Caenorhabditis elegans*. Virulence of the bacteria appears to be more associated with cell-associated pyomelanin than with the secreted form of the pigment. Furthermore, production of pyomelanin enhances the antifungal properties of the bacteria. Only melanized strains of the bacteria exhibit strong inhibition of Candida albicans in an agar plate bioassay.

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