Investigation of Potential Virulence Factors in a Novel *Pseudomonas* Species

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ABSTRACT

Pseudomonas sp. UC17F4 is a novel species that was isolated from the skin of red-backed salamanders collected in Central New York State. UC17F4 is a unique bacterial species that produces both extracellular pyomelanin and intracellular eumelanin. We have explored the possibility that bacterial melanins are a virulence factor utilizing the microscopic nematode *Caenorhabditis elegans* as a model host organism. *C. elegans* larvae were transferred to lawns of UC17F4 under conditions where the bacteria produced eumelanin. We observed that L1 and L2 stage larvae did not survive in the presence of this bacterium, while L3, L4, and adult worms did. In the present study, UC17F4 was grown on Lawrence minimal media (LMM), a citrate-enriched chemically-defined medium, that allows melanization. On LMM, UC17F4 caused complete loss of L1 stage worm viability between 12-24 hrs. When LMM was supplemented with 0.25% peptone (LMMP) to enhance melanogenesis, L1 worms died within 4 hours after transfer to bacterial lawns. We hypothesized that the rapid loss of viability on LMMP was due to the production of proteases, which could degrade the thin cuticle of larval worms, while the delayed loss of viability on LMM media was due to toxic effects of eumelanin as the worms consumed the bacteria. To test this hypothesis, protease production by UC17F4 biofilms was measured using a fluorimetric enzyme assay. Three media formulations were used: LMM, LMMP, and citrate-free LMMP (LMMP-CIT). All biofilm studies showed that the bacteria produced protease only in the citrate-free LMMP, suggesting that citrate represses protease production through carbon catabolite repression. These data also refute our hypothesis that proteases are responsible for the rapid death of worms on LMMP, because bacteria grown on citrate-free LMMP do not kill *C. elegans*. Alternatively, UC17F4 produces higher levels of extracellular pyomelanin on LLMP than on the other media, which could contribute to the rapid loss of viability in the worms. Our findings indicate that protease is not a potential virulence factor in UC17F4, and we are continuing to identify other factors that may be responsible for quick death in juvenile nematodes.

BACKGROUND

Pseudomonas sp. UC17F4 is a novel species isolated from red backed salamanders (van Kessel et al., 2003). Biochemical and molecular genetic evidence suggest that this isolate is the prototype of a previously undescribed species, for which the name *Pseudomonas uticensis* is being proposed (Lawrence et al., manuscript in preparation). UC17F4 produces two different forms of melanin; high molecular weight eumelanin, and low molecular weight pyomelanin (McHarris et al., 2015). Citrate is the favored carbon source in the production of both pyomelanin and eumelanin (Lawrence et al., 2015). Melanin has been seen as a virulence factor in several organisms, including bacteria and fungi (Nosanchuk and Casadevall, 2003; Plonka and Graback, 2006; Liu and Nizet, 2009). We utilized *Caenorhabditis elegans* as a model to explore the possibility of virulence in UC17F4. *C. elegans* are soil-dwelling nematodes that eat bacteria. In the laboratory, they are fed OP50 (*E. coli*) (Marsh and May, 2012). *C. elegans* have a cuticle composed of 80% collagen (Cox et al., 1981). We previously demonstrated that UC17F4 exhibits virulence in L1 and L2 larval stage *C. elegans,* but not in L3, L4 or adult worms (Morreale et al., 2014; Brockett et al., 2015). Initially, we hypothesized that melanin was a virulence factor; however, due to the quick kill of the L1 and L2 larvae, we hypothesized that proteases that are produced by UC17F4 may be a potential virulence factor for the killing of L1 and L2 *C. elegans*, as it may degrade the collagen-rich cuticle. To measure protease activity, we cultured UC17F4 in Lawrence Minimal Media (LMM), LMM supplemented with peptone (LMMP), and LMM salts supplemented with peptone but without citrate (LMMP-CIT), and then performed fluorimetric assays for protease activity in cells grown in the different media.

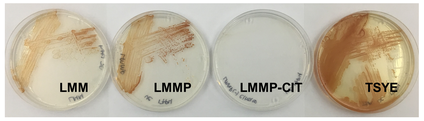


Fig 1. Pigment production in UC17F4 on different growth media. UC17F4 was streaked on LMM, LMMP, LMMP-CIT and TSYE agar plates. A brown pigment is produced on all media except LMMP-CIT, where bacterial growth was greatly reduced.

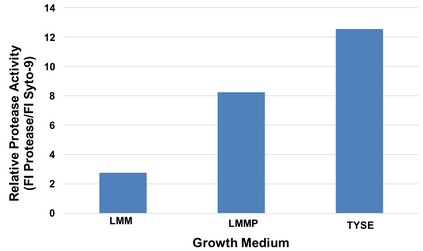


Fig 2. Protease activity of UC17F4 was the highest when cultured in protein enriched broth media. UC17F4 was grown in LMM, LMMP, and TSYE media overnight in an illuminated 30°C incubator. Then we centrifuged cultures and collected the supernatant to perform EnzChek® Peptidase/Protease Assay to measure protease activity. We added sterile broth to resuspend the cells and then performed a Syto-9 assay to determine cell counts. Protease activity is expressed as the ratio of protease assay fluorescence to Syto-9 fluorescence.

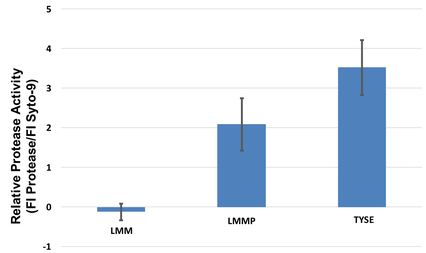


Fig 3. Increased protease activity present in UC17F4 biofilms grown on nitrocellulose filters on TSYE agar. UC17F4 was grown on LMM, LMMP, and TSYE with three nitrocellulose filters on each agar plate overnight in an illuminated 30°C incubator. Then we removed the nitrocellulose filters, added sterile broth to remove the cells and then performed a Syto-9 Assay. EnzChek® Peptidase/Protease Assay was performed to measure protease activity. Protease activity is expressed as the ratio of protease assay fluorescence to Syto-9 fluorescence.

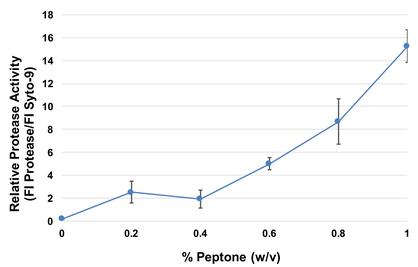


Fig 4. As citrate concentration decreased and peptone concentration increased protease activity increases. We cultured UC17F4 in LMM (1.0% citrate & 0.0% peptone), LMM (0.8% Citrate & 0.2 peptone), LMM (0.6% Citrate & 0.4% Peptone), LMM (0.4% citrate & 0.6% Peptone), LMM (0.2% Citrate & 0.8% Peptone), LMM (0.0% Citrate & 1.0% Peptone) overnight in an illuminated 30°C incubator. Then we centrifuged cultures and collected the supernatant to perform EnzChek® Peptidase/Protease Assay to measure protease activity. We added sterile broth to re-suspend the cells and then performed a Syto-9 assay. Relative protease activity is expressed as the ratio of protease assay fluorescence to Syto-9 fluorescence.

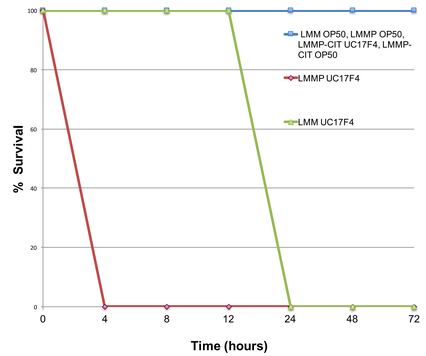


Fig 5. Kinetics of L1 *C. elegans* viability when cultured on UC17F4 grown on different LMM compositions: L1 *C. elegans* die within 4-24 hours after exposure to UC17F4 when grown on LMM and LMMP. Wild-type (N2) L1 worms (n = 10/group) were transferred to lawns of UC17F4 or OP50 (control *E. coli* feeding strain) grown on LMM, LMMP, or LMMP-CIT agar media. Touch assays were used to determine viability at 0, 4, 8, 12, 24, and 72 hours after transfer. Kaplan-Meier survival curves were generated and pairwise comparisons (log-rank test) by strain and media composition were as follows: $p < 0.0001$ for LMM UC17F4 vs. LMM OP50, LMMP UC17F4 vs. LMMP OP50, LMM UC17F4 vs. LMMP UC17F4, LMM UC17F4 vs. LMMP-CIT UC17F4, and LMMP UC17F4 vs. LMMP-CIT UC17F4; $p > 0.05$ for LMMP-CIT UC17F4 vs. LMMP–CIT OP50.

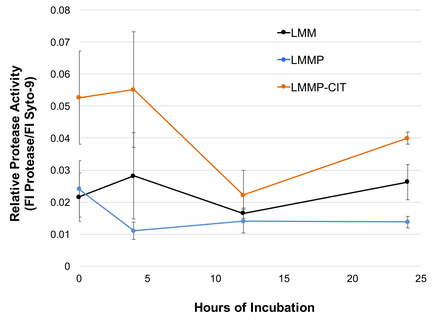
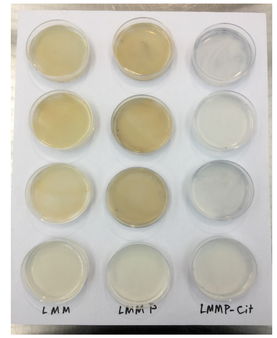


Fig 6. Protease activity is not a virulence factor for L1 *C. elegans*. UC17F4 was cultured in LMM, LMMP, and LMMP-CIT broth media overnight in an illuminated 30°C incubator. Then we performed a Syto-9 Assay and EnzChek® Peptidase/Protease Assay to measure protease activity. Relative protease activity is expressed as the ratio of protease assay fluorescence to Syto-9 fluorescence. Four hours post incubation, protease activity was the lowest in LMMP, while at this time point UC17F4 grown on LMMP showed the most lethality to L1 *C. elegans*. Furthermore 24 hours post incubation, protease activity was greatest in LMMP-CIT but there was no lethality.



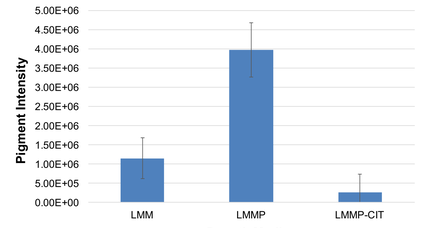


Fig 7. Pigment intensity of pyomelanin and eumelanin produced by UC17F4 was the highest when cultured in LMMP media. UC17F4 was cultured in LMM, LMMP, and LMMP-CIT broth media overnight in an illuminated 30°C incubator. Plates were photographed under identical lighting conditions, converted to grayscale, inverted in color, and pigment intensity was determined using ImageJ software. The bottom row of plates in the photograph are uninoculated controls, and used to subtract background pigmentation.

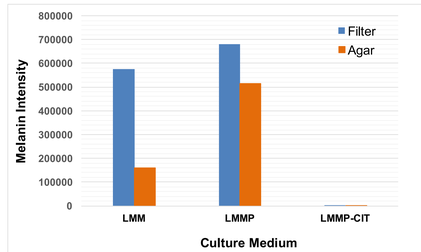


Fig 8. UC17F4 produces the highest levels of intracellular melanin and secreted pyomelanin on LMMP medium. UC17F4 was grown on LMM, LMMP, LMMP-CIT agar with nitrocellulose filters in an illuminated incubator overnight at 30°C. The culture plates were illuminated for an additional 24 hours at room temperature. Plates were photographed under identical lighting conditions, converted to grayscale, inverted in color, and pigment intensity was determined using ImageJ software. Filters were removed and plates were photographed again to measure pyomelanin that had diffused into the agar under the filters.

CONCLUSION

Pseudomonas sp. UC17F4 (*P. uticensis*) exhibits virulence when exposed to L1 and L2 larvae of the nematode, *C. elegans*. We hypothesized that bacterial proteases may be a virulence factor, causing degradation of the thin collagenous cuticle of the worms. However, our data refute this hypothesis, as protease activity is repressed under the bacterial growth conditions where killing of the larval worms is the most rapid. It is probable that protease activity is under carbon catabolite control in UC17F4, since relative activity is highest in citrate-free medium. Our data do confirm that bacterial melanin production is highest in the culture conditions where virulence is greatest, suggesting that one or both types of melanin may be a virulence factor.

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