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Elucidating Polymyxins' Mode of Action and PrpA's (Polymyxin resistance protein A) Function in *Acinetobacter baumannii*

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
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Bachelor of Sciences with Honors

Department of Biology, Emory University

2013

Abstract

Elucidating Polymyxins' Mode of Action and PrpA's (Polymyxin resistance protein A) Function in *Acinetobacter baumannii*

By Xiang 'Sean' Liu

Antibiotic resistance has become a major public health concern as the prevalence of multi-drug resistant pathogens continues to rise. *Acinetobacter baumannii* is a highly antibiotic resistant, nosocomial pathogen, that is a significant cause of ventilator-acquired pneumonia and other infections. Its ability to survive for prolonged periods under harsh conditions and to subvert the host immune system makes it difficult to combat. Although seldom used for decades due to their nephrotoxicity, polymyxins are now used for the treatment of *A. baumannii* infections as the last-resort drug therapy. Thus, it is crucial to understand how polymyxins kill bacteria, and the mechanisms the bacteria use to acquire resistance to these drugs and handicap the host innate immune system. I have shown that polymyxins kill *A. baumannii* through induction of a hydroxyl radical death pathway. Furthermore, I investigated the genetic basis of polymyxin B resistance in *A. baumannii* and identified PrpA (Polymyxin resistance protein A) as an important determinant. In the absence of *prpA*, *A. baumannii* was more susceptible to killing by polymyxin B. The mutant strain also has a defect in biofilm formation. Thorough analysis of PrpA will provide insight into a new mechanism that *A. baumannii* uses to evade killing, and potentially provide us with a new therapeutic target to treat future infections. Since PrpA is highly conserved, it is very likely that the same mechanism is utilized by other Gram-negative pathogens as well.

Elucidating Polymyxins' Mode of Action and PrpA's (Polymyxin resistance protein A) Function in *Acinetobacter baumannii*

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TABLE OF CONTENTS

INTRODUCTION

PART I: Polymyxins' Mode of Action

Acinetobacter baumannii is a Gram-negative, aerobic, non-motile, nonfastidious, and nonfermenting coccobacilli (28). This opportunistic pathogen has emerged as one of the most problematic microorganisms in causing nosocomial infections globally (28). *A. baumannii* is responsible for causing skin and blood infections, as well as pneumonia and meningitis (28). Patients in intensive-care units and military hospitals are most vulnerable to infections caused by *A. baumannii*, and these infections often have a very high mortality rate (8, 28). This bacterium is especially troublesome because it is able to survive for prolonged periods of time on dry surfaces and is difficult to eliminate with disinfectants, which contributes to its spread (35). In addition, *A. baumannii* has the remarkable ability to up-regulate its antibiotic resistance systems and acquire resistance determinants from other species, making it hard to treat with antibiotics (28).

In recent years, many strains of multi-drug resistant (MDR) *A. baumannii* have been identified in clinical settings (8, 28). Thus, we are quickly running out of antibiotics to treat infections by *A. baumannii*. Therefore, it is crucial to study the pathogenesis of *A. baumannii* in order to combat future infections caused by this bacterium (30).

Polymyxins have not been used clinically for decades because of their nephrotoxicity (30). Because of the increase in the prevalence of MDR infections, they are now used as a last-resort drug therapy (21). Polymyxins that are used clinically include polymyxin B and polymyxin E (28). Polymyxins are cationic antimicrobial peptides that disrupt the outer membrane integrity of Gram-negative bacteria by binding to lipid A in the outer leaflet of the

outer membrane (7, 26). A ring formed by the positively charged amino acid residues in the polymyxins interacts with the negatively charged residues within the lipid to cause perturbations in the membrane (7). It has also been shown that polymyxins contain hydrophobic residues that can be inserted into the outer membrane to increase membrane leakiness (7). The killing of bacteria by polymyxins has often been associated with cell lysis due to membrane disruption; however, reports from the 1970s indicate that other mechanisms might be involved in the killing, as some bacteria were killed by polymyxins without evident cell lysis (5, 17).

Recently, some studies have shown that bacteria are killed by hydroxyl radicals that are induced by the treatment of several classes of bactericidal antibiotics (9, 18). Lethal doses of hydroxyl radicals are produced within bacteria by the Fenton reaction (9, 18). In this reaction, peroxides created from superoxide by superoxide dismutase react with ferric iron and oxidize the iron to form hydroxyl radicals in bacterial cells (18). These hydroxyl radicals interact with DNA, lipids, and proteins to cause oxidative damage that eventually leads to cell death (9, 18). Studies have shown that antibiotics that target bacterial enzymes induce hydroxyl radicals (18); however, no studies have demonstrated that antibiotics such as polymyxins, which are assumed to kill bacteria through membrane disruption, cause killing through a similar method (30).

Through this thesis, I will demonstrate that polymyxin B and colistin cause rapid killing of both sensitive and MDR strains of *A. baumannii* through the induction of hydroxyl radicals (30). *A. baumannii* treated with polymyxins showed an increase in hydroxyl radical production (30); however, in the presence of hydroxyl radical inhibitors, a delay of killing was observed. The inhibitors, thiourea and dipyridyl, quench radicals directly or prevent the production of radicals by indirectly interfering with the Fenton reaction (30). This is the first study to show that polymyxins kill *A. baumannii* through the induction of lethal hydroxyl radicals and explains the

previous observation of polymyxin-induced cell death without cell lysis (30). It provides the knowledge previously not demonstrated to show how last-resort drug therapy, polymyxins, induces cell death (30). Potentially, this study provides support to utilize therapeutic methods that exploit hydroxyl radical-mediated cell death to combat drug resistance in both *A. baumannii* and other MDR pathogens (30).

PART II: PrpA (Polymyxin Resistant Protein A)

I sought to elucidate the genetic basis of polymyxin resistance in *A. baumannii.* Anna Llewellyn et al (2012) demonstrated that in another Gram-negative pathogen, *Francisella novicida*, a protein called NaxD is involved in resistance to polymyxins and contributes to virulence in mouse infections (23). In *Francisella*, NaxD is a deacetylase and is required for the modification of outer membrane lipid A (23). A genetic analysis led us to find an uncharacterized, highly homologous gene to an unpublished *Francisella* protein in *A. baumannii*. I named the protein product of this gene PrpA (Polymyxin Resistance Protein A). The amino acid sequence of this protein resembles that of GtrA-like proteins. GtrA-like proteins are predicted to be integral membrane proteins that are involved in the modification of the bacterial outer membrane (13). They participate in cell surface polysaccharide synthesis by translocating undecaprenyl phosphate linked glucose (UndP-Glc) across the cytoplasmic membrane (13). In addition, they are also involved in O-antigen modification of lipopolysaccharide (LPS) by *Shigella flexneri* bacteriophage X (SfX) (13). Cell-surface polysaccharide and O-antigen modifications contribute to the increased resistance to antibiotics and virulence in Gram-negative bacteria (13).

Here, I demonstrate that PrpA indeed plays a significant role in *A. baumannii*'s resistance to polymyxins. I have also shown that the *prpA* mutant has a defect in the ability to form a biofilm. Biofilms are formed when bacterial cells aggregate together and adhere to a surface when stimulated by multiple environmental factors utilizing pilli and exopolysaccharides (34). The biofilm provides a protective niche for the group of bacterial cells living within the biofilm, defending against harmful external factors and allowing better attachment to biotic and abiotic surfaces (16). In fact, *A. baumannii*'s ability to persist in harsh environments and on medical equipment could be dependent on its ability to form biofilms. A correlation between biofilm formation on polystyrene materials and antibiotic resistance has been shown as well (20). I have demonstrated thus far that PrpA is a very important resistance determinant and a virulence factor in *A. baumannii*. I seek to further explore the properties of PrpA and how it affects the outer membrane structure of *A. baumannii*. An improved understanding of this protein could provide us with a new drug target to treat future MDR *A. baumannii* infections and allow a more efficient elimination of *A. baumannii* in clinical settings to help prevent the spread of this bacterium.

RESULTS AND DISCUSSION

PART I: Rapid Killing of *Acinetobacter baumannii* by Polymxins is Mediated by a Hydroxyl Radical Death Pathway

Polymyxins induce rapid hydroxyl radical production. It has been observed that bactericidal antibiotics induce hydroxyl radical production in bacterial cells, eventually leading to cell death by oxidation of important cellular components (18). Therefore, I sought to demonstrate whether polymyxins kill *A. baumannii* through induction of hydroxyl radicals as well (30). First, I established the concentration of polymyxins for my time-kill assays using the MIC (minimal inhibitory concentration) of type strain, ATCC 17978 (30). Both polymyxin B

and colistin, at the MIC, mediate a rapid killing of *A. baumannii* (Fig.1) (30). Within 30 minutes, viable cells of *A. baumannii* treated with polymyxin were reduced by about 1000-fold in comparison to the untreated sample (Fig. 1) (30).

Tim Sampson then sought to determine whether the rapid killing of *A. baumannii* by polymyxins is associated with the induction of hydroxyl radicals (30). To quantify the formation of hydroxyl radicals, he used cell permeable, hydroxyl radical-specific fluorescent dye, 3′-(p-hydroxyphenyl) fluorescein (HPF) (9, 30). He observed a significant ~2-fold increase of hydroxyl radicals in polymyxin treated samples after 30 minutes (Sup. Fig. 1) (30). Hydrogen peroxide and kanamycin have previously been shown to induce hydroxyl radicals; therefore, he used them as controls for the experiments (30). To show that cell lysis alone does not produce increased HPF fluorescence, he sonicated *A. baumannii* (30). His data indicated that hydroxyl radicals were induced by the treatment of polymyxins and not bacterial lysis (30). He has also shown that polymyxins induce radical production in other Gram-negative bacteria to show that the mechanism of killing is not only limited to *A. baumannii* (30).

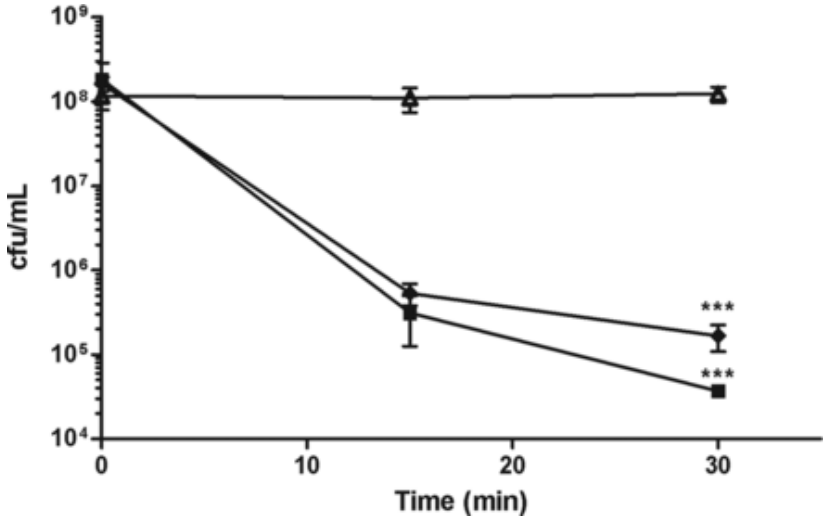
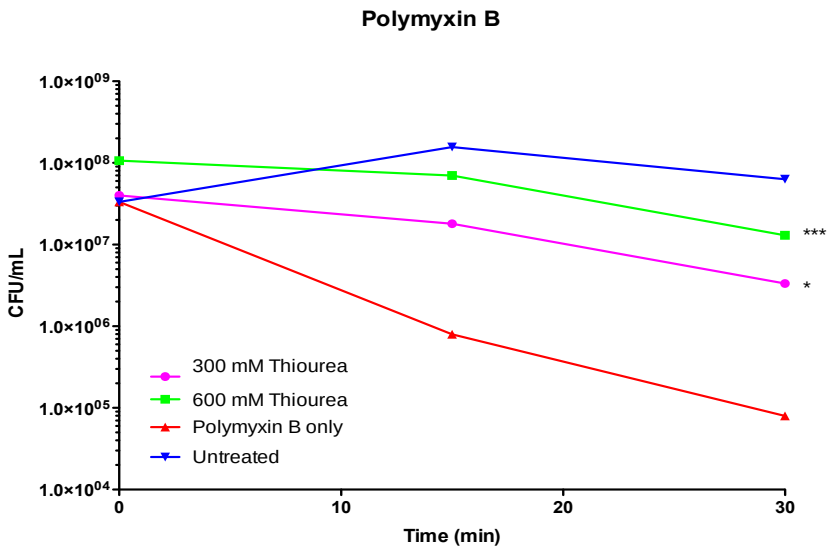


Figure 1. Polymyxins induce rapid killing of *A. baumannii*. *A. baumannii* cultures were treated with 2 µg/ml of polymyxin B (■) or colistin (□), or left untreated (△). At 0, 15, and 30 min, cultures were plated, and the CFU were enumerated. The data are representative of three independent experiments. Points represent the means and bars represent the standard deviation of triplicate samples. ***, P < 0.0001. Significance was obtained by comparison to untreated cultures. (Adapted from Sampson T.R, Liu X., et al. *Antimicrob. Agents Chemother*. 2012;56:5642-5649.)

Rapid killing of *A. baumannii* by polymyxins is mediated by the production of hydroxyl radicals. After he observed that the treatment of polymyxins induces high levels of hydroxyl radical production and the induction is correlated with the rapid killing of *A. baumannii*, I utilized a hydroxyl radical scavenging compound, thiourea, to determine if cell death is caused by hydroxyl radicals (29, 30). Thiourea can convert peroxides to more stable diol compounds and prevent the formation of hydroxyl radicals. If hydroxyl radicals were required for the killing of *A. baumannii* by polymyxins, then the addition of thiourea would prevent the killing of *A. baumannii* by inhibiting hydroxyl radical production (30). Thiourea delayed killing of *A. baumannii* significantly in a concentration-dependent manner (Fig. 2) (30). Thiourea delayed killing by polymyxin treatment in other Gram-negative bacteria as well (30). I demonstrated that thiourea did not alter growth kinetics of *A. baumannii* in broth (30). My data suggested that hydroxyl radicals play a crucial role in initiating the rapid killing of *A. baumannii* when treated with polymyxins (30)

A.



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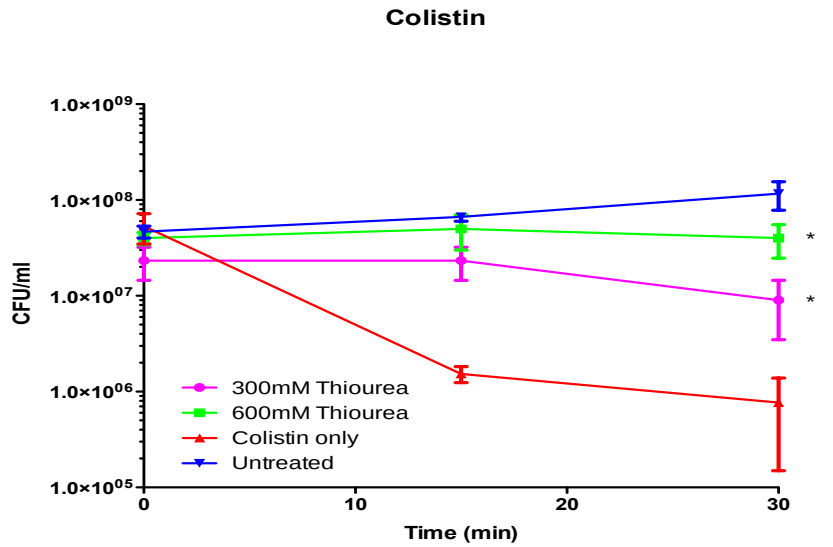
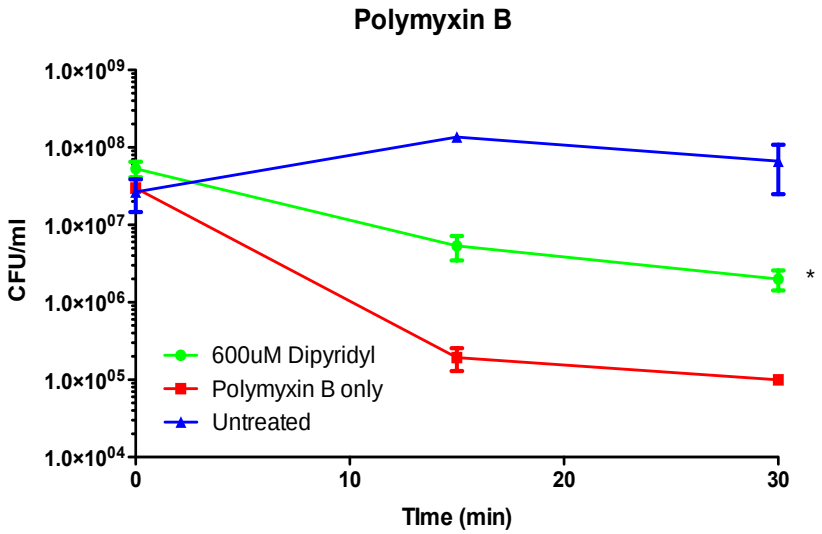


Figure 2. Polymyxin killing is delayed by hydroxyl radical quenching. *A. baumannii* cultures were treated with either 2 µg/ml of polymyxin B or colistin alone or in combination with either 300 mM thiourea or 600 mM thiourea , or they were left untreated in culture medium. At 0, 15, and 30 min, the cultures were plated, and the CFU were enumerated. The data are representative of three independent experiments. Points represent the means and bars represent the standard deviations of triplicate samples. ***, P < 0.0001; *, P < 0.05. (A) 300mM : t=3.688, df=4. 600mM: t=7.459, df=4 (B) 300mM: t=1.486, df=4. 600mM : t=2.566, df=4. Significance was obtained by comparison to polymyxins-only cultures. (Adapted from Sampson T.R, Liu X., et al. *Antimicrob. Agents Chemother*. 2012;56:5642-5649.)

Fenton reaction inhibition delays killing of *A. baumannii* by polymyxins. It has been observed that the intracellular Fenton reaction is responsible for the large increase in hydroxyl radical production initiated by reactive oxygen species (9, 15, 20). To determine if Fenton chemistry play a role in the killing of *A. baumannii* by polymyxins, I utilized 2,2′-dipyridyl (dipyridyl) (30). Dipyridyl is a reagent that chelates intracellular iron and inhibits the Fenton reaction by limiting the availability of iron that is required by peroxides for radical production (15). When dipyridyl was added to *A. baumannii* treated with polymyxin B or colistin, a significant delay of killing by polymyxins was observed (Fig. 3) (30). In order for dipyridyl to work properly, samples were pretreated with dipyridyl for 20 minutes prior to performing the time-kill assays. Dipyridyl does not prevent growth of *A. baumannii* in broth. My data suggest that radicals produced through the Fenton reaction are indeed involved in the rapid killing of *A. baumannii*. Together with the delay of killing by the addition of thiourea, my data strongly indicate that polymyxins induce killing of *A. baumannii* through the production of hydroxyl radicals (30)

A.



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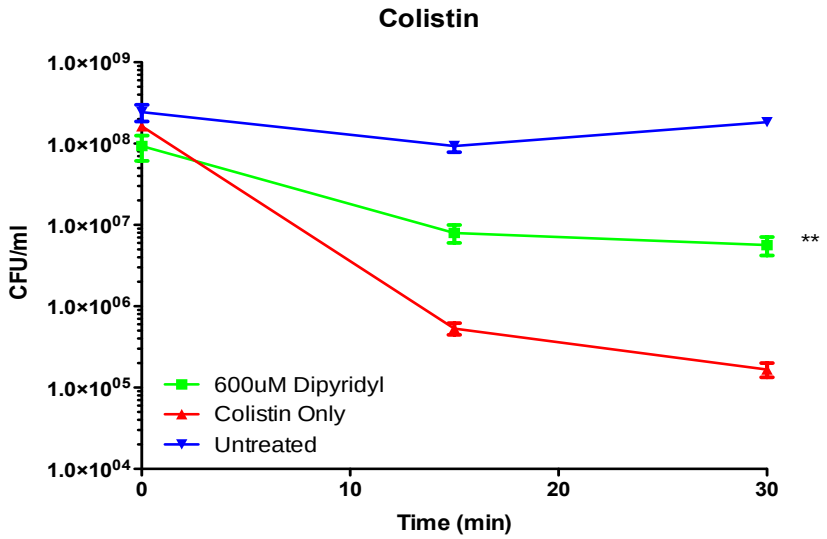


Figure 3. Polymyxin killing is mediated by iron. *A. baumannii* cultures were treated with either 2 µg.ml of polymyxin B or colistin alone or in combination with 600 µM dipyridyl , or they were left untreated in culture medium. At 0, 15, and 30 min, the cultures were plated, and the CFU were enumerated. The data are representative of three independent experiments. Points represent the means and bar the standard deviations of triplicate samples. **, P < 0.005; *, P < 0.05. (A) t=3.291, df=4. (B) t=3.784, df=4. Significance was obtained by comparison to polymyxins-only cultures. (Adapted from Sampson T.R, Liu X., et al. *Antimicrob. Agents Chemother*. 2012;56:5642-5649.)

Drug-resistant clinical isolates of *A. baumannii* are also killed by polymyxins through hydroxyl radical induction. The increasing prevalence of multi-drug resistant strains of *A. baumannii* is becoming problematic in hospital settings; therefore, Tim Sampson sought to examine whether polymyxins kill these clinical isolates through the hydroxyl radical death pathway (30). Consistent with previous results, colistin sensitive clinical strains, CI-2 and CI-3, have a significant increase in the level of hydroxyl radicals after 30 minutes of treatment with colistin (Sup. Fig. 2) (30). However, there was no change in the level of hydroxyl radicals for CI-4, a colistin-resistant isolate (Sup. Fig. 2) (30). This is consistent with other studies demonstrating that sub-lethal dosage of antibiotics is not sufficient to induce hydroxyl radical production and killing (18). Furthermore, I utilized thiourea and dipyridyl to demonstrate whether hydroxyl radicals were responsible for the killing of these clinical isolates (30). Indeed, the killing of CI-2 and CI-3 was significantly delayed by the treatment of thiourea or dipyridyl (Fig. 4) (31). Again, CI-4 did not demonstrate a significant change in the treated sample because its MIC was much higher (30). Together, our data suggest that the rapid killing of *A. baumannii* by polymyxins through the induction of hydroxyl radicals is conserved in MDR clinical isolates that are sensitive to colistin (30). Furthermore, our findings support the ongoing effort to utilize hydroxyl radical therapy as a new therapeutic treatment to combat deadly MDR nosocomial infections (30).

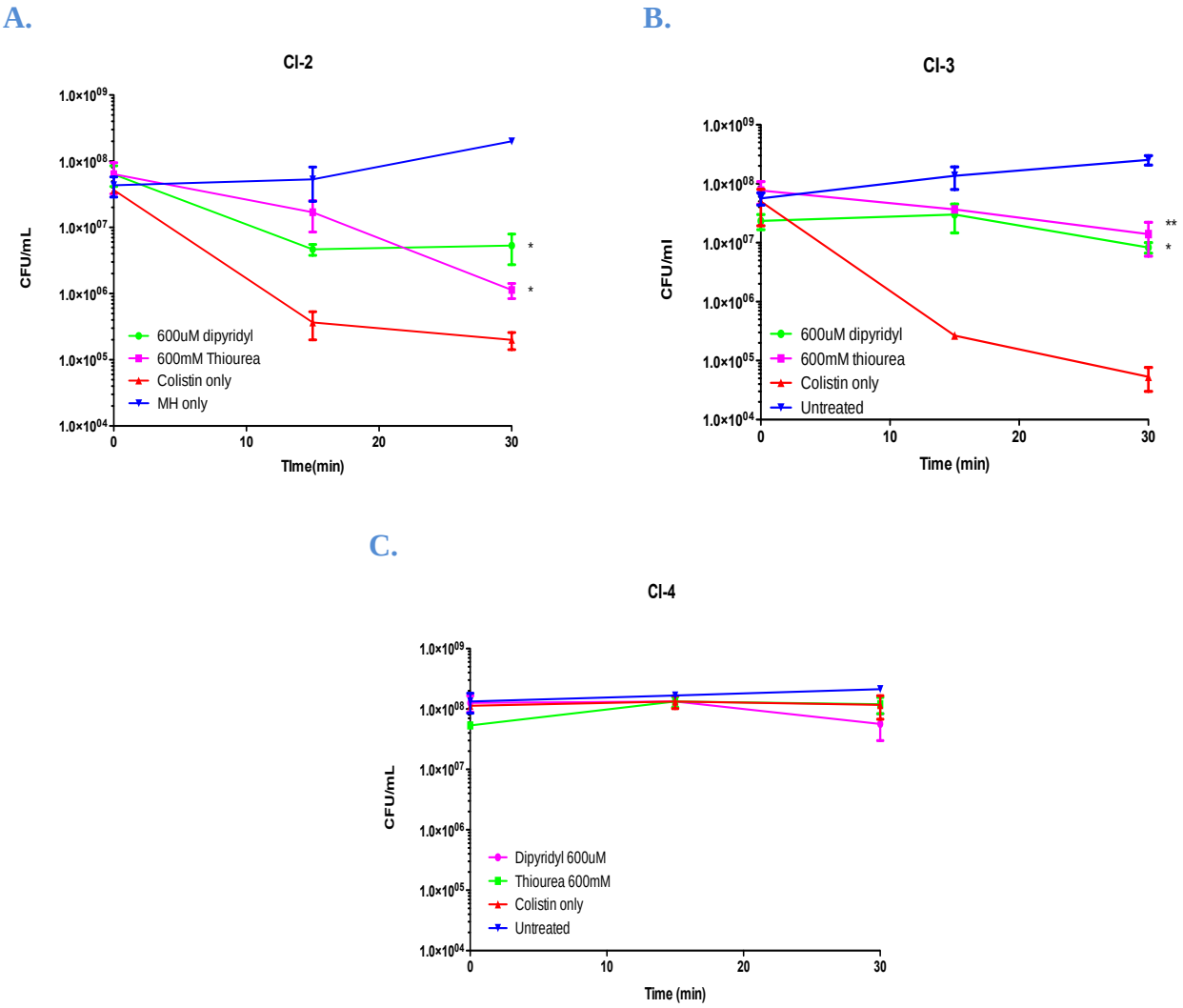


Figure 4. Clinical isolates are killed through hydroxyl radical production during polymyxin treatment. The colistin-sensitive MDR clinical isolates CI-2 (A) and CI-3 (B) or the colistin-resistant PDR *A. baumannii* strain CI-4 (C) were treated with 2 µg of colistin/ml alone or in combination with either 600 µM dipyridyl or 600 mM thiourea , or they were left untreated in culture medium. At 0, 15, and 30 min, the cultures were plated, and the CFU were enumerated. The data are representative of two independent experiments. Points represent the means and bars represent the standard deviation of triplicate samples. **, P < 0.005; *, P < 0.05. (A) Thiourea: t=3.150, df=4. Dipyridyl: t=1.971, df=4. (B) Thiourea : t=1.725, df=4. Dipyridyl t=1.518 df=4. Significance was obtained by comparison to polymyxins-only cultures. (Adapted from Sampson T.R, Liu X., et al. Antimicrob. Agents Chemother. 2012;56:5642-5649.)

Here, we demonstrated that the treatment of *A. baumannii* with polymyxins induced a rapid production of hydroxyl radicals through the Fenton reaction, which ultimately led to bacterial cell death by oxidation of cell membranes, DNA, and proteins (30). My study further augmented the classical explanation for the killing of Gram-negative bacteria by polymyxins; cell lysis is not the only means of killing by polymyxins (5, 17).

Bactericidal antibiotics that directly interact with enzymes that are involved in bacterial physiology have previously been shown to kill bacteria through the induction of hydroxyl radical production (9, 18). Polymyxins, however, have been known to target bacterial cell membrane and not the bacterial enzymes (8, 27). As a result, it was interesting to find that polymyxins also kill *A. baumannii* through the induction of hydroxyl radicals (30). Furthermore, this study is the first, to my knowledge, to demonstrate that polymyxins utilize the oxidative cell death pathway to kill Gram-negative bacteria (30).

Although my data suggest that killing by polymyxins is a result of hydroxyl radical production, what promotes the initiation of the radical production is unclear. Production of hydroxyl radicals is likely caused by the increased activity of the electron transport chain (ETC) and the rapid depletion of NADH after bactericidal antibiotic treatment (9, 18). NADH is an important electron carrier for the ETC; however, when unregulated, produces superoxide by donating its electron to oxygen (18). When the tricarboxylic acid cycle that was responsible for the production of NADH was prohibited, bacterial survival was increased after antibiotic treatment (18). Superoxide produced from the up-regulated ETC initiate the Fenton reaction, ultimately leading to a large production of hydroxyl radicals (9, 15, 18). It has been demonstrated that bacterial envelope stress response, such as that of the CpxAR two-component system in

E.coli, triggers the rapid utilization of NADH and subsequently the production of hydroxyl radicals following treatment with aminoglycosides (19). Therefore, I infer that polymyxins may utilize a similar mechanism to initiate the production of hydroxyl radicals (30). A study using *Vibrio cholera* showed an increase in the transcription of sigma factor, *rpoE*, which is involved in envelope and oxidative stress responses, after treatment with polymyxin B (31). In addition, other studies have shown that polymyxin B can induce aberrant oxidative respiration (25, 32, 33). Although *A. baumannii* lacks the two-component CpxAR system, I speculate that its envelope stress response is also triggered through an unidentified sensory system after the binding of polymyxin B (30). Together with my observations and Sampson's findings, we suggest that killing of *A. baumannii* and other Gram-negative bacteria is caused by the induction of the hydroxyl radical death pathway (30).

I further elucidated that the polymyxins, last line antibiotic treatments for infections with MDR *A. baumannii*, also induce the production of hydroxyl radicals in MDR clinical isolates (30). Furthermore, the killing of theses MDR strains was delayed by thiourea and dipyridyl. However, I observed that the killing by colistin is prohibited in the clinical isolate resistant to colistin. Resistance to colistin has been linked to the modification of lipid A on the outer membrane, preventing the binding of the antibiotic to the cell outer membrane (30). In addition, it has been shown that some bacterial pathogens that limit their intracellular iron level have increased resistance to oxidative killing (22). This suggests that bacteria can prevent polymyxin killing through dampening the Fenton reaction by limiting intracellular iron concentration, further reinforcing the utilization of the oxidative radical death pathway (30).

As the number of nosocomial infections caused by *A. baumannii* and the need for the treatment with polymyxins continue to increase, identifying the mechanisms by which the

antibiotics cause killing is imperative (2, 30). My findings improve our knowledge of the mechanism of polymyxin action and also provide support to current concepts of utilizing hydroxyl radical-inducing agents as therapies against extensively drug-resistant pathogens (6, 30, 37).

PART II: PrpA (Polymyxin Resistant Protein A) and Biofilm Formation

PrpA (Polymyxins Resistant Protein A) contributes to *A. baumannii*'s resistance to polymyxin B. I identified a gene in *A. baumannii* that could potentially be responsible for the bacterium's resistance to polymyxins and its ability to modify the outer membrane structure, leading to increased virulence. First, a mutant derivative of strain ATCC 17978, lacking *prpA*, was created with the help of a graduate student, Brooke Napier, in my lab. She created the mutant by replacing *prpA* with a kanamycin cassette. Once the mutant was created and verified by sequencing, I sought to identify the function of PrpA. Since polymyxin resistant strains have already been identified, I first examined if this protein is involved in resistance to polymyxin B. I determined that PrpA is involved in resistance to polymyxin B by performing time-kill assays, which showed that the PrpA mutant has ~100 fold less viable cells than that of the wild-type (Fig. 5). Polymyxins kill Gram-negative bacteria by binding to lipid A on the outer leaflet of the bacterial outer membrane (7). This class of antibiotics is positively charged and bind to the negatively charged lipid A (7). By altering the net charge on the outer membrane, sensitivity to polymyxins can be affected (7). PrpA's potential involvement in the modification of the outer membrane to resist killing by antibiotics could contribute to the increased sensitivity to polymyxin of *A. baumannii* lacking the protein.

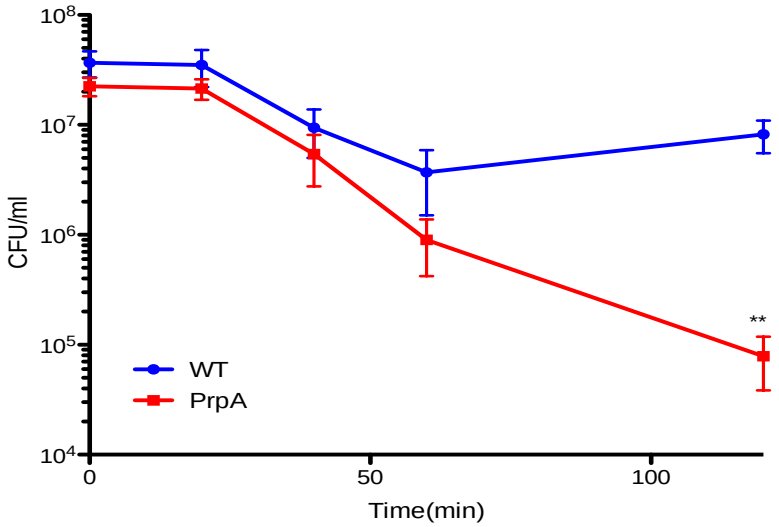


Figure 5. The *prpA* mutant is more sensitive to killing by polymyxin B. *A. baumannii* cultures were treated with 1ug of polymyxin B/ml. At 0, 15, 30, 45, 60, 90 min, cultures were plated, and the CFU were enumerated. The data are collection of three independent experiments. Points represent the means and bars represent the standard deviation. **, P<0.01. t=3.020, df=16. Significance was obtained by comparison to WT cultures.

The *prpA* mutant is defective in its ability to form a biofilm. *A. baumannii*'s ability to form biofilms has been reported as one of the most critical problems due to its contribution to the increased resistance to antibiotics and prolonged survival. Since exopolysaccharides are involved in the process of biofilm formation, I sought to determine whether PrpA, which is thought to be involved in sugar translocation, could affect *A. baumannii*'s ability to form biofilms on abiotic surfaces. To visualize whether PrpA is responsible for biofilm formation, I stained *A. baumannii* cultures with crystal violet after allowing the bacteria to grow and form biofilms for 24 hours (27). I observed that *A. baumannii* lacking PrpA makes less biofilm than the wild-type (Fig. 6). To quantify the visualized difference in biofilm formation, I measured the level of biofilm formation by solubilizing the stained biofilm in 33% acetic acid. Consistent with the visualization data, the mutant lacking *prpA* showed a significant, ~2 fold less, biofilm production (Fig. 7). It has been reported that bacteria produce different levels of biofilm on different abiotic surfaces; therefore, I sought to determine whether *A. baumannii* lacking PrpA express the same phenotype in other abiotic surfaces as well (27). When I quantified biofilm formation of *A. baumannii* on polystyrene and polypropylene 96-well plates, the mutant lacking PrpA also showed significantly less formation of biofilms (Fig. 8). *A. baumannii* is problematic in clinical settings because of its ability to survive on dry surfaces for a prolonged period of time (10). Biofilm formation has been shown to help bacteria attach to abiotic surfaces and allow them to persist for a longer duration of time after being subjected to disinfection (10). My data suggest that PrpA is responsible for *A. baumannii*'s ability to form biofilm on various abiotic surfaces. This study provides a new genetic basis for *A. baumannii*'s ability to form biofilms. Potentially, PrpA could be targeted to help remove *A. baumannii* in the clinical setting since the mutant lacking *prpA* is defective in forming biofilms on various surfaces.

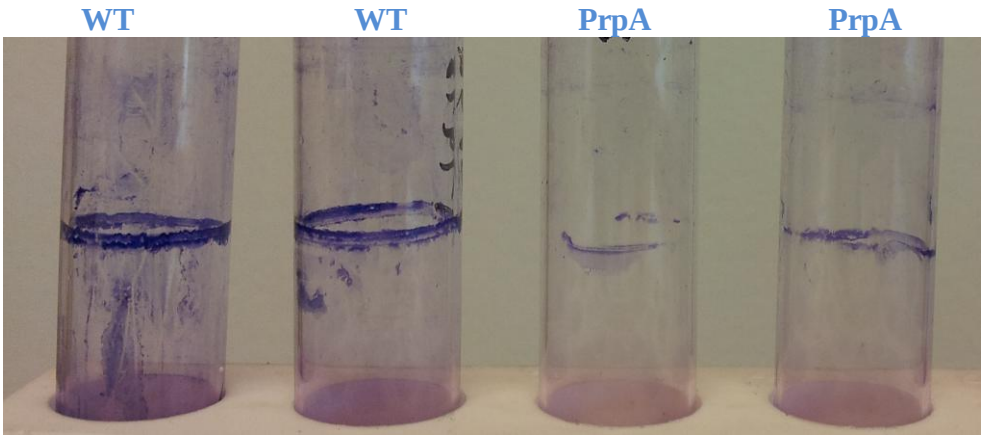


Figure 6. The *prpA* mutant has a defect in the ability to form biofilms. All samples were treated with 1% CV and washed with water. Photograph was taken with a normal camera.

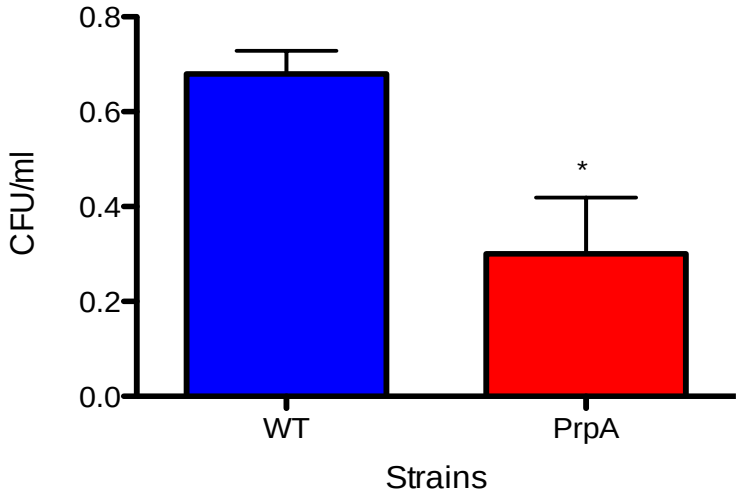
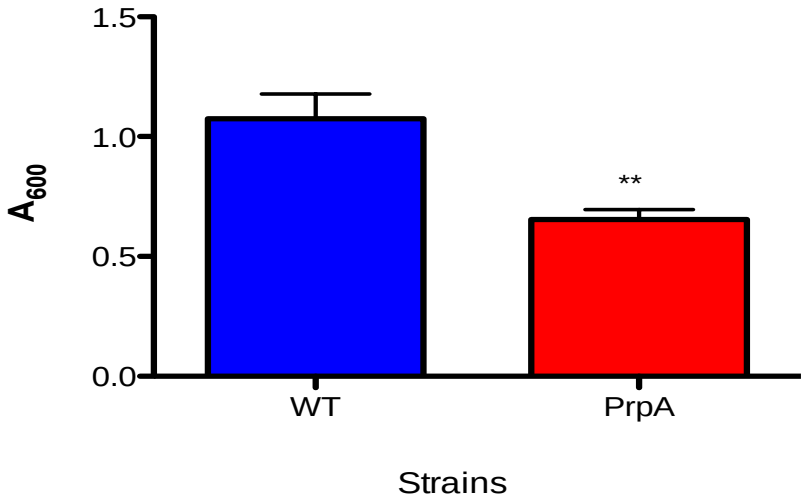


Figure 7. The *prpA* mutant has impaired ability to form biofilm on glass tube. All samples were treated with 1% CV and washed with water. After the CV-stained biofilms are solubilized in 33% acetic acid, absorbance of samples was measured (600nm). Bars represent the means and standard deviations of triplicate samples. **, P<0.01. t=2.968, df=4.

A.



B.

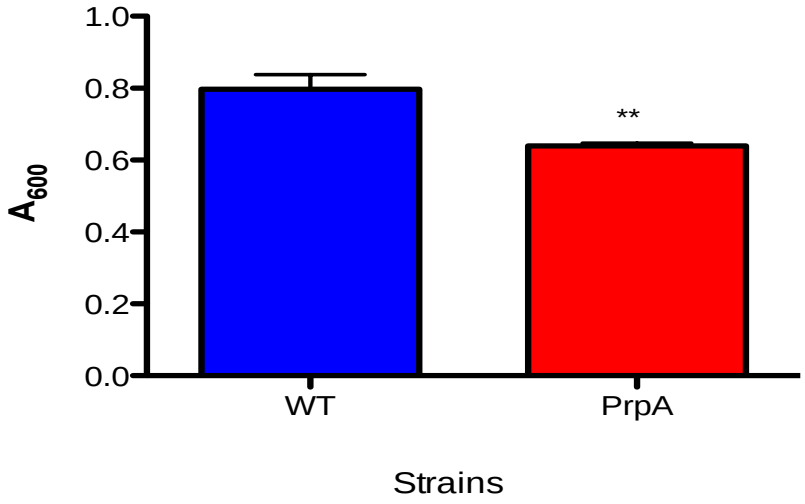


Figure 8. *prpA* mutant has lower level of biofilm formation on polystyrene plates (A) and polypropylene plates (B). All samples were treated with 1% CV and washed with water. After the CV-stained biofilms are solubilized in 33% acetic acid, absorbance of samples was measured (600nm). Bars represent the means and standard deviations of triplicate samples. **, P<0.01. (A) t=3.747, df=8. (B) t=3.784 df=8.

PrpA is involved in *A. baumannii* growth. After the construction of the *prpA* mutant, I sought to determine whether this gene is essential for *A. baumannii*'s growth. *A. baumannii* cultures were diluted to $O.D._{600}$ of .03 and grown for 24 hours with periodic sampling to collect colony forming unit (CFU) data at different time points. The mutant had no apparent difference in morphology and its $O.D._{600}$ reading to colony forming unit conversion was not significantly different from the WT (Fig. 9), confirming the amount of bacteria I added to the experiments described above was the same. The growth curve showed that the mutant is unable to reach the same level of CFU as the wild-type (Fig. 110). Studies have shown that quorum sensing and exopolysaccharides are responsible for initiation of biofilm formation (10). My data suggest that the large defect in biofilm formation in the mutant is due to the lack of PrpA; however, I am not able to infer the mechanism behind the observed phenotype. Further study is required to exploit PrpA as a new therapeutic target, as my data suggest that it is involved in antibiotic resistance and problematic biofilm formation.

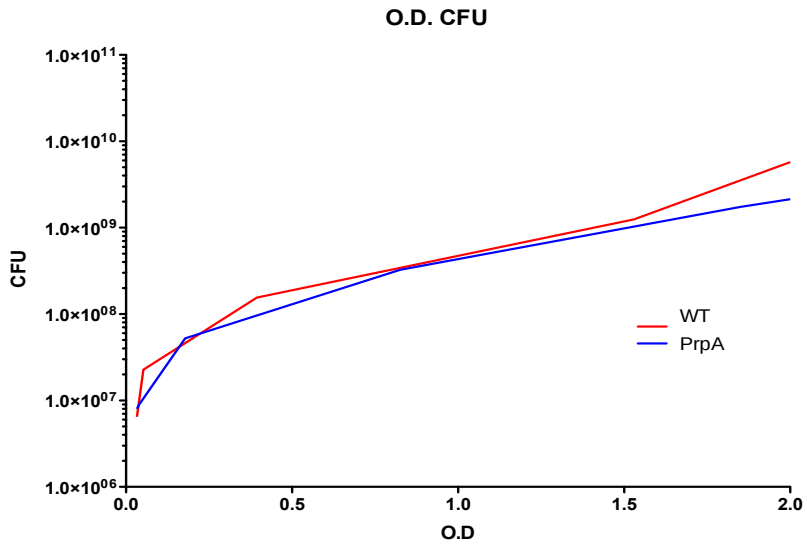


Figure 9. Wild-type and mutant O.D.-CFU Comparison

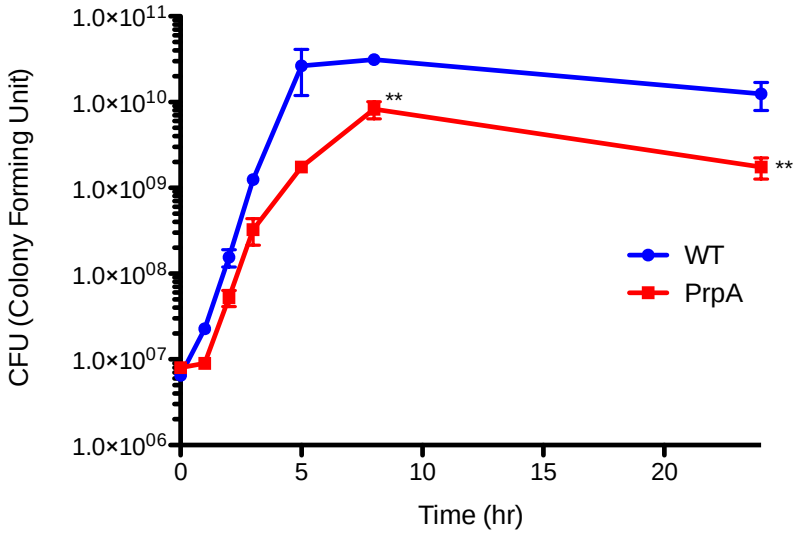


Figure 10. Comparison of wild-type and mutant growth. **, P<0.01. t=5.282, df=6.

I sought to identify the genetic basis of polymyxin resistance in *A. baumannii*. I discovered that *prpA* was responsible for polymyxin B resistance in *A. baumannii*. It has been shown that modifications of lipid A that mask the negatively charged phosphate moieties could provide increased resistance to polymyxin (1, 3, 14, 24). As a GtrA-like protein, PrpA is involved in resistance to polymyxin and could potentially be involved in lipid A modification.

Surprisingly, I found that PrpA is also involved in biofilm formation. A biofilm is formed by the close interaction of multiple bacterial cells aggregating on a surface within an extracellular matrix (4). Formation of a biofilm is strictly regulated by a wide variety of environmental factors (10). Common factors include: nutrient availability, bacterial appendages, outer membrane proteins, quorum sensing, and secretion of polysaccharides (11). *A. baumannii* poses a serious threat in clinical settings because of its ability to attach to medical equipment and dry surfaces and survive for a long duration of time (16, 36). Jawad and colleagues have shown that *A. baumannii*'s mean survival time when subjected to desiccation is over 27 days (16). Such prolonged survival increases the chance that *A. baumannii* would be spread (16). Previous studies have demonstrated a positive correlation between biofilm formation and antibiotic resistance (20). Not only do biofilms help bacteria resist killing by antimicrobial agents, but they also improve their persistence on biotic and abiotic surfaces. (20). The mechanisms utilized by PrpA are unclear. Since the mutant shows a slight growth defect, it is possible that *A. baumannii* does not grow to an optimal concentration to initiate biofilm formation. In addition to quorum sensing, that mutant's defect in biofilm formation could be due to the absence of PrpA that is likely involved in polysaccharide secretion. PrpA is crucial for the production of biofilms and could serve as a potential therapeutic target to reverse *A. baumannii's* ability to resist antibiotics and to form biofilms and persist (12).

I have shown that PrpA is not only responsible for polymyxin resistance, but also required for biofilm formation. Further study of this protein is crucial as it could potentially provide a new therapeutic target to treat future MDR *A. baumannii* infections and combat the troublesome persistence of *Acinetobacter* species.

METHODS

Bacterial strains and growth conditions.

All *A. baumannii* strains (ATCC 17978, PrpA Mutant, CI-2, CI-3, and CI-4 were routinely grown from frozen stock in Mueller-Hinton (MH) broth (BD Biosciences, Sparks, MD), or Luria Broth (LB) at 37°C with aeration. CI-2, CI-3, and CI-4 were kindly provided by Brandi Limbago, Division of Health Care Quality Promotion, Centers for Disease Control and Prevention, Atlanta, GA. CI-2 was isolated in the District of Columbia in 2005, CI-3 was isolated in Ohio in 2006 by endotrachial aspirate, and CI-4 was isolated in Mississippi in 2010 from sputum. PrpA mutant was created from ATCC 17978 strains using a kanamycin cassette.

Time-kill assay (Hydroxyl Radical Experiments).

To determine the levels of killing by antimicrobial compounds, time-kill experiments were performed as previously described (28). Overnight cultures were subcultured as described above and then diluted to a final concentration of 10^7 CFU/ml in MH broth. Samples were treated with 2 μg of either polymyxin B or colistin/ml and incubated with aeration at 37°C. At the indicated time points, aliquots of treated cells were harvested, suitable dilutions were performed, and then the cells were plated onto MH agar plates. After overnight incubation of the plates at 37°C, CFU were enumerated. Thiourea (Sigma-Aldrich) was added to cultures

concurrently at the indicated doses. When 2,2′-dipyridyl (MP Biomedical, Solon, OH) was utilized, the cells were pretreated for 20 min at 37°C with the indicated doses, before treatment with polymyxins.

Time-kill Assays (PrpA Experiments)

Conditions used were the same as described above. However, LB broth and agars were used instead of MH and the concentration of polymyxin B used was 1ug/ml.

Bacterial Growth Curve

Overnight cultures were subcultured to an $O.D._{600}$ of .03. Cultures were taken at time points 0hr, 1hr, 2hr, 3hr. 5hr, 8hr, and 24hr, and plated on LB agar plates after serial dilution in PBS. Plates were incubated at 37^{o}C overnight and CFUs were counted the next day.

Biofilm formation assay

To determine difference in wild-type and PrpA mutant's abilities to form biofilm on different abiotic surfaces, biofilm formation assays were performed using the modified method previously described (30). Adherence was tested on either 96-well styrene plates or borosilicate glass tubes. Overnight cultures were diluted to $O.D._{600}$ 0.1 in LB. For 96-well plates, 100ul of diluted culture were used and incubated with aeration at 37°C for 10 hours. 2ml of cultures were addition to glass tubes and incubated with aeration at 37°C for 24 hours. Samples were stained with 1% crystal violet (CV) in ethanol for visualization and quantification. For quantification on 96-well plates, 25ul of of 1% CV were added. For glass tubes, 2ml of 1% CV were added. Each sample was washed 5 times with water.

Quantification of biofilm formation.

CV-stained biofilm was solubilized by the addition of 200ul and 2ml of 33% acetic acid to each of the CV-stained samples in 96-well plates and glass tubes, respectively. To quantify the level of biofilm formation, 125ul of the solubilized samples were transferred to a new 96-well plate, and the absorbance was determined at 600nm using a plate reader.

Statistical Analysis

Analysis was done in Graphpad. Two-tailed, unpaired T-test with 95% confidence interval was used. All data sets passed the D'Agostino and Pearson omnibus normality test.

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SUPPLEMENTARY FIGURES

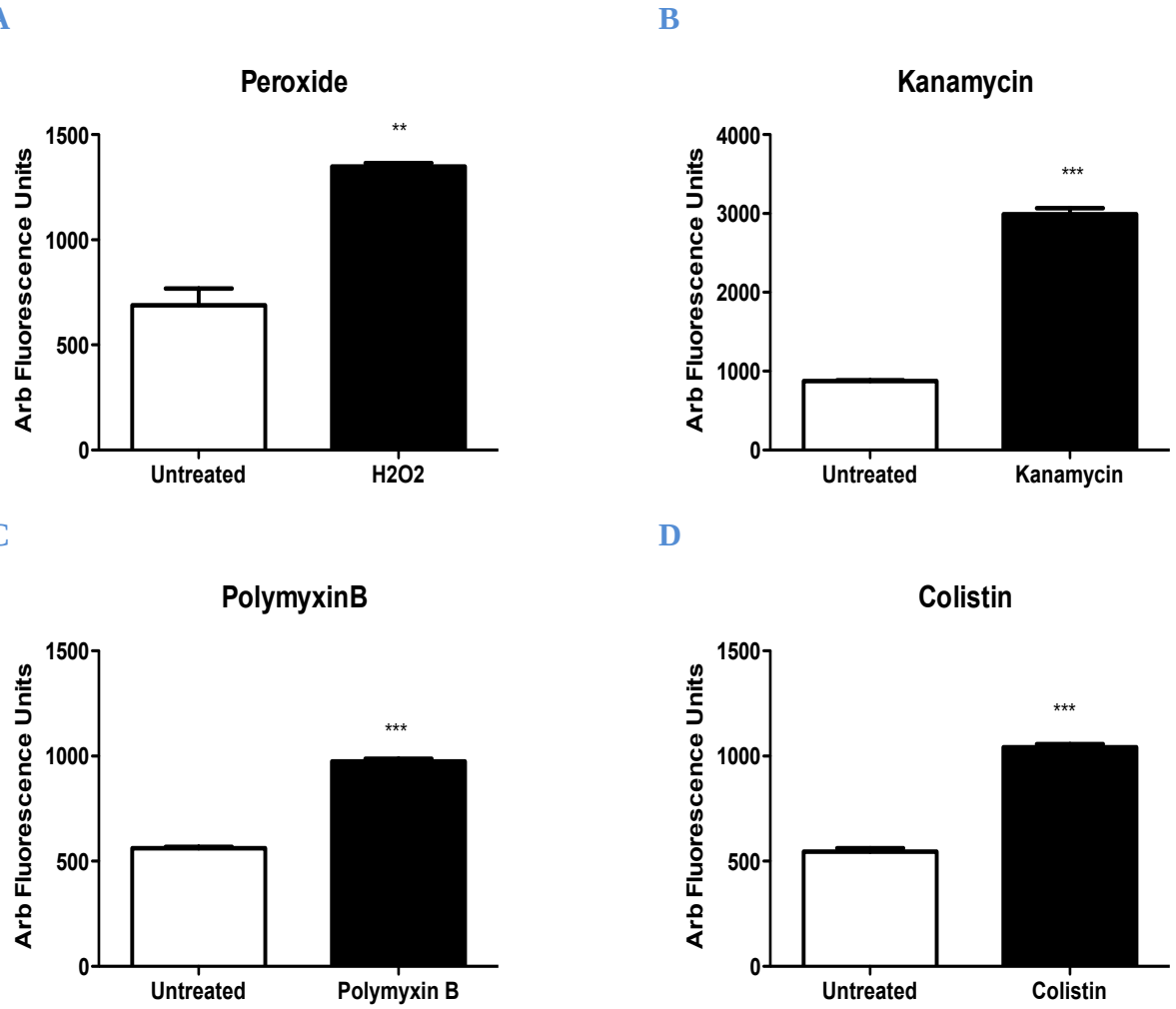

Figure 1. Polymyxins induce hydroxyl radical production. *A. baumannii* cultures were left untreated or were treated with 0.15% hydrogen peroxide (H_2O_2) (A), 5 µg of kanamycin/ml (B), 2 µg of polymyxin B/ml (C), or 2 µg of colistin/ml (D) for 30 min. After treatment, the hydroxyl radical specific fluorescent dye 3′-(p-hydroxyphenyl) fluorescein was added, and the fluorescence was measured (490 nm/515 nm). The data are representative of three independent experiments. Bars represent the means and standard deviations of triplicate samples. ***, P < 0.0001. (Adapted from Sampson T.R, Liu X., et al. Antimicrob. Agents Chemother. 2012;56:5642-5649.)

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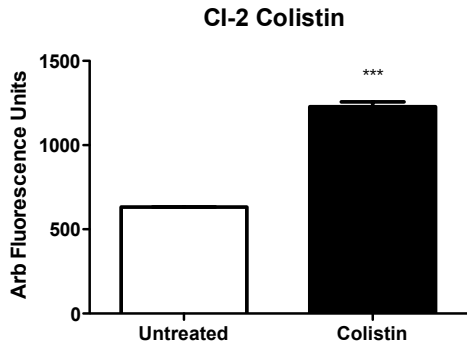

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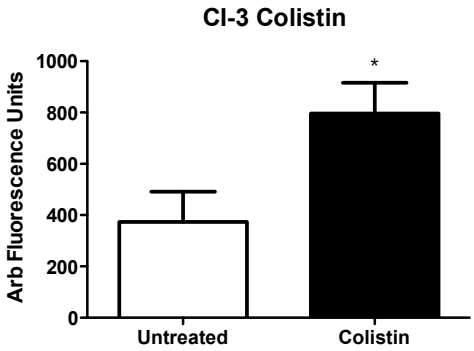

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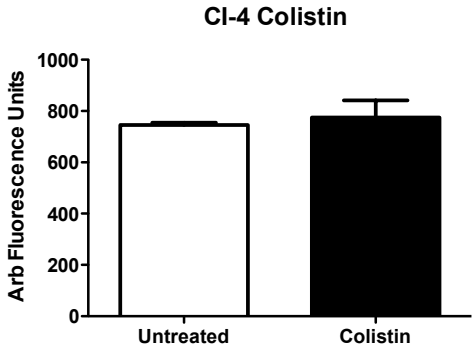

Figure 2. Colistin induces hydroxyl radical production in MDR clinical isolates. Cultures of colistin-sensitive MDR strain CI-2 or CI-3 or a colistin-resistant PDR *A. baumannii* strain CI-4 were treated with 2 µg of colistin/ml or left untreated for 30 min. After treatment, the hydroxyl radical specific fluorescent dye 3′-(p-hydroxyphenyl) fluorescein was added, and fluorescence was measured (490 nm/515 nm). The data are representative of two independent experiments. Bars represent the means and standard deviations of triplicate samples. ***, P < 0.0001; *, P< 0.05. (Adapted from Sampson T.R, Liu X., et al. Antimicrob. Agents Chemother. 2012;56:5642-5649.)