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Light Effects on Pyomelanin Production in a Novel *Pseudomonas* Species

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ABSTRACT

Some *Pseudomonas* species produce pyomelanin in order to protect the cell against damage from ultraviolet light. Several years ago we isolated a novel species of Pseudomonas that produces a dark reddish-brown pigment on nutrient-rich media. This pigment is dependent on tyrosine in the medium, and has been identified as pyomelanin (PM). Recent work in our lab showed that PM production also is light-dependent and not produced when bacteria are grown in complete darkness. To further investigate photoinduction of PM in a novel *Pseudomonas* species, this two-part study focused on quantifying the effects of light intensity and the length of exposure to light on PM production. *Pseudomonas* sp. UC17F4 was cultured at high and low density on TSYE agar plates. The goal of the first part of the study was to control the amount of time the bacteria were subjected to a constant light intensity (300 lux). The plates were subjected to light in intervals, from 5 to 45 min., with plates remaining in complete darkness and in 300 lux for 24 hours as controls. The second part of the study focused on varying light intensities over a constant time period. Freshly spread plates were placed under lights at 100, 200, 250, and 300 lux (with a control in complete darkness) for 24 hours. After incubation, bacteria were suspended by gently scraping the bacteria off the agar in PBS. The samples were centrifuged to pellet the cells, which were then weighed. Cell pellets were resuspended in 1% SDS and disrupted by boiling. Cell lysates were analyzed by UV spectrometry, and data were expressed as A_{335}U/g wet wt. PM production tended to be dependent on the density of cells, with more PM produced in cells plated at higher density. A two-fold increase of PM content was exhibited in time exposures up to 35 min. The light intensity study showed an increase of melanin up to 250 lux. Lastly, it was shown that a significant amount PM was produced in the dark controls. The results show that PM production is dependent on the duration of exposure to light and on the light intensity. The fact that PM production declines above 250 lux suggests that photobleaching may occur. Also, the fact that time- and intensity-dependent changes in PM levels were of lesser magnitude suggests that quorum sensing may also induce PM production in this organism.

BACKGROUND

Microorganisms can be found in a variety of environments – in the guts of animals, on inanimate objects, or in every type of terrestrial or aquatic habitat. 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 two bacterial strains, UC179D3 and UC17F4, from the cutaneous microbial flora of female red-backed salamanders. These bacterial strains have the ability to produce potent antifungal compounds. Biochemical and DNA sequence analysis aided the team in discovering that the strains are species of *Pseudomonas.* They were able to identify a number of genes in UC179D3 matching the homology of *P. fluorescens.* However, 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 the result of pyomelanin production. 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). These observations suggest that pyomelanin production in *Pseudomonas sp.* UC17F4 is photoregulated.

In the present study, we investigate the effects of visible light intensity and time of exposure on the production of pyomelanin in *Pseudomonas* sp. UC17F4.

METHODS

Cultures of *Pseudomonas* sp. UC17F4 were maintained by weekly transfer on tryptic soy – yeast extract (TSYE) or Luria-Bertani (LB) agar. Seed cultures for experiments were prepared by inoculation of bacteria into Nutrient Broth (which minimized pyomelanin production), and incubation overnight at 30°C in a water bath shaker.

For experimental cultures, seed cultures were diluted, stained with crystal violet solution and counted on a hemocytometer. Aliquots containing 10^8 cells were transferred to 8.5 cm Petri dishes containing 15-20 ml TSYE agar, and were spread with a sterilized glass "hockey stick". In some experiments, plates were inoculated by streaking bacteria with a sterile metal wire loop. In different experiments, plates were exposed to room light (50 lux), or more intense light from fluorescent light sources. Experiments were conducted in a temperature-controlled room at 30°C. Different light intensities were accomplished by adjusting the distance from the light source, and monitored using a light meter. "Dark" cultures were inoculated in diminished room light, wrapped in aluminum foil and incubated at 30°C.

At the conclusion of light exposure, 3 ml of phosphate buffered saline (PBS) was added to each of the agar plates and the cells were gently scraped off the agar using the tip of a sterile 10 ml pipette. The suspended cells were pipetted from the plates into pre-weighed microcentrifuge tubes. The tubes were centrifuged for 2 min at 14,000xG. The resulting supernatants were discarded and microcentrifuge tubes containing the cell pellets were weighed to determine the mass of the pellet. 1 ml of 1% sodium dodecyl sulfate (SDS) was then added to each tube to resuspend each pellet. Tubes were vortexed for approximately 3 seconds to ensure distribution of the pellet, then sealed with parafilm and placed in boiling water for approximately five minutes, until the lysates became clear. Lysates were transferred to glass calibrated test tubes, and the volume was brought to 3 ml with 1% SDS. The samples were analyzed for absorbance at 335 nm in a Spectronic 21D UV/VIS spectrophotometer, using 1% SDS as a blank. Pyomelanin content was expressed as absorbance at 335 nm per gram of wet cell pellet weight.

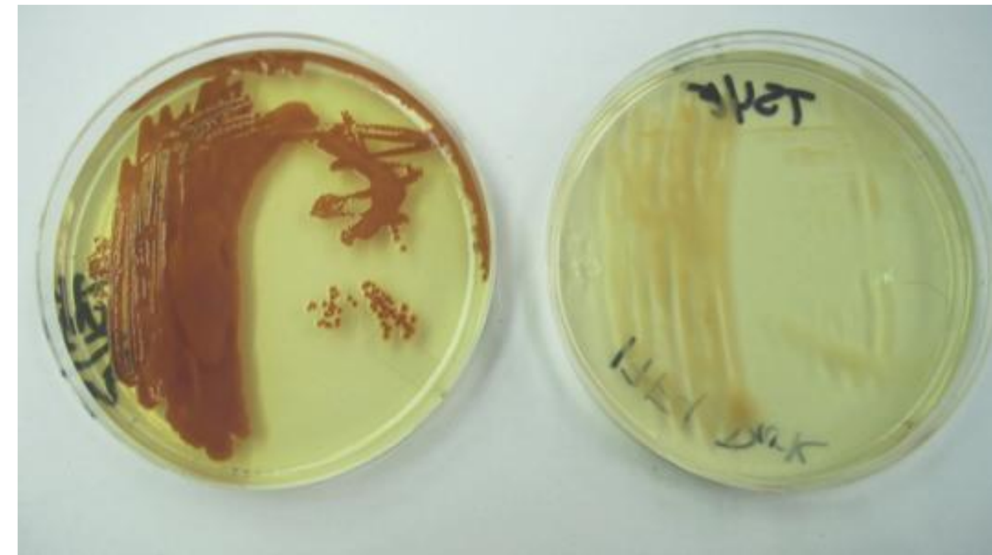

Fig. 1 Pyomelanin production in *Pseudomonas* sp. UC17F4 is light dependent. Bacteria were cultivated on TSYE agar for 72 hrs at 30°C. The plate on the left was exposed to room light during the incubation period, while the plate on the right was incubated in the dark. Dark brown pigmentation is clearly evident only in the light-exposed bacteria.

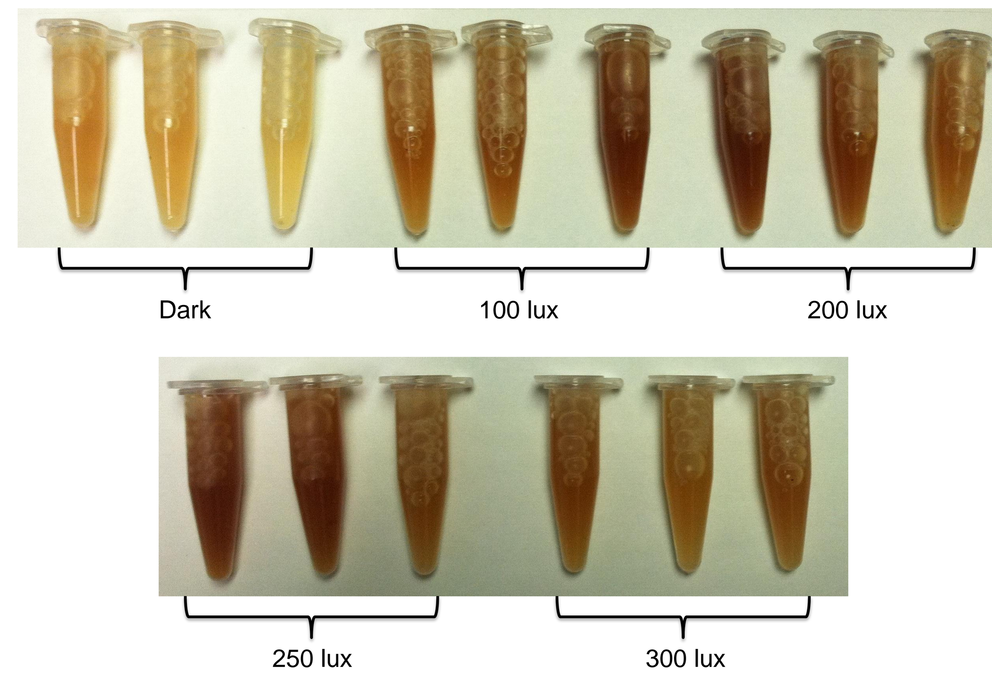

Fig. 2. Visual evidence of stimulation of pyomelanin production in UC17F4 exposed to bright light of varying intensities. Bacteria were plated as a lawn onto TSYE agar plates in low light, then exposed to constant light of intensities up to 300 lux for 24 hours. Bacteria were harvested by rinsing plates with PBS, pelleted by centrifugation, weighed and then lysed by boiling in 1% SDS as described in "Methods". Lysates in tubes were photographed before spectrophotometric analysis. Results reveal that exposure to bright light as low as 100 lux is sufficient to stimulate pyomelanin production.

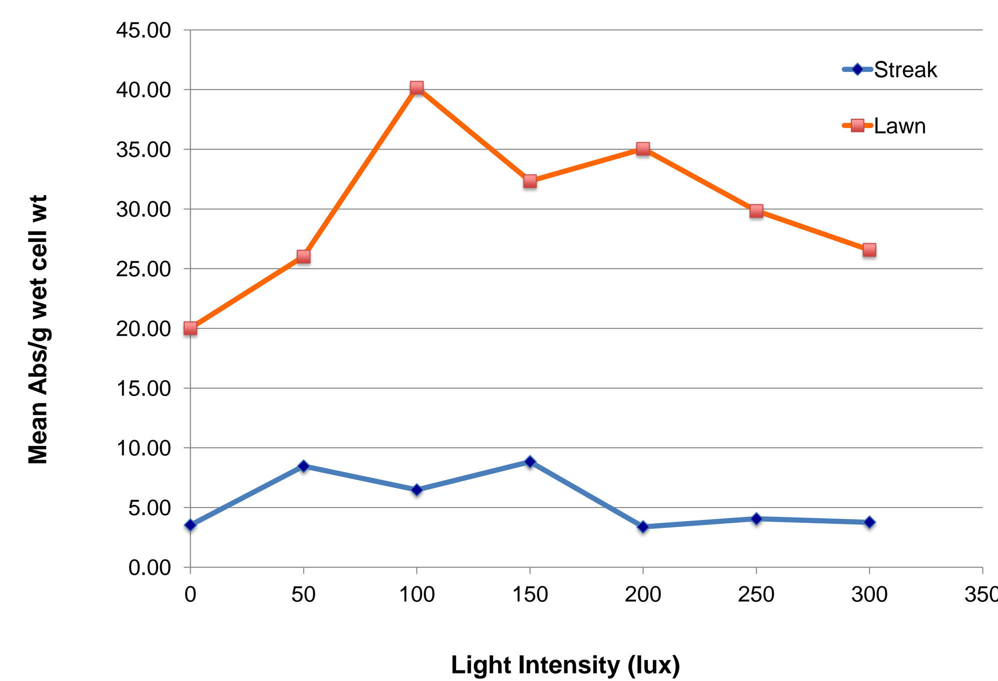

Fig. 3. Pyomelanin content in UC17F4 is dependent on light intensity. Bacteria were either streaked or spread by the hockey stick method at a density of approx. 10^8 cells/plate and incubated at 30°C for 24 hrs at the specified light intensity. Data reveal lower melanin content at intensities greater than 150 lux. Data also indicate that bacterial cells growing at higher density in lawns produce more pyomelanin than those streaked on plates.

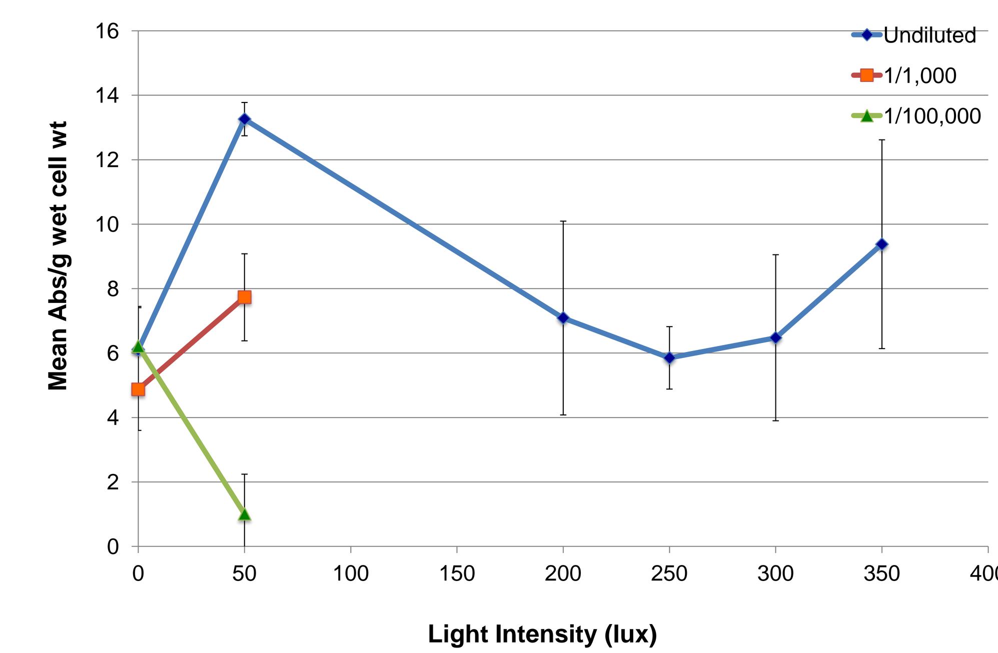

Fig. 4. Increasing light intensity inhibits growth of diluted cultures of UC17F4. Approx. 10^8 cells were spread on TSYE agar plates designated as "Undiluted", as well as equivalent volumes of 1/1,000 and 1/100,000 dilutions of the stock culture, and plates were incubated for 24 hrs at 30°C under illumination of varying intensity. "0" controls were covered in aluminum foil during the incubation period. Results indicate that pyomelanin production occurs optimally at 50 lux in the undiluted plates, as well as in the 1/1,000 dilution plates. Pyomelanin production appears to be inhibited in the 1/100,000 dilution at 50 lux. In both diluted cultures, no growth occurred on plates incubated at light intensities greater than 50 lux.

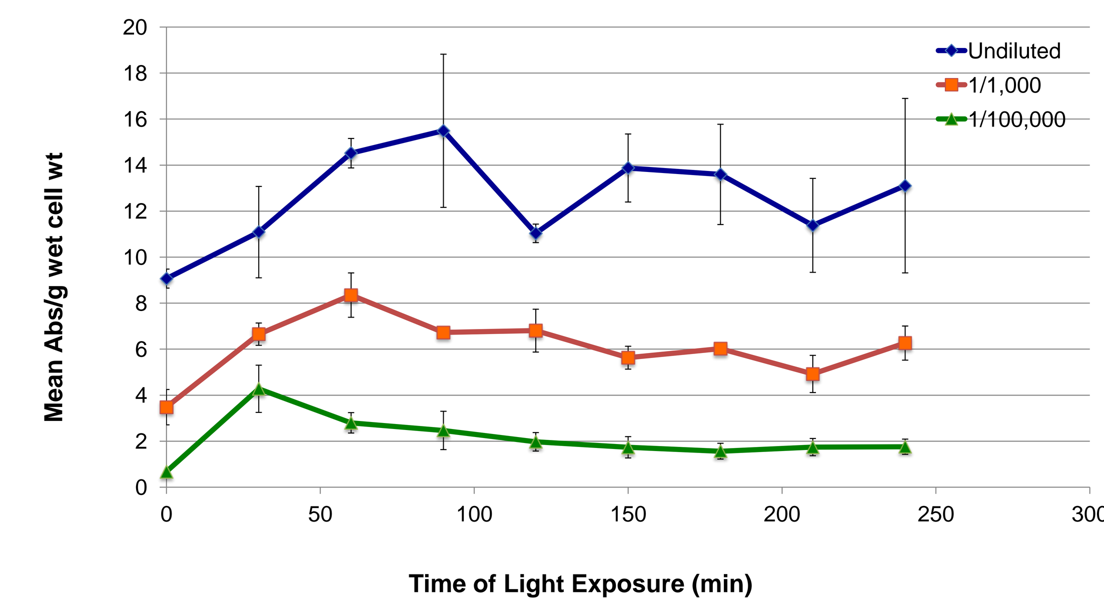

Fig. 5.. Exposure to intense light stimulates pyomelanin production, but also causes photobleaching. UC17F4 was spread on TSYE agar plates and incubated at 30°C under 50 lux illumination for 24 hrs, then exposed to 350 lux illumination for up to 4 hrs prior to harvest and extraction. Data show that the rate of increase in pyomelanin content is dependent upon cell density, and that a decline in pyomelanin content due to photobleaching occurs more rapidly and to a greater extent in more diluted cultures.

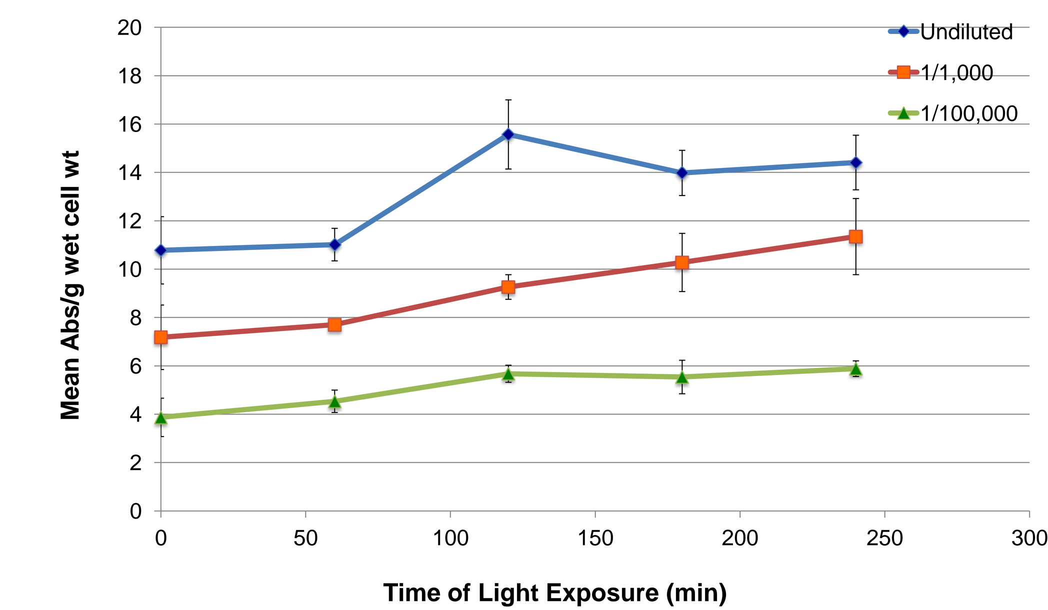

Fig. 6. Extended exposure to intense light light stimulates pyomelanin production in UC17F4 broth cultures. Bacteria were grown in 3 ml TSYE broth in 3.5 cm diameter Petri plates for 24 hrs at 30°C under 50 lux illumination. Plates were then exposed to 350 lux illumination for up to 4 hrs prior to harvest and extraction. Results are consistent experiments conducted on agar medium, showing that bright light further stimulates pyomelanin production in the bacteria. No photobleaching was apparent with extended exposure to bright light in this experiment.

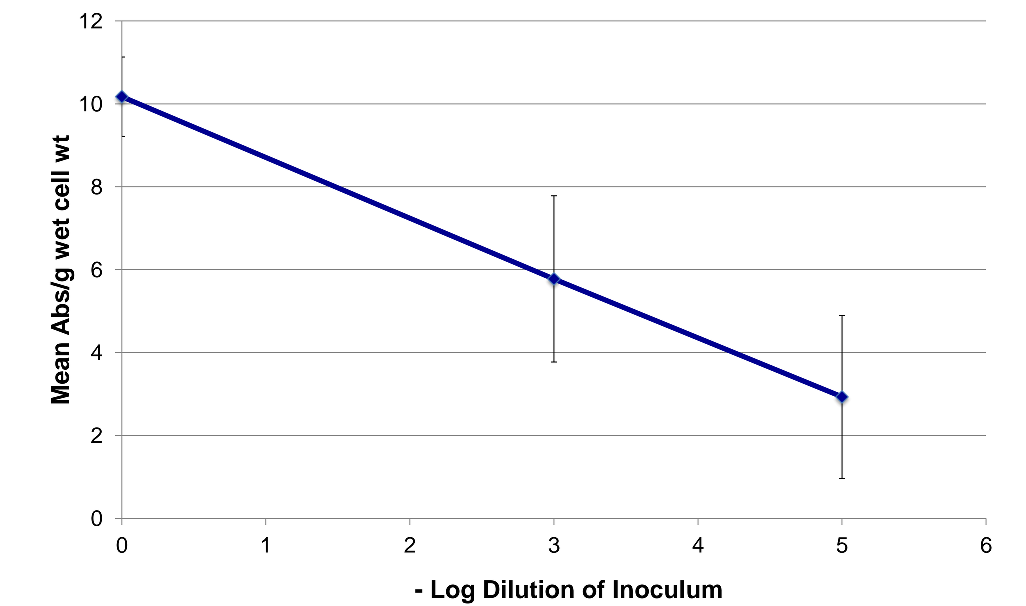

Fig. 7. UC17F4 cell density affects pyomelanin content. Data for "time 0" points from three separate experiments where cultures were grown for 24 hrs at 30°C under 50 lux illumination were averaged and expressed as means $\pm$ standard error of the mean. These data show that under identical conditions of illumination, pyomelanin content in UC17F4 varies with respect to culture cell density. This suggests that quorum sensing pathways may also contribute to the stimulation of pyomelanin synthesis in *Pseudomonas* sp. UC17F4.

CONCLUSION

Based on the results of these studies, we are able to conclude that pyomelanin production and degradation in *Pseudomonas* sp. UC17F4 are dependent on both light and cell density. Photobleaching of pyomelanin occurs in cultures grown on agar media and exposed to high intenisty light after the pyomelanin was produced; more diluted cell cultures exhibit a greater decline in pyomelanin content. Evidence of quorum sensing in these studies leads us to a greater understanding of the magnitude differences in pyomelanin between different dilutions. Future studies will focus on determining if there is any structural damage to the bacteria during exposure to high intensity light by transmission electron microscopy studies and viability staining methods. Another future goal is to produce a gene knock-out library to determine the genes involved in photoregulation and cell density-dependent control of pyomelanin production in *Pseudomonas* sp. UC17F4..

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