INFLUENCE OF BACTERIAL OUTER MEMBRANE VESICLES ON STRUVITE CRYSTAL GROWTH

by

Ashley Shanaé Summers, B.S.

A thesis submitted to the Graduate Council of Texas State University
in partial fulfillment of the requirements for the degree of
Master of Science with a Major in Biology
December 2018

Committee Members:

Robert J. McLean, Chair

Manish Kumar

Jeffrey W. Schertzer

FAIR USE AND AUTHOR'S PERMISSION STATEMENT

Fair Use

Duplication Permission

ACKNOWLEDGMENTS

First and foremost, I'd like to thank my parents for always supporting my dreams. I would not be where I am today without them. Thank you, mom, for always being my rock (and my chauffer!) whenever I need you. You are my best friend!

Dr. McLean, thank you so much for designing this project for me. I had so much fun doing this research and I learned so many invaluable lessons. Thank you for being such a great advisor and answering my millions of questions!

Dr. Kumar and Dr. Schertzer, thank you so much for agreeing to be on my committee. Your support and direction were always so helpful. Thank you for all of your insight and advice! You both were crucial to the success of my paper.

Dr. Cardona and Dr. Chiung, thank you so much for letting us use the flow cytometer at UTSA and for teaching me how to use IDEAS. I would not have been able to complete my thesis if it weren't for your graciousness.

Dr. Weigum, thank you for allowing me to use your sonication probe. I am so appreciative of your willingness to help during that section of my research.

To my lab mates, especially Starla and Quentin, thank you so much for answering all of my questions! Especially during the time that Dr. McLean was gone. You guys helped me so much and kept me calm through the process. I am grateful for our friendships!

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

ABSTRACT

Urinary tract infections (UTIs) are a very serious health concern, affecting millions of people each year. They lead to almost 10 million doctor's visits and hundreds of thousands of hospital admissions every year in the United States (Mittal 2009). One of the common causatives of UTIs is *Pseudomonas aeruginosa*, a gram negative, aerobic, rod-shaped bacterium (Balcht 1994). As a potential way to deliver virulence factors, *P. aeruginosa* secrete outer membrane vesicles (OMV), which are formed through direct interaction of *Pseudomonas* quinolone signal (PQS) with the lipopolysaccharide component of the outer membrane (Schertzer 2012, Mashburn-Warren et. al. 2008). Strains used in this research have mutations in these genes in the pathway that result in the inhibition of the formation of OMV. Commonly seen in bacterial urinary tract infections is the presence of struvite crystals. These crystals precipitate in alkaline urine, and eventually form larger stones. Alkaline urine is caused by the hydrolysis of urea to ammonia via the enzyme urease (Le Corre et. al. 2005). The purpose of this research is to study if the presence of outer membrane vesicles produced in *P. aeruginosa* induce struvite crystal formation. Various strains of *P. aeruginosa,* with and without OMV, were grown in BHI (Brain Heart Infusion agar) and suspended in artificial urine. Since *P. aeruginosa* lacks urease, the urease action was mimicked by titrating artificial urine with ammonium hydroxide, inducing struvite crystal formation. The presence and shape (crystal habit) of struvite crystals was confirmed via phase contrast microscopy.

Transmission electron microscopy (TEM) was used to confirm the presence of OMV. When crystals were measured using imaging flow cytometry, there was no significant difference in crystal numbers in intact and fragmented *P. aeruginosa* cells regardless of the presence or absence of OMV; although the number of crystals formed was elevated in controls lacking bacteria. This in vitro data suggests that *P. aeruginosa,* regardless of OMV production, does not enhance struvite formation in artificial urine.

I. INTRODUCTION

Nanobacteria

Nanobacteria is defined as a proposed class of living organisms, specifically cell-walled microorganisms with a size much smaller than the generally accepted lower limit for life (Kajander 2006). Nanobacteria (which range in size from 80-500nm) were initially proposed as unique independent life forms (Uwins et. al. 1998, Folk 1993) and objects interpreted as fossilized nanobacteria within a Martian meteorite were postulated to be evidence of extraterrestrial life (McKay et. al. 1996). Recently a number of studies have been performed to address the origin of nanobacteria. Several studies have shown that under sterile abiotic conditions, some minerals can form small nano-sized particles that morphologically resemble nanobacteria (Garcia-Ruiz et. al. 2003). Nano-sized objects can also arise from the interaction of bacterial cell surface capsule polysaccharides with elevated calcium (Kirkland et. al. 1999).

Although the generally accepted minimal diameter for independent life-forms is now considered to be ~300 nm, nano-sized objects are found in environments that are compatible with microorganisms. A plausible hypothesis is that nano-sized objects may represent fragments of bacteria and other microorganisms. One common feature seen in several gram-negative bacteria is the formation of outer membrane vesicles (Schooling 2006). Outer membrane vesicles (OMV) are made of lipids released from the outer membranes of gram-negative bacteria to communicate among themselves and with other

microorganisms in their environment (Biller et. al. 2014). OMV are approximately the size of nanobacteria. Also seen in gram-negative bacteria during infection in the urinary tract is the formation of mineral crystals, such as struvite (magnesium ammonium phosphate). Given the observation of nano-sized objects during many instances of bacterial-associated mineral formation, the goal of this thesis was to investigate whether nano-sized outer membrane vesicles had any influence on mineral formation and crystal growth.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a gram negative, aerobic, rod-shaped bacterium that is found in the natural flora of soil, water, plants, and animals. It is an opportunistic pathogen known to cause many diseases such as pneumonia, enterocolitis, and most importantly to this research, urinary tract infections (Balcht 1994). As a potential way to deliver virulence factors, *P. aeruginosa* secrete outer membrane vesicles (OMV) that contain many factors important for infection. OMV can be detected by looking under a light or electron microscope. OMV formation occurs through direct interaction of *Pseudomonas* quinolone signal (PQS) with the lipopolysaccharide component of the outer membrane (Schertzer 2012, Mashburn-Warren et. al. 2008). *Escherichia coli* is the most common cause of uncomplicated cystitis, meaning that there are no foreign objects present or changes in tissue structure. *Proteus mirabilis* is commonly associated with complicated cystitis, meaning that some type of tissue disruption occurs, in the case of *Proteus* – struvite calculi (Jacobsen et. al. 2008). However, *P. aeruginosa* was chosen because of its known outer membrane vesicle formation mechanism.

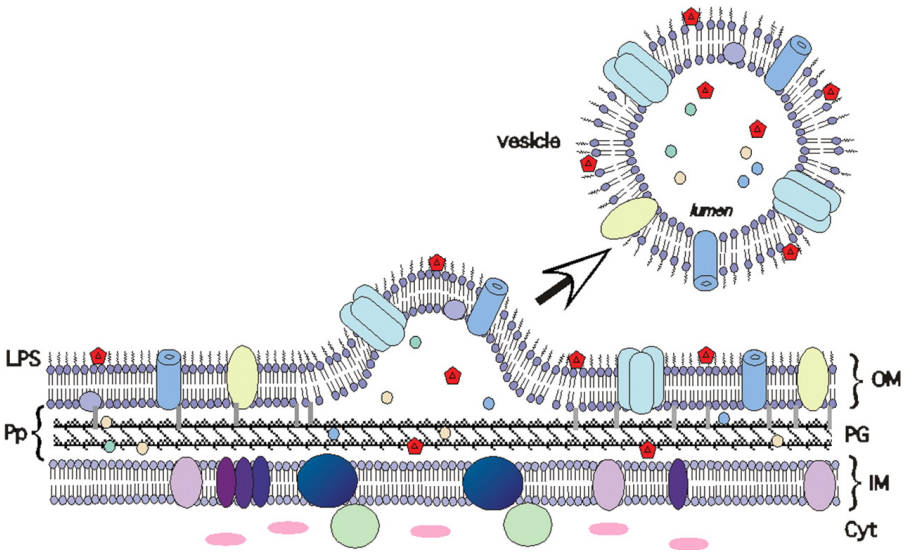

Figure 1: Formation of outer membrane vesicles (Kuehn 2005).

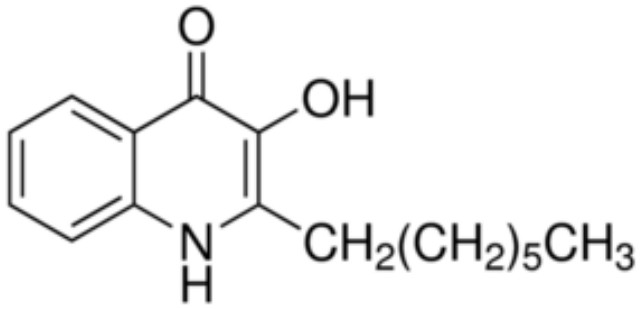

Figure 2: Structure of *Pseudomonas* quinolone signal (PQS).

PQS Pathway and OMV Formation

P. aeruginosa houses a unique system that produces a *Pseudomonas* quinolone signal (PQS), 2-heptyl-3-hydroxy-4-quinolone, that is necessary for OMV formation. Although it was previously known to be an important signal for controlling group behavior, it has been found to be a multifunctional compound (Schertzer 2012).

PQS is synthesized via the *pqsABCDE* operon, which is also responsible for the synthesis of other antimicrobial quinolones, including the PQS precursor 2-heptyl-4-quinolone (HHQ). HHQ is derived via the condensation reaction of anthranilic acid and β-ketodecanoic acid. This is aided by *pqsABCDE*, where PqsA is a coenzyme A ligase that helps with anthranilate activation, and PqsB, PqsC, and PqsD act as β-keto-acyl-acyl carrier protein synthetases. The FAD-dependent monooxygenase PqsH converts PQS precursor HHQ into PQS (Schertzer et. al. 2010). Anthranilic acid can be derived via two different pathways: either by PhnAB anthranilate synthetase from shikimic acid via chorismic acid or tryptophan degradation (Dubern 2008).

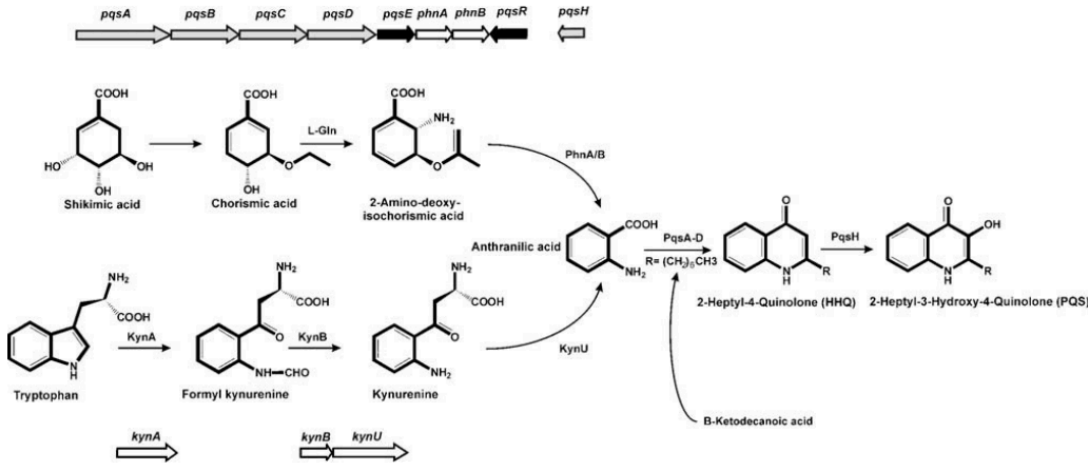

Figure 3: PQS synthesis in *P. aeruginosa.* The top pathway depicts anthranilic acid synthesis via PhnAB anthranilate synthetase from shikimic acid. The bottom pathway depicts anthranilic acid synthesis via tryptophan degradation (Dubern 2008)

A bilayer-couple model was suggested for the mechanism of PQS in OMV formation. This model proposes that PQS activates OMV formation through asymmetric expansion

of the outer membrane. Hydrophobic PQS has a high affinity for the lipopolysaccharides (LPS) found in the membrane making it easy to attach. This causes a tight interaction with lipid A, which in turn causes the membrane to expand and curve out. Once the membrane curves in a large enough area, it is forced to buckle and pinch off, creating an OMV (Schertzer 2012).

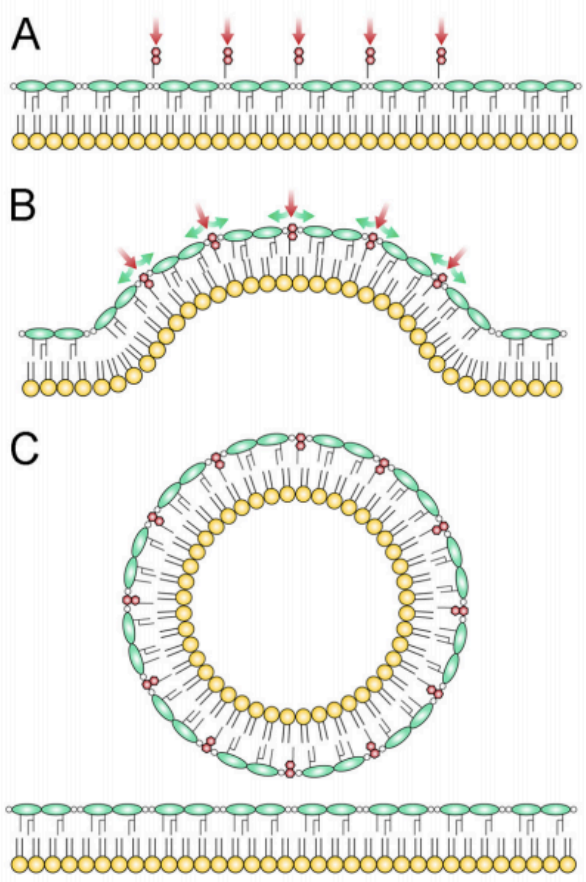

Figure 4: Bilayer-couple model of OMV formation. (A) PQS interaction with LPS. (B) Curvature of membrane. (C) Buckling and pinching of membrane to form OMV (Schertzer 2012).

Urinary Tract Infections

Urinary tract infections (UTIs) are a very serious health concern, affecting millions of people each year. They lead to almost 10 million doctor's visits and hundreds of thousands of hospital admissions every year in the United States. Because of these statistics, UTIs are the second most common infection of all the organ systems in the body. In strains associated with UTIs, high production of elastase and protease were seen. *P. aeruginosa* also has the tendency to form biofilms, which highly correlates with UTIs (Mittal et. al. 2009).

Struvite ($MgNH_4PO_4 \cdot 6H_2O$) crystal formation and associated urolithiasis (kidney stone formation) is a common complication of urinary infections by *Proteus mirabilis* and other urease-producing bacteria (McLean et. al. 1988). Here the main virulence factor is urease, which hydrolyzes urea and generates an alkaline pH, which in turn causes precipitation of Mg^{2+} ions as struvite (Griffith et. al. 1976.). However, struvite crystal growth rates have been shown to be influenced by *P. mirabilis* biofilm growth (Clapham et. al. 1990, McLean et al. 1991) and capsule polymers (Dumanski et. al. 1994). In order to test whether outer membrane vesicles had an influence on struvite formation, we used *Pseudomonas aeruginosa* as a model organism in that the genetics and mechanism of its outer membrane vesicle formation are known (Schertzer 2012). Urease activity was modeled using ammonium hydroxide titration of artificial urine (Downey et. al. 1992).

II. METHODS

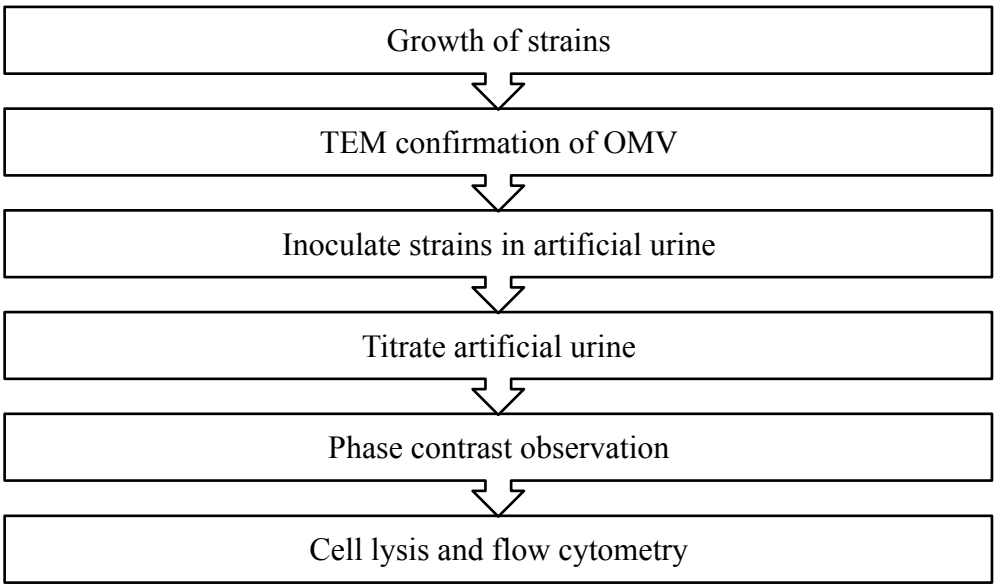

Figure 5: Flowchart of methods.

Growth of Strains

Various strains of *P. aeruginosa* bacteria were grown in BHI (blood heart infused agar). There was one parent strain and one strain with a mutation occurring in a gene associated with the conversion of PQS precursor HHQ into PQS (Mashburn 2005). The strains are summarized in the following table.

Table 1: Strains of *P. aeruginosa* used.

Strain	Description
PA14 wt	Parent strain
PA14 Δ *pqs*H	Mutant strain

Strains were kept frozen at -80°C while in storage. They were then grown on BHI agar overnight (~17 hours) at 37°C. Next, they were grown in BHI broth overnight (~17 hours) at 37°C in a shaking incubator set at 150 rpm. In a recent article published, an experiment was described where they grew *P. aeruginosa* in LB medium and in brain heart infused (BHI) medium. Their results showed that simply using the BHI medium as opposed to LB medium caused *P. aeruginosa* to produce more OMV (Florez et. al. 2017).

TEM Confirmation of OMV

Transmission electron microscopy (TEM) was used to confirm the presence of OMV. *P. aeruginosa* strains were grown using the method previous described. A 1% solution of filter-sterilized uranyl acetate was used to suspend bacteria. The 0.22 μm filter sterilization as intended to remove any large, undissolved particles that would interfere with examination under the microscope. *P. aeruginosa* was picked up using formvar-coated grids and viewed using a JEOL 1200ex ii transmission electron microscope.

Artificial Urine & Phase Contrast Microscopy

Artificial urine (AU) was made using the following recipe. It was made in two parts. Part A was made and autoclaved, while Part B was made and filter-sterilized. After

aseptically combining the two parts, the AU was adjusted to a pH of 5.8 using hydrochloric acid (HCl) as an acid and sodium hydroxide (NaOH) as a base.

Table 2: Recipe for artificial urine.

Chemical	Amount
Part A	
MilliQ Water	900 mL
Magnesium chloride (hexahydrate)	0.65 g
Sodium chloride	4.60 g
Sodium sulfate	2.30 g
Sodium citrate	0.65g
Sodium oxalate	0.002 g
Potassium phosphate (monobasic)	5.60 g
Potassium chloride	1.60 g
Tryptic soy broth	10.0 g
Part B	
MilliQ Water	100 mL
Ammonium chloride	1.00 g
Calcium chloride (dihydrate)	0.65 g
Urea	25.0 g
Creatinine	1.10 g
Total	**1000 mL (1 L)**

15 mL of artificial urine was transferred to a sterile 30 mL beaker. 1 mL of *P. aeruginosa* was inoculated in the AU. Since *P. aeruginosa* is not known to have urease, urease was recreated by titrating artificial urine with ammonia hydroxide to reach a pH of 8 (Griffith et. al. 1976).

Using a glass pipette, one drop of inoculated AU was transferred to a clean microscope slide. Once a coverslip was placed on top, crystal struvite formation was observed under phase contrast microscopy. The actual shape of the crystal structure is indicative of the rate of growth. Crystals that are X-shaped have a very high growth rate, while trapezoidal crystals indicate slow growth (Clapham et al. 1990).

Cell Lysis and Flow Cytometry

A flow cytometer was used to determine the ratio of cell particles to crystal structures. Lysed bacteria were used as a control because they can produce material that is roughly the same size and count as OMV secreted by *P. aeruginosa*.

P. aeruginosa was lysed using a sonication probe. In a 1.5 mL microcentrifuge tube, cells were suspended in artificial urine. The cells were sonicated at a frequency of 20 kHz for 3 X 10 seconds with approximately 5 seconds between blasts. After sonication, cells were spun down to pellet debris. Debris may contain unlysed cells, as well as cell and membrane fragments. The supernatant was transferred to a new microcentrifuge tube and labeled appropriately. Samples were stored at -80 °C.

10 scenarios were created to test the strains under various conditions. The PA14 wildtype strain was separated into two groups, one that was sonicated to create cell fragments and one that remained intact. These subgroups were further separated into two more groups, one that was titrated to form crystals and one that was not titrated so that crystals would not be seen. The *pqsH* mutant strain was also divided into the same groups. An additional background control group was created, where artificial urine with no bacteria present was separated into two groups, titrated and not titrated.

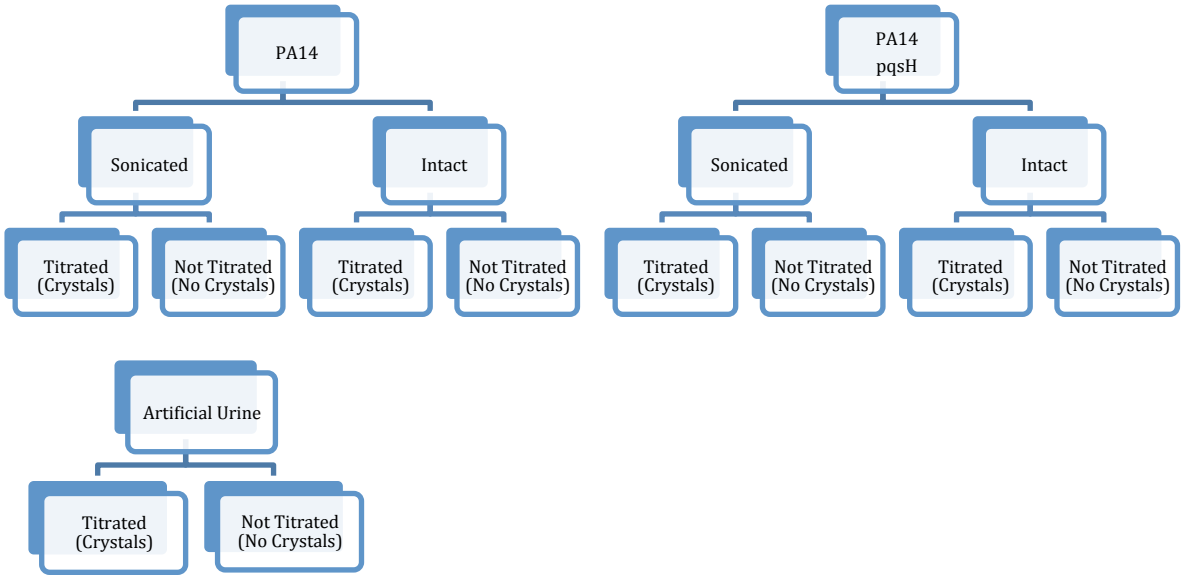

Figure 6: Summarization of strain scenarios used in the flow cytometry experiments.

An imaging flow cytometer was used for quantitative analysis of each scenario with the help of University of Texas – San Antonio. First, 1 mL of bacteria/bacteria sonicate was spun down and the supernatant was discarded. The pellet was resuspended in 50 µL of artificial urine. 5 µL of fluorescent beads at a known concentration were added in each sample. The sample was analyzed with the flow cytometer. Two imaging channels were

used: a brightfield channel for visualization of each data point coming through the nozzle and a fluorescent channel (400 nm) for identification of the control beads. The goal count for each scenario was 50,000 data points. The data was analyzed using the Imaging Data Exploration of Analysis Software (IDEAS) by AMNIS. The data was further analyzed using Sigma Plot.

III. RESULTS

TEM Confirmation of OMV

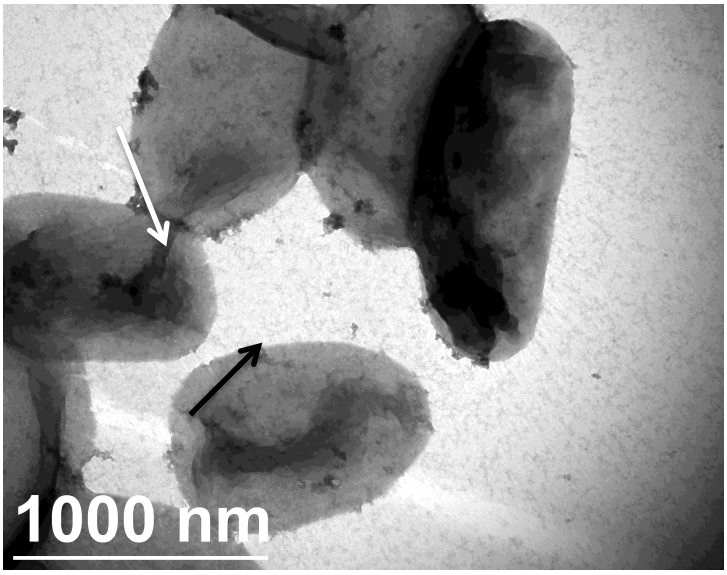

Figure 7: Negative stain preparation of *P. aeruginosa* strain PA14 wt under transmission electron microscopy. OMV can be seen blebbing from the surface of the bacteria and floating freely between.

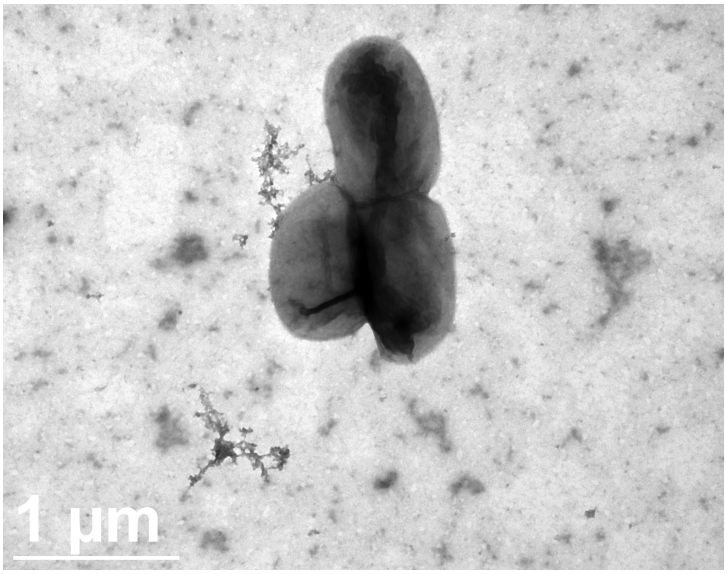

Figure 8: Negative strain preparation of *P. aeruginosa* strain PA14 *pqsH* under transmission electron microscopy. There are no OMV present.

Phase Contrast Microscopy

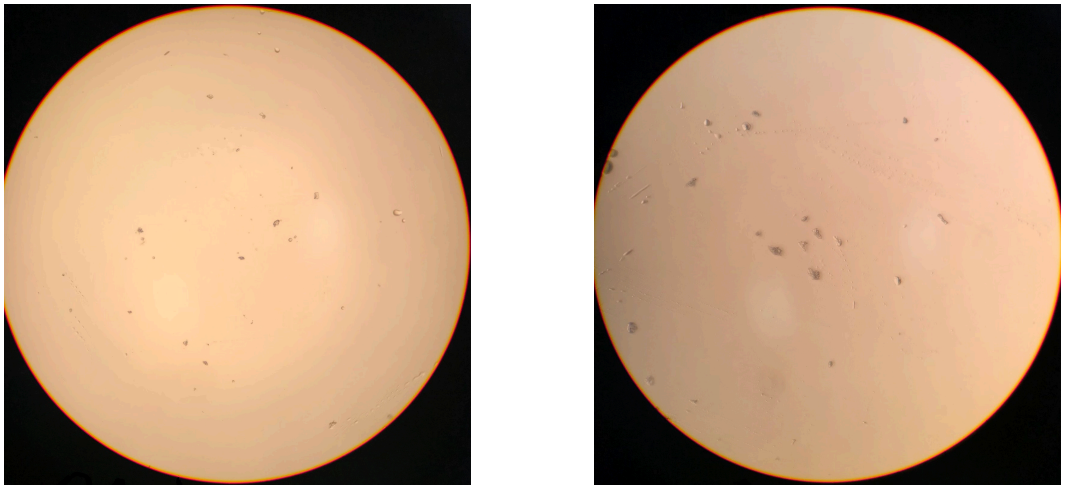

Figure 9: Both images show *P. aeruginosa* strain PA14 wt under phase contrast microscopy. Crystal formation can be clearly seen.

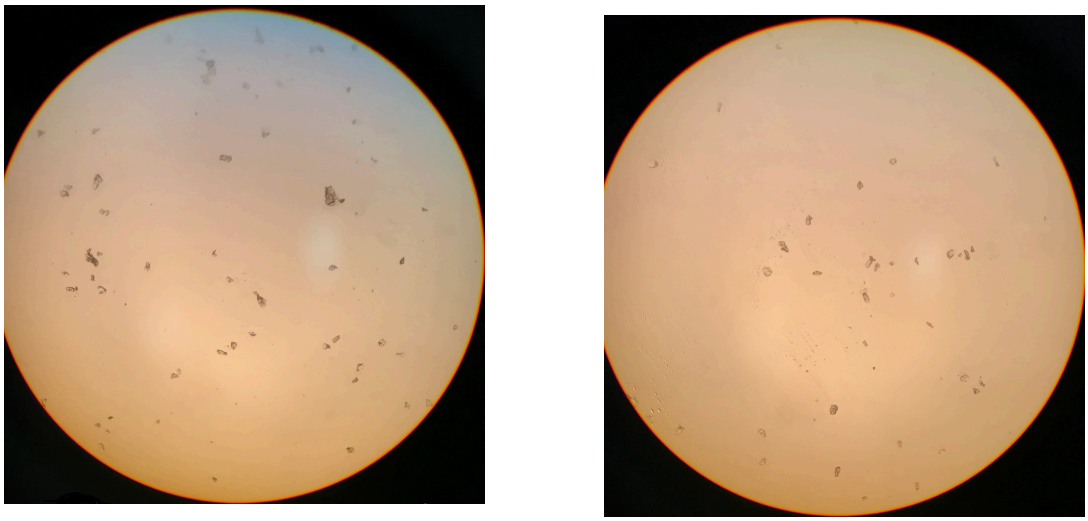

Figure 10: Both images show *P. aeruginosa* strain PA14 *pqsH* under phase contrast microscopy. Crystal formation can be clearly seen.

Flow Cytometry

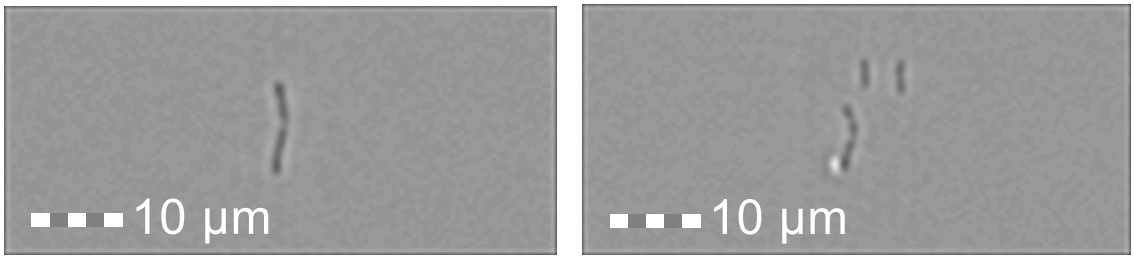

Figure 11: Numerous *P. aeruginosa* cells forming chains as they pass through the imaging flow cytometer.

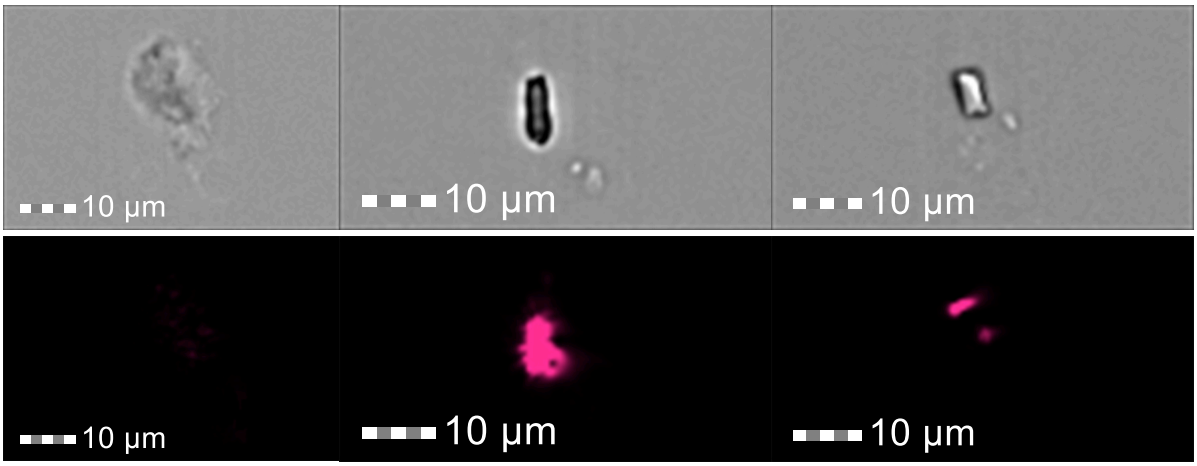

Figure 12: Various crystal formations as they pass through the imaging flow cytometer. The picture above shows the bright field channel, while the image on the bottom shows the fluorescent channel. The more formed crystals reflect light back, which is shown in the fluorescent signal.

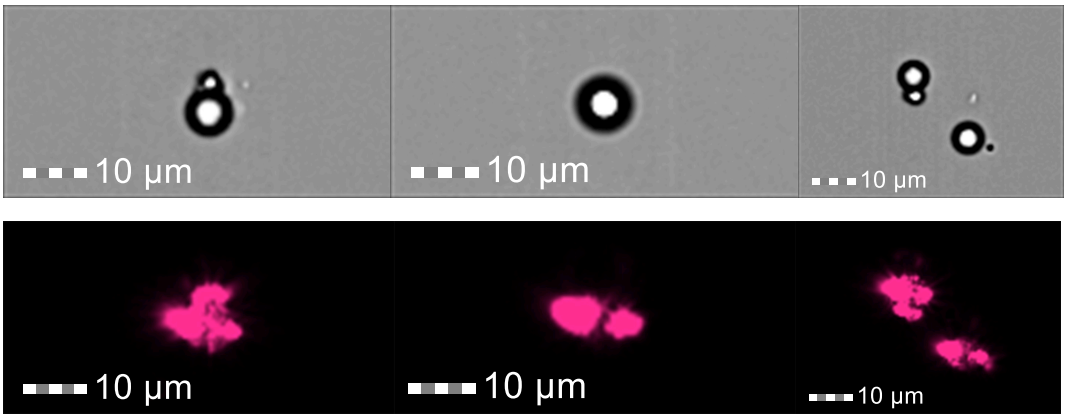

Figure 13: Fluorescent beads used as control as they pass through the imaging flow cytometer. The fluorescent signal is clearly seen on the bottom.

Table 3: Ratios of beads, bacterial particles, and crystals. All scenarios were done in triplicates. Scenarios noted with a C at the end were titrated with ammonium hydroxide to induce crystal formation. Scenarios noted with NC at the end did not undergo titration. Scenarios labeled "intact" are whole cells that did not undergo sonication. Scenarios labeled "son" are cell debris created by sonication. AU: artificial urine with no bacteria present.

Population	All	Beads	Bacterial Particles	Crystals
AU C	100	0.68	20.44	46.39
AU C	100	6.32	17.65	40.03
AU C	100	2.17	20.78	43.72
AU NC	100	50.52	40.31	3.21
AU NC	100	32.89	55.65	3.22
AU NC	100	47.99	40.61	2.65
PA14 Intact C	100	0.51	82.87	10.74
PA14 Intact C	100	0.77	79.75	11.47
PA14 Intact C	100	0.75	71.79	16.93
PA14 Intact NC	100	1.66	72.33	13.19
PA14 Intact NC	100	5.53	83.65	6.63
PA14 Intact NC	100	3.53	81.58	7.08
PA14 Son C	100	0.54	81.5	11.17
PA14 Son C	100	1.5	79.03	11.87
PA14 Son C	100	1.67	75.11	14.18
PA14 Son NC	100	2.8	69	11.15
PA14 Son NC	100	12.25	80.88	4.68
PA14 Son NC	100	0.97	81.7	8.89
pqsH Intact C	100	0.99	71.2	17.84
pqsH Intact C	100	0.92	76.2	14.59

Table 3: Continued.

pqsH Intact C	100	1.75	68.82	18.68
pqsH Intact NC	100	0.33	83.45	11.31
pqsH Intact NC	100	0.28	82.72	11.89
pqsH Intact NC	100	0.97	87.68	8.02
pqsH Son C	100	1.4	67.08	17.34
pqsH Son C	100	2.8	19.61	49.52
pqsH Son C	100	2.69	25.04	40.48
pqsH son NC	100	13.69	78.73	5.02
pqsH son NC	100	12.89	48.54	29.12
pqsH son NC	100	4.78	32.65	50.12

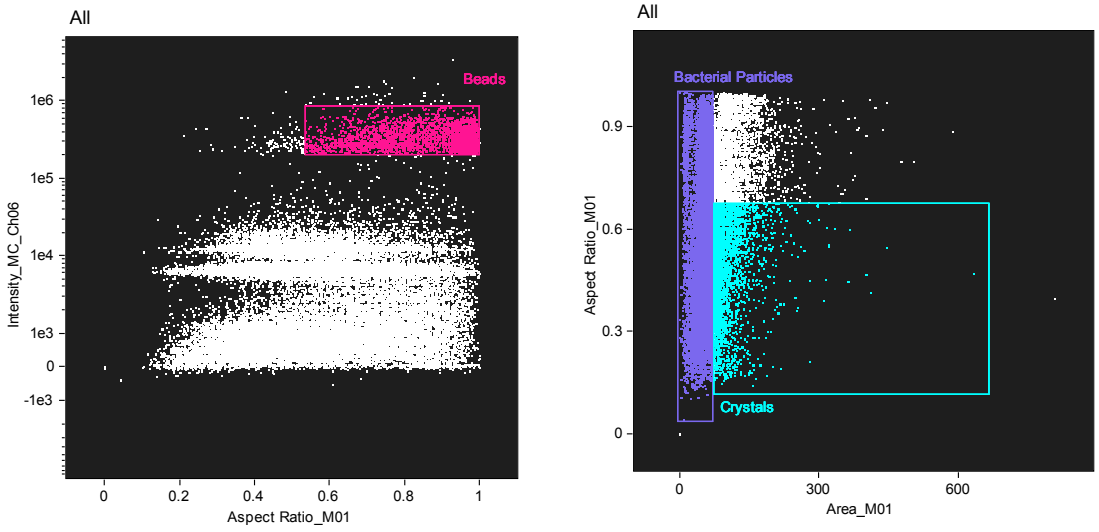

Figure 14: Parameters used to create populations for the calculation of ratios. The bead population was created using data from fluorescent intensity and aspect ratio. The bacterial particle and crystal populations were created using data based on area and aspect ratio.

Table 4: Summary of the averages and standard deviations for each scenario.

Cell Strain		Beads	Bacterial Particles	Crystals
PA14 Intact C	Avg	0.68	78.14	13.05
	StDev	0.14	5.71	3.38
PA14 Intact NC	Avg	3.57	79.19	8.97
	StDev	1.94	6.03	3.66
PA14 Son C	Avg	1.24	78.55	12.41
	StDev	0.61	3.22	1.58
PA14 Son NC	Avg	5.34	77.19	8.24
	StDev	6.05	7.11	3.28
pqsH Intact C	Avg	1.22	72.07	17.04
	StDev	0.46	3.77	2.16
pqsH Intact NC	Avg	0.53	84.62	10.41
	StDev	0.38	2.68	2.09
pqsH Son C	Avg	2.30	37.24	35.78
	StDev	0.78	25.98	16.60
pqsH son NC	Avg	10.45	53.31	28.09
	StDev	4.93	23.41	22.57
PA14 Intact C	Avg	0.68	78.14	13.05
	StDev	0.14	5.71	3.38
PA14 Intact NC	Avg	3.57	79.19	8.97
	StDev	1.94	6.03	3.66

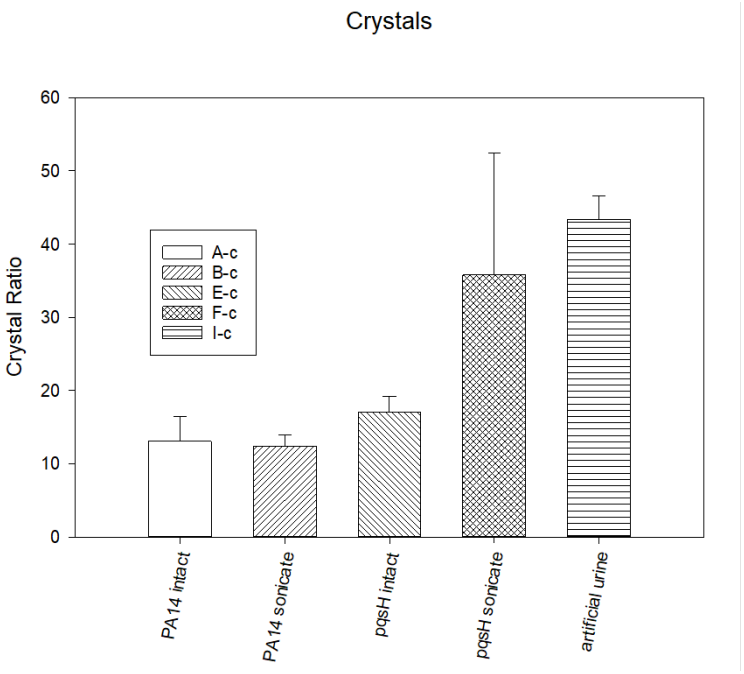

Figure 15: Ratio of crystals in various samples that underwent titration.

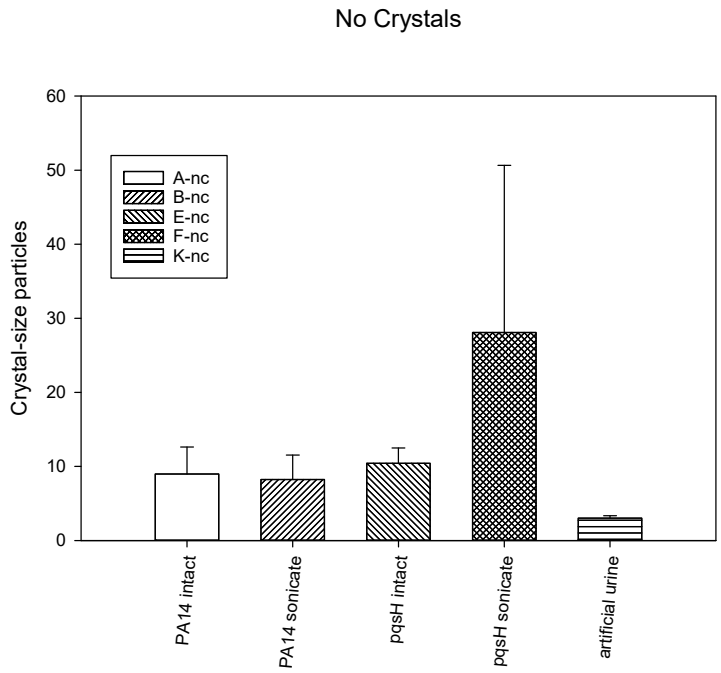

Figure 16: Ratio of crystals in various samples that did not undergo titration.

P. aeruginosa PA14 wt and *pqsH* strains were grown on BHI media, suspended in filtered 2% (w/v) preparations of uranyl acetate, and suspended on formvar-coated electron microscopy grids. TEM examination of these negative-stain preparations confirmed the presence of OMVs in *P. aeruginosa* PA14 wt (Figure 7) and absence of OMVs in *P. aeruginosa pqsH* (Figure 8).

When *P. aeruginosa* cells were suspended in artificial urine and the mixture titrated with ammonium hydroxide to mimic urease activity, struvite crystals could be observed by phase contrast microscopy in both the wt (Figure 9) and *pqsH* (Figure 10) cell suspensions.

In order to get quantitative data of struvite crystal formation, flow cytometry was used. The original plans were to obtain crystal growth at two time points: initial crystal formation (within 30 minutes of artificial urine titration), and longer term crystal growth (16-24 h following titration). However, the crystals that formed and aggregated into larger clusters after 16-24h, caused repeated clogging of the flow cytometer and so long-term measurements could not be performed (data not shown). As a result, only the short-term crystal formation (within 30 minutes of artificial urine titration with NH_4OH) is shown. In setting up the flow cytometry analysis, the portions of the readout showing *P. aeruginosa* cells (Figure 11), struvite crystals (Figure 12), and fluorescent beads (Figure 13) were selected and used to create parameters (Figure 14) used for flow cytometry calculations. The ratios of crystal signals in titrated and not titrated preparations are shown in Figures 15 and 16 respectively.

IV. DISCUSSION

Phase Contrast Microscopy

Phase contrast microscopy was performed to observe and confirm the presence and shape (crystal habit) of struvite crystals. Microscopy using both *P. aeruginosa* strains PA14 and *pqsH* showed a myriad of crystal growth at different rates. While this experiment was only for visual confirmation, it was noted that strain *pqsH* seemed to increase crystal growth. This observation was studied more in depth with flow cytometry.

TEM Confirmation of OMV

Transmission electron microscopy was performed to confirm the presence of outer membrane vesicles. This was needed because one of the strains used (*pqsH*) contained a mutation in the PQS pathway and, therefore, would not produce high levels of OMV. The wildtype strain (PA14) would produce OMV as normal. TEM confirmed the presence of OMV in the PA14 strain and confirmed the absence/low level of OMV in the *pqsH* mutant strain.

Flow Cytometry

All experiments up to this point were purely for confirmation purposes. Most of the data that was used to analyze the hypothesis was collected during the use of the imaging flow cytometer.

When looking at the averages for each scenario, it seemed like the titrated groups always had a higher ratio of crystals as opposed to the groups that were not titrated. However, when analyzed by ANOVA, the p values for the crystal groups and no crystal groups ($p = 0.093$; $p = 0.105$, respectively) showed that the differences between groups was not great enough to exclude the possibility that the difference is due to random sampling variability. In other words, there is not a statistically significant difference.

The *pqsH* mutant strain sonicate provided extreme data and could be considered an outlier. This could be due to the fact that higher HHQ levels are seen in *pqsH* mutant strains because the precursor HHQ is not converted into PQS. There could also be some chemical interference during the titration. Since the titration was done over a short period of time, this exposed the bacteria to a harsh chemical environment. This could have had an effect on the behavior that was exhibited. Another thing to consider is that although the strains were growth at 37C, the titrations were done at around 25C (room temperature). This also may have affected crystal growth.

One thing that should be addressed is the relatively high levels of crystal ratios seen in non-titrated groups. It was hypothesized that these groups would not see any

crystal growth at all since they did not undergo titration. However, it is thought that these crystals are actually undissolved particles from the making of the artificial urine. The artificial urine was made in two parts (Parts A and B), and mostly crystallized chemicals were used. The protocol used only called for the filter sterilization of part B, leaving part A susceptible to contain undissolved crystallized chemicals.

While gathering flow cytometry data, many problems occurred, but the most relevant to the outcome of this research is when the cell flow nozzle would get clogged with all of the crystals coming through. Since the goal count was 50,000, this would slow down the process immensely. The machine would need to be purged, which would stop the current reading. Once the purge was complete, the count would have to start all over. The experiments that were done in the beginning gathered 50,000 data points, or at least somewhere close to that. As experiments continued, the data points that were gathered diminished. The *pqsH* sonicate was one of the last experiments to be ran, and only gathered about 2,000 data points. This caused the data to be skewed.

The biggest problem that was encountered during analysis of the flow cytometry data was the designation of parameters for the population groups. Since the goal amount for data points was 50,000, a template analysis was used for each scenario to make things quick and efficient. The beads were easy to define because they had fluorescent capabilities. A graph was created using the aspect ratio data on the X-axis and the intensity of the fluorescent channel on the Y-axis. Since the beads were round, they were expected to have an aspect ratio of around 1. An aspect ratio of 0 indicates an oblong

object. Sometimes, multiple beads would come through the flow cytometer at once in a cluster, so the population group encompassed objects with aspect ratios from about 0.5 to 1 and high fluorescent intensity. The crystals and bacterial particles were extremely difficult to identify. These were identified on a graph using data for the area on the X-axis and aspect ratio on the Y-axis. Since *P. aeruginosa* is very small and its sonicated fragments would be even smaller, only a small portion of the x-axis was used for the population parameter. Because it is known that *P. aeruginosa* has a bacillus morphology, the intact cells would have an aspect ratio of close to 0 (more oblong). However, the bacterial sonicates could have an aspect ratio of any value since they are random cell fragments. This is why the entire Y-axis was used for the bacterial particle population. Crystals were seen growth at various rates, therefore, there were different sizes and shapes of crystals. This group was the most difficult to define because they could have any value of size or aspect ratio. Since we used an aspect ratio of 0.5-1 as the range for the beads, I used an aspect ratio of 0-0.5 for the crystals to avoid including beads in two different population groups. Because of this difficulty, all of the population groups were not exclusive. The bead population group contained some crystals and bacterial particles, and vice versa. This caused the ratios to not be entirely accurate.

In conclusion, strains exhibited OMV formation, as they were supposed to. PA14 wildtype strain produced OMVs, whereas the *pqsH* sonicate produced little to no OMVs. Crystal formation was accomplished via titration, as seen under phase contrast microscopy. While research has been done on nanobacteria and their role in inducing calcium crystal growth, this particular subject is in an unprecedented area of research.

Looking at the data, the *pqsH* sonicate could be considered an outlier If you ignore *pqsH* sonicate, other data shows no significant difference, as confirmed by ANOVA testing.

Future Considerations

In furthering this research, there are many considerations that should be taken into account. First, PQS production is an area that isn't very well studied. There is a gene called *pqsL*, whose mechanism is not known. However, a mutation in *pqsL* has shown to cause an overproduction of PQS. This research only studied normal production of PQS and inhibited of production of PQS. In the future, a strain of *P. aeruginosa* with a mutation in *pqsL* could be used for further comparison.

P. aeruginosa is not the only OMV-producing, UTI-causing, gram negative, bacillus bacteria. Another example of this type of bacteria is *Escherichia coli*. It would be interesting to run the experiment again using *E. coli* instead of *P. aeruginosa*. However, the mechanism of OMV formation in *E. coli* would have to be studied and a strain with a mutation that causes underproduction of OMV would have to be chosen to be an accurate comparison with this research (McBroom et. al. 2006).

As previously stated, the titration of artificial urine with ammonium hydroxide was done over a short period of time. This exposed the bacteria to harsh chemicals very quickly and could have affected the results. It would be interesting in the future to see if a titration would even be needed. The artificial urine could be inoculated with *P. aeruginosa* and set out over a long period of time. Perhaps some component of *P.*

aeruginosa could lower the pH of the artificial urine by itself without having to use a harsh base such as ammonium hydroxide.

Another aspect to consider is the analysis of data using IDEAS. Data was batch analyzed using the same template for all 10 scenarios. While this was the most efficient way to analyze almost 50,000 data points for most experiments, the confidence for the results was very low. In the future, it may be more beneficial to gather less data points per experiment, perhaps around 3,000, and to manually add each data point to its correct population. While 3,000 data points may not provide the most accurate representation of the population, the confidence for the results would be very high.

WORKS CITED

Balcht A, Smith R (1994). Pseudomonas aeruginosa*: Infections and Treatment*. Informa Health Care. pp. 83–84.

Bauman, S. J., & Kuehn, M. J. (2006). Purification of outer membrane vesicles from Pseudomonas aeruginosa and their activation of an IL-8 response. *Microbes and infection*, *8*(9), 2400-2408.

Biller, S. J., Schubotz, F., Roggensack, S. E., Thompson, A. W., Summons, R. E., & Chisholm, S. W. (2014). Bacterial vesicles in marine ecosystems. *Science*, *343*(6167), 183-186.

Chauhan, C. K., Joseph, K. C., Parekh, B. B., & Joshi, M. J. (2008). Growth and characterization of struvite crystals.

Clapham, L., McLean, R. J. C., Nickel, J. C., Downey, J., & Costerton, J. W. (1990). The influence of bacteria on struvite crystal habit and its importance in urinary stone formation. *Journal of crystal growth*, *104*(2), 475-484.

Downey, J. A., Nickel, J. C., Clapham, L., & McLean, R. J. C. (1992). In vitro inhibition of struvite crystal growth by acetohydroxamic acid. *British journal of urology*, *70*(4), 355-359.

Dubern, J., & Diggle, S. P. (2008). Quorum sensing by 2-alkyl-4-quinolones in Pseudomonas aeruginosa and other bacterial species. *Molecular BioSystems*, *4*(9), 873-956.

Dumanski, A. J., Hedelin, H., Edin-Liljegren, A., Beauchemin, D., & McLean, R. J. (1994). Unique ability of the Proteus mirabilis capsule to enhance mineral growth in infectious urinary calculi. *Infection and immunity*, *62*(7), 2998-3003.

Fiocca, R., Necchi, V., Sommi, P., Ricci, V., Telford, J., Cover, T. L., & Solcia, E. (1999). Release of Helicobacter pylori vacuolating cytotoxin by both a specific secretion pathway and budding of outer membrane vesicles. Uptake of released toxin and vesicles by gastric epithelium. *The Journal of pathology*, *188*(2), 220-226.

Florez, C., Raab, J. E., Cooke, A. C., & Schertzer, J. W. (2017). Membrane Distribution of the Pseudomonas Quinolone Signal Modulates Outer Membrane Vesicle Production in Pseudomonas aeruginosa. *mBio*, *8*(4), e01034-17.

Folk, R. L. (1993). SEM imaging of bacteria and nannobacteria in carbonate sediments and rocks. *Journal of Sedimentary Research*, *63*(5), 990-999.

García-Ruiz, J. M., Hyde, S. T., Carnerup, A. M., Christy, A. G., Van Kranendonk, M. J., & Welham, N. J. (2003). Self-assembled silica-carbonate structures and detection of ancient microfossils. *Science*, *302*(5648), 1194-1197.

Griffith, D. P., Musher, D. Á., & Itin, C. (1976). Urease. The primary cause of infection-induced urinary stones. *Investigative urology*, *13*(5), 346-350.

Jacobsen, S. Á., Stickler, D. J., Mobley, H. L. T., & Shirtliff, M. E. (2008). Complicated catheter-associated urinary tract infections due to Escherichia coli and Proteus mirabilis. *Clinical microbiology reviews*, *21*(1), 26-59.

Kajander, E. O. (2006). Nanobacteria–propagating calcifying nanoparticles. *Letters in applied microbiology*, *42*(6), 549-552.

Kajander, E. O., & Ciftcioglu, N. (1998). Nanobacteria: an alternative mechanism for pathogenic intra-and extracellular calcification and stone formation. *Proceedings of the National Academy of Sciences*, *95*(14), 8274-8279.

Keenan, J., Day, T., Neal, S., Cook, B., Perez-Perez, G., Allardyce, R., & Bagshaw, P. (2000). A role for the bacterial outer membrane in the pathogenesis of Helicobacter pylori infection. *FEMS microbiology letters*, *182*(2), 259-264.

Kirkland, B. L., Leo Lynch, F., Rahnis, M. A., Folk, R. L., Molineux, I. J., & McLean, R. J. (1999). Alternative origins for nannobacteria-like objects in calcite. *Geology*, *27*(4), 347-350.

Klimentová, J., & Stulík, J. (2015). Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria. *Microbiological research*, *170*, 1-9.

Kuehn, M. J., & Kesty, N. C. (2005). Bacterial outer membrane vesicles and the host–pathogen interaction. *Genes & development*, *19*(22), 2645-2655.

Le Corre, K. S., Valsami-Jones, E., Hobbs, P., & Parsons, S. A. (2005). Impact of calcium on struvite crystal size, shape and purity. *Journal of Crystal Growth*, *283*(3), 514-522.

MacDonald, I. A., & Kuehn, M. J. (2013). Stress-induced outer membrane vesicle production by Pseudomonas aeruginosa. *Journal of bacteriology*, *195*(13), 2971-2981.

Mashburn-Warren, L., Howe, J., Brandenburg, K., & Whiteley, M. (2009). Structural requirements of the Pseudomonas quinolone signal for membrane vesicle stimulation. *Journal of bacteriology*, *191*(10), 3411-3414.

Mashburn‐Warren, L., Howe, J., Garidel, P., Richter, W., Steiniger, F., Roessle, M., ... & Whiteley, M. (2008). Interaction of quorum signals with outer membrane lipids: insights into prokaryotic membrane vesicle formation. *Molecular microbiology*, *69*(2), 491-502.

Mashburn, L. M., & Whiteley, M. (2005). Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature*, *437*(7057), 422.

McBroom, A. J., Johnson, A. P., Vemulapalli, S., & Kuehn, M. J. (2006). Outer membrane vesicle production by Escherichia coli is independent of membrane instability. *Journal of bacteriology*, *188*(15), 5385-5392.

McKay, D. S., Gibson, E. K., Thomas-Keprta, K. L., Vali, H., Romanek, C. S., Clemett, S. J., ... & Zare, R. N. (1996). Search for past life on Mars: Possible relic biogenic activity in Martian meteorite ALH84001. *Science*, *273*(5277), 924-930.x

McLean, R. J., Nickel, J. C., Cheng, K. J., Costerton, J. W., & Banwell, J. G. (1988). The ecology and pathogenicity of urease-producing bacteria in the urinary tract. *CRC Critical reviews in microbiology*, *16*(1), 37-79.

McLean, R.J.C., Downey, J., Clapham, L., and Nickel, J.C. (1989). A simple technique for studying crystal growth in vitro. *Urological Research*. 18:39-43.

McLean, R. J., Lawrence, J. R., Korber, D. R., & Caldwell, D. E. (1991). Proteus mirabilis biofilm protection against struvite crystal dissolution and its implications in struvite urolithiasis. *The Journal of urology*, *146*(4), 1138-1142.

Mittal, R., Aggarwal, S., Sharma, S., Chhibber, S., & Harjai, K. (2009). Urinary tract infections caused by Pseudomonas aeruginosa: a minireview. *Journal of infection and public health*, *2*(3), 101-111.

Sams, T., Baker, Y., Hodgkinson, J., Gross, J., Spring, D., & Welch, M. (2016) The Pseudomonas quinolone signal (PQS). *Israel Journal of Chemistry*, *56(5),* 282-294.

Schertzer, J. W., & Whiteley, M. (2012). A bilayer-couple model of bacterial outer membrane vesicle biogenesis. MBio, 3(2), e00297-11.

Schertzer, J. W., Brown, S. A., & Whiteley, M. (2010). Oxygen levels rapidly modulate Pseudomonas aeruginosa social behaviours via substrate limitation of PqsH. *Molecular microbiology*, *77*(6), 1527-1538.

Schooling, S. R., & Beveridge, T. J. (2006). Membrane vesicles: an overlooked component of the matrices of biofilms. *Journal of bacteriology*, *188*(16), 5945-5957.

Uwins, P. J., Webb, R. I., & Taylor, A. P. (1998). Novel nano-organisms from Australian sandstones. *American Mineralogist*, *83*(11-12_Part_2), 1541-1550.

Wessel, A. K., Liew, J., Kwon, T., Marcotte, E. M., & Whiteley, M. (2013). Role of Pseudomonas aeruginosa peptidoglycan-associated outer membrane proteins in vesicle formation. *Journal of bacteriology*, *195*(2), 213-219.