Video Article

Methods to Inhibit Bacterial Pyomelanin Production and Determine the Corresponding Increase in Sensitivity to Oxidative Stress

Laura M. Ketelboeter[1], Sonia L. Bardy[1]

[1]Department of Biological Sciences, University of Wisconsin Milwaukee

Correspondence to: Sonia L. Bardy at bardy@uwm.edu

URL: https://www.jove.com/video/53105
DOI: doi:10.3791/53105

Keywords: Immunology, Issue 102, Microbiology, *Pseudomonas aeruginosa*, pyomelanin, NTBC, oxidative stress, minimum inhibitory concentration

Date Published: 8/31/2015

Citation: Ketelboeter, L.M., Bardy, S.L. Methods to Inhibit Bacterial Pyomelanin Production and Determine the Corresponding Increase in Sensitivity to Oxidative Stress. *J. Vis. Exp.* (102), e53105, doi:10.3791/53105 (2015).

Abstract

Pyomelanin is an extracellular red-brown pigment produced by several bacterial and fungal species. This pigment is derived from the tyrosine catabolism pathway and contributes to increased oxidative stress resistance. Pyomelanin production in *Pseudomonas aeruginosa* is reduced in a dose dependent manner through treatment with 2-[2-nitro-4-(trifluoromethyl)benzoyl]-1,3-cyclohexanedione (NTBC). We describe a titration method using multiple concentrations of NTBC to determine the concentration of drug that will reduce or abolish pyomelanin production in bacteria. The titration method has an easily quantifiable outcome, a visible reduction in pigment production with increasing drug concentrations. We also describe a microtiter plate method to assay antibiotic minimum inhibitory concentration (MIC) in bacteria. This method uses a minimum of resources and can easily be scaled up to test multiple antibiotics in one microtiter plate for one strain of bacteria. The MIC assay can be adapted to test the affects of non-antibiotic compounds on bacterial growth at specific concentrations. Finally, we describe a method for testing bacterial sensitivity to oxidative stress by incorporating H_2O_2 into agar plates and spotting multiple dilutions of bacteria onto the plates. Sensitivity to oxidative stress is indicated by reductions in colony number and size for the different dilutions on plates containing H_2O_2 compared to a no H_2O_2 control. The oxidative stress spot plate assay uses a minimum of resources and low concentrations of H_2O_2. Importantly, it also has good reproducibility. This spot plate assay could be adapted to test bacterial sensitivity to various compounds by incorporating the compounds in agar plates and characterizing the resulting bacterial growth.

Video Link

The video component of this article can be found at https://www.jove.com/video/53105/

Introduction

Pseudomonas aeruginosa is a Gram negative bacterium that produces a variety of pigments including pyomelanin, a red-brown pigment that helps provide protection from oxidative stress[1-4] and binds a variety of compounds, including aminoglycoside antibiotics[5-7]. Pyomelanin production is caused by a defect in the tyrosine catabolism pathway[4,8], either through deletions or mutations of the gene encoding homogentisate 1,2-dioxygenase (HmgA)[1,9] or through imbalances in the various enzymes in the pathway[10]. Homogentisate accumulates due to inactivation of HmgA, and is secreted and oxidized to form pyomelanin[11]. Production of pyomelanin can be abolished or reduced in a dose dependent manner through treatment with the herbicide 2-[2-nitro-4-(trifluoromethyl)benzoyl]-1,3-cyclohexanedione (NTBC)[12], which inhibits 4-hydroxyphenylpyruvate dioxygenase (Hpd) in the tyrosine catabolism pathway[13]. Hpd is required for the formation of homogentisate, and therefore pyomelanin[11].

We describe in detail three techniques that were important in our studies of NTBC treatment of pyomelanin producing strains of *P. aeruginosa*. These techniques include titration of NTBC to determine the concentrations that will abolish or reduce pyomelanin production in laboratory and clinical pyomelanin producing strains, determination of the minimum inhibitory concentration (MIC) of antibiotics when bacteria are treated with NTBC, and the resulting sensitivity to oxidative stress with NTBC treatment.

The titration assay we developed serves two purposes. First, the assay will allow the user to determine if NTBC can abolish or reduce pyomelanin production in the bacterium being studied and at which concentrations. This will allow the user to determine sensitivity to NTBC, since different strains of bacteria may have different sensitivities to this compound, as observed in *P. aeruginosa*[12]. Second, the NTBC titration assay will allow the user to determine the appropriate concentration of NTBC to use in subsequent assays, such as antibiotic MIC and oxidative stress response assays, if the goal is to abolish or reduce pyomelanin production and determine the effects of pigment reduction.

The titration assay works because a visible difference in pyomelanin production can be seen in strains treated with NTBC and the differences in pyomelanin production are dose dependent[12]. Additionally, this technique can be applied to the study of other compounds that may eliminate or enhance pigment production in bacteria.

Antibiotic MICs are used to determine the sensitivity of bacteria to antibiotics. There are several methods to determine MICs, including agar dilution plates and broth dilutions[14]. Broth dilutions can be performed in small test tubes or in a 96-well microtiter plate. The microtiter plate method of MIC determination described herein will allow the user to test a wide range of antibiotics using a minimum of resources. The assay

Copyright © 2015 Journal of Visualized Experiments

provides reproducibility as well as flexibility in the number of antibiotics and strains tested by this method. Additionally, with the incorporation of NTBC in the assay, the user can determine if elimination or reduction of pyomelanin production alters antibiotic sensitivity in bacteria that produce pyomelanin.

Bacterial response to oxidative stress can be tested in several ways. The most common methods described are either viable counts of bacteria subjected to oxidative stress for a period of time[1], or oxidative stress disc diffusion assays[15]. These methods tend to use high concentrations of oxidative stressors to examine the effects of oxidative stress in bacteria and results can be quite variable between biological replicates. The viable count assay also tends to use more agar plates than the other methods. The spot plate assay we describe uses low concentrations of H_2O_2 and allows the user to test the oxidative stress response of multiple strains using a minimum of plates. The assay is also consistently reproducible between technical and biological replicates. As pyomelanin is involved in resistance to oxidative stress, the incorporation of NTBC in the assay allows the user to determine the effects of elimination of pyomelanin production on oxidative stress resistance.

Protocol

1. Preparation of Culture Media, Antibiotics, and 2-[2-nitro-4-(trifluoromethyl)benzoyl]-1,3-cyclohexanedione (NTBC)

1. Make LB broth (1% tryptone, 0.5% yeast extract, 0.25% NaCl in H_2O) and aliquot into appropriate volumes. Sterilize by autoclave. Store at room temperature.
2. Make 100 ml LB agar (1% tryptone, 0.5% yeast extract, 0.25% NaCl, 1.5% agar in H_2O) in 250 ml flasks. Sterilize by autoclave and store at room temperature. Ensure that the agar is melted before pouring into plates.
   NOTE: Flasks containing 100 ml of LB agar will yield 4 plates. The amount of LB agar can be altered to correspond to the number of plates needed for the assay.
3. Make PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4)[16]. Sterilize by autoclave or filtration and store at room temperature.
4. Prepare the antibiotic stock solutions for gentamicin, kanamycin, and tobramycin.
   1. Prepare appropriate antibiotic stock concentrations for *P. aeruginosa* strains containing 100 mg/ml gentamicin, 30 mg/ml kanamycin, and 10 mg/ml tobramycin. Dissolve the antibiotics in water, filter sterilize (0.2 µm), and store at 4 °C. Alter the antibiotics and concentrations depending on the bacterium studied.

5. Prepare the NTBC stock solutions. Dissolve 10 mg of NTBC in 400 µl of DMSO. This yields a concentration of 75.9 mM NTBC. Store NTBC stock solutions at -20 °C. Thaw solutions at room temperature as needed.
   NOTE: Different sources of NTBC have differences in solubility. Determine the appropriate vehicle in which to dissolve the NTBC based on the manufacturer's recommendations and adjust Step 1.5 accordingly.

2. NTBC Titrations of Bacterial Strains

1. Set up overnight cultures of the strains to be tested. Add 2 ml LB broth to 16 x 150 mm test tubes (one per strain) and inoculate with 1 isolated colony from each strain. Incubate overnight at 37 °C with aeration on a tissue culture rotator in an air incubator.
2. The following day, prepare titrations of NTBC in LB broth. Use an initial range from 0 to 900 µM NTBC since different strains have differences in sensitivity to NTBC.
   1. Add 1 ml LB broth to 4 to 5 test tubes (16 x 150 mm) per strain.
   2. Add the NTBC stock solution (75.9 mM) to the test tubes (16 x 150 mm) in a range of concentrations. See **Table 1** for NTBC concentrations and corresponding stock volumes to add to 1 ml of LB broth.

3. Measure the OD_{600} of the overnight cultures. Wash cultures before taking OD_{600} readings to eliminate pyomelanin present in the media.
   1. Wash the cultures by centrifuging 1 ml of culture in a microcentrifuge at 16,000 x g for 2 min. Remove the supernatant and any loosely pelleted cells with a micropipettor and resuspend the solid cell pellet in 1 ml LB.

4. Inoculate titration tubes at OD_{600} 0.05. Calculate the amount of washed culture needed to inoculate the tubes.
   NOTE: Use the washed cultures for inoculations since pyomelanin should not be present.
5. Incubate the titration tubes for approximately 24 hr at 37 °C with aeration using a tissue culture rotator in an air incubator.
6. Photograph the titration tubes and compare pigment production within and between strains to determine the amount of NTBC to use for MIC and oxidative stress assays. Use OD_{600} readings to determine the amount of pyomelanin in cell free culture supernatant and to determine cell density.
   NOTE: The OD_{600} ratio of pyomelanin in culture supernatant to cells can be calculated to quantify differences in pyomelanin production after treatment with NTBC.

3. Antibiotic Minimum Inhibitory Concentration (MIC) Assay in 96-well Plates

1. Set up overnight cultures of the strains to be tested in LB with and without NTBC.
   NOTE: This protocol is described using the representative level of 300 µM NTBC. The appropriate level of NTBC to be used is determined in Step 2.6.
   1. Add 300 µM NTBC to 2 ml LB. Add an equivalent volume of vehicle (DMSO) to 2 ml LB for the no NTBC condition.
   2. Using sterile toothpicks, inoculate tubes with one isolated colony of bacteria. There will be one culture with NTBC and one culture without NTBC for each strain. Incubate overnight at 37 °C with aeration using a tissue culture rotator in an air incubator.

Copyright © 2015 Journal of Visualized Experiments

2. The following day, make LB + NTBC and LB + DMSO master solutions for setting up the MIC assay. Add NTBC at a concentration of 600 µM as this will be diluted two fold when inoculum is added, yielding a final concentration of 300 µM.
    1. To test one antibiotic for one strain, add 600 µM NTBC to 2 ml LB and mix to make the NTBC master solution. Add an equivalent volume of vehicle (DMSO) to 2 ml LB and mix to make the no NTBC master solution. Use these solutions for creating antibiotic stock solutions as well as for setting up the dilution series in 96-well plates.
    NOTE: The master solution formulations will yield extra solution to account for pipetting errors. The master solutions can be scaled up or down as required depending on the number of antibiotics and strains tested.

3. Prepare the antibiotic solutions in the LB + NTBC or LB + DMSO master solutions.
NOTE: The antibiotic concentration in these solutions should be double the final desired concentration. Enough solution should be made to transfer 100 µl to four wells in a 96-well plate.
    1. Prepare gentamicin +/- NTBC stock solution at 64 µg/ml. To make this solution, add 0.288 µl of gentamicin stock (100 mg/ml) to 450 µl LB + NTBC or LB + DMSO master solution.
    NOTE: The maximum concentration of gentamicin for *P. aeruginosa* PAO1 is 32 µg/ml.
    2. Make the kanamycin +/- NTBC stock solution at 256 µg/ml. To make this solution, add 3.84 µl of kanamycin stock (30 mg/ml) to 450 µl LB + NTBC or LB + DMSO master solutions.
    NOTE: The maximum concentration of kanamycin for *P. aeruginosa* PAO1 is 128 µg/ml.
    3. Prepare tobramycin +/- NTBC stock solution at 8 µg/ml. To make this solution, add 0.36 µl of tobramycin stock (10 mg/ml) to 450 µl LB + NTBC or LB + DMSO master solutions.
    NOTE: For *P. aeruginosa* PAO1, the maximum concentration of tobramycin is 4 µg/ml.
    NOTE: The antibiotics and concentrations can be adjusted for the bacteria to be tested.

4. Add 100 µl of each 2x antibiotic solution to four wells in a 96-well plate. Place these solutions in row A. For example, gentamicin should be placed in A1 through A4, kanamycin should be placed in A5 through A8, and tobramycin should be placed in A9 through A12. See **Figure 1A** for a diagram of a 96-well plate set up.
NOTE: Multiple antibiotics can be tested in one plate, but only one strain should be tested per plate to eliminate the potential for cross-contamination from other strains.
5. Add 50 µl of the LB + NTBC or LB + DMSO master solution to rows B through H of the 96-well plate. Ensure that one plate is LB + NTBC and one plate is LB + DMSO. See **Figure 1A.**
    1. Use LB + NTBC for the antibiotics in LB + NTBC. Use LB + DMSO for the antibiotics in LB + DMSO.

6. Using a micropipettor perform two fold serial dilutions of the antibiotics by transferring 50 µl of the solution from row A to row B. Mix the solution, change the pipet tips, and transfer 50 µl of the solution from row B to row C. Repeat for the remaining rows. After diluting row G, remove 50 µl of the solution from that row and discard. Use row H as a no antibiotic control for bacterial growth. See **Figure 1B**.
NOTE: Each well in rows A through G now contains 50 µl of antibiotic in LB + NTBC or LB + DMSO at 2X the final desired concentration. Row H contains LB + NTBC or LB + DMSO with no antibiotics.
7. Measure the OD_{600} of the overnight cultures. Wash all cultures before taking OD_{600} readings to eliminate pyomelanin present in the media.
    1. Wash the cultures by centrifuging 1 ml of culture in a microcentrifuge at 16,000 x g for 2 min. Remove the supernatant with a micropipettor and resuspend the cell pellet in 1 ml LB.

8. Dilute the overnight cultures to $2.75x10^5$ CFU/ml in LB.
NOTE: Assume that one OD_{600} unit is the equivalent of $1x10^9$ CFU/ml for *P. aeruginosa*. OD to CFU/ml conversions may be different in other bacteria.
9. Add 50 µl of the diluted bacterial culture to the appropriate well.
NOTE: Cultures grown in NTBC should be added to the wells containing NTBC and cultures grown in DMSO should be added to the wells containing DMSO. See **Figure 1B**.
    1. Add bacteria to three wells for each strain and antibiotic concentration. Add 50 µl of LB to the fourth well to act as a control for bacterial contamination. See **Figure 1B**.
    2. Use a multi-channel micropipettor to inoculate the wells. Ensure that pipet tips are near the bottom of the wells when adding inoculum to prevent contamination of neighboring wells.
    NOTE: Adding bacterial culture to the wells will dilute the antibiotic and NTBC concentrations two fold.

10. Cover the 96-well plates with parafilm and incubate approximately 24 hr at 37 °C. Incubate 96-well plates statically, in an air incubator.
11. Examine the plates for bacterial growth in the wells. The MIC is the lowest concentration of antibiotic in which no bacterial growth is seen for all three replicates of each strain.
    1. Visually examine the plate for growth or read using a plate reader set to OD_{600}.

4. Spot Plate Assay for Oxidative Stress Response

1. Set up overnight cultures of the strains to be tested in LB with and without NTBC as described in step 3.1.
2. The next day, prepare LB agar plates containing H_2O_2 as an oxidative stressor. A range of H_2O_2 concentrations from 0 to 1 mM is a good starting point.
    1. Melt the LB agar flasks. Cool media to approximately 50 °C at room temperature.
    2. Add H_2O_2 directly to the cooled media at the desired concentrations. Swirl flasks to mix. See **Table 2** for concentrations of H_2O_2 and volumes of concentrated H_2O_2 to add. These values are based on 100 ml of LB agar.
    3. Pour plates immediately after adding H_2O_2 and flame the surface to remove bubbles. The yield is 4 plates per 100 ml of LB agar. Mark the plates with the H_2O_2 concentration.
    4. Place the plates uncovered in a biological flow hood with the fan running for 30 min to remove excess moisture from the plates.
    NOTE: Use the plates the same day they are prepared. Failure to do so may result in inconsistent data.

NOTE: Oxidative stressors such as paraquat can be substituted for H_2O_2 in this assay. The concentrations used for other oxidative stressors may be different than those used for H_2O_2.

3. Wash and measure the OD_{600} of the overnight cultures as described in step 3.7.
4. Normalize the OD_{600} of all the overnight cultures to the lowest value for the set of strains being tested. *P. aeruginosa* generally has an OD_{600} of approximately 2.5 when grown overnight in LB + NTBC or LB + DMSO.
    1. Determine the volume of culture needed to dilute the culture to the lowest OD_{600} in a total volume of 1 ml. For example, if a culture has an OD_{600} of 3 and the lowest OD_{600} for the set of strains is 2.5, perform the following calculation: (2.5)(1 ml) = (3)(x). x = 0.833 ml. 0.833 ml of culture will be placed in a microfuge tube.
    2. Calculate the amount of LB + NTBC (300 µM) or LB + DMSO needed to bring the culture volume to 1 ml. For the example in step 4.4.1, the amount of LB + NTBC or LB + DMSO added to the culture would be 0.167 ml (1 ml total volume – 0.833 ml culture). Make stock solutions of LB + NTBC and LB + DMSO to use for these dilutions based on the volume needed for diluting all strains.
    3. Mix the culture and LB + NTBC or LB + DMSO by vortexing.

5. To maintain cultures in a constant concentration of NTBC or DMSO, perform ten fold serial dilutions of the normalized overnight cultures in PBS + NTBC or PBS + DMSO.
    1. Make stock solutions of PBS + NTBC and PBS + DMSO. For one set of dilutions for one strain, mix 300 µM NTBC or an equivalent volume of DMSO with PBS to yield a total volume of 720 µl. Scale these stocks up or down depending on how many strains are tested.
    2. Label microfuge tubes for 10^{-1} through 10^{-7} serial dilutions. Add 90 µl of PBS + NTBC or PBS + DMSO to the appropriate tubes. Use PBS + NTBC for strains grown in LB + NTBC and use PBS + DMSO for strains grown in LB + DMSO.
    3. Add 10 µl of culture to the appropriate 10^{-1} dilution tube. Mix by vortexing and transfer 10 µl of the 10^{-1} dilution to the 10^{-2} dilution tube. Repeat until all dilutions have been performed. Change pipet tips between dilutions.

6. Spot 5 µl of the 10^{-3} through 10^{-7} dilutions on LB + H_2O_2 plates in duplicate for each strain. Use one pipet tip if spots are plated from most dilute to least dilute (10^{-7} to 10^{-3}). Do not tip or tilt the plate until the liquid has dried into the plate.
7. Incubate the plates for 24-48 hr at 37 °C (air incubator), depending on the strain.
   NOTE: *P. aeruginosa* PAO1 will have good sized colonies on LB after 24 hr of incubation. Incubate strains until they have colonies approximately the same size as PAO1.
8. Photograph the plates using a CCD camera above a transluminator. Optionally, edit the photos for contrast and crop to the same size. Count the number of colonies in each spot to determine changes in sensitivity to oxidative stress.

Representative Results

NTBC titrations

The NTBC titrations were used to determine if NTBC was able to reduce pyomelanin production in *P. aeruginosa*, and also identify the concentration of NTBC that eliminates or reduces pyomelanin production for use in additional assays. There may be variations in the levels of pyomelanin produced in different replications, but general trends remain constant. The NTBC titration assay could also be modified to test other compounds that may affect pigment production in other bacteria. This will only work, however, if there is a phenotypic change that can be visually determined or quantified.

Treatment of pyomelanin producing strains of *P. aeruginosa* with NTBC resulted in a dose dependent decrease in pigment production[12]. **Figure 2** shows that different strains of *P. aeruginosa* have differences in sensitivity to NTBC, as indicated by levels of pyomelanin production. Higher concentrations of NTBC were required to reduce pyomelanin production in the clinical isolate PA1111[17] (obtained from Dara Frank) compared to the laboratory strain *hmgA::tn*[18] (University of Washington PAO1 transposon mutant library). Strains that do not produce pyomelanin [PAO1 (obtained from Carrie Harwood) and *hpd::tn*[18] (University of Washington PAO1 transposon mutant library)] showed no change in pigmentation with NTBC treatment. We decided to use 300 µM NTBC for our assays because pyomelanin production was substantially reduced in the laboratory strain *hmgA::tn* using that concentration (compare 300 µM to 0 µM NTBC). All strains used in this study were stored at -80 °C in 15% glycerol.

Antibiotic MICs

Antibiotic MICs can be tested using several different methods. The microtiter plate method described here will allow the user to test a range of antibiotic concentrations and use a minimum of resources. Results are easily replicated and the assay can be modified to allow for differences in antibiotic sensitivity of the organism to be tested. Some variation may be seen between independent experiments, but trends are fairly consistent. Technical replicates should exhibit the same MIC.

Table 3 shows the results for different *P. aeruginosa* strains treated with and without NTBC and various aminoglycoside antibiotics. Three independent colonies were tested in triplicate following the method described. MICs were recorded as the lowest concentration of antibiotic that inhibited bacterial growth in all three technical replicates. NTBC treatment had no effect on the aminoglycoside MICs for the strains tested.

Oxidative stress spot plate assay

The spot plate assay for testing oxidative stress gives reproducible results using lower levels of H_2O_2 than those used in other assays. The plate without H_2O_2 is a control plate to determine the accuracy of the dilution series for each strain, as well as determine colony size and number of colonies in each spot without subjecting the cells to oxidative stress. In a proper dilution series, the final spot should have very few colonies, while the first spot will have an uncountable number of colonies on H_2O_2 free media. There should be a ten-fold difference in the number of colonies in each spot within a dilution series. For all strains tested, similar numbers of colonies should be seen in the same dilution on the H_2O_2 free control plate.

Copyright © 2015 Journal of Visualized Experiments

As different strains of bacteria may have different sensitivities to oxidative stress, a range of H_2O_2 concentrations should be tested. Increasing concentrations of H_2O_2 should show decreasing amounts of growth, as indicated by a reduction in colony size and numbers in each spot, assuming the bacteria are sensitive to H_2O_2 induced oxidative stress. The growth characteristics of different bacterial strains can be compared to determine the sensitivity to oxidative stress under a particular H_2O_2 concentration. The assay can be modified to determine the effects of a particular compound or reagent, such as NTBC, on a single bacterial strain when the bacteria are subjected to oxidative stress. Colonies can also be counted to determine the percent reduction in bacterial colony forming units between different experimental conditions.

Figure 3 shows a spot plate assay of pyomelanin and non-pyomelanin producing strains of *P. aeruginosa* treated with and without NTBC and exposed to H_2O_2 induced oxidative stress. There is a clear difference in sensitivity to oxidative stress when pyomelanin producing bacteria are treated with NTBC, and also between bacteria that do not produce pyomelanin compared to those that do produce pigment. Strains that did not produce pyomelanin, either naturally or due to NTBC treatment, were more sensitive to oxidative stress than strains that produced pyomelanin.

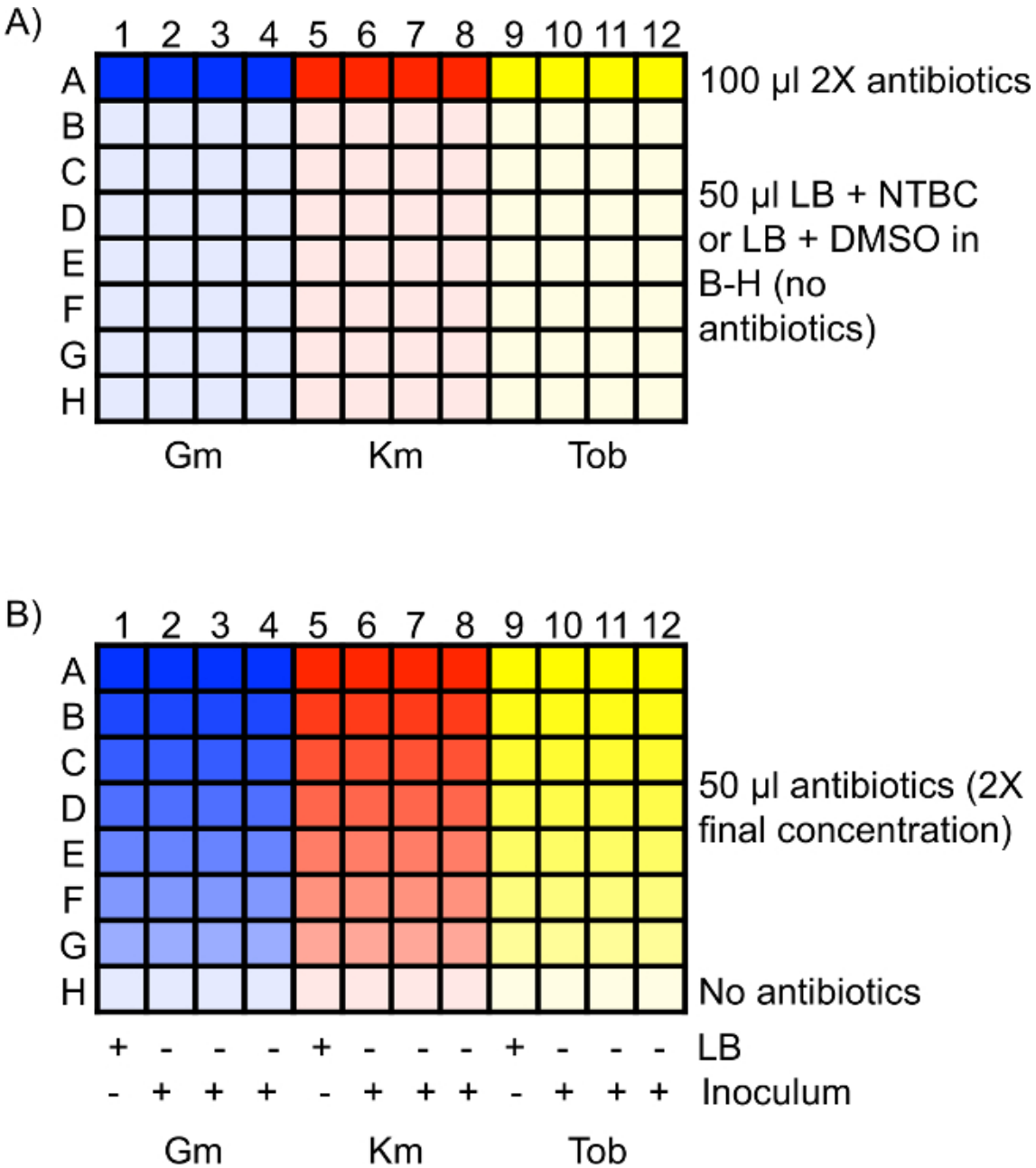

Figure 1: **Schematic of antibiotic MIC assay 96-well plate set up.** (**A**) 100 µl of 2x antibiotics of the highest starting concentration are in row A. Rows B through H are filled with 50 µl of either LB + NTBC or LB + DMSO without antibiotics. (**B**) Two fold serial dilutions are performed in rows A through G, resulting in 50 µl of diluted antibiotic in each well at 2x the final desired concentration. Row H is a control well for bacterial growth without antibiotics. 50 µl of LB or inoculum is added to the appropriate wells, diluting the antibiotics two fold to the final concentration. LB serves as a control for bacterial contamination in the antibiotics. Gm, gentamicin; Km, kanamycin; Tob, tobramycin.

Copyright © 2015 Journal of Visualized Experiments

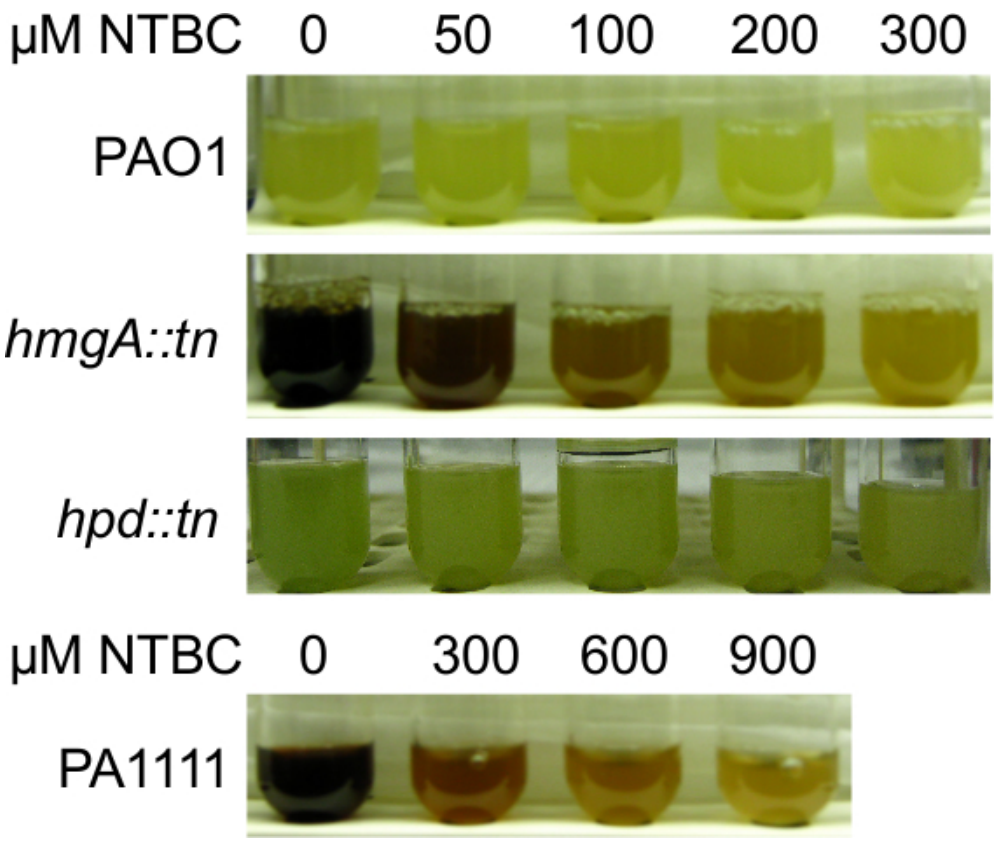

Figure 2: NTBC titrations of pyomelanin producers and non-producers of *P. aeruginosa*. NTBC reduced pyomelanin production in a dose dependent manner in laboratory and clinical pyomelanin producers, but had no effect on pigment production in strains that do not produce pyomelanin. Modified from reference[12].

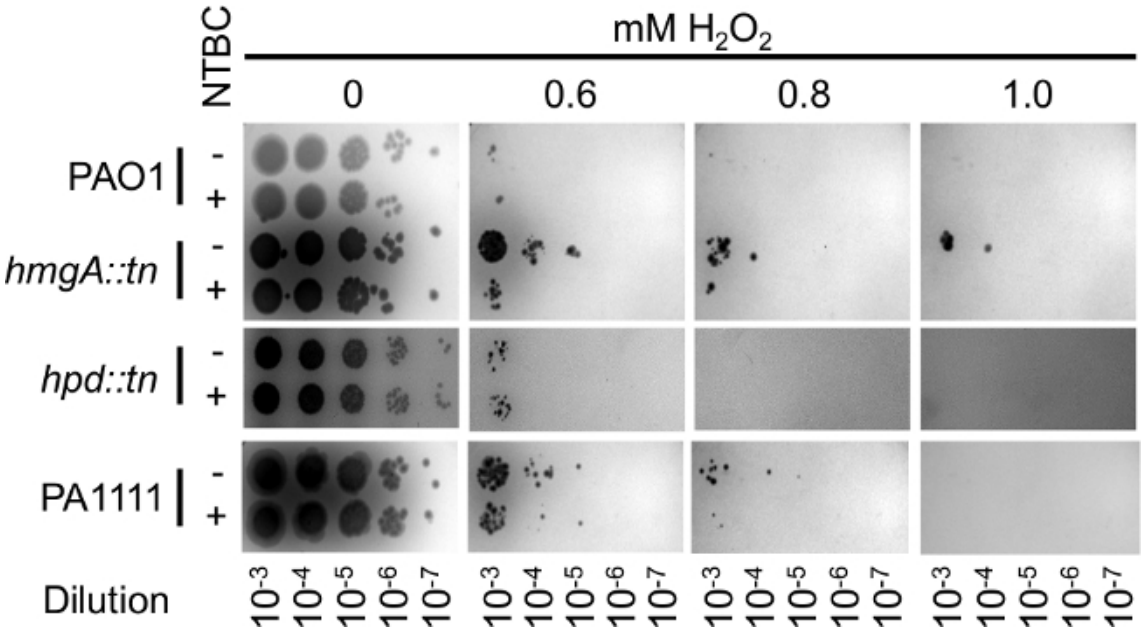

Figure 3: Spot plate assay for oxidative stress response. Bacterial strains were diluted to the same OD_{600}, serial diluted 10-fold, and spotted on LB plates containing various concentrations of H_2O_2. The 0 mM H_2O_2 plate results showed that all the strains were diluted properly, as indicated by a similar number of colonies in each of the spots for the same dilution for different strains. The plates containing H_2O_2 show that the strains were more sensitive to oxidative stress as the concentration of H_2O_2 increased. This is indicated by decreased colony counts in the spots compared to the no H_2O_2 condition, as well as a reduction in colony size. Modified from reference[12].

Final Concentration of NTBC (μM)	Volume of NTBC (75.9 mM) to add (μl)
0	0

50	0.659
100	1.318
200	2.64
300	3.95
600	7.91
900	11.86

Table 1: NTBC concentrations for setting up titrations in LB. This table gives various NTBC concentrations and the corresponding amount of NTBC stock to add to 1 ml LB.

Final Concentration of H_2O_2 (mM)	**Amount of 9.79 M H_2O_2 (30% wt) to add (µl)**
0	0
0.2	2.04
0.4	4.09
0.6	6.13
0.8	8.17
1	10.21

Table 2: Concentrations of H_2O_2 to add to LB agar for the oxidative stress spot plate assay. This table gives various H_2O_2 concentrations and the corresponding amount of concentrated H_2O_2 stock to add to 100 ml LB agar.

	PAO1 - NTBC	PAO1 + NTBC	*hmgA::tn* - NTBC	*hmgA::tn* + NTBC	*hpd::tn* - NTBC	*hpd::tn* + NTBC	PA1111 - NTBC	PA1111 + NTBC
Gentamicin	1	0.5	2	2	1	1	0.5	0.5
Kanamycin	16	8	32	32	32	32	16	16
Tobramycin	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.5

Table 3: Antibiotic MIC results. Three independent colonies were tested in triplicate for each strain. Re-printed with permission from reference[12].

Discussion

The NTBC titration method described in this protocol will allow the user to determine if NTBC can reduce or eliminate pyomelanin production in bacteria, and determine the concentration of NTBC required. The most critical step in the NTBC titration assay is determining the range of NTBC concentrations to use in the assay. Different strains of *P. aeruginosa* have different sensitivities to NTBC, and laboratory strains may be more sensitive to NTBC than clinical isolates[12] (**Figure 2**). Therefore, it is necessary to test a range of NTBC concentrations for each strain being assayed. It is advisable to start with lower concentrations and increase to higher concentrations as needed because the user will most likely want to determine the lowest concentration of NTBC that inhibits pyomelanin production in a particular strain. Our titration assay describes a qualitative method to determine inhibition of pigment production. Depending on the objective of the study, it may be necessary to determine pyomelanin levels. This could be accomplished through HPLC[3] or electron paramagnetic resonance (EPR) spectroscopy[19], which could be used to detect the presence of pyomelanin in the samples. Melanin produces a characteristic EPR spectrum, and samples lacking melanin should not produce that spectrum[20].

Modifications can be made to the titration assay by using minimal medium supplemented with tyrosine instead of LB. If a minimal medium (such as Defined minimal media[21]) is used, tyrosine must be supplemented in the medium since pyomelanin production will not occur in the absence of tyrosine. Minimal medium supplemented with tyrosine provides the advantage of having a defined concentration of tyrosine in the media and may eliminate some variation between biological replicates. The titration technique works quite well when one is looking for a change in pigment production, but will not work if the bacterium does not produce pigment. Other compounds could be substituted for NTBC in a titration series, provided there is a visual output to determine if the compound has an effect on the bacteria, such as reduction or enhancement of pigment production. In our assay, once the concentration of NTBC needed to eliminate or reduce pyomelanin production has been determined it is possible to use this concentration in other assays, including antibiotic MICs and oxidative stress response, in order to test the effects of NTBC on other bacterial phenotypes.

The antibiotic MIC method described in this paper will allow the user to determine antibiotic sensitivity of bacteria. Additionally, the incorporation of NTBC in the assay will allow the user to determine the effects of NTBC treatment on antibiotic sensitivity in pyomelanin producing bacteria (**Table 3**). Antibiotic sensitivity in bacteria can be quite variable and there are several techniques that can be used to determine MICs. Common techniques include agar dilution plates and large and small scale broth dilutions[14]. The microtiter plate broth dilution method described here is an easy way to determine MICs for various antibiotics, and it uses fewer resources than other methods since the protocol is designed to use small volumes. The most critical steps in the procedure are setting up the initial antibiotic stock solutions and performing accurate two fold dilutions in the 96-well microtiter plate (**Figure 1A**). It is important that the antibiotic stocks are two fold higher than the final desired concentration, since the antibiotics will be diluted two fold when the inoculum is added to the wells. The range of antibiotic concentrations used must be determined experimentally since different strains of bacteria have different sensitivities to antibiotics, and sensitivities are dependent on the antibiotic. The

concentration of NTBC used in the assay must also be two fold higher than the final desired concentration since it will be diluted two fold when the inoculum is added. Accuracy in performing serial dilutions will help ensure that there is a two-fold difference in antibiotic concentration in each row of the plate.

The 96-well microtiter plate MIC assay can be easily modified to incorporate as many antibiotics or bacterial strains as needed. While more than one antibiotic can be tested in a plate, only one bacterial strain should be tested per plate to avoid cross contamination from other bacterial strains. No more than three antibiotics should be tested in one plate to allow for testing of one bacterial colony in triplicate with one control column containing medium only for each antibiotic (**Figure 1B**). The medium only column will test for contamination in the antibiotic stocks. It is also advisable to include an LB only row inoculated with bacteria in the plate to test for bacterial growth in the absence of antibiotics. This will ensure that the bacteria is growing properly and in the event that there is no growth in any of the antibiotic concentrations tested, the possibility that there was something wrong with the inoculum can be ruled out. In addition, this will provide a turbidity control for unimpaired growth, should a plate reader be used to determine the optical densities of each well. The majority of issues arise in determining the MIC, which is defined as the lowest concentration of antibiotic in which no growth occurs in all three technical replicates. It is possible that technical replicates may have slightly different MICs. Differences in MIC within technical replicates for a strain should be no more than two fold. We found that placing the pipet tips in the antibiotic containing medium while adding inoculum helps reduce some of the MIC differences for technical replicates, as it eliminates some of the potential for inoculum splashes into adjacent wells. The MIC assay should be done with at least three biological replicates to determine that the MICs and trends are consistent between replicates. The assay as described gives a very clear method for setting up a 96-well plate with different antibiotics and strains of bacteria. A large range of antibiotic concentrations can be tested, as well as a variety of different antibiotics, which can be adapted as appropriate to the bacterium being tested. This technique can be modified to test the antibiotic sensitivities of a large range of bacteria, as well as to test sensitivities to non-antibiotic compounds.

Several oxidative stress response assays have been described in the literature, including viable counts after exposure to oxidative stress[1] and disc diffusion assays[15]. The spot plate assay described here determines bacterial sensitivity to oxidative stress when the oxidative stressor is in an agar plate. This technique allows low concentrations of H_2O_2 to be tested instead of the high concentrations typically used in the other assays. The spot plate technique has less variation between biological replicates than viable counts and disc diffusion methods. Trends in the spot plate assay tend to be very consistent, although there is still some variability between biological replicates. The variability is usually seen as slight differences in the H_2O_2 concentration that allows a clear difference in bacterial growth and sensitivity to oxidative stress to be observed. Sources for these inconsistencies are likely due to either pipetting errors when adding H_2O_2 to the plates or natural variations in sensitivity to oxidative stress in different colonies of bacteria. Technical replicates consistently show the same results. It is important that a range of H_2O_2 concentrations be tested, as different strains of bacteria may have different sensitivities to oxidative stress (**Figure 3**). It is also critical that the same dilutions be plated for all conditions so comparisons can be made between strains and test conditions. Additionally, a plate containing no H_2O_2 must be included as a control to ensure that a proper dilution series was created and to provide a comparison for bacterial growth in the absence of oxidative stress. If bacteria are treated with a compound, such as NTBC, it is necessary to include a no treatment control so a comparison can be made between the two conditions. This will allow the user to determine if the compound has an effect on oxidative stress response (**Figure 3**).

The spot plate protocol can be modified to include other oxidative stressors such as paraquat. However, the type of oxidative stress to be generated in the bacteria must be determined before adding the oxidative stressor, since different compounds generate different types of oxidative stress. This assay could also be modified to test bacterial sensitivity to other compounds. In that case, the compound would be included in the agar plates at various concentrations and the bacteria would be spotted onto the plates as described in the protocol. Differences in colony numbers and size in different dilutions compared to a control plate without the compound would indicate sensitivity to the compound.

We describe three methods to test the effects of NTBC on pyomelanin producing strains of bacteria. First, we use a titration containing various concentrations of NTBC to determine if NTBC will reduce or abolish pyomelanin production in bacteria, and then determine the minimum concentration of NTBC necessary to do so. The concentration of NTBC that eliminates or reduces pyomelanin production will be used in other assays to determine the effects of NTBC on antibiotic MICs and oxidative stress response. The MIC assay will allow the user to test the effects of NTBC on antibiotic sensitivity. Finally, the spot plate oxidative stress response assay will be used to test the effects of NTBC on sensitivity to oxidative stress.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

The authors thank Dara Frank and Carrie Harwood for their generous contribution of strains. University of Wisconsin Milwaukee Research Foundation holds patent no. 8,354,451; with claims broadly directed to treating or inhibiting the progression of infection of a microorganism in a patient by administering a 4-hydroxyphenylpyruvate dioxygenase-inhibiting compound such as 2-[2-nitro-4-(trifluoromethyl)benzoyl]-1,3-cyclohexanedione (NTBC). Inventors are Graham Moran and Pang He. This research was supported by the National Institutes of Health (R00-GM083147). The University of Washington *P. aeruginosa* transposon mutant library is supported by NIH P30 DK089507.

References

1. Rodriguez-Rojas, A. *et al.* Inactivation of the *hmgA* gene of *Pseudomonas aeruginosa* leads to pyomelanin hyperproduction, stress resistance and increased persistence in chronic lung infection. *Microbiology.* **155**, 1050-1057, doi:10.1099/mic.0.024745-0 (2009).
2. Keith, K. E., Killip, L., He, P., Moran, G. R., & Valvano, M. A. *Burkholderia cenocepacia* C5424 Produces a Pigment with Antioxidant Properties Using a Homogentisate Intermediate. *J Bacteriol.* **189**, 9057-9065, doi:10.1128/JB.00436-07 (2007).

3. Schmaler-Ripcke, J. *et al.* Production of Pyomelanin, a Second Type of Melanin, via the Tyrosine Degradation Pathway in *Aspergillus fumigatus*. *Appl Environ Microbiol.* **75**, 493-503, doi:10.1128/AEM.02077-08 (2009).
4. Turick, C. E., Knox, A. S., Becnel, J. M., Ekechukwu, A. A., & Milliken, C. E. Properties and Function of Pyomelanin. *Biopolymers In Tech.* (ed M Elnashar) 449-472, doi:10.5772/10273 (2010).
5. Bridelli, M. G., Ciati, A., & Crippa, P. R. Binding of chemicals to melanins re-examined: adsorption of some drugs to the surface of melanin particles. *Biophys Chem.* **119**, 137-145, doi:10.1016/j.bpc.2005.06.004 (2006).
6. Barza, M., Baum, J., & Kane, A. Inhibition of antibiotic activity in vitro by synthetic melanin. *Antimicrob Agents Chemother.* **10**, 569-570, doi:10.1128/AAC.10.3.569 (1976).
7. Nosanchuk, J. D., & Casadevall, A. Impact of Melanin on Microbial Virulence and Clinical Resistance to Antimicrobial Compounds. *Antimicrob Agents Chemother.* **50**, 3519-3528, doi:10.1128/AAC.00545-06 (2006).
8. Arias-Barrau, E. *et al.* The Homogentisate Pathway: a Central Catabolic Pathway Involved in the Degradation of L-Phenylalanine, L-Tyrosine, and 3-Hydroxyphenylacetate in *Pseudomonas putida*. *J Bacteriol.* **186**, 5062-5077, doi:10.1128/JB.186.15.5062-5077.2004 (2004).
9. Ernst, R. K. *et al.* Genome mosaicism is conserved but not unique in *Pseudomonas aeruginosa* isolates from the airways of young children with cystic fibrosis. *Environ Microbiol.* **5**, 1341-1349, doi:10.1111/j.1462-2920.2003.00518.x (2003).
10. Sanchez-Amat, A., Ruzafa, C., & Solano, F. Comparative tyrosine degradation in *Vibrio cholerae* strains. The strain ATCC 14035 as a prokaryotic melanogenic model of homogentisate-releasing cell. *Comp Biochem Physiol B Biochem Mol Biol.* **119**, 557-562, doi:10.1016/S0305-0491(98)00028-5 (1998).
11. Hunter, R. C., & Newman, D. K. A Putative ABC Transporter, HatABCDE, Is among Molecular Determinants of Pyomelanin Production in *Pseudomonas aeruginosa*. *J Bacteriol.* **192**, 5962-5971, doi:10.1128/JB.01021-10 (2010).
12. Ketelboeter, L. M., Potharla, V. Y., & Bardy, S. L. NTBC treatment of the pyomelanogenic *Pseudomonas aeruginosa* clinical isolate PA1111 inhibits pigment production and increases sensitivity to oxidative stress. *Curr Microbiol.* **69**, 343-348, doi:10.1007/s00284-014-0593-9 (2014).
13. Kavana, M., & Moran, G. R. Interaction of (4-Hydroxyphenyl)pyruvate Dioxygenase with the Specific Inhibitor 2-[2-Nitro-4-(trifluoromethyl)benzoyl]-1,3-cyclohexanedione. *Biochemistry.* **42**, 10238-10245, doi:10.1021/bi034658b (2003).
14. Andrews, J. M. Determination of minimum inhibitory concentrations. *J Antimicrob Chemother.* **48**, 5-16, doi:10.1093/jac/48.suppl_1.5 (2001).
15. Nikodinovic-Runic, J., Martin, L. B., Babu, R., Blau, W., & O'Connor, K. E. Characterization of melanin-overproducing transposon mutants of *Pseudomonas putida* F6. *FEMS Microbiol Lett.* **298**, 174-183, doi:10.1111/j.1574-6968.2009.01716.x (2009).
16. Sambrook, J., & Russell, D. W. *Molecular cloning: a laboratory manual.* 3 edn, Cold Spring Harbor Laboratory Press, (2001).
17. Roy-Burman, A. *et al.* Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. *J Infect Dis.* **183**, 1767-1774, doi:10.1086/320737 (2001).
18. Jacobs, M. A. *et al.* Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA.* **100**, 14339-14344, doi:10.1073/pnas.2036282100 (2003).
19. Youngchim, S., Pornsuwan, S., Nosanchuk, J. D., Dankai, W., & Vanittanakom, N. Melanogenesis in dermatophyte species *in vitro* and during infection. *Microbiology.* **157**, 2348-2356, doi:10.1099/mic.0.047928-0 (2011).
20. Khajo, A. *et al.* Protection of Melanized *Cryptococcus neoformans* from Lethal Dose Gamma Irradiation Involves Changes in Melanin's Chemical Structure and Paramagnetism. *PLoS ONE.* **6**, e25092, doi:10.1371/journal.pone.0025092 (2011).
21. Hancock, R. E. W. *Hancock Laboratory Methods. Department of Microbiology and Immunology.*, University of British Columbia, British Columbia, Canada, http://www.cmdr.ubc.ca/bobh/methods.htm, 1999-2015 (2015).

Copyright © 2015 Journal of Visualized Experiments