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Culture of Small Colony Variant of Pseudomonas Aeruginosa and Quantitation of its Alginate

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Dear Editor,

I would like to submit our revised manuscript to be considered for publication in JOVE. The title of the manuscript is as follows: “Methods for Culturing Small Colony Variant and Quantitation of *Pseudomonas aeruginosa* Alginate.”

Thank you so much for consideration.

Sincerely,

Hongwei D. Yu, Ph.D.

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TITLE:

Culture of Small Colony Variant of *Pseudomonas Aeruginosa* and Quantitation of its Alginate

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KEYWORDS:

Pseudomonas aeruginosa, cystic fibrosis (CF), small colony variant (SCV), pyrimidine biosynthesis, growth, mucoidy, alginate, uronic acid carbazole assay, alginate-specific mAb, ELISA

SUMMARY:

Here, we describe a growth condition to culture the small colony variant of *Pseudomonas aeruginosa.* We also describe two separate methods for the detection and quantitation of the exopolysaccharide alginate produced by *P. aeruginosa* using a traditional uronic acid carbazole assay and an alginate-specific monoclonal antibody (mAb) based ELISA.

ABSTRACT:

Pseudomonas aeruginosa, an opportunistic Gram-negative bacterial pathogen, can overproduce an exopolysaccharide alginate resulting in a unique phenotype called mucoidy. Alginate is linked to chronic lung infections resulting in poor prognosis in patients with cystic fibrosis (CF). Understanding the pathways that regulate the production of alginate can aid in the development of novel therapeutic strategies targeting the alginate formation. Another disease-related phenotype is the small colony variant (SCV). SCV is due to the slow growth of bacteria and often associated with increased resistance to antimicrobials. In this paper, we first show a method of culturing a genetically defined form of *P. aeruginosa* SCV due to pyrimidine biosynthesis mutations. Supplementation of nitrogenous bases, uracil or cytosine, returns the normal growth to these mutants, demonstrating the presence of a salvage pathway that scavenges free bases from the environment. Next, we discuss two methods for the measurement of bacterial alginate.

The first method relies on the hydrolysis of the polysaccharide to its uronic acid monomer followed by derivatization with a chromogenic reagent, carbazole, while the second method uses an ELISA based on a commercially available, alginate-specific mAb. Both methods require a standard curve for quantitation. We also show that the immunological method is specific for alginate quantification and may be used for the measurement of alginate in the clinical specimens.

INTRODUCTION:

Chronic lung infections with *Pseudomonas aeruginosa* are a major cause of morbidity and mortality in patients with cystic fibrosis (CF). During early childhood, patients are colonized by multiple bacterial pathogens including nonmucoid isolates of *P. aeruginosa*[1,2]. Emergence of the small colony variant (SCV) isolates as well as mucoid isolates is a marker for the onset to chronic infections. SCV isolates are highly drug resistant[3] due to their slow growth rates[4], which renders them a severe deterrent in the treatment regiments and other chronic infections[5] by *P. aeruginosa.* Work by Al Ahmar et al.[6] showed a link between SCV and mucoidy linked by de novo pyrimidine biosynthesis. Pyrimidine starvation, due to mutations in genes involved with pyrimidine production, resulted in SCV phenotype in the nonmucoid reference strain PAO1 and the mucoid derivative, PAO581 (PAO1*mucA25*).

Even though alginate overproduction is an important disease marker for chronic lung infections in CF, it is not clear whether there is a direct correlation between the amount of alginate and lung pathology, and it is unclear if alginate can be used as a prognosis marker for treatment[7]. Alginate production is mainly regulated by two operons, a regulatory operon (*algUmucABCD*)[8,9] and the biosynthetic operon (*algD* operon)[10,11]. Alginate production is tightly regulated by the sigma factor AlgU[9,12] (also known as AlgT) and the degradation of the anti-sigma factor MucA[13]. The ability to monitor the production of alginate in situ from the patients' sputum specimens can aid in the development of novel therapeutic options.

Here, we describe a growth condition that detects the presence of SCV caused by mutants that cannot synthesize the pyrimidine de novo. Supplementation of uracil and/or cytosine, the nitrogenous base of pyrimidine nucleotide, to the medium activates the salvage pathway, thus restoring the normal growth in mutants. This growth method for these specific SCV mutants may be used as a screening method to identify pyrimidine mutations in patient samples. In addition, we discuss two methods for detection and measurement of alginate produced and secreted by *P. aeruginosa*. The first is the traditional method[14-16] of degrading the polysaccharide using a high concentration of acid and then adding a colorimetric indicator to quantitate the concentration in the sample. The second method, developed in our laboratory, utilizes the Enzyme-Linked Immunosorbent Assay (ELISA) using an anti-alginate monoclonal antibody (mAb) developed by QED Biosciences. The ELISA method proves to be more specific and sensitive than the uronic acid assay and allows for safer use due to the avoidance of the highly concentrated sulfuric acid. With the ability of the ELISA to be used directly on patient sputum samples to measure alginate, it can be developed as a monitoring diagnostic tool to follow the amount of alginate present in the lungs at different periods of the infection.

PROTOCOL:

1. SCV growth conditions and physiological activation of the salvage pathway

1.1. Detection of SCV

1.1.1. Streak the *P. aeruginosa* strains PAO1, PAO1Δ*pyrD*, PAO581, and PAO581Δ*pyrD* on prewarmed *Pseudomonas* isolation agar (PIA) plates and grow at 37 °C for 48 h. On the growth plate identify a single colony isolate that has the SCV phenotype (colony size of 1−3 mm as opposed to the normal 3−5 mm colony size).

1.1.2. Repeat step 1.1.1 to obtain a pure isolate of the SCV.

NOTE: Growth may require up to 72 h due to the slow growth rate of the SCV colonies.

1.2. Physiological activation of the salvage pathway.

1.2.1. Using a sterile inoculation loop, pick the SCV colony from the PIA plate and streak on a prewarmed PIA supplemented with 0.1 mM of uracil. Grow plate at 37 °C for 24−48 h.

NOTE: All PIA plates used in this protocol contain 20 mL/L of glycerol added to the mixture before autoclaving. After autoclaving and after mixture temperature is below 55 °C add 11.21 mg/L of uracil (0.1 mM) to the liquid media before pouring plates. This method would help identify pyrimidine mutations that cause SCV phenotype in *P. aeruginosa*. If the SCV phenotype is a result of said mutation, then normal colony growth and size (3−5 mm) would be observed on the uracil supplemented PIA plates. If strains had the ability to produce alginate, then the mucoid phenotype will also return upon uracil supplementation.

2. Uronic acid carbazole assay

2.1. From a pure culture of desired strain to be tested, identify a single colony. Using a sterile toothpick, pick the colony, and place the toothpick in a culture test tube containing 5 mL of *Pseudomonas* isolation broth (PIB). Grow in a shaker incubator at 37 °C for 24 h.

NOTE: The following strains PAO581 (PAO1*mucA25*), PAO581*carA*, PAO581*carB*, and PAO581*pyrD* were grown on PIA plates or PIA plates supplemented with 0.1 mM uracil to harvest alginate for measurement.

2.2. Onto a prewarmed PIA plate add 150 µL of cultured PIB broth (for 15 mm x 100 mm plates) or 450 µL of broth (for 15 mm x 150 mm plates). Using a sterile cell spreader, spread the broth over the plate. Grow the plate at 37 °C for 24 h.

NOTE: Aspirate any excess fluid, if any, from the plate using a pipet by tipping the plate to one side. Both PIB and PIA plates used in the protocol contain 20 mL/L of glycerol to be used by cells

as a carbon source to aid in alginate production.

2.3. Using a pipette controller and a sterile 50 mL pipette, add 0.85% NaCl to the lawn grown and collect sample by scrapping the plate using a cell spreader. Aspirate the sample using a fresh 50 mL pipet into a 50 mL collection tube. Vortex the sample on high to mix and place the samples on ice.

NOTE: The volume of added 0.85% NaCl varies depending on the size of the plate. For 15 mm x 100 mm plates, use 10−30 mL, and for 15 mm x 150 mm plates, use 20−50 mL.

2.4. Measure the optical density at 600 nm (OD_{600}) of the samples by adding 1 mL of the sample to a disposable cuvette and reading the OD using a spectrophotometer. Repeat this step 2x to obtain a triplicate of reads for each sample.

2.5. Add 3 mL of the sulfuric acid/borate solution into culture tubes and let sit on ice. Add 350 µL of the collected sample SLOWLY to the acid mix in the test tubes and vortex on low briefly.

2.5.1. Prepare borate stock by adding 10.099 g of potassium hydroxide (KOH) powder to 45 mL of water. Add 24.74 g of boric acid (H_3BO_3) to the mixture and bring volume to 100 mL.

2.5.2. Place 1 L bottle in a sink with ice and fill bottle with 500 mL of concentrated sulfuric acid. SLOWLY add 15 mL of the prepared borate stock. Allow bottle to cool.

2.5.3. Add another 10 mL of borate stock for a total of 25 mL. Once bottle is cooled, bring the volume to 1 L.

CAUTION: This method relies on the use of highly concentrated sulfuric acid. Proper personal protective equipment should be used to ensure safety and proper disposal protocol of the sample should be followed.

NOTE: For a positive control a known concentration of *D*-mannuronic acid and/or a known bacterial alginate samples harvested and purified through alginate producing strains of *P. aeruginosa* (e.g.: PAO581-PAO1*mucA25*). For a negative control use 0.85% NaCl solution and/or PAO1Δ*algD* (In-frame deletion of *algD* gene that renders the bacteria completely unable to produce alginate). Multiple tubes can be made from each sample to increase the number of technical repeats to aid in statistical analysis. Prepare 3−5 tubes of each sample.

2.6. Add 100 µL of 0.1% carbazole in ethanol solution to the acid/sample mix. Cap the tube and vortex on medium setting for 5 s. Place in a dry bath at 55 °C for 30 min.

2.7. After incubation, remove the tubes and vortex briefly on high and allow to cool for 5 min.

NOTE: Color to be seen would be a shade of purple. Color will remain stable for measurement for 1−2 h.

2.8. Read the OD_{530} of each tube by adding 1 mL of the mixture to a clean cuvette and reading the OD of the samples at 530 nm on a spectrophotometer. Use the tube with 0.85% NaCl as a negative control to blank spectrophotometer.

2.9. Prepare a standard curve by measuring the OD_{530} of serial dilutions of known concentrations of *D*-Mannuronic acid (1,024 µg/mL, 512 µg/mL, 256 µg/mL, 128 µg/mL, 64 µg/mL, 32 µg/mL, 16 µg/mL, and 8 µg/mL). Repeat 2x. Extract a linear equation from these readings.

NOTE: Standard curve only needs to be done once. The linear equation extracted from it can be used for later testing.

2.9.1. Calculate the concentration of the alginate in each sample using the standard curve and divide the alginate concentration from linear equation by OD_{600} to obtain the total amount of alginate per OD_{600}.

3. ELISA for alginate quantitation

3.1. Repeat steps 2.1−2.4 to obtain samples for alginate measurement.

NOTE: Strains used were PAO1, PAO1Δ*algD*, and PAO1 carrying pHERD20T-*algU.*

3.2. Using a micropipette add 50 µL of the collected sample to an untreated 96-well plate. Add 50 µL of coating buffer (carbonate/bicarbonate buffer pH 9.6) to the wells. Incubate the plate at 37 °C for 2 h.

NOTE: For a positive control, a known concentration of *D*-Mannuronic acid and/or a known stable alginate producing strain (e.g.: PAO581-PAO1*mucA25*) can be used. For a negative control use 0.85% NaCl solution and/or PAO1Δ*algD* (in-frame deletion of *algD* gene renders the bacteria completely unable to produce any alginate). Multiple wells can be made from each sample to increase the number of technical repeats to aid in statistical analysis. Prepare 3−5 wells of each sample.

3.3. Using a squirt bottle, wash the plate wells 2x with 1x phosphate buffer saline (PBS) with 0.05% Tween 20 (PBS-T) by filling the wells, and then draining them by flipping the plate over.

3.4. Using a micropipette add 200 µL of blocking buffer (10% skim milk in PBS-T) to the wells. Incubate at 4 °C overnight.

NOTE: Wrap plate in paraffin film or shrink wrap to help avoid any evaporation in the refrigerator.

3.5. Using a squirt bottle wash the plate wells 2x with PBS-T by filling the wells, and then draining them by flipping plate over.

3.6. Using a micropipette add 100 µL of diluted primary antibody (mouse anti-alginate monoclonal antibody) to the wells and incubate at 37 °C for 1–2 h.

NOTE: Primary antibody (mouse anti-alginate monoclonal antibody) was provided at a concentration of 0.5 µg/mL (dilution of 1:2,000 of 1 mg/mL stock) in antibody diluent solution (1% skim milk in 1x PBS).

3.7. Using a squirt bottle wash the plate wells 3x with PBS-T by filling the wells, and then draining them by flipping plate over.

3.8. Using a micropipette add 100 µL of diluted secondary antibody to the wells and incubate at 37 °C for 1–2 h.

NOTE: Secondary antibody used was pierce goat anti-mouse poly-HRP antibody to a concentration of 0.25 µg/mL (dilution of 1:2000 of 0.5 mg/mL stock) in antibody diluent solution.

3.9. Using a squirt bottle wash the plate wells 3x with PBS-T by filling the wells, and then draining them by flipping plate over.

3.10. Using a micropipette, add 100 µL of TMB-ELISA solution (**Table of Materials**) and incubate at room temperature for 30 min in the dark.

NOTE: The color of a positive reaction would be a shade of blue.

3.11. Using a micropipette, add 100 µL of stop solution (2 N sulfuric acid).

NOTE: The color will turn from blue to yellow when the stop solution is added. Color is stable for up to 30 min after the reaction is stopped.

3.12. Using a plate reader, read the OD at 450 nm.

3.13. Produce a standard curve by measuring the OD_{450} of serial dilutions of known concentrations of *D*-Mannuronic acid (1,024 µg/mL, 512 µg/mL, 256 µg/mL, 128 µg/mL, 64 µg/mL, 32 µg/mL, 16 µg/mL, and 8 µg/mL). Repeat 2x. From these readings extract a linear equation.

NOTE: The standard curve needs to be only done once. The linear equation extracted from it can be used for later testing.

3.13.1. Calculate the concentration of the alginate in each sample using the standard curve and divide the alginate concentration from linear equation by OD_{600} to obtain the total amount of alginate per OD_{600}.

4. Statistical analysis of alginate measurement

4.1. In a graphical software sheet, set a column with the linear equation derived from the standard curve. Set the y-axis results as the alginate concentration and the x-axis results as the OD_{530} for uronic acid assay and OD_{450} for ELISA to obtain the alginate concentrations in each sample.

4.2. To normalize the amount of alginate in each sample to the amount per 5.5 $x10^8$ CFU/mL (1 OD_{600}), divide the concentration of each sample obtained above by its respective OD_{600} value. If multiple OD_{600} were measured for each sample, divide by the mean OD_{600}.

4.3. Perform statistical analysis using preferred statistical software (e.g., GraphPad Prism 7.02).

4.4. Open the software. Choose **Column** under **New Table & Graph** and choose the one-way ANOVA data set.

4.5. Name each column with the strain tested and add the alginate concentrations underneath.

4.6. After inputting all the data click on **Analyze** in the top menu. This will open an analysis settings window. Choose the appropriate parameters for testing and indicate whether any multiple comparisons need to be run.

NOTE: After analysis the data set will contain a p-value that would help determine the statistical significance of the data. Data would be considered statistically significant if the p-value is less than the set α value (normally α value is set for $p<0.01$).

4.7. Under the graph tab choose the Bar Graph type. This will automatically chart the data with the indicated title from the input data sheet. Indicate statistical significance of the data as needed by showing the * symbol above connective lines between the bars of interest.

REPRESENTATIVE RESULTS:

Figure 1 shows plates of PAO1 and PAO581 with or without in-frame deletion in the *pyrD* gene (a gene in the pyrimidine biosynthesis pathway) that results in SCV[6]. The PAO1 SCV mutant was restored to normal growth in response to uracil supplementation (**Figure 1A,B**). Furthermore, the PAO581Δ*pyrD*SCV mutant was returned to mucoidy with the same uracil treatment, because the parent strain PAO581 has an additional *mucA25* mutation (**Figure 1C,D**). The results for the uronic acid carbazole assay are shown in **Figure 2**[6]. The data represents samples of PAO581 and PAO581 with mutations in genes regulating pyrimidine de novo biosynthesis grown on PIA and PIA supplemented with 0.1 mM of uracil (**Figure 2A**). The data shows that the presence of uracil in the media results in the conversion of the mutant strain back to mucoidy (as seen by the increase/restored alginate production) (**Figure 2B**). The results for the anti-alginate monoclonal antibody based ELISA are represented in **Figure 3**. The data shows PAO1, PAO1 with an in-frame deletion of the *algD* gene encoding the key alginate biosynthetic enzyme GDP-mannose dehydrogenase, and PAO1 carrying an expression plasmid pHERD20T with the main alginate-specific sigma factor *algU* when grown on PIA plates with arabinose for the induction the pBAD

promoter in *p*HERD20T. This data shows the non-mucoid levels of alginate measured for PAO1, and PAO1Δ*algD* versus the mucoid levels of alginate measured for PAO1+*p*HERD20T-*algU*. **Figure 4** compares the two methods of alginate measurements together. The results were not statistically significant when compared to each other using a two-way ANOVA with $p<0.01$. **Figure 5A** compares the cross reactivity of the anti-alginate mAb against other polysaccharides including amylopectin, amylose, collagen, and glycogen. **Figure 5B** shows the comparison in specificity and sensitivity of the uronic acid carbazole assay to the newly developed anti-alginate monoclonal antibody-based ELISA with the control utilizing the highly purified seaweed alginate (**Table of Materials**). **Figure 6** shows direct testing of the ELISA on patient sputum samples that were positive for mucoid *P. aeruginosa* and patients that did not contain mucoid *P. aeruginosa*.

FIGURE LEGENDS:

Figure 1: Representative images of the de novo pyrimidine biosynthesis mutations that result in SCV phenotype in PAO1 and PAO581. Image shows PAO1 (*left*) and PAO1Δ*pyrD* (*right*) on PIA plates (**A**) and PIA plates supplemented with uracil (**B**) grown at 37°C for 48 h. Image shows PAO581 (*left*) and PAO581Δ*pyrD* (*right*) on PIA plates(**C**) and PIA plates supplemented with uracil (**D**) grown at 37 °C for 48 h. This figure has been modified from work done by Al Ahmar et al.[6].

Figure 2: Representative graph of uronic acid carbazole assay. (**A**) The image of mucoid *P. aeruginosa* strain PAO581(PAO1*mucA25*) grown at 37 °C for 24 h on PIA plates (*left*) and PIA plates with uracil (*right*). (**B**) Alginate production for PAO581, PAO581*carA*, PAO581*carB*, and PAO581*pyrD* when grown on PIA plates with and without uracil at 37 °C for 24 h. Alginate was collected and measured using the standard carbazole assay. Values shown are mean alginate ± standard deviation of triplicate reads. (****=$p<0.0001$). This figure has been modified from work done by Al Ahmar et al.[6].

Figure 3: Representative graph of anti-alginate mAb-based ELISA. Alginate production for PAO1, PAO1Δ*algD* and PAO1 carrying the expression vector pHERD20T-*algU* grown on PIA with 0.1% arabinose at 37 °C for 24 h. Alginate was collected and measured using the anti-alginate ELISA with the mouse anti-alginate monoclonal antibody. Values shown are mean alginate ± standard deviation of triplicate reads. ****$p < 0.0001$.

Figure 4: Comparison between the results obtained from uronic acid assay and anti-alginate mAb-based ELISA. Alginate production from five different mucoid *P. aeruginosa* proprietary strains grown on PIA plates at 37 °C for 24 h. Alginate was collected and measured by the uronic acid assay and anti-alginate ELISA. Values shown are mean alginate ± standard deviation of triplicate reads.

Figure 5: Specificity and sensitivity of the anti-alginate mAb based ELISA in comparison to the uronic acid carbazole assay. (**A**) ELISA was run with high (800 µg/mL) and low (100 µg/mL) internal assay controls of the seaweed alginate. This alginate was also used as a standard for the ELISA. Other polysaccharides tested that may cross react with anti-alginate mAb were amylopectin, amylose, collagen, and glycogen (500 µg/mL each). (**B**) Uronic acid carbazole assay and ELISA were run using the same range of standard concentrations with seaweed alginate: 50

µg/mL, 5 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.1 µg/mL, and 0.05 µg/mL. Values shown are mean alginate ± standard deviation of triplicate reads.

Figure 6: Direct patient sample testing. Anti-alginate ELISA was tested on patients' sputum samples without prior growth on plates. Three CF sputum samples that had growth of mucoid *P. aeruginosa* were used as well as two patient sputum samples that contained either non-mucoid *P. aeruginosa* (Neg 1) or no *P. aeruginosa* growth (Neg 2). Values shown are mean alginate ± standard deviation of triplicate reads.

DISCUSSION:

Both SCV and alginate are important disease markers implicated in several chronic infections. Therefore, the ability to grow SCV as well as study the regulation and production of alginate by *P. aeruginosa* is integral to the discovery of novel treatments for these chronic illnesses.

SCV strains are notoriously difficult to grow due to their slow growth rate[4] as compared to other *P. aeruginosa* strains, which aids in their antimicrobial resistance[3]. Our work identifies a specific form of *P. aeruginosa* SCV that are a result of mutations in the de novo pyrimidine biosynthesis. Here, we discuss a growth condition for such SCV to revert to a normal growth phenotype when nucleoside uracil is supplied. However, when UMP/UTP was added to the medium, the defective growth was not restored (data not shown). The porin(s) responsible for this selectivity needs to be further studied. Supplementation of the growth media with uracil aided in relieving the stress induced from pyrimidine starvation (**Figure 1**). Similarly, addition of free cytosine to the media in the same method of addition of uracil, aided in the relief of pyrimidine starvation (data not shown). Both free uracil and free cytosine in the media enter the cells and help return the normal levels of uridine monophosphate (UMP) and uridine tri-phosphate (UTP)[6] in the cell, which is how the SCV isolates reverted back to normal growth.

Several critical steps for the alginate measurements exist that might aid in reproducibility of the results. Initially the samples, after being scrapped from the plates, must remain on ice to help block the degradation of alginate in the sample and result in lower measured concentrations. In addition, prior to OD_{600} measurements, it is important to thoroughly mix the sample to obtain a homogeneous solution as clumps do form in samples that have been sitting for a while and that might interfere with the OD measurement and thereby with the final concentration calculations. When using the ELISA method, samples from growth plates may need to be diluted especially when using strains that produce a large amount of alginate since results might be outside of the standard curve range. When using the uronic acid, thoroughly vortex the culture tubes after addition of the carbazole and after the incubation to ensure a homogeneous sample. When working with the uronic acid assay, it is important to be cautious when handling the acid mixture and to dispose of the samples properly.

The traditional method of measurement requiring acid hydrolysis of alginate described above has shown great potential and has been utilized by many researchers for several years. In this work, we show the procedure for the traditional assay along with an in-house developed ELISA using an anti-alginate monoclonal antibody. These antibodies were developed in mice and can

recognize alginate at a yet to be identified epitope. The specificity of the ELISA method was thoroughly tested against alginate from *P. aeruginosa* as well as from seaweeds. Moreover, the ELISA was comparable to the uronic acid assay in quantifying alginate in bacterial samples tested (**Figure 3**). In addition, it was tested against other polysaccharides that might result in false positive results. Our work showed a high specificity of the antibody to alginate and its ability to distinguish between alginate and the other tested polysaccharides (**Figure 5A**). The ELISA protocol has higher sensitivity as compared to the uronic acid assay (**Figure 5B**) since we were able to detect trace levels of alginate using ELISA that were not detected using the uronic acid assay when testing patient sputum samples (**Figure 6**). The ELISA method can be adapted for in vivo measurement of alginate from the patients' sputum (**Figure 6**). Both the growth conditions using uracil supplementation as well as the newly developed ELISA would be powerful tools in better understanding *P. aeruginosa* pathogenesis in CF.

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DISCLOSURES:

The author Hongwei D. Yu is the Chief Science Officer and Co-founder of Progenesis Technologies, LLC.

REFERENCES:

1. Govan, J. R., Deretic, V. Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. *Microbiological Reviews.* **60** (3), 539-574 (1996).
2. Hogardt, M., Heesemann, J. Adaptation of Pseudomonas aeruginosa during persistence in the cystic fibrosis lung. *International Journal of Medical Microbiology.* **300** (8), 557-562 (2010).
3. Evans, T. J. Small colony variants of Pseudomonas aeruginosa in chronic bacterial infection of the lung in cystic fibrosis. *Future Microbiology.* **10** (2), 231-239 (2015).
4. Johns, B. E., Purdy, K. J., Tucker, N. P., Maddocks, S. E. Phenotypic and Genotypic Characteristics of Small Colony Variants and Their Role in Chronic Infection. *Microbiology Insights.* **8**, 15-23 (2015).
5. Pestrak, M. J. et al. Pseudomonas aeruginosa rugose small-colony variants evade host clearance, are hyper-inflammatory, and persist in multiple host environments. *PLoS Pathogones.* **14** (2), e1006842 (2018).
6. Al Ahmar, R., Kirby, B. D., Yu, H. D. Pyrimidine Biosynthesis Regulates Small Colony Variant and Mucoidy in Pseudomonas aeruginosa Through Sigma Factor Competition. *Journal of Bacteriology.* **201** (1), e00575-18 (2019).
7. Ramsey, D. M., Wozniak, D. J. Understanding the control of Pseudomonas aeruginosa alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. *Molecular Microbiology.* **56** (2), 309-322 (2005).
8. Mathee, K., McPherson, C. J., Ohman, D. E. Posttranslational control of the algT (algU)-encoded sigma22 for expression of the alginate regulon in Pseudomonas aeruginosa and localization of its antagonist proteins MucA and MucB (AlgN). *Journal of Bacteriology.* **179** (11), 3711-3720 (1997).
9. Schurr, M. J., Yu, H., Martinez-Salazar, J. M., Boucher, J. C., Deretic, V. Control of AlgU, a

member of the sigma E-like family of stress sigma factors, by the negative regulators MucA and MucB and Pseudomonas aeruginosa conversion to mucoidy in cystic fibrosis. *Journal of Bacteriology.* **178** (16), 4997-5004 (1996).
10. Rehm, B. H. A. Alginate Production: Precursor Biosynthesis, Polymerization and Secretion. In *Alginates: Biology and Applications.* Edited by Rehm, B. H. A., 55-71, Springer Berlin Heidelberg. Berlin, Germany (2009).
11. Remminghorst, U., Rehm, B. H. Bacterial alginates: from biosynthesis to applications. *Biotechnology Letters.* **28** (21), 1701-1712 (2006).
12. Potvin, E., Sanschagrin, F., Levesque, R. C. Sigma factors in Pseudomonas aeruginosa. *FEMS Microbiology Reviews.* **32** (1), 38-55 (2008).
13. Damron, F. H., Goldberg, J. B. Proteolytic regulation of alginate overproduction in Pseudomonas aeruginosa. *Molecular Microbiology.* **84** (4), 595-607 (2012).
14. Bowness, J. M. Application of the carbazole reaction to the estimation of glucuronic acid and flucose in some acidic polysaccharides and in urine. *The Biochemical Journal.* **67** (2), 295-300 (1957).
15. Fazio, S. A., Uhlinger, D. J., Parker, J. H., White, D. C. Estimations of uronic acids as quantitative measures of extracellular and cell wall polysaccharide polymers from environmental samples. *Applied Environmental Microbiology.* **43** (5), 1151-1159 (1982).
16. Knutson, C. A., Jeanes, A. A new modification of the carbazole analysis: application to heteropolysaccharides. *Analytical Biochemistry.* **24** (3), 470-481 (1968).

Figure 1

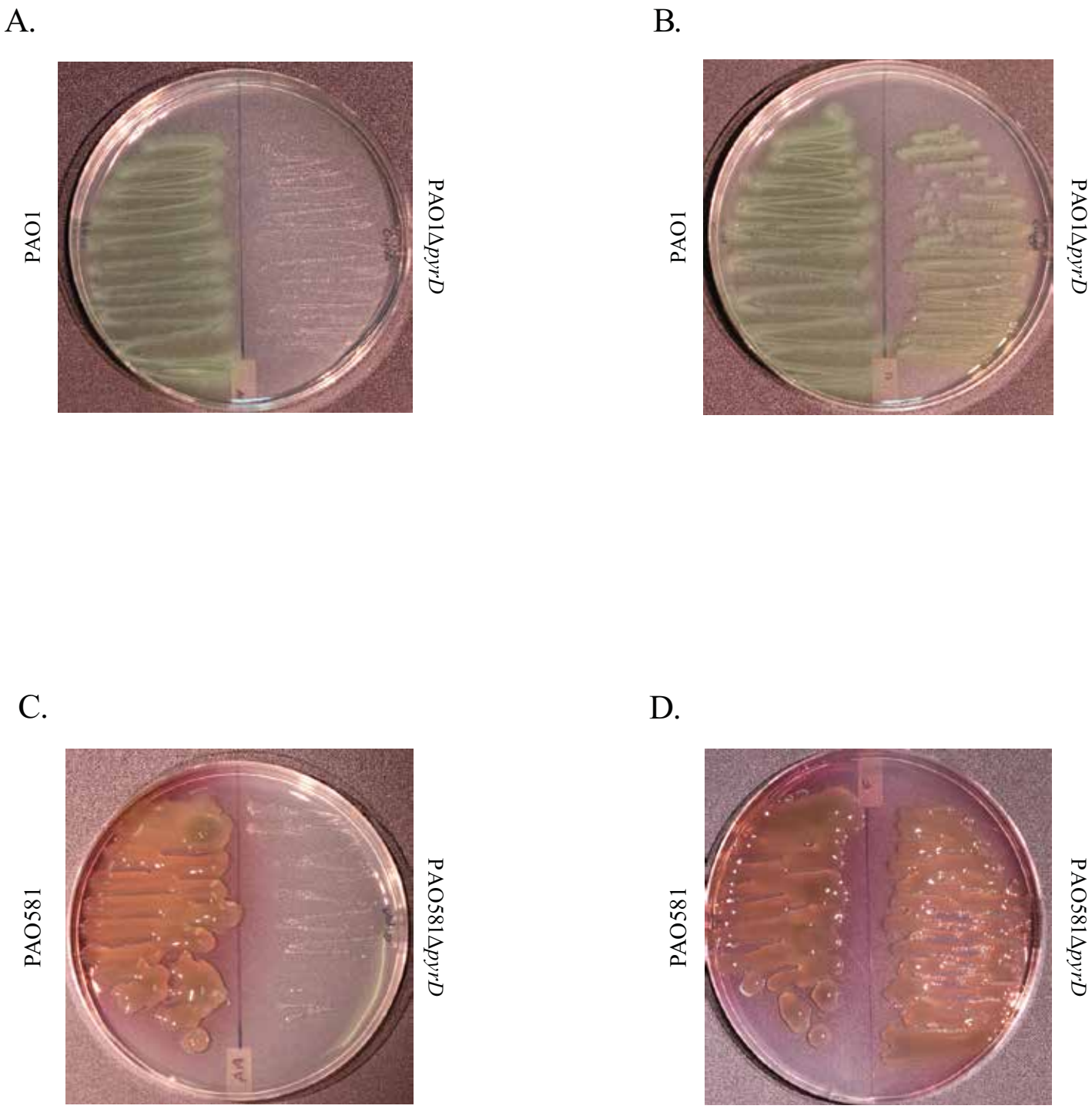
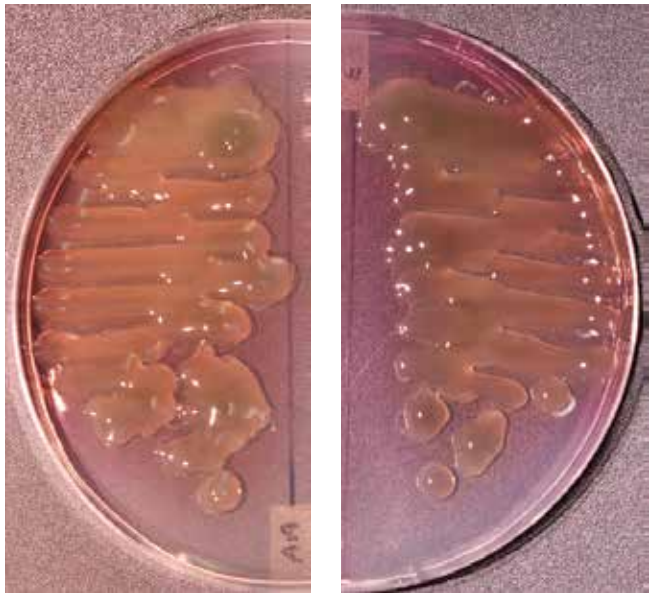


Figure 2

A.



PIA PIA + Uracil

B.

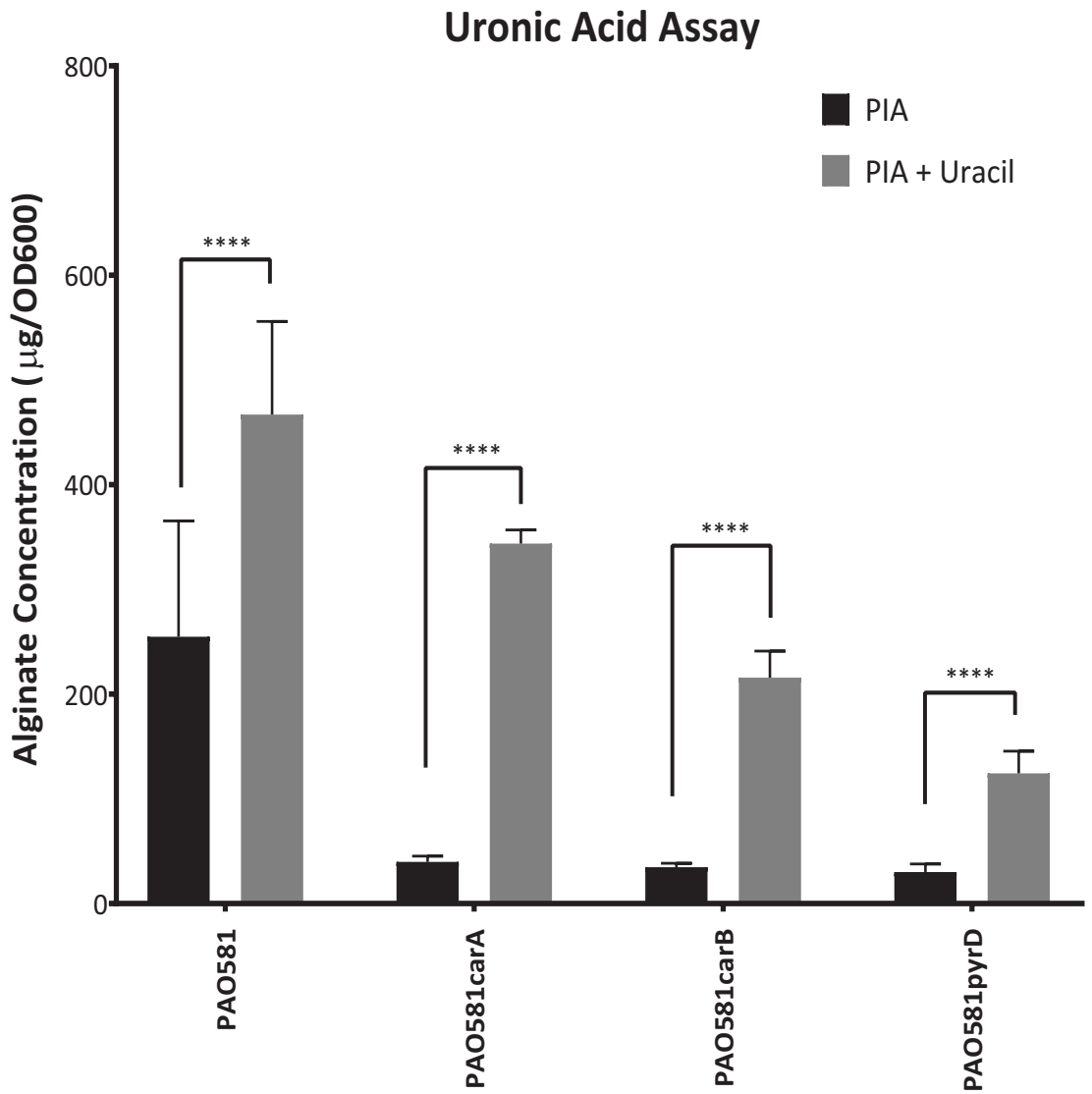


Figure 3

Click here to access/download;Figure;Figure 3.pdf

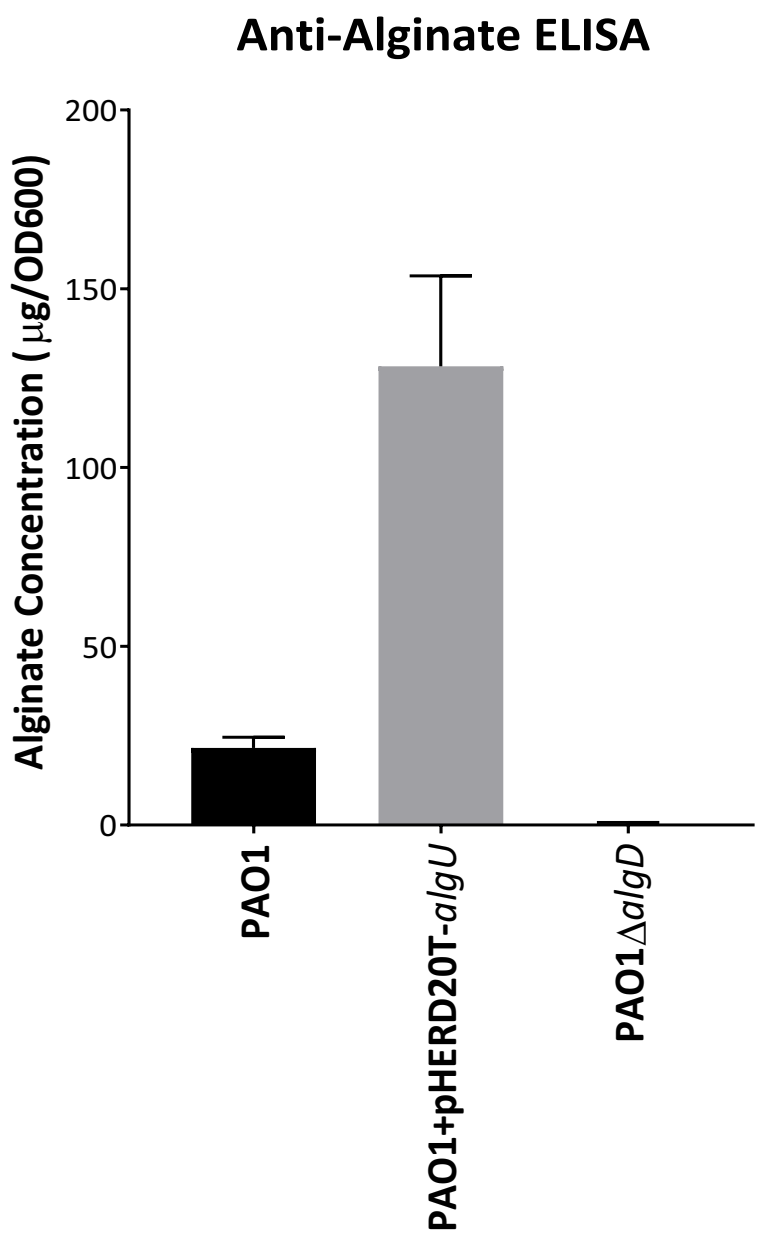


Figure 4

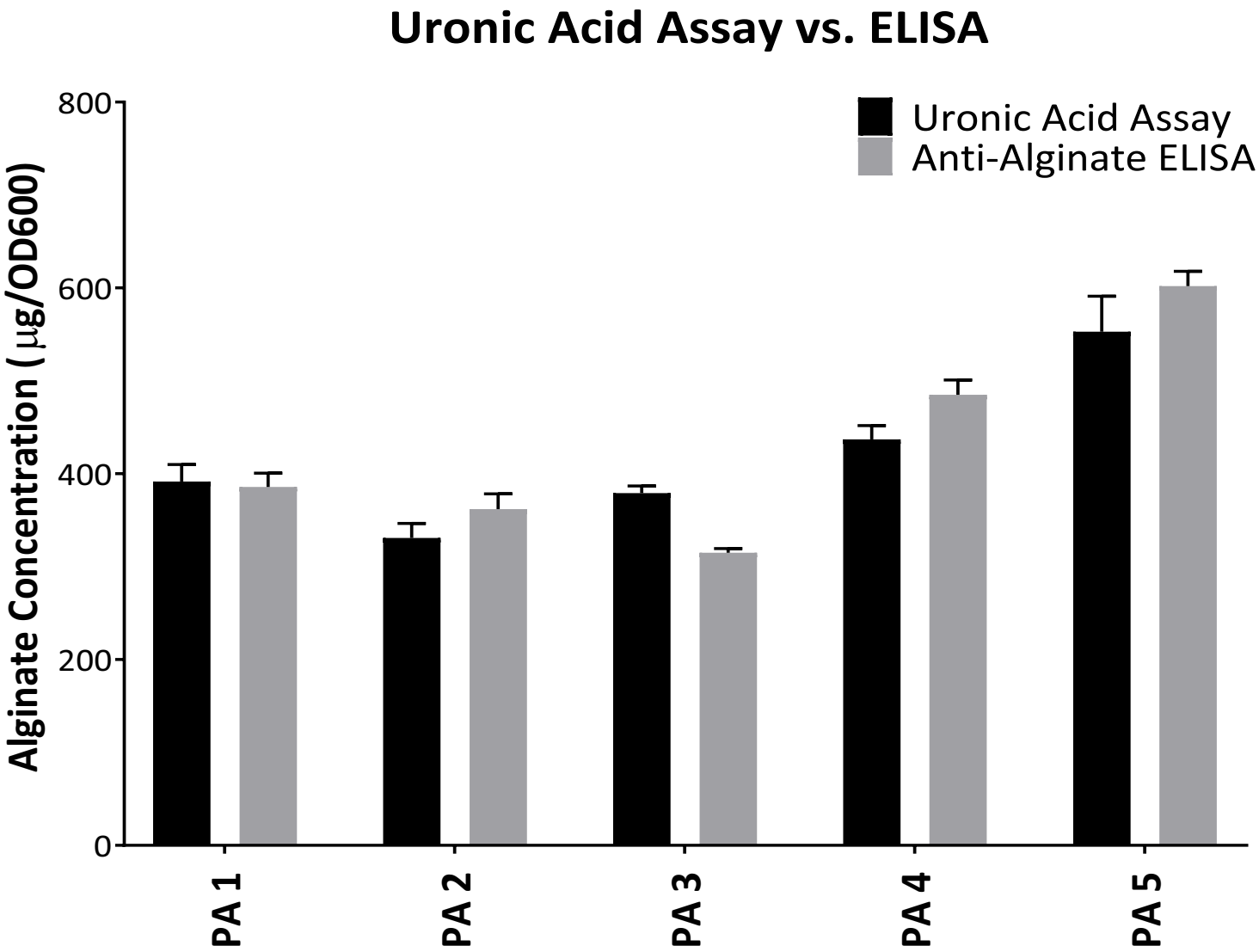


Figure 5

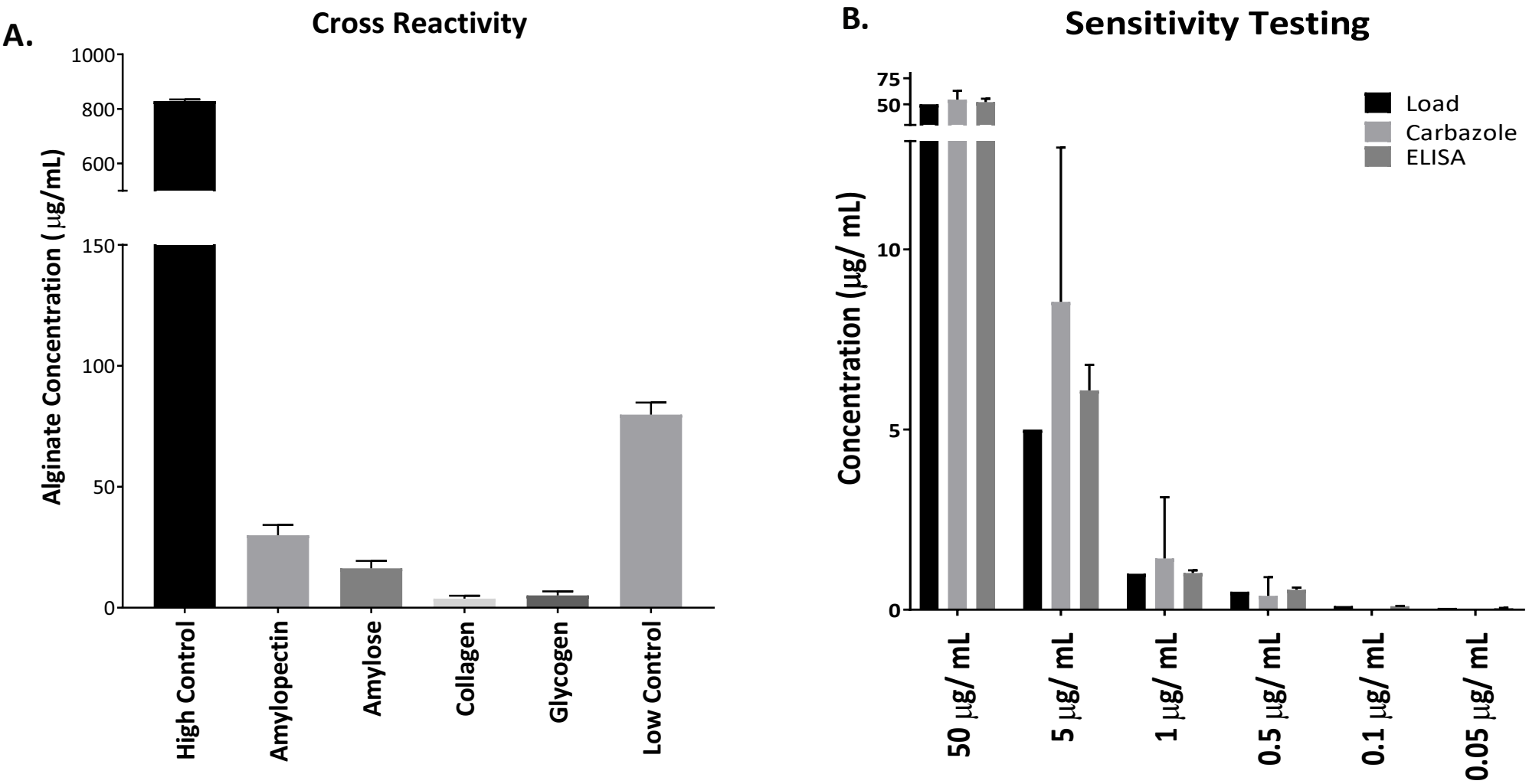


Figure 6

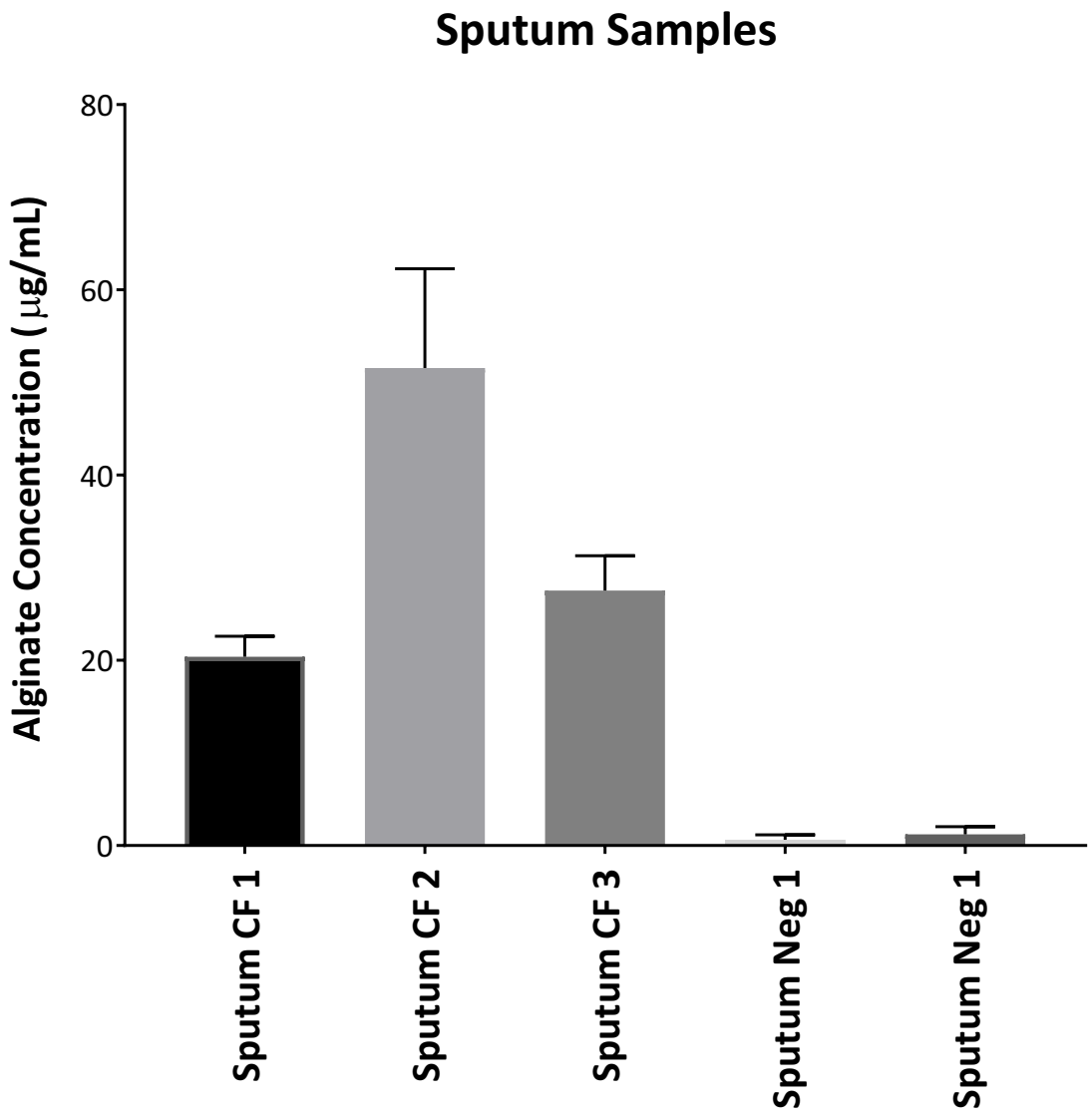


Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1-Step Ultra TMB-ELISA	Thermo Scientific	34028	via Fisher Scientific
Absolute Ethanol (200 Proof)	Fisher Scientific	BP2818-4	Molecular Bio-grade
Accu Block Digital Dry Bath	Labnet	NC0205808	via Fisher Scientific
Assay Plates 96-well	CoStar	2021-12-20	
Bench Top Vortex-Genie 2	Scientific Industries	G560	
Boric Acid	Research Products International Corp.	10043-35-3	
Cabinet Incubator	VWR	1540	
Carbazole	Sigma	C-5132	
Carbonate-Bicarbonate Buffer	Sigma	C3041	
Centrifuge Tubes (50 ml)	Fisher Scientific	05-539-13	via Fisher Scientific
Culture Test Tubes	Fisher Scientific	14-956-6D	via Fisher Scientific
Cuvette Polystyrene (1.5 ml)	Fisher Scientific	14955127	via Fisher Scientific
Cytosine	Acros Organics	71-30-7	
Diposable Inoculation Loops	Fisher Scientific	22-363-597	
D- Mannuronic Acid Sodium	Sigma Aldrich	SMB00280	
FMC Alginate	FMC	2133	
Glycerol	Fisher Scientific	BP906-5	For Molecular Biology
Mouse Anti-Alginate Monoclonal Antibody	QED Biosciences	N/A	Lot # :15725/15726
Phosphate Buffered Saline Powder (PBS)	Sigma	P3813	
Pierce Goat Anti-Mouse Poly-HRP Antibody	Thermo Scientific	32230	via Fisher Scientific
Potassium Hydroxide	Fisher Scientific	1310-58-3	via Fisher Scientific
Prism 7	GraphPad		
Pseudomonas Isolation Agar (PIA)	Difco	292710	via Fisher Scientific
Pseudomonas Isolation Broth (PIB)	Alpha Biosciences	P16-115	via Fisher Scientific
Round Toothpicks	Diamond		Any brand
Seaweed alginate (Protanal CR 8133)	FMC Corporation		
Skim Milk	Difco	232100	via Fisher Scientific
SmartSpec Plus Spectrophotometer	BioRad	170-2525	or preferred vendor
Sodium Chloride (NaCl)	Sigma	S-5886	
SpectraMax i3x Multi-mode MicroPlate Reader	Molecular Devices	i3x	or preferred vendor
Sterile Petri Dish 100mm x 15mm	Fisher Scientific	FB0875713	via Fisher Scientific
Sulfuric Acid	Fisher Scientific	A298-212	Technical Grade
Sulfuric Acid (2 Normal -Stop Solution)	R&D Systems	DY994	
Tween 20	Sigma	P2287	
Uracil	Acros Organics	66-22-8	

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Author(s):	Roy Al Ahmar, Brandon D Kirby, Hongwei D Yu

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Title:	**Professor**
Signature:	
Date:	**06/22/2019**

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Thank you to the reviewers for their comprehensive review of our manuscript. Find below the point by point rebuttal letter.

All changes made in the text have been highlighted with red text.

Editorial comments:

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2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: 1-Step Ultra, Excel, GraphPad Prism, QED Biosciences, etc.

*Commercial names have been removed from the manuscript. QED Biosciences was kept since they have developed the monoclonal antibody used in this work. The mAbs used in this study are available (*https://www.qedbio.com/product/pseudomonas-reactive-alginate-monoclonal-antibodies-2/).

3. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Fixed

4. Please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

Fixed

5. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

Fixed

6. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Fixed

Reviewers' comments:

All changes made in the text have been highlighted with red text.

Reviewer #1:

Manuscript Summary:

I commend authors for addressing all of my concerns. I have only a few additional minor comments, that once addressed I would recommend publishing the manuscript. I do not need to review the manuscript again.

Minor Concerns:
1. Step 2.4. I think my confusion with this step is the statement of 'culture tubes'. To me this implies adding the solution to a tube containing the bacterial culture. However, I think authors are referring to an empty tube that they use to culture bacteria in? Perhaps if authors clarify this by stating 'glass tube' or something similar instead?

Culture tubes has been changed to test tubes to avoid the confusion. (Line: 147)

2. L210-213. Note has been replicated

Removed from text.

3. Step 3.3. No need to indicate OD600 is an abbreviation of 'optical density at 600nm' as this has been introduced in the section above.

Removed from text.

4. L303. As each alginate method uses a different wavelength to measure the sample, carbazole

assay used OD530 and ELISA uses OD450, authors might want to change the x-axis to something more generic or specify the OD for each assay.

Fixed in text. (Line: 302)

5. L332. Italicize 'pyrD'.

Fixed in text. (Line: 331)

6. L356. I think authors mean PAO581ΔpyrD instead of PAO1ΔpyrD

Fixed in text. (Line: 356)

7. Figure 4. What are these strains? Are they clinical isolates?

Strains used for Figure 4 are proprietary strains produced by the company Progenesis Technologies LLC. They were derived from reference strains of P. aeruginosa PAO1. *We cannot disclose more details about them at this time.*

This has been noted in the figure legend in text. (Line: 374)

Reviewer #2:
Manuscript Summary:
Unfortunately, this revised manuscript still fails to address many of the previous concerns. The stated goals are to provide methods for culturing small colony variant (SCV) of Pseudomonas aeruginosa and quantifying the level of alginate using two different techniques. However, there are many problems with the justification of these approaches not to mention the techniques themselves. Furthermore, particular mis-statements pointed out in the prior review did not seem to be corrected or clarified and numerous typographical or grammatical errors still remain.

Major Concerns:
Line 37: Alginate is not the only polysaccharide involved in biofilm formation.

Changed to alginate in text. (Line: 37)

Line 40: As mentioned in the prior review, pyrimidine biosynthesis mutations are only ONE way that SCV can arise in P. aeruginosa. This needs to be stated explicitly.

Amended in text. (Line:40)

Line 62: It is not clear what is meant by this statement. What marker would be revealed by measuring alginate production? This needs to be clarified.

Fixed in the text (Line 63)

Line 67: What is the evidence that the level of alginate in sputum samples correlates with patients' quality of life?

This sentence was removed and amended in text. (Line: 70)

Line 71: It is not obvious how the techniques presented INDUCE the formation of SCV, they would only seem to detect them.

Amended in text. (Line: 72)

Line 84: The ELISA method IS NOT measuring alginate present IN THE LUNGS. Here the authors are presenting measuring alginate in sputum coughed up by patients.

We are using the sputum samples aspirated from the patients with chronic lung infections with P. aeruginosa. Since the ELISA developed can detect alginate in these samples directly, it may predict the alginate concentrations in the lungs of these patients.

Line 92: As mentioned, this does not seem to be ISOLATION, but rather DETECTION.

Amended in text. (Line: 93)

For the new data looking at the specificity of the monoclonal antibody, nothing seems to have been done quantitatively. There still doesn't seem to be any data showing that the ELISA is more sensitive than the chemical analysis.

Data added for sensitivity testing. (Figure 5B)

Figure 5 does not indicate how much of any polysaccharides were added. Why weren't the ug/ml of mannuronic acid vs. the levels of reactivity of the carbazole vs. the ELISA shown? What was the volume of sputum tested? Why wasn't the data for the sputum tested with the carbazole presented?

1. *Amount of polysaccharide added was 500 μg/mL. This value has been added to the figure legend. (Line: 382)*

2. *Volume of the sputum was 50 µl as any sample for the ELISA.*
3. *The sputum testing using carbazole did not generate any results. The background was too high to detect any alginate in the samples. Since no true measurements were taken, that data was not included.*

General comment: It is unclear how the authors envision any of these protocols being useful, if investigators do not have access to the appropriate positive or negative controls samples.

The usefulness of SCV protocol lies in the restoration of growth defect by adding uracil to the growth medium. This allows further characterization of the respective porins in the future studies, as mentioned in the amended discussion. The ELISA protocol shows an improved specificity and sensitivity compared to the traditional uronic acid method (new Figure 5). This mAb based ELISA may allow the measurement of alginate in patient's sputum (Figure 6). Our positive controls are highly purified seaweed alginate from FMC biopolymer (Protanal CR 8133).), and negative controls are a bacterial strain that produce zero alginate because the gene encoding the key alginate biosynthesis enzyme (AlgD) was in-frame deleted from the chromosome of the bacteria (PAO1ΔalgD. Both controls were clearly pointed out in the revised text.

Minor Concerns:
Line 27: Small Colony Variant does not need to be capitalized, but Pseudomonas should be written out the first time.

Fixed in text. (Line: 27)

Line 33: Pseudomonas should be written out the first time used.

Fixed in text. (Line: 33)

Reviewer #3:
Manuscript Summary:
The manuscript describes two methods to quantify alginate production in Pseudomonas aeruginosa and a method for culturing small colony variants. The alginate quantitation methods are very precise and will be useful to investigators interested in determining the amount of alginate produced by Pseudomonas aeruginosa. It might be interesting to know if the author's methods can be used for other organisms that produce alginate, like Rhizobium. The direct comparison of the uronic acid assay to the ELISA was informative as was the cross reactivity test performed by the authors.

Major Concerns:
The method for culturing small colony variants is very specific to a pyrimidine auxotroph. It isn't apparent that this method could be used to determine IF a colony is a small colony variant or if

the organism had another mutation that resulted in small colony variation. The proposed method is very specific to pyrimidine metabolism and it is known that there are many other pathways to small colony variants, e.g., cytochrome oxidase mutations, amino acid auxotrophy and central carbon metabolism mutations. It almost looks like the authors were trying to bolster a previous publication but the link between alginate and pyrimidine auxotrphy may simply be growth rate. In this reviewer's opinion, the alginate quantitation stands alone and is sufficient for publication. The small colony variant method should be removed from the document.

We agree with this reviewer on the characterization of one type of SCV as pyrimidine auxotrophs as described here. There are other forms of SCV. But the restoration of growth defect by adding uracil to the medium is a unique aspect of this phenotype. We add the following statement to the discussion to better explain this genetically defined SCV, and also point out the limitation and future direction of our studies.

Our work identifies a specific form of P. aeruginosa SCV that are a result of mutations in the de novo pyrimidine biosynthesis. Here we discuss a growth condition for such SCV to revert to a normal growth phenotype when nucleoside uracil is supplied. However, when UMP/UTP was added to the medium, the defective growth was not restored (data not shown). The porin(s) responsible for this selectivity needs to be further studied.

Minor Concerns:

1. Pg. 4, under 3.1.2. "Note" and "Note 1" are identical.

Removed from text.