Mutations to *ettA* and *lepA* Cause *Acinetobacter baumannii* to Overcome Growth Defects in the Δ*csrA* Strain in the Presence of Amino Acids

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July 2023

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Acinetobacter baumannii is a Gram-negative bacterial pathogen which often causes nosocomial infections. *A. baumannii* is known for its incredible ability to survive drying and its high rates of multidrug resistance. Carbapenem resistant *A. baumannii* (CRAB) has even been classified as a critical priority for new drug discovery by the World Health Organization. We found that the gene *csrA* is essential for *A. baumannii* to grow within a host, and that deletion of *csrA* results in growth inhibition in complex media or media containing amino acids. To try and understand this growth inhibition, suppressor mutants of a *csrA* deletion strain where generated which regained the ability to grow on complex media. Some of these suppressor mutants contained point mutations in either of the genes *lepA* or *ettA*, which both produce proteins thought to be involved in translational regulation. We generated deletion mutants of both genes individually in wild-type and Δ*csrA* backgrounds to explore their roles in growth

inhibition. We also recreated point mutations to both genes, identical to those seen in suppressor mutants to ensure these mutations were the cause of the rescued growth in Δ*csrA*. Analysis of growth for each strain was performed on lysogeny broth (LB) and chemically defined growth media (MOPS-A) with and without the addition of amino acids. Lysates of each strain were also analyzed by SDS-PAGE to compare protein expression. Long-term growth rates of wild-type and Δ*ettA* strains of *A. baumannii* were measured over 98 hours to see if EttA provided a survival advantage. Finally, EttA and the EttA point mutant (EttAE470V) were purified and ATPase activity was measured for each. We observed improved growth of the suppressor mutants and point mutants in Δ*csrA* on LB agar compared to Δ*lepA*Δ*csrA* and Δ*ettA*Δ*csrA* double mutants and observed similar results during growth in MOPS-A broth supplemented with amino acids. SDS-PAGE analysis showed different protein expression patterns for both suppressor mutants and point mutants of *ettA* when compared to the other strains. We observed higher CFU counts for the wild-type strain compared to Δ*ettA* strain after 74 hours of incubation in LB. Finally, we found a significantly lower rate of ATP hydrolysis for EttAE470V compared to EttA. Based on these results, we concluded that the rescued growth in the suppressors is the result of a partial change in the structure of the proteins LepA and EttA that altered their function. We also found that the point mutation in *ettA* results in altered translation of specific proteins in *A. baumannii* which may be involved in the growth phenotype. Overall, these results showed that LepA and EttA are involved in growth defects observed in Δ*csrA*, and that EttA is important for *A. baumannii* survival during starvation, which may help it persist in hospitals.

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A Thesis

Presented to the Faculty of the Department of Microbiology and Immunology

Brody School of Medicine at East Carolina University

In Partial Fulfilment of the Degree

Master of Science in Biomedical Sciences

By

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July 2023

Director of Thesis: John M. Farrow III, PhD

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Dedication

This work is dedicated to my grandfather William K. Hinton who supported me in furthering my education and seeking a graduate degree.

Acknowledgments

I first want to thank my thesis director, Dr. John Farrow, for sharing his knowledge of molecular biology techniques and bacterial genetics with me. As well as for being supportive of me during my research and writing efforts. Additionally, I would like to thank the other members of my thesis committee: Drs. Everett Pesci and Brandon Garcia for agreeing to sit on my committee as well as giving important advice and uplifting support.

I would also like to thank my fellow ECU Graduate students: Ryan Garrigues, Denise Rohlik, Connor Cribb, and Charles Booth for their advice and support.

I would also like to thank Greg Wells, the lab technician of the Pesci lab for sharing with me his knowledge of laboratory equipment and procedures which enabled me to undertake experiments required for this thesis.

Finally, I would like to thank my family and friends for their constant support, including my parents, Frank and Kellie Wood, and my grandmother Barbara Hinton.

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Chapter I

Introduction

1.1: *Acinetobacter baumannii*

Acinetobacter is a diverse genus of Gram-negative, aerobic, coccobacilli containing more than 50 species. While most *Acinetobacter* are non-pathogenic and persist in the environment, some are known to cause opportunistic nosocomial infections (1)(2). Of the pathogenic *Acinetobacter* that cause nosocomial infections, *Acinetobacter baumannii* is the most virulent (2). *A. baumannii* has an incredible ability to survive for long periods on dry surfaces along with possessing high rates of multi drug resistance. *A. baumannii* employs mechanisms of complement evasion to avoid phagocytosis during pathogenesis. It is proposed to evade alternative pathway complement killing through the binding of factor H to its outer membrane proteins. This neutralizes factor H which is an important component of the alternative complement pathway (2)(25)(26). Once the bacteria gain a foothold, it is believed that its lipopolysaccharides trigger Toll-Like Receptor 4 in macrophages which initiates septic shock (2)(23). When causing nosocomial infections, *A. baumannii* primarily causes pneumonia and urinary tract infections. Transmission of these infections often occurs through exposure to contaminated hospital equipment such as ventilators and catheters. Additionally, *A. baumannii* is known to infect wounds. *A. baumanni* gained the colloquial name “Iraqibacter” due to major outbreaks among wounded soldiers during the Iraq war (2003-2011) (2)(23). Increases in multidrug resistance and hospital acquired infections have led to a growing interest in *A.*

baumannii as a pathogen. This thesis will focus on gene regulation within *A. baumannii* and genes important for its growth in complex media and the host environment.

1.2: Bacterial defense systems

1.2.1: Multidrug resistance

Due to the persistent use of antibiotics and exposure to infections in hospitals, multidrug resistance has become an increasing threat. One 2019 study suggested that as much as 15.5% of hospital infections originate from multidrug resistant bacteria (4). A small group of bacterial species have been grouped together as the "ESKAPE" pathogens, which includes: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp. (5). These species all have a great ability to "escape" killing by drugs in the hospital setting.

Acinetobacter baumannii is among the ESKAPE pathogens due to its high rates of multidrug resistance. *A. baumannii* has adopted mechanisms such as the use of β-lactamases and efflux pumps, along with permeability defects and alteration of target sites (23). These mechanisms have given *A. baumannii* an incredible ability to survive many of the most effective antibacterial drugs.

One study found that between 2004 and 2014, the percent of *A. baumannii* clinical isolates carrying multidrug resistance increased from 23% to 63% globally, with the overall rate of multidrug resistance being approximately 44% (6). Additionally, carbapenem resistant *A. baumannii* (CRAB) has emerged as a particularly dangerous variation of *A. baumannii*, as carbapenems are a last line of defense. Thus, when carbapenems stop being effective, clinicians must turn to less effective antibiotics or

antibiotics with undesirable side effects (2)(27). This is important because rapidly clearing the bacteria early in the course of infection can result in better outcomes for patients compared to early ineffective therapy (2).

In the United States in 2017 the estimated number of hospital-acquired CRAB infections was approximately 8,500 with approximately 700 deaths (7). From 2019 to 2020, rates of hospital acquired multidrug resistant infections increased overall due to the COVID-19 pandemic. In this time, the rate of carbapenem resistant *Acinetobacter* infections increased by 78%, the highest increase of any drug resistant bacteria (8). Results like these have caused the World Health Organization (WHO) to label CRAB as a critical priority for new drug discovery, their highest priority level (21).

1.2.2: Drying resistance

Another mechanism which contributes to *A. baumannii* pathogenicity is its incredible ability to survive desiccation on dry surfaces for long periods. Most strains of *A. baumannii* can survive on dry surfaces for over 20 days (9) with some strains capable of surviving on dry surfaces for over 3 months (10). It is this phenotype which allows *A. baumannii* to persist and spread rapidly in hospitals. Additionally, *A. baumannii* cells have even been identified from air and water samples from hospitals (45)(46). Inanimate surfaces can act as reservoirs for contamination and spur hospital outbreaks (45)(46). A*. baumannii* can be spread to hospital equipment such as ventilators and catheters by patients or medical staff (3). The use of contaminated equipment on patients can lead to a vicious cycle where patients become infected and then further infect staff, other patients, or more equipment, leading to additional infections (3).

Previous research by this lab into *A. baumannii* desiccation tolerance has uncovered *csrA* as a gene important for drying survival. Mutations in the gene *csrA* resulted in increased sensitivity to drying in strains normally resistant to desiccation (11). This was discovered by screening for laboratory adapted strains with a reduced ability to survive drying after growth in LB broth for five to seven passages (11). The focus of this thesis will be on *csrA*'s impact on *A. baumannii's* growth.

1.3: Background of the project

1.3.1: Function of CsrA in *Acinetobacter baumannii*

The gene *csrA* encodes the protein Carbon Storage Regulator A (CsrA) which is an RNA binding protein shown to upregulate genes which are normally expressed in stationary phase and under stressful conditions while downregulating genes normally expressed in exponential phase (12)(44). CsrA has also been shown to be important for biofilm formation, quorum sensing, carbon metabolism, motility, and stress responses in other species (12)(44), but its role in *A. baumannii* is not well characterized. In most cases CsrA regulates its target genes through post transcriptional regulation by binding mRNAs and occluding the Shine-Dalgarno sequence. This blocks ribosomes from binding these mRNAs and prevents translation of the peptide they encode (12)(44). CsrA can itself be sequestered by small RNAs. Small RNA binding to CsrA reduces the amount of free CsrA within the cell and allows translation of CsrA target genes (12)(44).

When attempting to generate an in-frame deletion of *csrA*, it was discovered that *csrA* deletion mutants of *A. baumannii* strains AB09-003 and ATCC 17961 failed to grow on media containing amino acids (11). This was resolved through growth of *csrA* deletion mutants on 3-(N-morpholino) propane sulfonic acid-buffered defined medium

for *Acinetobacter* (MOPS-A), which contains no amino acids and is supplemented with succinate (11). It was shown that *csrA* mutants of *A. baumannii* strain AB09-003 and ATCC 17961 were more sensitive to drying. It was also shown that CsrA was important for biofilm formation and decreased the cell's ability to tolerate increased osmolarity in addition to being involved in regulating stress response and metabolism similarly to what had been observed in other species (11). Finally, the *csrA* deletion mutants were unable to infect a waxworm model or grow in human serum, suggesting *csrA* is important for virulence (11). Despite these observations, it remained uncertain why the *csrA* deletion mutant would not grow in the presence of amino acids (11). Similar growth defects had been seen before in *csrA* mutants in other species under similar conditions (19).

1.3.2: Suppressor mutants for *csrA* deletion

By growing *A. baumannii csrA* deletion mutants in LB media (a complex media), our lab generated suppressor mutants which regained the ability to grow on complex media, or in the presence of amino acids. A total of 21 suppressors were generated and analyzed by whole genome sequencing, and point mutations that altered the coding sequence for genes were identified in seven of them. Other suppressors had variants in repetitive regions such as phage-related genes or rRNAs. Three had premature stop codons in the gene *lepA*, and four had point mutations in the gene *ettA* which altered the EttA protein sequence so that glutamate 470 was converted to valine (Table 1). Both LepA and EttA are believed to be involved in regulation of bacterial translation. Additional suppressor mutants had no identifiable genetic changes. The goal of the

Table 1. Suppressor mutations in Δ*csrA* that rescued growth in complex media.

	Date of Isolation	Gene	DNA Change	Impact	Amino Acid change
Suppressor 1	2-18-21	*lepA*	638T>A	Early Stop Codon	L213*
Suppressor 4	2-24-21	*ettA*	1409A>T	Glutamate to Valine	E470V
Suppressor 5	2-24-21	*lepA*	339C>T	Early Stop Codon	Q447*
Suppressor 12	3-25-21	*ettA*	1409A>T	Glutamate to Valine	E470V
Suppressor 13	3-25-21	*ettA*	1409A>T	Glutamate to Valine	E470V
Suppressor 14	3-25-21	*ettA*	1409A>T	Glutamate to Valine	E470V
Suppressor 20	5-4-21	*lepA*	1262C>T	Early Stop Codon	Q421*

* Denotes premature stop codon.

studies performed for this thesis was to identify how the mutations discovered here led to rescued growth in complex media or media containing amino acids.

1.3.3: EttA

The *ettA* gene encodes the protein energy-dependent translational throttle A (EttA) (formerly Yjjk) and has been proposed to gate entry of the ribosome into the elongation cycle of translation based on ATP/ADP ratios (13). In 2014, Böel et al., solved the crystal structure of EttA in *Escherichia coli.* EttA is an ABC-F protein containing dual ABC domains in the active monomer form (13)(22). Proteins in the ABC-F family appear to function as translation factors which bind to the E site of the ribosome where they can regulate protein expression. Some members of this family of proteins have been shown to mediate antibiotic resistance to compounds which target the 50S subunit of the ribosome (47). The ABC-F family of proteins comprises proteins with 2 ATP binding cassettes (ABCs) and are not believed to be membrane bound as they have no membrane spanning domains (24). *EttA* encodes the protein energy-dependent translational throttle A (EttA) (formerly Yjjk) and has been proposed to gate entry of the ribosome into the elongation cycle of translation based on ATP/ADP ratios (13).

Böel et al., (13) proposed that EttA bound to ADP will bind to the E site within the ribosome and block movement of tRNAs from the P to E site. When EttA binds and hydrolyzes ATP, the resulting conformational change causes the protein to dissociate from the ribosome (13). EttA has been shown to be highly conserved, having orthologs in over half of all eubacteria species (13). The EttA homolog in *A. baumannii* has approximately 71% similarity with its *E. coli* counterpart based on results using the

sequence alignment tool on uniprot.org. Several key sequences such as the LSGGE signature sequences and the Walker A and B sites are conserved between the two homologs.

The Walker A motif (also called P-loop) is a conserved sequence generating a 3D structure on the protein capable of binding the gamma-phosphate of ATP. The Walker B motif is a conserved sequence with residues which stabilize cofactors such as water and Mg^{2+} which are necessary for ATP hydrolysis (13)(14)(28). The LSGGQ sequence (LSGGE in EttA) is believed to encapsulate the triphosphate and ribose of the ATP bound to the opposing subunit in a nucleotide bound conformation (13).

The sequences described above normally drive the ATPase power stroke through the formation of an ATP bound dimer or “ATP sandwich dimer” (13)(14). Because ABC-F proteins contain two ABC domains, they undergo the same process without forming a dimer, instead a simple change in conformation occurs (13). Within the Walker B motif, ABCs contain a “catalytic base” which is a glutamate located in the DEPT sequence (14)(15). This residue is vital to an ABCs ability to hydrolyze ATP because of the ability of its carboxylate to accept protons from a water molecule which then donates a pair of electrons to the gamma-phosphate of ATP resulting in hydrolysis (14)(15). When this glutamate residue is mutated to a glutamine the amine group on its side chain donates a hydrogen bond to this same water molecule and inhibits ATP hydrolysis (14).

In the EttA homolog in *E. coli*, when glutamate 188 and 470 are both mutated to glutamine (EttA-EQ_2), the protein is proposed to have lost its ability to hydrolyze ATP while continuing to bind ATP (13). Böel et al., (13) showed that upregulating EttA-EQ_2

inhibited cell growth and translation in *E. coli*. They also showed EttA-EQ_2 prevented the formation of a second peptide bond during translation. It was proposed that nucleotide bound EttA binds to the E site of the ribosome to prevent the first translocation during translation, preventing the first tRNA from exiting the ribosome and a third tRNA from entering the A site (13)(22). However, EttA prevented the formation of any peptide bonds when elevated levels of ADP were present relative to levels of ATP (13).

It was proposed that EttA may cause hibernation of active ribosomes in energy depleted cells. This was supported by the observation that *ettA* deletion mutants had a disadvantage compared to wild-type cells when grown in LB for 6 days and then grown for 24 hours in fresh LB (13). It was proposed that, while in an ATP depleted state, initiation complexes bound to EttA would form. These complexes would not allow translation to occur but would be poised to rapidly resume protein synthesis once ATP becomes available (13).

It is interesting to note that the suppressor mutants we identified in *ettA* all generated a glutamate to valine substitution in the catalytic base of the second Walker B motif, which is one of the same residues mutated to generate EttA-EQ_2. Like glutamine, valine also does not carry a carboxylate, suggesting that the EttA generated from this mutation in *A. baumannii* (EttA-E470V), may also lose its ability to hydrolyze ATP at this site. However, valine is very different from both glutamine and glutamate in that it is nonpolar and noncharged, suggesting structural changes may be occurring which are more significant than the glutamate to glutamine change. It is worth noting that the first catalytic base remained unmutated in our suppressor mutants.

1.3.4 LepA

LepA encodes the protein leader peptidase A (LepA) (also known as Elongation Factor 4 or EF4) which is a noncanonical GTPase. *LepA* is highly conserved, being present in most bacteria with exceptions such as *Streptococcus pyogenes* and *Carsonella ruddii*. *LepA* is also present in the mitochondria of eukaryotes, although it is absent in the cytosol of eukaryotes (16). Unlike the canonical translational GTPases (trGTPases) IF2, EF-TU, EF-G, and RF3 which all have known roles in translation, the exact function of LepA remains unknown, although several theories exist regarding its function (16).

LepA possesses much structural similarity to EF-G but unlike EF-G, LepA lacks domain IV and has a unique C-terminal domain (CTD) (17). Since EF-G drives translocation during translation and its domain IV acts as a doorstop preventing "back-translocation", it was proposed that LepA functioned to induce "back-translocation" (16)(17). Back-translocation is the reverse of the translocation process involving the movement of tRNAs back to previous sites in the ribosome (a tRNA in the E site will go to the P site and a tRNA in the P site will return to the A site). To support the back-translocation theory, it was shown that with the addition of LepA and GTP to the "POST" state complex (tRNAs in the E and P sites), the ribosome appeared to revert back to the "PRE" state (tRNAs in the P and A sites) (17).

As evidence for the transition from the Post to the Pre state, an experiment was done with puromycin. Puromycin is an aatRNA 3' end analog which binds to the A site in POST state ribosomes but not PRE state ribosomes (because the A site is occupied). When LepA and GTP were added to POST state ribosomes followed by puromycin, the

puromycin was unable to bind to the ribosomes suggesting that LepA caused the POST state to revert to the PRE state (17).

Additionally, a toeprinting assay was done by adding primers upstream from the ribosome and reverse transcribing the RNA. Reverse transcriptase will stop once it reaches the ribosome so the length of the reverse transcribed DNA will correspond with the location of the ribosome on the RNA. Longer DNA sequences will correspond to the ribosome being located at an earlier codon in the coding sequence. This was done with PRE and POST state ribosomes. When LepA and GTP were added to the POST state and the sequences were run on a gel, approximately 77% of the DNA was in the PRE state band (three nucleotides longer) compared to approximately 18% from spontaneous back-translocation when no LepA was added (17). Taken together these results seemed to suggest that the role of LepA was in back translocating the ribosome during translation. Back-translocation may be an important function in that it may give EF-G a “second chance” to translocate tRNAs properly after a defective translocation, thus ensuring proteins carry correct amino acid sequences (17).

Alternatively, it has been proposed that LepA functions in ribosome biogenesis. The results presented by Qin et al., where they proposed that LepA functions in back-translcation have been difficult for other researchers to replicate (18). In 2017, Gibbs et al., showed that deletion of *lepA* in *E. coli* led to the accumulation of ribosome small subunit particles (SSU) lacking S3, S10, S14, and S21 (18). Mutation of *lepA* in addition to *rsgA* (a protein responsible for ribosome assembly) exacerbated this problem leading to a growth defect (18). These results appear to show that LepA functions in ribosome biogenesis but that it is only critical in the absence of RsgA (18).

Finally, LepA has been proposed as a ribosome-sequestering factor. It was identified in 2011 by Liu et al., (20) that LepA binding to the PRE state resulted in the formation of a ribosome species called X_3. X_3 is identical to a similar structure called I_3 which was proposed to be generated due to LepA binding to the POST state (20). The X_3/I_3 complex presumably acts as an intermediate of the PRE and POST complexes based on levels of mRNA fluorescence and puromycin reactivity (20). Ultimately, it was proposed that LepA competes with EF-G for binding to the PRE state ribosome and may lead to "transient inhibition" of the ribosome under conditions of growth stress (20).

Regardless of LepA function, the gene *lepA* has been linked to stress response. Bejilsma et al., showed that *lepA* was one of the genes essential for *Helicobacter pylori* growth in low pH (31). Since *H. pylori* must be able to survive in low pH environments to invade the stomach and cause stomach ulcers, LepA is essential for its pathogenesis. In 2011, Pech et al., showed that Δ*lepA* strains of *E. coli* had impaired growth compared to wild type in 100 mM $MgCl_2$, at pH 6, and at 16°C (30). Additionally, Pech et al., (30) showed that LepA migrated from the membrane to the cytosol at pH 6, in 100 mM $MgCl_2$, or at 16°C. This was discovered using an anti-LepA antibody to tag proteins in the cytosolic and membrane fractions after lysis and centrifugation (30). Finally, they showed that wild-type cells had five-fold greater levels of protein synthesis compared to Δ*lepA* at 14 mM Mg^{2+} (30).

Pech et al., proposed that the membrane functioned as a storage holder for LepA under normal conditions and that LepA would be released under stress conditions (30). This would be a more rapid way of generating cytosolic LepA under stress conditions compared to increasing gene expression. During periods of stress, LepA may be

entering the cytosol to interact with the ribosome, either to support translation through initiation/ribosome biogenesis, or to stall the ribosome (16).

1.4: Project objectives

Based on the sequencing results of our suppressor mutants, and what is known about CsrA, EttA, and LepA, we set out to answer several questions. We wanted to know if deletion of either of the genes *ettA* and *lepA* would rescue growth for strain 17961- Δ*csrA,* or if regeneration the specific point mutations seen in our suppressors would be required. We also wanted to know if our suppressors would express proteins differently than wild type or strain 17961- Δ*csrA* considering that both *ettA* and *lepA* have been linked to translational regulation in *E. coli*. Because EttA had been shown to provide a competitive advantage during long term survival in *E. coli* (13), we wanted to know if the same would be true for *A. baumannii*. If so, then EttA could prove to be an important factor for the pathogens ability to proliferate in the hospital setting. Finally, we wondered if our specific glutamate to valine substitution of residue 470 in the EttA sequence would affect the protein's ability to hydrolyze ATP.

Chapter II

Experimental Materials and Methods

2.1: Bacterial strains and growth conditions

In the experiments performed for this thesis, all bacterial strains used are listed in Table 2. For each strain, stocks were maintained at -80°C in 15% glycerol. For each experiment, bacteria were freshly plated. If required for the experiment, bacteria were cultured in either lysogeny broth (LB; Lennox formulation) or MOPS-buffered defined medium for *Acinetobacter* (MOPS-A) (11). Cultures grew at 37°C and were subjected to shaking at 240 to 260 rpm. Media were supplemented with antibiotics for the purposes of selection at the concentrations indicated in the text.

2.2: Plasmid construction

Plasmids were designed with deletions of the genes *lepA* and *ettA* (pEX18Ap-Δ*lepA* and pEX18Ap-Δ*ettA*). For each, DNA fragments upstream and downstream from the deleted region were amplified using PCR with strain ATCC 17961 chromosomal DNA being used as a template. Primers were designed which added either BamHI or KpnI sites to the end of *ettA* and *lepA* fragments, respectively (Table 3). Fragments of approximately 1 kb flanking the deleted region were amplified by PCR. These fragments were spliced by overlap extension PCR before being digested with appropriate enzymes, gel purified, and ligated with pEX18Ap. The *ettA* sequence had 1484 bp removed, approximately 89% of the gene (from bp 2355 to 3839 with the A in ATG counting as the first bp) and the *lepA* sequence had 1692 bp removed, approximately 93% of the gene (from bp 2243 to 3935 with the A in ATG counting as the first bp).

Plasmids where also designed containing point mutations in the genes *lepA* and *ettA* (pEX18Ap-*lepA*L213* and pEX18Ap-*ettA*E470V). Segments of *lepA* were amplified by PCR. Primer P1 and P4 were used to amplify *lepA* creating a fragment 3,891 base pairs long. These fragments were ligated into pEX18Ap, and primers lepA primer A and lepA primer B (Table 3) were designed which generated a single nucleotide substitution in *lepA* identical to the one observed in suppressor mutant 1 (Table 1). Mutant sequences were amplified by PCR and circularized by ligation to produce the plasmid pEX18Ap-*lepA*L213*. For *ettA* we used chromosomal DNA from suppressor mutant #4 as a template and amplified a region of DNA containing the *ettA* point mutation using ettAP_up and ettAP_down (Table 3), generating a fragment 2,139 bp long. Fragments were cut with their corresponding enzymes, gel purified, and ligated with pEX18Ap.

Expression vectors were designed which encoded the wild-type and E470V forms of *ettA* in the plasmid pET28-b. For each, DNA fragments carrying *ettA* were amplified by PCR using DNA from either the wild-type strain or strain 17961-Δ*csrA* sup. 4. These were amplified with an N-terminal primer (NT primer) and a C-terminal primer (BA primer) (Table 3). NT primer contained an NdeI cut site followed by the cleavage sequence for Tobacco Etch Virus (TEV) protease, followed by the start codon and 8 more codons within the *ettA* genome. BA primer contained a BamHI cut site followed by a sequence located downstream from the stop codon for *ettA*. PCR products were digested, gel purified, and ligated with pET28-b to generate the plasmids pET28-b-*ettA* and pET28-b-*ettA*E470V.

2.3: Mutant generation

A. baumannii strains 17961-Δ*ettA* and 17961-Δ*lepA* were both generated using strain ATCC 17961. Plasmids carrying these deletions were transferred to *A. baumannii* via conjugation with *E. coli* strain SM10. Conjugation mixtures were plated on LB agar supplemented with 5 μg/ml chloramphenicol and 150 to 200 μg/ml carbenicillin. The chloramphenicol was meant to select for *A. baumannii* over *E. coli* and carbenicillin was meant to select for *A. baumannii* where plasmids containing mutated sequences were integrated into the chromosome. Homologous recombination was assumed to have taken place in colonies which grew on LB supplemented with carbenicillin but not on plates supplemented with carbenicillin and 10% sucrose due to the presence of the gene *sacB* on the plasmid. After culturing for 4 hours at room temperature, cells were screened on LB agar supplemented with 10% sucrose to resolve the vector sequences from the bacterial chromosome. Mutants were confirmed through DNA sequencing of PCR products. This same protocol was used to generate the strain 17961-*lepA*L213* using the plasmid pEX18Ap-lepAL213*.

A. baumannii strains 17961-Δ*csrA*Δ*lepA* and 17961-Δ*csrA*Δ*ettA* were generated using the same plasmids that generated strains 17961-Δ*ettA* and 17961-Δ*lepA.* Plasmids were transferred to 17961-Δ*csrA* through conjugation with SM10. The generation of these mutants was identical to the generation of 17961-Δ*ettA* and 17961-Δ*lepA* except it was carried out on MOPS-A plates in place of LB plates. Additionally, generation of 17961-Δ*csrAlepA*L213* and 17961-Δ*csrAettA*E470V followed this same technique using plasmids pEX18Ap-lepAL213* and pEX18Ap-*ettA*E470V, respectively.

Table 2. Strains used in this study.

Strain	Description	Reference or source
ATCC 17961	Clinical isolate from blood	ATCC
17961-Δ*csrA*	*csrA* deletion mutant of strain ATCC 17961	11
17961-Δ*lepA*	*lepA* deletion mutant of strain ATCC 17961	This study
17961-Δ*ettA*	*ettA* deletion mutant of strain ATCC 17961	This study
17961-Δ*csrA*Δ*lepA*	*ettA* deletion mutant of 17961-Δ*csrA*	This study
17961-Δ*csrA*Δ*ettA*	*ettA* deletion mutant of 17961-Δ*csrA*	This study
BL21 (DE3)	*E. coli* strain used for protein overexpression	Novagen
17961-Δ*csrA* sup. 1	Suppressor mutant of strain 17961-Δ*csrA*	This study
17961-Δ*csrA* sup. 4	Suppressor mutant of strain 17961-Δ*csrA*	This study
17961-Δ*csrA* sup. 5	Suppressor mutant of strain 17961-Δ*csrA*	This study
17961-Δ*csrA*lepAL213*	*lepA* point mutant of strain ATCC 17961-Δ*csrA*	This study
17961-Δ*csrA*ettAE470V	*ettA* point mutant of strain ATCC 17961-Δ*csrA*	This study
17961-lepAL213*	*lepA* point mutant of strain ATCC 17961	This study

Table 3. Plasmids used in this study.

Plasmids	Description	Reference or source
pEX18Ap	Suicide vector	48
pEX18Ap-Δ*lepA*	pEX18Ap carrying *lepA* deletion	This study
pEX18Ap-Δ*ettA*	pEX18Ap carrying *ettA* deletion	This study
pET28-b	Vector plasmid	Novagen
pET28-b-*ettA*	pET28-b carrying *ettA*	This study
pET28-b-*ettA*E470V	pET28-b carrying *ettA* E470V point mutant	This study
pEX18Ap-*lepA*L213*	pEX18Ap carrying *lepA* point mutant	This study
pEX18Ap-*ettA*E470V	pEX18Ap carrying *ettA* point mutant	This study

Table 4. Primers used in this study.

Purpose	Primers	Sequence (5' to 3')[a]
Deletion mutant generation	lepAP1	ATTATTAAGGTACCGCCTCTGCTTCTAAAGGTCC
Deletion mutant generation	lepApreP	TTCTCAAGGTAGCCCATGGCGCAA
Deletion mutant generation	lepAP2	CAAAACAGCTAAGAACGCTTCAGCCAAAGTAGACTTACCATG
Deletion mutant generation	lepAP3	CATGGTAAGTCTACTTTGGCTGAAAGCGTTCTTAGCTGTTTTG
Deletion mutant generation	lepAP4	ATTAATAAGGTACCAATATGCTGGCCAAACTGCAA
Deletion mutant generation	ettAP1	ATAATATTGGATCCGGAAACTTACCGCTATCGATA
Deletion mutant generation	ettApreP	TTGAGGAGTCCTGCGTGGCCCAA
Deletion mutant generation	ettAP2	TGAGCGTCATCACCTAGCTTACAAACCGAGAACAACAATTTT
Deletion mutant generation	ettAP3	AAAATTGGTGTTCTCGGTTTGTAAGCTAGGTGATGACGCTCA
Deletion mutant generation	ettAP4	AATATTATGGATCCCAACAAACGATTCCAACTCCA
Point mutant generation	ettA primer A	ACATCCAGTAAGATAACGTTTGC
Point mutant generation	ettA primer B	GCCATCAAACGACTTGGACG
Point mutant generation	lepA primer A	TAGTTATCAAACCATGAGTC
Point mutant generation	lepA primer B	TTAAGGTGTTGTCTCTT
Point mutant generation	ettAP_up	AATTATGGATCCATGCTGATTTTGATGCGCTTG
Point mutant generation	ettAP_down	AATTATGGATCCGACGGTAGCCCCTTCTCT

[a]Underlined sequences represent cut sites for restriction enzymes

2.4: Protein purification

The protein EttA in both the wild-type and mutant E470V form were purified from *E.* coli strain BL21 (DE3) containing plasmids pET28-b-*ettA* and pET28-b-*ettA*E470V, respectively. Purification was completed using the protocol developed in 2010 for purification of translation factors by Fei et al., with a few modifications (29). Cells were cultured in 500 mL LB broth containing 150 μg/ml carbenicillin and started at an OD_{600} of 0.05. Cultures were at 37°C until they reached an OD_{600} of approximately 0.6. Cultures where then moved to room temperature, allowed to cool for approximately 10 minutes, and induced with IPTG. For pET28-b-*ettA* we induced with a final concentration of 0.01 mM IPTG, and for pET28-b-*ettA*E470V we induced with a final concentration of 0.02 mM IPTG. Cells were cultured at room temperature for an additional four hours before being centrifuged at 5,000 times gravity for 15 minutes at 4°C. Pellets were stored in a -80°C freezer and once thawed, all remaining steps would take place at 4°C (29).

Thawed pellets were each suspended in 20 mL of lysis buffer (20 mM Tris-HCl ($pH_{4°C}$=7.5), 300 mM NaCl, 10 mM imidazole, 0.2 mM phenylmethanesulphonyl fluoride (PMSF), 2 mM beta-mercaptoethanol (BME), and 2 mg/mL lysozyme) (29) before being sonicated with seven rounds of 20 pulse sonications separated by 30 seconds. Lysates were spun at 12,000 times gravity for 30 minutes at 4°C. During centrifugation, 4 mL of Ni-resin was pre-equilibrated with 20 mL of TF buffer A (lysis buffer without lysozyme) (29). Lysate supernatant was mixed with equilibrated resin and rocked for 30 minutes at 4°C. Resin was allowed to settle in a column, and flowthrough was collected by gravity flow. The column was washed with 40 mL of TF buffer B (TF buffer A with 30 mM

imidazole) (29), adding 5 mL at a time. Proteins were eluted using 16 mL of TF buffer C (TF buffer A with 500 mM NaCl and 250 mM imidazole) (29) collected over four fractions.

The concentration of protein in each fraction was determined using Bradford assays with bovine serum albumin as a standard, before being concentrated down to 1-5 mg/mL of protein. For the concentration step, centrifugal concentrators from the Vivaspin 20 kit (Sartorius Stedim Lab Ltd. Gloucestershire, UK) were used. Eluted proteins were added to concentrators before being spun at 4,000 times gravity at 4°C in a swinging bucket centrifuge. Proteins were buffer exchanged with TF buffer E (20 mM Tris-HOAc ($pH_{4°C}$=7.5), 100 mM KCl, 10 mM BME) (29) over four exchanges. The volume of buffer added for each exchange was the same as the volume of eluted protein added. Finally, concentrated proteins were stored at -20°C and in 50% glycerol. Protein concentration was determined based on results from Bradford assays using bovine serum albumin as a standard. Purity of protein was determined to be greater than 95% by SDS PAGE.

2.5: Growth curves

To measure long term survival of strain ATCC 17961 compared to strain 17961-Δ*ettA*, colony forming units (CFU) were measured over 98 hours. Both genotypes were plated on LB and sub-cultured overnight at 37°C in 10 mL LB. In the morning, 40 mL cultures of both genotypes were made at an OD_{600} of approximately 0.08. Cultures were divided into two 20 mL cultures and grown at 37°C. LB media was shaken alongside the four cultures as a negative control. Serial dilutions were made at time points 0, 2, 4, 6, 26, 50, 74, and 98 hours and 50 μL of each was plated on LB plates. Plates were

incubated overnight at 37°C and plates containing between 20 and 200 colonies were counted. For each time point, CFUs per mL were calculated. No less than three repeats were performed for each time point.

All other growth curves were generated using a plate reader that assayed OD every 20 minutes from a 96 well plate over 8 hours. For growth curves examining *lepA* genotypes, strains 17961-WT, 17961-Δ*csrA*, 17961-Δ*lepA*, 17961-Δ*csrA*Δ*lepA*, and both suppressor mutants 1 and 5 of 17961-Δ*csrA* were inoculated into 10 mL MOPS-A and grown overnight. The next day, 10 mL subcultures were made at OD_{600} values of 0.08 for each culture except for 17961-Δ*csrA* which was made at an OD_{600} value of 0.12 due to this strain's slow growth. Cultures were grown for 3.5 hours at 37°C before new cultures were made of each at an OD_{600} of 0.15. For each culture along with blank MOPS-A media, 50 μL were added to 6 wells of a row. To the top three wells, 50 μL MOPS-A was added, and to the bottom three wells, 50 μL of MOPS-A containing two percent casamino acids was added resulting in a final volume of 100 μL per well. The plate was placed into a TECAN machine and incubated at 37°C with shaking.

The same protocol was followed for the growth curves examining *ettA* phenotypes. These used strains ATCC-17961, 17961-Δ*csrA*, 17961-Δ*ettA*, 17961-Δ*csrA*Δ*ettA*, and suppressor mutant 4 of 17961-Δ*csrA*. After subculturing overnight, the new cultures were all made at an OD_{600} value of 0.08 except for strain 17961-Δ*ettA* which was made at an OD_{600} value of 0.12. Once strains 17961-*lepA*L213* and 17961-Δ*csrAlepA*L213* where constructed, new growth curves were completed using the same protocol with the two new strains being measured along with strains 17961-WT and 17961-Δ*csrA*. Once strain 17961-Δ*csrAettA*E470V was constructed, new growth curves

were completed with this strain being measured along with strains 17961-WT and 17961-Δ*csrA*. Strain 17961-Δ*csrA*ett*A*E470V was subcultured at an OD_{600} value of 0.12 due its slow growth. For all graphs, averages and standard deviations were calculated using Microsoft Excel, while figures were made using Prism Graphpad. T-tests and linear regressions were performed using Prism Graphpad.

2.6: SDS PAGE

For SDS PAGE, cultures were grown overnight at 37°C before 1 mL of culture was centrifuged at 16,000 times gravity for one minute. Supernatant was decanted and the wet pellet weight was measured. For each mg of wet pellet, 10 μL of lysis buffer was added. The lysis buffer contained BugBuster Protein Extractoin Reagent along with 2 mg lysozyme and 1 μL benzoase nuclease per mL of solution. Cell suspensions were shaken on a platform for 20 minutes before centrifugation at 16,000 times gravity for 20 minutes. Supernatant was collected as the soluble fraction and kept on ice.

Pellet solubilization buffer (PSB) was made containing 50 mM Tris, pH 6.8, 0.1 M NaCl, and 0.1% SDS. 10 μL PSB was added to the pellet for each mg of wet pellet measured earlier. This solution was incubated at 55°C before grinding the pellet with a P1000 pipette tip, the ground pellet solution was then incubated at 55°C for an additional 5 minutes. This solution was collected as the insoluble fraction and kept on ice.

SDS sample buffer was made containing 250 mM Tris, pH 6.7, 10% SDS, 0.05% bromophenol blue, 50% glycerol, and 100 mM dithiothreitol (DTT). For each sample, an 8 μL of fraction, 3 μL of sample buffer, and 4 μL water were mixed before incubating 5 minutes in a boiling water bath. Afterwards, the samples were left on ice for 1 minute

and all 15 μL of each sample was added to their corresponding wells. All gels were run at 120 volts with one or more wells of Benchmark protein ladder. Gels were stained with Coomassie blue and photographed.

2.7: ATPase activity assay

ATPase activity was measured using the protocol of Jacquet et al., 2008 with some changes (32). [γ-^{32}P] ATP containing 3,000 Ci per mMol and 10 mCi per mL was ordered from PerkinElmer. The principle of the experiment is that enzymes with ATPase activity will hydrolyze the gamma phosphate of ATP and that activated charcoal suspended in phosphoric acid can be used to sequester the unhydrolyzed ATP. As a result, free P^{32} will be left in the supernatant after centrifugation of the charcoal suspension (32)(33). Acid washed charcoal (catalog C-5510) was ordered from Sigma Aldrich (34). For each experiment, two reactions were set up in 100 μL, each containing 2 μM of protein with one reaction containing wild-type EttA and one containing EttAE470V. Reactions were carried out in 25 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 5 mM $MgCl_2$ (32). A “no protein”, negative control was also used for each experiment (32).

Each reaction was caried out at 30°C and was started by addition of unlabeled ATP to a final concentration of 2 mM in addition to 0.95 μCi of [γ-^{32}P] ATP. Unlabeled ATP was added to ensure enzymes would be saturated with ATP (35). At time points 0, 5,15, 25, 35, 45, and 55 minutes, 10 μL of reaction was added to 400 μL of four percent activated charcoal suspended in 20 mM phosphoric acid before being placed in an ice bath (32). Charcoal suspensions representing each time point were kept at 4°C and spun at 16,000 times gravity for five minutes. Supernatant was removed and spun again at 16,000 times gravity for five minutes before being filtered. 200 μL of filtrate was

added to 3 mL of scintillation fluid and radioactivity was measured with a scintillation counter.

Three repeats were performed with averages and standard deviations being calculated for each time point. Values for the five-minute time point of the EttAE470V reaction were excluded because they were not above background values produced by the control reactions. Lines of best fit for both reactions were calculated and compared statistically via linear regression.

Chapter III

Experimental Results

3.1: Point mutations in *ettA* and *lepA* rescue growth of the Δ*csrA* strain in the presence of amino acids

Our initial screen identified point mutations in the genes *lepA* and *ettA* which allowed the Δ*csrA* strain to grow in the presence of amino acids. To confirm these results, we re-created point mutations in both genes individually in wild-type and strain 17961-Δ*csrA*. We also generated complete deletions of these genes individually in the wild-type strain and strain 17961-Δ*csrA*. We hypothesized that these new strains would have the same phenotype as the suppressor mutants. To test this, we plated strains of *A. baumannii* ATCC 17961 on both LB and MOPS-A media. Strain 17961-Δ*csrA*Δ*ettA* was plated next to strain 17961-Δ*csrA* on LB agar (figure 1A), and strain 17961-Δ*csrA*Δ*lepA* was plated next to strain 17961-Δ*csrA* on an additional LB agar plate (figure 1B). Both plates showed no growth for either strain while suppressor mutant #4 (17961-Δ*csrA* sup. 4) and suppressor mutant #1 (17961-Δ*csrA* sup. 1) grew well after 26 hours (figure 1 A and B).

These results suggested that the specific point mutations to *lepA* or *ettA,* and not inactivation of their encoded proteins, were responsible for the rescued growth on LB. Strain 17961-Δ*csrAettA*E470V was plated next to strain 17961-Δ*csrA* sup. 4 on an LB agar plate (figure 1A) and strain 17961-Δ*csrAlepA*L213* was plated next to strain 17961-Δ*csrA* sup. 1 on an additional LB agar plate (figure 1B). Both plates showed that strains 17961-Δ*csrAettA*E470V and 17961-Δ*csrAlepA*L213* grew equally as well as strains 17961-Δ*csrA* sup. 4 and 17961-Δ*csrA* sup. 1, respectively (figure 1). As

expected, all strains were able to grow on MOPS-A media (figure 1). These results confirmed that point mutations were able to rescue strain Δ*csrA* on complex media.

To examine the growth of mutant strains in the presence of amino acids, growth in broth culture was monitored using a TECAN plate reader. Cultures were grown on MOPS-A (figure 2 A and B) and in MOPS-A supplemented with 1% casamino acids (figure 2 C and D). When grown in MOPS-A alone, strains 17961-Δ*csrA*, 17961-Δ*csrA* sup. 1, 17961-Δ*csrAlepA*L213*, and 17961-Δ*csrA*Δ*lepA* grew as well as wild-type cells (figure 2A). Surprisingly, strain 17961-Δ*lepA* had a decreased growth rate on MOPS-A alone (figure 2B). This suggested that LepA is important for growth in MOPS-A, unless *csrA* has been deleted.

In MOPS-A supplemented with 1% casamino acids, strain 17961-Δ*lepA* grew similar to the wild-type strain (figure 2D). In the same media, strains 17961-Δ*csrA*Δ*lepA* and 17961-Δ*csrA* both had severely inhibited growth while strains 17961-Δ*csrA* sup. 1, 17961-Δ*csrA* sup. 5 and 17961-Δ*csrAlepA*L213* all grew much better but did not fully recover growth to the level of the wild-type strain (figure 2D).

Unlike strain 17961-Δ*lepA*, strain 17961-Δ*ettA* did not exibit growth inhibition in MOPS-A alone (figure 2A). In contrast, when grown in MOPS-A alone strains 17961-Δ*csrAettA*E470V and 17961-Δ*csrA* sup. 4, had a decreased growth rate compared to strain 17961-wt (figure 2A). For *ettA* strains, when grown in MOPS-A supplemented with 1% casamino acids, strain 17961-Δ*ettA* grew equally well as the wild-type strain (figure 2C). Strain 17961-Δ*csrA* had severely inhibited growth, while strain 17961-Δ*csrA*Δ*ettA* had slightly improved growth (figure 2C). Strains 17961-Δ*csrAettA*E470V and 17961-Δ*csrA* sup. 4 had equal growth rates, and both grew better than 17961-Δ*csrA*Δ*ettA*

without fully recovering growth to the levels of wild-type and strain 17961-Δ*ettA* (figure 2C).

Taken together, results from figures 1 and 2 showed that the point mutations in *ettA* or *lepA*, which were seen in the suppressor mutants, were sufficient to rescue growth of the Δ*csrA* mutant in the presence of amino acids. However, deletion of these genes does not rescue growth. This suggests that the proteins EttAE470V and LepAL213* are carrying out some function when CsrA is not present, which allows the cell to overcome the growth defect resulting from the presence of amino acids.

3.2: Protein expression is altered in strain 17961-Δ*csrAettA*E470V

To identify how our mutant proteins were affecting the Δ*csrA* mutant, we wanted to analyze protein expression in our point mutants and suppressor mutants to compare them with wild-type strain and strain 17961-Δ*csrA*. We wondered whether these strains would display altered protein expression, since the genes *lepA* and *ettA* both produce proteins which play a role in translational regulation in *E. coli*. To analyze protein expression, we ran soluble and insoluble fractions of lysates of *A. baumannii* strains: wild-type, 17961-Δ*csrA,* 17961-Δ*csrAettA*E470V, and 17961-Δ*csrA* sup. 4 on one SDS PAGE gel (figure 3A), and strains: wild-type, 17961-Δ*csrA*, 17961-Δ*csrAlepA*L213*, and 17961-Δ*csrA* sup. 1 on another SDS PAGE gel (figure 3B).

Two bands do not appear from the lysates of strains 17961-Δ*csrAettA*E470V and 17961-Δ*csrA* sup. 4, which did appear in lysates of the wild-type strain and strain 17961-Δ*csrA*. In the soluble lysate, a band greater than 70 kDa was no longer present in strains 17961-Δ*csrAettA*E470V and 17961-Δ*csrA* sup. 4. In the insoluble portion of the lysate, a band of between 60 and 70 kDa was no longer present in strains 17961-

Δ*csrAettA*E470V and 17961-Δ *csrA* sup. 4 (figure 3A). Additionally, a band of approximately 50 kDa, was overexpressed in the soluble lysate of strain 17961-Δ*csrA*, but not in strains 17961, 17961-Δ*csrAettA*E470V and 17961-Δ*csrA* sup. 4 (figure 3A).

Our examination of proteins from Δ*lepA* strains showed that all bands which appear in both wild-type and 17961-Δ*csrA* strains, also appeared in strains 17961-Δ*csrAlepA*L213* and 17961-Δ*csrA* sup. 1 (figure 3B). The band at approximately 50 kDa, which was overexpressed in the soluble lysate of strain 17961-Δ*csrA* is also overexpressed in the soluble lysates of strains 17961-Δ*csrA*, 17961-Δ*csrAlepA*L213* and 17961-Δ*csrA* sup. 1, but not in the wild type strain (figure 3B).

It may be the case that when CsrA is absent and amino acids are present, EttAE470V can inhibit translation of some specific genes while allowing translation to go forward for all other genes. This would explain why two specific bands are not expressed in lysates of cells expressing EttAE470V but not CsrA. The band overexpressed in strain 17961-Δ*csrA* may be a protein whose translation is normally repressed (directly or indirectly) by CsrA. The fact that this band is not overexpressed in strain 17961-Δ*csrAettA*E470V or 17961-Δ*csrA* sup. 4 could mean that the mutant protein EttAE470V is able to inhibit translation of this protein.

3.3: EttA is important for long term survival in *A. baumannii*

After identifying that *A. baumannii* stains 17961-Δ*csrAettA*E470V and 17961-Δ*csrAlepA*L213* regained growth phenotypes in the presence of amino acids and identifying that the translation of specific proteins was altered in strain 17961-Δ*csrAettA*E470V, we decided to focus our research on the gene *ettA* and the effect of the *ettA*E470V mutation in the Δ*csrA* background. This decision was also supported by

the uncertainty as to the function of LepA when compared to EttA, which regulates translation based on ATP/ADP ratios (13).

We wanted to test that EttA would function similarly in *A. baumannii* strain ATCC 17961 as it had in *E. coli*. To do this we compared the long-term growth of wild-type and strain 17961-Δ*ettA*. It had previously been shown that *E. coli* cells lacking EttA, had growth defects when grown for 144-hours in LB media and then transferred to fresh LB media (13). We performed a similar experiment in *A. baumannii*. We measured the number of cells in culture over 98 hours in LB by measuring colony forming units (CFUs) per mL (13).

Based on what was known about the EttA homolog in *E. coli* and the sequence similarity between homologs of EttA in *E. coli* and *A. baumannii* as discussed in chapter I, we hypothesized that the strain 17961-Δ*ettA* would have decreased long-term survival when compared to wild-type cells. Our first observation was that strain 17961-Δ*ettA* grew slower initially in comparison with wild-type based on there being significantly more CFUs/mL for the wild-type strain at the 4-hour time point (figure 4). This result was not surprising as we had already observed that strain 17961-Δ*ettA* grew slower in both solid and liquid media than most other strains.

Growth of strain 17961-Δ*ettA* did catch up to that of strain 17961-wt by the 6-hour time point, and both strains maintained similar density until the 74-hour time point. Once again, there were significantly more CFUs/mL for wild-type when compared to strain 17961-Δ*ettA* for both the 74 and 98-hour time points (figure 4). This result supported our hypothesis that EttA is important for survival during long term growth of *A. baumannii*. Based on this result and the alignment data mentioned in chapter I, we speculated that

the function of the EttA homolog in *A. baumannii* was similar to its counterpart in *E. coli*, where it is proposed to regulate translation based on ATP/ADP ratios.

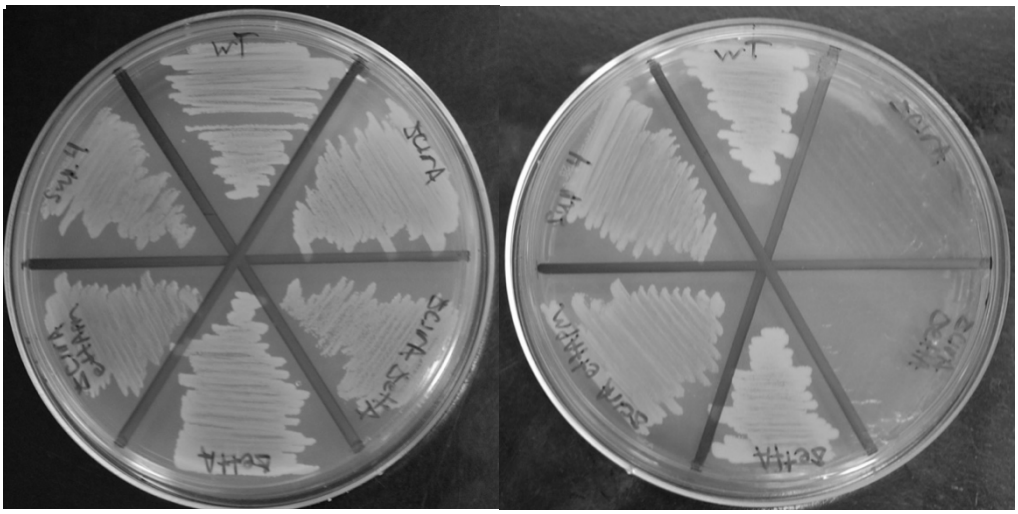
3.4: EttAE470V has reduced ATPase activity compared to wild type

To learn how our point mutation affected the function of EttA, we attempted to measure the ATPase activity of EttAE470V compared to EttA. Studies on a different ABC-F family protein, Vga(A), showed that a Vga(A) point mutant with a glutamate to glutamine mutation on the catalytic base of the second DEPT sequence resulted in a loss of approximately 70% of its ATPase activity (32). Based on this, we hypothesized that EttAE470V would lose a similar proportion of its ATPase activity since EttAE470V had a point mutation on the same base of the second DEPT sequence. We did recognize the possibility that, since our mutation was glutamate to valine and not glutamate to glutamine, EttAE470V may not have the same reduced ATPase activity as the Vga(A) point mutant, since the point mutation in EttAE470V could alter some other function of the protein.

ATPase activity measurements were taken by measuring ^{32}P released from [γ-^{32}P] ATP when placed in a reaction with purified protein. After three repeats were performed, a line of best fit was calculated and plotted for both proteins. Lines of best fit were compared using simple linear regressions comparing both slopes to zero and to each other (figure 5). The slope of the lines of best fit for EttA and EttAE470V were 57.88 and 16.15, respectively with r^2 values of 0.9941 and 0.9678, respectively (figure 5). According to simple linear regression, EttA and EttAE470V's lines of best fit were both statistically higher than zero ($p<0.0001$ and $p<0.0005$, respectively) and both lines of best fit were statistically different from each other ($p<0.0001$) (figure 5).

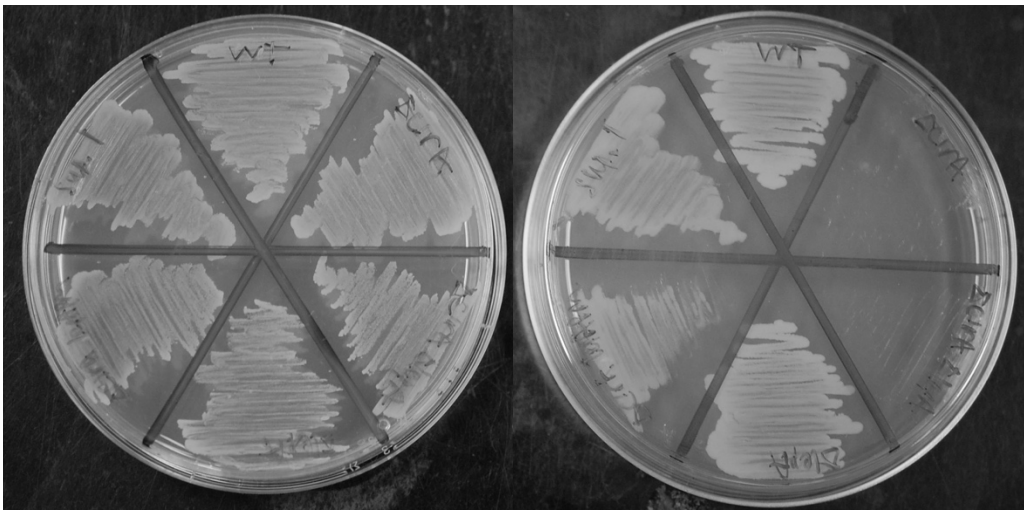
These results confirmed our hypothesis that EttA has ATPase activity and that EttAE470V had reduced ATPase activity compared to EttA. This was similar to the results published for Vga(A), with the rate of ATP hydrolysis for EttA being approximately 3.5-fold higher than that of EttAE470V (figure 5). This suggests that the glutamate to valine mutation of the catalytic site of the second DEPT sequence results in similar physiological alterations as the glutamate to glutamine mutation. It remains unclear how glutamate to valine mutations in both DEPT sequences would affect EttA. Based on the results of Böel et al., in 2013, it seems likely that ATPase activity would be completely or nearly completely inhibited by the protein continuously being in the ATP bound form (13).

A.



MOPS-A LB

B.



MOPS-A LB

Figure 1

C.

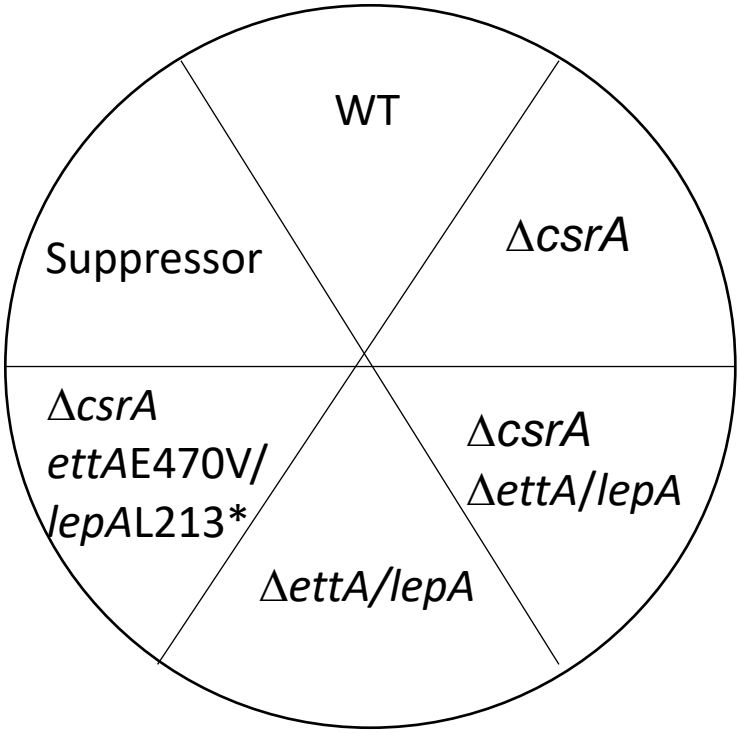
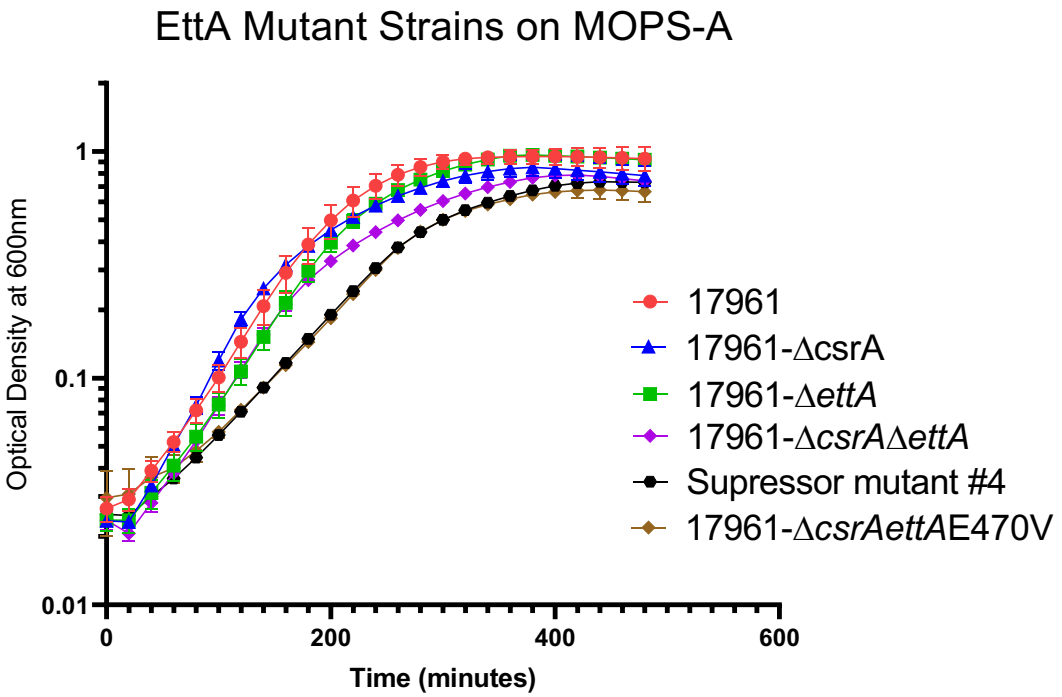


Figure 1 – Point mutations in *ettA* and *lepA* rescue growth of the Δ*csrA* mutant strain on LB agar. On the left are MOPS-A agar plates and on the right are LB agar plates. All plates were incubated at 37°C for approximately 26 hours. (A) Beginning at the top of each plate and moving clockwise: wild type, Δ*csrA*, Δ*csrA*Δ*ettA*, Δ*ettA*, Δ*csrAettA*E470V, and Δ*csrA* suppressor #4. (B) Beginning at the top of each plate and moving clockwise: wild type, Δ*csrA*, Δ*csrA*Δ*lepA*, Δ*lepA,* Δ*csrAlepA*L213*, and Δ*csrA* suppressor #1. (C) Model showing the position of each strain on all plates.

A.


EttA Mutant Strains on MOPS-A
Optical Density at 600nm
1
0.1
0.01
0
200
400
600
Time (minutes)
17961
17961-ΔcsrA
17961-Δ*ettA*
17961-Δ*csrA*Δ*ettA*
Supressor mutant #4
17961-Δ*csrAettA*E470V

C.

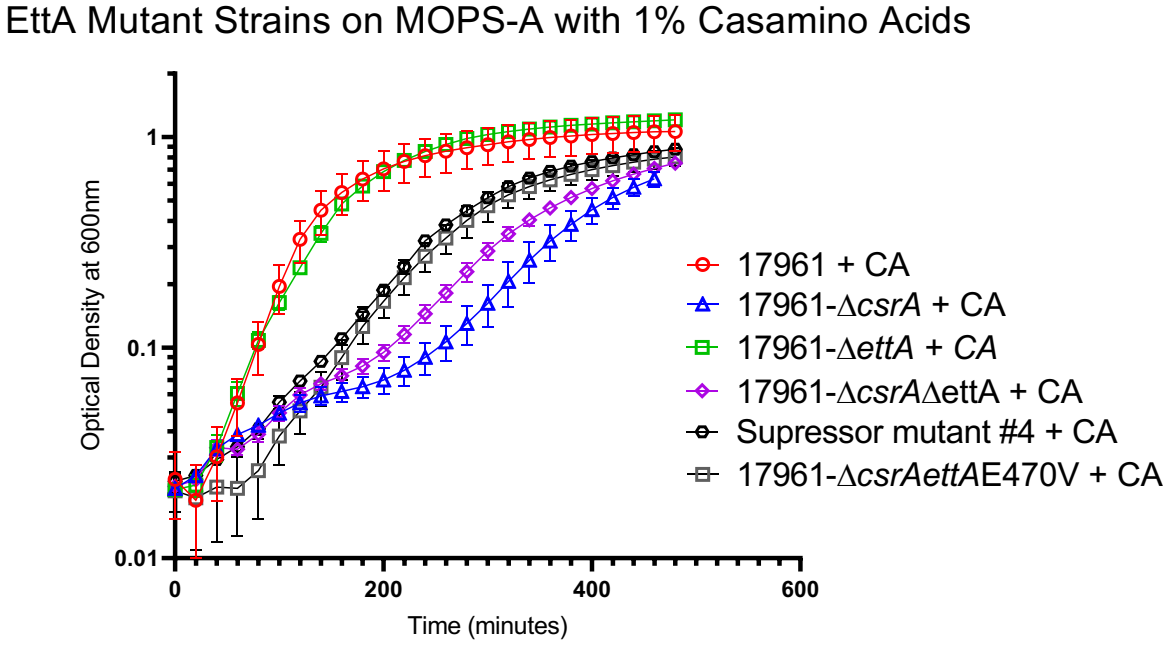
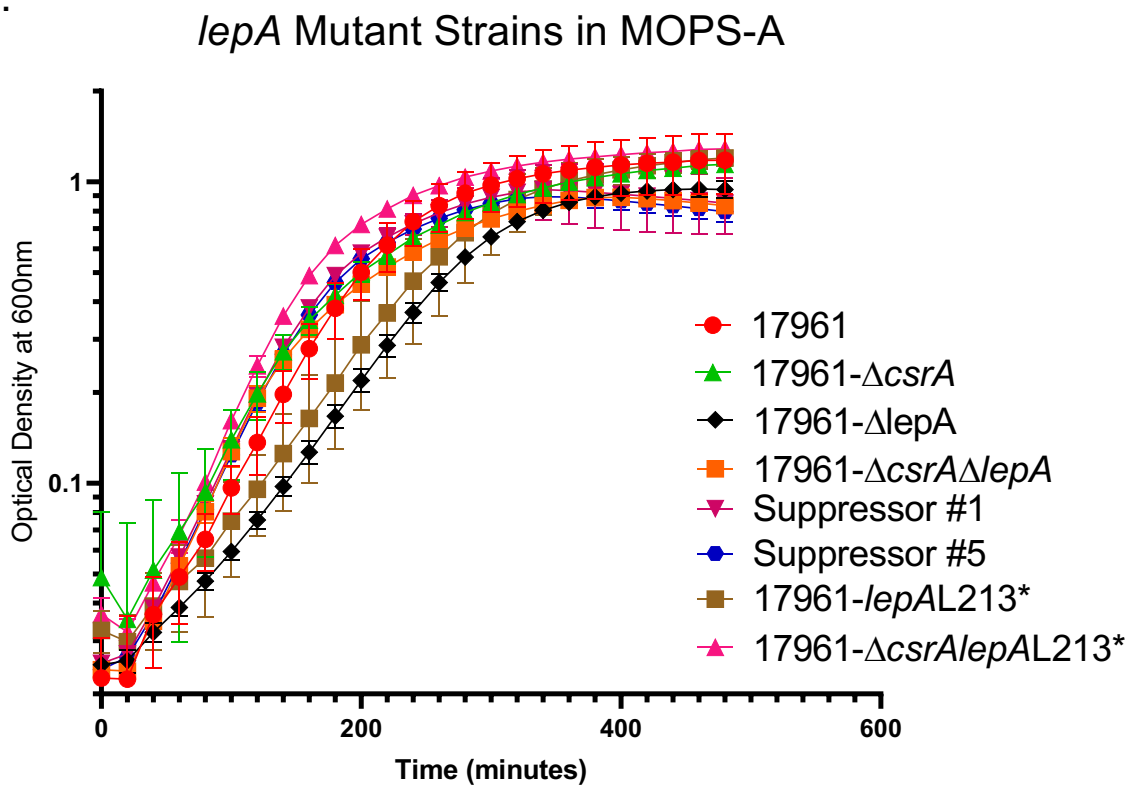

EttA Mutant Strains on MOPS-A with 1% Casamino Acids
Optical Density at 600nm
1
0.1
0.01
0
200
400
600
Time (minutes)
17961 + CA
17961-Δ*csrA* + CA
17961-Δ*ettA* + *CA*
17961-Δ*csrA*ΔettA + CA
Supressor mutant #4 + CA
17961-Δ*csrAettA*E470V + CA

Figure 2

B.


lepA Mutant Strains in MOPS-A
Optical Density at 600nm
1
0.1
0
200
400
600
Time (minutes)
17961
17961-ΔcsrA
17961-ΔlepA
17961-ΔcsrAΔlepA
Suppressor #1
Suppressor #5
17961-lepAL213*
17961-ΔcsrAlepAL213*

D.

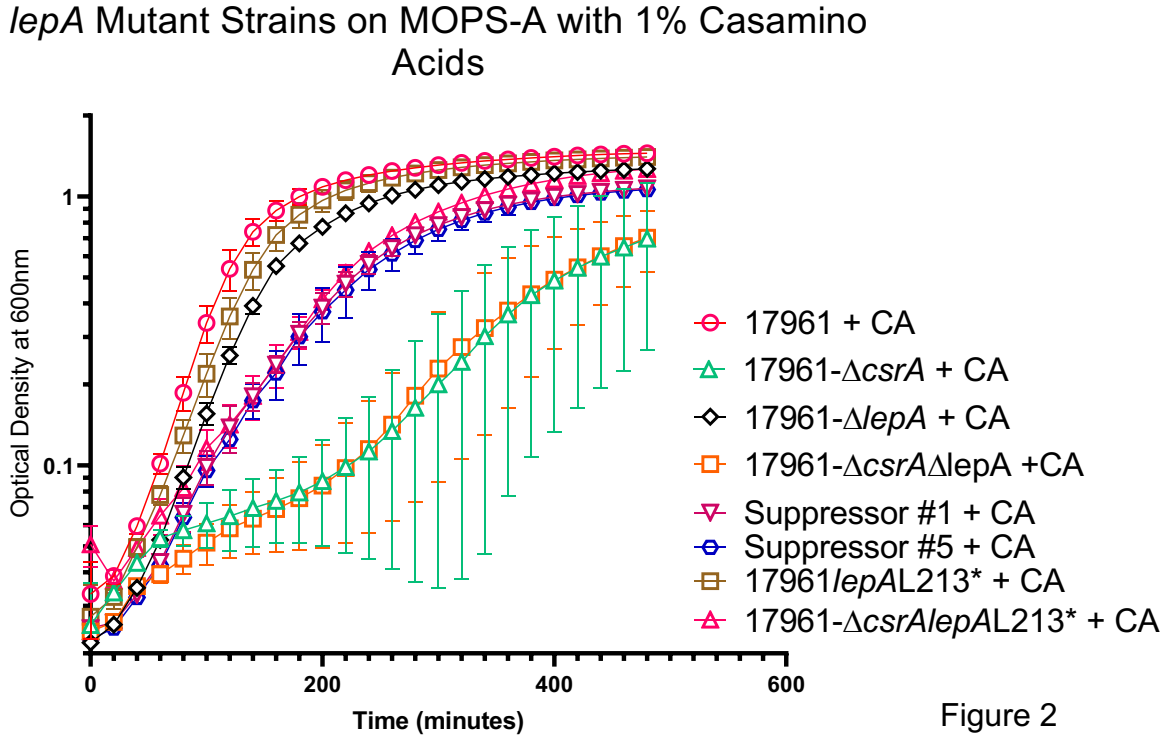

lepA Mutant Strains on MOPS-A with 1% Casamino Acids
Optical Density at 600nm
1
0.1
0
200
400
600
Time (minutes)
17961 + CA
17961-ΔcsrA + CA
17961-ΔlepA + CA
17961-ΔcsrAΔlepA +CA
Suppressor #1 + CA
Suppressor #5 + CA
17961lepAL213* + CA
17961-ΔcsrAlepAL213* + CA

Figure 2

Figure 2 – Point mutations in *ettA* and *lepA* rescue growth in the presence of amino acids. Growth curve data generated by a TECAN plate reader. Growth of different strains of *A. baumannii* ATCC 17961 were compared using optical density readings. (A and C) compare growth of the strains wild type, Δ*csrA*, Δ*lepA*, Δ*csrA*Δ*lepA*, Δ*csrA* sup. 1, Δ*csrA* sup. 5 and Δ*csrAlepA*L213*. Growth curves were performed in (A) MOPS-A media and (C) MOPS-A media supplemented with 1% casamino acids. (B and D) compare growth of the strains wild type, Δ*csrA*, Δ*ettA*, Δ*csrA*Δ*ettA*, Δ*csrA* sup. 4, and Δ*csrAettA*E470V. Growth curves were performed in (B) MOPS-A media and (D) MOPS-A media supplemented with 1% casamino acids. For each experiment, growth curve data was collected in triplicate and averaged. Data represents the mean $^{+}/_{-}$ standard deviation for each time point.

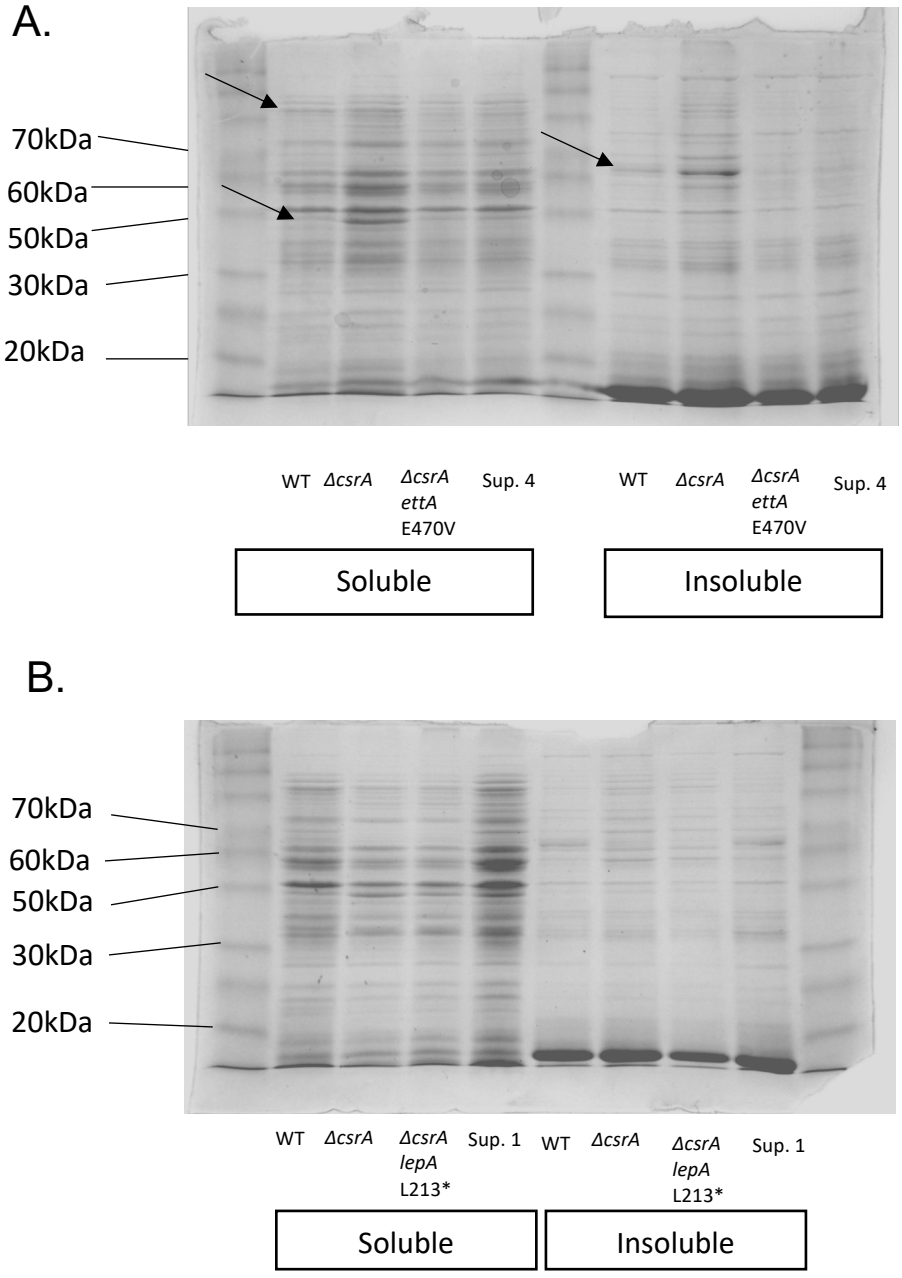


Figure 3 – Protein expression patterns are altered by *ettA* point mutations. SDS PAGE's containing soluble (left) and insoluble (right) lysates of different strains of *A. baumannii* ATCC 17961. On the far left are protein ladders and the corresponding kDa of select bands. (A) compares strains wild type, 17961-Δ*csrA*, 17961-Δ*csrA*ett*A*E470V, and Δ*csrA* sup. 4 (left to right). (B) compares strains wild type, 17961-Δ*csrA*, 17961-Δ*csrA*lep*A*L213*, and Δ*csrA* sup. 1 (left to right). Arrows point to bands which appear to be unevenly expressed among different lysates.

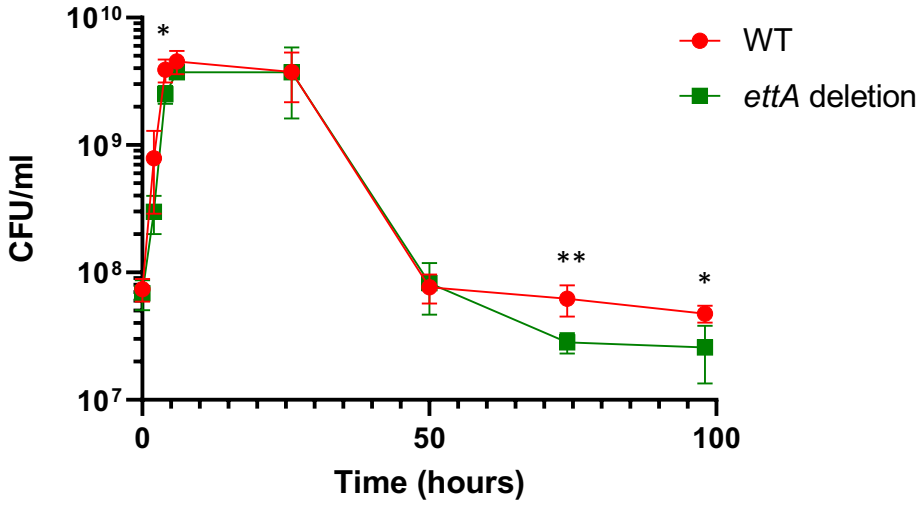


Figure 4 – EttA is important for long term survival. Colony forming units (CFUs) per mL were measured across eight time points for *A. baumannii* ATCC 17961 wild-type and strain 17961-Δ*ettA*. Serial dilutions of culture were plated on LB agar at 0, 2, 4, 6, 26, 50, 74, and 98 hours. Each time point represents averages from no less than three replicates, with error bars representing standard deviation. *t*-tests were done comparing values for each time point, with there being a statistically significant amount more CFUs/mL for wild-type compared to strain 17961-Δ*ettA* at times 4, 74, and 98 hours. *t*-test (*: $p<0.05$) (**: $p<0.005$)

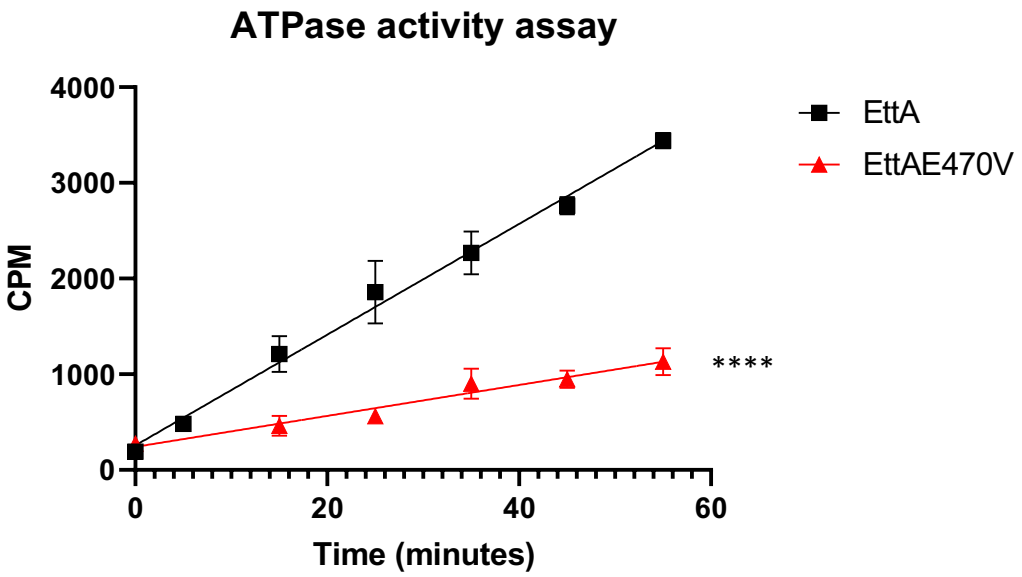


Figure 5 – WT EttA expresses greater ATPase activity compared to EttAE470V. Purified EttA, or EttAE470V, were incubated in a reaction with excess ATP at 2 mM and an additional 0.95 μCi of [γ-^{32}P] ATP. At the indicated time points, un-cleaved ATP was absorbed with activated charcoal, and liberated ^{32}P was measured by scintillation counting. Lines of best fit were calculated, plotted, and a simple linear regression was performed. Data represent the average CPM at indicated time points. Simple linear regression ($p<0.0001$: ****)

Chapter IV

Discussion

4.1: Rescued growth in the presence of amino acids

Our studies have shown that CsrA is vital to *A. baumannii*'s ability to survive in the presence of amino acids. Based on unpublished metabolomic data (J. Farrow), we proposed that strain 17961-Δ*csrA* enters a nitrogen deficient state in the presence of amino acids. This is based off the observation that the CsrA deficient strain showed low levels of glutamine and high levels of 2-oxoglutarate. Low glutamine to 2-oxoglutarate ratios are known to be indicators of nitrogen starvation (37). One proposal is that certain amino acids may be feedback inhibitors of a key enzyme in *A. baumannii*'s nitrogen metabolism, and that this negative feedback signal would further restrict nitrogen assimilation in the Δ*csrA* mutant strain, blocking growth. As of the time of this writing, no data have been gathered to definitively prove that nitrogen starvation is the cause of the inhibited growth phenotype, or that CsrA aids in overcoming any enzymatic inhibition promoted by amino acids.

We observed that strain 17961-Δ*csrA* could regain the ability to grow in the presence of amino acids through point mutations. Some of these point mutations altered the sequence of *lepA* through the introduction of premature stop codons, while some others altered the sequence of *ettA* through the mutation of glutamate 470 to valine (table 1). After constructing strains 17961-Δ*csrA*Δ*lepA* and 17961-Δ*csrA*Δ*ettA*, we observed that these strains did not regain the ability to grow in media containing amino acids (figures 1 and 2).

After constructing strains 17961-Δ*csrA*Δ*lepA*L213* and 17961-Δ*csrA*Δ*ettA*E470V, we observed that these strains regained the ability to grow in the presence of amino acids (figures 1 and 2). These results imply that specific alteration to the proteins LepA and EttA, but not deletion of these proteins, is responsible for rescued growth in the presence of amino acids. Specifically, these results indicate that the N-terminus of LepA, or a mutation to the second catalytic site of EttA, are vital for strain 17961-Δ*csrA* to grow in the presence of amino acids.

The earliest stop codon observed in *lepA* in our suppressor mutants was on residue 213 (table 1). It is likely that some residues upstream from residue 213 are important for LepA to carry out a vital function in strain 17961-Δ*csrA* when amino acids are present. Interestingly, in 2015, Laurentiis and Wieden showed that histidine 81 was vital to LepA catalysis (36). They generated a *lepA* mutant in *E. coli* with histidine 81 mutated to alanine and observed that this mutant protein exhibited GTPase activity indistinguishable from background but was still able to bind GTP at normal levels (36). These results appear to suggest that histidine 81 is vital to LepA's GTPase function. Because histidine 81 is present in strain 17961-Δ*csrA*Δ*lepA*L213*, but not strain 17961-Δ*csrA*Δ*lepA*, it may be that the GTPase activity of LepA is vital for *A. baumannii* to grow in amino acids when CsrA is not expressed. It remains unknown if the protein produced by *lepA*L213* would be capable of hydrolyzing GTP or interacting with ribosomes.

The second catalytic site in EttA is the glutamate on the second DEPT sequence. This residue is vital to ATP hydrolysis and when mutated from glutamate to glutamine, this protein has a reduced ability to hydrolyze ATP (14)(15). In our suppressor mutants of strain 17961-Δ*csrA*, glutamate 470 was mutated to valine and not glutamine.

However, our proteins exhibited lower ATPase activity than wild-type EttA (figure 5) in line with what is proposed to occur in glutamate to glutamine mutants of EttA (13), and in line with what had been observed in other ABCF proteins (32).

It was interesting to observe that strain 17961Δ*lepA* had inhibited growth compared to the wild-type strain in MOPS-A media but not in MOPS-A media supplemented with 1% casamino acids. It may be the case that when LepA is not expressed in strain ATCC 17961, the cells become deficient in a nutrient which is not present in MOPS-A. Strains which do not express CsrA or LepA do not experience this same growth inhibition. It may be that CsrA regulation along with a failure to express LepA is the reason for this nutrient deficiency.

4.2: Altered protein expression in mutant strains

To explain how mutations to *lepA* and *ettA* could rescue growth in the presence of amino acids, we observed the protein expression of strains 17961-Δ*csrAlepA*L213* and 17961-Δ*csrAettA*E470V. Because both LepA and EttA were known to be involved in translational regulation, we hypothesized that these strains would show some difference in protein expression compared to wild-type and strain 17961-Δ*csrA*. This hypothesis proved true for strain 17961-Δ*csrAettA*E470V as two proteins were observed in the lysates of wild-type and 17961-Δ*csrA* that were not in the lysates of strains 17961-Δ*csrAettA*E470V or 17961-Δ*csrA* sup. 4. One band appeared in the soluble lysates with a molecular weight of over 70 kDa and the other appeared in the insoluble lysate with a molecular weight of between 60 and 70 kDa (figure 3A). Our hypothesis was incorrect however regarding strains 17961-Δ*csrAlepA*L213 and 17961-Δ*csrA* sup. 1, as no

difference in protein expression were observed between wild-type and strain 17961-Δ*csrA* (figure 3B).

For now, the proteins expressed in wild-type and 17961-Δ*csrA* strains but not strains 17961-Δ*csrAettA*E470V or 17961-Δ*csrA* sup. 4, have not been identified. Because of what is known about EttA and its role in translational regulation, we proposed EttAE470V was halting translation of specific sequences and that this was, directly or indirectly, reversing the nitrogen starvation seen in strain 17961-Δ*csrA* in the presence of amino acids.

The other interesting observation which was made from SDS-PAGE analysis was that a single band of approximately 50 kDa appeared overexpressed in the soluble lysate of strain 17961-Δ*csrA*, but not in any of the other soluble lysates on the gel in figure 3A. Unpublished preliminary data (J. Farrow) had previously identified this band as an asparaginase enzyme. It appears likely that CsrA may (directly or indirectly) negatively regulate expression of this protein. It may be that EttAE470V may also negatively regulate expression of this protein as that would explain its presence in a strain lacking CsrA while not being present in a strain carrying CsrA. This putative asparaginase does not appear to play a role in strain 17961-Δ*csrA's* growth inhibition, since deletion mutants of the asparaginase gene in strain 17961-Δ*csrA* failed to effect growth (unpublished data, J. Farrow).

4.3: EttA is important during starvation

After identifying that *A. baumannii* strains carrying EttAE470V failed to express at least two proteins which were expressed in strains not carrying this mutant enzyme, we decided to focus our research on EttA. Research done in 2013 by Böel et al., suggested

that the EttA homolog in *E. coli* functioned to inhibit translation by binding to the E site of the ribosome while in the nucleotide bound form and dissociating from this site after hydrolyzing ATP. This dissociation is proposed to allow translation to move forward (13). However, at high concentrations of ADP and low concentrations of ATP, translation appeared to be inhibited (13). Additionally, they showed that cells expressing EttA had improved long term survival when compared to cells which did not express EttA. This is likely due to EttA preventing cells from wasting energy through translation while in an energy depleted state (13). We hoped to identify if EttA acted similarly in *A. baumannii* during starvation after long-term growth.

We observed that a strain of *A. baumannii* ATCC 17961 expressing EttA experienced better survival in LB media after 74 and 98 hours, compared with a strain lacking EttA (figure 4). This result appeared to be in line with what has been observed regarding EttA in *E. coli* (13). This result, in addition to the sequence similarity between the *E. coli* and *A. baumannii* homologs of EttA, allowed us to propose that the EttA homolog in *A. baumannii* had a similar function to that of EttA in *E. coli*.

4.4: Substitution of glutamate 470 to valine leads to altered EttA function

After establishing EttA's role in long term survival and proposing its function to be the same as its counterpart in *E. coli*, we next wondered how the specific mutations observed in our suppressor mutants would affect EttA's function (table 1). It is known that glutamate to glutamine mutations in both catalytic sites of ABCF proteins will lead these proteins to becoming stuck in nucleotide bound forms (13)(14)(15). It is also known that a glutamate to glutamine mutation of the second catalytic base alone will lead to a loss of approximately 70% of ATPase activity in the ABCF protein Vga(A) (32).

This implied that ATP is being hydrolyzed at one ATP binding site but not the other. To our knowledge, it was unknown how glutamate to valine mutations on catalytic bases effect ABCF proteins.

Based on what happened when the second catalytic site was mutated from glutamate to glutamine in Vga(A) (32), we hypothesized that the protein EttAE470V would experience a partial loss of ATPase activity. Our hypothesis was proven correct by the ATPase activity assay we performed on purified EttA and EttAE470V using [γ-^{32}P] ATP (figure 5). These results showed that EttAE470V was failing to hydrolyze ATP at one ATP binding site, but hydrolyzing it at the other ATP binding site. However, it remains unknown if EttAE470V can bind ATP at the same rate as EttA. An alternative explanation may be that the glutamate to valine mutation on residue 470 is not altering the enzyme's ability to hydrolyze ATP but is instead altering the structure of the protein in such a way that it loses its affinity for binding ATP. It may be important in the future for us to identify if ATP binding is being inhibited in EttAE470V.

4.5: Model

It seems likely that the alteration in ATPase activity seen in EttAE470V is leading to some proteins not being translated in strain 17961-Δ*csrA*. As a result of this, cells are regaining their ability to grow in the presence of amino acids. Based on this assumption we have proposed a model. This model attempt to explain how the presence of amino acids prevents the growth of strain 17961-Δ*csrA*, and how this can be overcome through the glutamate to valine mutation in residue 470 of the gene *ettA* (figure 6).

As mentioned earlier in this chapter, unpublished data suggest that when *csrA* is deleted, the availability of nitrogen in the cell becomes limited, with amino acids

exacerbating this effect. Whether this also results in the depletion of ATP remains unknown. In 2021 Doello et al., proposed that during nitrogen starvation both ATP and ATP/ADP ratios decline. This assumption was based off ATP and ADP measurements taken from *Synechocystis spp.* before and after inducing nitrogen deprivation (38). It remains uncertain if these results will extend to *A. baumannii*. Additionally, it has yet to be proven that nitrogen starvation is indeed the cause of strain 17961-Δ*csrA*'s growth defect in the presence of amino acids. For these reasons we have developed a model in which ATP levels become depleted in strain 17961-Δ*csrA* when amino acids are present. This model assumes that nitrogen starvation is taking place and that *A. baumannii* cells are responding to this in a similar way as *Synechocystis spp.* (38).

In our model, we assume that ATP becomes depleted in strain 17961-Δ*csrA* when amino acids are present due to nitrogen limitation. However, we assume this does not occur in wild-type cells (figure 6). This is caused by nitrogen starvation, but can be inhibited by CsrA (figure 6A). When CsrA is not present, amino acids lead to depleted levels of ATP and elevated levels of ADP. This triggers EttA to halt translation and results in loss of growth. When EttA is mutated to EttAE470V, translation is only partially halted, which results in all but a few proteins being expressed. These proteins failing to express lead, either directly or indirectly, to rescued growth (figure 6B). One explanation for how nitrogen starvation is occurring in strain 17961-Δ*csrA*, is that an enzyme involved in nitrogen metabolism is inhibited by specific amino acids and that expression of this enzyme is upregulated by CsrA counteracting the effects of amino acids. This is only one of several potential explanations for how amino acids cause nitrogen starvation

in Δ*csrA*. If this theory is correct, the identity of the protein involved in this regulation remains unknown. Because of this uncertainty, this proposal was left out of our model.

Alternatively, it may be the case that ATP levels remain high during strain 17961-Δ*csrA*'s exposure to amino acids. This is because we do not know for sure if our cells are undergoing nitrogen starvation or if nitrogen starvation will have the same effect that it did in *Synechocystis spp.* (38). We assume that EttA would allow translation to continue at normal levels. This would be detrimental to the cells which do not have the recourses available to translate normally. Once *ettA* is mutated and EttAE470V becomes expressed, the mutant protein may halt translation of some specific proteins. For unknown reasons, altered ATPase activity would likely be responsible for this partial blocking of translation. The result of this alternate proposal would be the same as in our model (figure 6) in that growth is restored either directly or indirectly due to some specific proteins failing to express.

4.6: Future directions

4.6.1: Measurement of ATP levels

There remain many unknowns in our proposed model. A major unknown is whether ATP is being depleted or maintained by cells of strain 17961-Δ*csrA* during exposure to amino acids. If we assume that nitrogen starvation is being triggered in these cells, as our preliminary metabolomic data suggest, then previous research suggests that ATP should become depleted (38)(43).

To confirm this hypothesis, a good next step for this project would be to measure the abundance of ATP in cells of strain 17961-Δ*csrA* before and after exposure to amino acids. This has been done in the past in *E. coli* cells using a BacTiter Glow kit (39). In

our proposed experiment, we would use the Bactiter Glo™ Microbial Cell Viability Assay (Promega; Madison, WI). This kit works by mixing Beetle Luciferin with recombinant fly luciferase, along with Mg^{2+} and O_2. This solution will react with ATP to produce visible light through the production of oxyluciferin which gives off a photon due to its high energy state (42). This solution can be combined with cell cultures to react with ATP produced by the cell, meaning luminescence will be correlated with ATP concentration.

For our proposed experiment, we should attempt to measure the amount of ATP in strain 17961-Δ*csrA* grown in MOPS-A media before and after exposure to 1% casamino acids. We could calculate the amount of ATP per μg of protein using BCA Protein Assay Kit (Thermo scientific; Rockford, IL). We hypothesize that more ATP per μg of protein would be observed in strain 17961-Δ*csrA* prior to exposure to casamino acids as opposed to 30 minutes after exposure to casamino acids. This is based off our speculation that cells undergo nitrogen starvation when exposed to amino acids, and the idea that nitrogen starvation leads to ATP depletion in bacterial cells (38).

4.6.2: DRaCALA

Once we establish whether strain 17961-Δ*csrA* is being depleted of ATP, our next experiment would be to identify if EttAE470V has a reduced ability to bind ATP compared to EttA. This would be an important step in identifying how the glutamate to valine mutation in *ettA* residue 470 is altering the protein. It may be the case that an inhibited ability to bind ATP is leading to the inhibited ability to hydrolyze ATP which we observed in figure 5.

To determine if EttAE470V has inhibited ATP binding compared to EttA, we propose performing a differential radial capillary action of ligand assay (DRaCALA)

developed by Roelofs et al, in 2011 (40). This assay works by mixing radiolabeled nucleotides with the enzymes being studied and adding droplets of the mixture to untreated nitrocellulose paper. Capillary action should cause unbound nucleotides to spread out in a circular pattern from the point of contact on nitrocellulose (outer circle). Nucleotides which remain bound to protein should stay closer to the point of contact on nitrocellulose (inner circle) (40).

The intensity of radioactivity, as well as the areas of the inner and outer circles can be calculated using a phosphorimager (40)(41). Once these calculations are made, an equation can be used to calculate the amount of ligand bound to proteins. This equation will determine the fraction bound (F_b), which is the percent of ligand bound to protein (40). The K_d can be calculated using this assay in order to determine a proteins affinity for its ligand. This is done by performing repeats with varying concentration of protein, while maintaining identical concentrations of ligand (40).

Our proposed experiment would use ATP labeled with ^{32}P and measure its binding to EttAE470V compared to EttA. First, you must establish concentrations of ^{32}P labeled ATP which can bind EttA, and then you test the binding of both EttA and EttAE470V to this concentration of ^{32}P bound ATP (41). Serial dilutions of highly concentrated EttA and EttAE470V could be mixed with identical concentrations of ^{32}P bound ATP to generate a Michalis-Menten plot of binding affinity for both enzymes. This would allow us to calculate the K_d for the binding affinity of both proteins to ATP and identify if EttAE470V has a lower ATP binding affinity than EttA (40)(41).

If EttAE470V has inhibited binding to ATP, then this could help explain how the mutant protein restores translation in ATP depleted cells. It may be that this inhibited

binding to ATP also causes inhibited binding to ADP. Since EttA is proposed to recognize high ADP levels relative to ATP, and inhibit translation (13), it may be that failure to bind ATP or ADP prevents EttA from functioning to block translation. With this being said, it is not known if inhibited ATP binding will necessarily imply an inhibited ability to bind ADP. Additionally, inhibited binding of ATP and ADP still does not explain how EttAE470V appears to be selectively inhibiting translation of some specific proteins.

4.6.3: Identifying the proteins not expressed in strain 17961-Δ*csrAettA*E470V

Once we can fill in our model with results from an ATP per cell assay and a differential radial capillary action of ligand assay (DRaCALA), a good next step would be to identify the proteins failing to express in strain 17961-Δ*csrAettA*E470V. To do this we can simply excise the bands from SDS-PAGE gels which were expressed in wild-type and strain 17961-Δ*csrA* but not strain 17961-Δ*csrAettA*E470V. These samples can be analyzed via mass spectrometry to determine their identities. Once we identify these proteins, we can generate deletion mutants of these genes in the strain 17961-Δ*csrA*. If this new strain can grow in the presence of amino acids, then we can propose that failure of strain 17961-Δ*csrAettA*E470V to express these proteins is leading to a reversal of the phenomena causing this growth defect.

4.6: Conclusions

Overall, it appears that mutation of *ettA* to *ettA*E470V, or the generation of a premature stop codon in *lepA*, after residue 213, can reverse the effects which amino acids have on *A. baumannii* strain 17961-Δ*csrA* (table 1) (figure 1 and 2). Mutation of *ettA* to *ettA*E470V appears to cause a partial loss of ATPase activity (figure 5). This altered ATPase activity of EttA appeared to lead to cells failing to express at least two

proteins normally expressed in the wild-type strain and strain 17961-Δ*csrA* (figure 3). It appears likely that nitrogen starvation is being induced by amino acids in strain 17961-Δ*csrA* and that this is being reversed by some proteins not being expressed in strain 17961-Δ*csrAettA*E470V. Further directions for this project will likely include calculating ATP levels in strain 17961-Δ*csrA* when amino acids are present in the growth media, determining if EttAE470V has inhibited binding to ATP compared to EttA, and identifying the proteins not expressed by strain 17961-Δ*csrAettA*E470V. The inability of strain 17961-Δ*csrA* to grow in the presence of amino acids is important because it implies this strain would experience inhibited virulence, since human serum should contain amino acids. These studies should help in furthering our understanding of *A. baumannii* and the ways in which CsrA regulates nitrogen metabolism within this species.

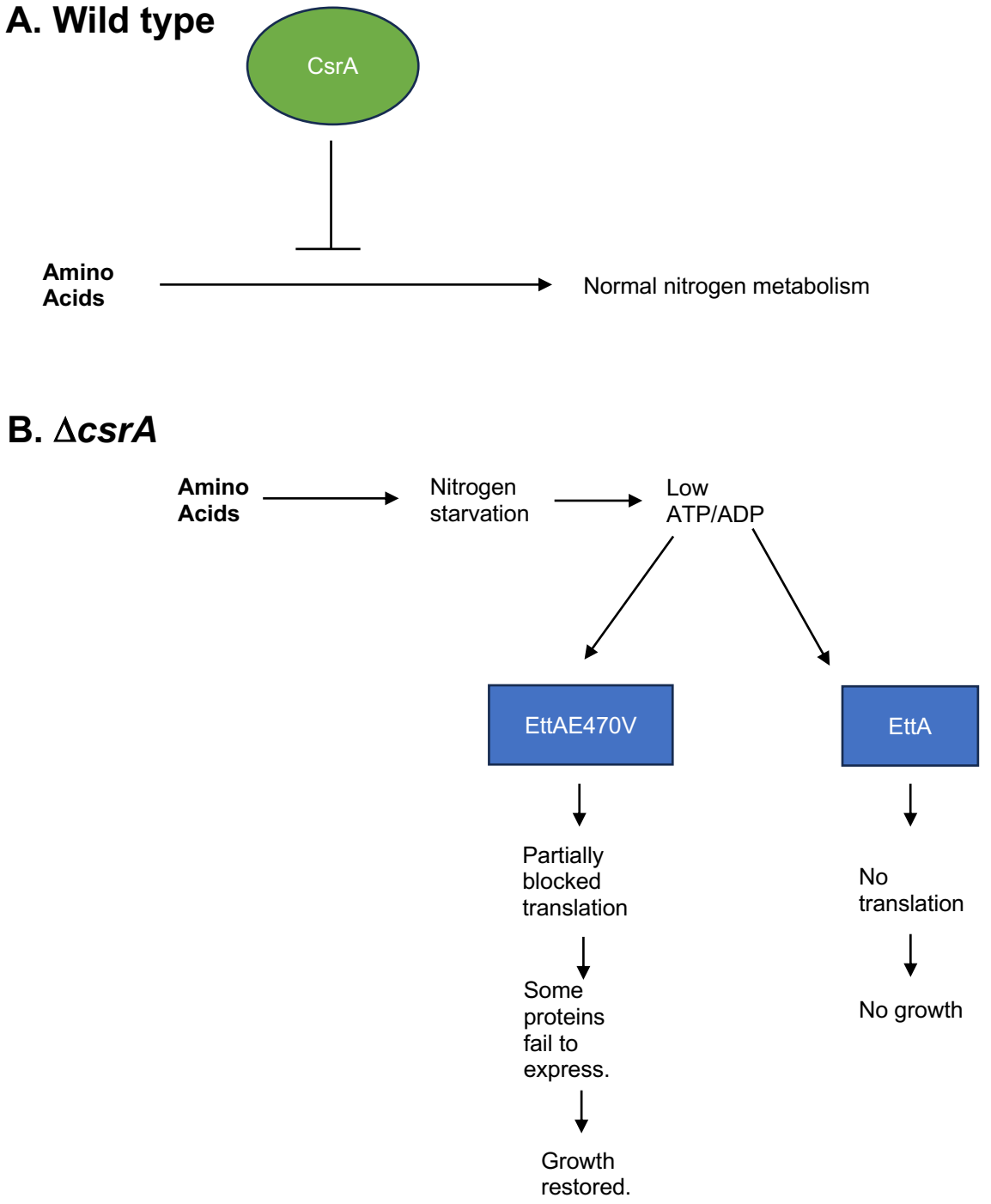

A. Wild type
CsrA
Amino Acids
Normal nitrogen metabolism
B. ΔcsrA
Amino Acids
Nitrogen starvation
Low ATP/ADP
EttAE470V
EttA
Partially blocked translation
Some proteins fail to express.
Growth restored.
No translation
No growth

Figure 6

Figure 6 – Model
Model for how mutations of EttA to EttAE470V is leading to rescued growth for strain 17961-Δ*csrA* in the presence of amino acids. (A) Shows a wild-type cell in which amino acids are present. Amino acids would lead to nitrogen starvation, but this effect is blocked by CsrA (green oval). (B) Shows a Δ*csrA* cell in which amino acids are present. Amino acids will cause cells enter a state of nitrogen starvation. This leads to low ATP/ADP ratios which lead EttA to block translation. EttAE470V mostly restores translation with only a few sequences being blocked. This results in some proteins failing to express and results in growth being restored.

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