Elongation Factor P is Required for Processes Associated with *Acinetobacter* Pathogenesis

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ABSTRACT
Antibiotic resistance is one of the world's fastest-growing and most prevalent problems today. The influx of antibiotics within our environment from inadequate antibiotic stewardship has led to a surge of drug-resistant microorganisms. The CDC has classified Carbapenem-resistant *Acinetobacter* (CRA) as an urgent threat within this crisis. New drug development is imperative to combat infections caused by drug-resistant pathogens such as CRA. Bacterial translation, the process of protein synthesis by the ribosome, is a common target for new antibiotic development. Elongation factor P (EF-P) is a universally conserved translation factor required for antibiotic resistance in many bacteria. In this study, we assessed the importance of EF-P in processes associated with *Acinetobacter* pathogenesis. In the absence of EF-P, *Acinetobacter baylyi* displays decreased biofilm formation, surface-associated motility, and resistance to beta-lactams and carbapenems. This data holds hope for future drug development targeting EF-P in pathogens closely related to *A. baylyi*.

KEYWORDS
Acinetobacter baylyi; Translation; Ribosome; Elongation Factor P; Polyproline; Biofilm; Surface Associated Motility; Antibiotic Resistance

INTRODUCTION

Antibiotic resistance is one of the world's most pressing problems today because of the difficulty of treating antibiotic-resistant infections. Antibiotic resistance refers to the ability of bacteria to develop resistance to particular drugs designed to kill them.[1] Thus, resistant bacteria will survive in the presence of current antibiotic treatments.[2] The growth of antibiotic-resistant bacteria can be attributed to antibiotic overuse and poor stewardship during hospital discharge.[3] The Center for Disease Control and Prevention (CDC) estimates a national total of 2,868,700 infections and 35,900 deaths each year from resistant fungi and bacteria.[2] The inability to effectively treat patients and the number of deaths display the importance of finding new ways to treat antibiotic-resistant bacteria.

The CDC characterizes bacteria on a threat level ranging from concerning, serious, and urgent based on factors including clinical impact, availability of effective antibiotics, transmissibility, and incidence.[2] Carbapenem-resistant *Acinetobacter* (CRA) bacteria jumped from a threat level of serious to an urgent threat level between the two most recent antibiotic resistance threat reports.[2,4] CRA cause bloodstream infections and pneumonia in patients with weakened immune systems, such as long-term hospital patients in intensive care units.[5] CRA microbes are often multidrug-resistant, meaning the bacteria are resistant to more than just the carbapenem class of antibiotics.[2] These resistant bacteria were responsible for nearly 8,500 hospitalizations and 700 deaths in 2017 according to the CDC. [2]

The increase in infections from multi-drug resistant microorganisms limits the treatments available to a patient.[3] New drug development is imperative to cope with the rise of multi-resistant microbes.[6] Many new drugs in development target translation.[7] Translation is the cellular process that allows both eukaryotic and prokaryotic cells to synthesize proteins using ribosomes.[8-9] Bacterial translation is an intriguing target for adjuvant therapies that could potentiate existing drugs, as bacteria are often hypersensitive to antibiotics in the absence of key a translation factor known as Elongation Factor P (EF-P).[10-13] Elongation factor P (EF-P) is a universally conserved protein that alleviates ribosomal pausing at polyproline motifs.[14-15] Polyproline motifs cause ribosomal pausing because steric hindrance of proline delays the formation of peptide bonds.[16] The strength of the pause is determined by the translation initiation rate, the surrounding amino acids, and the location of the pause on the transcript.[17-19] When the ribosome pauses at a polyproline motif, EF-P enters the vacant E site of the ribosome and aids in the peptide bond formation to allow translation to continue.[20]

In many organisms, EF-P is required for a wide range of cellular processes including growth, motility, and antibiotic resistance.[10-13, 21-22] Here, we characterize the role of EF-P in *Acinetobacter baylyi*. While it is generally non-pathogenic, *A. baylyi* is an established genetic model system that shares a significant genetic similarity with CRA species *Acinetobacter baumanii.*[23] *A. baylyi* is often used in drug resistance studies, and 80% of *A. baumannii* core genes have orthologs in *A. baylyi.*[23] Studying *A. baylyi* could lead to fundamental discoveries applicable to *A. baumannii* and the creation of therapeutics targeting EF-P in CRA. The shared genetic material in combination with being an ideal model organism makes *A. baylyi* an alternative way to study the dangerous opportunistic pathogen *A. baumannii.* In *A. baylyi,* loss of EF-P results in a decreased growth rate, but other phenotypic effects have not been characterized.[24] Here, the physiological significance of EF-P in *A. baylyi* was investigated, focusing on processes that are required for pathogenesis in *Acinetobacter* species.[25-26]

MATERIALS AND PROCEDURES

Bioinformatics

The EF-P protein sequences for *A. baylyi* (Q6FAA9) and *A. baumannii* (B2HVL6) were accessed through UniProt.[27] Alignments of the EF-P protein sequences were generated by a multiple sequence alignment tool on Clustal Omega, and the percent identity between the two sequences was noted.[28] Alignment was repeated comparing *A. baylyi* to the following microorganisms: *Bacillus subtills* (P49778), *Escherichia coli* (P0A6N4), *Pseudomonas aeruginosa* (Q9HZZ2), *Salmonella typhimurium* (P64036), *Erwinia amylovora* (D4I1J7), *Neisseria meningitidis* (P0DUK0), and *Staphylococcus aureus* (Q6G937). All percent identities relative to *A. baylyi* were noted.

The full proteomes for *A. baylyi* (UP000000430) and the other microorganisms of interest were accessed through UniProt. Proteins containing PPX motifs were identified using the Expasy ScanProsite tool.[29] The percentage of proteome containing PPX motifs was calculated by dividing the number of proteins on the PPX list by the total number of proteins indicated by UniProt. The percentage PPX was noted for all the following organisms: *A. baumannii* (UP000005740), *B. subtilis* (UP000001570), *E. coli* (UP000000558), *P. aeruginosa* (UP000002438), *N. meningitidis* (UP000000425), *S. typhimurium* (UP000001014), *S. aureus* (UP000008816), and *E. amylovora* (UP000001841).

Strains and Growth Conditions

Both the wild-type and *Δefp* mutant strains of *A. baylyi* were graciously donated by Dr. Valrie de Crecy-Lagard in the Microbiology department at the University of Florida.[24] The wild-type strain was ADP1 and the experimental strain was PS6433 (ADP1 *Δefp::sacBKanR*). Both bacterial cultures grew in Lennox formulation Luria broth (LB) (Carolina) liquid tubes with shaking. The wild-type culture grew at 30 °C in a liquid LB culture for 24 hours. The *Δefp* culture grew at 30 °C in liquid LB in the presence of 15 μg/mL kanamycin (Thermo Scientific) for 48 hours. All assays were conducted at 30 °C except for the surface-associated motility assay, which was conducted at 37 °C.

Surface Associated Motility

The motility testing was performed with semisolid agar plates containing 0.5% granulated tryptone, 0.25% NaCl, and 0.3% agarose.[25] A needle was used to inoculate the agar plates by puncturing the top surface of the agar. The bacteria grew in a dense halo formation with jagged edges displaying surface-associated motility. The plates grew in an incubator at 37 °C and were scanned after 24 and 48 hours. The assay was performed in triplicate using biological replication.

Biofilm Formation

Saturated starting cultures for both strains were diluted to an OD of 0.5. 30 μl of each dilution was added to a corresponding three mL LB liquid test tube and placed in the stationary incubator at 30 °C for 72 hours. The liquid media was removed, and adherent cells were stained for 10 minutes in three mL of 0.02% crystal violet dye. The stain was removed, and photographs were taken of each test tube's biofilm formations. One mL of PBS was added to each formation and vortexed for five minutes to collect adherent cells. The cell suspension was collected and the OD_{580} of each sample was measured. The assay was performed in triplicate using biological replication.

Disc Diffusion

Saturated wild-type and *Δefp* cultures were back diluted to an OD_{600} of 0.1. Bacterial lawns of *A. baylyi* were grown on LB plates using sterilized swabs for each sample. Paper discs saturated with 10μg of the following antibiotics were placed on the LB agar plates using forceps: ampicillin (Biogram), amoxicillin (Sensi-Disc), meropenem (Sensi-Disc), and imipenem (Sensi-Disc). The plates were incubated at 30 °C for 48 hours. Plates were scanned and the zones of inhibition for each antibiotic were measured using ImageJ software.[30] The assay was performed in triplicate using biological replication.

RESULTS

EF-P sequence and overall PPX content is similar between A. baylyi and A. baumannii

To support the notion that EF-P likely plays a similar role in both *A. baylyi* and *A. baumannii*, we began by analyzing both the EF-P protein sequence and the polyproline content of both organisms. EF-P in *A. baylyi* shares 92% sequence identity to *A. baumannii* (Table 1). *A. baylyi* and *A. baumanni* also had nearly identical polyproline content in their respective proteomes (26.5%) (Table 1). Taken together, this supports the idea that EF-P will likely have a similar role in both organisms.

To predict the physiological significance of EF-P in *A. baylyi,* we compared the EF-P sequence and polyproline content to organisms in which EF-P has been thoroughly characterized. To function efficiently, EF-P requires post-translational modification at a conserved residue. However, this modification varies widely between different organisms.[31] Organisms that employ *R*-β-lysine modification strategy shared the highest sequence identity and polyproline content with *A. baylyi* (*E. coli, S. typhimurium, E. amylovora*) (Table 1). In contrast, organisms that use *L*-rhamnose modification of EF-P maintained less sequence identity and substantially higher polyproline content in their proteomes (*N. meningitidis* and *P. aeruginosa*) (Table 1). Organisms that use 5-aminopentanol modification had relatively lower percent identity and polyproline content (*B. subtilis* and *S. aureus*) (Table 1). Taken together, these results suggest that in *A. baylyi* EF-P will have a similar functional and physiological role as it does in other organisms that also modify EF-P with the *R-β*-lysine modification.

Table 1. EF-P amino acid percent identity and percentage of proteome containing at least one PPX motif.

Organism	Percent Identity	Percent Proteome with PPX
A. baylyi	100	26.5
A. baumannii	92	26.7
E. coli	60	32.7
S. typhimurium	60	33.6
E. amylovora	59	31.3
B. subtilis	43	21.4
S. aureus	40	16.0
P. aeruginosa	35	45.0
N. meningitidis	31	30.8

EF-P is required for surface associated motility

To demonstrate whether EF-P is utilized in surface motility of *A. baylyi*, we collected data on wildtype and ⊿*efp* strain motility at 24 hours of incubation. Qualitative imaging at 24 hours displayed normal wildtype motility over time, while ⊿*efp* motility was completely abolished (**Figure 1a**). Quantitative measurements of the zones indicated that the difference in motility between wildtype and Δ*efp* were significant (**Figure 1b**). Since the surface associated motility assay is sensitive to differences in growth, we continued to allow all biological replicates to grow until a 48-hour timepoint. If the apparent ⊿*efp* motility defect was a result of decreased growth rather than decreased motility, we would expect the cells to eventually migrate from the inoculation point given sufficient growth time. For two of the ⊿*efp* biological replicates, no surface associated motility was observed; however, one ⊿*efp* replicate displayed a low number of cells that migrated from the inoculation point, included in **Figure 1 (labeled suppressor)**. Given the distinct morphology of the replicate, we believe this was a suppressor mutation to circumnavigate the EF-P requirement for surface associated motility.

EF-P is necessary for successful biofilm formation

To assess the role of EF-P in the biofilm formation, both wildtype and Δ*efp* strains were grown in cultures to promote biofilm formation. After three days of incubation, cells were stained and observed. The WT image indicates a faint stained ring of biofilm formation halfway up the image, whereas in ⊿*efp,* there is no visible indication of biofilm formation (**Figure 2a**). To quantitatively assess this phenotype, stained cells were harvested and the OD_{580} was recorded in triplicate. **Figure 2b** indicates that wildtype strain produced greater biofilm mass than the ⊿*efp* strain. It is possible that the decrease in biofilm mass in the ⊿*efp* strain could be the indirect result in the growth rate defect rather than a direct biofilm formation defect. While we cannot definitely exclude this possibility, it is important to note that there was no detectable biofilm biomass on the tube (absorbance readings were comparable to background). Given the time scale of the experiment, we would expect some level of biofilm formation to have occurred within this timeframe if the mutant strain was able to form a biofilm.

EF-P is required for antibiotic resistance

To determine the role EF-P plays in antibiotic resistance, we analyzed the antibiotic sensitivity of *A. baylyi* to beta-lactams and carbapenems. Each zone of inhibition was larger in *Δefp,* showing more sensitivity to each of the antibiotics than the wildtype control (**Figure 3**). The zones of inhibition for both classes of antibiotics indicate the *A. baylyi* cells lacking *efp* are significantly more sensitive to antibiotics.

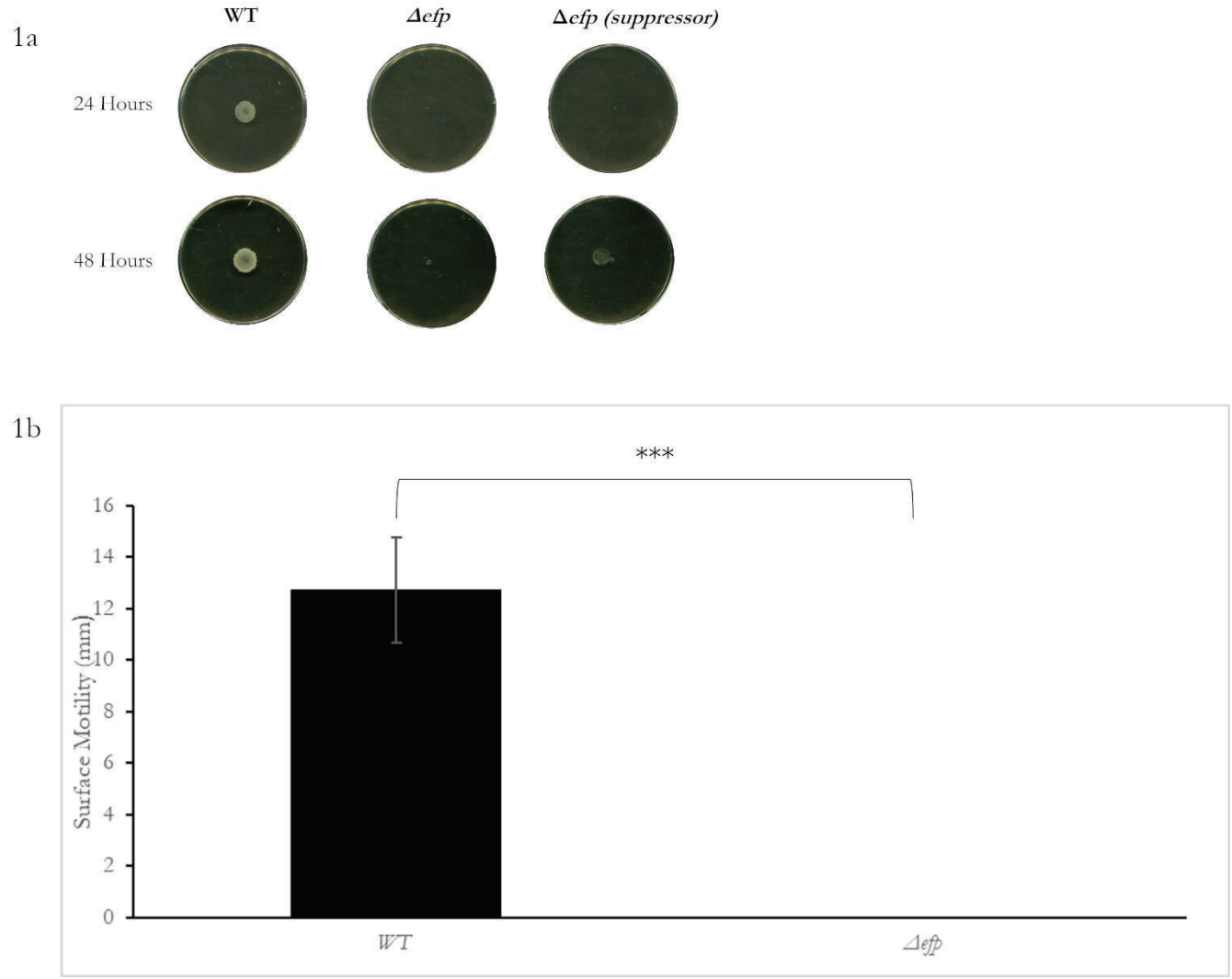

Figure 1. EF-P is required for surface associated motility. (a) WT and Δ*efp* cultures were inoculated in surface associated motility plates in triplicate. Motility was documented after 24 and 48 hours of incubation at 30° C. One replicate produced an atypical migration pattern only after 48 hours, suggesting it acquired suppressor mutation. (b) Quantitative data showing the zone of motility present in the WT and Δ*efp* after 24 hours of incubation. ImageJ was utilized to quantify the surface motility shown in both groups. Since the mutant lacked motility completely, no measurement was collected. A t-test analysis determined there is significance in the data, ***p<0.001. Data represents the mean of three biological replicates. The error bars represent the standard deviation between all three biological replicates.

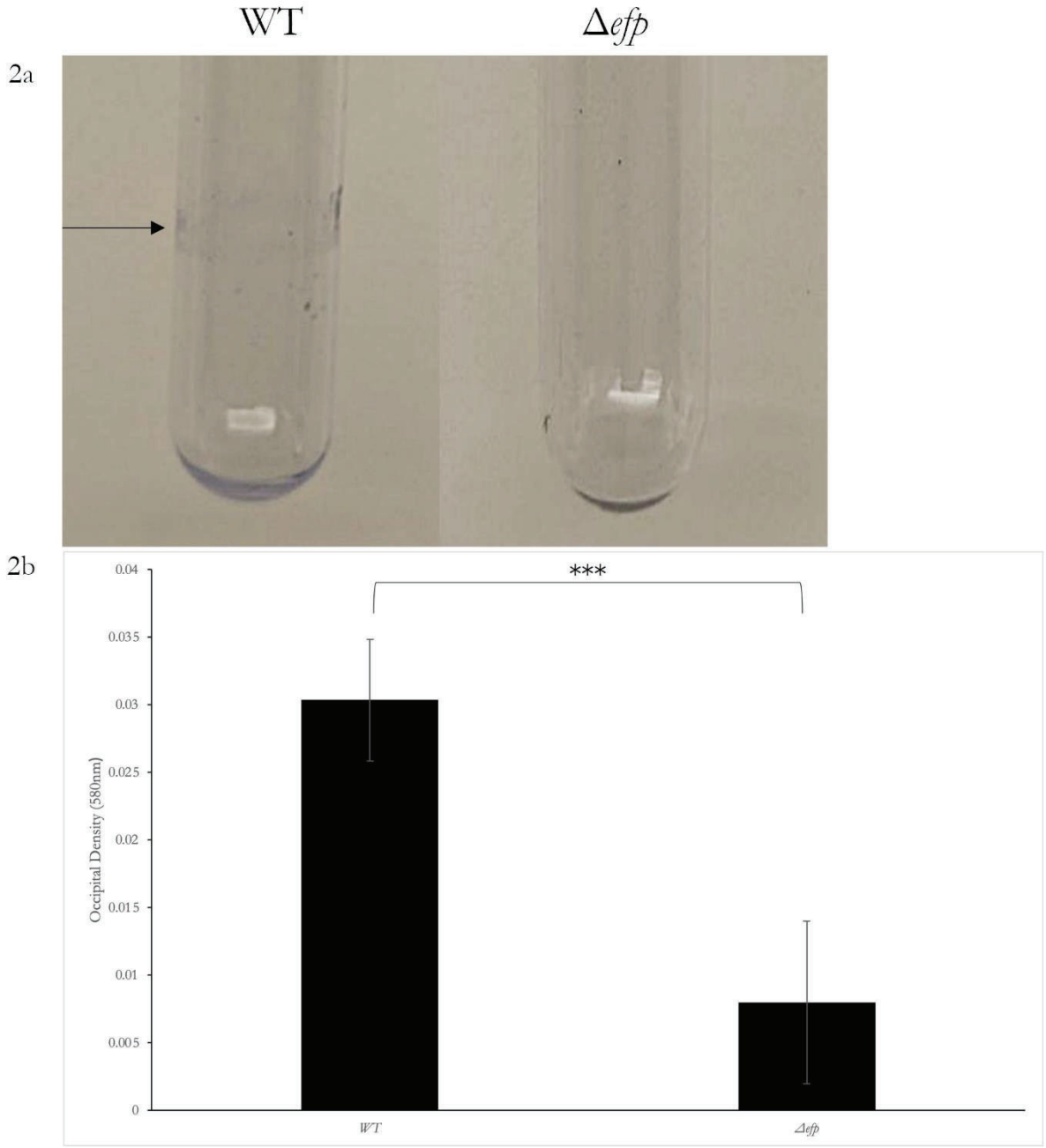

Figure 2. EF-P is required for biofilm formation. (a) Qualitative representation of biofilm ring formation of both wildtype and *Δefp* strains. The photographs were taken after 72 hours of incubation directly followed by 0.02% crystal violet staining. The *Δefp* replicate displays no stained formation, whereas wildtype shows a stained ring biofilm formation as indicated by the arrow. Similar results were obtained for all three replicates. (b) Optical densities at 580 nm shows wildtype biofilm has significantly greater biofilm mass compared to *Δefp*. After resuspending the biofilm cultures with 1 mL of PBS, absorbance readings at an OD of 580 nm were collected. The absorbance readings for *Δefp* were severely lower than wildtype. A t-test analysis determined there is significance in the data, ***$p<0.001$. Data represents the mean of three biological replicates. The error bars represent the standard deviation between all three biological replicates.

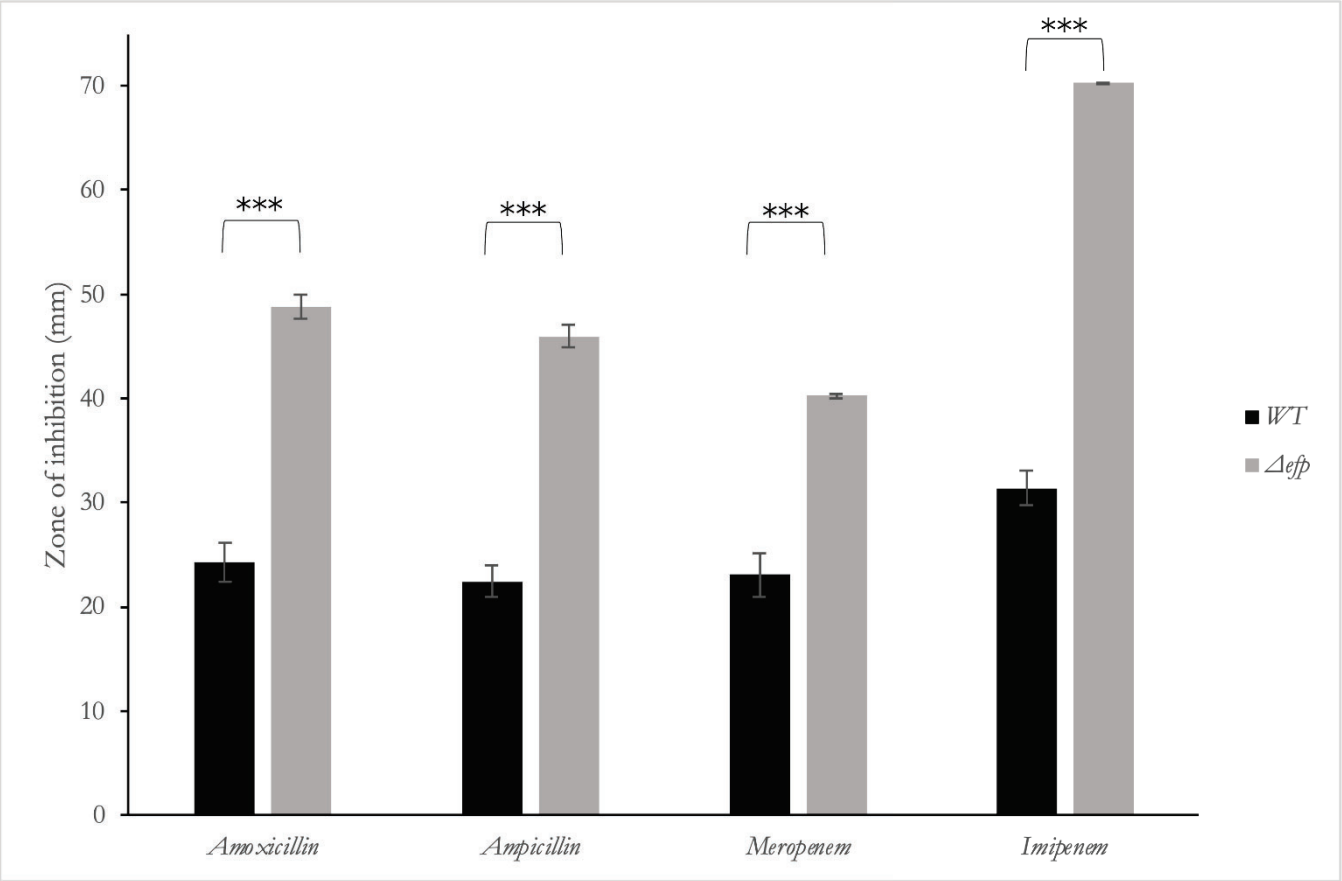

Figure 3. *A. baylyi* is hypersensitive to antibiotics without EF-P. *A. baylyi* zones of inhibition (mm) for amoxicillin, ampicillin, meropenem, and imipenem on both wildtype and Δ*efp* are shown. After growing a bacterial lawn of the strains in the presence of each antibiotic, ImageJ was used to quantify the zones of inhibition for all the samples. The zones of inhibition of each antibiotic are significantly larger in the Δ*efp* group showing less resistance to each antibiotic. A t-test analysis determined there is significance in the data, ***p<0.001. Data represents the mean of three biological replicates. The error bars represent the standard deviation between all three biological replicates.

DISCUSSION

CRA has been identified by the CDC as an urgent threat within the antibiotic resistance crisis.[2] Here, we aimed to characterize the role of EF-P, a potential drug target, in *Acinetobacter* species. Though it is rarely pathogenic, we characterized EF-P in *A. baylyi,* as it has been proposed to serve as a safer model system in studying *A. baumanii.*[23] The percent identity in the EF-P amino acid sequences between both *A. baumannii* and *A. baylyi* is 92%, suggesting EF-P likely functions similarly in both these microorganisms when compared to the other microorganisms surveyed (**Table 1**). After surveying multiple microorganism's proteomes containing a PPX motif, *A. baumannii* and *A. baylyi* contained nearly identical polyproline content within the proteome, displaying even more similarities between the two microorganisms and the likely importance of EF-P in each of these species. (**Table 1**).

In *A. baylyi,* surface associated motility is completely abolished in the absence of EF-P (**Figure 1**). The putative suppressor suggests a biological pathway the bacteria mutated to circumnavigate the requirement of the *efp* gene to successfully perform surface motility. Similarly, biofilm formation and antibiotic resistance are severely impacted by the absence of EF-P (**Figure 2** & **3**). Surface associated motility and biofilm formation are both processes known to be required for pathogenesis in *Acinetobacter* species.[25-26] Antibiotic resistance is of particular interest in a clinical setting considering the prevalence of CRA.[2] Taken together, these phenotypes suggest EF-P plays an important role in the ability of *Acinetobacter* to survive in a clinical setting.

One interesting finding is the disparity between the PPX content of *A. baylyi* and *P. aeruginosa.* Both species have severe growth defects without EF-P, but maintain disparate PPX content in their respective proteomes (**Table 1**).[10] Although one might assume the higher PPX percentage in the proteome is a causation for a greater need of EF-P in a microorganism, we observe *A. baylyi* contains a lower PPX percentage than *P. aeruginosa* but displays equally severe phenotypes without EF-P. However, it is important to also consider the context dependent nature of ribosomal pausing at PPX motifs in the absence of EF-P. The translation

initiation rate, distance from the N-terminus, and the amino acid in the third position of the motif all play a significant role in the severity of ribosomal pausing.[17-19] It is possible that these three factors are contributing to pausing in a high number of proteins in *A. baylyi*. Future ribosomal profiling studies will be required to accurately characterize pausing in the absence of EF-P in *A. baylyi.*

Previous literature has documented phenotypes associated with loss of EF-P within *A. baumannii.*[32] The biofilm results presented here concur with their findings that EF-P is required for biofilm formation. However, it was also established here that in *A. baylyi,* EF-P is also required for surface associated motility. According to Guo et al., the deletion of *efp* enhanced the surface associated motility of *A. baumannii.*[32] There are several possible explanations for this discrepancy. One explanation would be the length of time the surface associated motility tests we conducted. Guo et al. conducted the assay for 8 hours, while here data was collected after 24 and 48 hours. It is possible that the requirement for EF-P varies throughout the stages of this physiological state. A second explanation could be due to the nature of the strain construction in both studies. Here, a true knockout deletion strain of *A. baylyi* was used, while Guo et al. used a deletion that placed *efp* under the control of an inducible promoter. Inducible systems can be subject to leaky expression, allowing for some expression of the target protein.[33] Differences in EF-P expression in these two systems could lead to different results. Lastly, though *A. baylyi* and *A. baumanii* are highly similar, they are distinct species of *Acinetobacter*. Guo et al., has shown that in *A. baumannii,* EF-P interacts with c-di-GMP. However, this interaction does not appear to occur in other microbes. It is plausible that this may also be the case for *A. baylyi,* meaning EF-P is playing a unique role in *A. baumanii.*[32]

CONCLUSIONS

EF-P is required for biofilm formation, surface associated motility, and resistance to beta-lactams and carbapenems in *A. baylyi.* Bioinformatic analyses suggest that EF-P likely maintains a similar role in the closely related pathogen *A. baumanii.* This work shows that EF-P contributes to a wide range cellular processes related to pathogenesis, which may be applicable to *A. baumanii.*[25-26] Development of antibiotics targeting EF-P would likely have minimal off target effects due to a very minimal structural resemblance of eukaryotic EF-P (EIF5A).[34] However, we do not yet understand how exactly to target EF-P, nor do we understand the most important PPX motifs alleviated by EF-P in *A. baylyi*. Future ribosomal profiling studies must be conducted to better understand how each PPX motif impacts the severity of ribosomal pausing in *A. baylyi.*

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PRESS SUMMARY

Antibiotic resistance is one of society's most prevalent problems today. According to the Centers for Disease Control and Prevention (CDC), 35,900 people die each year from antibiotic-resistant infections. The CDC ranks Carbapenem-Resistant Acinetobacter (CRA) species as an urgent threat to global health. This study characterizes the physiological significance of a translation factor in *Acinetobacter* species to assess the potential for this protein to be a target in future drug development against CRA.