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Posted Date: 25 October 2024

doi: 10.20944/preprints202405.0730.v2

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Article

Cloning and Mutagenesis of AmpC β–Lactamase in *Pseudomonas aeruginosa* for the Identification of Targetable Allosteric Sites

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Abstract: The opportunistic pathogen *Pseudomonas aeruginosa* is responsible for numerous chronic infections and has a case fatality rate of 50%. All *P. aeruginosa* strains express β–lactamase, an enzyme capable of inactivating β–lactam antibiotics, including penicillins. However, β– lactams are still considered desirable therapeutics by physicians due to their low toxicity. The short term goal of this study was to create an optimal model for *ampC* expression in *P. aeruginosa* and determine the selecting antibiotic to be used during mutagenesis. In the long term, this study aims to renew effectiveness of β–lactams by identifying potential allosteric binding sites on β–lactamase for the development of novel β–lactamase inhibitors. To create a system for *ampC* expression, the chromosomal *P. aeruginosa ampC* gene encoding for β– lactamase was cloned into *Escherichia coli*, then conjugated into *P. aeruginosa* ΔampC. β– lactamase assays on recombinant *E. coli* and *P. aeruginosa* detected much higher production of β–lactamase in pMMB67HE, which was confirmed with antibiotic susceptibility testing (AST) to numerous β-lactams. From AST, carbenicillin was chosen as the antibiotic to select functional β–lactamase production during mutagenesis. Future work includes conducting mutagenesis on *ampC* to identify allosteric binding sites on the β–lactamase enzyme that may enable the discovery of new β–lactamase inhibitors. This work allows for the development of novel β–lactamase inhibitors that could renew the utility of β–lactams and drastically reduce morbidity and mortality from chronic *Pseudomonas* infections.

Keywords: *Pseudomonas aeruginos*; β–lactamase; *ampC*

Summary

The bacteria *Pseudomonas aeruginosa* is gaining notoriety in the clinical setting for causing deadly bacterial infections because of its resistance to antibiotics. *P. aeruginosa* produces an enzyme called β–lactamase that destroys most clinically prescribed antibiotics, including penicillins. Therefore, this study aims to investigate potential binding sites on the β–lactamase protein for novel inhibitors that inactivate this protein. The gene encoding for this protein was replicated into many copies and confirmed for its expression in bacteria. The antibiotic carbenicillin was found best measure the expression of this gene. Future work includes creating mutated copies of the gene and identifying the copies that cause the gene to be expressed incorrectly; this will elucidate on potential sites on the protein that drugs can target to inactivate this protein. This information will be crucial in developing novel therapeutics to treat *Pseudomonas* infections, which could save thousands of lives annually.

1. Introduction

Infections with bacteria resistant to commonly prescribed antibiotics represent a major public health crisis. In 2008, the Infectious Diseases Society of America designated a group of antibiotic-resistant bacteria, referred to by the acronym ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter*), as especially problematic [1]. The resistance mechanisms of these pathogens belong to three categories: 1. Acquisition and expression of genes encoding antibiotic-inactivating enzymes, 2. Mutations in the target of antibiotics, making them resistant,

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and 3. Presence and expression of pumps that expel the antibiotics from cells before they reach toxic levels [2]. The opportunistic pathogen *Pseudomonas aerug- inosa* (the P in ESKAPE), in particular, is responsible for a number of acute nosocomial infections and chronic infections such as chronic wounds, urinary tract infections, bacteremia, endocarditis, nosocomial infections, and infections in the lungs of cystic fibrosis (CF) patients [1,2]. Furthermore, this organism is difficult to treat due to its high levels of resistance to most clinically useful antibiotics.

P. aeruginosa is best characterized for its resistance to conventional *β*–lactam antibi- otics, including penicillins, carbapenems, and cephalosporins [3]. This is due to the ability of *P. aerugionosa* to secrete *β*–lactamase, an enzyme that hydrolyzes the *β*–lactam ring of *β*–lactam antibiotics, thereby inactivating the antibiotic [2–4]. Regardless, *β*–lactams are still considered desirable therapeutics by physicians due to their low cost and lack of toxicity even at high concentrations [3]. One solution to renew the effectiveness of *β*–lactam an- tibiotics is *β*–lactamase inhibitors that are designed to inhibit serine *β*–lactamases, thereby restoring the antimicrobial properties of *β*–lactams [4]. However, bacterial species are already beginning to acquire resistance to these inhibitors; avibactam is currently one of the only effective *Pseudomonas β*–lactamase inhibitors when prescribed with ceftazidime, and it is already in decline [5]. While the ceftazidime-avibactam (CAZ-AVI) *β*–lactamase inhibitor combination maintains efficacy against *Enterobacteriaceae*, *P. aeruginosa* has already de- veloped a resistance rate of up to 18% within the first five years of CAZ-AVI's clinical use (resistance rate defined by the percentage of non-sensitive bacterial isolates) [5]. This is likely due to *β*–lactamase inhibitors' inactivation mechanism; most *β*–lactamase inhibitors exert activity through competitive occupancy of its active site. However, mutations on this enzyme have developed to where the inhibitor can no longer bind to the protein but its respective substrate still maintains these capabilities, rendering the inhibitor ineffective [6]. Allosteric inhibition, on the other hand, induces a conformational change in the protein of interest, resulting in its complete loss of functionality; this property of allosteric inhibition makes it an ideal strategy for novel *β*–lactamase inhibitors. Thus, it is clinically relevant to study the chemical structure and functionality of the *β*–lactamase protein to identify new inhibitory sites for *β*–lactamase inhibitors.

It is thought that the chromosomal *ampC* gene in *Pseudomonas* is responsible for the encoding of *β*-lactamase, the main mechanism driving *β*-lactam antibiotic resistance in *P. aeruginosa* [4]. However, the *ampC* gene in *Pseudomonas* has not been thoroughly inves- tigated or confirmed for its expression of *β*–lactamase. This is likely due to the difficulties involved in studying genes present on bacterial chromosomal DNA; antibiotic resistance genes found on plasmids or other mobile genetic elements can be easily transformed into a host bacterium and is more likely to be expressed. However, it is difficult to study *ampC* because it is uncertain how this gene will be expressed when removed from its natural environment and cloned into a plasmid with a different and perhaps incompatible promoter system. For these reasons, the *ampC* in *Pseudomonas* has never been isolated or studied in a laboratory setting. Therefore, to study the *β*-lactamase protein in *Pseudomonas*, this experiment aimed to first isolate and clone *ampC* of *P. aerugionsa* into *E. coli*. Furthermore, considering the inefficiency of current *β*–lactam antibiotics, the long-term goal of this study was to perform random mutagenesis on *ampC* to identify potential allosteric inhibitor sites on *β*-lactamase for novel *β*-lactamase inhibitors.

2. Methods

2.1. Bacterial Strains, Plasmids, and Media

Wild type *Escherichia coli* and *Pseudomonas aeruginosa* were cultured in Luria-Bertani medium. Chromosomal *P. aeruginosa* PA01 DNA containing the gene of interest (*ampC*) was prepared via phenol-chloroform extraction. pDN19 and pMMB67HE plasmid was pu- rified from DH5*α* *E. coli* using a plasmid purification kit from Bio Basic (plasmids later utilized as host vectors for recombination of *ampC*). Recombinant plasmids were introduced in competent DH5*α* and BL21 *E. coli* cells then conjugated into *ampC* deficient strain of

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P. aeruginosa PAO1 (Δ*ampC*); conjugation was facilitated by *E. coli* transformed with pRK2013 plasmid.

For antibiotic susceptibility testing, the following strains of *P. aeruginosa* were utilized: wild type PAO1 (laboratory strain used to generate *ampC* deletion), PAO1 Δ*ampC*, PA14, PA14 Tn-*ampC* (*ampC* gene disrupted through insertion of a transposon), PAO1 seq (orig- inal sequenced isolate of *P. aeruginosa*, and PAO1 seq Tn-*ampC* (*ampC* gene disrupted through insertion of a transposon, isogenic to PAO1 seq).

2.2. Cloning the ampC Gene

Restriction enzymes EcoRI (forward) and XbaI (reverse) were utilized to amplify *ampC* in *P. aeruginosa* via PCR at an annealing temperature of 64° C and extension time of one minute and twenty seconds.

PCR reactions were carried out using a high fidelity polymerase kit (Q5) Purchased from New England Bio Labs, and molecular size of the amplified *ampC* insert (1.2 kb) was confirmed using gel electrophoresis (GE) on 1% agarose gels. Ligation of *ampC* into pMMB67HE and pDN19 was performed using the rapid ligation kit purchased from the same manufacturer.

Recombinant pDN19 plasmids were introduced into competent *E. coli* DH5*α* and BL21 cells and recombinant pMMB67HE plasmids were introduced into competent *E. coli* DH5*α* cells by transformation. Sixteen randomly selected *E. coli* BL21 and Dh5*α* transformants were then selected on Luria-Bertani agar supplemented with tetracycline (10 *μ*g/ml), X-Gal, and Isopropyl ß-D-1-thiogalactopyranoside (IPTG) to perform Blue-White screening [7–9] on transformed *E. coli* colonies. White colonies were then amplified via colony PCR and run on 1% agarose gels to verify the presence of recombinant plasmid containing the *ampC* gene insert.

2.3. Conjugation of P. aeruginosa

Donor recombinant DH5*α* *E. coli* cells containing recombinant pDN19 and pMMB67HE plasmids were grown in Luria-Bertani (LB) media supplemented with tetracycline (10 *μ*g/ml).

E. coli carrying prk2013 was cultured in kanamycin (10 *μ*g/ml) and recipient *P. aeruginosa δampC* was grown in LB media overnight. 1 ml of each cell culture was washed and re-suspended with LB media. Equal volumes of donor, recipient, and *E. coli* prk2013 cultures were mixed then spotted on LB and incubated for 10-12 at 37°C. Recombinant *P. aerug- inosa* Δ*ampC* containing the plasmid of interest were selected on LB agar supplemented tetracycline (30 *μ*g/ml) and irgasan (25 *μ*g/ml).

2.4. β–Lactamase Assay

Wild type *P. aeruginosa* PAO1 and PA14 strains (both induced and uninduced with 500 *μ*g/ml of penicillin G benzathine) as well as *E. coli* DH5*α* and BL21 cells and *P. aeruginosa* PAO1 Δ*ampC* transformed with recombinant pMMB67HE and pDN19 plasmids were directly assessed for their *β*–lactamase producing capabilities to test the expression of the cloned *ampC* gene. DH5*α* and BL21 *E. coli* recombinant cells were suspended in 100 *μ*M phosphate buffer, pH 7. 100 *μ*l of cell suspensions were mixed with equal volumes of colorometric substrate of *β*–lactamase (nitrocefin, 0.5 mg/ml) and color change of solutions (from yellow to red) were observed after a ten minute incubation at 37°C.

2.5. Antibiotic Susceptibility Testing

Antibiotic Susceptibility Testing (AST) was performed on *E. coli* isolates to confirm the expression of *ampC* through susceptibility to *β*–lactam antibiotics. AST was performed by the Disk diffusion by the Kirby-Bauer method [10]. Expression of the *ampC* gene in *P. aeruginosa* was first confirmed by testing pairs of isogenic *P. aeruginosa* isolates induced with 50 *μ*g/ml penicillin G benzathine for their susceptibility to *β*–lactam antibiotics, including ceftazidime (CAZ, 30 *μ*g/ml),

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piperacillin (TZP, 110 μg/ml), cefepime (FEP, 30 μg/ml), meropenem (MEM 10 μg/ml), cefotaxime (CTX, 30 μg/ml), and penicillin G benzathine (50 μg/ml).

To identify the antibiotic used for selection of expression of functional β–lactamase, *E. coli* DH5α and BL21 transformed with recombinant pDN19 and pMMB67HE plasmids were tested for their succeptibility to cefixime (CFM, 10 μg/ml), ampicillin (AMP, 50 μg/ml), carabanicillin (CB, 50 μg/ml), ceftazidime (CAZ, 10μg/ml), gentamicin (GEN, 15 μg/ml), and penicillin G benzathine (PEN 50 μg/ml).

Finally, functional expression of β–lactamase was confirmed in *P. aeruginosa* Δ*ampC* transformants, the model that will be used for testing *ampC* expression during mutagenesis. These strains were tested for their susceptibility to the same antibiotics and concentrations used for *E. coli* transformants.

A less than 1 cm difference in the inhibition zones between bacterial isolates transformed with plasmid carrying or lacking *ampC* was used as the criteria for resistance to the β–lactam antibiotics, therefore confirming β–lactamase production and *ampC* expression.

2.6. Mutagenesis

Random mutagenesis via error prone PCR was conducted on *ampC* insert in recombi- nant pMMB67HE plasmid [11] using the same PCR primers used in cloning of the gene. Mutageneis was conducted through procedures recommended by GeneMorph II random mu- tagenesis kid to yield approximately one mutation per thousand base pairs of DNA of each copy. The presence of the *ampC* PCR product was confirmed on 1% agarose gels and products were ligated into pMMB67HE then transformed into *P. aeruginosa* Δ*ampC*. Carbenicillin was used to select for *ampC* copies with expression of functional β–lactamase. *ampC* PCR product DNA was sequenced before and after this selection to identify mutations that affect the expression of functional β–lactamase.

3. Results

3.1. GE Confirms Successful Cloning of ampC

ampC was successfully amplified via PCR and ligated into pDN19 and pMMB67HE. Presence of PCR product after cutting and cleanup was confirmed on 1% agarose gels, as shown by Figure 1.

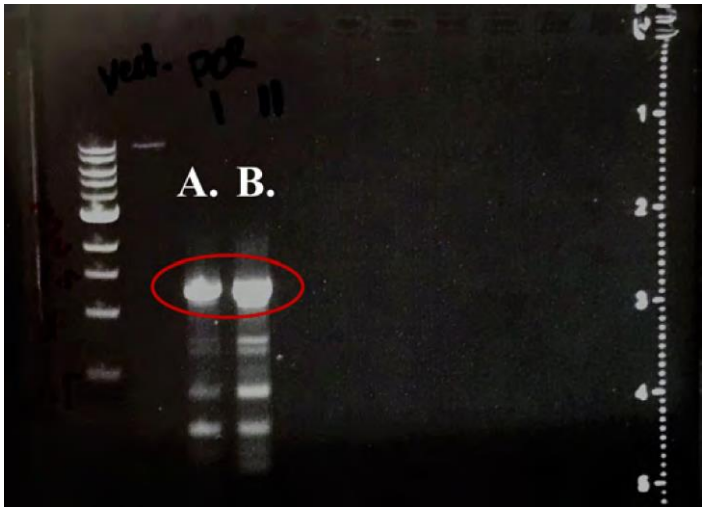

Figure 1. *ampC* gene amplified by PCR. Gel electrophoresis confirmed presence of *ampC* to be used for insertion (A.) and *ampC* to be used for analysis (B.), as demonstrated by the band located between 1.0 and 1.5 kb (circled in red).

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Colony PCR was conducted on 16 randomly selected *E. coli* transformants and products were run on 1% agarose gels to confirm presence of recombinant pDN19 and pMMB67HE plasmids and gene insert (Figure 2). 15 of the 16 randomly selected pMMB67HE colonies and 9 of the 16 pDN19 colonies were successfully transformed with their respective recombinant plasmids.

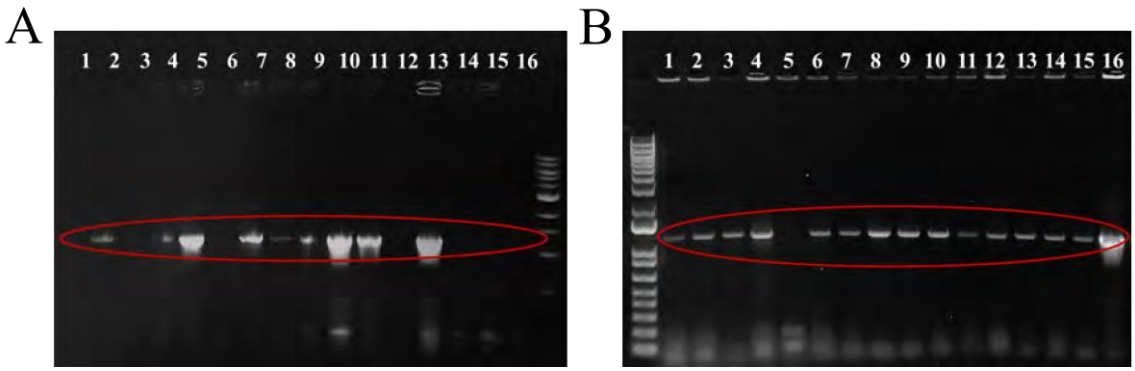

Figure 2. A. Cloning *ampC* into pDN19 and transformed into *E. coli* DH5*α*. Colony PCR of 16 randomly selected DH5*α* transformants confirmed the presence of the pDN19 recombinant plasmid containing the *ampC* gene insert, as demonstrated by the band located between 1.0 and 1.5 kb (circled in red). Colonies 5, 10, 11, and 13 were selected on Luria-Bertani agar supplemented with tetracycline (10 *μ*g/ml) and utilized for antibiotic susceptibility testing. **B.** Cloning *ampC* into pMMB67HE and transformed into *E. coli* DH5*α*. Colony PCR of 16 randomly selected DH5*α* transformants confirmed the presence of the pMMB67HE recombinant plasmid containing the *ampC*, as demonstrated by the band located above 1.5 kb (circled in red). Colonies 4, 9, and 10 were selected on Luria-Bertani agar supplemented with tetracycline (10 *μ*g/ml) and utilized for antibiotic susceptibility testing.

3.2. Successful Conjugation of P. aeruginosa

P. aeruginosa Δ*ampC* was successfully conjugated with pDN19 and pMMB67HE plas- mid. Little growth was observed on control plates without the helper *E. coli* prk2013, whereas individual colonies were seen in mating with all three strains, ensuring that selected *Pseu- domonas* colonies contained the recombinant plasmids (Figure 3).

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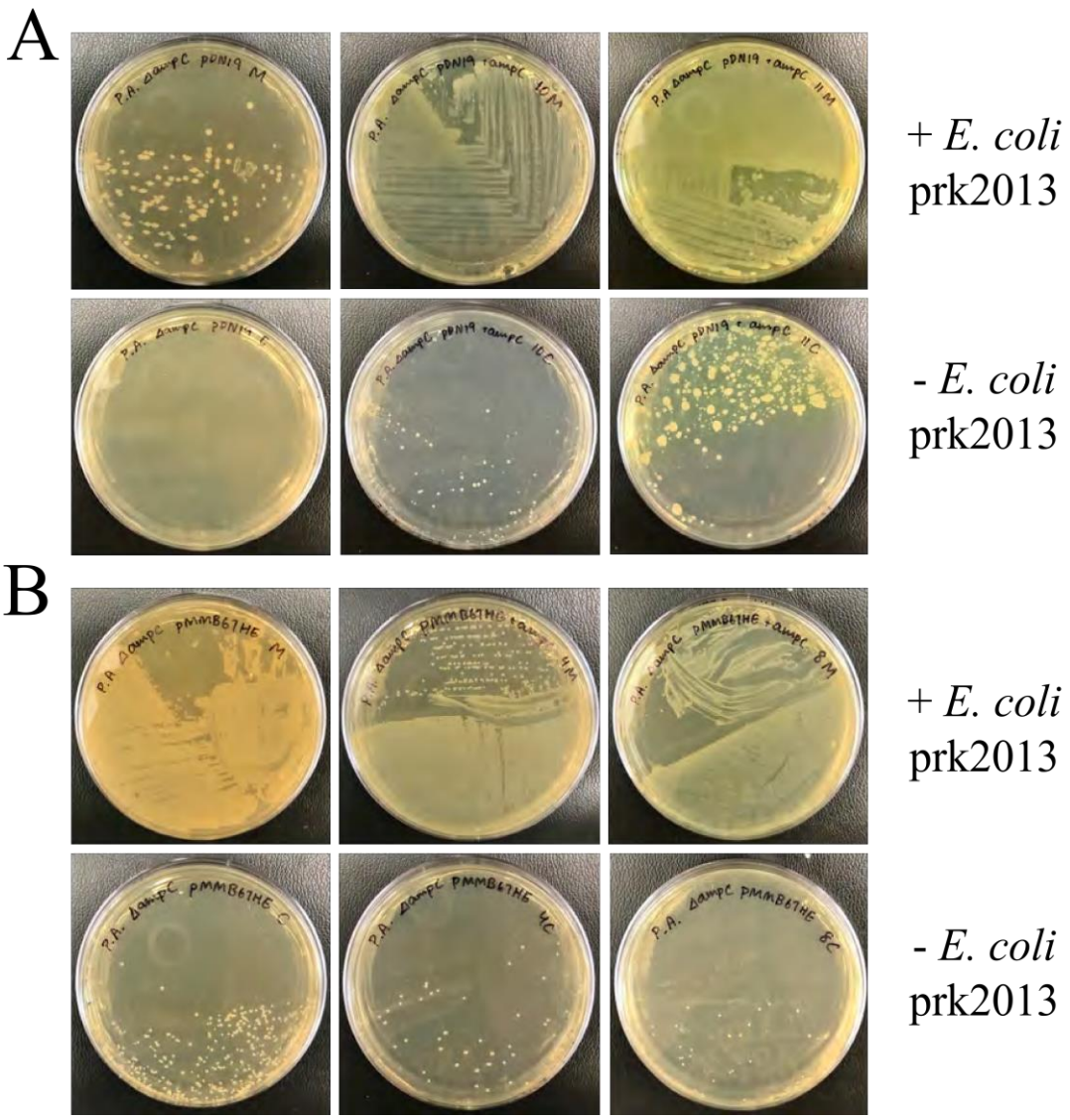

Figure 3. *P. aeruginosa* Δ*ampC* and *E. coli* matings. **A.** Matings of *P. aeruginosa* Δ*ampC* and *E. coli* carrying pDN19. Colony growth was significantly larger in matings supplemented with *E. coli* prk2013 (top row) compared to without (bottom row), indicating that most se- lected *P. aeruginosa* colonies were truly conjugated. **B.** Matings of *P. aeruginosa* Δ*ampC* and *E. coli* recombinant pMMB67HE (pMMB67HE + *ampC*). Colony growth was signifi- cantly larger in matings supplemented with *E. coli* prk2013 (top row) compared to without (bottom row), indicating that most selected *P. aeruginosa* were truly conjugated.

3.3. Induced ampC Produces β–Lactamase

P. aeruginosa PA14 and PAO1 induced with penicillin G produced *β*–lactamase, while uninduced strains did not (Figure 4A). This is likely because expression of *ampC* relies on penicillins that are capable of inducing damage to the cell wall without compromising the bacteria.

P. aeruginosa carrying pDN19 with *ampC* produced minimal quantities of *β*–lactamase, although likely not enough to result in a significant difference in sensitivity to *β*–lactams (Figure 4B). However, both *E. coli* and *P. aeruginosa* carrying *ampC* in the pMMB67HE plasmid both expressed the gene, producing a significant quantities of *β*–lactamase (Figures 4C and 4D, respectively). pMMB67HE displayed early signs of promise as a host vector for *ampC* ; this is likely because pMMB67HE has its own expression system (tac promoter) within the plasmid, which can lead to much higher system expression.

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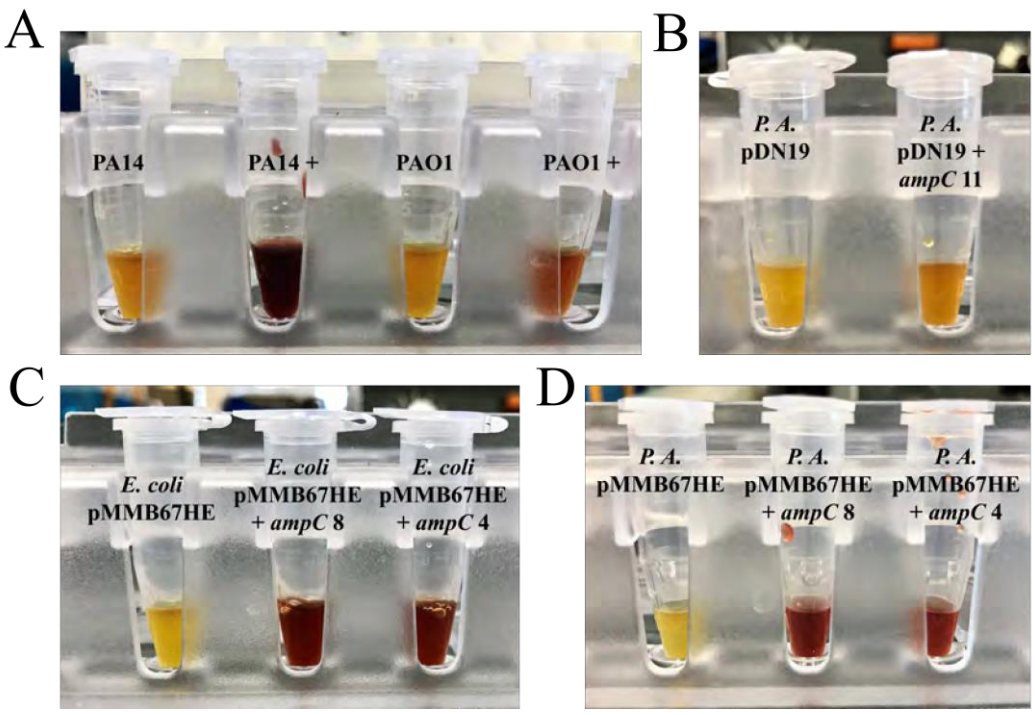

Figure 4. Color change from yellow to red after ten minutes upon addition of nitrocefin revealed production of *β*–lactamase. **A.** *P. aeruginosa* PAO1 and PA14 induced with peni- cillin G (+) changed colors from yellow to light red and deep red (respectively), confirming their production of *β*–lactamase. **B.** *P. aeruginosa* Δ*ampC* conjugated with pDN19 did not change colors while *P. aeruginosa* conjugated with pDN19 containing *ampC* changed colors slightly from yellow to light orange. This color change was insufficient to confirm *β*– lactamase production. **C.** *E. coli* carrying pMMB67HE did not change colors while *E. coli* containing pMMB67HE with *ampC* changed colors significantly to dark red. This confirmed *β*–lactamase expression in *E. coli* transformants carrying *ampC*. **D.** *P. aeruginosa* Δ*ampC* carrying pMMB67HE did not change colors while *P. aeruginosa* carrying pMMB67HE with *ampC* changed colors significantly to dark red. This confirmed *β*–lactamase expression in *P. aeruginosa* conjugates with *ampC*.

3.4. Antibiotic Susceptibility Testing Confirms ampC Expression

Antimicrobial susceptibility testing suggested expression of *ampC* through susceptibil- ity to penicillin G, as demonstrated by Table A3, but demonstrated equal susceptibility to cephalasporin antibiotics. This was likely due to inadequate induction of *ampC* (50 *μ*g/ml of penicillin G). The additional 50 *μ*g/ml of penicillin added to *P. aeruginosa* during AST was likely enough to induce *ampC*, explaining why penicillin G was the only antibiotic *P. aeruginosa* was resistant to. When *P. aeruginosa* was induced with 500 *μ*g/ml of penicillin G, it produced much greater quantities of *β*–lactamase (Figure 4) If antibiotic susceptibility testing was reconducted on isogenic *Pseudomonas*, strains containing *ampC* would likely confer resistance to most cephalosporins.

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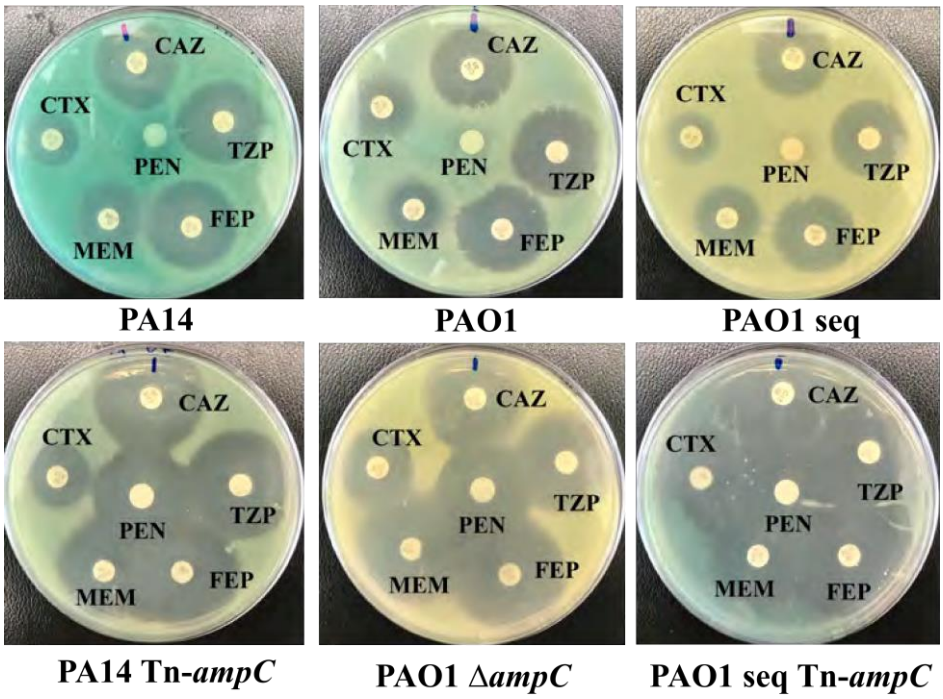

Figure 5. Isogenic *Pseudomonas* strains treated with ceftazidime (CAZ), priperacillin (TZP), cefepime (FEP), meropnem (FEP), cefotaxime (CTX), and penicillin G (PEN) (ori- entation of antibiotics was the same for all plates). All *Pseudomonas* strains with *ampC* were not susceptible to penicillin G compared to strains without *ampC*.

Table 1. Zones of inhibition (cm) of isogenic *P. aeruginosa* strains with or without *ampC* treated with ceftazidime, piperacillin, cefepime, meropenem, ceftotaxime, and penicillin G (pen-G). Pen-G met the requirement for resistance (>1 cm difference in zones of inhibition), thus the expression of *ampC* was detected.

	Strain					
***β*-lactam**	PA14	PA14 Tn-*ampC*	PAO1	PAO1 Δ*ampC*	PAO1 seq	PAO1 Tn-*ampC*
Ceftazidime	2.5	3.0	2.4	3.0	2.1	4.2
Piperacillin	2.5	3.2	2.5	3.4	2.2	3.4
Cefepime	2.6	3.2	2.7	3.4	2.4	3.1
Meropenem	1.9	2.8	2.7	3.1	2.7	3.0
Cefotaxime	1.5	1.7	1.9	2.2	2.4	2.8
Pen-G	0	2.6	0	3.0	0	2.8

ampC expression was not detected in *E. coli* DH5*α* and BL21 or *P. aeruginosa* Δ*ampC* transformants containing pDN19 with *ampC*, as revealed in Table 2. This result is supported by the low levels of *β*–lactamase produced by *P. aeruginosa* Δ*ampC* containing pDN19 with *ampC* (Figure 4B). There did not appear to be a signficant difference in *ampC* expression between *E. coli* DH5*α* and the higher expressing BL21 strain (Table 2).

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Table 2. Average zones of inhibition (cm) of *E. coli* DH5*α* and BL21 and *P. aeruginosa.* Δ*ampC* transformants with cefixime, gentamicin, ceftazidime, carbenicillin, ampicillin, and penicillin G (Pen-G). No *β*–lactam met the criteria for resistance, thus expression of *ampC* was not detected in either *E. coli* DH5*α* or BL21.

	Strain					
	DH5*α*		BL21		Δ*ampC*	
***β*-lactam**	pDN19	pDN19 + *ampC*	pDN19	pDN19 + *ampC*	pDN19	pDN19 + *ampC*
Cefixime	3.5	3.9	5.2	5.0	0	0
Gentamicin	2.4	2.1	2.7	2.4	2.8	2.7
Ceftazidime	2.8	3.5	3.6	4.2	2.4	2.6
Carabanicillin	3.0	3.1	3.6	3.9	2.0	1.2
Ampicillin	1.7	1.8	2.4	2.5	1.0	1.0
Pen-G	3.0	2.8	5.0	5.2	2.6	1.4

ampC expression was detected in *E. coli* DH5*α* and *P. aeruginosa* Δ*ampC* containing pMMB67HE with *ampC*, supported by their resistance to ampicillin, penicillin G, and car- benicillin (Table 3). pMMB67HE was determined as the more effective host vector for *ampC* gene expression in both *E. coli* and *P. aeruginosa*.

Carbenicillin was chosen as the antibiotic used for selection of functional *β*–lactamase production during mutagenesis. This is because both *E. coli* and *P. aeruginosa* displayed resistance to carbenicillin and it had the largest difference in zones of inhibition between strains with and without *ampC* (Table 3).

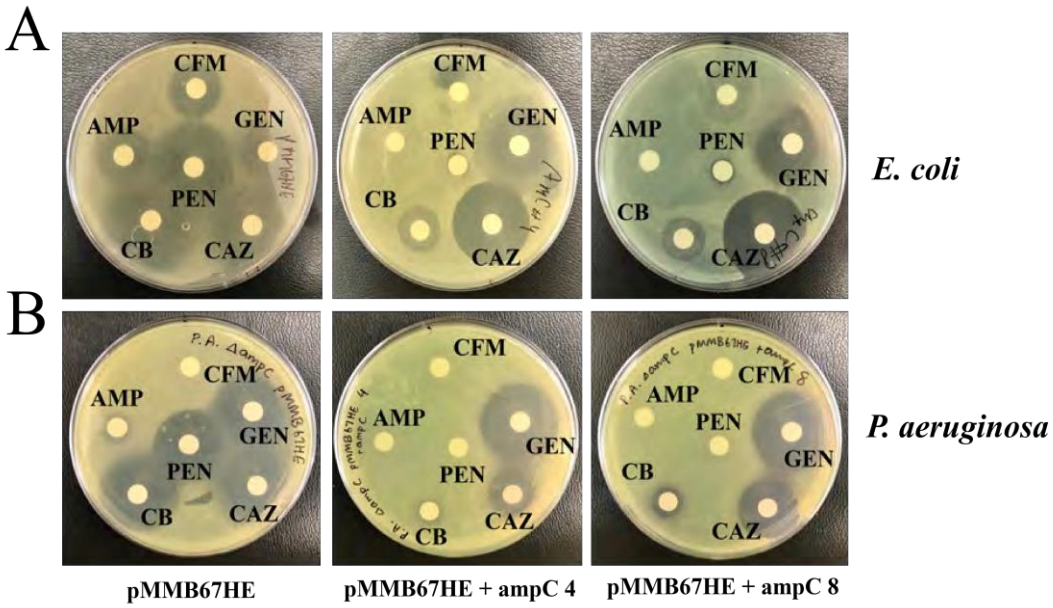

Figure 6. A. *E. coli* pMMB67HE recombinants with and without *ampC* treated with ce- fixime (CFM), gentamicin (GEN), ceftazidime (CAZ), carbenicillin (CB), ampicillin (AMP), and penicillin G (PEN) (orientation of antibiotics was the same for all plates). All *E. coli* re- combinants with *ampC* were not susceptible to penicillin G, ampicillin, and carbenicillin com- pared to strains without *ampC*. **B.** *P.*

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aeruginosa Δ*ampC* pMMB67HE recombinants with and without *ampC* treated with cefixime (CFM), gentamicin (GEN), ceftazidime (CAZ), carbenicillin (CB), ampicillin (AMP), and penicillin G (PEN) (orientation of antibiotics was the same for all plates). All *P. aeruginosa* recombinants with *ampC* were not susceptible to penicillin G and carbenicillin compared to strains without *ampC*.

Table 3. Zones of Inhibition (cm) of *E. coli* DH5*α* and *P. aeruginosa* Δ*ampC* pMMB67HE transformants treated with cefixime, gentamicin, ceftazidime, carbenicillin, ampicillin, and penicillin G (Pen-G). Carbenicillin and pen-G met the criteria for resistance for both *P. aeruginosa* and *E. coli* transformants while carbenicillin met the criteria for resistance in *E. coli*; expression of *ampC* was thus detected in both bacteria.

	Strain			
	DH5*α*		Δ*ampC*	
***β*–lactam**	pMMB67HE	pMMB67HE + *ampC*	pMMB67HE	pMMB67HE + *ampC*
Cefixime	2.0	0	0	0
Gentamicin	1.8	2.9	2.6	2.9
Ceftazidime	3.4	3.0	2.5	2.0
Carbenicillin	3.8	1.5	2.8	1.0
Ampicillin	1.9	0	0.9	0
Pen-G	3.2	0	2.4	0

4. Discussion

AmpC *β*–lactamase produced by *E. coli* was the first bacterial enzyme that destroyed penicillins, the most widely prescribed antibiotic [12]. However, since then, few studies have explored *ampC* -regulated *β*–lactamase production in *P. aeruginosa*, which is more deadly (responsible for millions of deaths) thus its mechanism is relatively unknown.

In this study, the chromosomal *ampC* gene was confirmed to express *β*–lactamase in *P. aeruginosa*, putting to rest alternative theories of *P. aeruginosa* *β*–lactamase produc- tion being primarily plasmid-mediated [13]. This study is also the first of its kind to isolate chromosomal *ampC* in *P. aeruginosa*, clone the gene into a vector, and create a success- ful, self-sustaining system for *ampC* expression in both *E. coli* and *P. aeruginosa* Δ*ampC*.

Because pMMB67HE contains its own tac promoter, its expression is not reliant on tran- scription regulators inherent to a specific bacterium [14]. Furthermore, this vector can easily be regulated by IPTG; for these reasons, this is likely why PMMB67HE was more effective than pDN19. pMMB67HE could be a useful tool to induce expression of *ampC* and other chromosomal genes.

pMMB67HE being utilized as the host vector will allow for *ampC* expression to be de- tected during mutagenesis. If mutagenesis was conducted on *ampC*, potential allosteric bind- ing sites on *β*–lactamase would provide groundbreaking insights into developing *β*–lactamase inhibitors that inactivate *β*–lactamase. Due to the nature of allosteric inhibition, this type of inhibitor would mitigate the possibilities of *P. aeruginosa* conferring resistance to it, offer- ing a sustainable solution in combating *Pseudomonas* infections. Furthermore, in developing countries where penicillins are more cost-effective, a *β*–lactamase inhibitor that renews the efficacy of *β*–lactam antibiotics would increase accessibility of *P. aeruginosa* therapeutics. This could propel drug discovery in the direction of developing safer therapeutics that remain effective in the long-term. [15].

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5. Future Work

Because *ampC* was experimentally confirmed as the gene expressing for *β*–lactamase in *P. aeruginosa*, and *ampC* has been isolated and cloned into *E. coli*, further tests may now be conducted on *β*–lactamase produced by *P. aeruginosa*.

Because the pMMB67HE plasmid was identified as the optimal host vector for *ampC* expression, this plasmid will be used to express *ampC* during mutagenesis. Furthermore, since the expression in pMMB67HE can be regulated by IPTG, this is a suitable template for mutagenesis. During mutagenesis, copies of *ampC* will be ligated into pMMB67HE then transformed in *P. aeruginosa* Δ*ampC* to test for expression, using carbenicillin as the selecting antibiotic.

The minimum inhibitory concentration (MIC) of carbenicillin on *P. aeruginosa* will need to be determined in order to identify the concentration of antibiotic required for selection of *P. aeruginosa* cells producing functional *β*–lactamase. Mutated copies will be sequenced before and after selection to identify mutations that affect the expression of functional *β*–lactamase. Relevant mutation sites located in close proximity to one another could act as potential allosteric binding sites for novel *β*–lactamase inhibitors.

6. Conclusion

ampC was first verified as the gene encoding for *β*–lactamase in *P. aeruginosa* by a *β*–lactamase assay. *ampC* was then successfully isolated and cloned into *E. coli* in pDN19 and pMMB67HE plasmids. *β*–lactamase assays on *E. coli* recombinants found that *ampC* produced more *β*–lactamase when in pMMB67HE compared to pDN19, suggesting greater *ampC* expression in this pMMB67HE.

Antibiotic susceptibility testing (AST) conducted on recombinant *E. coli* confirmed this result: no *β*–lactamse met the criteria for resistance (thus no *β*–lactamase expression detected) for pDN19 containing *ampC* whereas three *β*–lactams (ampicillin, pen-G, carbeni- cillin) met the criteria for resistance (thus *β*–lactamase expression detected) in pMMB67HE. Both recombinant plasmids were conjugated into *ampC* deficient strain of *P. aerug- inosa* and *β*–lactamase assay supported greater production of *β*–lactamase of *ampC* in pMMB67HE compared to pDN19 within *Pseudomonas*. AST on these strains confirmed this result, finding *β*–lactamase to provide most specificity to carbenicillin in *P. aeruginosa*. It was concluded that the pMMB67HE plasmid was the optimal host vector for *ampC* ex- pression, and carbenicillin was selected as the antibiotic used for selection. A functional model of measuring *β*–lactamase for mutagenesis was thus developed: pMMB67HE ligated with *ampC* in *P. aeruginosa* with carbenicillin acting as the selecting antibiotic.

Appendix A

XbaI-RBS-ampC F:	5’	TTTTTCTAGAGACAAAGGACGCCAATCCTCATG	3’
EcoRI-ampC R:	5’	TTTTGAATTCTCAGCGCTTCAGCGGCACCTTG	3’

Figure A1. Primer sequences of forward (EcoRI) and reverse (XbaI) restriction enzymes used for the cleaving of the ampG gene. The underlined segment of the primer sequence highlights sequence of the enzyme’s restriction site.

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Table A1. Zones of Inhibition (cm) of selected *E. coli* DH5*α* pDN19 transformants (10, 11, and 13) treated with cefixime, gentamicin, cephtazadine, carabanicillin, ampicillin, and pen-G.

	Strain			
***β*-lactam**	pDN19	pDN19 + ampC 10	pDN19 + ampC 11	pDN19 + ampC 13
Cefixime	3.5	3.7	3.7	4.2
Gentamicin	2.4	2.0	2.0	2.2
Cephtazadine	2.8	3.8	3.2	3.5
Carabanicillin	3.0	3.0	3.2	3.1
Ampicillin	1.7	2.0	1.6	1.9
Pen-G	3.0	2.7	3.0	2.8

Table A2. Zones of Inhibition (cm) of selected *E. coli* BL21 pDN19 transformants (10 and 11) treated with cefixime, gentamicin, cephtazadine, carabanicillin, ampicillin, and pen-G.

	Strain		
***β*–lactam**	pDN19	pDN19 + ampC 10	pDN19 + ampC 11
Cefixime	5.2	5.0	5.0
Gentamicin	2.7	2.0	2.8
Cephtazadine	3.6	4.2	4.2
Carabanicillin	3.6	3.4	4.4
Ampicillin	2.4	2.4	2.6
Penicillin G Benzathine	5.0	5.0	5.4

Table A3. Zones of Inhibition (cm) of selected *E. coli* DH5*α* pMMB67HE transformants (4 and 8) treated with cefixime, gentamicin, cephtazadine, carabanicillin, ampicillin, and pen-G.

	Strain		
***β*–lactam**	DH5*α* pMMB67HE	pMMB67HE + ampC 4	pMMB67HE + ampC 8
Cefixime	2.0	0	0
Gentamicin	1.8	2.9	2.8
Cephtazadine	3.4	2.9	3.0
Carabanicillin	3.8	1.5	1.5
Ampicillin	1.9	0	0
Penicillin G Benzathine	3.2	0	0

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Table A4. Zones of Inhibition (cm) of selected *P. aeruginosa* ΔampC pDN19 transformants (10 and 11) treated with cefixime, gentamicin, cephtazadine, carabanicillin, ampicillin, and pen-G.

	Strain		
Beta-lactam	pDN19	pDN19 + ampC 10	pDN19 + ampC 11
Cefixime	0	0	0
Gentamicin	2.8	2.5	2.8
Cephtazadine	2.4	2.6	2.6
Carabanicillin	2.0	1.2	1.1
Ampicillin	1.0	0.9	1.1
Pen-G	2.6	0	1.9

Table A5. Zones of Inhibition (cm) of selected *P. aeruginosa* ΔampC pMMB67HE transfor- mants (4 and 8) treated with cefixime, gentamicin, cephtazadine, carabanicillin, ampicillin, and pen-G.

	Strain		
***β*-lactam**	pMMB67HE	pMMB67HE + ampC 4	pMMB67 + ampC 8
Cefixime	0	0	0
Gentamicin	2.6	2.9	2.8
Cephtazadine	2.5	1.8	2.2
Carabanicillin	2.8	0.8	1.1
Ampicillin	0.9	0	0
Penicillin G Benzathine	2.4	0	0

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