The Color of Survival: An Inquiry-based Inter-disciplinary Study of Bacterial Pigments

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Abstract. Pigments are light-absorbing substances that are abundant in nature, serving roles in coloration, camouflage, mate/pollinator attraction and photosynthesis among higher life forms. Among microbial organisms, pigments can also be found in a wide range of phyla. While some of these pigments function in photosynthesis, namely among algae and some bacteria, the great majority of microbial pigments play entirely different roles. The exploratory study presented here will stimulate students to think about the cost and benefit of a heritable trait (pigment production) of microscopic organisms and its effects on survival in a competitive and hostile environment. Exploring and understanding the roles these pigments play allows for a number of cross-disciplinary learning opportunities that combine physics, chemistry, biology and even art, and can be set up as an inquiry-based learning module suitable for small-group and active learning experiences.

Keywords: Interdisciplinary; inquiry-based; bacterial pigments; microbiology; physics

Introduction

Pigment production in bacteria. If you have ever had the opportunity to grow bacteria in a classroom, you will likely have noticed that these microbes can be surprisingly colorful. I am fascinated by the colors produced by bacteria, both in teaching undergraduate microbiology and through my own research. Many bacteria have the ability to produce pigmentation, spanning the spectrum of color from dark purple to yellow. Pigments include violacein (purple), prodigiosin (red), carotenoids (orange-yellow), fluorescein (greenish-yellow), and pyocyanin (blue-green). Colorful bacteria can be isolated from many common sources, including soil, water and human skin. The frequency with which such pigmented bacteria can be isolated from environmental samples suggests that pigments must play an important role, since each pigment requires enzymatic pathways to synthesize them that can be quite complex and therefore costly. The production of prodigiosin by Serratia marcescens, for instance, requires more than a dozen different enzymes (Harris et al., 2004)! Have you ever wondered what function these pigments really play?

The following is an inquiry-based approach to studying pigments that can be used in diverse settings, including non-majors biology, ecology, chemistry, and physics courses as well as general microbiology classes. The above-mentioned question is an excellent starting point for having students think about science at multiple levels, including structure – function relationships, heritability and expression of biological traits, natural selection and the cost/benefit of pigment production, physical and chemical properties of pigments, and so on.

Pigment studies as an inter-disciplinary STEM project. Although the most obvious questions about bacterial pigments relate to their biological role, don't miss the opportunity to delve into inter-disciplinary ideas! Pigments are often quite complex chemical compounds composed of one or more cyclic hydrocarbon skeletons. The spectrum of light that is absorbed by a pigment (a.k.a. its absorption spectrum), and hence its color, depends on the chemical structure of the pigment, and changes to this structure can change the color. Use this pigment study to integrate concepts of organic chemistry and physics of light into your class. Likewise, the calculations of dilutions and bacterial numbers integrate math skills.

An inquiry-based learning module using pigmented bacteria

Do bacterial pigments have a purpose beyond the merely decorative? Your students are sure to have some good ideas. An open-ended group discussion is going to produce a variety of possible answers, many of which are testable in a class setting. The following sequence of hands-on activities can be adapted to your classroom setting by selecting those for which you have the time and resources available. Alternatives are suggested at each level, and detailed protocols included at the end of this article. Although students should be encouraged to come up with their own ideas, prior lessons or readings could be used to suggest some possibilities, namely that bacteria produce pigments for: 1) photosynthesis, 2) antibiotic properties, 3) UV/light protection, 4) competition/predation protection and 5) as antioxidants. Water samples often contain cyanobacteria ('blue-green algae') whose green pigmentation consists of chlorophyll.

Chromobacterium violaceum and other purple-pigmented bacteria can be isolated from water and soil samples (Agate et al., 2016), and its deep purple pigment (violacein) has antibiotic (Durán & Menck, 2001) and anti-oxidant properties (Konzen et al., 2006) and protects against predation (Matz et al., 2004). Other pigments protect bacteria from harmful photodamage (Rajagopal et al., 1997). Soil and water samples may contain *Pseudomonas*, whose blue-green pyocyanin pigment is a factor in causing infections (Lau et al., 2004), as is *Staphylococcus aureus*' golden pigment (Liu et al., 2005). *Serratia marcescens' prodigiosin* (red) has anti-microbial, energy-spilling, and cancer-killing properties (Williamson et al., 2006; Haddix et al., 2008; Vijayalakshmi & Jagathy, 2016). Yellow, orange, pink and red pigments are common among *Micrococcus* species (Fig. 2) and may be anti-microbial or protect them from oxidants and radiation (Arrage et al., 1993; Mohammadi et al., 2012; Mohana et al., 2013; Rostami et al., 2016).

pigment	source	Color	Putative functions
Prodigiosin	*Serratia marcescens*	Red	Antibiotic, energy management
Violacein	*Chromobacterium violaceum*	Purple	Anti-microbial, antioxidant
Carotenoid	*Micrococcus luteus*	Yellow	UV absorption
Carotenoid	*Micrococcus agilis*	Red	?
Pyocyanin	*Pseudomonas aeruginosa*	Green/blue	Virulence
Carotenoid	*Micrococcus nishiominensis*	Orange	?
Staphyloxanthin	*Staphylococcus aureus*	Golden	virulence
Canthaxanthin	*Micrococcus roseus*	Pink	antimicrobial?

Table 1. Bacterial pigments and some of their putative functions. Citations are given in the text that follows.

A. Isolation of naturally occurring pigment-producing bacteria. You may wish to start by having your students culture bacteria from soil, water, or skin swabs, having students "discover" these colorful bacteria as they go along. Alternatively, many of the pigmented bacteria can be purchased from biological supply companies. You can easily isolate and grow pigment producers from soils, water, air, plants, and human skin through a simple swab inoculation or streak plating of samples on agar (Fig. 1). Once students have grown their own bacterial colonies, have them compare plates to select the most colorful colonies.

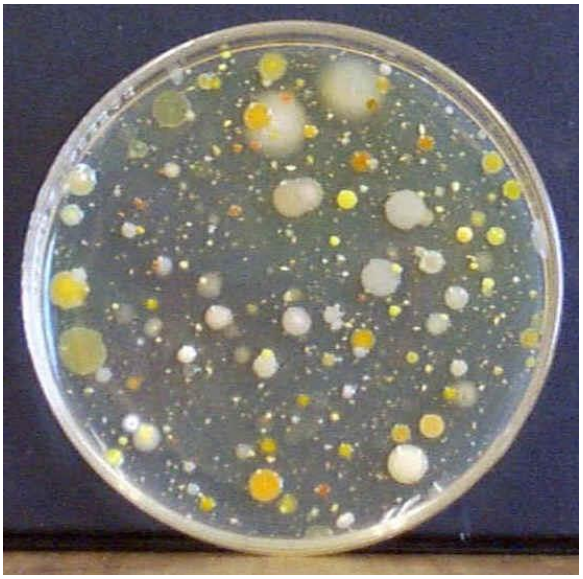


Figure 1. Bacterial colonies from a water sample growing on agar include many pigmented species.

B. Functions of pigments. Once students have the opportunity to observe colorful bacteria, you can have them come up with hypotheses about their function. Some good questions to start a discussion may be:

- How common are pigmented bacteria (as a percentage of the entire population)?
- Which colors predominate?
- Why do you think bacteria produce pigments?

Figure 2. A palette of pigmented bacteria, clockwise from bottom: *Micrococcus* (yellow, orange, pink and red), *Serratia* (dark red) and *Chromobacterium* (purple)

Although not all ideas that students are likely to come up with are testable, many can be investigated

and will make for a colorful lesson! Alternately, you can provide readings for students to explore and discuss. The summary above includes many references that can serve as a starting point.

C. Preparing pure cultures of bacteria. To study pigments, bacteria must first be grown in quantity as a pure culture; either an agar plate or liquid medium can be used. Have your class work in groups to prepare cultures of growth medium each containing a different-colored microbe. These cultures can be used to extract pigments, paint pictures on agar (Fig. 3), or study pigment functions. By observing cultures under different conditions, students can study the effect of temperature, light, or nutrients on color development. You can also freeze portions of these for future use.

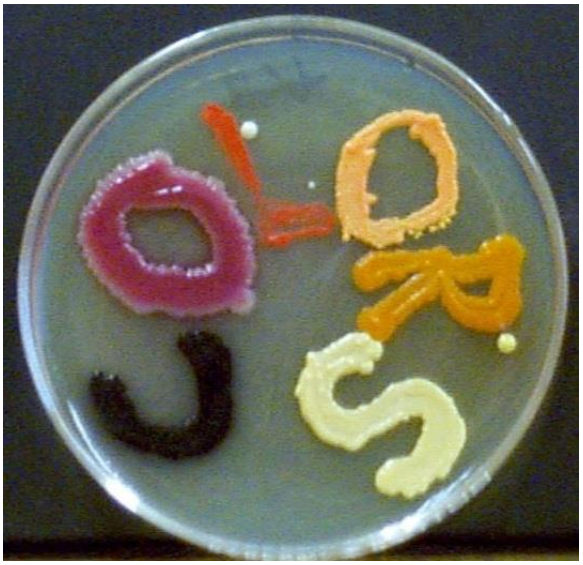


Figure 3. Agar plate with bacterial growth illustrates the beauty of bacterial pigmentation

D. **Extraction and analysis of pigments.** Your students can now extract the pigments from the cells using solvent extraction. The solvent to be used depends on the type of pigment (Dunn et al., 2004). The extracted pigments can be analyzed with a spectrophotometer by measuring the amount of light absorbed at different wavelengths (absorption spectrum; Fig. 4). Each pigment's absorption spectrum is unique and can help identify specific bacteria (Sahin, 2011).

E. Examining pigment function in bacteria. Some of the possible roles of pigments can be studied in the laboratory with minimal resources, such as antimicrobial properties and UV light resistance. Other interesting concepts you can tie in to your lessons include quorum sensing, a phenomenon where pigment production in *Serratia* and *Chromobacterium* requires a certain cell number, or "quorum", to take place (Mcclean et al., 1997; Van Houdt et al., 2007); temperature-dependent pigment production (*Serratia* produces pigment only below 35°C; competition (many pigmented bacteria out-compete non-pigmented rivals); and predation.

F. The physics and chemistry of light and color. Interwoven with the chemical and biological aspects of pigmentation is the understanding of how light works. The visible spectrum of light consists of a range of wavelengths (400 – 700 nm) which, when impinging on a molecule, may be absorbed, reflected or transmitted. Wavelengths which are absorbed or reflected by an object determine the color that is perceived by the human eye; in the case of chlorophyll, those reflected wavelengths are predominantly green light, hence the color of most plants. In other cases, it is the complementary color of the absorbed wavelength that is seen. The chemical structure of pigments determines the wavelengths that are absorbed, and is influenced by chemical bonds, pH, and molecular size (see Clark, 2019 in the online resources section).

G. **A place for math.** While all of the experiments can be set up for students, don't miss the opportunity to engage them in the calculations they may need to perform. Serial dilutions are a frequent necessity in microbiology as the number of bacteria in samples often measures in the billions. Calculating dilution factors and using scientific notation are incorporated into this exercise. Analyzing and graphing data, even if done using software, builds analytical skills such as scaling, normalizing data, and comparing populations using statistical tests.

Procedure

Protocols for culturing and analyzing pigmented bacteria. Whether using bacteria for artistic or scientific experiments, here are a few protocols to get you started. Students should always practice safe laboratory techniques when handling bacteria – handle these soil/water bacteria as if they were hazardous, even if they are perfectly safe. Sterilize and dispose of all cultures properly after the experiments are done.

Materials needed for a class of 24

General: per group: Bunsen burner or bacticinerator; inoculating loops; 100 ul and 1 ml micropipettors and pipette tips; balance; weigh paper

Soil culturing: Saline solution (9 g sodium chloride in 1 L distilled water), 3 Erlenmeyer flasks (250 ml size), stoppers or tin foil, 24 Trypticase Soy Agar (TSA) plates (can be purchased pre-made), sterile screw-cap test tubes for collecting soil

Skin Swabs: 24 TSA plates, 24 sterile cotton swabs, 100 ml sterile saline or water

Lake or stream water: 24 TSA plates, sterile bottles for collecting water sample

Pure culture study: 24 TSA plates, pure cultures of *Chromobacterium violaceum*, *Micrococcus luteus*,

Micrococcus roseus, Micrococcus agilis, Pseudomonas putida, Pseudomonas aeruginosa, Rhodospirillum rubrum, and/or Serratia marcescens

Spectrophotometry: 100 ml of various solvents (ethanol, acetone, methanol or isopropanol), screw-cap tubes, spectrophotometer, cuvettes. Solvents should be selected based on availability, safety, and effectiveness. While we have found methanol to be most effective, other less toxic solvents such as acetone can also give satisfactory results (Fig. 4). Pigment extraction may be more effective at different concentrations of solvents (70 – 100%).

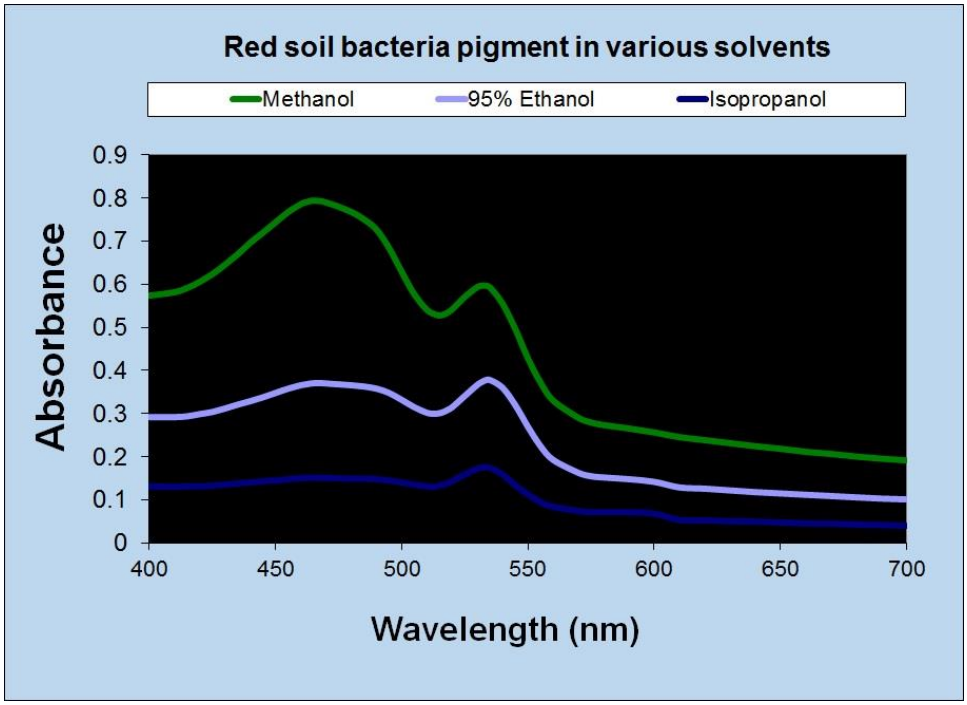


Figure 4. Absorption spectrum of pigment isolated from a red soil bacterium after extraction with methanol, ethanol or isopropanol.

Protocols

Sterilization. Sterilize all liquids and pipettes by autoclaving for 20 minutes at 121°C at 15 psi pressure. Alternately, pre-sterilized solutions and media may be purchased. Glassware can be heat-sterilized (2h at 160°C). Surfaces as well as stoppers can be adequately disinfected with 70% ethanol.

Culturing soil samples. Since bacteria are abundant in soils, a dilution is necessary to avoid having all the colonies grow into each other. Weigh out 1 gram of soil and add it to 99 ml of sterile saline solution. Stopper the flask and shake vigorously for 1 minute. Using a sterile pipette, remove 1 ml from this flask and add it to a second 99 ml flask of saline. Stopper the flask and shake vigorously for 1 minute. Transfer 1 ml from flask #2 to a third flask, stopper the flask and shake vigorously. Students now have 3 dilutions available: Flask #1 (1:100 or 10^{-2}), Flask #2 (1:10,000 or 10^{-4}), and Flask #3 (1:1,000,000 or 10^{-6}). Add 1.0 ml of dilution #3 to the surface of an agar plate and spread out the water sample using a bent (L-shaped) glass rod sterilized in ethanol or boiling water. The agar plates should be taped shut and allowed to incubate at room temperature for 2 – 7 days.

Culturing water samples. Most lake, pond or stream samples contain far fewer bacteria per ml than soils, and 0.1 – 1.0 ml of the water sample can be placed directly onto the surface of an agar plate. Samples are spread out and incubated as noted above.

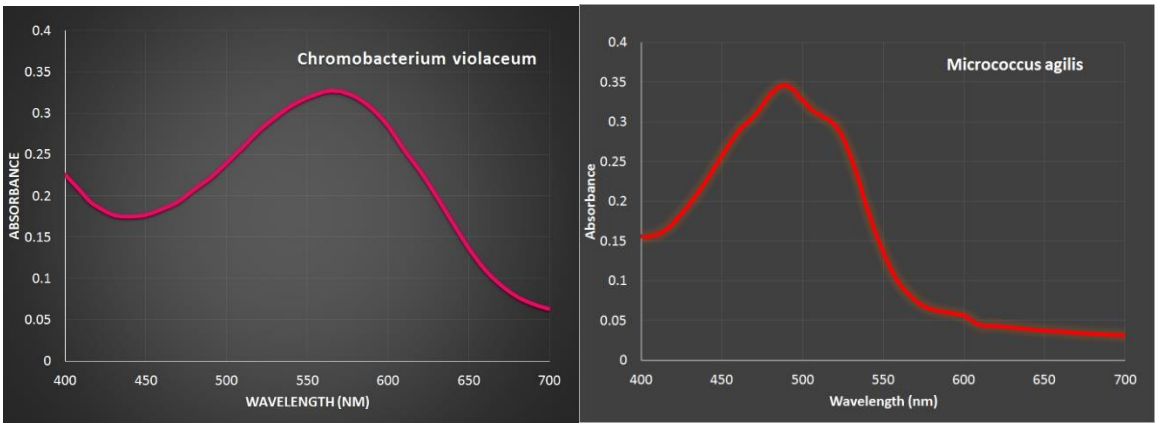
Culturing skin bacteria. Moisten a sterile cotton swab by dipping it into a beaker of sterile water or saline solution. Vigorously rub the swab over a 2 cm x 2 cm area of skin (forearm works nicely) and, with gentle pressure, rub and roll the swab over the surface of a TSA plate. Incubate 2 – 7 days at room temperature.

Culturing commercial strains of bacteria. Chromobacterium violaceum, Micrococcus luteus, Micrococcus roseus, Micrococcus agilis, Pseudomonas putida, Pseudomonas aeruginosa, Rhodospirillum rubrum, and Serratia marcescens can be purchased from Biological Supply companies such as Presque Isle Cultures (Erie, PA). These cultures will be shipped in agar slant tubes and will be ready for use. Aseptic transfer of bacteria to the agar plate is done with inoculating loops. Metal loops are heated (Bunsen burner or bacticinerator) for 10 seconds to sterilize them before and after transfers. Alternatively, pre-sterilized plastic loops can be purchased and

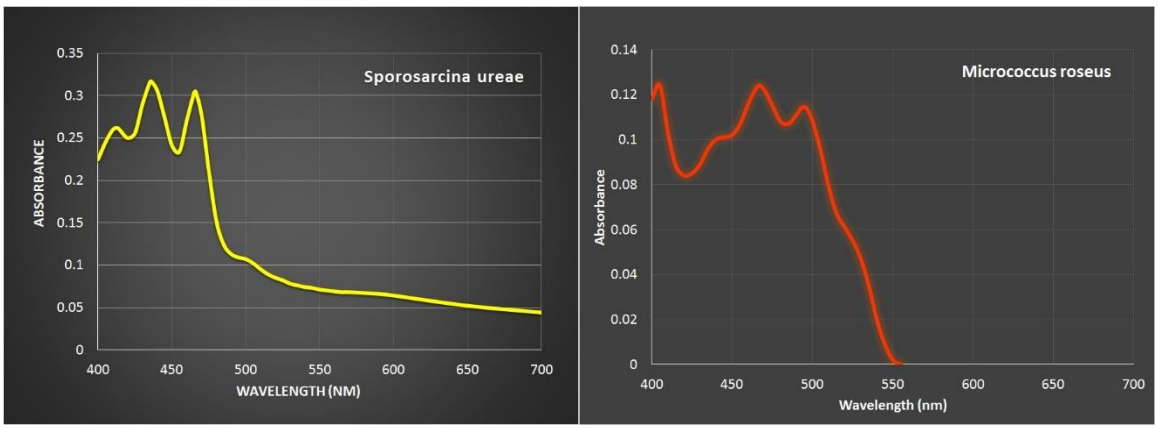
disposed of when done. The bacteria picked up with one loop are sufficient for an experiment. Transfer the bacteria to their agar plate and coat from ¼ to ½ of the agar plate surface with each strain selected. Incubate the plates for 2 – 7 days at room temperature.

Producing pure cultures from environmental samples. Once the individual colonies of bacteria from soil, water, or skin have grown to a size of 1 – 5 mm (see Fig. 1), select one colony of interest. Describe the colony (color, size, shape). Using an inoculating loop, carefully transfer part of the colony to a sterile agar plate as described above. Incubate for 2 – 7 days at room temperature. After incubation, examine the plate carefully to ensure that only the desired bacteria are growing on this plate.

Painting with bacteria. For each "color" (pigmented bacterial strain) and group of students, add 10 ml of 0.9% saline to sterile screw-cap test tubes. Using a sterile inoculating loop, add 3 loops of a pure bacterial culture (bought or prepared as described above) to a tube, tightly seal the tube with the screw cap and shake well to mix. You may wish to prepare larger volumes to divide up your stock into test tubes for several lab groups to use. Label flasks and tubes. Allow each student to 'paint' on a sterile agar plate using two or more 'colors'. Remind students to use a different loop for each culture. Seal each agar plate with tape and incubate the plates for up to one week at room temperature. At the end of the class, collect bacterial culture flasks and tubes and sterilize. Particularly creative artwork can be submitted to the annual Agar Art contest (see online resources).



Panel A Panel B



Panel C Panel D

Figure 5. Absorption spectra of pigments isolated from *Chromobacterium violaceum* (A), *Micrococcus agilis* (B), *Sporosarcina ureae* (C) and *Micrococcus roseus* (D).

Spectrophotometry. Add 5 ml of solvent to a sterile screw-cap tube. Solvents should be selected based availability, safety and disposal considerations, and effectiveness (often based on the lipid solubility of the pigment). Suggested solvents include acetone (80% or 100%), ethanol (70%, 95% or 100%), methanol (100%), or isopropanol (70% or 95%). Once sufficient bacterial growth is observed on the pure culture plate, transfer a pea-sized clump of cells (~ 1 g) of the culture into the solvent by scraping the agar surface with a sterile inoculating loop. Several loop transfers may be necessary. Cap the tube and shake or vortex (if a vortexer is available) for 5 minutes until the pigment is well dissolved in the solvent. Water-soluble pigments will easily dissolve in alcohols, but lipid-soluble pigments may require chloroform, benzene or other non-polar solvents. Additional disruption of cells may help release pigment, including mashing the cells with a mortar or using sonication. Turn on the spectrophotometer 15 minutes before taking measurements. Follow the manufacturer's instructions for calibrating and using the instrument. Most spectrophotometers accept 13 mm x 30 mm cuvettes. One such cuvette should be filled with pigment-free solvent used in the extraction. This cuvette can be shared among lab groups and is used to calibrate the baseline (zero) absorbance before each reading and at each wavelength. Take the first absorbance reading at the lowest wavelength allowed by the instrument (usually ~ 350 nm) and take readings in increments of 10 nm, up to the maximum of ~ 700 nm (Fig. 5). Some instruments will automatically scan the entire spectrum, allowing more rapid data collection. If compatible with data collection software such as LoggerPro, data will be saved and graphed automatically. Other skills that can be practiced here include graphing, comparison of the spectra of several different-colored bacteria, and normalizing data relative to each spectrum's maximum value. This will allow students to understand the difference between absorbed and reflected light and to better discuss the role of pigments in biological process such as photosynthesis.

Further studies

Although brief in nature, the following ideas can easily be adapted to your classroom setting, and we have tested and tried these ideas already.

Antibiotic properties. A simple test of the antibiotic potential of the pigment can be carried out using the Kirby-Bauer method (Bauer et al., 1966) with the pigment extract. Transfer 5 ml of the pigmented solvent to a clean test tube. Place 6 small paper disks (I use the cardboard backing from notepads and a single hole-punch, then sterilize the disks in an autoclave) into the tube and allow the solvent to evaporate – this may take several days. Place each dried paper disk on an agar plate inoculated with Escherichia coli or Staphylococcus aureus and observe the plates for the absence of growth around the paper disk. Suitable controls should be conducted with solvent-exposed disks.

Study of pigment-less mutants. The importance of the pigments can be further studied by using non-pigmented mutants. Pigmented bacteria are suspended in 10 ml of 0.9% NaCl and then exposed to UV light for 1 – 5 minutes. 1,000,000-fold dilutions are spread on Petri plates and cultured in an attempt to find non-pigmented mutants. Using this approach, we were able to isolate mutant strains of M. roseus, P. putida and soil bacteria (Fig. 6). Such mutants can also be used in a genetic analysis of the pigment-synthesizing pathways (Schmidt, 1993). Non-pigmented mutant strains are also available from researchers who are often willing to provide such cultures for teaching purposes, and some bacteria (e.g. Serratia) will be non-pigmented at temperatures above 35°C.

Panel A

Panel B

Panel C

Figure 6. Pigmented bacteria and non-pigmented mutant strains obtained by UV exposure. (A) Red soil bacteria (unidentified), (B) *Pseudomonas putida*, (C) *Micrococcus roseus*.

Protection from UV light or chemicals. To evaluate the importance of pigmentation in protecting bacteria from oxidative stress, UV radiation or other challenges, pigmented and unpigmented strains of the same species can be subjected to UV light (Fig. 7), ozone (de Ondarza, 2017), hydrogen peroxide or antiseptics/disinfectants. This experiment will require good dilution skills and enumeration of surviving bacteria.

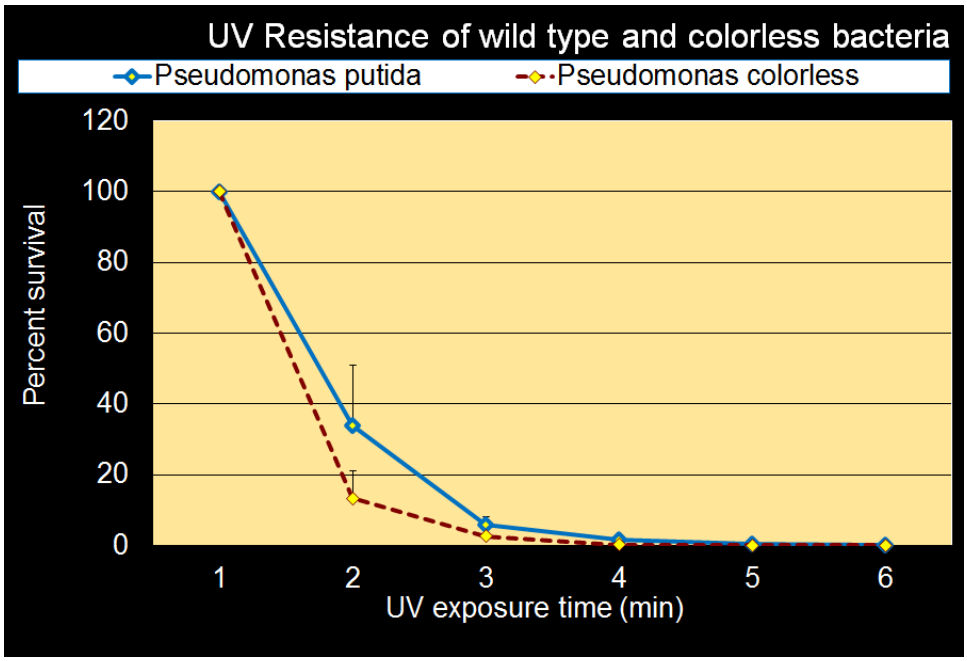


Figure 7. Effect of UV irradiation on survival of pigmented and non-pigmented (mutant strain) bacteria.

Safety considerations. The bacterial strains selected for this study are generally harmless to human health. Nonetheless, many bacteria are opportunistic pathogens and may cause an infection if in contact with a scratch or open wound. Students should handle all bacteria with the utmost caution. Lab benches should be disinfected before and after class with commercially available disinfectants. Students should wash their hands and wear protective gloves, lab coats and goggles. Absolutely no mouth pipetting, eating or drinking should be allowed.

Disposal. All biological materials and disposable objects that came in contact with them (agar plates, plastic pipettes, plastic loops, paper towels, filter paper) should be disposed of in a large biohazard bag. Seal the bag and sterilize by autoclave. (Other arrangements may be available to you for the safe disposal of biohazard materials). Disposal of solvents usually requires special arrangements, such as flammable waste containers.

Discussion

As science and scientific research become more specialized and focused, we often miss out on the inter-disciplinary nature of most biological studies and experimentation. By designing projects to intentionally include lessons in other disciplines such as physics, chemistry and ecology, students can be engaged in multiple ways while gaining a deeper understanding of the ways different STEM disciplines interact. Some of the most insightful ideas in my classroom have come from students outside of the traditional Biology curriculum, including art, education, history, nutrition and physics. Bacterial pigments are ideally suited to such inter-disciplinary projects; they are at once visually enticing and generate curiosity. Bacteria that produce pigments are readily isolated from natural habitats such as soil and water and even the human body and can be cultured with very little material and equipment. The potential to engage students in "living art" through painting pictures on agar plates is stunningly illustrated by the many entries into the annual Agar Art contest of the American Society for Microbiology. Pigments from bacteria are extracted with minimal difficulty or expense and allow for further study of their possible function. Mostly, having students discuss the "why" of bacterial pigments engages them in the physics of light absorption, the energy cost of biosynthesis, the potential selective advantage of a biological trait, the ecological interactions (predation, antibiosis) involved, and the chemistry of complex pigments. A learning module that incorporates lessons in physics, chemistry, biology and ecology can be implemented over a 3-4-week span in one class or even engage instructors and courses outside of biology in a common-problem project.

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