Cloning and Expression of Putative Antibiotic Resistance Gene, *eamA*, from Environmental Plasmid pTRE-2011

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ABSTRACT

With the fast-paced evolution of antibiotic-resistant bacteria around the globe comes the emergence of novel genes which confer these resistances. Many of these genes are found on large conjugative plasmids as opposed to the bacterial chromosome. These mobile plasmid accessory genes continue to evolve not only in clinical settings, but environmental as well. Many of such plasmids have been isolated from the Tijuana River Estuary (TRE), and confer multiple resistances, plasmid pTRE-2011 being one of them. On this plasmid, unknown gene *eamA*, suggested in the literature to be involved in metabolite/drug efflux, is located next to a known resistance gene, *tetA*, whose main mechanism of action is efflux of tetracycline antibiotics. For this reason, it was hypothesized that *eamA* might also play a role in antimicrobial resistance (AMR). To determine if *eamA* was involved in AMR, it was isolated from pTRE-2011 by PCR and cloned into the expression vector pSF-OXB11. A successful clone was confirmed with Oxford Nanopore sequencing. Its phenotype was then expressed in *E.coli* JM109 on its own, and in conjunction with *tetA*. As expected, *tetA* provided resistance to tetracyclines. Unexpectedly, it also decreased susceptibility to piperacillin, ampicillin, and ciprofloxacin. *eamA* did not provide resistance to any tested antibiotic on its own, only slightly decreased susceptibility to ampicillin. Attempts to clone and express both genes in the same vector have not yet been successful, hindering our ability to assess any synergistic effects.

Keywords: antibiotic resistance genes, plasmids, wetlands, conjugation, *eamA,* molecular cloning, drug/metabolite exporter

INTRODUCTION

Antibiotic resistance is an ever-present and increasing issue throughout the world (Zaman et al., 2017) and the cause of 1.27 million deaths worldwide in 2019 (Antimicrobial Resistance Collaborators, 2022). With antibiotics as a necessary means of treatment for a multitude of bacterial infections, this increase in resistance is cause for serious concern . Not only are these superbugs present in clinical settings, but also in the environment, specifically wetlands around the globe (Martínez et al., 2009). The speed of this evolution is in part due to conjugation, or the exchange of plasmids between bacteria independent of reproduction (Falagas et al. 2005; Giske et al. 2008; Strahilevitz et al. 2009; Nordmann et al. 2011). Conjugative plasmids are independent, circular DNA elements that replicate separately from the bacterial chromosome and provide some sort of benefit to their host. Sewage runoff into these wetlands provides large amounts of new DNA in the form of resistance plasmids. Selective pressure in the form of antibiotics in the sewage allows bacteria with antibiotic resistance plasmids to persist and evolve

(Borgogna et al. 2016; Botts et al. 2017; Cummings et al. 2011). One such plasmid is pTRE-2011, a large (144 kb) IncF plasmid, isolated from the Tijuana River Estuary (TRE), a coastal wetland near San Diego, California, USA on the border of the United States and Mexico (Zedler et al. 1992). This plasmid has been shown to provide resistance to a multitude of antibiotics, encoded on the following antibiotic resistance genes (ARGs): *sulII, sulIII, floR, tetA/R, blaCTX-M-55, blaTEM-1b, aadA1, aac(3)-IIa,* and *strA/B*. Among these genes we find resistances to aminoglycosides, beta-lactams, amphenicols, sulfonamides, and tetracyclines, many of which are vital to fighting a multitude of clinical infections. pTRE2011 was also shown to decrease susceptibility to colistin when in *E. coli* JM109, however no mcr1 gene for colistin resistance was found in the sequence. It was hypothesized that a general efflux gene could be responsible for this increased resistance (Botts et al. 2017). Figure 1 below displays pTRE-2011 and the resistance genes mentioned above.

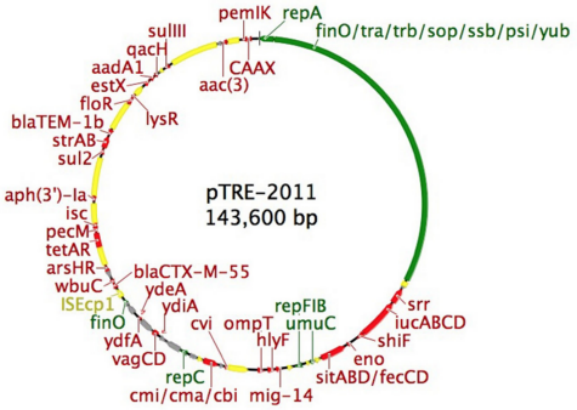


FIGURE 1. Map of pTRE-2011.Green represents backbone genes; gray, unidentified open reading frames; yellow, mobile genetic elements; red, accessory genes (antibiotic and heavy metal resistance).

Within these plasmids, there are common structural and organizational elements. Similar genes are clustered together giving them an evolutionary advantage, as multiple ARGs can be copied together and transferred between plasmids (Thomas et al. 2000). Two common regions of organization include the backbone and accessory regions. The backbone region includes genes for replication, partitioning, mobilization, transfer, mating pair formation and stability, while the accessory region regularly includes antibiotic and heavy metal resistances. These accessory regions are often interlaced with transposons and insertion sequences, which are indicative of their evolution and mobility. In the TRE specifically, there have been multiple plasmids found with backbones nearly identical to that of pTRE2011 and many similar resistance genes such as blaCTX-M and *tetA* (Botts et al. 2022). The prevalence of this AMR is of much concern to geographical areas surrounding the TRE, including the San Diego and Tijuana communities. Increased difficulties are faced by physicians treating bacterial infections when the antibiotics regularly used to treat patients infections are not functioning. While the mechanism by which these resistance plasmid containing bacteria enter the greater population is unknown, there is evidence that animal vectors such as shorebirds could play a role (Botts et al., 2022; Luo et al., 2022).

The gene, *tetA,* listed above is a prevalent ARG that persists among many of these environmental plasmids, providing resistance to tetracycline antibiotics. This resistance gene is a well known tetracycline efflux gene, meaning its main mechanism of action is pumping solutes, specifically tetracycline, out of the cell, or efflux. On average, efflux pumps make up about 10% of the transporters in many bacterial species and usually can efflux a wide range of different compounds with varying structures (Martinez et al. 2009). Within antibiotic-resistant bacteria, efflux genes thought to be specific to a certain class of drugs are sometimes able to transport drugs beyond an individual class.

In this study, the gene *eamA*, along with its associated cysteine-hydrolase, was selected for cloning and characterization. The explicit function of the *eamA* gene on pTRE-2011 is unknown. It is known to be part of the DMT superfamily which is a class of drug/metabolite exporters and hypothesized to function in amino acid transport, specifically the export of cysteine and O-acetylserine (Gene ID: 946081) (Jack et al. 2001; NIH). This hypothesis is supported by *eamA*'s proximity to the cysteine hydrolase on the plasmid pTRE-2011. The two genes, *eamA* and the cysteine hydrolase are on the same integron and combined, will be referred to as *CHeamA*. These genes are also right next to a *tetA* gene, whose main mechanism of action is efflux, giving its host resistance to tetracyclines. This specific location is what gave rise to the question of its involvement in antibiotic resistance. The idea that this gene could be involved in transport makes it more likely that it is involved in antibiotic resistance as efflux of antibiotics is a common mechanism of resistance. This is further supported by the fact that similar genes are clustered together on resistance plasmids. We hypothesized that *eamA* could possibly be involved in AMR, either on its own as a drug exporter, or working in conjunction with the *tetA* to increase resistance.

To test this hypothesis, molecular cloning was utilized to isolate and express the *CHeamA* pair on its own and then, in conjunction with *tetA*, and tested against various antibiotics. By cloning the genes in this manner, function can either be associated with each gene individually, or due to synergy among the set. With this cloning trio, we can also assess the specificity of this particular *tetA* gene to tetracyclines.

MATERIALS AND METHODS

Antibiotic Abbreviations

AN, amikacin; AMP, ampicillin; ATM, aztreonam; AZM, azithromycin; C, chloramphenicol; KF, cephalothin; CIP, ciprofloxacin; CT, colistin; CTX, cefotaxime; CRO, ceftriaxone; DO, doxycycline; ETP, ertapenem; CN, gentamicin; K, kanamycin; LEV, levofloxacin; MEM, meropenem; PRL, piperacillin; PB, polymyxin b; S, streptomycin; SAM, ampicillin/sulbactam; SXT, sulfamethoxazole/trimethoprim; TE, tetracycline; TOB, tobramycin; TZP, piperacillin/tazobactam.

pSF-OXB11

The constitutive expression vector pSFOXB11 (Sigma Aldrich) was chosen for the selectable marker Kanamycin that would not interfere with selection for our genes of interest, more specifically the use of tetracycline. It also included cut sites that were not located anywhere on our genes of interest, as well as an intermediate strength promoter. (Oxford Corporation; depicted in Figure 2)

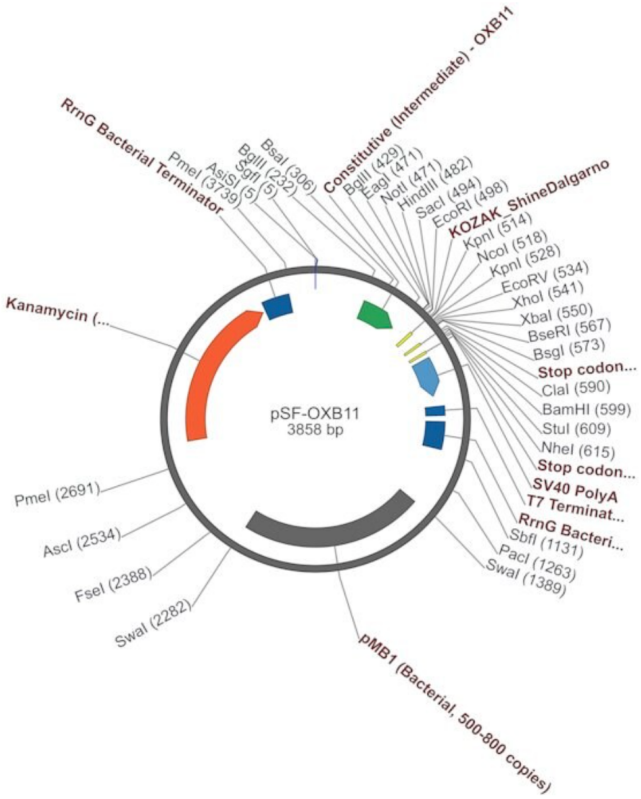


FIGURE 2. Map of pSF-OXB11 from Sigma Aldrich. Kanamycin selectable marker shown in orange. RE cut sites shown in gray.

Gene Isolation Utilizing Polymerase Chain Reaction

Primers were designed to isolate CHe*amA*, *tetA* and combination gene with *CHeamA* and *tetA* together, or "cassette". Locations of the genes in question were determined previously by Botts et al. in 2017 [Accession number: ON943059]. Cut sites for restriction enzymes were included in primer design in order to create complementary ends for ligation. A double digest system was utilized in order to increase specificity and ensure the correct directional orientation of the gene within the expression vector. AccuPrime Pfx DNA Polymerase (Invitrogen) was used along with the accompanying protocol for thermocycling parameters.

TABLE 1. Cut sites for restriction enzymes added to forward and reverse primers

KpnI	Cut sites added to forward primer	GGTACC
XbaI	Cut sites added to reverse primer	TCTAGA

Cysteine Hydrolase and *eamA*

Primers were designed to isolate genes from position 39225 to 40657 as seen in Table 2. Total gene size is 1432 base pairs (bp). The neighboring cysteine hydrolase was included when isolating this gene because it is on the same integron as *eamA*. The *eamA* with the associated cysteine hydrolase will be referred to as *CHEamA*. The promoter from the vector was used, so there could be a slight difference in expression level compared to in nature. The thermocycling parameters for the PCR reaction are detailed in Table 3.

TABLE 2. *CHeamA* primers. KpnI cut sites in blue and XbaI cut sites in green. Start codon in red. Tm is melting temperature and Ta is annealing temperature.

Forward Primer	5’ GCGGTACCCGTGAAACCCAACAGACC 3’	Tm (ºC)	Ta Used in PCR Rxn (ºC)
		81.6	68
Reverse Primer	5’ GCTCTAGAGTTTCCACGATCAGCGATC 3’	69.5	

TABLE 3. Thermocycling parameters for the isolation of *CHeamA*.

Thermocycling for *CHeamA*			
Segment	Number of cycles	Temperature (°C)	Duration
1	1	95	5 min
2	30	95	45 sec
		68	45 sec
		68	1.4 min
3	1	72	10 min

tetA

Primers were designed to isolate the gene *tetA* from pTRE-2011 from position 40687 to 41887 for a total of 1200 bp as seen in Table 4. The natural promoter was included to mimic the expression level of that in nature most closely, rather than the intermediate strength promoter of the vector pSFOXB11. These primers were then used to isolate the gene with PCR using the thermocycling parameters below (Table 5).

TABLE 4. *tetA* primers. KpnI cut sites in blue and XbaI cut sites in green. Start codon in red. Tm is melting temperature and Ta is annealing temperature.

Forward Primer	5’ GCGGTACCCGTGAAACCCAACAGACC 3’	Tm (ºC)	Ta Used in PCR Rxn (ºC)
		63.3	62
Reverse Primer	5’ GCTCTAGAGTTTCCACGATCAGCGATC 3’	63.5	

TABLE 5. Thermocycling parameters for the isolation of *tetA*.

Thermocycling for *tetA*			
Segment	Number of cycles	Temperature (°C)	Duration
1	1	95	5 min
2	30	95	45 sec
		62	45 sec
		68	1.2 min
3	1	72	10 min

Cassette

A combination of the previous genes was isolated and the natural promoter included in the sequence. This gene including the Cysteine Hydrolase, *eamA*, *tetA* and the natural promoter was coined the "cassette". PCR primers were designed to amplify from position 39225 to 41989 of pTRE-2011, for a total of 2764bp, seen in Table 6. The thermocycling parameters, Table 7, were used for PCR amplification. The natural promoter was included to mimic the expression level of that in nature just like above.

TABLE 6. Cassette primers. KpnI cut sites in blue and XbaI cut sites in green. Start codon in red. Tm is melting temperature and Ta is annealing temperature.

Forward Primer	5' GCGGTACCCTTGCGCGGCCGGCCCGT 3'	Tm (ºC)	Ta Used in PCR Rxn (ºC)
		81.6	53
Reverse Primer	5' GCTCTAGACATGCTTGACACTTTATC 3'	54.6	

TABLE 7. Thermocycling parameters for the isolation of the cassette.

Thermocycling for Cassette			
Segment	# of cycles	Temperature (°C)	Duration
1	1	95	5 min
2	30	95	45 sec
		53	45 sec
		68	2.7 min
3	1	72	10 min

PCR Purification
PCR products and the digested vector were isolated using gel electrophoresis. Wizard PCR Preps DNA purification System from Promega was used and protocol followed exactly to purify each gene insert from previous buffers, and eliminate any undigested vector.

Restriction Digest
Restriction enzymes (REs) KpnI from Thermo Fisher Scientific and XbaI from Invitrogen were used to digest the expression vector. These were chosen because they do not cut the middle of the gene and there was a restriction site for each of them on the vector. Kpn1 was shown to exhibit 100% activity in NEB's rCutSmart Buffer, and Xba1, 50%, with no star activity (New England Biolabs). The ability of the enzymes to digest in rCutSmart was confirmed by the digestion of pSF-OXB11 and visualization of such digestion with gel electrophoresis. Digestions were performed at 37 °C for one hour, followed by a kill run at 80 °C for 20 min per standard, to ensure the enzymes were inactive and would not cut the final product after ligation. Digestion was also performed post cloning, after isolation of DNA from tranformants to verify the presence of the insert genes.

Molecular Cloning
Standard procedure for cloning and subsequent heat shock transformation was followed per Bagdasarian, M., with a few modifications (Bagdasarian,1994). Standard Ethanol Precipitation of digested vector was used to eliminate any undigested product and prevent background. Dephosphorylation of the vector was completed with Calf Intestinal Alkaline Phosphatase from Thermo Fisher Scientific. An insert to vector ratio of 10:1 and overnight ligation at 16 °C were found to optimize results and minimize background. The DNA was transformed via the heat shock method into Single-use JM109 Competent Cells from Promega, and plated on KAN plates at 50 μg/mL to select for colonies with the vector, given the selectable marker. The ligation and transformation procedures were completed with the vector only, no insert, to act as a negative control and observe background growth. Each gene, *CHeamA, tetA* and the cassette were inserted individually into the expression vector.

Plasmid Preps
QIAGEN CompactPrep Plasmid Kits were used according to the protocol for high-copy plasmids to isolate cloned and transformed DNA and verify the presence of the insert gene in the expression vector. Large, isolated, and central colonies were chosen from plates (n=5 and n=10) and grown up in LB medium for 16 hours at 37 °C. The remaining procedure was followed to standard and the samples eluted in water. From here a digest was run with the above mentioned restriction enzymes and visualized on a gel to confirm the gene insert was present within the vector.

Sequencing
Each of the three separate samples were sequenced by Eurofins Genomics using Oxford Nanopore sequencing. Samples were prepared according to the company's specifications. Reported accuracy rate is 98.3%. Snap Gene was used to view the sequence and create figures. The DNA between KpnI and XbaI from each sequence was input into NCBI-BLAST to determine the presence of the correct gene.

Antimicrobial Susceptibility Testing

E. coli JM109 with and without plasmids was subjected to antimicrobial susceptibility testing (AST) repeatedly (n=3) against a broad range of antibiotics via the disk diffusion method according to CLSI protocols and standards (Clinical and Laboratory Standards Institute, 2015, 2017) with disks obtained from BD or Oxoid. The antibiotics tested against include AN, AMP, ATM, AZM, C, KF, CIP, CT, CTX, CRO, DO, ETP, CN, K, LEV, MEM, PRL, PB, S, SAM, SXT, TOB, and TZP.

Statistical Analysis

Averages of the triplicate halo diameters for each separate gene were tested against that of E. coli JM109 in a standard, independent, two tailed t-test. Significance was determined by a p value of less than 0.05.

RESULTS

CHeamA, tetA and cassette genes were successfully isolated from pTRE2011 by PCR. This was confirmed using standard gel electrophoresis. Bands of the correct corresponding base pair lengths were visualized for each sample as seen in Figure 3.

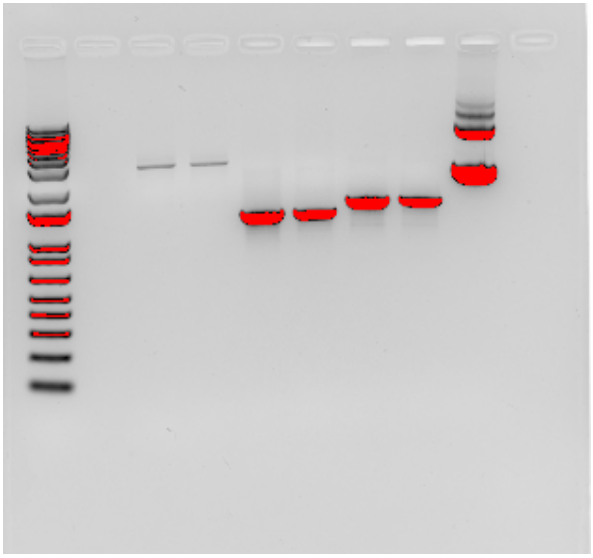
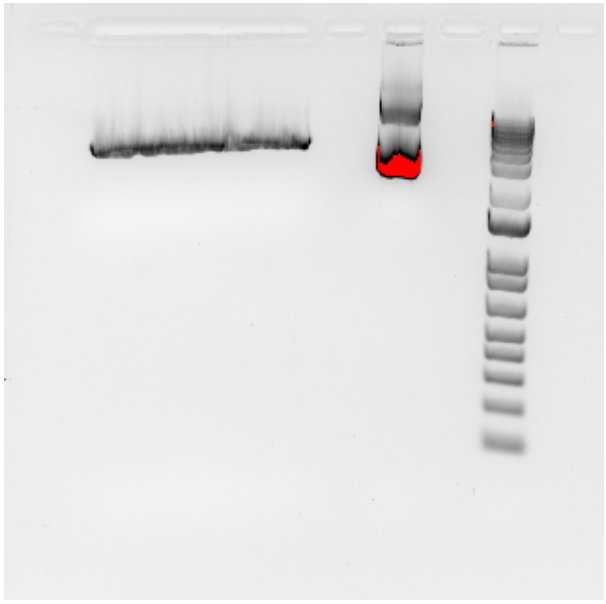


FIGURE 3. (above) Gel electrophoresis of genes isolated via PCR. From left to right the lanes contain: 1 Kb Plus DNA Ladder, cassette (2x), *tetA* 2x, *CHeamA* (2x), and the pSFOXB11 mini prep. Each band corresponds to the correct base pair length when compared to the ladder.

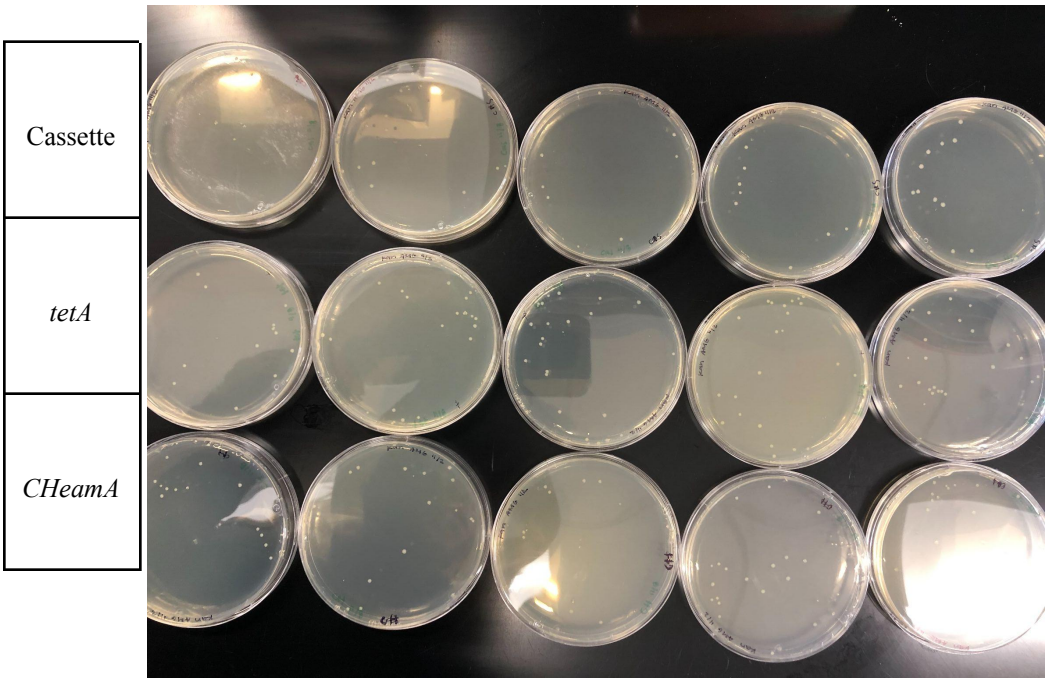
FIGURE 4. (below) Gel electrophoresis of vector prep post restriction digest. From left to right the lanes contain: digested and purified pSFOXB11, pSFOXB11 mini prep, and the 1 Kb Plus DNA Ladder. The digested vector corresponds to the correct number of base pairs (about 3850 bp) when compared to the ladder.



The vector digest was confirmed via gel electrophoresis as well when a single band was visualized post Ethanol precipitation in Figure 4.

Transformation of the cloned DNA samples into E. coli JM109 was confirmed by growth on kanamycin plates, and limited background, as seen in Figure 5.

FIGURE 5. Kanamycin plates containing E. coli JM109 with successfully transformed DNA.



Multiple colonies (n=5 or n=10) were selected for screening from the plates seen in Figure 5, and mini prepped. Isolated, and digested DNA showed successful clones of *CHeamA*, *tetA*. However the cassette was not clear enough to confirm. Vector (top) and insert (bottom) bands were present at respective sizes for *tetA* and *CHeamA* clones shown in Figures 6 and 7.

FIGURE 6. Gel electrophoresis of cloned DNA isolated from above colonies and digested with KpnI and XbaI. From left to right the lanes contain: 1 Kb Plus DNA Ladder, and *tetA* colonies 1-5. Colony 4 was successful as the band indicating the insert is at the correct length of about 1200bp.

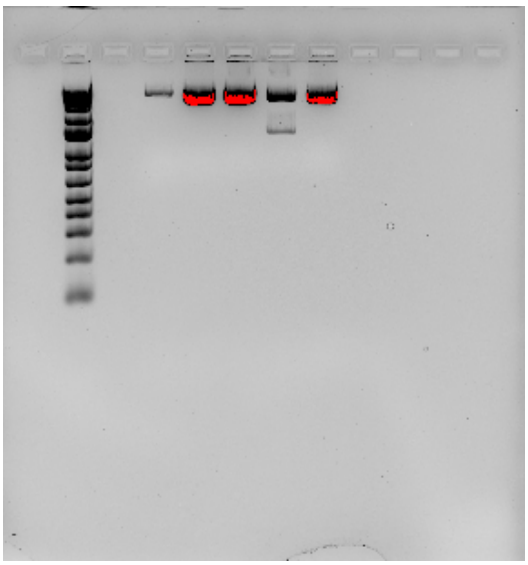
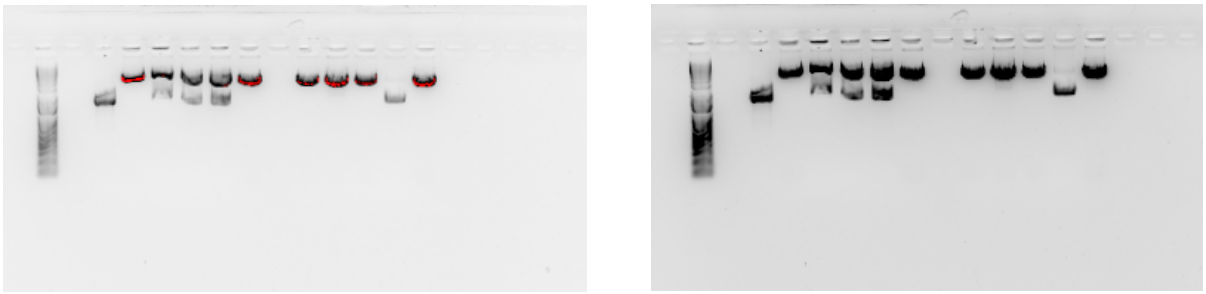


FIGURE 7. Gel electrophoresis of cloned DNA isolated from above colonies and digested with KpnI and XbaI. From left to right the lanes contain: 1 Kb Plus DNA Ladder, *CHeamA* gene isolate via PCR, *CHeamA* colonies 1-5, and cassette colonies 1-5. Colony 4 of *CHeamA* looks most successful as the band indicating the insert is at the correct length of about 1400bp, and aligns with the reference gene perfectly. Cassette colony 4 also looks like the insert is present with a lower band around 2750bp. When overexposed (right), it seems as if the vector band is very faint.



These gel results were further confirmed with sequencing of each sample. *CHEamA* and *tetA* sequences were input into NCBI-BLAST which showed the genes were indeed correct and in full. The cassette however was not present in the sequence; the bases between the restriction enzymes on the vector, plus a few more, had been deleted. The smaller band seen on the gel in Figure 7 was just the half of the vector left which is similar in size to the cassette.

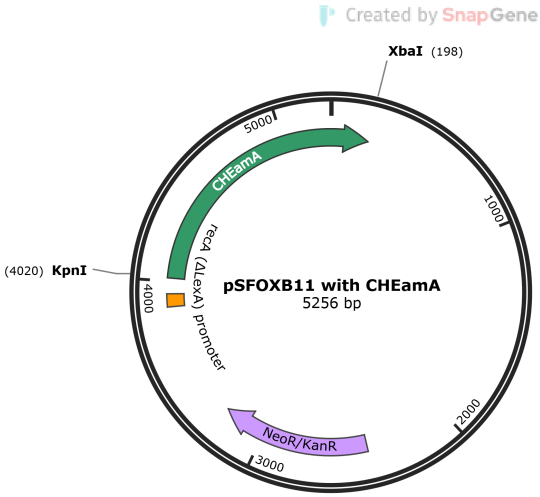
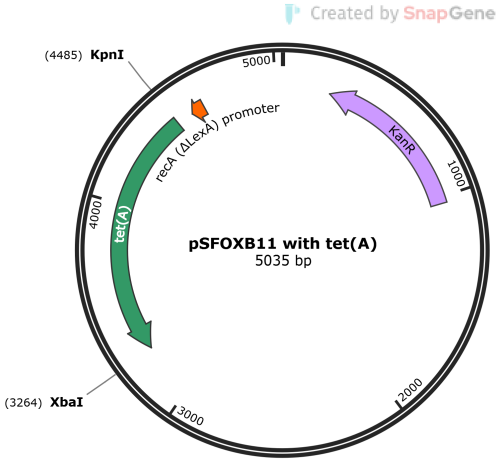


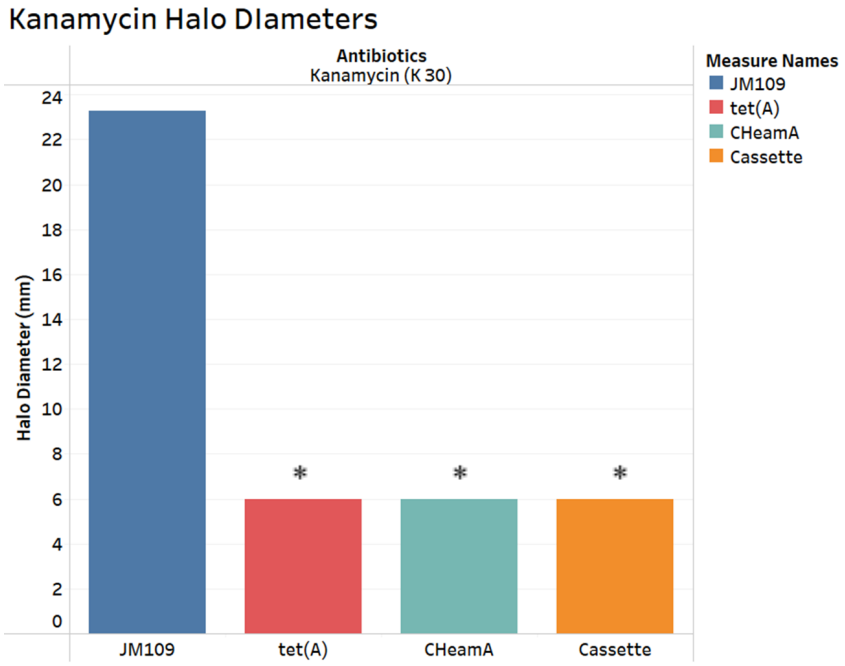
FIGURE 8. *CHeamA* insert sequence map. The insert gene is shown in green, between the restriction enzymes KpnI and XbaI. The intermediate strength promoter is shown in orange just before the insert. The Kanamycin selectable marker is shown in purple.

FIGURE 9. *tetA* insert sequence map. The insert gene is shown in green between the restriction enzymes KpnI and XbaI. The intermediate strength promoter is shown in orange just before the insert. The Kanamycin selectable marker is shown in purple.



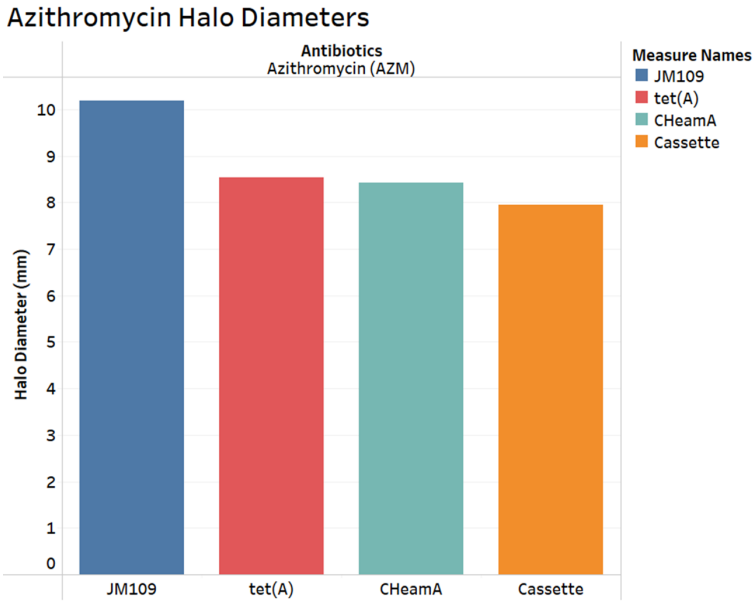
AST provided data regarding the phenotype of each individual insert, as well as the control E. coli JM109. KAN resistance was displayed by each transformed sample, indicating successful expression of the selectable marker on the expression vector. E. coli JM109 did not display resistance to KAN.

FIGURE 10. Selectable marker function displayed in all three insertions when expressed in E. coli JM109. Each clone is significantly different from the control E. coli JM109 without a plasmid. * indicates statistically significant



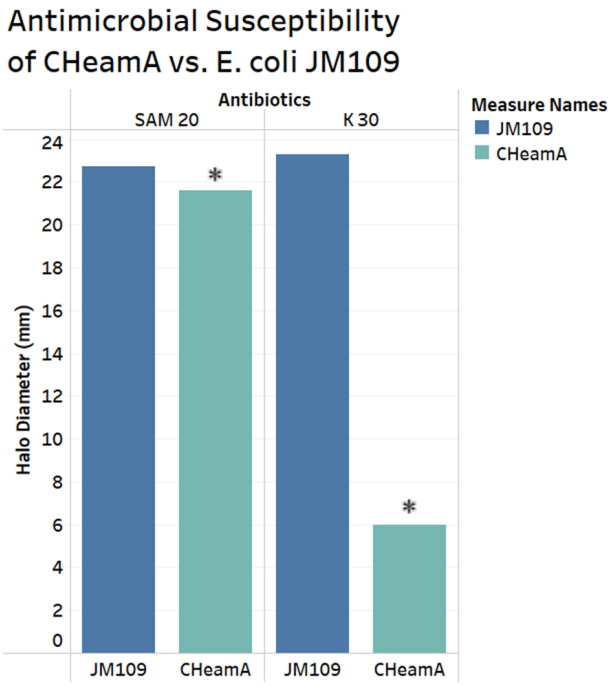
JM109 was found to provide resistance to AZM, which is not noted in the genome, and therefore unexpected. This was consistent throughout all replicates and different gene inserts. Each transformed gene was shown to reduce susceptibility to SAM as well- even the attempted cassette which only had the selectable marker gene remaining.

FIGURE 11. Displayed resistance to AZM indicated by halo diameter < 11mm.



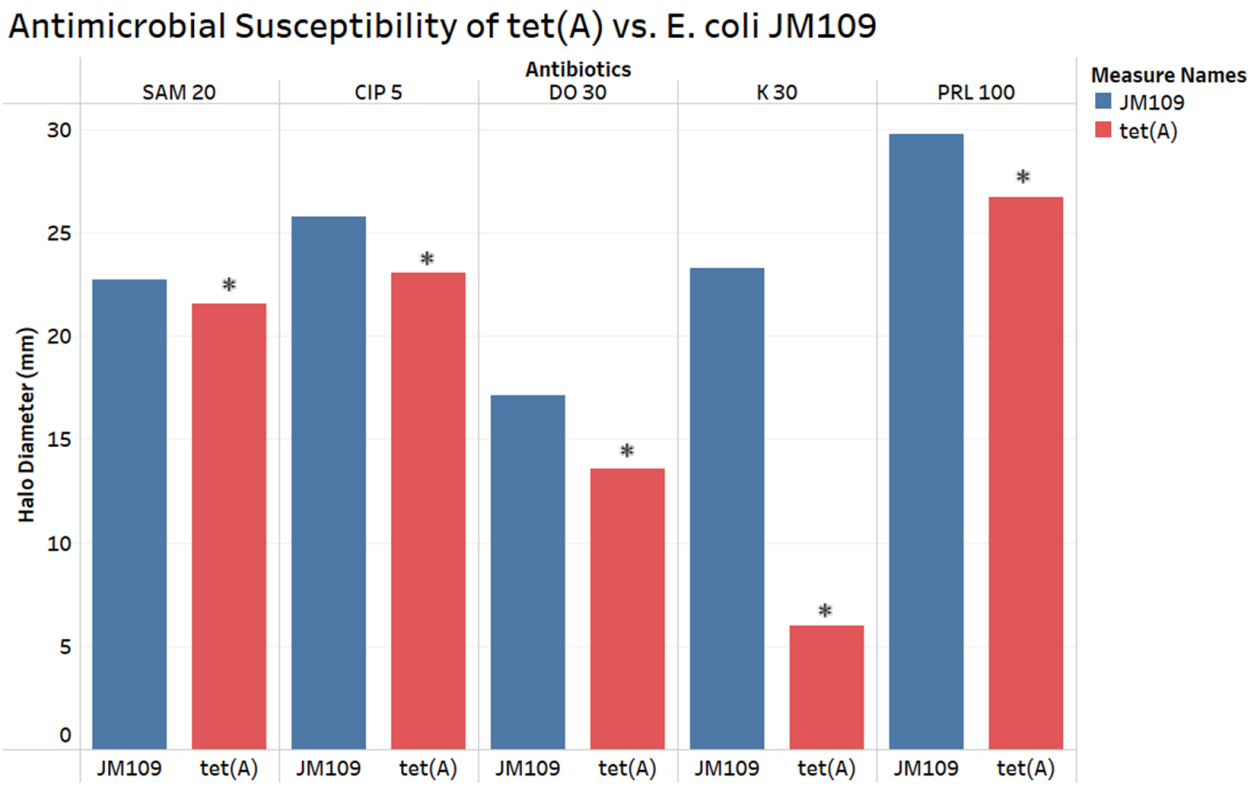
CHeamA did not provide resistance to any of the tested antibiotics on its own. However it slightly, yet significantly reduced susceptibility to SAM.

FIGURE 12. AST of *CHeamA* showed displayed resistance to KAN and significantly decreased susceptibility to SAM, and a functioning selectable marker. * indicates statistically significant



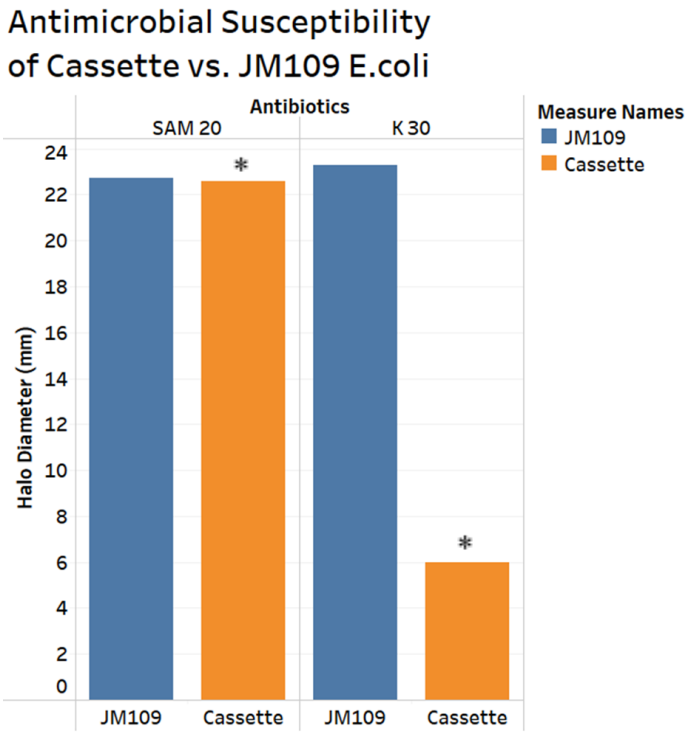
tetA provided resistance to DO as expected. It also unexpectedly reduced susceptibility to PRL, SAM, and CIP by a statistically significant margin.

FIGURE 13. AST of *tetA* showed displayed resistance to DO and significantly decreased susceptibility to PRL, SAM, and CIP, and a functioning selectable marker. * indicates statistically significant



Even with the unsuccessful clone of the cassette, the remaining vector was able to express resistance to Kanamycin, showing the selectable marker was functioning, as well as significantly decreased susceptibility to SAM.

FIGURE 14. AST of cassette showed significantly decreased susceptibility to SAM and a functioning selectable marker. * indicates statistically significant



DISCUSSION

The results above are indicative of the successful isolation of the *CHeamA*, *tetA*, and the cassette from pTRE-2011. *tetA* and *CHeamA* were also successfully cloned into the expression vector pSFOXB-11 but the cassette was not. The successful insertion of *CHeamA* and *tetA* and subsequent AST revealed their respective resistance phenotypes. As expected *tetA* was shown to provide resistance to tetracyclines. The fact that the resistance was seen indicates a functioning clone and therefore, gives us reason to believe that *CHeamA* is also functioning, as the same procedure was followed for each. Therefore, the lack of resistance seen in the phenotype of *CHeamA* can be assumed to be accurate and the hypothesis that it can function as a drug exporter on its own can be rejected. The resistance to colistin associated with pTRE-2011 found by Cummings et al. must be the results of a different efflux gene as there was no significant change to CT resistance in any of the clones. The hypothesized function of *eamA* as a drug/metabolite exporter still holds true, however, it is most likely solely exporting metabolites.

The *tetA* clone, in addition to DO, was seen to decrease susceptibility to PRL, SAM, and CIP. As decreased susceptibility to SAM was seen in all three clone phenotypes, including the unsuccessful cassette, this can most likely be attributed to the expression vector. This would have to be confirmed with a vector-only control, as the cassette clone had about 1200 bp removed and therefore cannot function as a perfect control. The decrease in susceptibility to PRL and CIP, however, can quite confidently be attributed to the *tetA* gene. This was not expected as *tetA* is supposedly specific to the tetracycline class of antibiotics. Decreased susceptibility to other classes means the efflux pump could be gaining function to resist drugs other than solely tetracyclines. This decreased specificity to a certain drug class brings with it grave implications for the clinical treatment of bacterial infections, significantly limiting the options physicians have to treat these resistant infections.

The cassette clone was not inserted successfully as the gene was not present in the sequencing data. About 1200 base pairs were removed, but no gene inserted, the reason for which is unclear as the same cloning procedure was followed. Despite this, the remaining vector still displayed resistance to KAN in addition to significantly decreased susceptibility to SAM. This decreased susceptibility to SAM was seen with *CHeamA* and *tetA* as well. Because the selectable marker was functioning for KAN as well, this means the expression vector must have recircularized after digestion without the insert gene. The decrease in SAM susceptibility cannot confidently be attributed to the expression vector because there was no control in the AMST that included the unedited vector. However, it is a possibility that the selectable marker has some function beyond KAN which accounts for the decrease in susceptibility seen with SAM. Because the clone of the three genes together was not successful, further research is needed to determine if *CHeamA* could work synergistically with *tetA* to increase resistance in any way.

With the grim data of an ever increasing evolution of antibiotic resistance microbes, the fact *eamA* is not involved in such resistance is a small victory. As these antibiotic resistance plasmids evolve and persist, more and more resistance genes are discovered regularly but here, that is not the case.

In conclusion, the Tijuana River Estuary is a reservoir for evolving ARGs which are found on many resistance plasmids that make their way into the surrounding communities and can affect patient health outcomes. Many of these genes within the TRE have yet to be fully characterized. The understanding of these genes and their associated phenotypes is crucial to better approach the issue of resistance seen clinically. Here we provide a tested approach for the cloning and expression of antibiotic resistance genes from larger plasmids for individual characterization. We also determined the function of the unknown gene *eamA,* alone is not related to antibiotic resistance. In order to confirm whether there is any increased resistance seen with the three genes together, a successful clone of the cassette would need to be produced.

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