Identification of Determinants that Allow Maintenance of High-Level Fluoroquinolone Resistance in *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii is associated with multidrug resistant (MDR) infections in healthcare settings, with fluoroquinolones such as ciprofloxacin being currently ineffective. Clinical isolates largely harbor mutations in the GyrA and TopoIV fluoroquinolone targets, as well as mutations that increase expression of drug resistance-nodulation-division (RND) efflux pumps. Factors critical for maintaining fitness levels of pump overproducers are uncharacterized despite their prevalence in clinical isolates, so proteins that contribute to fitness of FQR strains overexpressing three known RND systems were identified using high-density insertion mutagenesis. Overproduction of the AdeFGH efflux pump caused hypersensitization to defects in outer membrane homeostatic regulation, including lesions that reduced peptidoglycan recycling and LOS biosynthesis, and interfered with outer membrane lipid asymmetry driven by the Mla system. In contrast, AdeAB pump overproduction, which does not affect the outer membrane pump component, was relatively tolerant to loss of these functions, consistent with outer membrane protein overproduction being the primary disruptive component. Surprisingly, overproduction of proton-transporting efflux pumps had little impact on cytosolic pH, consistent with a compensatory response to pump activity. The most striking transcriptional changes were associated with AdeFGH pump overproduction, resulting in activation of the phenylacetate (PAA) degradation regulon. Disruption of the PAA pathway resulted in cytosolic acidification and defective expression of genes involved in protection from peroxide stress. These results indicate that the RND outer membrane protein overproduction is compensated by cytoplasmic buffering and maintenance of outer membrane integrity in *A. baumannii* to facilitate fitness of FQR isolates.

Introduction

Acinetobacter baumannii is a Gram-negative bacterium primarily associated with diseases in healthcare settings (1-3). The microorganism has the potential to cause a diverse range of diseases including pneumonia, wound, and urinary tract infections (1-3). Of great concern is its role in causing ventilator-associated pneumonia, which may be closely tied to its ability to grow in biofilms on indwelling devices (2-6). Particularly challenging is the high incidence of multidrug resistant (MDR) *A. baumannii* in hospitalized patients, leaving afflicted individuals with few treatment options (1-3). Although there was a reduction in nosocomial cases in the US from 2013 to 2019, the SARS-CoV2 pandemic saw a large jump in cases that illustrates the difficulties in suppressing diseases by this organism (3). Furthermore, the increasingly common resistance profiles of clinical isolates that are refractory to most antimicrobial therapy regimens indicate that the pathogen poses a continued threat to successful treatment (1-3, 7-10). These clinical isolates include a high proportion that are multi-drug resistant (MDR) or have decreased susceptibility to various single agents (1-3, 7-10). This has prompted the World Health Organization to report that *A. baumannii* is a high priority candidate for research and development of new antimicrobials (8). For this reason, there is a high demand for novel antimicrobials or improved combination therapies with which we can combat this pathogen.

There are a variety of means by which *A. baumannii* acquires resistance, including the acquisition of drug resistance elements via horizontal gene transfer (HGT), the selection of mutations that inactivate drug target sites, and hyper-expression of preexisting drug egress pumps (11-15). Pathogens not only harbor a multitude of efflux systems, some of which are

acquired by HGT, but many have broad substrate specificities (11-15), stimulating efforts to discover effective pump inhibitors (16). Unfortunately, there has been no indication of progress in bringing these inhibitors to the clinic, although several have proven effective in interfering with drug action or synergizing with clinically useful antibiotics using laboratory culture conditions or in animal models (16). Nevertheless, given the relentless patterns of resistance acquisition after the use (and misuse) of single-target antibiotics, a treatment regimen that uses a combination of readily available antimicrobials working in synergy could be effective in interfering with resistance evolution. This study approaches this concept by determining candidate proteins that could be targeted together with clinically-proven classes of antibiotics.

One drug class that has reduced effectiveness against nosocomial pathogens is the fluoroquinolones (2, 17). Resistance in clinical isolates, or after growth in the presence of antibiotic under laboratory conditions, primarily selects for mutations that disrupt drug binding to the targets topoisomerases II and IV, encoded for by *gyrA* and *parC* genes respectively (17). In *A. baumannii*, the highest levels of fluoroquinolone resistance (FQR) are found in isolates that have increased expression of drug egress pumps in addition to mutations in the drug targets. These drug egress pumps belong to the resistance-nodulation-division (RND) family, in which overexpression is commonly driven by mutations in regulatory elements that result in constitutive expression (18-23). The three major efflux pumps commonly associated with FQR in *A. baumannii* clinical isolates are encoded for by *adeAB*, *adeFGH*, and *adeIJK*, which are controlled by *adeRS* (encoding a two-component system), *adeL* (a LysR type regulator), and *adeN* (a TetR repressor) regulators respectively (18-23). Among these three, the AdeN-regulated pump is associated with intrinsic resistance to fluoroquinolones, as this is the only RND pump in

A. baumannii that has measurable levels of expression in the absence of activating mutations (17, 22, 23).

These pump systems traverse both membranes (24-30), and overproduction requires that the envelope accommodates a large population of protein complexes not present in drug-sensitive isolates, putting potential stress on the inner and outer membrane. Therefore, the stability and assembly of these proteins, as well as the fitness of *A. baumannii* during exponential growth may rely upon bacterial functions that work to maintain cell envelope integrity or prevent intrinsic toxicity of the overexpressed efflux system. Our work focuses on identifying genetic interactions in these high-level FQR strains that allow them to maximize fitness, with the goal of identifying proteins necessary to buffer the deleterious effects of pump hyperexpression that could lead to decreased fitness levels in these strains. Such mutations are predicted to identify targets for drugs that are specifically toxic for pump overproducers.

In this study, we used dense transposon sequencing and transcriptomics strategies to identify proteins necessary to maintain fitness of *A. baumannii* efflux pump overproducing strains. We demonstrate that strains harboring overproducing mutations in *adeN* and *adeS* show surprising robustness and identify targets of vulnerability in overproducers of the AdeL-regulated pump. These targets are central players in maintaining cell envelope homeostasis. We also connect the expression of the phenylacetate catabolic pathway as a response to pump overproduction that leads to maintaining cytosolic pH and maximizing protection from reactive oxygen species.

Results

Identification of mutations that depress fitness of fluoroquinolone-resistant strains overexpressing RND efflux pumps. To identify genes that are important for maintaining the fitness of strains having high-level fluoroquinolone resistance (FQR), we isolated mutations in the ATCC 17978UN *A. baumannii* strain background that are viable in high concentrations of ciprofloxacin (Materials and Methods). These strains were then subjected to a mutagenesis strategy (Fig. 1A) that identified insertion mutations that had lowered viability in an FQR strain background compared to the wild type (called strain WT) (Fig. 1B). Three FQR strains were interrogated, each of which harbored *gyrA*(S81L) and *parC*(S84L) mutations that are responsible for fluoroquinolone resistance in most Gram-negative organisms and are commonly found in the clinic (called strain GP). The three strains were distinguished from each other by having distinct mutations in loci that regulate the expression of clinically relevant *A. baumannii* resistance-nodulation-division (RND) drug egress pumps. The mutation in the two-component regulatory component *adeS*(R152S) upregulates the AdeAB pump, the activating *adeL*(ΔI335A336) mutation upregulates the AdeFGH pump, while a knockout in the repressor *adeN*(::ISAba1) increases expression of the AdeIJK pump (Fig. 1C). It should be noted under conditions of AdeAB pump overproduction, AdeK is used as the outer membrane component and is expressed at wild type levels in the strain background used in this study.

Transposon (Tn) mutagenesis was used to determine candidates important for viability or fitness in either the absence or presence of drug. Between 110,000-150,000 unique transposon mutants were created in all three FQR and the WT strain backgrounds, reaching pool saturation levels of about 37-50% based on targets predicted for the *himar-1* transposon (Materials and Methods). Each bank was sequenced post-harvest and transposon-proximal sequences were

amplified to identify the locations of insertions in each strain background and to determine overall pool saturation. This sequence data was also utilized to identify insertion site distributions that were present in each strain, because insertions in genes predicted to be essential or cause fitness defects in a specific pump overproducer strain are predicted to be underrepresented relative to the WT strain. Per our first approach (Fig. 1A), insertion abundance throughout the genome was compared between strain backgrounds using the TRANSIT resampling method to identify genes that have fewer insertions relative to WT in the absence of drug (31) (Fig. 2A, Supp. Table 2), as candidates for genes that are putative conditionally essential targets. These were then displayed by the statistical significance as a function of the insertion abundance in each of the pump overproducer strains relative to WT (Fig. 2A). Among genes identified in this fashion (passing significance criteria adj. $p<0.05$) in the absence of fluoroquinolones were *cyoAB* (*adeL* background), *sodB* (*adeN* background) and *psiE* (*adeS* background) encoding components of the ubiquinol oxidase complex, superoxide dismutase, and a phosphate starvation inducible locus (Fig. 2B). In the case of the ubiquinol oxidase, its function during aerobic respiration as a generator of the proton motive force may play a role in allowing the bacterium to tolerate proton influx resulting from the drug-proton antiport activity of the RND efflux pump (32, 33).

To provide support that lowered Cyo activity reduces fitness in the presence of *adeL*ΔI335A336, we used CRISPRi to knock down expression of the *cyoABCDE* operon in the GPL and WT strains and determined growth rates in broth culture. To this end, an anhydrotetracycline (aTc) -inducible dCas9 was inserted at the *att*Tn7 locus in both strains and transformed with a plasmid harboring a constitutive single guide RNA (sgRNA) (Materials and Methods). As a control, the original vector containing a scrambled 20nt sequence (labeled NC-

sgRNA) was used. Growth was monitored in cultures grown for 5.5 or 6 hours after dilution to low density in LB and aTc (Supp. Fig. 1A). We saw a fitness defect when expression of the ubiquinol oxidase system was knocked down in both the WT and the GPL strains, with the defect enhanced in the WT treated with 100ng/mL aTc (Supp. Fig. 1A). The extent of effect was difficult to evaluate, in part because the presence of aTc appeared to cause toxicity in the WT strain at this concentration. Quantification of *cyoABCDE* operon transcript levels after aTc addition showed that expression was silenced in both strains at comparable efficiency compared to controls (Supp. Fig. 1B).

Notably, there were very few insertions in either the WT or the GPL strain. As a consequence, although the locus was distinguished from others by showing the most highly significant difference in insertion efficiencies (Fig. 2, Supp. Fig. 1C), these differences were measured by degrees of essentiality (Fig. 2B, Supp. Fig. 1C). Therefore, a second strategy was pursued to measure fitness defects during growth in broth culture to attempt to identify more clearcut differences in the two strain backgrounds.

The FQR mutants are more vulnerable to disruption of constituents controlling cell envelope homeostasis. To attack this problem using a second approach, multiple small Tn insertion pools (~8-16k unique insertions) were constructed in each background, and each was grown for 7-8 generations in the absence of antibiotic to determine the relative fitness levels of individual gene disruptions (>50,000 unique insertions in total for each background; Materials and Methods). The difference in fitness levels between an individual pump overproducer and the wild type was then determined, and statistical significance was plotted as a function of fitness differences in the two backgrounds (Fig. 3A). In the absence of drug, the GPS mutant was

particularly tolerant of insertion mutations relative to the WT control, as only insertions in *lnt*, encoding lipoprotein acyltransferase, showed reduced fitness in the GPS mutant compared to WT (Fig. 3A; Supp. Table 3). In the GPS and GPL strains, loss of function of this gene similarly reduced fitness, but in addition, factors involved in lipid trafficking, LOS synthesis, as well as other functions involved in maintaining outer membrane integrity were identified (Fig. 3A, B). These data indicate that in the absence of antibiotic treatment, the pump overproducers showed differential sensitivity to disruption of pathways that support cell envelope homeostasis. Notably, the GPS strain that shows no upregulation of the outer membrane component of the RND systems was the most tolerant of disruption in these pathways (Fig. 3A,B).

To determine if antibiotic treatment increased the spectrum of mutations that sensitize pump overproducers, each of the strains was treated at various ciprofloxacin concentrations to identify conditions that resulted in 20-30% growth rate inhibition (Fig. 3C). Using drug concentrations that reproduced these growth rates, each of the insertion banks was grown 7-8 generations in the presence of CIP (Fig. 3C) (17), cultures were harvested, gDNA was isolated, insertion sites were selectively amplified and sequenced, and the abundance of each insertion was determined using the MAGenTA pipeline (17, 34, 35) (Materials and Methods). From these datasets, as in the absence of drug, the differences in fitness levels between an individual pump overproducer and the wild type were used to identify mutants that preferentially hypersensitize the pump overproducer to ciprofloxacin (Figs. 3D, 3E).

In general, treatment with drug increased the number of insertion mutations that resulted in fitness defects in the pump overproducers compared to WT (compare Figs. 3B and 3D). Similar to results in the absence of drug, the primary targets for preferential fitness defects were concentrated in proteins involved in the maintenance of outer membrane integrity, with the GPS

strain showing higher tolerance than the GPL strain for mutations in the same pathways (Fig. 3D). Furthermore, sensitization of pump overproducers became apparent in the presence of CIP in the presence of a hyperactive BfmRS global regulatory system, which controls envelope homeostasis (36), and disruption of the peptidoglycan interface with the outer membrane (*pbpG* (35)) (Figs. 3D, E). Even so, the magnitude of defects was consistently higher for mutations in the GPL compared to the GPS backgrounds, with mutations in the GPN background often showing intermediate effects (Fig. 3D). These effects correlated well with levels of transcriptional upregulation of the gene for the individual RND outer membrane component in each of the pump overproducer strains and were consistent with the lack of upregulation of the GPS outer membrane component.

Knockout mutations of candidates were generated in both the FQR and parent strains via homologous recombination to verify their phenotypes (Materials and Methods). Bacterial growth was then evaluated in the presence of Cip (Figs. 4, 5) at concentrations that yielded comparable growth inhibition across strains (Fig. 3C). Based on this analysis (Fig. 3D, E), knockouts in GPN and GPL (*bfmS* knockouts) and in GPL ($ompA_{Ab}$ knockout) showed increased CIP sensitivity (Fig. 4A). Consistent with the deep sequencing analysis in Fig. 3D, growth defects caused by these deletion mutations were particularly exacerbated in the GPL strain after Cip treatment as compared to the WT strain background (Fig. 4B).

To ensure the growth inhibition was a result of pump overexpression, the consequences of crossing the mutations into the lower-level resistance GP strain were analyzed in comparison to GP carrying each of the pump overproducers (Supp. Fig. 2A). This allowed a direct comparison to strains that only differ in their levels of pump overproduction. The increase in drug sensitivity when comparing GP carrying the Δ*bfmS* and $\Delta ompA_{Ab}$ to the same lesions in the pump

overproducers was again more pronounced in the pump overproducers relative to GP, indicating that pump overproduction requires BfmS and $OmpA_{Ab}$ to effectively tolerate Cip exposure (Supp. Fig. S2). Of particular interest was the behavior of each of the mutants in the GPL background. Both the Δ*bfmS* and Δ*ompA* mutants were highly attenuated for growth in the presence of Cip, with growth plateauing far below that observed for controls (Fig. 4B). For instance, when Δ*bfmS* was crossed into the GPL background, growth plateau was observed at about 10X lower culture densities than that observed for the GPL parent, and 12-20X for the same mutation crossed into WT (Fig 4B). Similarly, when the Δ*ompA* mutation was crossed into GPL, plateaus occurred at 5-8X lower culture density than the GPL parent, and about 8X lower than the same mutation crossed into WT (Fig. 4B; Supp. Fig. S2).

Given the strong defects observed with these two mutants, other candidate mutations predicted to interfere with envelope biogenesis were interrogated, including LpsB and PbpG, which showed lowered insertion mutation fitness in the pump hyper-expressors when compared to WT, particularly in the GPL strain (Fig. 5A). Deletion mutations were constructed in WT, GP, GPN and GPL backgrounds, and growth of the eight mutant strains was monitored in the presence of Cip. Loss of PbpG from either the GPN or GPL strain background was particularly detrimental to growth in the presence of low Cip concentrations (Fig. 5). In both GPN and GPL backgrounds, strains having the Δ*pbpG* mutation initiated growth, but culture densities stopped increasing, and there was a dip in densities consistent with disruption of bacterial morphology or lysis (Fig. 5B). It is likely that in the presence of Cip, this mutation selectively hypersensitizes efflux pump overproducers, as the parental GP mutant did not show this phenotype (Supp. Fig. 2B). In the case of the Δ*lpsB* mutant, growth plateaued at lower levels when the deletion was analyzed in the GPL mutant (8X lower than parent GPL) as compared to GPN (5-7X lower than

GPN), consistent with the *adeL*(ΔI335A336) mutation causing more envelope stress than the *adeN* knockout (Fig. 5B). To ensure that this phenotype was directly due to these null mutations, both deletions were complemented *in trans*. The presence of plasmids harboring these genes restored the fitness of both pump hyperexpressers, with growth in the presence of Cip indistinguishable from the appropriate parents harboring empty vector (Fig. 5C).

Both PbpG and LpsB have been described to influence overall levels of lipooligosaccharide (LOS) and full-length LOS of *A. baumannii* (35). We wanted to demonstrate that this was observed in the strain backgrounds described here, so the LOS profiles of these strains were investigated (Fig. 6). Lysates from bacteria grown in broth cultures were separated by SDS-PAGE, LOS was quantified by staining with Pro-Q 300 Emerald and normalized to total protein content (Materials and Methods). No significant differences in LOS was detected between the WT or FQR strain backgrounds in regards to profiles or quantities of products (Fig. 6A). The LOS bands were classified as “full” (F) or “intermediate” (I) as representative for their glycosylation levels. The unspecified (U) low molecular mass (LMM) species may be a lipid A precursor such as glycosylated undecaprenyl phosphate lipid carrier/precursor (M.S. Trent, University of Georgia, personal communication).

For the various strains harboring the Δ*pbpG* mutant, there were decreased levels of the “U”, unspecified LMM species, when compared to WT, with slightly higher yields of the LMM band in the GPL background (Fig. 6B). Furthermore, each showed a very similar migration profile on fractionation when compared to either WT or the various parental strains. In contrast, as expected, Δ*lpsB* mutations resulted in truncated LOS (Fig. 6C). Therefore, LOS is altered by these mutations as previously described that was independent of the strain background tested (35).

Maintenance of pH homeostasis in the presence of pump overproduction. The RND-class egress pumps are known to function as drug-proton antiporters (37). With this in mind, we investigated whether FQR pump hyper-expression causes *A. baumannii* cytosolic pH to become more acidic in the presence or absence of Cip substrate. WT, the high-level FQR strains, and the GP precursor strains were grown to mid-exponential phase and loaded with BCECF-AM dye to determine internal pH (pHi). The pHi values were then determined from a standard curve of cells incubated in pH-adjusted buffers in the presence of CCCP to equilibrate external and internal pH (Materials and Methods). In the absence of antibiotic, the cytosol of GPN, the AdeIJK pump over-expressor, was significantly more basic than the GP parent strain (Fig. 7A), while the cytosolic pH values of the other pump over-producers were otherwise comparable to GP or WT controls (Fig. 7A; one-way ANOVA, multiple comparisons between all strains and GP, $p<0.05$). To determine if the presence of Cip, which should result in proton-Cip antiport, affects internal pH, the experiment was repeated after exposure to Cip at concentrations yielding comparable levels of growth inhibition across strains. Cip treatment lowered the pHi compared to the untreated conditions for all strains. The cytosols of the GPS and GPL strains were fractionally more acidic than the GP strain after CIP addition, but the differences did not rise to the level of statistical significance (Fig. 7B; same tests as in 7A). These data indicate there may be homeostatic mechanisms to neutralize the effects of pump overexpression that act at either transcriptional or post-transcriptional levels. Therefore, the global transcriptional effects of pump overexpression were analyzed in the presence and absence of ciprofloxacin (Fig. 1A, third approach).

Large transcriptional changes in response to pump overproduction are limited to the AdeL-regulated pump. To determine if pump overproduction results in transcriptional changes that support fitness, the FQR, low-level resistance GP, and WT strains were cultured in the absence or presence of Cip at concentrations causing 20-30% growth inhibition and analyzed by RNAseq (Materials and Methods). Samples were collected during the exponential phase (A_{600} ~0.5) as well as early post-exponential phase (A_{600} ~1), and differentially regulated genes were identified by comparing the high-level FQR strains and the GP parent strain (Fig. 7C-F; GP vs GPL (C, +Cip) and (D, -Cip), GP vs GPN (E), and GP vs GPS (F), Supp. Table 4). In either the presence or absence of Cip, the GPL strain had the greatest number of differentially expressed genes relative to the control GP strain when compared to the other pump-overexpressing strains (Fig. 7C-F). In fact, hyperexpression of either the AdeS- or AdeN-regulated pumps only altered the gene expression patterns of very few genes, either in the presence or absence of Cip (Fig. 7E, F). This result echoes the Tn-seq analysis of the three overproducer strains, as the *adeL* mutant had the largest set of mutations that lowered fitness relative to WT (Figs. 2, 3, and Supp. Fig. 3).

Among the various transcriptional changes resulting from AdeL-regulated pump hyper-expression, the most notable was upregulation of the *paa* operon, which codes for proteins involved in degradation of phenylacetate (Fig. 7C). As confirmation, RNA was isolated from parallel cultures of GP and GPL grown in the absence of drug, and cDNA was prepared for q-rtPCR analysis. Consistent with the RNAseq results, transcript levels of the first gene of the operon, *paaA*, were significantly elevated in the GPL strain compared to the GP parent, with a 23-fold increase during exponential phase and a 6-fold increase in post-exponential phase (Fig. 8A, B). The other transcriptomic change of note in the GPL strain was the downregulation of the *atpE* gene encoding a component of the F_1F_0 proton translocating ATPase/synthase (Fig. 7D).

This parallels other work, which shows that depletion of the ATPase/synthase increases fitness of the GPL strain, consistent with lowered expression acting to allow proper homeostatic regulation of either membrane potential or the intracellular proton environment (unpublished).

Depletion of *paaA* alters expression of stress-related genes and lowers intracellular pH in the GPL pump hyperexpressor. The phenylacetate catabolic pathway breaks down the substrate phenylacetate to generate end products that feed into the TCA cycle (38). Previous studies indicate that induction of the *paa* pathway in *A. baumannii* may be a response to stress, as observed after antibiotic treatment (39). Therefore, upregulation of this operon may similarly support the fitness as a response to overproduction of the AdeL-regulated pump. It should be emphasized that *paa* mutants showed no apparent fitness defect in our TnSeq screen that was performed on exponentially-growing bacteria. Consistent with the Tn-seq findings, targeting *paaA* by CRISPRi caused no growth defect in the GPL strain as compared to a parallel non-targeting control strain. Depletion was efficient, as there was a 20-fold knockdown in exponential phase and almost 200-fold in early post-exponential phase compared to the negative control (Fig. 8C, D). This argues that in the absence of phenylacetate degradation that other compensatory pathways may allow fitness to be maintained.

The *paa* pathway has been shown to promote resistance to hydrogen peroxide (39, 40), so enhanced expression of the *paa* pathway in the AdeFGH pump hyperexpresser may facilitate a response to oxidative stress and/or other stressors. To test this idea, transcription of a set of oxidative stress genes was probed in the GPL strain by q-rtPCR analysis. Knockdown of *paaA* expression in early post exponential phase yielded reduced transcript levels of *katG*, *katE*, and *sodC* (encoding two peroxidases and superoxide dismutase, respectively; Fig. 8D). This

diminished expression is consistent with previous observations that *paa* null mutants are more sensitive to hydrogen peroxide stress through some previously uncharacterized mechanism (39, 40).

Internal pH was determined after *paaA* knockdown in the GPL mutant (Fig. 8E, F). Knockdown of *paaA*, the first gene of the large operon, resulted in a significant acidification during exponential growth compared to a nontargeting control (Fig. 8E) (A_{600} ~0.52-0.62). By early post-exponential phase (A_{600}~1.22-1.39), the effect of the knockdown was no longer statistically significant compared to the non-targeting control (Fig. 8F). When the *paa* knockdown cultures were grown to exponential phase and exposed to sub-MIC levels of Cip for 45min, the pHi was lower than the non-targeting control, but the difference was not determined to be statistically significant (Fig. 8G; significance determined via unpaired t tests). These data indicate a potential role for this pathway in maintaining pH homeostasis during exponential growth of strains overproducing the AdeL-regulated pump.

Given that the *paa* operon has been documented to be associated with a drug stress response, we determined if the expression of the *paa* operon is altered in response to Cip in each of the strains. Based on the RNAseq data in exponential (Fig. 9A) and post-exponential (Fig. 9B) phase, *paaA* transcript levels in all the strains were significantly upregulated in response to Cip exposure as compared to growth in the absence of drug (Fig. 9A, B). These results are consistent with previous results showing that upregulation of the *A. baumannii paa* catabolon occurs in response to drug stress (39). Notably, upregulation of *paa* transcript levels in the absence of drug is a special feature of the GPL strain when compared to the GP parent, and this is not observed in the GPS or GPN strains (Fig. 7D).

Previous work has shown that pump hyper-expression and the oxidative stress response are contributors to the development of persistence in the presence of supra-MIC levels of antibiotics (41-44). In particular, the single AdeL mutant promotes persistence in the presence of about 20X MIC levels of Cip (44). We therefore determined if depleting *paaA* expression would interfere with persistence. Depleting *paaA* transcript did not alter the ability of a single *adeL* mutation in the WT background to persist in high levels of Cip when compared to the nontargeting control in AdeL (Fig. 9C). WT with the nontargeting control (WT+NC) was included as a negative control to demonstrate the non-persister phenotype. It is evident that the phenylacetate catabolic pathway responds to stress, modulates the oxidative stress response and pH homeostasis, and is documented as maintaining virulence in a number of Gram-negative organisms (39, 45-47).

Discussion

In this work, we set out to identify proteins that were required to maintain fitness of *Acinetobacter baumannii* strains that overproduce drug efflux pumps. The most surprising result was that *A. baumannii* strains that overproduced the AdeN and AdeS regulated efflux pumps appeared to tolerate overproduction, with few mutations causing specific defects in these strain backgrounds (Fig. 3) and little compensatory transcriptional changes (Fig. 7). In contrast, high fluoroquinolone resistant (FQR) strains overproducing the AdeL-regulated pump showed a range of mutants that lowered fitness relative to the WT control and a more pronounced shift in its transcriptome relative to a *gyrAparC* FQR mutant (GP) as a result of increased pump expression. Whether this is due to overproduction of this AdeFGH pump, which is 1000-fold overexpressed relative to that observed in the GP strain, and/or due to additional downstream effects resulting

from the mutation in the regulator AdeL is unclear. An obvious difference between these mutants is that *adeL*-activating mutations results in far higher overproduction of the outer membrane component AdeH than the AdeS- and AdeN-regulated outer membrane components. Most notably, the AdeS-regulated pump was the most tolerant of insertion mutations and had the least transcriptional disruption of the strains studied. With this pump, the outer membrane component AdeK is not overproduced in response to the AdeS activating mutation, so it is predicted to have little effect on outer membrane integrity.

We have previously seen the AdeL mutation confers persistence in the face of high levels of Cip (44). We initially encountered mutations in this gene during successive passaging of *A. baumannii* in a neutropenic mouse pneumonia model subjected to Cip treatment. Single *adeL* activated mutants were eventually outcompeted by mutations that allow increased Cip resistance as well as increased fitness in the absence of antibiotic (44). Therefore, *adeL* activating mutations impart a fitness cost. We suspect that the lowered growth rate is a consequence of envelope stress (Fig. 3) and could have clinical corollaries. The inability of *adeL* mutants to efficiently compete in immunocompetent animals (44) may mirror drug resistance evolution in hospitals, where immunocompromised patients may act as reservoirs for these variants that eventually act as evolutionary precursors to more fit and higher resistance variants. In this fashion, evolutionary precursors that cause cell envelope disruptions may be compensated by further mutations that allow spread in individuals with intact immune systems.

Fitness costs and resistance drivers are important for the evolution of MDR clinical isolates and it is common for a pathogen to employ multiple drug egress systems at a time, which has the potential for accumulated envelope stress (19). Of note, while it appears there is no advantage to overexpressing both AdeIJK and AdeFGH efflux systems simultaneously during

Cip challenge (GPNL mutant, Supp. Fig. 5), this was not investigated further in the presence of other drugs nor with the third transporter described in this body of work, AdeAB. This work does emphasize, however, that there is an interplay between fitness costs and fractional differences in drug resistance, and that strains having multiple antibiotic efflux systems may require compensatory mutations to reverse fitness costs.

The work described here identified points of vulnerability in the high-level FQR strains, especially the AdeL (GPL) mutant, that may be exploited to re-sensitize MDR strains to this drug. It is evident that GPL heavily relies on determinants that stabilize the cell envelope and the induction of specific stress responses to alleviate stress likely imposed by pump hyper-expression. Furthermore, it may explain why *adeS* and *adeN* pump overproducer mutants appear to be particularly common in clinical isolates, while the presence of *adeL* overproducer mutants, have been difficult to demonstrate.

BfmS was one of the candidates identified in our Tn-seq screen. It is one half of the BfmRS two-component regulatory system (TCS), functioning as the histidine kinase sensor which negatively regulates the BfmR response regulator. BfmR in turn controls a large transcriptional network including proteins linked to cell wall biogenesis, capsule production, and outer membrane protein assembly among others (36). It is intriguing that disruption of *bfmS* is detrimental to the fitness of the RND pump hyper-expressors, because disruption had been previously shown to promote *A. baumannii* virulence, drug resistance, and the expression of candidate genes such as *lnt* and *pbpG* (36). Presumably, genes known to be preferentially downregulated when the TCS is activated by loss of BfmS may, in bulk, contribute to the fitness of the FQR strains. Another such protein is OmpA, a major outer membrane protein of *A. baumannii* with a periplasmic domain that interacts with peptidoglycan (48, 49), that apparently

functions as a low-efficiency non-selective ion channel in a fashion similar to that of *E.coli* OmpA and *Pseudomonas* OprF (50, 51). Transcription of *ompA* is controlled by the BfmRS regulon, indicating that increased sensitivity of a *bfmS* mutant in pump overexpressing strains may be tightly linked to lowering *ompA* gene expression (52, 53).

The peptidoglycan recycling factor PbpG, also influenced by BfmS, is a candidate of interest predicting that pump overproduction may cause envelope stress. How proteins that function in cell wall dynamics and LOS biosynthesis interface is still unclear, but both are integral to supporting cell structure and the membrane permeability barrier against external stressors. Peptidoglycan (PG) has been shown to play an important role in supporting the fitness of LOS deficient *A. baumannii* (54). It has been postulated that PG-associated proteins may affect the transport and synthesis of LOS (35). Therefore, mutations in this gene could reduce fitness of pump overproducers either due to direct effects on PG or composition of the outer membrane (OM). Interactions between RND transporters and PG had been previously observed, supporting a direct model for this defect (55, 56). On the other hand, there is also good reason to posit that the effects on LOS or composition of the outer membrane otherwise are the primary reason that the absence of *pbpG* reduces fitness of the overproducers. We have found that loss of the LOS biosynthesis gene *lpsB* causes a fitness defect for pump overproducers that is similar to loss of *pbpG*. Therefore, we hypothesize that the FQR strains are highly susceptible to disruptions in *pbpG* and *lpsB* because of their impact on the composition of the OM and LOS levels. Loss of PbpG may interfere with the proper translocation and assembly of the RND transporter's OMF at the cell surface. The changes in LOS levels and composition could impede the stabilization and intra-pump complex interactions important for their functionality. Also, it has been demonstrated that LPS binds OMPs, and likely mediates the associations between

OMPs and the outer membrane, reinforcing membrane integrity (57, 58). Therefore, the pump overproducers may be more susceptible to disruptions in $ompA_{Ab}$ because its absence may disturb the permeability barrier as well as hinder the stability of the RND outer membrane component.

Other envelope determinants that were identified in our screen have either not shown strong defects or deletion mutants could not be constructed. The most significant was the lipoprotein acyltransferase *lnt* (59). We were unable to acquire a *lnt* deletion mutant in any strain background, indicating total deletions may be lethal, so other strategies are necessary to analyze the locus. The Δ*mlaFEDCB* mutants, however, were constructed and showed weaker phenotypes than anticipated, with only a small defect in the GPL mutant during Cip exposure (Supp. Fig. 4A, B). Other loci, such as candidates like *lpxM* which is integral to the synthesis of hepta-acylated lipid A, which support outer membrane barrier function, have yet to be analyzed (60, 61).

If there is a single feature linking together the Tn-seq mutant candidates that have lowered fitness in the presence of efflux pump overproduction, it is that they target proteins associated with cell envelope maintenance. Various studies have demonstrated that cell envelope components can compensate for defects in the membrane to facilitate survival, exemplifying the intricate and interconnected nature of the determinants controlling envelope biogenesis (59, 60). The interplay between the identified targets that control the cell wall and membrane integrity likely controls the functionality of the RND transporters. We postulate that PG, LPS, and other OMPs not identified in this study may serve to support the outer membrane component of the RND pump complexes in the cell membrane while simultaneously supporting membrane barrier integrity. As the cell membrane provides a selective permeability barrier against antimicrobials, any destabilization of this barrier may compromise drug efflux pump function as well as lead to

drug influx across a similarly compromised barrier. It should be noted that several of the candidates identified in this work show promise as drug targets that could sensitize RND overproducers to Cip treatment. We saw a 3-fold drop in Cipro MIC in the GPL strain when $OmpA_{Ab}$, LpsB, and PbpG were deleted (Supp. Fig. 4C).

Analysis of the transcriptome of the RND overproducers provides further evidence for the induction of cell stress as a consequence of overproduction of the AdeL-regulated pump (Fig. 7). Most notably, the transcription of the phenylacetate operon (*paa*) is induced in response to pump overexpression in the absence of antibiotic treatment. This catabolon has been documented to protect against antimicrobial treatment (39), linking pump overproduction to drug stress. Null mutations in the *A. baumannii*, *Burkholderia cenocepacia*, or *Pseudomonas aeruginosa paa* operons were shown to result in bacterial attenuation in a murine CAUTI or *C. elegans* infection models (39, 45-47). This attenuation may be due to its influence on the expression of virulence associated traits, and/or a putative negative signaling role of the substrate phenylacetate which accumulates in the null mutants (46, 47, 62, 63). *A. baumannii paa* null mutants which exhibited a consequent increase in phenylacetate were shown to attract neutrophils in a zebrafish infection model, promoting clearance (62).We reasoned that the production of substrates from this pathway which feed into the TCA cycle could provide proton relief from proton influx produced by the overproduced pump. Another link between the drug and pump overproduction stress responses is the fact that, although the GPS and GPN overproducer strains do not upregulate the *paa* operon expression in the absence of drug treatment, Cip treatment of these strains does result in activation of *paa* (Fig. 9A, B). This result mimics the effect on *paa* transcription caused by overproduction of the AdeL-regulated pump in the absence of antibiotic. The consequences of loss of *paaA* expression appear mild, as we have no evidence its loss leads to a fitness defect

under any condition tested here, and knockdown of this gene had no effect on persister formation by an AdeL-regulated pump overproducer (Fig. 9C). There may be redundant pathways that come into play in the presence of drug pressure or other stress when this pathway is absent that explain our failure to observe a clear phenotype.

One clear difference is that the absence of *paaA* led to lowered cytoplasmic pH (Fig. 8E, G). We do not know whether the substrate of *paa*, phenylacetate, is transported out of cells by the RND transporter. If it is not, then it is possible that accumulation of phenylacetic acid may be the basis for the lowered cytosolic pH upon knockdown of the *paa* operon. It may be that this substrate when accumulated can also be a signaling molecule. Indole and paraquat are examples of compounds previously shown to stimulate the expression of genes such as *oxyR* and *soxR* that serve to protect *E. coli* from oxidative stress. The accumulation of these compounds also promotes persistence of the bacterium in the face of antibiotic challenge and contributes to population survival, although coming at a fitness cost (41-43). Additional transcriptomic adaptations in the GPL mutant from loss of *paaA* expression should be explored to better understand the role of the catabolon. Finally, it should be pointed out that loss of the proton-pumping ubiquinol oxidase complex is predicted to cause lowered fitness of the GPL strain, consistent with maintenance of membrane protonmotive force being critical for pump overproduction.

Our work indicates that targets involved in supporting the integrity of the *A. baumannii* envelope are associated with maintaining fitness of strains that overproduce efflux pumps. The pump that was most highly overproduced in FQR mutants, the AdeL-regulated pump, was associated with the largest number of genetic requirements for fitness, as well as most disruptive to the host cell transcriptome. The genetic loci identified can be viewed as drug targets for

synergy with fluoroquinolones as well, defining pathways that may buffer the stress accompanying increased pump activity. Still unclear is how the phenylacetate degradation pathway is induced in response to the stress identified here, and what physiological role, if any, it plays in supporting the fitness of the strains analyzed in this study. Future work will attempt to expand these studies into analyzing the role of essential proteins in supporting pump overproduction in hopes of identifying potential targets for multidrug therapy targeting drug resistant mutants.

Materials and Methods

Bacterial strains and growth conditions. Bacterial strains used in this work are listed in Table 1. All *Acinetobacter baumannii* strains are derived from ATCC 17978UN. Cultures were grown in lysogeny broth (LB) (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) or on LB agar plates (LB supplemented with 15g/L agarose) and incubated at 37°C with aeration, as well as shaking or on a roller drum if grown in broth. Unless grown the same day, cultures were typically diluted, incubated to recover after overnight growth, then diluted to A_{600}=0.1-0.001 into either 5-10mL broth or into 96-well plates (Costar), and growth was monitored by measuring A_{600} every hour (broth culture) or every 15min (Epoch 2 or SynergyMx, (Biotek) plate readers.) Superoptimal broth with catabolite repression (SOC) was used for recovery following all electroporations of *E. coli* and *A. baumannii* during cloning or library preparations. Electroporations of *A. baumannii* were performed using 0.1cm gap cuvettes (0.2cm for *E. coli*) and the Gene Pulser (Bio-Rad) at the settings 200 Ω, 25 µF, and 1.8 kV (at 2.2kVd). As needed,

LB was supplemented with 10% Sucrose or antibiotics at the following concentrations: ampicillin (Amp): 100ug/mL; carbenicillin (Carb): 50-100ug/mL; gentamicin (Gent): 10ug/mL; chloramphenicol (Cm): 10ug/mL; kanamycin (Kan): 20µg/mL; or ciprofloxacin (Cip): varying concentrations (Sigma).

Molecular cloning and mutant construction. Primers (IDT) and plasmids described in this study as well as their sources are listed in Supplementary Table 1. The AdeS (R152S) mutant was selected for by drug selection in a precursor mutant strain, via plating on LB+Gent10. Single colonies were purified on LB agar plates, and then *adeR* and *adeS* amplified by colony PCR and sequenced (Genewiz) to identify what *adeRS* mutations were acquired. The selected *adeS* mutation (R152S) was introduced into the *gyrA(S81L)parC(S84L)*, or GP (GVA37), *A. baumannii* strain background to create *gyrA(S81L)parC(S84L)adeS(R152S)*, or GPS (EHA105), in a similar fashion as that described before (64). Briefly, the mutant allele was amplified with upstream and downstream flanking sequences to generate a product of about 1500bp in length and introduced first into pUC18 by restriction enzyme digestion and T4 DNA ligation. Once propagated in DH5α and sequence confirmed (Genewiz), the mutant allele was introduced into the pJB4648 plasmid by restriction enzyme digestion and T4 DNA ligation and propagated in DH5α λpir. The mutant allele in pJB4648 was then introduced into *A. baumannii* by electroporation and homologous recombination with two selection steps, the first selecting for transformants on LB+Gent10ug/mL agar plates and the second counterselection for $Gent^S$ on LB+Sucrose10% agar plates (during which time these plates were incubated either at room temperature or at 30^oC). The internal, in-frame gene deletion mutants were previously created or constructed during this study via three-way restriction enzyme digestions and ligations (35, 36,

64). GPL (EHA23), *gyrA(S81L)parC(S84L)adeL(ΔI335A336)*, was similarly constructed. The *adeL(ΔI335A336)* allele was PCR amplified from gDNA isolated from P9 Cy3.2 (44) with flanking regions, cloned into pUC18 then moved into pJB4648 as described before, propagated in XL1 Blues and DH5α λpir *E. coli*, sequence confirmed, then introduced into the GP background by electroporation and homologous recombination. GP was created previously (17) and *gyrA(S81L)parC(S84L)adeN(::ISAba1)* (GVA47), or GPN, generated by plating GP on LB+Cip32ug/mL.

LOS analysis. LOS profiles were examined using Tricine gel electrophoresis of cell lysates as previously described, using lysates from cells grown to mid-log phase, gels stained with Pro-Q Emerald 300 (Invitrogen), and Coomassie Brilliant Blue for total protein staining for sample quantification normalization (35).

pHi assay. Buffers of varying pH were made using 50mL combinations of 0.2M Na_2HPO_4 and 0.2M NaH_2PO_4 (65). 5mM BCECF/AM (Thermofisher) and 0.5mM carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma) were prepared in DMSO and stored at -20oC until use. Methods were adapted from previous literature with modifications (66). Overnight cultures made from individual colonies were diluted the next day for recovery before being diluted again to A_{600} ~0.003 in 8mL LB broth (with or without drug as indicated). In the case of dCas9 strains, single colonies were inoculated into 2mL LB in snap cap tubes and incubated to late exponential phase before diluting into 10mL LB + 100ng/mL anhydrotetracycline (aTc). Cultures were grown to exponential phase (reaching A_{600}=0.4-0.7) after which they were pelleted

in 15mL conical tubes at 7500g for 5 min, resuspended in sterile phosphate-buffered saline (PBS, Thermo Scientific), loaded with 20µM BCECF/AM in PBS followed by incubation at 30°C for 30 min in a 96-well v-bottom plate protected from light.

Cells in the 96 well plate were diluted 1:4, pelleted at 2000rpm for 5 min, resuspended in LB broth and incubated at 37°C for 5 min, before pelleting once more and re-suspending in 200-400µl previously prepared mono- and di-basic sodium phosphate buffer at neutral pH (experimental samples) or at a range of pH values (*in situ* standard curve samples). Samples were diluted 1:1 in the neutral or pH adjusted buffers to a final volume of 200µl in a 96-well black plate with a clear, flat bottom (Costar). About 10µM CCCP stock was added to the *in situ* standard curve wells. All samples were mixed via pipetting before fluorescence was read at 490ex/535em and 440ex/535em after 5 minutes using a spectrophotometer (Biotek SynergyMx with Gen5 software). Background fluorescence from buffer alone was subtracted from each sample before calculating the ratiometric fluorescence of the calibration curve samples and experimental samples. The experimental sample internal pH was interpolated from the *in-situ* calibration curve using standard curve fitting.

Preparation of transposon mutant banks. Transposon banks with *Himar-1* transposon were created as described (17, 35). 50µl electrocompetent *A. baumannii* cells were electroporated with 300ng pDL1100 (*Himar-1*-Kan^{R}). Cells were recovered in 1mL SOC broth, placed in 14mL snap cap tubes (Corning) and aerated on the drum roller for 15min at 37°C. The cells were pelleted and resuspended in 400µl SOC, then spread onto two 150 mm culture dishes containing mixed cellulose ester filters (0.45µm pore size, 137mm diameter; Advantec) that had been placed on top of the SOC agar. Plates were incubated for 1hr at 37°C before filters were

transferred to 150 mm culture dishes containing LB+Kan20µg/mL and incubated overnight (~16hrs) at 37°C.

For mini-Tn10 banks, electrocompetent cells were transformed with 75ng pDL1073 (mTn10, Kan^{R}), with subsequent steps following the same procedure as used for *Himar-1*. CFU counts were determined by grabbing images, determining thresholds and quantifying using Image J. Mutants were pooled from filters by gentle shaking in 50-75mL sterile PBS. Glycerol was added for a final concentration of around 10% and aliquots stored at -80oC until use. About 21 independent subpools were created in each strain background (producing around 100,000-150,000 cumulative unique mutants).

Cip challenge of transposon banks. Independent subpools comprising of approximately 7,500-16,000 unique transposon mutants were challenged as described (17, 35). Mutant banks were thawed on ice, diluted in 25mL LB broth to an A_{600}=0.1, grown to A_{600}=0.2 at 37°C, then diluted to A_{600}=0.003 in 10mL LB broth with or without ciprofloxacin of varied concentrations added in parallel cultures. The remaining culture (T_1) was harvested to determine what mutants were present prior to outgrowth and drug challenge. Cultures were incubated for about 8 generations (around 170min if untreated, 225min if Cip treated), then harvested (T_2). Drug-treated cultures that exhibited 20-30% growth inhibition were harvested. Cultures were pelleted and stored at -20°C until all samples were collected. The concentration ranges at which comparable growth inhibition was met for each strain background as well as the average population expansions between T_1 and T_2 with each condition (according to A_{600}) are noted in Supplementary Table 3.

Preparation of transposon libraries for sequencing. As described (17, 35), the gDNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen). Samples were treated with proteinase K during resuspension and incubated at 56°C with shaking for 1hr before treatment with RNase cocktail (Fisher Scientific) for 8min at 62°C. The gDNA was quantified with SYBR green then diluted to 7ng/µl in UltraPure water (Fisher Scientific). About 28ng of gDNA of each sample was tagmented using TD buffer and TDE1 enzyme (Illumina) and incubated in a thermocycler at 55°C for 5min prior to melting at 95°C for 0.5min. Transposon insertion sites were then amplified using Q5 hot start polymerase (NEB) and 0.6µM Nextera 2A-R and olj638 (for *Himar-1* libraries), or olj928 (for Tn10 libraries). The 50µl reactions were incubated at 98°C for 1min, cycled 30 times through 98°C for 10sec, 65°C for 20 sec, and 72°C for 1min, then finally at 72°C for 2min. 0.5µl of the first PCR products were used for a second PCR reaction used to introduce indexes with leftward *Himar-1*- or Tn10-specific indexed primers and rightward Nextera indexed primers. The reaction and settings were the same except cycling was shorted to 12 cycles. Amplified products were quantified via 2% agarose tris-acetate-EDTA (TAE) gels using SYBR Safe (Invitrogen) for visualization. The gels were loaded with a known amount of 100bp DNA ladder (NEB) alongside samples of each reaction to quantify their concentrations based on band intensity around 250-600bp using a quantitative gel imaging system. Equal measures of all samples were thereafter pooled and purified via PCR column clean-up (Qiagen). About 17.5 pmol of each pooled sample was reconditioned in an additional 50µl reaction with primers P1 and P2. Thermocycler settings were set with a heated lid and incubations at 95°C for 1min, with slow ramp of 0.1^{o}C/sec to 64°C then held for 20sec, and finally 72°C for 10min. The products were purified once more with the PCR column clean-up kit

(Qiagen). Primer olj115 (Tn10) or mar512 (*Himar-1*) was used for sequencing at the Tufts University Core Facility of Genomics with the Illumina HiSeq 2500 (SE50, post pippin size selection for 250-600bp).

Sequence analysis and assessment of mutant fitness. Sequenced reads were processed and analyzed as done previously (17, 34, 35, 67). After demultiplexing, the Nextera adapter sequence was clipped from reads using the fastx_clipper, quality filtered, and reads shorter than 20nt were discarded. Reads were mapped to the A. baumannii ATCC 17978 reference genome (https://genomes.atcc.org/genomes/e1d18ea4273549a0) using Bowtie 1.2.2 formatted so only unique and best mapped reads are aligned allowing up to 1 maximum mismatch. Fitness scores were then calculated for each transposon mutant using MAGenTA (67) and scores averaged and aggregated at the gene level across replicates. Insertions in the last 10% of genes were excluded and fitness scores normalized to a set of neutral/pseudo genes. The difference in fitness scoring for each gene disruption was assessed between each FQR strain and the WT drug susceptible strain. Significant fitness differences yielded from the same gene disruption between the different strain backgrounds were identified via multiple t test analysis using the original FDR method of Benjamini and Hochberg with significance falling within the FDR of 5% ($q<0.05$).

RNAseq experiment to uncover transcriptional differences between FQR and parent strains. Cultures were grown overnight, diluted to an A_{600} of 0.1 in 10mL LB broth, grown for about 1.5hrs at 37°C on a drum wheel to approximately mid-log growth, then diluted once more to A_{600} of 0.003 in fresh LB broth with or without ciprofloxacin. Some of these were

grown to approximately mid-log phase by incubation for 150-165min (untreated) or for 195-225min (ciprofloxacin treated) as similarly done before (17) and reaching A_{600}~0.445-0.68 and A_{600}~0.39-0.66 respectively. Others were grown to about early stationary for 195-210min (untreated) reaching A_{600}~0.85-1.195 or for 255-270min (ciprofloxacin treated) to A_{600}~0.82-1.125. Of the cultures treated with ciprofloxacin, those that exhibited 20-30% growth inhibition (compared to the untreated culture grown in parallel) were harvested (details provided in Supplementary Table 4). About 1-1.5 A_{600} units of all cultures were harvested and frozen at -80°C until all samples were collected.

Preparing RNA for sequencing. RNA was isolated from the samples using the RNeasy Mini kit (Qiagen) and prepared for sequencing as described (17, 68, 69) following published protocols (70). Approximately 600ng of RNA was fragmented at 94°C with 10x FastAP buffer for 2.5min then treated with TURBO DNase, FastAP (Thermo Scientific) (and murine RNase inhibitor; NEB) for 30min at 37°C. Samples were then purified using Agencourt RNAClean XP (Beckman Coulter) and eluted with 12.5µl of DEPC UltraPure water (Fisher Scientific). 5µl of sample was incubated with 1µl of 100µM barcoded RNA adapters at 72°C for 2 min before mixed with DMSO (American Bioanalytical), 10x T4 RNA ligase buffer, 100mM ATP, RNase inhibitor murine, T4 RNA ligase, and 50% PEG8000 (NEB) to allow for 3' linker ligation at room temperature for 1.5 hours. The 60 samples were pooled into 5 groups of 12 samples each during purification using RNA Clean & Concentrator (Zymo Research) and all eluted with 16µl DEPC UltraPure water.

Ribosomal rRNA was depleted using RNase-H as described (71). Probes complementary to *A. baumannii* ATCC 17978UN 23S, 16S, and 5S rRNA sequences (CP079931.1) were created

using 0.design_probe.py and off-target binding predicted using 2.predict_probe_offtarget.py (https://github.com/hym0405/RNaseH_depletion). The 89 devised oligos (IDT) were resuspended into 100µM in UltraPure water, and a master-mix created with 5µl of each probe. For each depletion the RNA was mixed with 1µl of the probe mixture, 0.6µl 1M Tris-HCl pH 7.5, 1.5µl 5M NaCl, and DEPC UltraPure water for a final 15µl reaction. They were incubated in a thermocycler with a heated lid at 95°C for 2min, temperature reduced to 45°C in 0.1^{o}C/sec increments and incubated a further 5 min at 45°C. Meanwhile, RNase-H master mix was prepared for each depletion (3µl Hybridase Thermostable RNase H; Epicentre), 0.5µl 1M Tris-HCl pH=7.5, 0.2µl 5M NaCl, 0.4µl 1M MgCl2, and 0.9µl DEPC UltraPure water) and preheated to 45°C. Post probe and RNA hybridization, 5µl prewarmed RNase-H mixture was added to each probe-RNA mixture and incubated at 45°C for 30min. These were then purified twice with 2x SPRI RNAClean XP (Agencourt) and eluted with 13µl DEPC UltraPure water.

The RNA preparation was then used as a template for cDNA synthesis. 12µl of sample was incubated with 2µl of 25µM AR2 primer at 72°C for 2min using 10x AffinityScript RT buffer and enzyme (Agilent Technologies), 0.1M DTT, 100mM dNTPs, and 40U/µl murine RNase inhibitor. After 55min at 55°C, RNA was then denatured in a 10% reaction volume (adding 2µl to the 20µl cDNA reaction product) of 1N freshly prepared NaOH at 70°C for 12min before neutralization with 4µl of 0.5M freshly prepared acetic acid. The products were then cleaned with Agencourt RNAClean XP, eluted with 5µl DEPC UltraPure water, incubated with 2µl of 40µM 3Tr3 adaptor at 75°C for 3min with the beads still present, and mixed with 10x T4 ligase buffer, DMSO, ATP, PEG8000, and T4 RNA ligase for an overnight 20µl ligation reaction at room temperature. The products were purified with SPRI RNAClean XP (Agencourt) beads twice and finally eluted with 25µl $H_2$0. DNA was amplified from cDNA using 10µl of 5x

Q5 reaction buffer and 2.5µl Q5 enzyme (NEB), 0.5µl 100mM dNTPs, 10µl cDNA, 2.5µl primers RTS_Enr_P5 and RTS_Enr_P7_BCXX, and water for 50µl reaction. The reactions were incubated at 98°C for 3min, cycled at 98°C for 30 sec, 67°C for 25sec, and 72°C for 15sec for 14 cycles, and completed at 72°C for 2min. PCR products were purified once with AxyPrep Magnetic PCR beads (Axygen; Corning). DNA was then quantified by Qubit with the dsDNA HS Assay kit (RNA HS Assay kit was used previously for the quantification of the RNA samples; Thermo Scientific) and about 100nM of each of the 5 subpools was pooled together into 1 sample for sequencing.

Throughout sample preparation, 1µl SUPERase-IN RNase inhibitor (Thermo Scientific) was added to samples if they were stored at -80°C in between steps. RNAse Zap was used continuously on surfaces until cDNA was synthesized. Samples were routinely analyzed by submitting samples for QC fragment analysis at the TUCF Genomics Core. The pooled samples were sequenced at this facility, using a NextSeq550 Single-End High output, with 75 cycles following nanoQC.

RNAseq analysis. Sequences that had been de-multiplexed for the i7 barcodes were then de-multiplexed for the i5 barcodes using fastx_barcode_splitter.pl (https://github.com/agordon/fastx_toolkit/). The 5’ anchored adaptor sequences were then trimmed *in silico* using Cutadapt (72). Truseq adaptor sequences were also trimmed *in silico*, disallowing any error, and dropping resulting sequences shorter than 20nt. Sequence data was thereafter analyzed following and modifying published scripts (https://github.com/huoww07/Bioinformatics-for-RNA-Seq). The Acinetobacter_baumannii_ATCC_17978.fasta and Acinetobacter_baumannii_ATCC_17978.gbk

files were downloaded from the ATCC genome portal (https://genomes.atcc.org/genomes/e1d18ea4273549a0?tab=overview-tab) to create the STAR genome index (via genomeGenerate with STAR). Annotation GTF files were constructed using Geneious then cufflinks gffread for conversion to strict gtf file. Alignments were then performed using STAR. Subread/1.6.3 was used to get a summary of the sequence data features, then featurecount_stat.R as well as mapping_percentage.R were adapted to analyze and visualize features including mapping efficiency and overview of mapped vs unmapped reads. Using the Tufts cluster on demand R studio app, DESeq2, ggplot2, dplyr, tidyverse, ggrepel, DEGreport, and pheatmap tools were employed to analyze the quality of the data set as well as visualize and deconvolve the contrasting transcriptomic data between the high-level FQR pump overproducing strains and the GP parent strain.

qPCR analysis for transcript abundance. Cultures derived from individual colonies were grown in LB broth to early post-exponential phase. About 1 A_{600} unit of culture was harvested and RNA was extracted using RNeasy (Qiagen). cDNA was synthesized after ezDNase treatment using the SuperScript VILO cDNA kit (Thermo Fisher), then amplified using PowerUp SYBR Green Master Mix (Applied Biosystems) and StepOnePlus Real-Time PCR system (Applied Biosystems) per manufacturer's instructions. The efficiency of target amplification was tested using a standard curve of cDNA at varying dilutions and consequently verified to be greater than 95% for each primer pair used. Three biological replicates were evaluated for each strain and three technical replicates examined per biological sample. To confirm a lack of gDNA contamination, control samples tested in parallel which lacked reverse-transcriptase during cDNA synthesis were included. Transcript levels for each indicated target

were compared between each mutant strain and the parental strain (via the comparative $2^{-\Delta\Delta CT}$ method) after normalization to that of 16S, the endogenous control. The data is represented in bar plots showing the mean fold difference in transcript levels ($2^{-\Delta\Delta CT}$) ± SEM.

MIC determination. MICs were determined by broth microdilutions. Overnight cultures were diluted to A_{600}=0.1 in 3mL LB broth, incubated to early exponential phase (A_{600}=0.2-0.3), then diluted into a final A_{600}=0.003 in 200ul LB broth in the presence of different dilutions of Cip in a 96-well plate (Costar). Growth was monitored by measuring A_{600} every 15min (Epoch 2 or SynergyMx, (Biotek) plate readers). At least three replicates were tested for all 29 strains except for Δ*mlaFEDCB* in the WT strain background (n=2). Cip MIC was determined as the lowest concentration that prevented growth up to 12 hours, where mean A_{600} units did not exceed 0.035, and they look like the LB only negative controls. Cip MICs were 256µg/mL (GPS and GPN) and 384µg/mL (GPL).

Persistence assay. The *A. baumannii* strains EHA417 (*adeL*ΔI335A336P_{Tet}*::dCas9* (pWH1266-*paaA*-sgRNA)), EHA418 (*adeL*ΔI335A336P_{Tet}*::dCas9* (pWH1266-NC-sgRNA)), and EHA254 (WTP_{Tet}*::dCas9* (pWH1266-NC-sgRNA)) were incubated in 1.5mL LB in triplicate for 2-3 hours. They were then diluted 1/1000 in 8mL LB + 100ng/mL aTc to induce dCas9 expression and grown to mid-exponential phase. Each culture was then normalized to A_{600}~0.5 with fresh LB containing inducer aTc, then treated with 10ug/mL Ciprofloxacin. Samples of culture (500µl) were removed at timepoints 0, 1, 6, and 24 hours, washed with PBS,

then resuspended in PBS and serial dilutions were spotted on LB agar plates for overnight incubation at 37°C and CFU enumeration the next day.

Accession numbers

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Author Contributions

R.R.I and E.G. designed the TnSeq study and provided training and support with experimental methodologies. E.H. and R.R.I devised the research. S.S. created mariner banks in the WT strain. A.C. created the mariner banks in the GPN mutant and performed TnSeq experiments with GPN and WT. J.B. performed the LOS experiments and analyses. W.H. provided bioinformatic support during trouble shooting, evaluated the frequency of pump regulator mutants among genomes in the PATRIC database, and ran the DESeq2 bioinformatic workflow for the comparative RNAseq study. E.G. created the vectors used to make two of the deletion mutants

and the backbone vector used for gene complementation. E.H. performed the experiments not listed here. E.H. and R.R.I interpreted the results. E.H. and R.R.I wrote the manuscript. E.G reviewed and revised the manuscript.

Figure legends

Figure 1. Uncovering the limitations of high-level FQR strains with enhanced RND pump expression. (A) Diagram for the three experimental approaches as described (Materials and Methods; Biorender). 1) Approach #1: Transposon insertion libraries were created in FQR strains overproducing efflux pumps and the drug susceptible WT strain. Insertion abundance across the genome between the two strain types was compared to identify genes with fewer insertions in the FQR strain background than in WT (see B for example). 2) Approach #2: Transposon insertion banks were challenged with sub-MIC levels of Cip, parallel to those grown without drug. Fitness of all transposon mutants was calculated and compared between the strain types. 3) Approach #3: Strains were grown in presence or absence of Cip to exponential or post-exponential phase in presence or absence of drug and transcriptomic data was obtained to discover differentially regulated pathways. (B) Example of insertion abundance in a single gene cluster in two strain backgrounds. (C) Activating mutations in efflux pump regulatory mutants specifically activate their cognate pumps. RNA was isolated from the noted strains and relative transcription determined by q-rtPCR (Materials and Methods). Displayed is mean fold change ± SEM of each target as compared to the parental GP strain. At least three independent biological replicates were used for each strain. FQR: fluoroquinolone resistant. Mid: mid exponential phase. EPE: early post-exponential phase. Cip: Ciprofloxacin. Untx: untreated. WT: wildtype. GPL: *gyrAparCadeL*. GPN: *gyrAparCadeN*. GPS: *gyrAparCadeS*. GP: *gyrAparC*.

Figure 2: Discovery of genes with lowered tolerance for insertions in the high-level FQR strains. (A) Volcano plots in which statistical significance is plotted as a function of the differences in insertion abundance between each FQR strain and WT based on TRANSIT algorithm (31). All adjusted p-values = 0 were assigned the value of 0.005 to allow graphing. Blue: genes with adjusted p-values<0.05. Values for all other genes are shown in grey. (B) Number of reads plotted as a function of insertion site for noted genes as well as flanking regions. Shown are: FQR: fluoroquinolone resistant. WT: wildtype. GPL: *gyrAparCadeL*. GPN: *gyrAparCadeN*. GPS: *gyrAparCadeS*.

Figure 3: Maintenance of cell envelope homeostasis supports the fitness of efflux pump overproducer strains. (A, E): Statistical significance plotted as a function of relative fitness comparing identical mutations in FQR and WT strains grown in the absence (A) or presence (E) of sub-MIC levels of Cip. (C) Growth rates at noted Cip concentrations to identify conditions allowing 20-30% growth inhibition in the FQR and WT strains. (A) Blue or (E) Red (B): Mutations above the FDR = 5%, q<0.05 as determined by Benjamini and Hochberg (73). Notable findings are labeled. All q-values < 0.000001 assigned the value of 0.000001 for graphing purposes. (B, D) Heatmaps showing fitness of insertions in each gene in noted strain backgrounds. Selected genes with Tn-disruptions yielding lowered fitness in the FQR strains are shown, in the absence of drug treatment (B) or in the presence of Cip (D). Gene names are followed by accession numbers which are preceded by "NDEONHPJ_" per the ATCC 17978 strain annotations. Shown are: WT: wildtype. GP: *gyrAparC*. GPS: *gyrAparCadeS*. GPN: *gyrAparCadeN*. GPL: *gyrAparCadeL*.

Figure 4: OmpA and BfmS are critical fitness determinants for *adeL*ΔI335A336 strains during Cip exposure. (A) The Single Fitness pipeline of MAGenTA was used to evaluate the fitness of Tn mutants at each position across the loci shown all post Cip treatment. (B) Null mutants in the FQR strain backgrounds grown in the presence of Cip compared to the WT background. Growth was monitored by taking absorbance measurements every 15 minutes in 96 well plates in presence of Cip. Displayed are means ± SEM (n≥3). Ciptx: Ciprofloxacin treated. WT: wildtype. GPL: *gyrAparCadeL*. GPN: *gyrAparCadeN*.

Figure 5: LpsB and PbpG are imperative for the AdeN (GPN) and AdeL (GPL) mutants to withstand Cip treatment. (A) Transposon mutant fitness post Cip treatment at each insertion position across the selected loci. (B) Growth of the indicated strains in the presence of Cip was monitored as in Fig. 4, displayed as mean ± SD (n≥3). (C) Growth of deletion mutants in LB + 65 µg/ml Cip + 1mM IPTG to induce expression of the indicated genes complemented *in trans* in GPN (column 2) or GPL (column 4) backgrounds. Data are means ± SEM (n≥6). Ciptx: Ciprofloxacin treated. IPTG: Isopropyl β-d-1-thiogalactopyranoside. WT: wildtype. GP: *gyrAparC.* GPL: *gyrAparCadeL*. GPN: *gyrAparCadeN*. EV: empty vector.

Figure 6: The absence of either PbpG or LpsB alters LOS levels. (A) Extracts of noted strains were fractionated on Novex 16% tricine gel and stained with Pro-Q Emerald 300 to reveal LOS. Representative images of three independent experiments are shown (left). Quantification of gel fractionated LOS, normalized to total protein (right). Shown are means relative to WT ± SD

(n=3), individual LOS fractions in FQR strains vs in GP are compared using two-way ANOVA with Dunnett's multiple comparisons (right). (A) Parent strains. (B) *pbpG* deletion mutants. (C) *lpsB* deletion mutants. Significant differences are marked with an asterisk, *=p-value of 0.0498. F: Full length LOS. I: Intermediate. U: Unspecified LMM (low molecular mass) species. WT: wildtype. GPL: *gyrAparCadeL*. GPN: *gyrAparCadeN*. GPS: *gyrAparCadeS*. GP: *gyrAparC*.

Figure 7: Transcriptional and physiological response to pump overproduction. (AB) Alterations in intracellular pH (pHi) in response to pump overproduction. Noted strains grown to mid-exponential phase in the absence (A) or presence (B) of Cip. Internal pH (pHi) was measured using 20µM BCECF-AM as described (Materials and Methods). Displayed are the means ± SEM for each strain from n=5-8 biological replicates, with P values derived from one-way ANOVA making multiple comparisons. (C-F) Transcriptional alterations in response to pump overproductions are magnified in presence of *adeL*ΔI335A336 mutation. Transcriptomic data (n=3) was analyzed comparing high-level FQR pump hyper-expressing strains and the GP parent strain (Materials and Methods). Differences in transcript levels for each comparison are shown via the heat maps with GP vs GPL (C, +Cip) and (D, -Cip), GP vs GPN (E) and GP vs GPS in (F). * = $p < 0.05$ (0.0259); ns=not significant. WT: wildtype. GPL: *gyrAparCadeL*. GPN: *gyrAparCadeN*. GPS: *gyrAparCadeS*. GP: *gyrAparC*. Mid: mid-exponential phase. EPE: early post-exponential phase.

Figure 8: The presence of *adeL*ΔI335A336 in GPL results in overexpression of the *paa* operon. RNA was isolated from GP and GPL strains and transcription of *paaA* gene was

quantified by q-rtPCR. Strains grown to exponential (A) or early post-exponential phase (B) in absence of drug. Data are mean fold change in transcript levels ($2^{-\Delta\Delta CT}$) ± SEM of each target (n=3) compared to parental GP strain, analyzed by two-tailed t test. Significance marked by asterisks; **, p=0.0099; ***, p=0.0004. (C, D) Alterations in transcript levels in CRISPRi-depleted *paaA* strain, with knockdown verified as described (Materials and Methods). RNA was prepared from cultures grown to mid exponential (C) or early post-exponential phase (D). Data are transcript levels of each target relative to nontargeting negative control ($2^{-\Delta\Delta CT}$) ± SEM (n=3), analyzed by multiple unpaired t test analyses following the two-stage step-up Benjamini, Krieger, and Yekutieli, false discovery rate of 5.00% (74). Significance: *, p=0.020-0.034; **, p=0.002-0.003; ****, p<0.001. (E-G). Acidification of cytosol as a consequence of *paaA* depletion. Internal pH (pHi) determined during mid-exponential phase in absence (E, F) or presence (G) of Cip. (F). Intracellular pH in early post-exponential phase. Data are mean ± SEM from n=5. P values were derived from an unpaired t test comparing the mean pHi values between the two strains. (***, p<0.0009 in E) & (ns, p=0.8479 in F or 0.1821 in G). Untx: Untreated. Ciptx: Ciprofloxacin treated at 30ug/mL. Mid: grown to mid-exponential phase. EPE: grown to early post-exponential phase. GPL: *gyrAparCadeL*. GP: *gyrAparC*.

Figure 9: *paaA* is significantly upregulated during Cip exposure. (A,B) The *paaA* transcript (shown with L2FCSE) is upregulated after Cip exposure independently of strain background. Strains were grown to noted growth phases, with data derived from RNAseq experiments (Fig. 7; Materials and Methods). Significance determined by s values from triplicate cultures for each (s ≤ 1.11E-03). (C) Persistence of *adeL*ΔI335A336 single mutant (in WT strain background) is unaffected by depletion of *paaA*. The indicated strains were grown to exponential phase with aTc

inducer to deplete *paaA* transcript, then exposed to Cip at ~20X MIC for the noted times prior to plating on LB agar plates in absence of drug to determine CFU/mL. The mean CFU/mL ±□SEM are shown (n=3). The dotted line indicates LOD, the limit of detection for the assay (375 CFU/mL). Mid: grown to mid-exponential phase. EPE: grown to early post-exponential phase. aTc: anhydrotetracycline. WT: wildtype. GPL: *gyrAparCadeL*. GPN: *gyrAparCadeN*. GPS: *gyrAparCadeS*. GP: *gyrAparC*.

Supplemental Figure 1. CyoA-targeted KD causes a fitness defect. (A) Indicated strains grown in LB broth + 100ng/mL aTc. A_{600} for culture density are displayed and their mean ± SEM (n=4-5). *cyoA*-KD: strain harboring pWH1266 plasmid with *cyoA*-sgRNA vs NC-sgRNA is with nontargeting sgRNA. (B) Culture samples harvested in exponential phase and transcript levels determined by q-rtPCR (Materials and Methods). Displayed is mean fold change in transcript levels ± SEM of each target compared to NC-sgRNA strain (n=3). (C) Read number plotted as function of insertion site. Shown are: KD: knockdown. aTc: anhydrotetracycline. WT: wildtype. GPL: *gyrAparCadeL*.

Supplemental Figure 2. OmpA, BfmS, LpsB, and PbpG are critical fitness determinants for *adeL*ΔI335A336 strains during Cip exposure. Null mutants in FQR strain backgrounds compared to the GP parent strain background. Growth was monitored every 15 minutes in 96 well plates. Displayed are means ± SEM (n≥3). Ciptx: Ciprofloxacin treated. GP: *gyrAparC*. GPL: *gyrAparCadeL*. GPN: *gyrAparCadeN*.

Supplemental Figure 3. Heatmaps of the normalized fitness of each transposon mutant in the listed FQR strain and WT strain grown in indicated conditions relative to the fitness of the population, alongside difference in fitness scores of the two strain backgrounds in that growth condition (fitness in the high-level FQR strain – fitness in the WT drug susceptible parent). Displayed are selected genes in which Tn10- and *Himar-1*-disruptions caused significantly lowered fitness in the FQR strains in the absence or the presence of Cip (Significance described in Materials and Methods). Gene names are followed by accession numbers which are preceded by "NDEONHPJ_" per the ATCC 17978 strain annotations. Cip treated are those exposed to Ciprofloxacin (Cip) at concentrations yielding 20-30% growth inhibition in each parent strain compared to the untreated grown in parallel. WT: wildtype. GPL: *gyrAparCadeL*. GPN: *gyrAparCadeN*. GPS: *gyrAparCadeS*.

Supplemental Figure 4. The observed minimum inhibitory concentrations (MIC) of Cip. Growth conditions described in Fig. 4. A_{600} values are displayed with mean ± SEM shown (n≥3). The observed MIC of Ciprofloxacin (Cip) for the indicated null mutation strains are exhibited as relative to their parent strains' MIC (given a value of 1) (C). MIC exceeded this value (arrowhead). WT: wildtype. GPL: *gyrAparCadeL*. GPN: *gyrAparCadeN*. GP: *gyrAparC*.

Supplemental Figure 5: Nonadditive effects of overproducing two efflux pumps. At least three biological replicates of each strain were tested, all grown in LB with Ciprofloxacin concentrations yielding comparable growth inhibition of the parent strains, 0.12µg/mL or 60µg/mL (A) or 0.14µg/mL (B). After growing to early post-exponential, all cultures were

diluted to A_{600}~0.003 in LB broth and culture density monitored every hour for five hours (A) or every 15min overnight in a 96-well plate, though the first 8 hours are shown (B). Displayed is the mean of the replicates ± SEM.

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