Identifying how drug efflux mechanisms impact *Acinetobacter baumannii* evolutionary paths to ciprofloxacin resistance

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Abstract

Acinetobacter baumannii is a multi-drug resistant pathogen commonly found in clinical settings. This pathogen frequently uses efflux pumps to mitigate antibiotic treatment stress and eliminate the drug. When *A. baumannii* is exposed to antibiotics, it often develops mutations in the efflux pump regulator genes, causing an increase in efflux pump production. We hypothesize that efflux is a key pathway that leads to treatment failure in *A. baumannii* infections. The extent to which increasing drug efflux impacts other cellular functions remains unknown. To identify how efflux pump mutations impact growth, resistance, and evolvability, wildtype *A. baumannii* laboratory strain 17978UN, along with four mutants of this strain, each with a single nucleotide polymorphism (SNP) in an efflux pump regulator (*adeL L341R, adeN I49N, adeR D23Y,* and *adeS R152S),* were propagated in the presence of antibiotic and an efflux pump inhibitor to place selective pressure on the isolates. SNPs increase the production of efflux pumps; inhibiting efflux ability will determine if the effect of each SNP is nullified. All evolved populations demonstrated differences in fitness and antibiotic resistance in comparison to their respective ancestors; the extent of adaptation affecting each phenotype was highly dependent on the regulator that was mutated. Counterintuitively, efflux inhibitors also placed stress on wild-type *A. baumannii* which leads to antibiotic resistance. These results demonstrate that efflux regulator mutations can influence population adaptability and cause treatment failure. Understanding the role that different efflux systems play in treatment failure and drug resistance evolution will be instrumental in developing treatment strategies that hinder the development of antibiotic resistance.

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Introduction

For decades, antibiotics have been a reliable way to help treat bacterial infections. However, within the last few years, bacteria have developed an increased resistance to antibiotics; this can cause longer and more severe infections resulting in a large economic burden[1]. One example of a bacteria known to evolve resistance quickly is *Acinetobacter baumannii*, a gram-negative pathogen that commonly affects immunocompromised individuals in hospital settings. *A. baumannii* is part of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species) pathogen group, which is comprised of bacteria that are often resistant to multiple drugs and thus are a top priority for new therapeutics and increased research. These pathogens evade antibiotics through three broad resistance mechanisms: 1) directly modifying the proteins targeted by the antibiotic, 2) actively transporting the drug out of the cell, and 3) inactivating the antibiotic. Resistance mutations that arise in the presence of one antibiotic class can cause a strain to be resistant to other antibiotic classes it has not been exposed to, which may contribute to the ability to develop multi-drug resistance quickly during treatment[2,3].

One antibiotic to which *A. baumannii* can develop resistance to is ciprofloxacin (CIP), part of the fluoroquinolone drug class. CIP kills cells by forming covalent bonds with two proteins necessary for DNA replication, DNA gyrase (*gyrA*) and topoisomerase IV (*parC*). These bonds allow the two proteins to cleave DNA strands but prevent the proteins from ligating the DNA back together, which causes DNA fragmentation and prevents further cell proliferation[4]. In response to this stress, *A. baumannii* evolves resistance through well-documented mutations to *gyrA* and *parC*, preventing ciprofloxacin from binding to these two proteins[5]. Another key mechanism of drug resistance is increasing drug efflux. This pathogen encodes several resistance-nodulation-division (RND) efflux pump systems, including *adeABC*, *adeIJK*, and *adeFGH*[6]. RND family pumps are comprised of an outer membrane channel, an inner membrane transporter, and a periplasmic lipoprotein, which make up a complex that can use the proton motive force to efflux toxic compounds out of the cell.

Each efflux pump system also contains a regulator that controls the transcription of the proteins that make up each efflux pump: *adeL* is a negative regulator of *adeFGH*, *adeN* is a negative regulator of *adeIJK*, and two-component regulator *adeR* and *adeS* is a positive regulator of *adeABC*[7,8]. In the two-component adeRS, *adeR* serves as a regulator and *adeS* serves as a sensor[9]. The SNP in each regulator

[1] Lee and Lee, *Clinical and Economic Evaluation of Acinetobacter baumannii*
[2] Hawkey et al, *Evolution of carbapenem resistance in Acinetobacter baumannii*
[3] Santos-Lopez et al, *Evolutionary pathways to antibiotic resistance*
[4] Ardebili et al, *Effect of Efflux Pump Inhibitor*
[5] Ardebili et al, *Association between mutations in gyrA and parC genes*
[6] Darby et al, *AdeIJK is the universal efflux pump*
[7] Leus et al, *Inactivation of AdeABC and AdeIJK*
[8] Coyne et al, *Overexpression of AdeFGH*
[9] Ouyang et al, *Proteolysis and multimerization*

increases efflux pump production; for instance, a SNP in a negative regulator will decrease the activity of the regulator, while a SNP in a positive regulator will increase activity. Each mutant encoded a regulator that moderates the production of the proteins that make up the efflux pump (Figure 1A). *A. baumannii* uses these pumps to push out antibiotics using the proton motive force (Figure 1B)[4]. Clinical evolution studies have demonstrated that mutations that increase RND efflux pump formation in *A. baumannii* affect other components of a cell besides resistance[10]. For instance, the overproduction of efflux pump inhibitor *adeRS* has been correlated with decreased biofilm formation due to changing membrane permeability[10]. This has increased interest in how the overexpression of efflux pumps can affect other aspects of the pathogen such as cellular fitness.

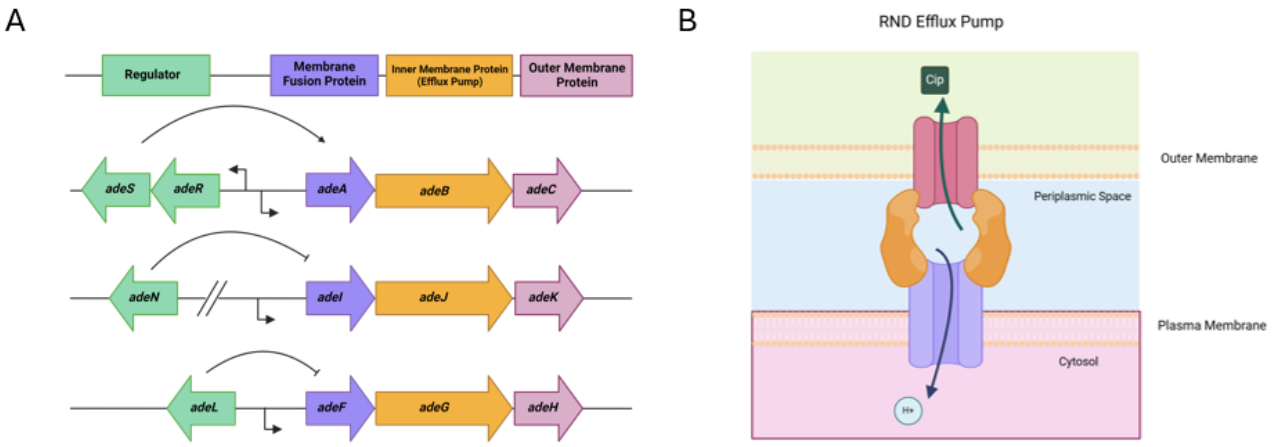

Figure 1. Schematic of efflux pumps encoded by SNPs.

A: Diagram featuring regulators of efflux pump components. *adeS* and *adeR* make up a 2-component system used to positively regulate adeABC. *adeN* is a negative regulator of adeIJK. *adeL* is a negative regulator of adeFGH. Diagram made using BioRender. B: Schematic of resistance-nodulation-division (RND) efflux pumps in *A. baumannii*. RND efflux pumps consist of proteins that span both the plasma and outer membranes and use the proton motive force system to expel drug.

In addition, more information is needed on how the presence of efflux pumps affects the evolutionary pathway toward resistance and how other cellular phenotypes may change because of the cell developing antibiotic resistance on an efflux mutant background. To study how lack of efflux capability may influence resistance evolvability, we selected the ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) as an efflux pump inhibitor. CCCP can move protons across the cell membrane, which uncouples oxidative phosphorylation and prevents the development of a proton

[4] Ardebili et al, *Effect of Efflux Pump Inhibitor*
[10] Yoon et al, *Contribution of resistance-nodulation-cell division efflux systems*

gradient. As a result, RND pumps are unable to expel drug[4,11]. Inhibiting the ability to use efflux pumps in each mutant and WT and subjecting them to ciprofloxacin creates stressful conditions that highlight new evolutionary paths that are taken toward developing resistance; we predicted that these paths would differ based on the efflux pump that is upregulated. This method helps uncover the mechanisms employed while evolving resistance as well as identify the phenotypes affected at different time points during the process. Previous research on *A. baumannii* multi-drug resistance has demonstrated the ability of this pathogen to develop clinical resistance under selective pressure from antibiotics[2,3]. When populations are exposed to a drug, isolates that are susceptible to the antibiotic are killed off, while isolates that have a pre-existing mutation that confers resistance can survive and reproduce. This process promotes the selection of cells that are resistant to the antibiotic, causing the resulting population to have a greater frequency of cells with resistant phenotypes.

In this study we used CCCP and CIP, individually and in combination, to assess the effects of varying selective pressures on the importance of efflux for resistance evolution. We used four isolates, each with a single nucleotide polymorphism (SNP) in an efflux pump regulator (*adeL L341R, adeN I49N, adeR D23Y*, and *adeS R152*, hereafter known as L, N, R, and S). These samples, along with WT ATCC 17978UN *A. baumannii* with no regulator mutations, were propagated for eight days in four environments (CCCP only, CIP only, CCCP + CIP, and no drug). We measured growth, fitness, and resistance for samples of each isolate taken throughout the experiment and compared to their respective ancestors.

Materials and Methods

Strains and media

All mutated isolates were derived from *A. baumannii* laboratory reference strain ATCC17978UN. L mutant was sourced as a clone from a CIP-resistant population propagated in the Cooper Lab. N mutant was sourced from a clone prepared by Dr. Francine Arroyo. R and S mutants were sourced from the Isberg lab at Tufts University. All bacterial cultures were grown in M9+ media, which contains 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 42.2 mM Na_2HPO_4, 22mM KH_2PO_4, 21.7 mM NaCl, 18.7 mM NH_4Cl, 11.1 mM glucose, 20 mL/L MEM essential amino acids (Gibco 11130051), 10 mL/L MEM nonessential amino acids (Gibco 11140050), and 1 mL of trace mineral solutions A, B, and C (Corning 25021-3 Cl). Bacterial cultures were placed on a roller-drum at 250 rpm for 24 hours at 37°C. Ciprofloxacin (98%, ACROS Organics, New Jersey, USA) and Carbonyl Cyanide m-Chlorophenylhydrazone (END Millipore Corp, Darmstadt, Germany) were used during the evolution experiment and for assays.

qRT-PCR amplification

Expression levels of genes *rpoB, adeB, adeG,* and *adeJ* were measured using real-time quantitative PCR. Three replicates of WT, L, N, R, and S were used per gene. A control without reverse transcriptase

[2] Hawkey et al, *Evolution of carbapenem resistance in Acinetobacter baumannii*
[3] Santos-Lopez et al, *Evolutionary pathways to antibiotic resistance*
[4] Ardebili et al, *Effect of Efflux Pump Inhibitor*
[11] Sanchez-Carbonel et al, *CCCP on the susceptibility to imipenem and cefepime*

mix was used to confirm no DNA contamination. Amplifications were performed using the 2X Power SYBR Green PCR Master Mix (Applied Biosystems), and qRT-PCR was done using a QuantStudio 3 real-time PCR machine (ThermoFisher scientific) Thermal cycling was performed at 95°C for 15 seconds and 60°C for 40 cycles. Melt curves were performed at 95°C and 60°C, confirming single target amplification. The resulting data was analyzed for ΔCt of gene expression fold changes and normalized to rpoB levels as housekeeping control.

Resistance analysis via minimum inhibitory concentrations (MICs)

The MICs of each sample were calculated using M9+ media and increasing concentrations of ciprofloxacin in a 96-well round bottom plate. Ciprofloxacin concentrations were doubled in each row, starting from 0.0625 mg/mL to 64 mg/mL. Three technical replicates were done per biological replicate. Two biological replicates were done per sample. Each replicate was inoculated into plain M9+ media to make 0.5 McFarland Standard and diluted further by 1:100 before being added to the prepared MIC plate. Plates were incubated for 24 hours at 37°C. Optical density was measured at 600 nm in a plate reader with the lid removed. MICs were determined as the concentration of antibiotic that inhibited 90% of bacterial growth (IC90). A paired t-test (*p-value* < 0.05) was used to determine the statistical significance of the effect of CCCP on MICs for unevolved strains. The statistical significance of the effect of strain and drug on MIC values for evolved isolates were evaluated by two-way ANOVA (*p-value* < 0.05). Resistance to CIP was also measured under a standard concentration of CCCP (6.25 μg/mL).

Evolution Experiment Conditions

WT, L, N, R, and S were propagated in 4 different conditions (CIP, CCCP, CIP + CCCP, no drug). Five lineages were used per sample per condition (Figure 5B). The no drug condition used plain M9+ media. CIP condition contained 0.125 μg/mL of ciprofloxacin in M9+ media for 5 days and was raised to 4 μg/mL of ciprofloxacin in M9+ media from day six onwards. CCCP condition contained 6.25 μg/mL of CCCP in M9+ media. CIP + CCCP condition used 0.125 μg/mL of ciprofloxacin and 6.25 μg/mL of CCCP in M9+ media for five days and was raised to 4 μg/mL of ciprofloxacin and 6.25 μg/mL of CCCP in M9+ media from day six onwards.

Evolution Experiment Propagation

Frozen samples of WT, L, N, R, and S were streaked on LB agar plates and grown in a 37°C incubator for 24 hours (Figure 5A). Five different colonies of each sample were inoculated into five individual test tubes containing five mL of plain M9+ media, representing the no drug condition. This step was repeated with the CIP, CCCP, and CIP + CCCP M9+ media. One tube per sample per condition was filled with five mL of media with no bacteria added, designated as a negative control. All tubes were incubated on a roller-drum at 250 rpm for 24 hours at 37°C. Every 24 hours, a 1:100 dilution was performed for each tube into a new tube of five mL fresh media. This was repeated for eight days.

20% cryovial freezer stock collection from evolution experiment

One mL of culture was taken every 24 hours from every biological replicate and added to 660 µL of 50% glycerol. The mixtures were vortexed and frozen at -80°C to create a frozen fossil record of the population evolution.

Cell pellet collection from evolution experiment

One mL of culture was taken every 24 hours from every biological replicate and spun at max centrifuge speed for one minute. Pellets were frozen at -20°C for later DNA extraction and sequencing.

Optical density from evolution experiment

200 µL of culture were taken every 24 hours from every biological replicate and placed in flat bottom 96-well plates. Optical density was read at 600 nm as a readout for population productivity/survival.

Fitness analysis via growth curves

Growth curves were performed using 100 µL of plain M9+ media in each well of a 96-well flat bottom plate. Two technical replicates were done per biological replicate. Three biological replicates were used per sample from day six. Two technical replicates were also performed for non-evolved versions of the samples. Streak plates were done on LB agar plates for each biological replicate from frozen cryovials. Two to three colonies were inoculated from each streak plate into 5 mL of plain M9+ media and incubated for 24 hours at 37°C. 1:500 dilution was performed with each culture into fresh M9+ media. 100 µL of each diluted culture was inoculated into the prepared plate. Plates were incubated for 48 hours at 37°C. Area under the curve was used as a measure of a population's absolute fitness and was represented as relative to each lineage's respective ancestor's fitness. Growth curves were analyzed in RStudio (R v4.2.1) using previously published pipelines (https://github.com/mjfritz/Growth_Curves_in_R) [12].

Results

Evolved SNPS in efflux pump regulators increase efflux pump expression

Our first aim was to determine how the SNPs individually affected efflux pump expression. We hypothesized that each mutation would produce different quantities of the respective efflux pump genes. To measure this, we quantified how much of each efflux pump gene is produced in the WT and mutant (L, N, R, S) strains by looking at the gene expression levels of the main efflux component of each efflux operon using qRT-PCR (Figure 2). As expected, L displayed elevated levels of *adeG* gene expression. While R and S are both parts of the two-component system that regulate the *adeB* efflux pump, there is significantly higher expression of *adeB* in the S mutant, showing that the sensor (S) plays a stronger role in regulating the *adeABC* pump gene than the response regulator (R). Gene expression of *adeJ* was similar across all the samples that were assessed; this was unexpected since we thought N would have a higher

[12] Santos-Lopez et al, *Evolved resistance to a novel cationic peptide antibiotic*

level of *adeJ* gene expression than the other mutants. These results show that increased levels of efflux pump production can occur if a SNP is present in the regulator associated with a specific pump.

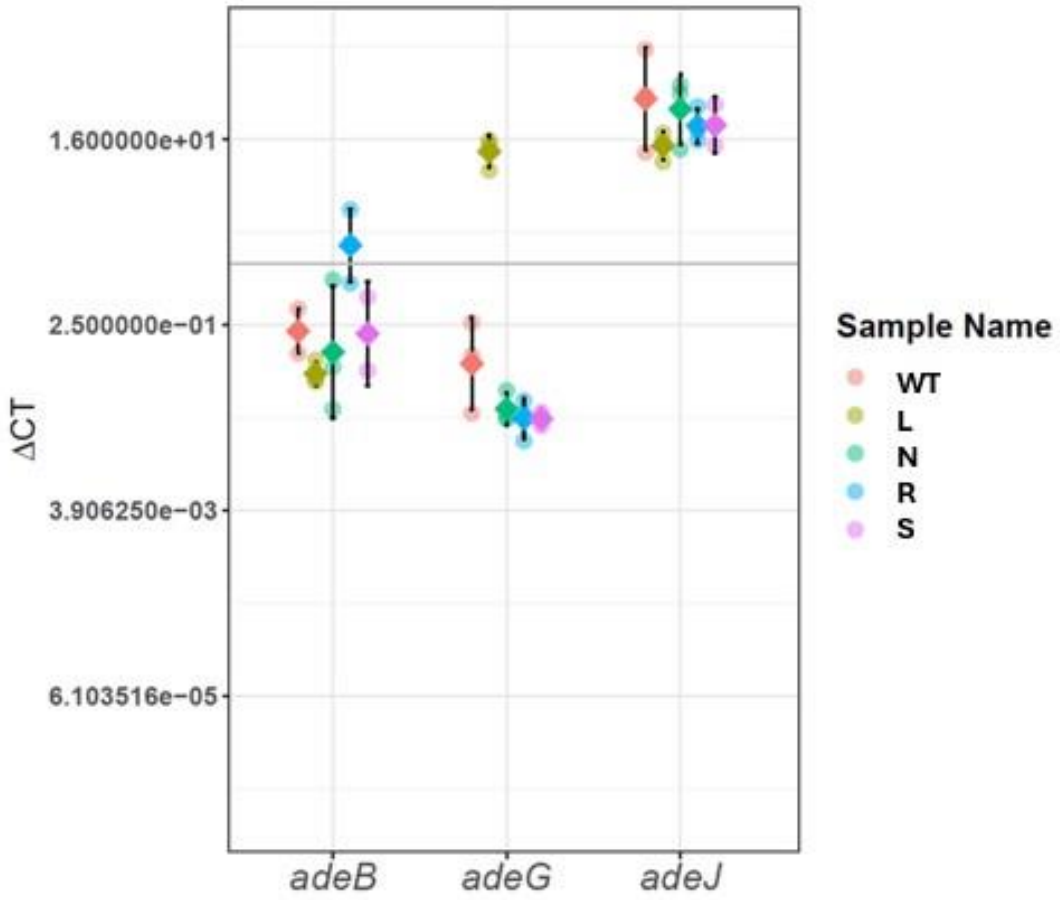

Figure 2. Evolved SNPs in efflux regulators *alter pump expression*

Quantitative reverse transcription PCR (QRT-PCR) was used to measure RNA expression levels for genes *adeB*, *adeG*, *adeJ*, and *gyrB* in WT strain ATCC 17978UN and 4 SNPs (*adeL*, *adeN*, *adeR*, and *adeS*). *adeB*, *adeG*, and *adeJ* are the main pump genes, while *gyrB* encodes DNA gyrase. Pump expression was normalized to the *rpoB* housekeeping gene. Each diamond represents an average of 3 biological replicates. Error bars depict standard deviation of the mean. Cycle threshold (CT) was found from the pcr machine and represented here as 2^{dCT} for ease of visualization

CIP and CCCP had different degrees of selective pressure on each sample

While designing the 8-day evolution experiment, we had to figure out the concentration of CIP and CCCP that would provide adequate stress on each isolate. We wanted the drug concentration to inhibit the efflux pumps without directly killing the strains. We performed growth curves using WT and mutants in six different concentrations of CCCP (Figure 3A). An ideal concentration of CCCP would allow

differences in growth for each sample so we can see the influence of each mutation on growth; we found that growing the samples in 6.125 µg/mL of CCCP had the desired effect. With the same rationale, we did growth curves also with six different concentrations of CIP (Figure 3B). We found that 0.125 µg/mL of CIP placed enough stress that each sample had a varied growth pattern while still growing to a high density. We selected these CIP and CCCP concentrations to use in the evolution experiment. Based on these results, we saw that each mutant had varied fitness when grown in differing concentrations of CIP and CCCP and that 0.125 µg/mL of CIP and 6.125 µg/mL of CCCP highlight these fitness differences without killing the mutants.

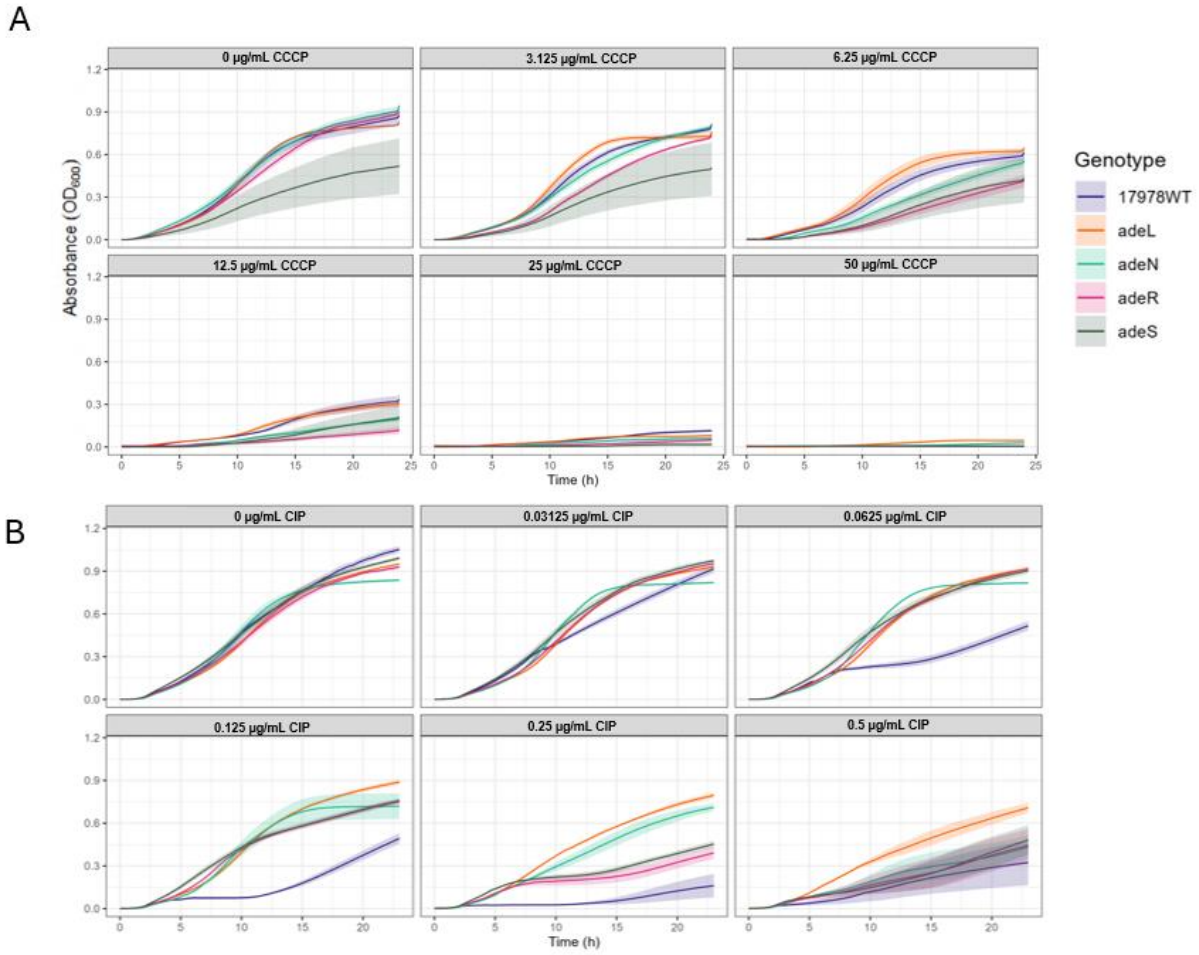

Figure 3. 6.25 µg/mL of CCCP and 0.125 µg/mL CIP places a moderate amount of stress on the samples.

A: Growth curves done in M9+ media and increasing CCCP concentrations up to 50 µg/mL with non-evolved WT *A. baumannii* 17978 and mutants *adeL*, *adeN*, *adeR*, and *adeS*. Samples grown in 6.25 µg/mL experienced varying degrees of stress that provided selective pressure without killing off the population. B: Growth curves done in M9+ media and increasing ciprofloxacin concentrations up to 50 µg/mL with non-evolved WT *A. baumannii* 17978 and mutants *adeL*, *adeN*, *adeR*, and *adeS*. Samples

grown in 0.125 μg/mL experienced stress that provided selective pressure without killing off the population.

SNPs in efflux pump regulators increase resistance to CIP

Our next aim was to determine how each efflux pump regulator mutation would affect *A. baumannii* resistance to CIP. We performed a minimum inhibitory concentration (MIC) assay using the WT and mutated isolates. We quantified the difference in resistance levels between WT and mutant samples and found all mutant samples to have a higher resistance susceptibility than the WT sample, as expected. The L mutant showed the highest resistance (Figure 4A), at almost 2-fold higher than WT. R had the smallest increase in resistance in comparison to wildtype, at only 0.5-fold higher than the WT isolate. All samples we assessed displayed a MIC level higher than 4 μg/mL, which indicates that these samples are clinically resistant to CIP[13]. This indicates that all efflux pump regulator mutations evaluated are associated with a clinically relevant increase in resistance to CIP.

To assess the effect of inhibiting efflux on CIP resistance, we performed MICs of the samples in the presence of increasing concentrations of CIP and a constant concentration of CCCP (Figure 4B). As CCCP blocks efflux, we expected the addition of CCCP to reduce CIP resistance as antibiotic efflux would be less effective and there would be more CIP in the cells when CCCP is present. As predicted, the presence of CCCP reduced the resistance of all mutants. Statistical analysis via a paired t-test (*p-value* <0.05 showed that R had a statistically significant change in MIC when grown in the presence of both CIP and CCCP (Figure 4B). WT, L, N, and R experienced slight decreases in MIC. This demonstrates that the CCCP inhibition of efflux pumps significantly affects CIP resistance in R.

[13] CLSI, *Performance Standards for Antimicrobial Susceptibility Testing*

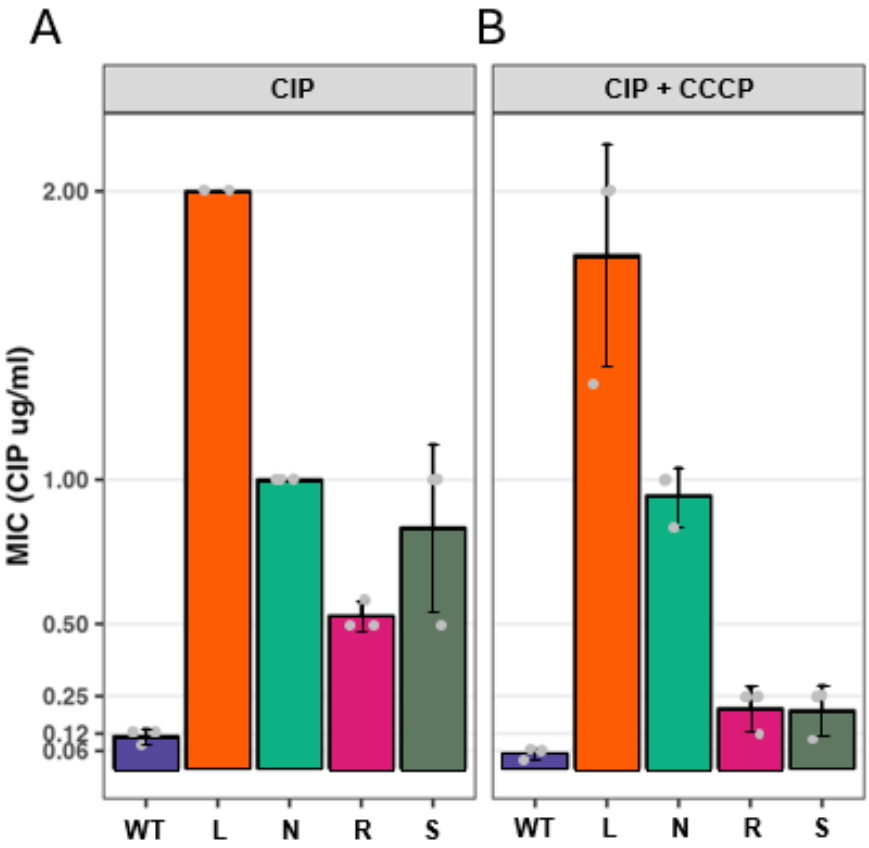

Figure 4. CCCP inhibitor causes a statistically significant reduction in MIC in R. Minimum inhibitory concentrations (MICs) performed using un-evolved WT *A. baumannii* 17978, *adeL*, *adeN*, *adeR*, and *adeS* mutants. Error bars indicate standard deviation. Averages of 2 biological replicates and 3 technical replicates were used for each sample. A: MICs were done with M9+ media in ciprofloxacin concentrations up to 64 mg/mL B: MICs were done in ciprofloxacin concentrations up to 64 mg/mL and in CCCP concentrations of 0.06 mL. Statistical significance was calculated using a paired T-test, with *p-values* <0.05 considered significant.

Efflux pump regulator mutants grew better than WT in the presence of drug

The next aim we had was to determine the effect of efflux pump regulator mutations on *A. baumannii* adaptability. We propagated WT and mutants in four different environments over eight days under four conditions: CIP only, CCCP only, CIP + CCCP, and NONE (Figure 5A, 5B). To measure population size and productivity, we measured the optical density of each lineage each day (Figure 6). We expected the samples to grow the best under condition NONE and the condition with only CCCP. We predicted that more lineages would survive in the presence of CIP only, compared to both CIP and CCCP. In contrast, we believed that the surviving lineages in CIP + CCCP would be able to grow to a higher

density than the ones grown in only CIP due to developing more adaptations that confer an increase in growth density.

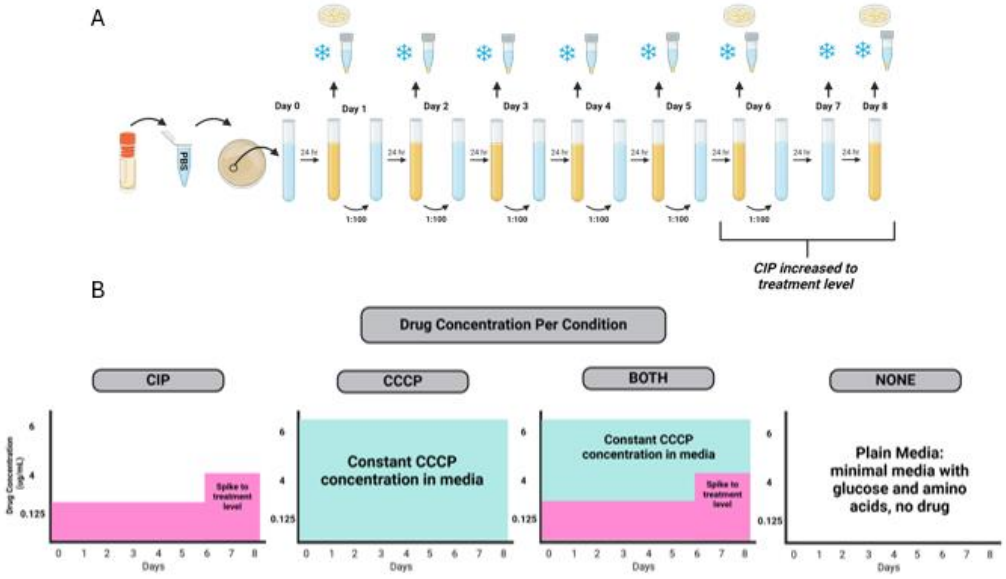

Figure 5. Schematic of the 8-day evolution experiment and conditions

A: *Acinetobacter baumannii* isolate 17978UN, *adeL* mutant, *adeN* mutant, *adeR* mutant, and *adeS* mutant were grown on LB plates for 24 hours. A colony from each plate was inoculated into four individual tubes of 5 mL M9+ media with a specific condition: CIP, CCCP, CIP + CCCP, or no drug. 5 different cultures were made per isolate type per condition and grown for 24 hours. 1/100th of each grown culture (represented by the yellow tube) was taken and reinoculated into fresh media (represented by the blue tube) every 24 hours. On day six, the concentration of ciprofloxacin in media was raised to 4 μg/mL. Cultures were plated on days one, six, and eight to count colony forming units. Samples of each fully grown culture were taken every 24 hours and frozen down in glycerol to create a freezer stock for storage at -80°C (represented by snowflake), as well as pelleted and frozen at -20°C. B-E: WT and mutant samples were propagated in M9+ media with CIP only, CCCP only, CIP + CCCP (BOTH), and no drug (NONE). All conditions with CIP started with media containing 0.125 μg/mL of ciprofloxacin and was raised to media containing 4 μg/mL of ciprofloxacin on the sixth day. Schematics created with BioRender.

All samples grew well in plain M9+ media (NONE). R and S mutants, however, showed more variability in density between replicate lineages grown in CCCP than in no drug. Under the initial CIP concentration, WT, L, N, and R demonstrated a consistent optical density, with WT having the highest density under this condition by day six. One biological replicate of N, however, displayed a decrease in density by day six. After we increased the CIP concentration to treatment level, all samples experienced a decreased optical density, highlighting the stress of the drug increase. WT and L displayed the highest density, or population recovery, in CIP by day eight, while N showed a consistent but lower density. R and S mutants propagated in CIP showed no growth by day eight. The combined CIP + CCCP condition

placed the most stress on the samples. Only one WT lineage grew until day six, while all lineages of the mutants survived until this time point. When we increased CIP to treatment level, all S populations died. Only one biological replicate per WT, L, and R populations was able to grow until day eight. However, all biological replicates of N were able to grow by day eight. The differences in population survival between mutants highlights the differences in selective pressures felt depending on the efflux pump that is utilized.

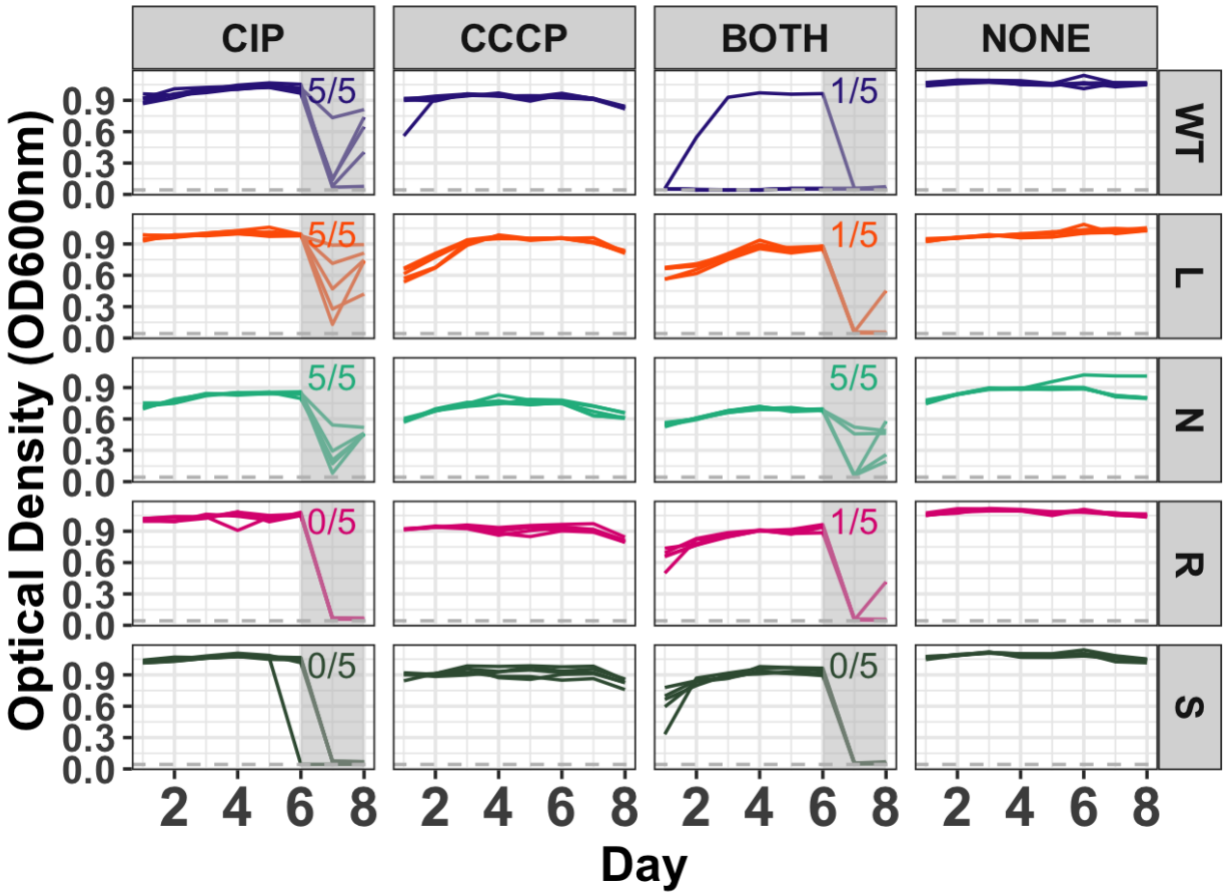

Figure 6. Population density decreases in the presence of drug.

Optical densities taken for evolved WT *A. baumannii* 17978 and mutants *adeL*, *adeN*, *adeR*, and *adeS* under all conditions. ODs were read at 600 nm after 24 hours of growth in culture. Numbers in corner represent number (out of the five replicate lineages) that survived the treatment level CIP spike (gray shading). Survival was defined as OD600 greater than 0.03 above background.

Fitness of evolved populations increased due to adaptation to CIP and CCCP stress

After evolving the samples in each condition, we wanted to see how the presence of each SNP affected the ability of the pathogen to adapt to the environment in which it was grown and any subsequent fitness tradeoffs. To determine this, we measured the fitness of evolved lineages by performing growth curves in plain M9+ media on populations from day six of the evolution experiment. We then compared them to the fitness of the ancestral version of each sample. Regardless of efflux mutation, we expected

fitness in plain M9+ media to decrease more for strains evolved under the stress of CIP. We also expected the samples to have the most challenging time adjusting to the CIP + CCCP condition. When comparing WT-evolved samples to the ancestral WT samples, we found that samples evolved in CIP experienced a decrease in fitness levels, which is what we expected. However, the samples evolved in CCCP showed the greatest increase in fitness among all WT *A. baumannii* used in the experiment, while WT samples evolved in no drug had a moderate increase in fitness (Figure 7). This means that WT was able to adapt best to the presence of CCCP.

The fitness levels of L increased under all conditions, with the greatest difference shown in L samples evolved in the presence of CCCP and the no drug condition (Figure 7). N also showed an improvement in fitness when grown in CIP, CIP + CCCP, and no drug; however, N samples evolved in CCCP experienced a decrease in fitness when compared to ancestral N (Figure 7). This may mean that the mutated N isolate is unable to compensate as quickly as L for the loss of the proton motive force.

R and S samples both experienced the most variability in fitness among biological replicates evolved in both CIP + CCCP as well as CCCP only (Figure 7). The R samples improved fitness when evolved under all conditions (Figure 7). The fitness of S increased when evolved in CIP, no drug, and in CIP + CCCP conditions, while S evolved in CCCP displayed similar fitness levels to ancestral S.

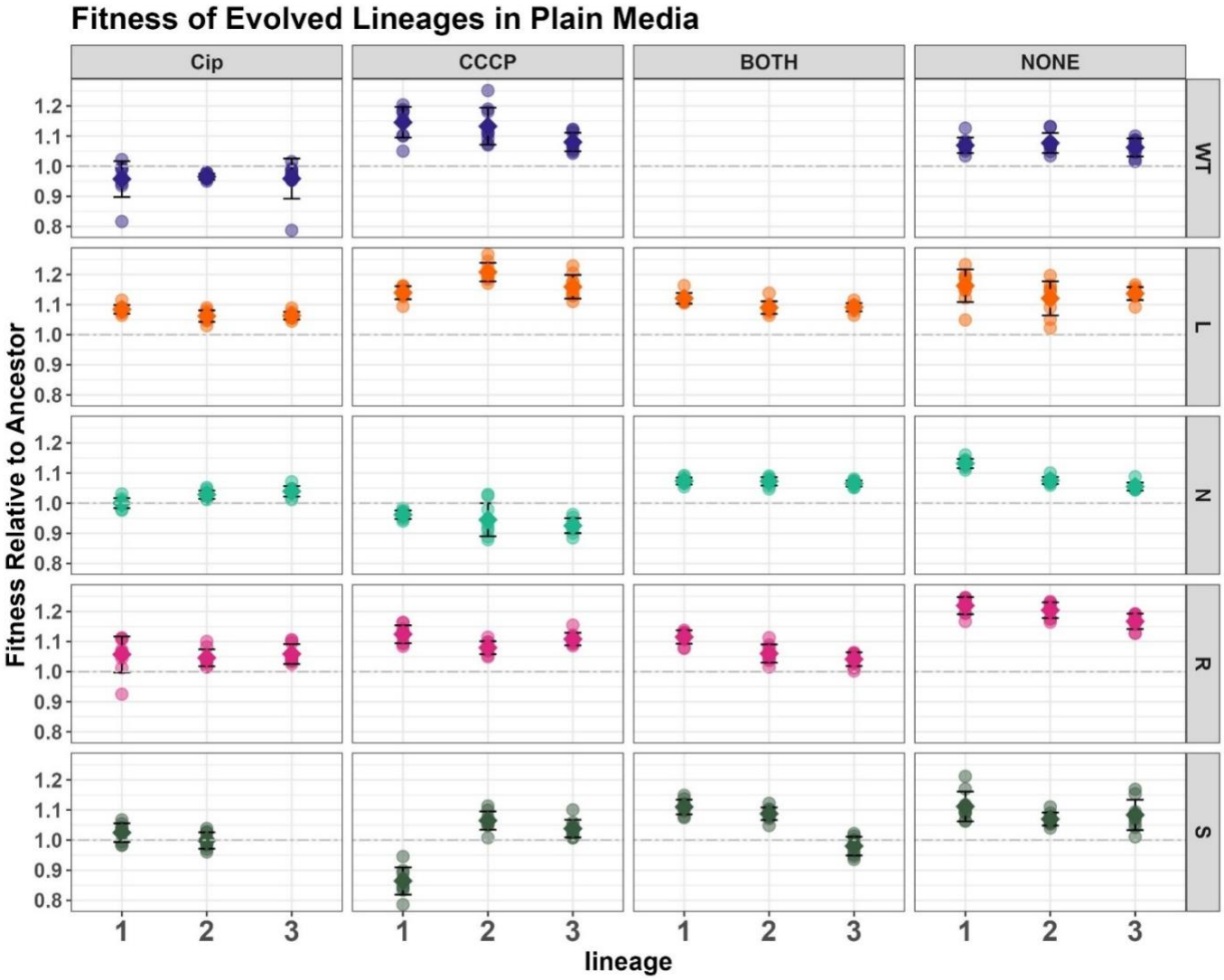

Figure 7. CIP and CCCP placed the most stress on mutants.
Growth curves done in M9+ media with WT *A. baumannii* 17978 and mutants evolved in ciprofloxacin only, CCCP only, and no drug, compared to non-evolved ancestral WT *A. baumannii* 17978 and mutants. Three technical replicates were performed per strain.

Evolved L and N increased resistance to CIP above clinical threshold

We expected CIP resistance to evolve in lineages propagated in conditions containing CIP. However, we did not know how the addition of CCCP to CIP-containing media (BOTH condition) would affect CIP resistance adaptation. We neither expected CCCP selection to result in CIP resistance nor did we expect propagation in plain M9+ (NONE) to select for CIP resistance.

To observe if CIP resistance was evolving in each condition, we took samples of each lineage from day six and day eight of the experiment and performed a minimum inhibitory concentration (MIC) assay with ciprofloxacin. We determined, via a two-way ANOVA (p-*value* < 0.05), that strain and condition produced a statistically significant effect on MIC values. Then, we analyzed MIC changes based on day. We first compared the fold changes relative to ancestors for day six only. When comparing WT MIC

levels, we found the lineages grown in CIP to have the highest increase in CIP MIC, as seen by a 4-fold change, in comparison to the ancestral isolate (Figure 8). WT samples grown in CCCP unexpectedly experienced a 3-fold increase in CIP MIC. Only one biological replicate of WT survived until day six when grown in both CIP + CCCP, but this surviving isolate showed a 3.4-fold increase in MIC. We considered this replicate as an outlier for being able to survive in the presence of both drugs. L, R, and S also displayed a substantial increase in MIC when evolved in the presence of CIP, but to a lesser extent than WT, likely because they started with an initially higher CIP MIC in the ancestors.

Next, we compared day six MICs to day eight MICs to see if any changes occurred due to increasing CIP concentrations (Figure 8). We expected all the samples evolved in CIP to have a higher resistance to CIP, allowing them to survive the treatment level drug spike. Though WT, L, and N had increased MICs, the evolved R and S samples displayed MICs similar to the ancestral MICs. R and S do not meet the MIC threshold for clinical resistance but may be able to tolerate antibiotics, which could explain how the samples are not completely dead under the high CIP levels. In addition, all samples evolved in the presence of CCCP continued to display CIP resistance on day eight, which was surprising since we did not expect proton motive force disruptions to lead to adaptations to CIP. As predicted, the presence of both CIP and CCCP killed most samples; N samples had the highest population of surviving replicates, and all displayed an increase in CIP resistance. The samples grown in no drug also developed a slightly higher resistance to CIP in comparison to ancestral isolates; this could be due to not changing the media between day seven and day eight, causing the samples to be stressed from a lack of nutrients.

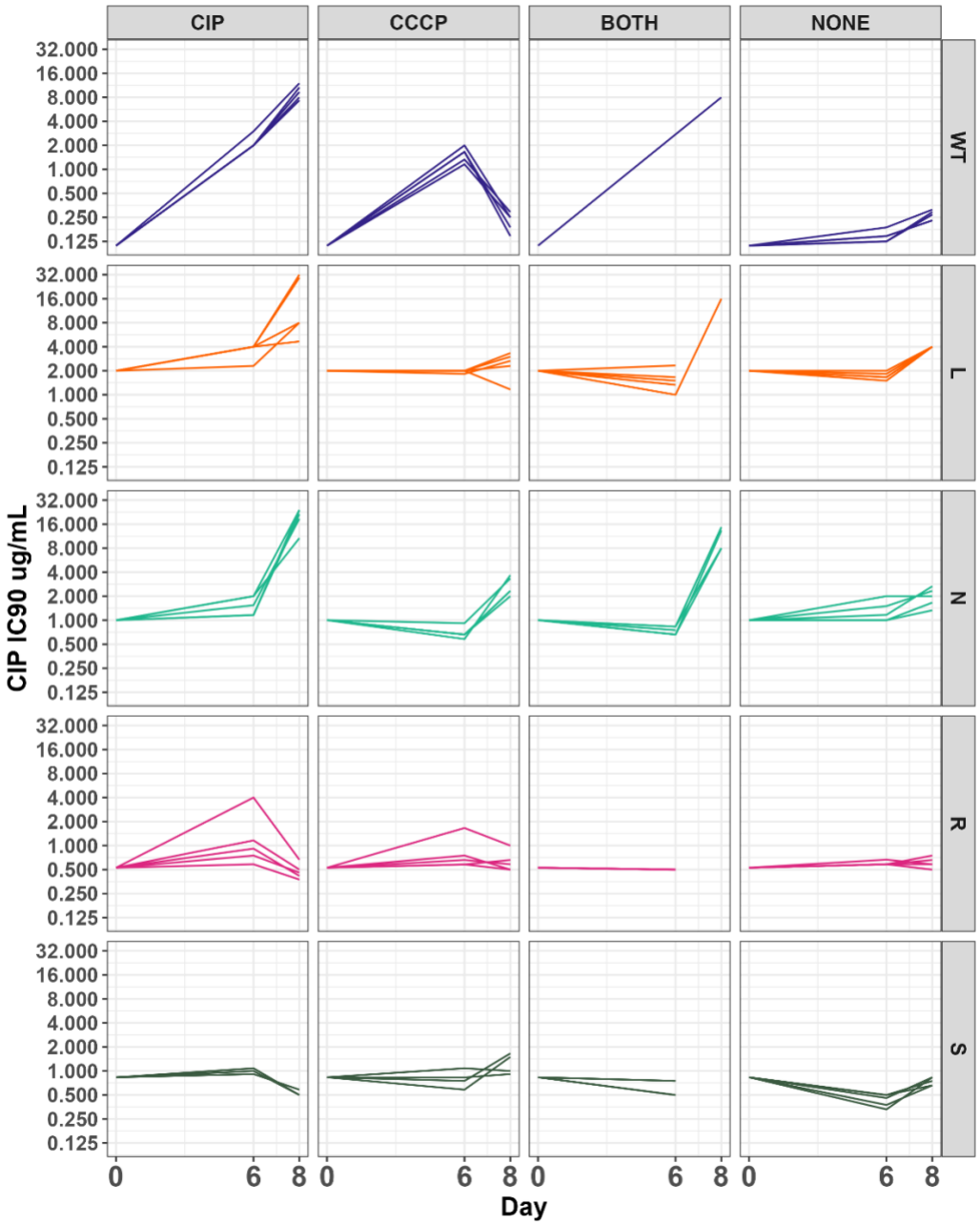

Figure 8. The extent of CIP resistance evolved depended on the SNP present. Minimum inhibitory concentrations (MICs) performed using evolved WT *A. baumannii* 17978, *adeL*, *adeN*, *adeR*, and *adeS* mutants in all four conditions (ciprofloxacin only, CCCP only, both ciprofloxacin and CCCP, and no drug). MICs were done in M9+ media with ciprofloxacin concentrations up to 64 mg/mL. Fold changes calculated using three biological replicates and three technical replicates for samples from day six and day eight of the experiment and normalized to ancestral WT and mutant strains. The statistical significance of the effect of strain and drug on MIC values was by two-way ANOVA (*p-value* < 0.05).

Discussion

Acinetobacter baumannii multi-drug resistance is attributed to the pathogen's ability to adapt quickly under stressful conditions imposed by presence of drug. Through propagating WT *A. baumannii* and four isolates with efflux pump regulator SNPs in multiple environments, we observed mutation-dependent adaptations to growth, fitness, and resistance to ciprofloxacin. This demonstrates that SNPs in efflux regulators affect the evolutionary path taken, which causes differing phenotypic effects that may be due to the SNP or secondary mutations that may have developed.

Although we hypothesized that CCCP would place stress on the isolates during the evolution experiment, we were surprised at the adaptations developed in isolates propagated only in the presence of the inhibitor. Initial MICs performed with the un-evolved isolates that CCCP had a stronger effect on decreasing MICs in WT, R, and S samples; however, L and N did not experience as large of a decrease (Figure 4B). This could mean that CCCP does not inhibit *adeL* and *adeN* regulated pumps as well as the other pumps, indicating that these mutants tolerate the presence of CCCP better. Although CCCP initially had varying success in decreasing resistance based on the efflux pump regulator mutated, we did not expect that CCCP would place a great enough stress to cause the bacteria to increase CIP resistance after being evolved only in the presence of this inhibitor. One explanation for the increase in resistance and fitness in response to the stress of CCCP could be that later generations exhibit a reduction of porins in the outer membrane to prevent fluoroquinolone entry[14]. However, porin-related antibiotic resistance has primarily occurred in response to carbapenems in *A. baumannii*, rather than fluroquinolones[15]. Determining how large of a role porin reduction plays within antibiotic resistance in *A. baumannii* is an area that will need future research.

As we compared the evolution of CIP resistance across all conditions, we noticed that the L lineages consistently displayed high resistance (Figure 8). This is unsurprising, as previous literature has shown mutations in *adeL* rising to high frequencies quickly and persisting for prolonged periods within the population[3], suggesting its role as a primary route for resistance. We noticed that populations of L continued to increase resistance despite already having a high resistance to ciprofloxacin in the respective ancestor; this could point toward L being the most adaptable mutant under stressful conditions. This idea is also supported by the fitness levels observed in the evolved L mutants, as they were shown to increase under all conditions. Overall, these results support the theory that L can quickly develop adaptations in subsequent generations regardless of the presence of stressful conditions.

While analyzing the fitness and resistance of L and N, we also considered the effects of diminishing returns epistasis on the potential benefits of this mutation within these populations. Diminishing returns epistasis refers to when the advantages associated with a mutation differ based on the fitness of the microbe[16]. Typically, as a population increases in fitness, fewer beneficial adaptations with

[3] Santos-Lopez et al, *Evolutionary pathways to antibiotic resistance*
[14] Shariati et al, Resistance mechanisms of bacteria against ciprofloxacin
[15] Prajapati et al, *Bacterial Porins*
[16] Chou et al, *Diminishing returns epistasis*

diminishing magnitude of benefit occur[17]. We had predicted that L would slow in speed of adaptation during later parts of the evolution experiment since the evolved L mutants would be fitter than the ancestor by day six. Although the evolved L mutants grown in all conditions were more fit than the ancestor by this time point, L continued to display increases in CIP resistance by day eight (Figure 8). Similarly, N also continued to establish higher CIP resistance by day eight while having increased fitness (Figure 8). These observations argue against diminishing returns since both populations continued to adapt in fitter populations. As a result, L and N may be very adaptable regulators that can continuously adapt even in fitter populations; however, it is important to note that the mechanisms that cause diminishing returns epistasis are still relatively unknown[16,17].

Most interestingly, we noticed that R and S mutants evolved in CIP displayed MIC levels similar to ancestor levels by day eight, which was unexpected (Figure 8). The evolved R and S samples in CIP were determined as optically dead based on optical densities taken from day eight (Figure 6); however, we were able to resurrect cells from the frozen-down samples to use in the MIC assays. This means that R and S did not grow very well by the end of the experiment and did not develop clinical resistance but may be tolerant of ciprofloxacin through mechanisms such as growth arrest. These strains have been shown to play a key role in the evolutionary pathway toward clinical resistance; through elongating the amount of time an isolate can survive in the presence of antibiotics, more time is provided for resistance mutations to develop.[18,19]

Moreover, when looking at R and S, we noticed that these samples experienced the most variability in fitness among the biological replicates evolved in both CIP + CCCP as well as CCCP only (Figure 7). This points toward R and S having more evolutionary pathways for adaptation that result in different fitness outcomes. In addition, the fitness of S increased when evolved in CIP, no drug, and in CIP + CCCP conditions, while S evolved in CCCP displayed similar fitness levels to ancestral S (Figure 7). In contrast, the fitness of R increased under all conditions. These results suggest that mutations that confer adaptations to proton motive force disruption without the presence of other stressors may be more likely to occur in the R regulator than the S sensor.

When comparing the phenotypes that resulted from each SNP, we could see trade-offs between fitness, resistance, and growth. For instance, N mutants evolved under all conditions displayed a slight decrease in CIP resistance (Figure 8), but improved fitness when evolved in CIP, no drug, and in CIP + CCCP (Figure 7). N mutants also had the most surviving biological replicates evolved in CIP + CCCP by day eight of the experiment, indicating that these mutants could maintain their population density by adapting the best to the disruption of the proton motive force in the presence of CIP (Figure 6). In contrast, R had lowered resistance and growth when evolved in CIP, but higher fitness. These results are consistent with

[17] Kryazhimskiy et al, *Global epistasis makes adaptation predictable*
[3] Santos-Lopez et al, *Evolutionary pathways to antibiotic resistance*
[16] Chou et al, *Diminishing returns epistasis*
[17] Kryazhimskiy et al, *Global epistasis makes adaptation predictable*
[18] Huo et al, *Immunosuppression broadens evolutionary pathways*
[19] Levin-Reisman et al, *Epistasis between tolerance, persistence, and resistance*

the tradeoffs seen in previous experiments performed with *A. baumannii*, which observe higher antibiotic resistance alongside lower fitness[3].

In addition, higher fitness coupled with slower growth may be an indication of tolerance[20]. Tolerant isolates can survive antibiotic treatment through mechanisms that do not directly push out, evade, or destroy the drug; these isolates instead go through growth arrest when placed under antibiotic stress. This could be observed when looking at *adeRS* resistance and population growth. Although both R and S evolved under CIP and CIP + CCCP were considered optically dead by day eight, samples could still be revived from these lineages for phenotypic assays (Figure 6). The evolved samples of R and S from the aforementioned conditions also demonstrated a resistance profile similar to their ancestors, demonstrating that resistance mutations have likely not developed in these mutants. We concluded that R and S may not have developed mutations conferring resistance, but instead developed tolerance through decreasing growth when placed under stress. Tolerant pathogens are known to be precursors to more resistant pathogens since they can persist in higher concentrations of antibiotics; as a result, *A. baumannii* isolates that contain the *adeRS* two-component system could develop multiple resistance mechanisms as a result of being able to persist in the presence of multiple rounds of antibiotic treatment[20].

In the future, we plan to sequence the evolved samples from day six and day eight of the evolution experiment to determine what mutations are present. We predict that there will be more resistance-related mutations in WT, L, and N such as increasing the presence of efflux pumps that do not rely on the proton motive force. In addition, we predict that R and S will have fewer mutations present that confer resistance and more mutations that confer a fitness advantage; however, we would still expect resistance-related mutations to be present, like an increase in efflux pump expression, as this has been demonstrated in other research[21]. Additionally, we plan to perform biofilm experiments with the evolved samples to determine if the mutations to each regulator have a role in biofilm formation. We also plan to observe if any changes in colony morphology and colony forming units by plating the evolved samples. The results from this experiment are integral to determining the evolutionary pathways that lead to clinical resistance in *A. baumannii*. These results can also inform future research on improving current antibiotic treatments to prevent persistent and resistant isolates from proliferating within infected individuals.

[20] Levin-Reisman et al, *Antibiotic tolerance facilitates the evolution of resistance*
[20] Levin-Reisman et al, *Antibiotic tolerance facilitates the evolution of resistance*
[21] Lari et al, *AdeR-AdeS mutations*

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