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Identification of a small molecule inhibitor of virulence factors in multidrug resistant acinetobacter baumannii

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Thesis

IDENTIFICATION OF A SMALL MOLECULE INHIBITOR OF VIRULENCE FACTORS IN MULTIDRUG RESISTANT *ACINETOBACTER BAUMANNII*

by

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ABSTRACT

Acinetobacter baumannii is an opportunistic pathogen prevalent in nosocomial infections, most commonly infecting humans with compromised immune systems during their hospital stays. The organism's success in such circumstances has to do with its ability to survive on dry, abiotic surfaces (e.g. catheters, bed railings, and other medical equipment) and its increasingly apparent antibiotic resistance. These factors make *A. baumannii* a serious problem for healthcare professionals and in public health generally. *A. baumannii* is paradigmatic and representative of the issues confronting healthcare in the ongoing antibiotic crisis, and many strains are showing multidrug resistant (MDR) phenotypes. Given that the patients infected by *A. baumannii* tend to be very vulnerable and traditional antibiotic treatment seems to be getting less and less effective, it is imperative to explore alternative treatment options that may lead to better outcomes, especially if their mechanisms are not the same as the traditional antibiotics that exert the selective pressures that have led to the current antibiotic crisis. A small molecule called M64 is known to inhibit a LysR-type transcription regulator (LTTR) important for virulence, but not cell growth or viability, in another opportunistic pathogen, *Pseudomonas aeruginosa*. The experiments presented here show that M64 was able to rescue mice infected

with *A. baumannii* and to downregulate the expression of important metabolic genes downstream from *A. baumannii* LTTRs BenM and CatM *in vitro* while having no effect on bacterial growth. BenM and CatM regulate genes involved in the metabolism of benzoate and catechol respectively, both of which are parts of tryptophan metabolism and are eventually broken down to form acetyl-CoA and succinyl-CoA for energy production in the citric acid cycle. Such a pharmacodynamic profile offers a starting point in the design of alternative treatments of MDR bacterial infections, as successful outcomes are observed without the direct killing of cells *in vitro* seen in traditional antibiotics. In this case, as catechol metabolism is important for siderophore biosynthesis and thus bacterial virulence, inhibition of the transcription of genes involved in catechol metabolism may be playing a role in the observed rescue of infected mice. Further studies are required to ascertain the nature of the inhibitor's effect, however.

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LIST OF ABBREVIATIONS

CFU ..... Colony-forming unit

DMSO..... Dimethyl sulfoxide

IP ..... Intraperitoneal

IV .....Intravenous

LB .....Lysogeny broth

LTTR .....LysR-type transcription regulator

MDR ..... Multidrug resistant

MHB .....Müller-Hinton broth

MIC..... Minimal inhibitory concentration

OD .....Optical density

Shr Cl *X* ..... Shriner's clinical strain *X*

SM .....Formedium™ SM Broth

TSB .....Tryptic soy broth

INTRODUCTION

Acinetobacter baumannii

A. baumannii was described as a distinct species within the genus *Acinetobacter* in 1986, when it was also noted that "[m]ost *Acinetobacter* strains isolated from human patients belong to this species" (Bouvet and Grimont, 1986). *A. baumannii* is increasingly being recognized as an important opportunistic pathogen in humans, especially in nosocomial settings (Scott et al., 2004; Pimentel et al., 2005; Geurrero et al., 2010). Immunocompromised patients have the highest risk of developing disease due to this opportunistic pathogen, and lethal cases have been described (Charnot-Katsikas et al., 2009; Ali et al., 2014; Sinha et al., 2014). Between 2002 and 2004, there was a notable rise in the number of cases of *A. baumannii* bloodstream infection seen in patients at US military medical facilities treating service members injured in Iraq, Kuwait, and Afghanistan (Scott et al., 2004), and occupational transmission of the bacterium from wounded military personnel to healthcare workers has been documented (Whitman et al. 2008).

The two most important features of *A. baumannii* that represent problems to healthcare are its increasing antibiotic resistance (Fournier et al., 2006; Maragakis and Perl, 2008; Gordon and Wareham, 2010; Castanheira et al., 2014) and its ability to survive for long periods of time on dry, abiotic surfaces (Jawad et al., 1998), evidenced by surveillance culture results in hospitals where

outbreaks have occurred (van den Broek et al., 2006) and probably related to the bacterium's marked ability to form biofilms (Longo et al., 2014). In turn, biofilm formation increases the persistence and antimicrobial resistance of the bacteria. Thus, *A. baumannii* colonization of surfaces in the hospital environment can pose very serious public health issues (Figure 1). It has also been included amongst the ESKAPE pathogens (Rice, 2008; Table 1), which are noted to be opportunistic pathogens that are highly resistant to currently available antibiotic treatments, presenting "new paradigms in pathogenesis, transmission, and resistance" (Dijkshoorn et al., 2007). As such, these bacteria pose particularly difficult challenges to investigators trying to develop the novel therapeutic options that are so desperately needed.

Curiously, although *A. baumannii* may be the most common species of *Acinetobacter* isolated from humans, "the existing data indicate that *A. baumannii* has a low prevalence in the community and that its occurrence in the environment is rare" (Dijkshoorn et al., 2007). In 1968, unspeciated acinetobacters were isolated from soil and water samples (Baumann, 1968), while unspeciated clinical isolates were known even earlier, but *Acinetobacter baumannii* specifically has not been shown to be a commonly found soil bacterium.

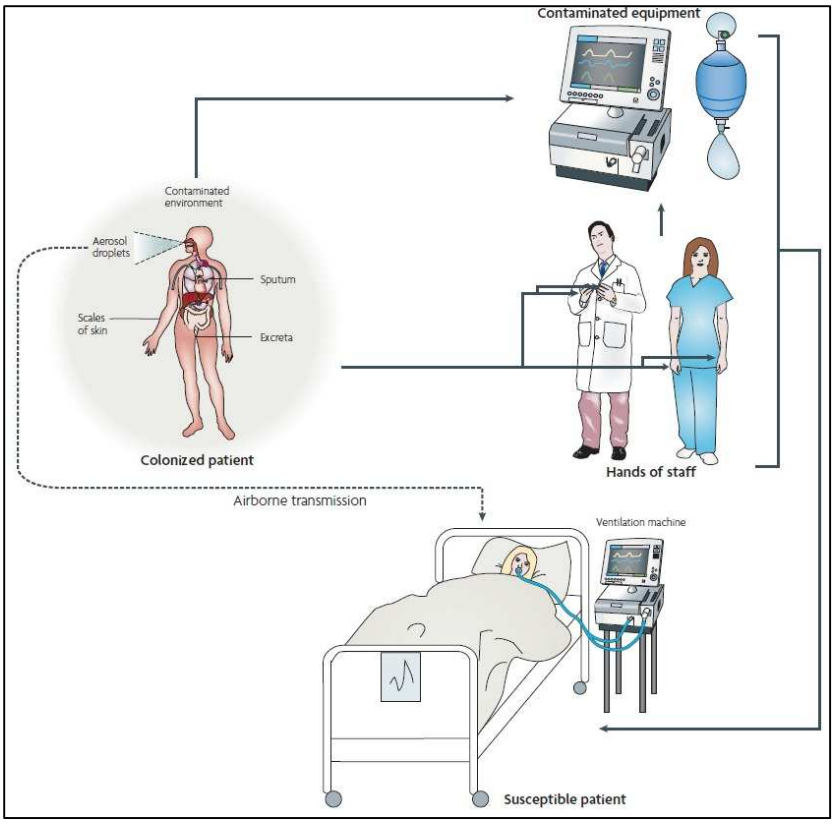

Figure 1: Dynamics of bacterial transmission in a hospital environment, adapted from Dijkshoorn et al. 2007.

Table 1: The ESKAPE Pathogens, as described by Rice (2008)

Letter	Bacterium
E	***E****nterococcus faecium*
S	***S****taphylococcus aureus*
K	***K****lebsiella pneumonia*
A	***A****cinetobacter baumannii*
P	***P****seudomonas aeruginosa*
E	***E****nterobacter* spp.

Some of the ESKAPE pathogens have been more thoroughly studied than others. For example, well-established model systems exist for studying *P. aeruginosa* in *Drosophila* (Apidianakis and Rahme, 2009) and in a variety of infection models in mice (Hoffmann et al., 2005; Kukavica-Ibrulj and Levesque, 2008). On the other hand, in the case of *A. baumannii*, immunocompromised murine models are often employed (Harris et al., 2013; Thompson et al., 2014) and *Galleria mellonella* has been utilized as a non-vertebrate model organism (Peleg et al., 2009) while *Drosophila* models have not been described.

Conveniently, for the studies described herein, several strains of *A. baumannii* were available in the lab, some being relatively well-established lab strains and others being newly-acquired clinical isolates from patients hospitalized at Shriner's Hospital. This availability of several strains meant that studies could be broad enough to show potentially far-reaching effects that could be applied to yet other *Acinetobacter* strains or even species.

The aforementioned *Drosophila* and murine infection models were also conveniently well-established and would offer a diversity of ways to study the available *Acinetobacter* strains, including the previously unstudied local clinical isolates shown below (Table 2). Reliable observation of virulent phenotypes (animal death) in infected flies or mice would allow for the screening of other strains for virulence, the testing of possible treatments to rescue infected animals, and analysis of bacterial proliferation in an *in vivo* setting.

Table 2: Strains of *A. baumannii* available for study

A. baumannii strain	Source	Notes
Shriner's Clinical (Shr Cl) strain 69	Shriner's Hospitals for Children, Boston	Local clinical isolates
Shr Cl 70		
Shr Cl 71		
Shr Cl 72		
AYE VB-1	Fournier et al., 2006	Multidrug resistant
SDF		Sensitive to antibiotics
AB 5075	Jacobs et al., 2014	Multidrug resistant; mutant library available
ATCC 17978	ATCC	Multidrug resistant; transposon library available

Inhibitor M64 and its Action

With an eye towards investigating potential treatments of *A. baumannii* infections, it was decided to explore the effects of a molecule recently found to disrupt bacterial quorum-sensing regulated pathogenicity in *Pseudomonas*

aeruginosa (Starkey et al., 2014) in experimental *A. baumannii* infections and *in vitro*. This molecule, termed M64 (Figure 2), presents great promise in treatment of *P. aeruginosa* infections because while it attenuates acute bacterial virulence and so increases the likelihood of host survival, it is not a classic antibiotic (targeting bacterial growth or cell viability, eg) and so does not kill cells directly. This means that *P. aeruginosa* is less likely to develop a resistance to this compound in the same way that bacteria develop resistance to classic antibiotics. Ultimately, such a strategy offers many advantages over classical antibiotics, as it could treat potentially lethal infections while sidestepping the current antibiotic resistance crises. If M64 shows similar or otherwise disruptive effects in experimental *A. baumannii* infections and cultures, it could prove an important first step towards the development of both next-generation antimicrobials and new treatment plans for human infection with this bacterium.

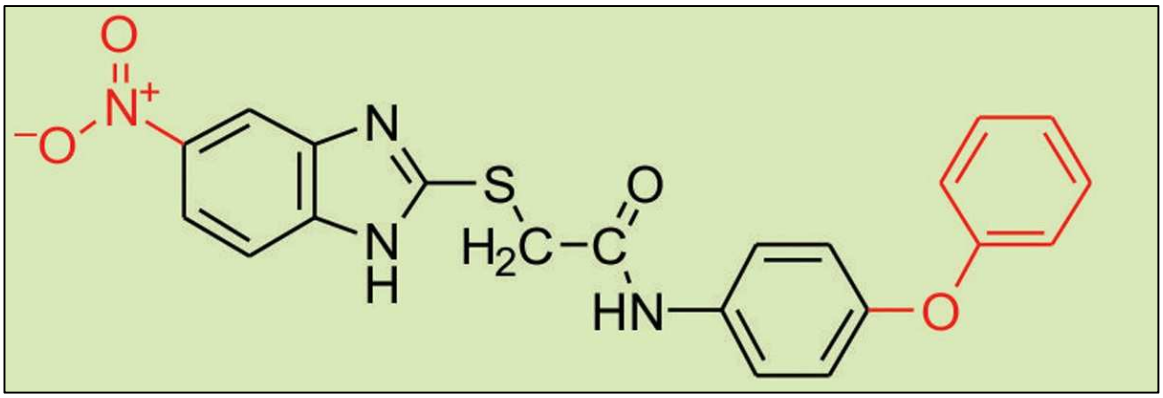

Figure 2: M64, small molecule inhibitor of quorum-sensing regulated pathogenicity in Pseudomonas aeruginosa. Adapted from Starkey et al. 2014.

In *P. aeruginosa*, M64 has been shown to inhibit a transcription factor known as MvfR (multiple virulence factor regulator), which ultimately regulates several functions related to the quorum-sensing regulated virulence of the pathogen but is not essential to cell viability (Starkey et al., 2014). Thus, targeting MvfR with a drug does not exert the same evolutionary pressure as classical antibiotics with their classic targets do, as cells are not being killed. Instead, an inhibitor of MvfR can inhibit pathogenicity by disrupting quorum-sensing regulated functions without outright killing of the pathogen: an infected patient treated with such a drug would see their symptoms alleviated while their own immune system eventually eradicates the pathogen itself.

It is important to note that MvfR is a LysR-type transcriptional regulator (LTTR), a class of regulators which “control the expression of a diverse array of virulence regulons in Gram-negative and Gram-positive pathogens, and are the largest family of homologous regulators in prokaryotes” (Starkey et al., 2014). This indicates that a promising potential starting point in investigating the effects of M64 on *A. baumannii* would be any known LTTRs present in that bacterium.

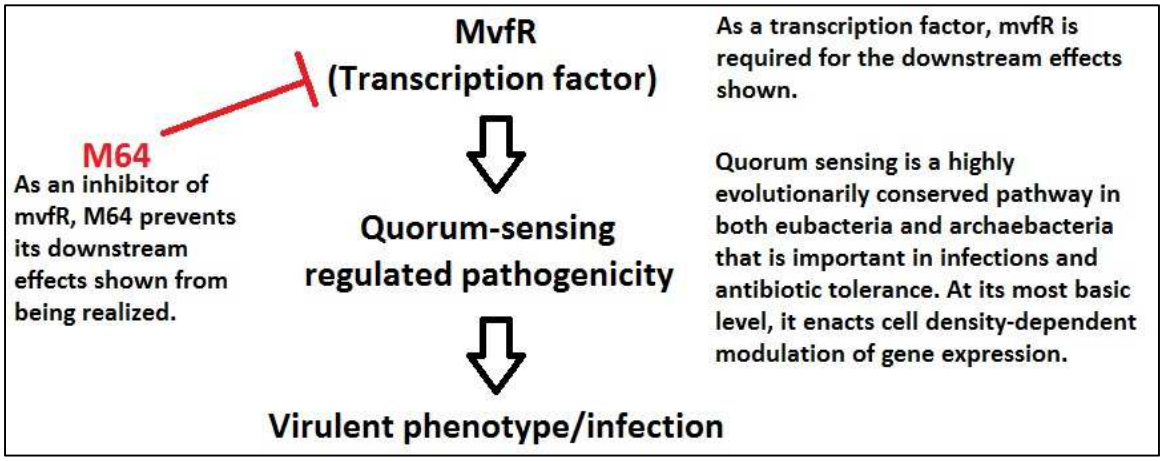

Figure 3: A simplified overview of quorum-sensing regulated pathogenicity in *P. aeruginosa* and the point of action of M64.

To be specific, two of the most studied LTTRs in *A. baumannii* are BenM and CatM, which are involved in the regulation of benzoate and catechol metabolism (Collier et al., 1998; Ezezika et al., 2006; Ezezika et al., 2007; Zhang et al., 2012). If M64 can be shown to have some disruptive effect on bacterial metabolism and if this metabolic defect is connected to less prominent virulence phenotypes, M64 could be a promising place to start in the investigation of non-traditional antibiotic treatments of *A. baumannii* infection.

Research Objectives and Project Outline

The overarching objective of the research project was to investigate the effects of M64 on *A. baumannii*. As the non-bactericidal M64 was known to inhibit virulence factors in *P. aeruginosa* by acting on MvfR, special attention early in the project was devoted to *in vivo* studies where animal models were used to test

if M64 could rescue animals from what would otherwise be lethal infections. A decrease in virulent infection phenotypes in experimental groups receiving M64 as treatment would provide a reason for further investigation of the effects of M64 on *A. baumannii*.

Growing *A. baumannii* in the presence and absence of M64 in different media yielded clues not only about the inhibitor's effects on and potential targets in *A. baumannii* but also on relevant characteristics intrinsic to the bacterial strains in question that might explain the later observed *in vivo* phenotypes. When the *in vitro* studies seemed to show M64 interfering in certain aspects of *A. baumannii* metabolism, *in vivo* studies were attempted to inquire into the live anti-virulence functions of the inhibitor.

METHODS

Bacterial Cultures

A sterile loop was used to scrape a 25% glycerol frozen (-80°C) bacterial stock. The loop was then dipped into 5 mL lysogeny broth (LB) in a culture tube, which was then set into an incubator at 37°C and 200 rpm shaking to allow the culture to grow overnight (16 hours), after which a 1:100 dilution (in LB) of the culture was grown over the course of the day ("overday" culture), in the same conditions.

Another medium used in some of the experiments was M63gcm, which was assembled as shown below (Table 3 and Table 4). Components were mixed via shaking or stirring in a sterilized bottle and the solution was then sterilized again via filtration into a new sterilized bottle.

Chemical	Quantity
$(NH_4)_2SO_4$	10 g
KH_2PO_4	68 g
$FeSO_4 \cdot 7H_2O$	2.5 mg
Deionized H_2O	1 L

Table 3: 1 L 5x M63 stock solution components

Chemical	Quantity
5x M63 stock	100 mL
Deionized H_2O	382.5 mL
20% glucose	5 mL
20% w/v casamino acids	12.5 mL
1M $MgSO_4 \cdot 7H_2O$	0.5 mL

Table 4: 500 mL 1x M63gcm working solution components

Assessments of Virulence/Animal Infections

Much insight into how virulent and in what ways *A. baumannii* can be virulent in humans can come from simply infecting animal model organisms and analyzing the results. Two animal models were attempted to study *A. baumannii in vivo*, one invertebrate and one vertebrate: *Drosophila melanogaster* and *Mus musculus*.

Drosophila melanogaster

Drosophila infection was carried out as described previously for infection with *P. aeruginosa* (Apidianakis and Rahme, 2009). Five- to seven-day-old male wild type (Oregon-R) flies were selected for each experiment. Overday bacterial cultures were allowed to grow until reaching an optical density (600 nm; OD_{600nm}) of 3.0. Aliquots were taken from this overday culture and centrifuged for three minutes at 8000 rcf. The supernatant LB was removed and the pelleted bacteria were resuspended in an equal volume of 10 mM $MgSO_4$ to wash them. After repeating the centrifugation and removing this wash, the pelleted bacteria were again resuspended in an equal volume of 10 mM $MgSO_4$ and this inoculum was considered ready for further dilution or experimental use.

Actual fly infection was carried out by dipping a disinfected needle into the inoculum and pricking flies, anesthetized with carbon dioxide, in the thorax. Infected flies were removed from anesthesia and put back into an incubator at 21°C, where their survival could be monitored over time. Flies in the control groups were pricked with a needle dipped into plain 10 mM $MgSO_4$.

An alternative to the needle pricking experiment was the glass micropipette microinjection infection model. Both involved thoracic pricking of the flies, thereby modelling the infection in a physically traumatic case, but the microinjector allowed for more controlled doses of bacteria to be delivered at the site of the wound by determining the bacterial inoculum CFU/mL, since the microinjector delivers a user-defined volume of inoculum with each injection. For microinjector experiments, overday bacterial cultures were grown until reaching OD_{600nm} 3.0. Aliquots of these cultures were centrifuged, supernatant LB was removed, and the cells were resuspended in 10 mM $MgSO_4$. A new pulled glass micropipette was attached to the instrument for each experiment, and the pipette was filled with the bacterial inoculum. (Or plain 10 mM $MgSO_4$ for control group injections.) 9.2 nL of solution were injected into each fly.

The third infection model tested was a non-traumatic gut infection model. In this assay, the control groups of flies were sorted into tubes where they were provided with 5 mL filter-sterilized 4% sucrose solution as food, pipetted onto sterile cotton balls at the bottoms of the tubes. Infection groups received 5-*x* mL of 4% sucrose solution and *x* mL of a bacterial overday culture that had been centrifuged, had the supernatant *x* mL LB removed, and the pelleted bacteria resuspended in *x* mL 4% sucrose. The sucrose solutions with and without bacteria were mixed together into a 5 mL solution and pipetted onto cotton balls at the bottoms of the tubes. The amount of bacterial inoculum *x* used could be tailored to provide the flies with any number of CFUs with their sucrose food,

though this experiment is inherently more variable, as it is somewhat difficult to control for the amount each fly may eat. In any case, the bacterial inoculum always came from an OD_{600nm} 3.0 overday culture.

Mus musculus

As mentioned before, mouse models had been widely employed in previous studies of *A. baumannii* (Luo et al., 2012; Lin et al., 2012; Jacobs et al., 2014; Thompson et al., 2014). Part of the appeal of the mouse model is in how it lends itself to the study of various types of infections, including open wounds, burns, and septicemia (via intraperitoneal [IP] injection). Mice have also been used to study pneumonia secondary to lung infection (Joly-Guillou et al., 1997; Harris et al., 2013). Methods to immunocompromise mice, thus raising their susceptibility to infection and better representing the sickly patients most at risk of becoming infected with *A. baumannii* in hospitals, have also been described (Zuluaga et al., 2006; Manepalli et al., 2013).

It was decided to test the lung infection model using seven-week old male CD-1 mice (Charles River Laboratories, MA, USA). The mice were immunocompromised with two IP injections of cyclophosphamide in 0.9% NaCl saline, one dose (150 mg/kg) four days before infection and another dose (100 mg/kg) the day before infection. Lung infection was accomplished via intranasal inoculation of 25 µL of 10 mM $MgSO_4$ containing the desired amount of CFUs for testing. The bacteria came from aliquots from overday liquid cultures grown to OD_{600nm} 2.0. The aliquots were centrifuged at 8000 rcf for three minutes, the

supernatant LB was removed, and the cells were resuspended and washed once with 10 mM $MgSO_4$, followed by a second centrifugation at 8000 rcf for three minutes and resuspending in 10 mM $MgSO_4$, with cells being diluted or concentrated as desired for testing. For the purposes of assessing CFUs from lungs or measuring lung weights, mice were sacrificed two days after infection at the earliest. Lung CFUs were measured by homogenizing the retrieved lung tissue in 1 mL PBS and plating serial dilutions of the resulting mixture on LB agar plates.

Minimal Inhibitory Concentration (MIC) experiments

Rich media

One night before the experiment, a sterile loop was used to scrape a 25% glycerol frozen bacterial stock of the strain(s) of interest and streak a LB agar plate. After 16 hours incubation at 37°C, isolated colonies were picked from the plates using a sterile loop. The colonies were immediately suspended in the medium being tested in the experiment via pipetting repeatedly and vortexing, with each strain's newly-formed liquid culture then being appropriately diluted so that each culture featured a similar number of colony-forming units (CFUs) per mL.

The inhibitor of interest (M64) was serially diluted from a concentrated stock solution to achieve the final concentrations of inhibitor to be tested, taking into account that the inhibitor was to be further diluted when all the experimental

components were finally plated (below). With these dilutions, the experiment was controlled for the volume of inhibitor solvent (DMSO) placed in each well of the plate.

Finally, 100 µL of empty medium was placed in each well of a 96-well clear, sterile, round-bottom assay plate. In each column of wells, 6 µL of appropriately-diluted inhibitor was added. Across each row of wells, 100 µL of bacterial culture (suspended colonies) was added so that, across one row of the plate, different concentrations of inhibitor were present in each well with the same amount of bacteria. As a negative control, each row also featured one well with no inhibitor added but rather with 6 µL 0.1 mg/mL polymyxin B, an antibiotic, dissolved in water. The positive control in each row was one well with 6 µL of plain DMSO added without inhibitor or antibiotic. The OD_{600nm} of each well was measured every 15 minutes by a Tecan Infinite 200 microplate reader (Männedorf, Switzerland) over the span of at least 24 hours.

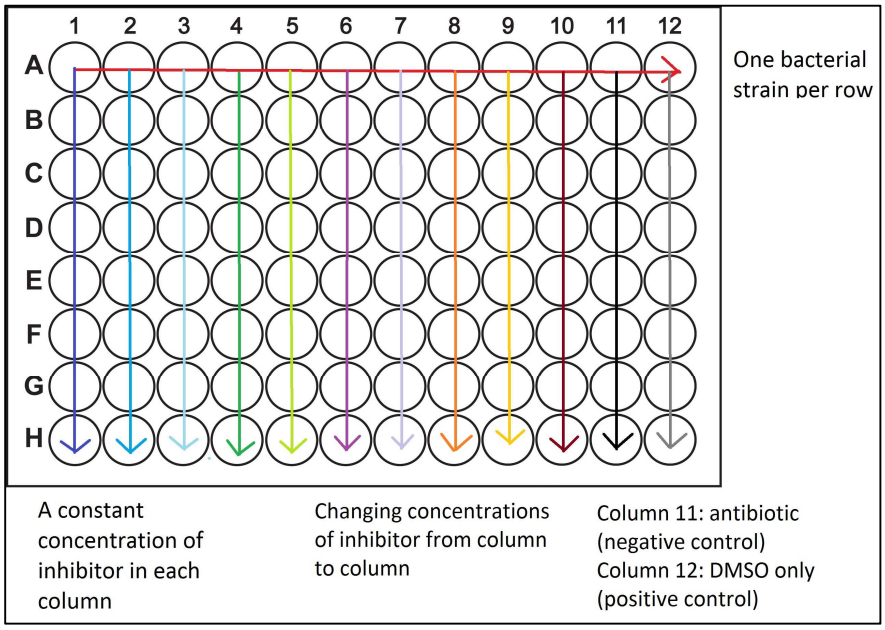

Figure 4: Setup of the 96-well plate for the MIC experiments

Minimal media

Original experiments were entirely similar to the MIC experiments in rich media, only substituting the minimal medium M63gcm for bacterial culture dilutions and on the 96-well plate. Later experiments did not use isolated colonies from a streak plate but rather overday liquid cultures that had been allowed to grow to an OD_{600nm} of 2.0 and which were then serially diluted to 1:1000. These 1:1000 dilutions were used in the 96-well plate, meaning that the final amount of bacteria in each well amounted to approximately a 1:2000 dilution of an overday OD_{600nm} 2.0 culture for each strain, accounting for the 100 µL of empty M63gcm media that was added to each well along with 100 µL of the 1:1000 diluted

overday culture. The different tested concentrations of inhibitor M64 remained the same as in the rich media MIC experiments.

qPCR

Real-time polymerase chain reaction (qPCR) was used to explore the effects of M64 on gene expression in *A. baumannii*. Overday liquid cultures were grown to the desired OD_{600nm}, still in LB but this time also in the presence of either plain DMSO or 50 μM M64. Aliquots from these cultures were then used for RNA extraction and the subsequent steps.

RNA extraction from the cells was achieved using the Qiagen RNeasy® Mini Kit and following the included instructions. Synthesis of cDNA from bacterial RNA was achieved using Invitrogen Superscript® III First-Strand Synthesis System (Thermo Fischer Scientific, MA, USA) and following the included instructions. The qPCR reactions were carried out using Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies, CA, USA) and following the included instructions, while the sequences of the primers used to amplify each gene are shown in Table 5 below. Primers for the 16S RNA were the same as used previously by Peleg et al., 2007. Primers for *benA* were the same as used previously by Zhan et al., 2009. The remaining primers were designed using the primer design tools available from the National Center for Biotechnology Information (NCBI).

Gene	Forward primer	Reverse primer
benM	GACCAAGCGTATTGGTCTCA	TCGAGGCAGCAACCCATAAA
catM	TTCGAAAAAGGCTCTCGTCC	GCGTCAAAATTTGCACCGCA
benA	GGTGGCTCCTATGGCTTTGA	TTTCGGTGTATTCATCTGCT
benB	AAGCCACAATATTGCCAACG	CCAGTTAAAGCGAACAGTGAC
catB	CGTTCGGCACTGAATTAT	GTAACGTAGCGGCTCTTTTA
catC	TTCCACTTGATATGCCAGCC	TGTGACGCCATTTACCTTGG
16S RNA	CAGCTCGTGTCGTGAGATGT	CGTAAGGGCCATGATGACTT

Table 5: Primers used to amplify the *ben* and *cat* genes via qPCR.

Statistical Analyses

Results from the mouse model pneumonia and infection studies were analyzed for statistical significance ($P \leq 0.05$) using two-tailed *t*-tests (equal sample sizes, equal variance). Results from the qPCR gene expression studies were analyzed by computing the fold change in gene expression compared to 16s ribosomal RNA and using a two-tailed *t*-test (equal sample sizes, equal variance) to check for a statistically significant ($P \leq 0.05$) difference. Results of the killing kinetic study where one group was treated with M64 and one was not (Figure 8) were analyzed using the log-rank test.

RESULTS

In vivo Experiments

Drosophila melanogaster *Infections*

Early experiments involved attempts to establish a host-pathogen model system using *D. melanogaster* and *A. baumannii*. Such a system had not been previously described in the literature, but was convenient to attempt in the lab since the bacteria and the flies were both readily available. Many experiments with several different strains and modes of infection were attempted, but none showed any significant effects from bacterial infection in healthy, wild type (Oregon-R) flies. Infected flies, regardless of the number of CFUs used to infect, did not die any more quickly than their non-infected counterparts. A representative sample of this data is shown in Figure 5.

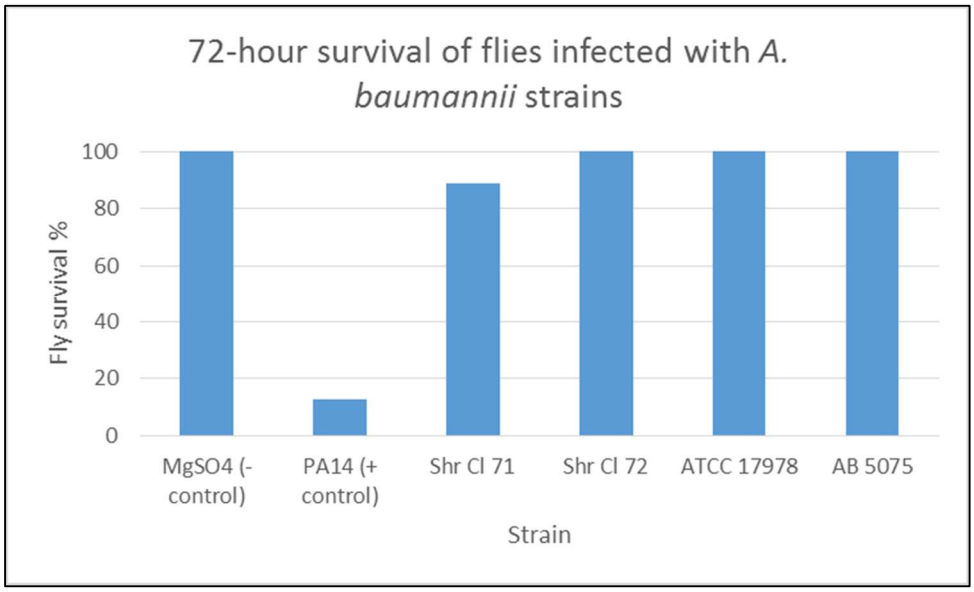

Figure 5: Survival of flies infected with A. baumannii strains via thoracic pricking. The positive control group were infected with PA14, a strain of P. aeruginosa known to be highly virulent in flies.

Mouse Infections

The immunocompromised mouse lung infection model provided reliable phenotypes indicative of bacterial-induced pneumonia, even in immunocompetent mice. Shriner's Clinical Strain 69 was used to infect immunocompetent mice to see if the strain was virulent enough to kill mice. Although no immunocompetent mice succumbed to the infections, some indices provided evidence that the mice were coming down with pneumonia (Figures 6, 7) despite a lack of cultivable CFUs in lung tissue from sacrificed mice.

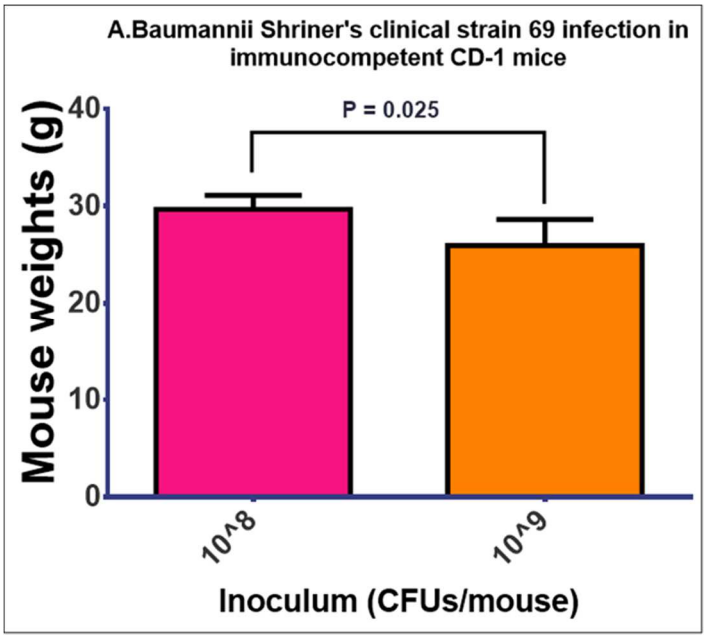

Figure 6: Weights of immunocompetent mice infected intranasally with Shr CI 69, four days post-infection. Mice infected with a higher number of CFUs exhibited more weight loss, indicating some degree of sickness despite that no mortality was observed.

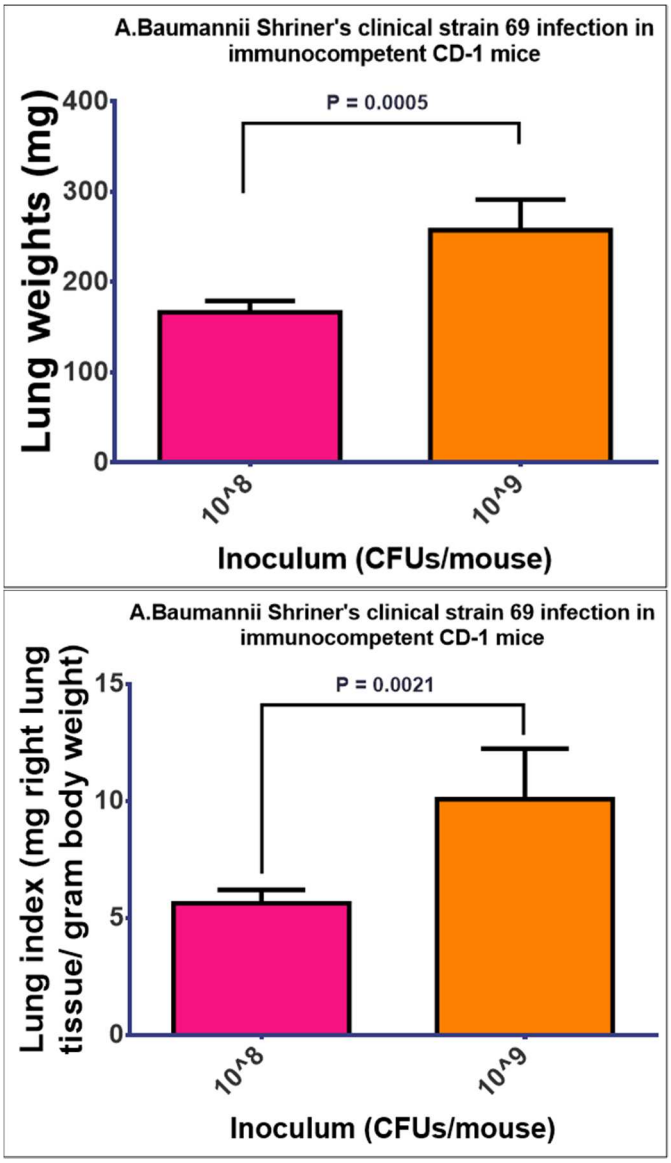

Figure 7: Indicators of pneumonia four days post-infection in immunocompetent mice infected intranasally with Shr CI 69. Mice infected with a higher number of CFUs exhibited significantly greater raw lung weight and greater lung index (mg right lung tissue/g body weight), consistent with a more advanced case of pneumonia.

A. baumannii was seen to be much more virulent in other experiments where the mice were immunocompromised using cyclophosphamide prior to inoculation. In these experiments, significant mouse mortality was observed (Figures 8, 9), and more concentrated inocula produced higher mortality (Figure 8).

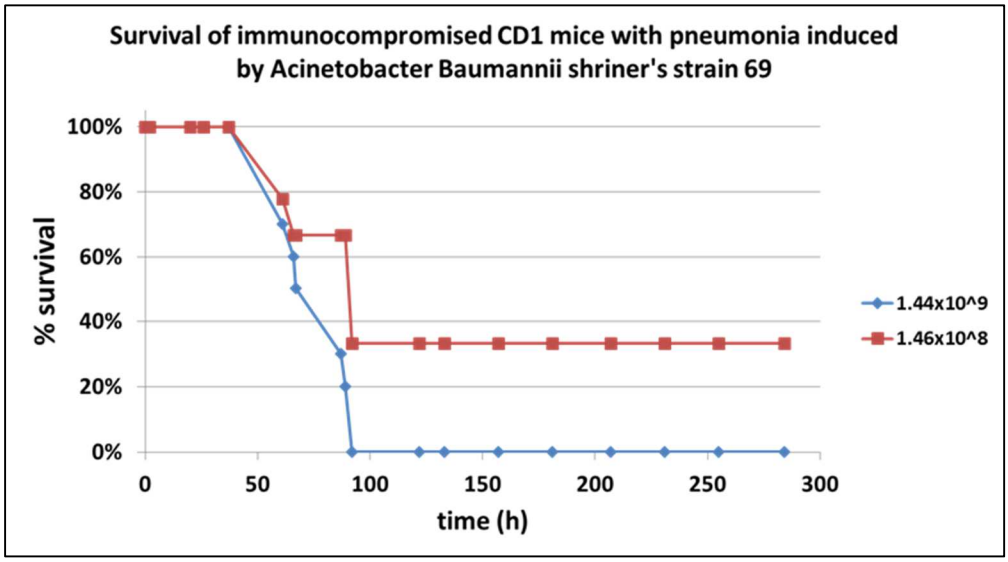

Figure 8: Killing kinetic of immunocompromised mice infected intranasally with Shr CI 69. (Infection occurred at t = 0)

Shr Cl 69 was not the only strain that produced such a virulent phenotype in infected mice. AB 5075, a more established lab strain, was also tested and showed a high degree of virulence. Most importantly, significantly less mortality and morbidity were observed in mice treated with M64 compared to mice that received plain DMSO (Figures 9, 10).

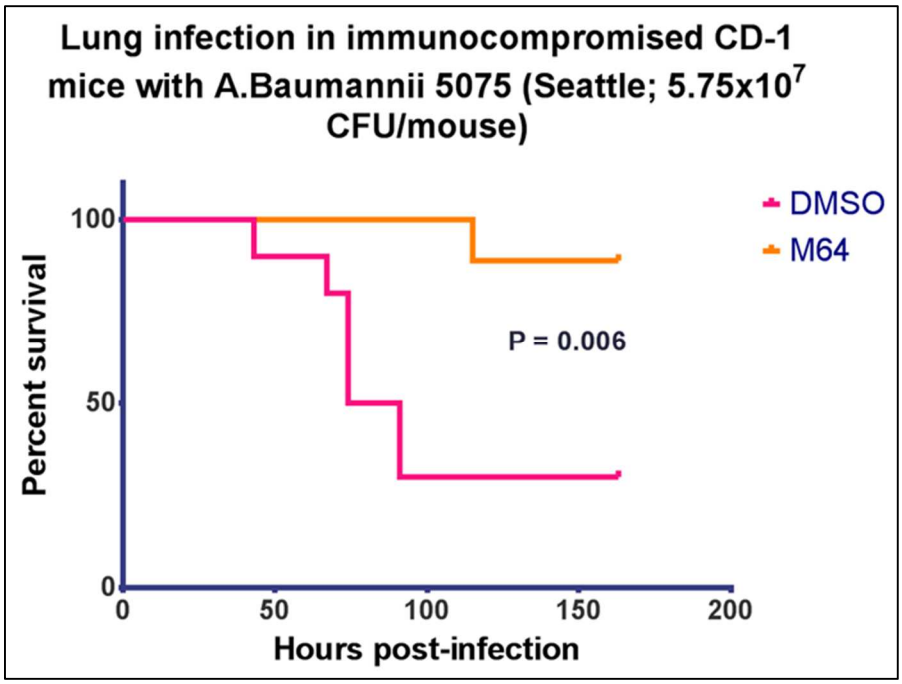

Figure 9: Kaplan-Meier killing kinetic for immunocompromised mice infected intranasally with $5.75x10^7$ CFUs of AB 5075. Only 30% of untreated mice (recieving IV DMSO injections) survived whereas 90% of mice given IV M64 in DMSO survived. Analyzed using the log-rank test.

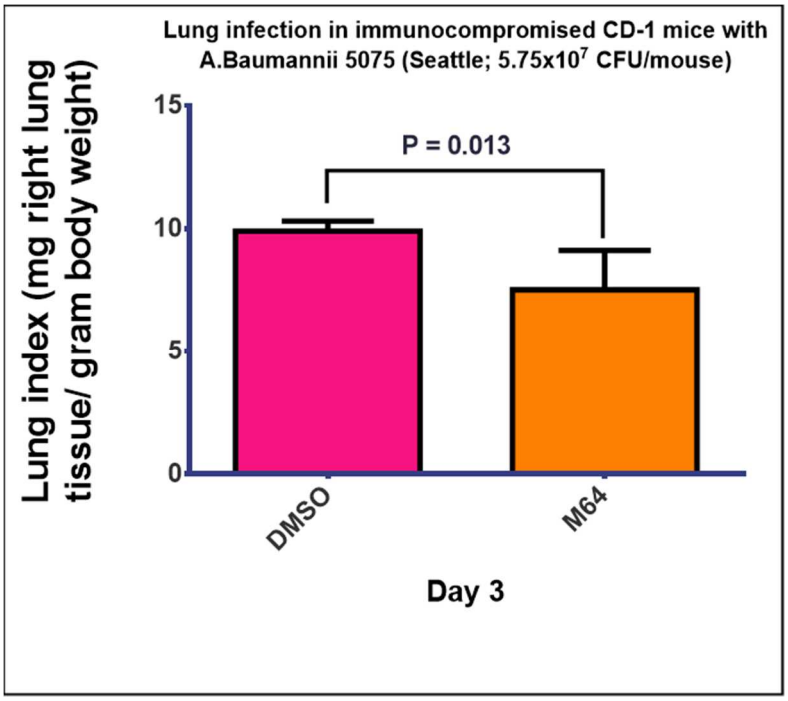

Figure 10: Lung index in immunocompromised mice three days after intranasal infection with AB 5075 and treatment with DMSO or M64 in DMSO.

In a different lung infection experiment where a different *A. baumannii* strain was being tested, lung tissue was collected from sacrificed mice and homogenized into a solution for serial dilution, plating on LB agar, and counting lung CFUs as an indicator of the level of infection. This data is shown in Figure 11.

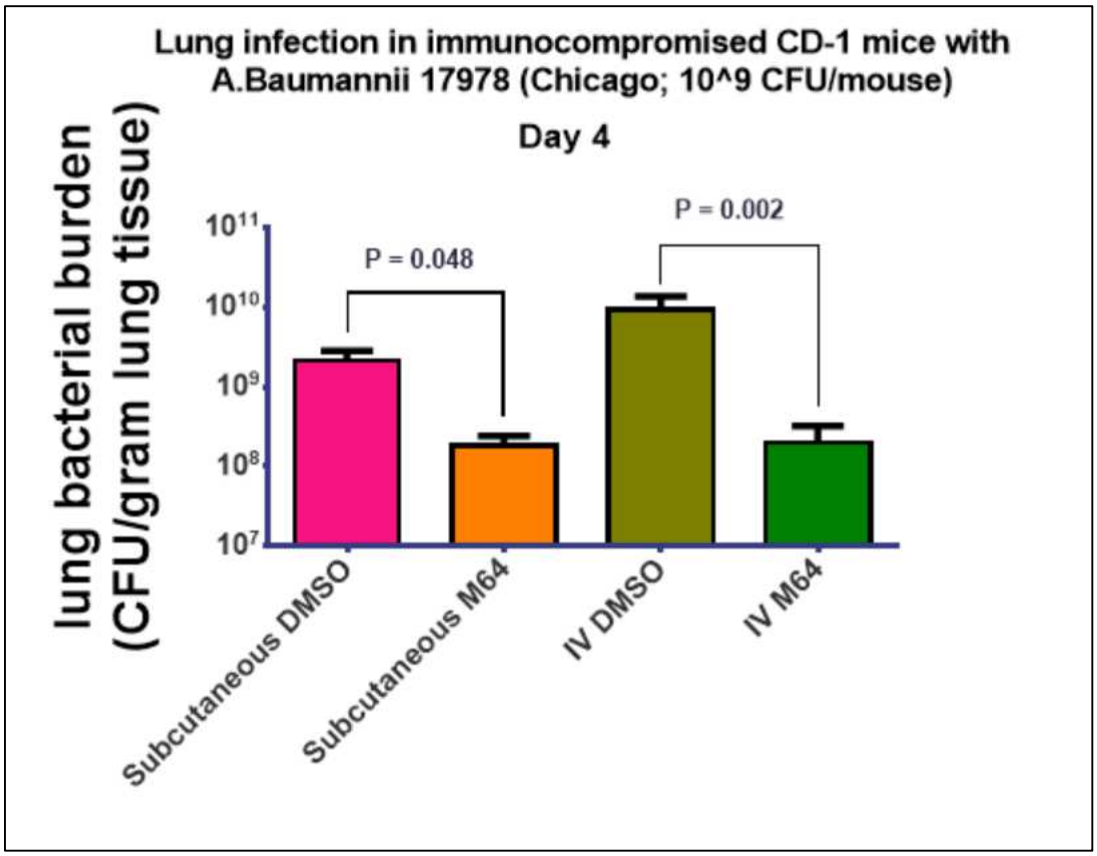

Figure 11: Lung bacterial burden four days after infection with ATCC 17978 in immunocompromised mice with lung infections treated with subcutaneous or intravenous 50 μM M64 in DMSO compared to controls treated with plain DMSO. Each mouse was infected with $1x10^9$ CFUs at the start.

In vitro Experiments

Effects of M64 on A. baumannii *Growth*

Equally important as figuring out the effects of M64 treatment of *A. baumannii* infection *in vivo* was the investigation of the effects of M64 on the bacterium itself. Figure 12 shows the effects of M64 on the growth of AB 5075 in various rich media, and is representative of what was seen in other strains in the same media with similar concentrations of M64.

Although the strains used in this study exhibited no growth defects in rich medium (LB), numerous attempts to grow the strains in M63gcm minimal medium exhibited that with the exception of AYE and SDF strains, all other *A. baumannii* strains tested simply will not grow in this medium (Figure 13).

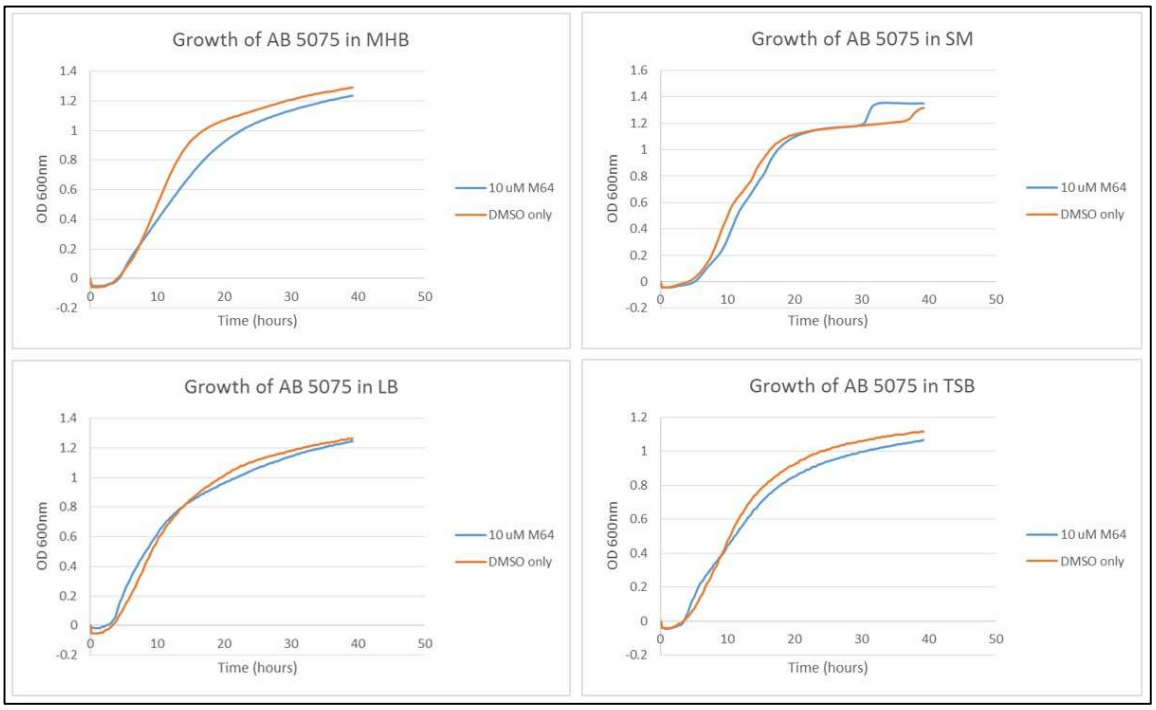

Figure 12: Growth of AB 5075 in various rich media in the presence of DMSO and 10 µM M64 in DMSO. Negative control (antibiotic) wells exhibited flat lines, with no increase from near-zero OD_{600nm} (not shown), indicating that the increases in OD_{600nm} in the other wells were due to bacterial growth.

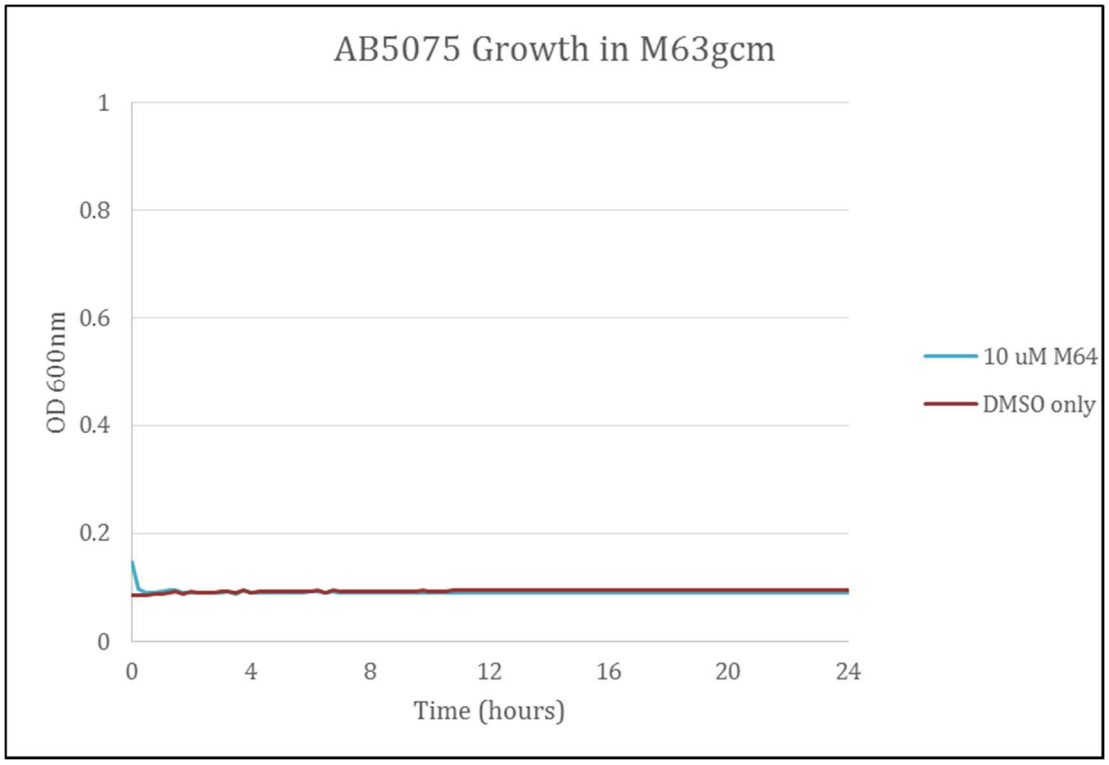

Figure 13: Growth kinetic of AB5075 in minimal medium M63gcm. The failure of this strain to grow in this medium is characteristic of several of the *A. baumannii* strains tested, including ATCC 17978.

Additionally, CFUs were assessed as part of investigating how *A. baumannii* grows in different media in the presence or absence of M64. This data is shown in Figure 14, where M64 again shows no effects on *A. baumannii* growth in rich medium but accelerates cell death in the minimal medium.

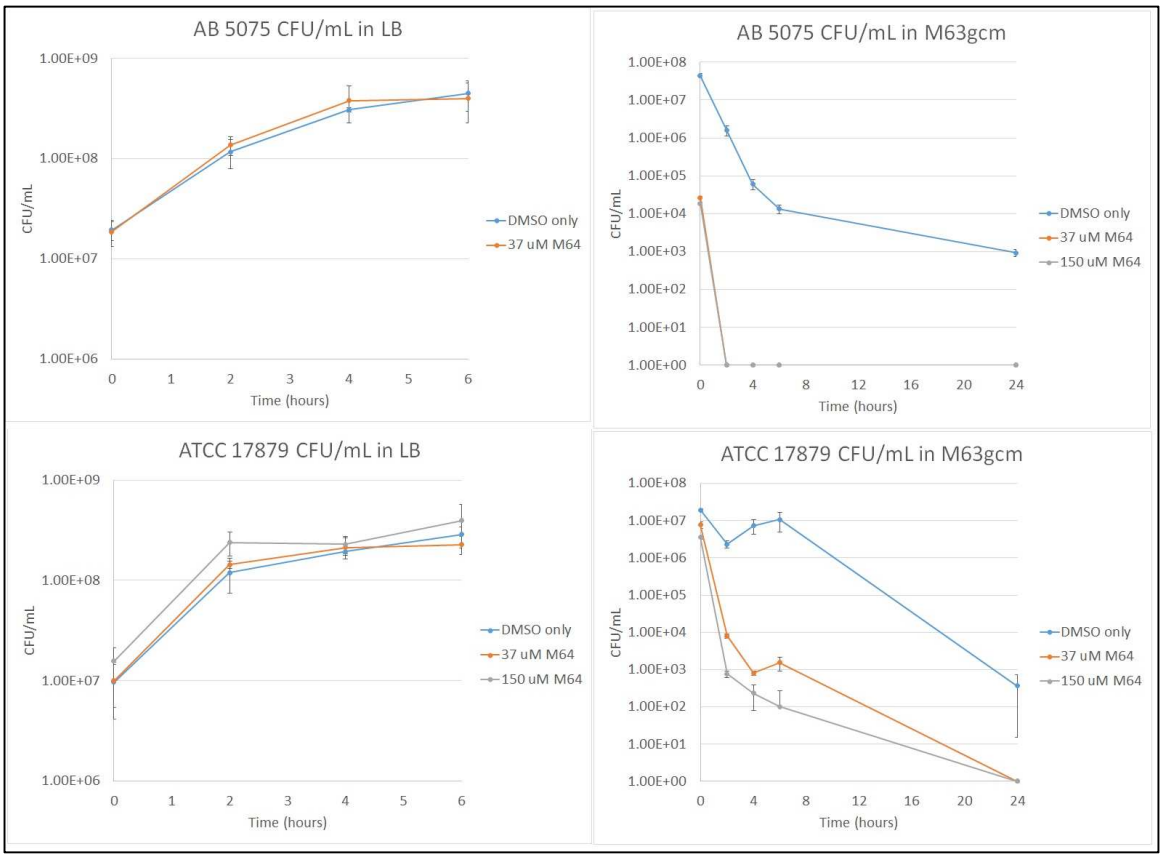

Figure 14: *A. baumannii* growth measured in CFU/mL in LB (top row) and M63gcm.

Knowing that most of the *A. baumannii* strains could not grow in M63gcm and that the only essential amino acid lacking from that medium is tryptophan, attempts were made to supplement M63gcm with tryptophan and other downstream metabolites to see if growth in the presence of M64 could be observed. If growth could be observed in these conditions, it would be strong evidence for the target of M64, since the supplemented metabolite would need to be downstream of M64 inhibition. No rescue has been observed with any of the tested metabolites to date, but other candidates still remain to be tested. The data collected to date are summarized in Table 6.

M63 media	tryptophan	M64 inhibitor	anthranillic acid	benzoic acid	growth?
+	+	+			no
+	+	+	+		no
+	+	+		+	no

Table 6: Attempts to grow AB 5075 in supplemented M63gcm and circumvent the metabolic interference of M64.

Effects of M64 on A. baumannii *Gene Expression*

LysR-type transcriptional regulators (LTTRs) are a highly abundant and thoroughly-studied family of prokaryotic transcriptional regulators that share a conserved structure. It was previously discovered that M64 acts on *P. aeruginosa* by inhibiting MvfR, an LTTR important for *P. aeruginosa* virulence. Such being the case, the LTTRs known in *A. baumannii* provided a good starting point for further investigation.

Two of the best-studied LTTRs in *A. baumannii* are the proteins BenM and CatM. They are involved as regulators in the pathways of benzoate and catechol metabolism respectively, though there may also be cross-regulation that occurs (Ezezika et al., 2006). If M64 is inhibiting these LTTRs in *A. baumannii*, it would be expected to see downregulation of their regulons, namely *benABCDE* and *catABCIJFD*, respectively. To investigate this possibility, RNA was purified from *A. baumannii* grown in the presence of 50 µM M64 in DMSO and cells grown in plain DMSO. cDNA was synthesized from the purified RNA and then a qPCR assay was done using primers for *benM*, *catM*, *benAB*, and *catBC*. The results, shown in Figure 15, are quite telling.

First, it is seen that transcription levels of BenM and CatM in the cells grown in the

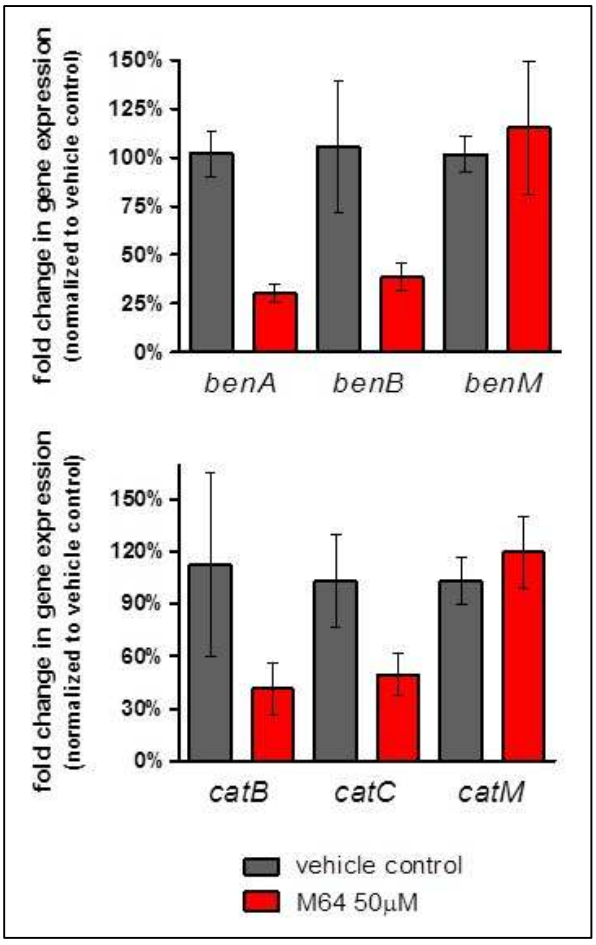

Figure 15: Transcription levels of BenM, BenA, BenB, CatM, CatB, and CatC in A. baumannii strain ATCC 17978 grown to OD_{600nm} 4.0 in LB the presence of plain DMSO or 50 µM M64 in DMSO.

benA P = 0.017

benB P = 0.088

benM P = 0.670

catB P = 0.198

catC P = 0.12

catM P = 0.512

presence of M64 are not significantly different from what they are in cells grown in the presence of plain DMSO (P = 0.670 and P = 0.512, respectively). Indeed, there is a statistically insignificant upwards trend in their transcription levels in the treated cells: if anything, this would be consistent with a feedback mechanism increasing transcription of these genes in cells where the corresponding protein product has been inhibited. The lack of significant decrease in transcription of these genes also indicates that M64 is not inhibiting the transcription of some other regulator even further upstream from BenM and CatM.

Although the transcription of these regulators is not affected, M64 could interfere with the activity of the BenM and/or CatM proteins. In such a case, one would expect a decrease in the levels of transcription of the genes they regulate. Indeed, in all of the tested genes, such a downward trend was observed. In the case of *benA*, this trend was statistically significant (P = 0.017) while in other genes statistical significance was not quite achieved, but the downward trends were still apparent and the levels of significance (P = 0.088 for *benB*, P = 0.198 for *catB*, and P = 0.12 for *catC*) were much closer to statistical significance than what was seen for *benM* (P = 0.670) and *catM* (P = 0.512). It seems likely that statistical significance could be achieved with a greater number of experimental replicates, which should decrease the variability seen in the transcription levels of certain genes. This overall result—the four downward trends in the transcription of downstream genes combined with no significant change in transcription of

their regulators—suggests that M64 is inhibiting the activity of LTTRs BenM and CatM.

DISCUSSION

The study began with an investigation into the effects of M64 on the growth of *A. baumannii* strains, while the choices of media used to grow the bacteria also seemed to reveal something about the bacteria's metabolism and the possible action of M64. First of all, M64 was not seen to have a significant effect on the growth of *A. baumannii* strains in rich media. The lack of killing and growth inhibition in these cases rules out traditional bactericidal or bacteriostatic action on the part of M64.

Second, most of the *A. baumannii* strains failed to grow in M63gcm minimal medium. Rather than an abject failure, this result actually provided a clue about the bacterial metabolism. An important difference between the rich media and minimal medium used for these experiments is that, where the rich media such as LB contain yeast extract and thus are considered to contain all essential amino acids, M63gcm is made with casamino acids. Casamino acids are a nearly complete source of amino acids, but lack in tryptophan.

Thus, the failure of the bacteria to grow in this medium hints at tryptophan auxotrophy in these *A. baumannii* strains. When assessing the CFUs, it was also seen that CFUs will diminish over time when most of the *A. baumannii* strains are grown in M63gcm, but they will diminish even faster if grown in M63gcm in the presence of M64 (Figure 14). Taken together, these data allowed for rapid identification of the putative target(s) of M64.

At this time, a clearer picture of the possible overall situation was starting to develop: it was known that the bacteria could not grow (and in fact would die off) in a medium lacking tryptophan, that this effect was even more profound in the presence of M64, and that M64 was previously known to interact with an LTTR in *P. aeruginosa*. These three points focused the next experiments on two relatively well-studied LTTRs in *A. baumannii* that were also known to be involved in tryptophan metabolism: BenM and CatM, transcription factors involved in the regulation of genes involved in benzoate and catechol metabolism respectively (Figure 16). Both of these are metabolites found downstream from tryptophan in the tryptophan degradation pathway (overview in Figure 19).

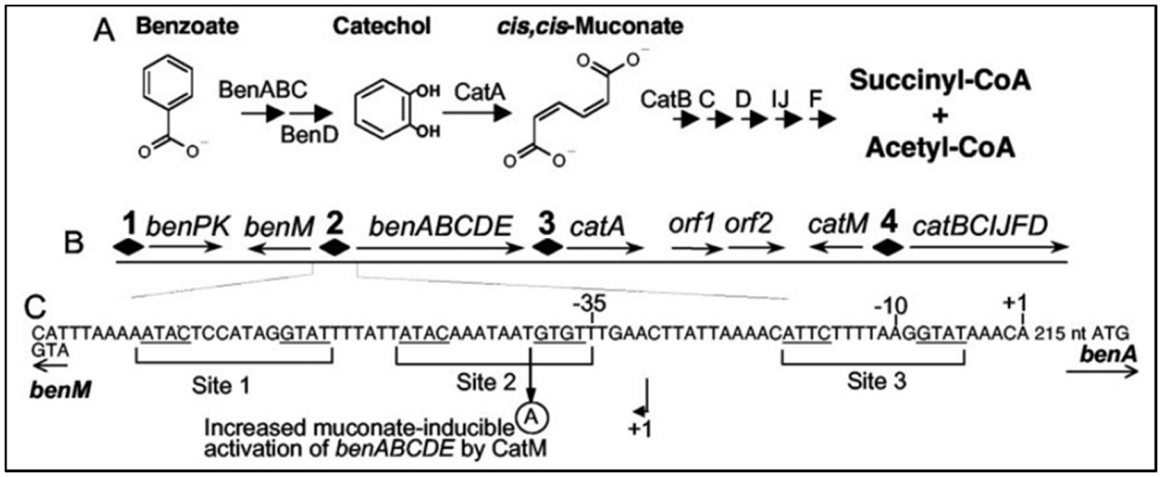

Figure 16: ben and cat genes and their gene products and their involvement in benzoate and catechol metabolism. benABCD, catA, and catBCDIJF gene products are enzymes involved in the catabolism of these substances (A). BenM and CatM regulate transcription initiation in four regions of the DNA (B: diamonds 1 to 4), where the proteins bind to the DNA and activate transcription. Adapted from Ezezika et al., 2006.

According to Figure 16, one of the most direct implications that inhibition of *benABCDE* and *catABCIJFD* transcription may have is a decrease in the

amounts of succinyl-CoA and acetyl-CoA available to the cell. These two compounds are important substrates in the citric acid cycle, which means that decreasing their availability to the cell could cause a decrease in the availability of energy and thus biological stress, a slowing of cell metabolism, or even eventual cell death. This concept makes even more sense when considering that benzoate and catechol can both be considered downstream products of tryptophan metabolism. A general inability to catabolize an amino acid could have many implications for the organism beyond the production of energy, such as deficiencies in biosynthesis of other molecules that require downstream products of tryptophan metabolism.

One such molecule could be the siderophore acinetobactin (Figure 17), which facilitates iron acquisition and thus is crucially important in *A. baumannii* growth and virulence (Gaddy et al., 2012), as iron plays a crucial role in cell survival and the formation of biofilms (Gentile, 2014). Notably, acinetobactin features a catechol moiety, though catechol alone is not itself a siderophore. Consequently, a compound that inhibits the cellular metabolism of the catechol required to synthesize such a prominent virulence factor would be properly called an inhibitor of a virulence factor (or factors) in *A. baumannii*. As such, with its putative inhibitory action on BenM and CatM, M64 is considered as an inhibitor of virulence factors in MDR *A. baumannii*. This is one possible explanation of the apparent rescue of infected mice by M64 (Figure 8).

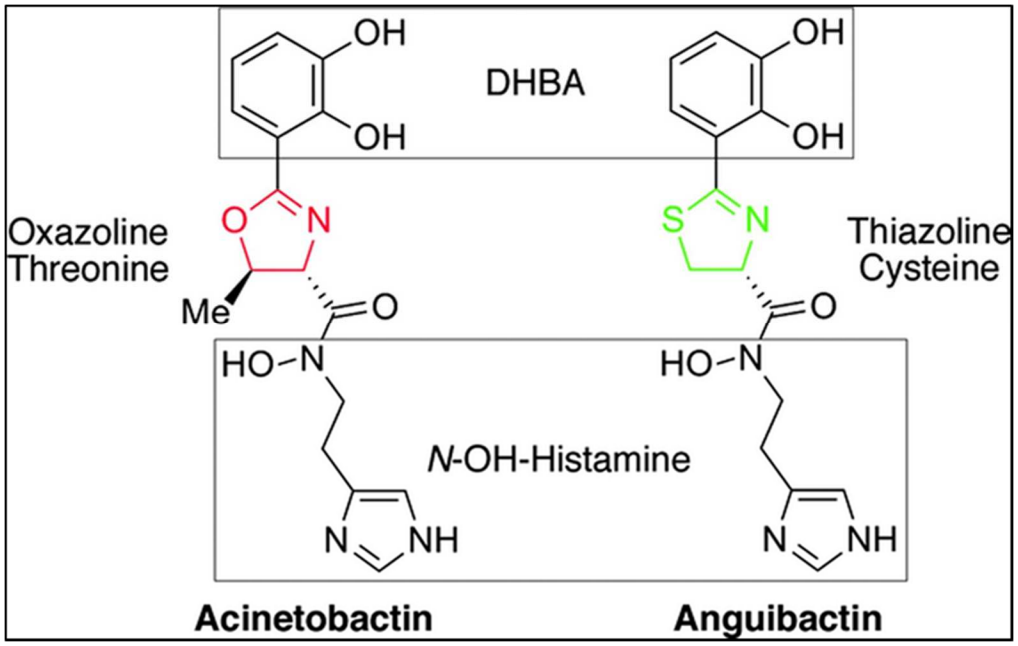

Figure 17: Structures of the siderophores acinetobactin (left) and anguibactin (produced in Vibrio anguillarum for comparison. The catechol moieties are seen at the top of the figure, labelled DHBA (dihydroxy benzoic acid). This illustrates the highly conserved features of these functionally crucial molecules. Figure adapted from McConnell et al., 2013.

Further experiments are required, however, to ascertain the nature of the observed inhibitory effects of M64. Although BenM and CatM are known to be LTTRs, LTTRs seem to share a conserved domain structure rather than a conserved amino acid sequence, as they regulate a remarkably diverse collection genes across a great number of bacterial species (Maddocks and Oyston, 2008; Figure 18). Thus, although M64 certainly binds to MvfR (Starkey et al., 2014), an LTTR in *P. aeruginosa*, there was never a guarantee that it would also bind to BenM or CatM. The data presented here seem to indicate that it does, but the proteins should be purified to have their binding affinities for M64 analyzed.

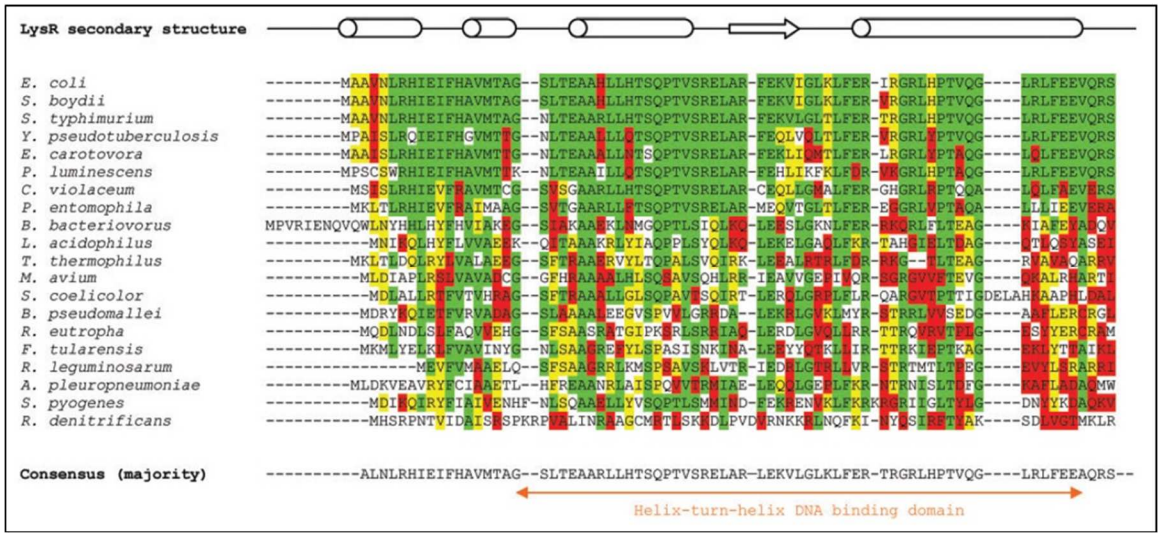

Figure 18: Amino acid homology in the helix-turn-helix DNA binding domain of LTTRs in various bacterial species. Adapted from Maddocks and Oyston, 2008.

The greater statistical significance of the decreased BenA and BenB transcription compared to CatB and CatC (Figure 15), for example, may be explained by M64 having a higher affinity for binding to BenM than to CatM. It may also be that the compound binds to both BenM and CatM with similar affinities but that transcription of the benABCDE operon, potentially being regulated by both of the LTTRs rather than only one (Ezezika et al., 2006) is more strongly inhibited than is transcription of the cat genes. Such cross-regulation of genes between the two transcription factors may also explain some of the difficulty in obtaining statistical significance for their decreased transcription levels.

As for the experiments *in vivo*, *A. baumannii* has never been shown to exhibit virulence in *D. melanogaster* in the literature. Repeated infection of wild type *D. melanogaster* with various *A. baumannii* strains did not produce statistically significant levels of fly death, so fly infection modeling was abandoned. It may be that *Drosophila*, especially in comparison to a mouse model, is more representative of a minimal nutrient environment than a rich one. This would have a similar effect on the bacteria as was seen in their growth in M63gcm, and would limit the bacterial ability to able to multiply, infect, and kill flies.

It was known from the literature, however, that mice are often used to model *A. baumannii infection*, so the next attempt in establishing a model system in the lab was to use mice. Although immunocompetent mice did display some levels of sickness after *A. baumannii* infection (even in quantifiable ways—Figures 6 and 7), the murine immune system generally seemed capable of fighting off the infections and no mice succumbed the infections.

Mouse death was observed in greater frequencies, however, when using relatively high inocula of bacteria to infect immunocompromised mice (Figures 8 and 9). The immunocompromised mouse was seen not only as a more powerful model system where death due to infection could be reliably observed, but also a more relevant model of one of the human populations that typically falls victim to *A. baumannii* infection: immunocompromised hospital patients. A now widely-acknowledged nosocomial pathogen, *A. baumannii* is increasingly seen as the

cause of potentially devastating medical complications that befall vulnerable patients who entered the hospital for other reasons, completely unrelated to their new infection. The immunocompromised mouse model reflects this patient population more accurately than the immunocompetent one.

Thus, immunocompromised, neutropenic mice were used to model *A. baumannii* infection and to test the efficacy of M64 in rescuing them. The results (Figure 9) were remarkable: mice that otherwise would have likely succumbed to their infections (as seen in the control group that received intravenous [IV] injections of plain DMSO) were being rescued with several IV injections of 50 μM M64. 70% of mice in the control group died within five days, compared to just 10% of the treated mice. This led to the development of a hypothesis that M64 was having anti-infective activity in the immunocompromised mice infected with *A. baumannii*.

Another metric used to compare the two experimental groups, the lung index, also proved to be significantly higher in untreated mice than in mice that received M64 (Figure 10). As the lung index is defined as the quotient of a sacrificed mouse's right lung weight (mg) divided by the total weight of the mouse (g), a mouse with pneumonia, a disease condition which increases the weight of the lungs (due to local inflammation) while promoting general weight loss in the mouse (due to the stress of disease), should have a relatively high lung index compared to a healthy mouse.

Since the infected mice treated with M64 proved to have a significantly lower lung index than the untreated mice, this seemed to be a second reason to believe that M64 was successfully treating bacterial-induced pneumonia in immunocompromised mice. The third hit that strongly supported the research hypothesis was seen when it was shown that *A. baumannii*-infected mice treated with M64 showed lower levels of lung bacterial burden compared to mice that did not receive M64 (Figure 11).

At the very least, the observations from the *in vivo* studies in mice seemed to demonstrate that M64 was indeed inhibiting virulence factors in *A. baumannii*. *In vitro* studies ruled out bactericidal or bacteriostatic antibacterial activity while implicating some involvement of tryptophan metabolism and BenM and CatM as potential targets of the inhibitor. Thus, the improvement seen in mouse survival is putatively attributed at least to inhibition of virulence factors, BenM and CatM. These proteins are involved in downstream pathways of tryptophan metabolism where they regulate production of benzoate and catechol, catechol forming a crucial part a siderophore important for *Acinetobacter* virulence (Figure 19).

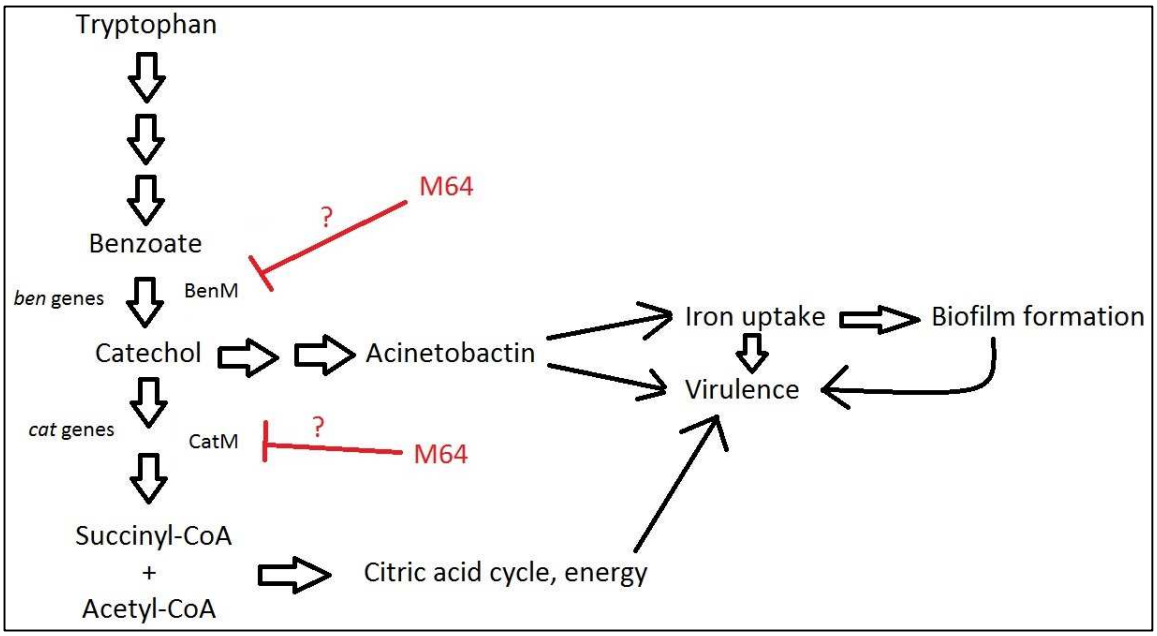

Figure 19: Hypothetical activity of M64 in inhibiting *A. baumannii* virulence factors.

To pinpoint the target of M64, however, it would be most useful to supplement M63gcm with various metabolites downstream from tryptophan to see which ones could promote *A. baumannii* growth in that medium and also which ones could overcome the inhibition exhibited by M64. Tryptophan itself, as well as anthranilic acid and benzoic acid, have been tried at various concentrations, but none of these supplements were enough to get *A. baumannii* to grow in M63gcm. A deficiency in utilization of these metabolites caused, for example, by inability for them to get inside the cells cannot be excluded as a possibility, however. Other metabolites, such as kynurenine, 3-hydroxy-kynurenine, and catechol are soon to be tested.

In summary, multidrug resistant *A. baumannii* continues to present serious problems to healthcare professionals and is a major player in the current

antibiotic crisis. Traditional antibiotics generally target cellular components that are required for bacterial viability or growth. Many infective cells die, but such treatments exert selective pressures and fail to eradicate the resistant mutants they select for. The infective symptoms perhaps subside but then return, unable to be treated with the same antibiotic. To circumvent the direct selective pressures of traditional antibiotics that have led to the emergence of multidrug resistant strains of bacteria, alternative therapeutic methods should be explored. Drugs targeting bacterial virulence factors that are not essential to growth or survival have shown some promise in treatment of *P. aeruginosa* infection by inhibition of an LTTR that regulates virulence factors. Demonstrated here is evidence of a similar *in vitro* inhibition by M64 of LTTRs that regulate the metabolism of compounds that could be important in energy production and virulence in *A. baumannii*, as well as evidence of the *in vivo* efficacy of the compound in diminishing *A. baumannii* virulence. The compound does not seem to significantly affect bacterial growth. As such, M64 seems to hold some promise as a putative treatment in infections by MDR *A. baumannii* while at the same time not exerting the same direct selective pressure of traditional antibiotics, making it more difficult for the bacteria to develop a resistance. Such treatments are especially desirable given the situation of the current antibiotic crisis and could present a new paradigm in the treatment of infectious disease, with further study.

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