

Genetic and biochemical analyses of pigmented ring formation in colonies of *Pseudoalteromonas sp.* 15DK1

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Abstract

Microbial growth on solid media can lead to the formation of a diverse array of colony morphologies, and these morphologies can be useful in understanding how microbes grow and respond to various challenges presented by such a dense and dry style of growth. A new isolate from seawater in Garbage Beach, *Pseudoalteromonas sp.* 15DK1, was isolated in the laboratory and found to form concentric pigmented rings. A genetic approach was undertaken to identify potential biological aspects of this pattern formation, including mechanisms that drive the formation of the rings from pigment-producing strains, as well as the production of the pigment itself. Mutants were isolated that show deficiency in ring formation, which may reveal information about how these concentric rings are formed. Extraction of the pigments revealed that different pigments may be produced during different growth phases on solid media, or in specific conditions in liquid media. Future analyses will be necessary to determine the identity of the pigments, as well as how they influence the formation of the ringed colonies.

Background

Patterns in microbial colony formation can range from simple, pale, circular colonies to a range of diverse colony morphotypes based on varied growth parameters. *Bacillus subtilis*, for example, can form a variety of different colony morphotypes depending upon nutrient availability and agar concentration, ranging from complex fractal-shaped colonies at high agar concentration and low nutrient availability, to colonies with periodic ring-shaped growth patterns at medium nutrient availability and medium agar concentration (Fujikawa, 1994). *Pseudomonas aeruginosa* can form extensively wrinkled colonies in an apparent attempt to maximize surface area for oxygen consumption when the redox balance inside the cells are altered due to genetic manipulation in their ability to produce phenazines (Dietrich *et al.*, 2013). Although the growth patterns of bacterial colonies under laboratory conditions may not be representative of the style of growth typical of the natural environment, analysis of these patterns and attempts to perturb them can reveal new features of microbial cell physiology as well as how cells interact with each other and the environment.

Pseudoalteromonas sp. 15DK1, isolated in the 2015 Microbial Diversity course in Woods Hole, MA, shows a colony morphology consisting of many concentric pigmented rings interspersed by non-pigmented rings. The similarity between this strain and a similar strain, *P. nigrifaciens*, described in 1969, suggests that this new strain may produce an indigoidine-like molecule (Norton and Jones, 1969). However, it is unclear why the pigment does not present throughout the entire colony, but instead in rings. The phenomenon could be explained by diffusion of the pigment out from the center of the colony, or by the formation of Liesegang rings, where a supersaturated solution of a chemical precipitates in a ring-like pattern in a gel matrix. Alternatively, the ring shapes could be produced by some aspect of the organism's biology, such as motility of pigment-producing cells away from the center of the colony, diffusion of signaling molecules to specific points in the colony to stimulate pigment production, or reactions of cells to metabolic signals such as oxygen concentration or redox potential.

The usage of genetics offers a powerful tool to assess whether biological functions play a role in the formation of rings. By mutagenizing cultures and looking for non-pigmented, or ring-deficient mutants, one can trace back mutations to the genes important for the maintenance of these traits. The following experiments are a mixture of chemical mutagenesis to attempt to find genes responsible for pigment production and ringed colony formation, while also using biochemical approaches to extract the pigments and determine some of their properties.

Methods

Media composition and growth conditions

Table 1 – Media composition

	SWC	MLTY
1x Seawater Base		
NaCl	20g/L	
MgCl ₂ ·6H ₂ O	3g/L	
CaCl ₂ ·2H ₂ O	0.15g/L	
KCl	0.5g/L	
Tryptone	5g/L	0.1g/L
Yeast Extract	1g/L	0.1g/L
Glycerol	3mL/L	-
MES pH 5.5	-	1mL/L
Notes	pH to 7.0	-

Plates were incubated at 30°C, while liquid cultures were shaken at 30°C unless otherwise noted. Plates contained an added 15g/L of agar.

Chemical mutagenesis

Log-phase cultures of *Pseudoalteromonas sp.* 15DK1 were exposed to 80mM methylmethanesulfonate for 40 minutes while shaking at 30°C. Cultures were pelleted and resuspended in 20mM sodium thiosulfate, followed by three washes in 20mM sodium thiosulfate. After the final wash, the pellet was resuspended in MLTY liquid medium and diluted for plating on MLTY. Colony counts were used compared to control plates without MMS treatment to confirm that the mutagenesis had imparted a lethal effect on approximately 50% of the cells. Mutant colonies were identified by visualizing under the Zeiss stereoscope.

Extraction of pigments

For cultures grown on solid media, colonies were picked from the surface of the agar plate and placed into a 1.5mL Eppendorf tube. Typically between 50-200 colonies were picked and added to the tube. For old and very dense regions of growth, cell material was scraped off the surface of the agar plate using an inoculating loop and placed in the tube. For liquid cultures, cells were pelleted for 5 minutes at 10,000xg and the supernatant was discarded. 5-10 mL of culture were used to obtain cell pellets. Once the cell material had been obtained in the tubes, they were mixed with 100% dimethyl sulfoxide (DMSO) and fully re-suspended by pipetting and vortexing. Remaining cell material was cleared from the extraction by centrifuging for 5 minutes at 10,000xg and transferring the supernatant.

Absorption spectrophotometry

Absorption spectra were obtained by shining a light source through a narrow (approximately 0.3 cm) slit into a 0.2cm plastic cuvette containing the sample, and finally into the detector. The machine was blanked using DMSO, and baseline values were obtained from the mean of five measurements of DMSO alone. Absorption values were taken as the mean of five replicate spectra.

Results and Discussion

Isolation of Pseudoalteromonas sp. 15DK1

The isolate was grown from seawater taken from Garbage Beach in Woods Hole, MA, using seawater complete (SWC) agar plates. The colony was found after three days incubation at 30°C as a dark-pigmented colony in a depressed region of the agar plate, indicative of agar-lytic activity. The organism was streaked three times for isolation, once on SWC plates, and twice on marine-LTY plates (MLTY). Generally, colonies formed after 24-36 hours incubation, eventually beginning to become pigmented after approximately 48 hours. Cultures grown in liquid medium did not show any pigmentation. With shaking at 30°C in rich medium, cultures grew with an approximate doubling time of 47 minutes. The cells are motile rods.

Pigmentation and formation of concentric rings

Colonies grown on SWC or MLTY plates would become pigmented after 3 days of incubation at 30°C. Colonies grown on SWC would typically become pigmented throughout the colony, and especially toward the edges of the colony, while colonies growing on MLTY plates would become pigmented in the center of the colony first, and eventually throughout after 4-6 days. After incubation for 3-5 days, colonies showed distinct pigmentation patterns wherein pigmented rings were interspersed with non-pigmented rings (Figure 1).

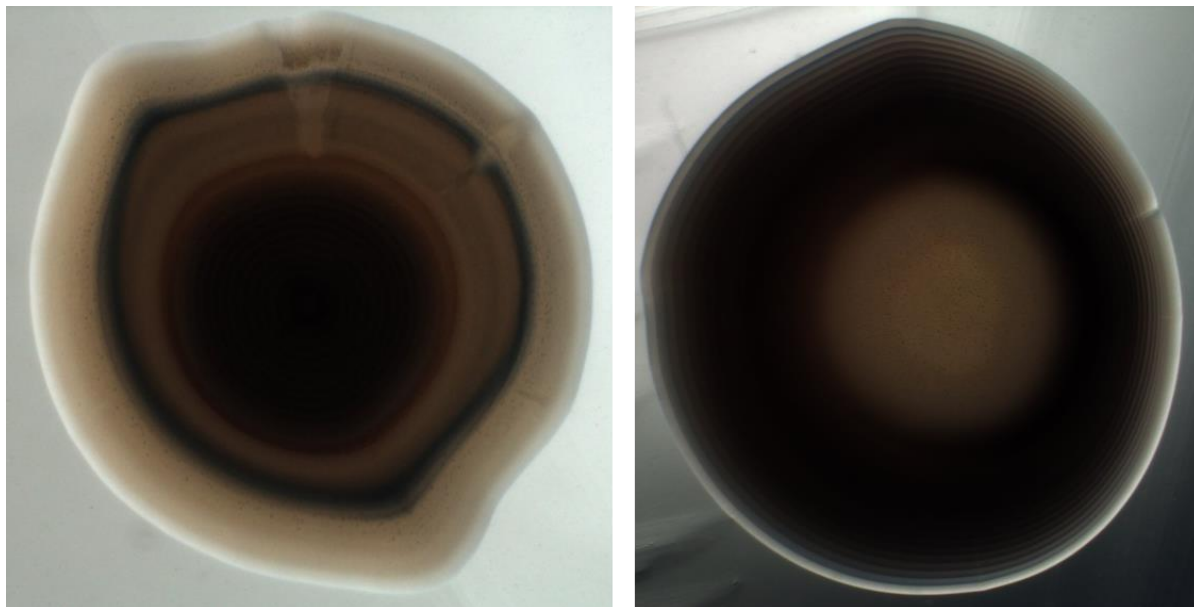


Figure 1 – Representative colony morphologies of *Pseudoalteromonas sp.* 15DK1 on MLTY plates (a) and SWC plates (b). Colonies are both approximately 3-4mm in diameter at the time of imaging. Imaging performed using a Zeiss stereoscope.

Mutagenesis of Pseudoalteromonas sp. 15DK1

Transposon mutagenesis was attempted using multiple transposon-carrying *Escherichia coli* strains, but none of the conjugations were successful. For chemical mutagenesis, log-phase cultures of 15DK1 were exposed to 80mM MMS for 40 minutes, followed by neutralization in 10mM sodium thiosulfate. Mutagenized cultures were plated on MLTY to observe colony morphologies. Major phenotypes that were investigated were pigmentation mutants that failed to produce pigment, and ring mutants that produced pigments but failed to generate concentric ring structures. Three mutants were identified in genetic screens of colony morphology.

While most colonies form on MLTY plates as white colonies with dark central ring structures, 15DK2 was discovered as a colony that was slightly pigmented throughout. Magnified viewing of the colony revealed that there was still a ringed structure of pigmentation, but the ‘non-pigmented’ rings of the colony were a grey color, rather than the typical white. This could be the result of a regulatory mutation that has caused the overproduction of pigment molecules. It could also be a separate regulatory mutation that causes leaky production of the pigment in rings of the colony that normally do not produce pigment. This genome has been placed in a queue for genome sequencing, which may offer future insights into this mechanism, yet time constraints have not

15DK3 was discovered as a pale colony that exhibited very little pigmentation. Magnified viewing of the colony revealed that the colony is producing pigment, but with a very poorly defined ring structure (Figure 2a). There is production of what are presumably granules of pigment forming a circle in the center of the colony. At the very center the colony appears light brown, with a diffuse blue ring surrounding the brown area. This mutant is especially interesting, because it maintains the ability to produce pigment without forming rings. It may be producing lower amounts of pigment, which do not form rings because the concentrations are too low when it diffuses away from the pigmented center. Alternatively, there is some missing biological function that is crucial for ring formation. Further analysis of this colony will be necessary to determine what biological functions are perturbed, as well as genome sequencing to

determine the corresponding genetic mutations. Currently, genomic DNA has been extracted, but time limitations did not allow for sequencing and further analysis.

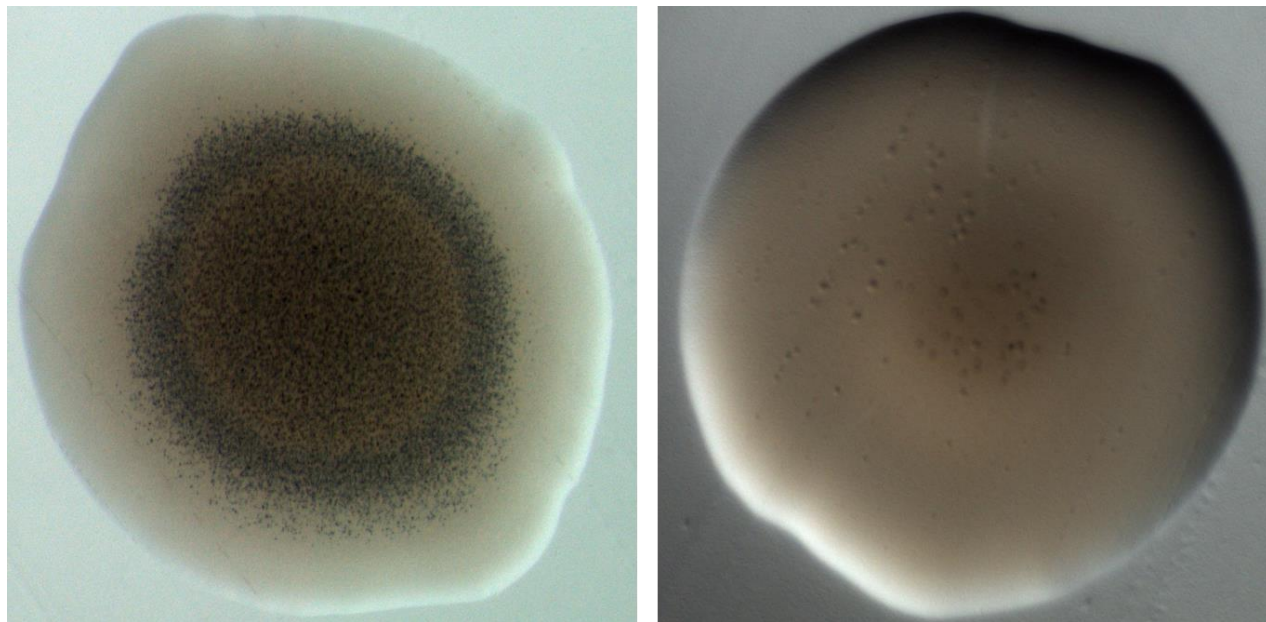


Figure 2 – Chemical mutants of *Pseudoalteromonas sp.* 15DK1. a) Ring formation mutant 15DK3, showing production of pigment without multiple concentric rings. Colony is approximately 3mm in diameter. b) Pigment-deficient mutant 15DK4. Colony is approximately 1mm in diameter.

15DK4 was discovered as a pigment-deficient strain without any coloration. Magnified viewing of the colony showed potentially very minimal production of pigment granules (Figure 2b). However, this neither of the two colonies that exhibited this phenotype could be cultured in liquid media. These colonies also remained quite small on the MLTY plates, even given that they had plenty of space around them to grow. If the pigment is required for later stages of growth on solid media, perhaps as some adaptation to metabolic stresses, then these cells could have been dead after 6-8 days of incubation without the pigment, and therefore they could not be grown further in liquid media. Alternatively, the lack of pigment production could be a side effect of some other metabolic mutation that precludes this strain from growing under certain conditions.

Extraction and analysis of pigments

The indigoidine-like molecule of *P. nigrifaciens* had been previously found to be soluble in concentrated H_2SO_4 , pyridine, dimethylformamide (DMF), and dimethyl sulfoxide (DMSO), while slightly soluble in glacial acetic acid, acetone, and quinoline (Norton and Jones, 1969). Pigment was extracted from 15DK1 by re-suspending colonies picked from the surface of agar plates in a solution of 100% DMSO. After a brief (1-5 minute) incubation, the DMSO becomes colored. When the extraction is performed on colonies, the resulting solution is blue (Figure 3). However, when the extraction is performed on old colonies (10+ days), especially in very dense regions of growth, the resulting solution is a brownish purple (Figure 3). The same purple color can be derived from cell pellets of pigmented liquid cultures grown in a chemostat in nutrient-limited medium under slow-growing conditions (doubling time of approximately 11.5 hours).

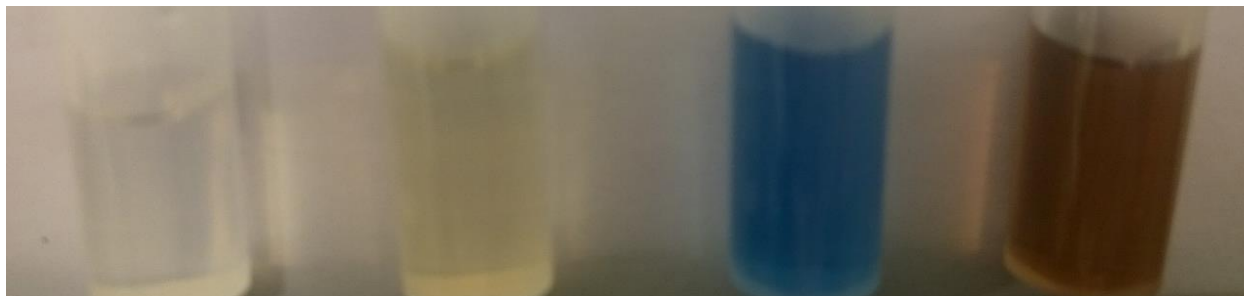


Figure 3 – DMSO extractions of pigment. From left to right: DMSO, DMSO + non-pigmented culture, DMSO + colonies, DMSO + chemostat culture.

Absorption spectra were obtained for both the blue and violet pigmented extracts (Figure 4). The violet pigment shows a broad absorption peak with a maximum value at 481nm. The blue pigment shows a more narrow peak with a maximum value at 609nm. The latter value is very close to previously observed values of indigoidine and the *P. nigrifaciens* indigoidine-like molecule with maximum absorption values at 613nm (Norton and Jones, 1969). The difference in peaks is distinct, and it is interesting given that the production of these different pigments may depend on growth phase. The continuous culture growing at a doubling time of 11.5 hours is likely very similar to a culture in stationary phase, being highly limited for nutrients. The production of the violet pigment may be a response to nutrient limitation. It is unclear what the function of the blue pigment is, given that it can be extracted from colonies throughout most of their growth.

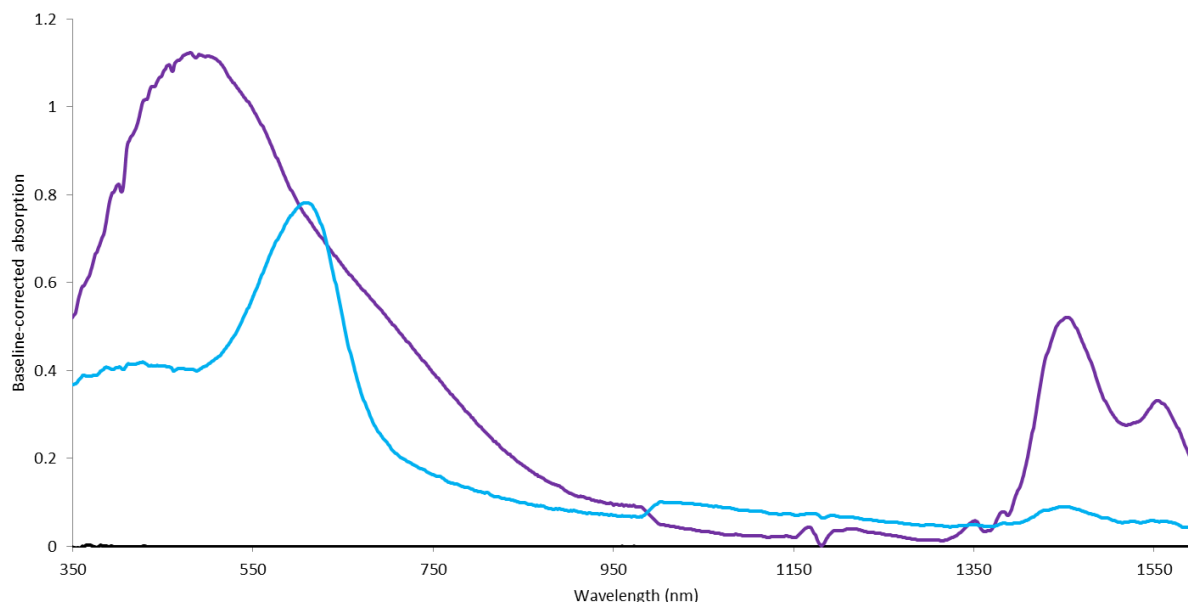


Figure 4 – Absorption spectra of DMSO-extracted pigments. The purple line is the brown-violet pigment extracted from old colonies and slow-growing chemostat culture. The blue line is the blue pigment extracted from colonies.

The pigments were also tested for the possibility that they could be reduced by dithiothreitol (DTT). DTT was added to samples to a final concentration of 0.1M, then incubated at room temperature overnight. The following morning samples were analyzed for any changes. Attempts to reduce the violet pigment with DTT showed no obvious change in coloration. However, reduction of the blue pigment with DTT caused the solution to become colorless. No attempt was made to re-oxidize the pigment, using chemicals such as H_2O_2 , but this would be an interesting future experiment to see if the pigment can be reversibly reduced.

and oxidized chemically. Studies have suggested that the reduction of indigoidine to its colorless form, leucoindigoidine may be enzymatically catalyzed in the cell (Cude *et al.*, 2012).

Indigoidine biosynthesis genes are present in the Pseudoalteromonas sp. 15DK1 genome

Indigoidine is synthesized by the cyclization of glutamine via a non-ribosomal peptide synthetase, often annotated as either *igiD* or *indC* (Cude *et al.*, 2012; Reverchon *et al.*, 2002). The homolog of this gene can be found in the *Pseudoalteromonas sp. 15DK1* genome via a blastp search of the genome using the *igiD* gene from *Phaeobacter sp.* with 44% identity and 62% query coverage (Cude *et al.*, 2012). The gene can be found from at positions 3,611,264 - 3,614,992. Running this gene through the PKS/NRPS Analysis tool (<http://nrps.igs.umaryland.edu/>) suggests by sequence homology that this gene should be specific for glutamine as a substrate, as would be expected for an indigoidine synthase (Bachmann and Ravel, 2009). If this enzyme does utilize glutamine as a substrate, it will need other enzymes to modify the indigoidine molecule to the putative structure inferred from previous studies of *P. nigrifaciens* (Norton and Jones, 1969). These modifying genes may be in the region directly surrounding the *indC* homolog, and further analysis using mass spectrometry and mutagenesis are needed to elucidate the mechanism of pigment formation.

Obligate aerobic growth

Pseudoalteromonas sp. 15DK1 was unable to grow anaerobically in a GasPak system, meaning that it cannot sustain metabolism through fermentation. Genome annotation suggested that this strain may contain genes required for nitrate reduction, so the strain was incubated on MLTY medium containing added 1mM KNO₃. The strain grew normally in the presence of nitrate and oxygen, but no growth was observed on plates after two weeks in the anaerobic chamber (10% N₂, 10% CO₂, 80% H₂) with nitrate but no oxygen. The cells also showed aerotaxis when grown in liquid media without shaking, such that the cells formed a thin layer at the top of the tube with no growth at the bottom. Cultures growing with shaking could also be left at room temperature without shaking and would form films at the air-water interface after a few hours.

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References

- Bachmann, B.O. and Ravel, J. 2009. In silico prediction of microbial secondary metabolic pathways from DNA sequence data. *Methods in Enzymology*. 458:181-217.
- Cude, W.N., Mooney, J., Tavanaei, A.A., Hadden, M.K., Frank, A.M., Gulvik, C.A., May, A.L., and Buchanan, A. 2012. Production of the antimicrobial secondary metabolite indigoidine contributes to competitive surface colonization by the marine Roseobacter *Phaeobacter sp.* Strain Y4I. *Appl. Environ. Micro.* 78:4771-4780.
- Dietrich, L.E.P., Okegbe, C., Price-Whelan, A., Saktah, H., Hunter, R.C., and Newman, D.K. 2013. Bacterial community morphogenesis is intimately linked to the intracellular redox state. *J. Bacteriol.* 195:1371-1380.

Fujikawa, H. 1994. Diversity of the growth patterns of *Bacillus subtilis* colonies on agar plates. FEMS Micro. Ecol. 13:159-168.

Norton, C.F. and Jones, G.E. 1969. A marine isolate of *Pseudoalteromonas nigrifaciens*: II. Characterization of its blue pigment. Arch. Mikrobiol. 64:369-376.

Reverchon, S., Rouanet, C., Expert, D., and Nasser, W. 2002. Characterization of indigoidine biosynthetic genes in *Erwinia chrysanthemi* and role of this blue pigment in pathogenicity. J. Bacteriol. 184:654-665.