CHARACTERIZATION OF THE ROLE OF EXOPOLYSACCHARIDES IN THE INFECTION CYCLE OF BACTERIOPHAGE PETTY

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Characterization of the Role of Exopolysaccharides in the Infection Cycle of Bacteriophage Petty. (May 2015)

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Acinetobacter baumannii is a Gram-negative nosocomial pathogen that has been difficult to eradicate from hospital settings due to its ability to form biofilm and colonize biotic and abiotic surfaces. Phages may represent an alternative to antibiotic therapy and prophylaxis for biofilm-forming bacteria, but the interaction between phages and encapsulated bacteria such as *A. baumannii* is not well understood. Recently, bacteriophage Petty was isolated against *A. baumannii* strain AU0783. Analysis of its genome suggested that it encoded a depolymerase that degrades the host capsular exopolysaccharide (EPS). The gene was cloned and the purified protein was shown to reproduce the halo phenotype observed around phage Petty's plaque, degrade purified host EPS, and affect biofilm formation of 5 different *A. baumannii* isolates. It is our aim to determine the cellular requirements for Petty to infect the host cell by characterizing the EPS of bacterial mutants of AU0783 that are resistant to phage infection. We isolated 42 mutants of AU0783 resistant to Petty and determined the titer of the phage. The capsule of these mutants and the wild-type strain was observed and its thickness measured. The ability of the mutants to produce biofilm was assessed using a microtiter plate assay. The EPS of 8 mutant strains was purified; the ability of the phage's depolymerase to degrade EPS and produce reducing ends was measured using a colorimetric assay and the effect of the enzyme on the

viscosity of EPS solutions was established. No plaques were observed under the given conditions in 91% of the Petty-resistant mutants isolated (EOP $<10^{-7}$). The titer of the phage on the remainder of the mutant strains was reduced 3 to 7 orders of magnitude, depending on the mutant. Eight mutants were selected for further studies, based on biofilm production and on capsule formation compared to the wt strain AU0783. Purified EPS isolated from mutants B11, D6, and E8 was unable to be degraded by the depolymerase of Petty, as judged by colorimetric assay, and by the viscosity of the capsular polysaccharide when treated with the enzyme. The EPS viscosity of mutant D14 was reduced when treated with active depolymerase, but the halo phenotype was not reproduced on a bacterial lawn. Studies aimed at finding phage host range mutants that would infect these selected strains are underway. The differences between these phages and the mutant strains will give insight on the mechanism of phage infection of encapsulated *A. baumannii.*

DEDICATION

To my parents,

Dr. Brent and Julie Lancaster,

And all my friends,

Without whom none of my success would be possible.

ACKNOWLEDGEMENTS

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Last but not least I would like to thank my friends here in College Station. Moving 500 miles away from home was somewhat of a culture shock but having people here who really cared about me made it all worth it!!!

Thanks & Gig 'Em!!!

Class of 2015 A – WHOOP!!!

CHAPTER I

INTRODUCTION

Acinetobacter baumannii

Acinetobacter baumannii is a nonmotile, Gram-negative, opportunistic pathogen, with some strains being multi-drug and pan-drug resistant (1) Though this bacterium is thought to be commonly found in soil and water, due to the fact that it is opportunistic most of the infections due to *A. baumannii* are found in places with immunocompromised patients, such as a hospital intensive care unit (ICU) (2). Resistance to unfavorable environmental conditions, such as desiccation, as well as the ability to survive on biotic and abiotic surfaces due to its production of biofilm, makes it a frequent cause of outbreaks (3). Also due to its natural competency and resistance to beta lactams and some disinfectants, the widespread growth of the bacteria allows for easy transmission to patients and infection that is hard to treat (4). Some of the infections caused by the bacterium include bacteremia, pneumonia, meningitis, urinary tract infection, and wound infection (5). Up to 1% of the nosocomial infections in an ICU are due to multidrug resistant *A. baumannii* strains (2).

Biofilm

Biofilm is defined as a community of cells characterized by their irreversible attachment to one another and their embedding in an extracellular matrix that the group of cells has produced (4). This extracellular matrix is composed mostly of capsular EPS, proteins, DNA, polysaccharides, and even cell debris (6). Biofilm gives microorganisms an increased tolerance to antimicrobial

agents such as antibiotics or chemicals due to the physical barrier between the substance and the cells (6). However, not all strains of *A. baumannii* form biofilm (7), 74% of ICU *A. baumannii* isolates form biofilm and are multi drug (8). The production of biofilm by *A. baumannii* is a multiple step process (3). The first step occurs when pili and surface proteins form and adhere to a surface allowing for the formation of micro-colonies. Once the micro-colonies form, quorum sensing directs biofilm formation. The production of acyl-homoserine lactone by a feedback loop acts as an up-regulator of biofilm formation. When the acyl-homoserine lactone is inactivated the reduction in biofilm is approximately 30-40% meaning that bacterial cell communication with respect to cell density is an integral part of biofilm formation (3). The most abundant of the substances in biofilm is capsular EPS. The capsule in *A. baumannii* is secreted in the *wzy*-dependent process, and is responsible for a slight resistance to phage as well as an increase in viscosity of the medium that leads to a decrease in phage spreading (9, 10).

Bacteriophage Petty

A novel bacteriophage was recently isolated against the clinical *A. baumannii* isolate AU0783 in the Center for Phage Technology. The phage has podophage morphology and was thus named Petty (Figure 1).

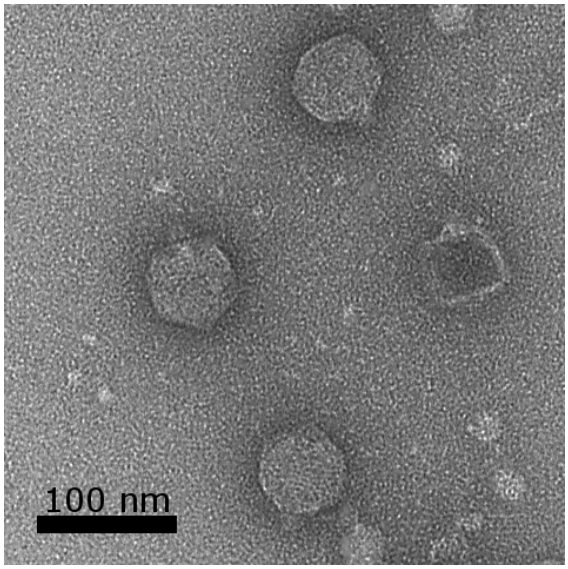

Figure 1. The transmission electron microscope image of podophage Petty. The bacteriophage has a capsid diameter of approximately 60 nm.

When bacteriophage Petty infects AU0783, a halo is observed around the plaques (figure 2), indicating the presence of a depolymerase - an enzyme that degrades capsular EPS (11). Previously reported proteins with depolymerase activity have been found to be tail fibers or tail spikes (12). After sequencing the 40.3 kb genome, gene 39, a putative tail fiber, was hypothesized to have EPS depolymerase activity. The 101.4 kDa Gp39, named Dpo1, was expressed using a hyper-expression clone and purified. The purified protein product was able to reproduce the halo phenotype on lawns of AU0783, as shown in Figure 3, and degrade purified EPS, reducing viscosity. The EPS polymerase is predicted to play a role in adsorption and infection of the host cell (11). The current hypothesis about Dpo1's role in phage infection is that the bacteriophage uses the depolymerase to degrade the EPS layer that is blocking the surface receptors so that Petty can bind to the receptors and continue with infection (11).

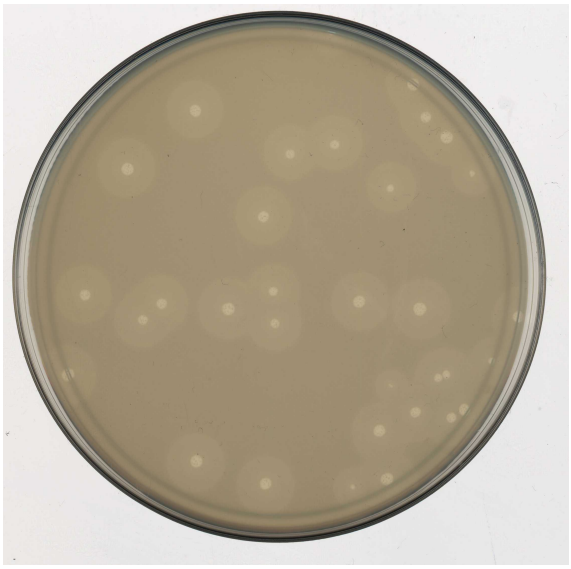

Figure 2. Tail fiber depolymerase causes halo phenotype formation around bacteriophage plaque. Phage Petty plated on AU0783 producing the halo phenotype due to EPS degradation.

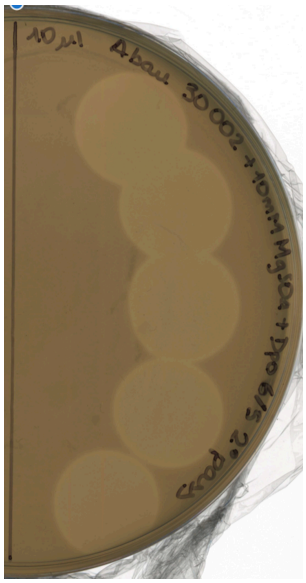

Figure 3. Degradation of capsular EPS by purified Dpo1. Using the Dpo clearing assay, dilutions of Dpo1 were spotted on a lawn of wtAU0783.

Depolymerase

Depolymerases are described as enzymes with the ability to degrade capsular exopolysaccharides. Bacteriophage PK1E, which also encodes a depolymerase, produces an Endo-N-acetylneuraminidase (Endo N) from a family of closely related phage-encoded glycosidases. As part of the tail structure, the glycosidases can degrade the homopolymeric **α**-2,8-linked sialic acid (PSA) that has been found as components of the capsule in sepsis- and meningitis- causing bacterial pathogens, such as PK1E's host *Escherichia coli* K1. Endo N acts like a polymer when in solution, and when Dpo1 is in solution it acts as a hexamer. The degradation of PSA also causes a halo phenotype like that seen in the degradation of EPS by Dpo1 (11, 12). Thus, we can infer that Dpo1 is in fact a depolymerase.

Bacteriophage Therapy

There are currently very few effective procedures for removing the persistent biofilm producing strains of the opportunistic pathogen, *A. baumannii,* from hospital environments. This causes an increased risk of immunocompromised patients acquiring an antibiotic resistant infection (1). d'Herelle successfully used bacteriophages as an alternative to antibiotics in 1919 when treating dysentery (13). The narrow range of the bacteriophage is advantageous to the wide host range of antibiotics because it does not disrupt other normal gastrointestinal flora. Not only can bacteriophage be used as therapeutic agents but so can phage gene products and components. Such an example is the phage encoded lysin enzyme that was used to treat dairy starter cultures to remove *Listeria monocytogenes* (14). Specific criteria are required for phage to be eligible for phage therapy, these require that the phage be lytic, infect faster then the rate at which they are removed from the site of treatment, and have no deleterious factors (15). Today, bacteriophages

are being widely used in Russia, Georgia, and Poland though elsewhere in the world bacteriophage still remains an unutilized source of antibacterial treatment (16). Theoretically, the purified depolymerase of bacteriophage Petty can be used like the lysin enzyme of *Listeria monocytogenes* and remove *A. baumannii* from both abiotic and biotic surfaces (14).

CHAPTER II

MATERIALS AND METHODS

Strains media and general methods

Tryptic soy broth (TSB) was used for all cultures and agar plates. For all plaque assays, a .75% overlay (top agar) was plated on 1.5% underlay (bottom agar). The host strain, *A. baumannii* AU0783, was used as a wild type standard for all experiments.

Host strain mutant hunt

Mutants of the host strain, AU0783, were discovered by preforming a standard pre-absorption assay (5) using 100 μL each of AU0783 and Petty. After incubating overnight (O/N) at 37°C, 42 colonies that survived the bacteriophage infection were collected. Mutants were passed three times and titer (17) of phage Petty was tested for each pass on the mutant versus the wt strain to determine stability of the resistance phenotype.

Evaluation of Dpo1 effect on mutant bacterial lawns

The depolymerase of phage Petty, Dpo1, was expressed using a hyper-expression clone and purified. When spotted on a lawn of wtAU0783 cells, the purified enzyme alone is able to reproduce the halo phenotype as seen in Figure 3. To test the susceptibility to the depolymerase, a Dpo clearing assay was done by spotting 10-fold depolymerase dilutions, beginning with an 8.5 μg/mL stock, till the halo phenotype was no longer observed. The highest dilution at which the enzyme was able to produce a halo was recorded.

Microtiter dish biofilm formation assay

All procedures from the O'Toole's methodology (18) were followed with the exception of biofilm growth and quantification. To grow biofilm, the mutant and host strains were incubated

O/N at 37°C on a shaking platform. The samples were diluted 10-fold in lysogeny broth (LB) and the absorbance at OD 550 nm was measured. The cultures were adjusted to an absorbance of 1 at OD 550 nm. The newly diluted cultures were then diluted again by 100-fold in LB, and 100 μL of the dilution was added in 3 wells of a 96 well plate. The plate incubated at 37°C for 3 days to allow for biofilm growth. To clean the wells, the cultures were emptied and the wells were rinsed with 150 μL of dH_2O three times. After cleaning the wells, 150 μL of .01% crystal violet stain was added and allowed to set for 10 minutes. The wells were cleaned again using 150 μL of dH_2O to rinse three times. To quantify the biofilm, 150 μL of 95% ethanol was added to each of the wells and left at room temperature for 15 min. The content of the 96 well plate was then transferred into a new 96 well plate and the absorbance was read at 590 nm.

EPS Purification

O/N cultures were made for the mutant and host strains. 150 μL of each culture were spread onto 20-40 150 x 50 mm petri dishes and incubated at 37°C for 5-7 days depending on the strain. To harvest the bacteria, the plates were flooded with 10 mL of 0.9% saline (w/v) then the bacteria were scraped off of the plate and transferred into a 500 mL centrifuge bottle using a cell scraper. 5% phenol (v/v) was added and stirred for 5 hours. The mixture was then centrifuged for 50 minutes at 5,000 rpm in a Beckman J2-21 centrifuge to pellet the insoluble material. The supernatant was then aliquoted into two 500 mL centrifuge bottles and 5 volumes of 95% ethanol was added and left O/N at 4°C. The EPS precipitate was pipetted out and washed twice with 25 mL of 95% ethanol, centrifuging in a Sorvall legend XTR centrifuge for 20 minutes at 11,500 rpm at 4°C each time. After the last wash, the EPS was allowed to dry O/N. EPS pellets were resuspended in 25 mL sterile dH_2O and centrifuged for 20 minutes at 11,500 rpm at 4°C. The supernatant was decanted and saved and the pellet was resuspended in 25 mL sterile dH_2O, and

spun down again. The two supernatants were pooled and centrifuged for 40 minutes at 11,500 rpm at 4°C to pellet the last of insoluble material. DNase and RNase were added to the pooled supernatant mixture to the following final concentrations: 25 mM Tris HCL pH7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 μg/mL DNase, and 1 μg/mL RNase. This mixture was incubated at 37°C for 1.5 hours then heat inactivated in an 80°C water bath for 10 minutes. Once heat inactivated, the EPS mixture was dialyzed O/N with 6-8 kDa MWCO tubing in 4L dH_2O. The water was changed three times. Upon completion of dialysis, the samples were transferred into 50 mL falcon tubes and freeze-dried using a Labconco lyophilizer for 5 days or until EPS was dry and fluffy.

Capsule visualization and measurements

Maneval's solution (Carolina Labs, NC) was used to stain cells growing in liquid culture. The staining protocol described by Maneval (19) was followed and capsule images were taken at 1000X magnification on a Nikon Eclipse Ti. Using these images along with the NIS- Elements AR Analysis program the capsular EPS thickness was measured.

Viscosity assay

Three types of solutions were prepared to test viscosity using an Ostwald viscometer. The first solution was 3.5 mL of untreated EPS at a concentration of 2.5 mg/mL diluted in 1X assay buffer (2.5 mM Tris, 15 mM NaCl, pH 7.2). In the second solution, EPS (2.5 mg/mL) in 1X assay buffer was mixed with active purified enzyme (15 μg/mL). As a control, a third EPS solution (2.5 mg/mL) was mixed with purified enzyme (15 μg/mL) that had been previously incubated at 95°C for 10 minutes to inactivate the enzyme. The three solutions were incubated at 37°C for 1 hour in water bath; after incubation, the enzyme was heat inactivated at 95°C for 5 minutes to

stop the assay. The viscosity of these solutions, and that of the 1X assay buffer, was measured at 20°C, in 3 independent assays.

Bicinchoninic acid assay for reducing ends

Two reagents were used in this assay: reagent A contains 5 mM bicinchoninic acid (BCA), 513 mM Na_2CO_3, and 288 mM $NaHCO_3$, reagent B contains 5 mM $CuSO_4$ and 12 mM L-serine. The BCA working reagent was made fresh in a 1:1:2 ratio of reagents A, B, and dH_2O. A master mix was made that included 100 μL of 10X assay buffer (25 mM Tris, 150 mM NaCl, pH 7.2), 100 μL of 5 mg/mL EPS, and 750 μL dH_2O. 95 μL of the master mix was aliquoted into 8 microcentrifuge tubes. The tubes were left open and placed into a 37°C heat block and allowed to sit for 5 minutes. 5 μL of the enzyme stock (200 nM) was added to each tube, and was then mixed by flicking. The tubes were placed back at 37°C. At 5, 10, 20, 30 minutes, a pair of tubes was removed and placed into the 95°C heat block for 5 minutes. The tubes were removed and cooled to room temperature. After all time points had been taken and tubes had been cooled, the tubes were reopened and 1 mL of BCA assay reagent was added to each tube. The samples were mixed by vortexing and the tubes were placed into a 95°C heat block for 15 minutes. The samples were cooled to room temperature for 20 minutes and absorbance was read at 560 nm. Two controls (negative and positive) were run alongside the EPS sample. The negative control had 10 μL 10X assay buffer and 90 μL mqH_2O. The positive control had 10 μL 2 mM glucose, 10 μL 10X assay buffer, and 80 μL mqH_2O. This assay was repeated for all working mutants and host strains.

CHAPTER III

RESULTS

Identification of phage Petty resistant AU0783 mutants

42 mutants of wtAU0783 were selected from bacterial colonies that survived infection by phage Petty. These bacteria were selected to help understand the mechanism of phage resistance. To determine if the phage resistance phenotype was stable in the 42 selected mutants, mutant bacteria were platted successively, phage Petty was spot tittered on the lawns, and titer was determined (Table 1). The susceptibility of the mutants to pure Dpo1 enzyme was also determined. The Dpo clearing assay helped to determine if the mutants would produce a halo phenotype and the lowest dilution at which the halo phenotype was observed is recorded in Table 1. By comparing the three-titer trials, 33 of the mutants were determined to be stable and 9 were false positives. Dpo1 also had an effect *in vivo* causing the halo phenotype to occur with of the 9 mutants, all of which were either unstable or seemed to be wild type.

Phage resistance in bacteria can occur at three main steps. The first is adsorption; during adsorption the phage will have some sort of physical barrier such as a capsule or the loss of a phage receptor. The second step affected is the uptake of the phage genome into the cytoplasm. During this step, if a prophage is present it will block the genome of the bacteriophage that is currently infecting the cell from entering the cytoplasm. The last step is host take-over. This is the last time the cell can stop infection. The presence of restriction enzymes, a CRISPR system, and/or a toxin-antitoxin system may destroy the bacteriophage genome once it enters the cytoplasm (20).

In the case of the 42 mutants selected, phage resistance is most likely due to defects in adsorption. When the capsule changes, the depolymerase may no longer be as effective or the bacteria may have lost a receptor recognized by the depolymerase

Table 1. Identification of phage Petty resistant AU0783 mutants. 42 independent bacterial colonies that survived infection by Petty were selected and each of the mutants was named with an arbitrary letter and number. Phage titer was determined on each of the 42 mutants as well as wtAU0783. The ability of Petty's depolymerase to reproduce the halo phenotype on lawns of the mutant strains was also assessed. Starting at a concentration of 8.49 µg/mL and diluting 10 fold until extinction, the highest dilution at which Dpo1 produced the halo phenotype was recorded. Those mutants that were not affected by the enzyme are marked with a zero.

	Titer			DPO		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
B11	$>10^9$	$>10^9$	$>10^9$	0	0	0
B5	$>10^9$	$>10^9$	$>10^9$	0	0	0
B7	$>10^9$	$>10^9$	$>10^9$	0	0	0
B16	$>10^9$	$>10^9$	$>10^9$	0	0	0
B15	$>10^9$	$>10^9$	$>10^9$	0	0	0
B13	$>10^9$	4.6×10^8	3.6×10^7	0	10^{-5}	10^{-4}
D2	$>10^9$	$>10^9$	$>10^9$	0	0	0
D5	$>10^9$	$>10^9$	$>10^9$	0	0	0
D6	$>10^9$	$>10^9$	$>10^9$	0	0	0
D7	$>10^9$	$>10^9$	$>10^9$	0	0	0
D14	$>10^9$	$>10^9$	$>10^9$	0	0	0
D16	$>10^9$	$>10^9$	$>10^9$	0	0	0
E11	$>10^9$	$>10^9$	$>10^9$	0	0	0
E9	$>10^9$	$>10^9$	$>10^9$	0	0	0
E13	$>10^9$	$>10^9$	$>10^9$	0	0	0
E8	$>10^9$	$>10^9$	$>10^9$	0	0	0
E6	$>10^9$	$>10^9$	$>10^9$	0	0	0

Table 1. Continued

	Titer			DPO		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
E3	$>10^9$	$>10^9$	$>10^9$	0	0	0
F3	$>10^9$	$>10^9$	$>10^9$	0	0	0
F4	$>10^9$	$>10^9$	$>10^9$	0	0	0
F5	$>10^9$	$>10^9$	$>10^9$	0	0	0
F10	$>10^9$	$>10^9$	$>10^9$	0	0	0
F8	$>10^9$	$>10^9$	$>10^9$	0	0	0
F7	$>10^9$	$>10^9$	$>10^9$	0	0	0
A15	$>10^9$	$>10^9$	$>10^9$	0	0	0
A11	4×10^7	2×10^9	1.8×10^9	10^{-3}	10^{-5}	10^{-4}
A2	$>10^4$	$>10^6$	8.8×10^7	10^{-3}	10^{-4}	10^{-4}
A6	$>10^9$	$>10^9$	$>10^9$	0	0	0
A7	$>10^4$	4.6×10^7	3.6×10^7	10^{-3}	10^{-4}	10^{-5}
A14	2.6×10^6	7×10^7	7.6×10^7	10^{-3}	10^{-5}	10^{-5}
C2	$>10^9$	$>10^9$	$>10^9$	0	0	0
C3	$>10^9$	$>10^4$	$>10^4$	0	10^{-4}	10^{-4}
C6	$>10^9$	$>10^9$	$>10^9$	0	0	0
C9	$>10^6$	5.2×10^9	2.8×10^9	10^{-3}	10^{-5}	10^{-5}
C12	$>10^4$	1.8×10^8	1.12×10^8	10^{-3}	10^{-4}	10^{-4}
C15	$>10^9$	2×10^9	$>10^9$	0	10^{-4}	0
G9	$>10^9$	$>10^9$	$>10^9$	0	0	0
G1	$>10^9$	$>10^9$	$>10^9$	0	0	0
G8	$>10^9$	$>10^9$	$>10^9$	0	0	0
G6	$>10^9$	$>10^9$	$>10^9$	0	0	0
G10	$>10^9$	$>10^9$	$>10^9$	0	0	0
G11	$>10^9$	$>10^9$	$>10^9$	0	0	0
AU0783 (WT)	6.5×10^9	6.4×10^9	4×10^9	10^{-5}	10^{-5}	10^{-5}

Characterization of biofilm formation of AU0783 mutants

To determine the ability of the mutants selected to form biofilm, a colorimetric biofilm assay was preformed. This assay allowed for the indirect determination of the amount of biofilm by staining it with crystal violet and viewing the normalized cell absorbance ratio, A_{590}/A_{550} (Figure

4a,b). Approximately 80% of the mutants produced no halo phenotype indicating a possible change in capsular EPS thus biofilm production. Mutant E8 produces the same amount of biofilm as AU0783 and is also resistant to phage infection. This lead to the hypothesis, that there may be other causes of resistance rather than just a change in the amount of biofilm produced.

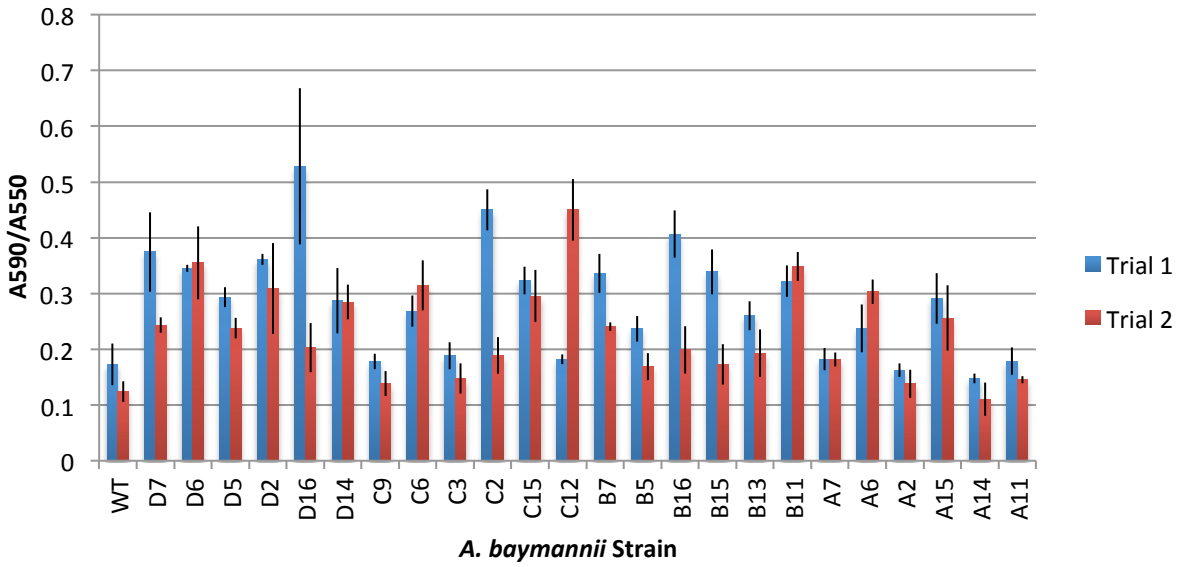

Figure 4a. Characterization of biofilm formation of AU0783 mutants. Cells were grown for three days at 37°C in a 96 well plate and absorbance was measured (A_{550}). Biofilm formation was assessed by staining with crystal violet and measuring absorbance (A_{590}), crystal violet absorption was measured in each well and each measurement was done in triplicate. The graph was plotted using the ratio of A_{590}/A_{550} to normalize to cell growth. The error bars show the standard deviation of the mean for each of the trials. Once again the stability of the most of the mutants by their biofilm production is seen. Table continued in next figure.

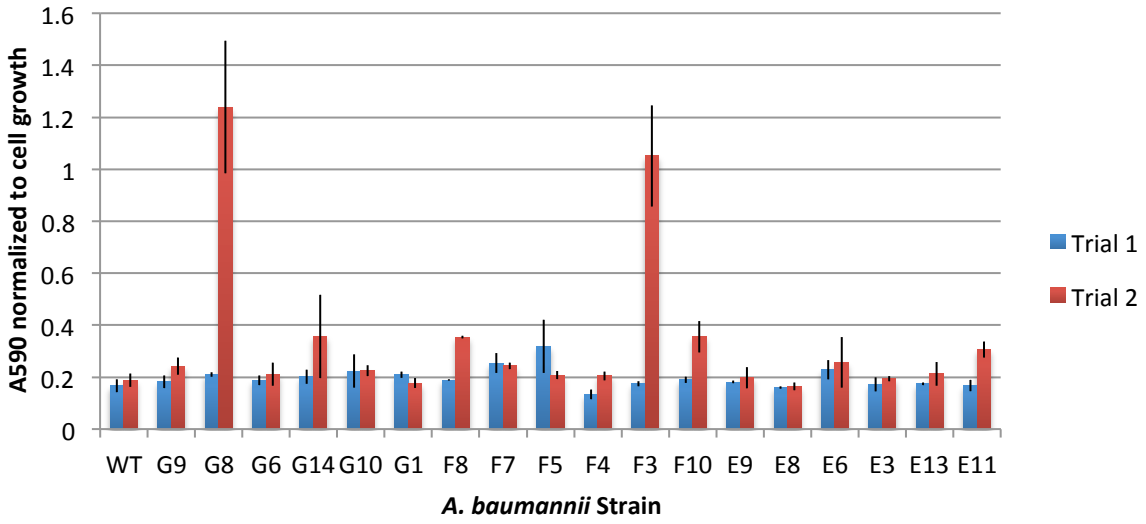

Figure 4b. Continuation of Figure 2a.

Characterization of mutant capsular EPS

While the biofilm assay can be used to determine if the mutant strain produces more or less biofilm than the wt strain, a more exact measurement of the capsular EPS of the mutants was needed. To visualize this, the mutants were stained with Congo red and Maneval's solution then images were taken at a magnification of 1000x using a Nikon Eclipse T*i*. Using these images, measurements of the capsular EPS thickness were taken using the program NIS- Elements AR Analysis (Figure 5). Approximately 200 cells per mutant were measured (Figure 6). While most of the mutants produce more EPS than the wt, strains E8, C9, and A11 produce a thinner capsule. Using the size of the capsule is important when determining why the bacteriophage is not infecting the bacterial mutants. As described earlier, one of the major types of phage resistance is due to a physical barrier blocking phage adsorption (20). If the capsule is getting larger due to a mutation it could cause the phage receptor site to be blocked by EPS, and if the capsule is getting smaller the loss of EPS could cause the loss of the receptor all together (21).

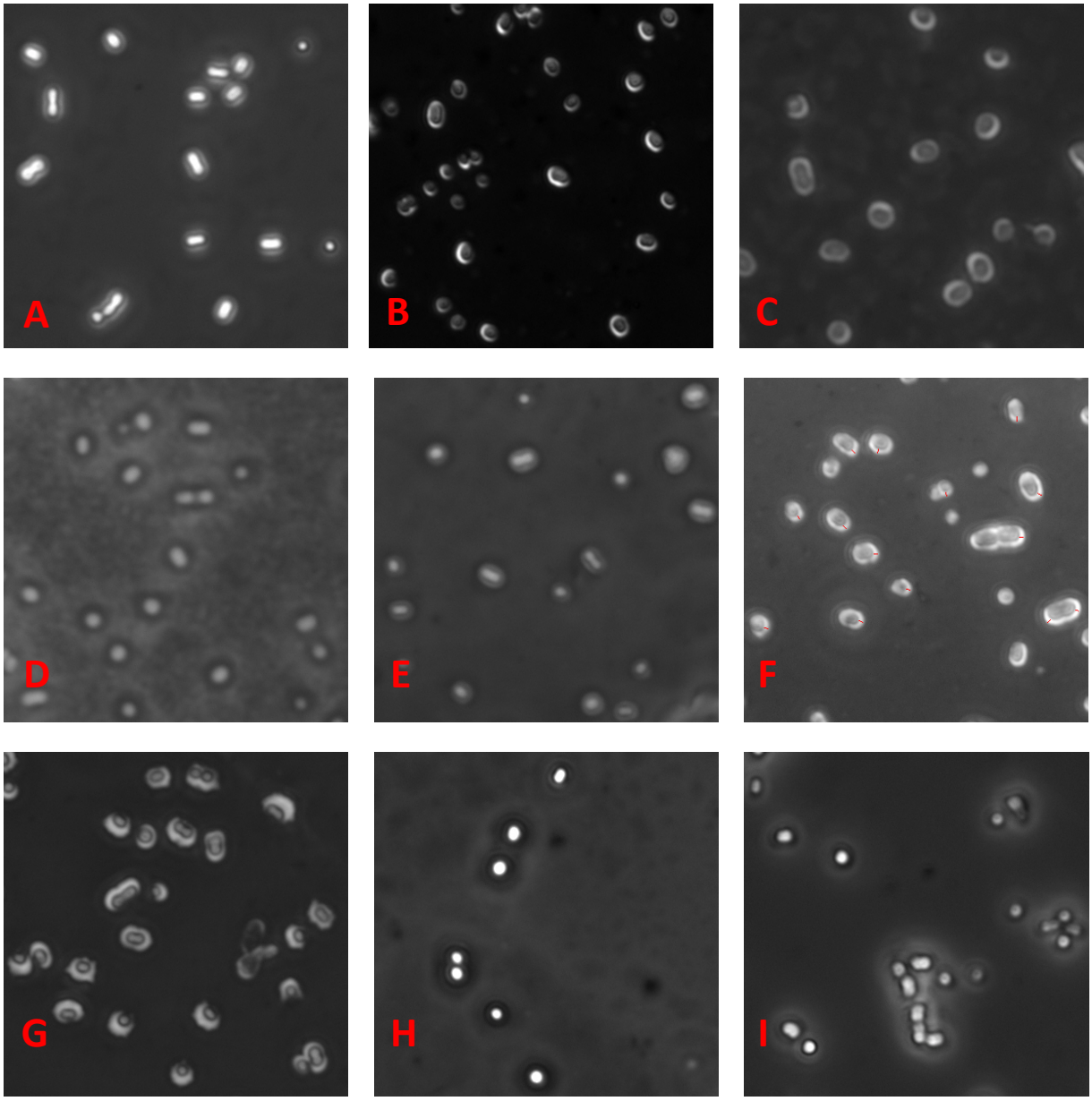

Figure 5. Characterization of mutant capsular EPS**.** The host and mutants were visualized by Maneval's stain and magnification at 1000x using a Nikon Eclipse T*i*. The capsule thickness was measured using the program NIS- Elements AR Analysis. Representative images from the strains

are as follows A. AU0783, B. All, C. B11, D. A7, E. A14, F. C9, G. D6, H. D14, and I. E8.

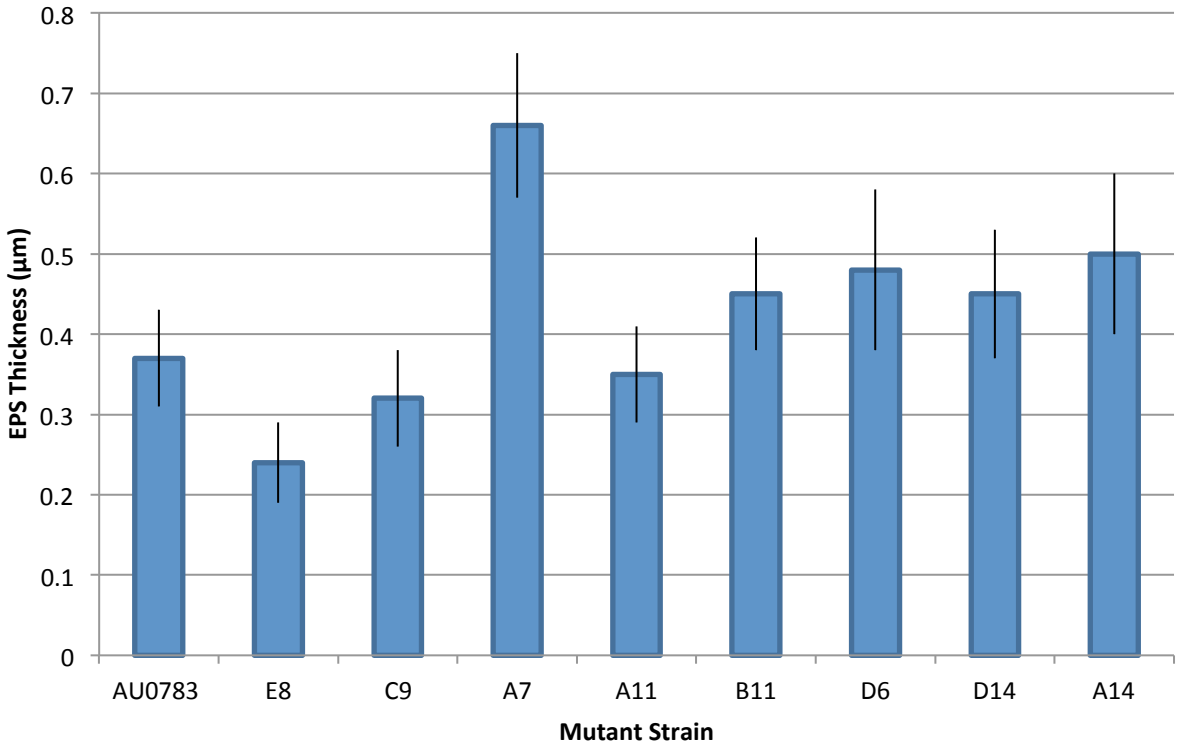

Figure 6. Characterization of mutant capsular EPS**.** The width of the EPS was averaged by measuring the capsular EPS thickness of 200 cells per mutant using the program NIS- Elements AR Analysis.

Evaluation of Dpo1 degradation of mutant EPS

Further evaluation of the mutant EPS was done using both a viscosity and BCA assay. It was shown from the Dpo1 clearing assay that E8, B11, D6, and D14 are not affected by Dpo1 *in vivo* while C9, A7, A11, and A14 are. To determine if the EPS was affected *in vitro*, purified EPS was treated with an active enzyme as well as a heat inactivated enzyme. The viscosities were measured using an Ostwald viscometer in triplicate (Figure 7).

The viscosity of mutant D14 is comparable to wild type and is brought to that of buffer when treated with active enzyme. This is interesting because *in vivo* D14 has no visible halo phenotype indicating that Dpo1 is not degrading the EPS. One of the possible explanations as to why no halo is seen when *in vivo*, is that the bacterial mutant is producing capsular EPS faster than the depolymerase can degrade it.

While as of now bacteriophage are not being used to their full potential in the United States, it is important to note that phage products are allowed and are being used today, and because of this there is a possibility for Dpo1 to be used as a disinfectant in settings where *A. baumannii* is prevalent such as the ICU (14).

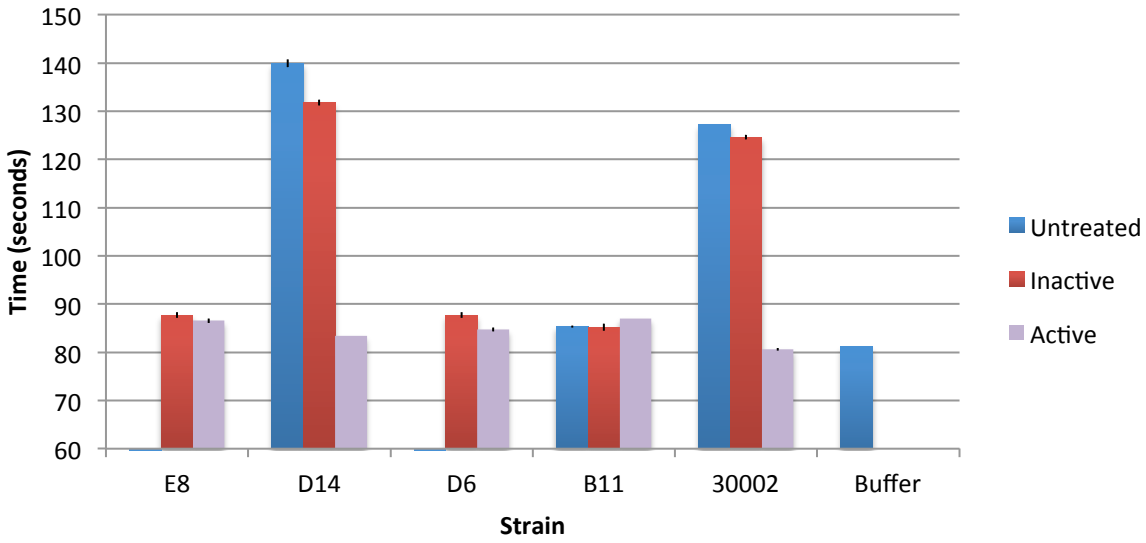

Figure 7. Evaluation of Dpo1 degradation of mutant EPS. The viscosity of the 3 solutions, untreated, inactive, and active, for each mutant were measured using an Oswald Viscometer. For mutants E8, D6, and B11, Dpo1 had no effect *in vitro* on the EPS, while for the D14 mutant Dpo1 degraded the EPS to the viscosity of buffer. This is similar to the effect that Dpo1 has on the wt host AU0783.

Purified recombinant, Dpo1, the depolymerase of phage Petty, generates reducing ends when degrading purified EPS from the host strain, AU0783. To further assess the effect of the purified enzyme on the purified EPS of the mutants, the BCA assay was preformed. The assay's working reagent's copper component is reduced from Cu^{2+} to Cu^{+} giving a color change from blue to purple. The extent of the color change proportionally indicates the amount of reducing ends in solution. Presumably, if the mutant EPS is degraded, the amount of reducing ends will increase allowing for a more exact visualization of EPS degradation then that seen in the viscosity assay. Figure 8 shows the results for D14, D6, and E8. As seen in the viscosity assay, the purified D14 EPS is degraded; so is the purified D6 EPS.

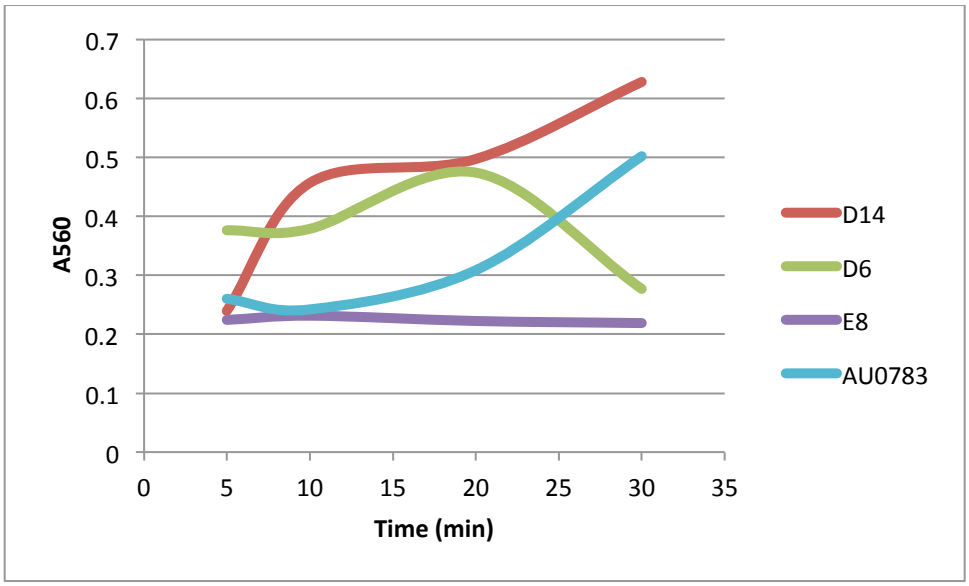

Figure 8. Evaluation of Dpo1 degradation of mutant EPS. The purified EPS of 3 mutants and host were put into solution at a concentration of 5mg/ml. From these diluted EPS samples, a master mix was produced. 5µl of depolymerase was added and two samples were taken for each time point of 5, 10, 20, and 30 minutes at 37°C. The samples were heat inactivated for 5 minutes at 95°C then cooled to room temperature. Once all time points were taken, 1 ml of BCA assay

reagent was added and the samples were heated at 95°C for 15 minutes. The graph was plotted using the average of A_{560} for two independent assays.

CHAPTER IV

CONCLUSIONS

To begin, 42 candidate mutants of AU0783 were selected from colonies that survived exposure to phage Petty. Of these, we continued with 8 based on biofilm production and capsule formation compared to the wt strain AU0783. In theory there are three different possibilities as to why phage resistance is occurring: 1) the amount of capsule is reduced impairing phage absorption; 2) due to a mutation, the capsular EPS structure has changed; 3) some sort of internal insensitivity to bacteriophage Petty has emerged, again as a result of mutation. These mutants of the clinical strain AU0783 were shown to be resistant to infection by phage Petty due to changed capsule characteristics. Phage resistance could be explained by a change in capsular composition, thus causing the insensitivity of the EPS of the mutants to depolymerase activity. The amount of capsule produced by the bacterial mutants could also affect the absorption of the bacteriophage Petty. Further adsorption assays will assess the ability of the phage to absorb to mutant cells.

Dpo1 was not effective against mutant D14 when plated with phage Petty. However, the purified EPS of this mutant is sensitive to the phage's depolymerase and its degradation produces reducing ends. The mechanism of resistance of this mutant cannot be explained by changes in the capsular characteristics that were assessed.

Genomic DNA of these mutants has been sent for sequencing to further investigate which of the three resistance possibilities is occurring for each mutant.

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