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Characterization of Fluoroquinolone Resistance Plasmid p1471 Isolated from Leech Symbiont, Aeromonas hydrophila

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I. Abstract

The plasmid p1471 was isolated from the ciprofloxacin (Cp) resistant *Aeromonas hydrophila,* medicinal leech isolate JG1471, which is a strain with a minimum inhibitory concentration (MIC) of > 32 µg/mL Cp. JG1471 carries a 6.8 Kb plasmid which contains Cp resistance gene *qnrS2*. *qnrS2* is a plasmid mediated resistance gene that has been isolated from clinical and environmental sources and has been implicated in increased resistance to the fluoroquinolone (FQ) Cp. To determine the FQ resistance conferred by *qnrS2*, primers were designed to perform a Gibson Assembly to insert a 1.99 Kb fragment from pKAS46, containing an *Escherichia coli* origin of replication (R6K), multiple cloning sites (MCS) and a kanamycin (Km) resistance gene into the 6.8 Kb p1471. The R6K origin of replication allows successful plasmid replication in *E. coli*, while the Km resistance factors allow for selection of both *Aeromonas* and *E. coli* strains containing the construct. The presence of the MCS in the construct makes it possible for the plasmid to be used as a shuttle vector in future experiments. The assembled construct, pEL1, was transformed into the *E. coli* DH5α-λ *pir* strain. Several assays were then performed to confirm that the plasmid construct was assembled correctly. *E. coli* showed a marked increase in ciprofloxacin resistance upon addition of pEL1, with a ~8-fold increase in MIC from 0. 032 µg/mL to 0.25 µg/mL. When pEL1 was transformed into a Cp^S strain of *Aeromonas veronii,* the MIC showed a 3,000-fold increase, from 0.002 µg/mL to 6 µg/mL, passing the 4 µg/mL clinical benchmark for resistance. This data suggests that the presence of *qnrS2* in p1471, a high copy plasmid, may play a substantial role in the Cp^R of the strain.

II. Introduction

Leeches have been used as medicinal tools since 200 BCE, though substantial changes have occurred in their prescribed use[1]. The European medicinal leech, *Hirudo verbana*, is no longer used to remove 'evil spirits' as it was in medieval times[2]. Instead, leech therapy employing *H. verbana* leeches is now a commonly used procedure for a broad spectrum of purposes that include application after reconstructive and plastic surgery such as digit replantation and graft transplants[2]. Leeches for hirudotherapy are purchased from Leeches USA, which is the only FDA-approved supplier of the medicinal leech for hirudotherapy. During and after hirudotherapy, venous congestion in the transplanted or reconstructed tissue wound site can present a real challenge to attaining successful outcome of the surgery. Venous blood return from the wound site dramatically decreases due to blood clotting and disruption of veins[3]. Leeches secrete vasodilators and anticoagulants like hirudin, which allow for improved blood flow to the surgically reconstructed sites and inhibit the activity of thrombin, an enzyme with critical roles in coagulation of blood[4]. Several other proteins secreted by the leech allow for increased blood flow to the area and increased prevention of blood clotting so that venous congestion does not occur and the surgical area can continue to receive enough blood flow to eventually heal[4]. When post-operative venous congestion was observed in digit replantation performed by Dr. Tamai, he reported 17% salvage rate without any use of leeches[5]. When Dr. Iain Whitaker studied digit replantation with use of leeches, he observed a 70% salvage of the tissues[6]. A 77.98% salvage rate was observed as a result of leech therapy after reconstructive surgery in 277 cases overall[3]. Since physicians have begun to use hirudotherapy, tissue salvage has been substantially improved[3].

While hirudotherapy is well established as a tool for tissue salvage in reconstructive surgery, this procedure is not without its risks; one consequence of hirudotherapy is an increased risk of infection. In some cases, infection as a result of leech therapy can lead to cellulitis, a simple bacterial infection of the skin. In more extreme cases, tissue loss and septicemia can occur as a result of infection, leading to

failure of the reconstruction or even death[7]. Without prophylactic antibiotic treatment, Whitaker observed a 26% infection rate of the reconstructed tissue after hirudotherapy. Upon addition of prophylactic treatment with antibiotics, however, Whitaker observed that the infection rate can go from 26% to 12.5% of patients[6]. With the decreased infection rate comes an increased success rate of the surgical reconstruction[6].

Infections following leech therapy are a result of the bacterial symbionts that reside in the gut of the medicinal leech. *H. verbana* has two main bacterial symbionts: *Aeromonas* species and *Mucinivorans hirudinis*[8]. *Aeromonas hydrophila* is one of the *Aeromonas* species isolated from the gut of the leech, and is the primary isolate published in case reports describing infection with leech therapy. *A. hydrophila* is a member of the family *Aeromonadaceae*[9]. It is Gram-negative, rod shaped, motile, and a facultative anaerobe[9]. *M. hirudinis* is an anaerobic bacteria of the family *Rikenellaceae* that was isolated and cultivated on mucin-containing plates[10].

Symbiosis is a close association between two biological partners. In the case of *H. verbana,* it has been suggested that its symbionts provide the leech host with extra nutrients, such as acetate and B vitamins that the host is unable to produce by itself[11,12]. It has also been hypothesized that the gut symbionts play a role in prevention of pathogens in the blood meal from colonization of the gut of the leech[13]. On the other hand, the leech host provides the symbionts with nutrients and an environment for successful growth. Host-produced mucin is thought to allow *Mucinivorans* to proliferate[8]. Sialic acid utilization genes in *Aeromonads* allow them to digest and utilize the sialic acid provided by the host[14]. Certain genetic markers that allow *Aeromonas* to be pathogenic are utilized as colonization factors when *Aeromonas* is present in the leech. For example, the normally pathogenic Type II secretion system (T2SS) in *Aeromonas* species is necessary for colonization of the gut of the leech by the bacteria[15]. When the T2SS was inactivated by transposon mutagenesis, the *Aeromonas* strain was initially unable to colonize the gut of the leech[15]. The T2SS allows hemolysis of red blood cells in the blood meal, and when it is

knocked out, the mutant is unable to colonize this niche[15]. While these bacteria are beneficial symbionts in the leech gut, they can act as pathogens in other settings: the T2SS found in *A. veronii* has been implicated in pathogenicity and *Aeromonas hydrophila,* in particular, is the bacteria most commonly associated with wound infections following hirudotherapy[15,7].

Standard procedure in clinical hirudotherapy includes prophylactic treatment with fluoroquinolones (FQs) such as ciprofloxacin (Cp) to prevent infection of the wound with *Aeromonas* species[3]. FQs are useful drugs when it comes to treatment of bacterial infections because they target the process of replication, reducing the ability of the bacteria to survive[16]. FQ's enter the bacterial cell by passive diffusion (specifically through porins in the outer membrane in Gram-negative bacteria and through the lipid bilayer)[17]. Once inside the cell, FQs bind to and inhibit the actions of topoisomerase proteins such as DNA gyrase (made up of protein subunits *gyrA* and *gyrB*) and topoisomerase IV (consisting of subunits *parC* and *parE*) which bind to the DNA to introduce supercoils that allow the DNA to be unwound downstream and then remove negative supercoils from the DNA when ATP is no longer present[16]. FQ's prevent DNA replication by direct interference in the replication process, with bactericidal effects on both Gram-negative and Gram-positive bacteria[16]. With the FQ bound to the enzyme, the supercoiling process will not continue and DNA cannot be replicated[16] In Gram-negative bacteria, DNA gyrase is the primary FQ binding site, while topoisomerase IV is the main target in Gram-positive bacteria[18,16]. In Gram-negative bacteria, FQ's bind to DNA gyrase while it is bound to the DNA[16]. In Gram-positive bacteria, FQs bind to DNA type IV topoisomerase and produce bactericidal effects by binding to the DNA-topoisomerase complex while there is a break in the DNA, just as it does with DNA gyrase, making FQs very useful antimicrobials[16].

The enhanced bactericidal effects of the synthetic drug, Cp, against Gram-negative bacteria make it the treatment of choice for *Aeromonas* infections resulting from hirudotherapy. While there are multiple FQs on the market, Cp is meant for human use[19]. Hirudotherapy is not the only route for

human acquisition of Cp^R bacterial infections. Resistance to enrofloxacin can also lead to Cp^R and human illness. In 2005, pharmaceutical companies withdrew enrofloxacin from use in poultry farms in the United States due to lack of FDA approval. Studies have shown that resistance to enrofloxacin developed in chickens treated for widespread infection of *Campylobacter* species[20]. Reports of FQ resistant *Campylobacter* bacteria associated with human consumption of infected poultry became more frequent. The patients acquired the resistant bacteria and experienced infection that could not be treated with clinically determined levels of ciprofloxacin[21].

Infections due to Cp^R *Aeromonas* bacteria, however, did not occur until 2011. Before 2011, prophylactic treatment of the patient with Cp was effective in prevention of infection. That year, however, Cp-resistant strains of *Aeromonas hydrophila* were isolated from wound infections following hirudotherapy, in some cases leading to tissue necrosis and loss of the reconstructed area[22]. When patients experience wound infections following hirudotherapy, the salvage rate of their tissue is 37.4%, while the tissue salvage rate is 88.3% when infection does not occur[3]. These statistics do not differentiate between patients who experience prophylactic treatment with Cp and those who don't, but resistance to Cp, the commonly used prophylactic treatment, will likely continue to increase the rate of infection and subsequent tissue failure following hirudotherapy[3].

Cp resistance can be a result of several cellular changes. One of the first steps toward resistance is a mutation in the *gyrA* or *parC* subunits of DNA gyrase and topoisomerase IV, the two targets of Cp's bactericidal mechanisms. When mutations occur in *gyrA* and *parC,* they primarily lead to amino acid changes in the active sites of the enzyme[18]. With a single point mutation in the quinolone resistance determining region (QRDR) of *gyrA*, resistance to ciprofloxacin can occur[18]. Bacteria with high-sensitivity DNA gyrase or topoisomerase IV enzymes have lower minimum inhibitory concentrations (MIC) of FQ's, which is the concentration of antibacterial compound required to inhibit visible growth. These bacteria with highly sensitive enzymes have much lower MIC values than those with more Cp resistance, so it is

hypothesized that a mutation in both *gyrA* and *parC* that makes the replication enzymes less sensitive would lead to an even higher MIC than a single point mutation in either region would provide[18]. FQ's can diffuse into the cell through porins, so downregulation or mutations in porin proteins can contribute to resistance as well[23]. While chromosomal mutations and the resistance provided by the QRDR are important factors in Cp resistance, Cp resistance can also be increased as a result of plasmid-mediated resistance genes[23].

Plasmid-mediated factors are commonly found in Cp^R bacteria. One example of a plasmid-mediated resistance factor is an efflux pump, a transmembrane pump that actively transports bactericidal compounds such as Cp out of the cell[23]. Genes encoding efflux pump proteins such as QepA and OqxAB are also thought to factor into Cp resistance. QepA provided a 32-fold increase in MIC of Cp when conjugated into *E. coli*[23]. QepA is a transmembrane protein that actively pumps out Cp from the cell[23]. QepA is just one of several plasmid-mediated resistance mechanisms. Another mechanism involves modification of Cp itself through a mutated aminoglycoside acetyl-transferase gene called *aac(60)-Ib-cr*, which acetylates and therefore deactivates Cp[23]. Observation of Cp^R strains of *Aeromonas* have shown an increase in MIC upon addition of more resistance factors[24,25].

One of the most recently determined plasmid-mediated resistance genes belongs in the *qnr* family[23]. The mechanism for FQ resistance as a result of Qnr proteins is not fully understood. One hypothesis is that the pentatpeptide repeat regions that make up Qnr act to destabilize the complex between Cp and DNA gyrase and allow DNA replication to occur[23]. The binding of Qnr proteins to DNA gyrase before it can bind to DNA allows prevention of FQs from binding the complex at all[23]. The specific gene *qnrS2* has been found in both environmental samples and leech-associated *A. hydrophila* infections[26].

Plasmid-mediated resistance factors are important for the spread of antibiotic resistance. Without the ability to transfer plasmids and plasmid-associated genes, *qnr* genes would not be so

widespread. Several *qnr* genes have been found in association with transposons that would allow them to be mobilized to different areas along the bacterial genome[23]. It has been hypothesized that *qnrS* genes are close to and potentially derived from *qnr*-like determinants from *Vibrio splendidus*[27]. Because *Aeromonads* are present in a variety of differing environments, further study could help determine if they are an important reservoir for the horizontal transmission of *qnr* genes[27]. While the mechanism by which *qnr* genes act may not be particularly well characterized at present, further research will provide more information about their role in FQ resistance as a whole. Table 2 shows a list of *qnrS2* isolates in the scientific literature and the MIC values that the presence of the gene provides in *Aeromonas, E. coli,* and *Salmonella* isolates.

A. hydrophila is one of the species implicated in Cp-resistant wound infections. One strain, JG1471, was isolated from a leech shipped from Leeches USA, the only FDA-approved leech supplier in the United States. JG1471 has an MIC of >32 µg/mL likely due to both chromosomal and plasmid-mediated mechanisms. JG1471 has point mutations in both *gyrA* and *parC,* as well as a plasmid-mediated resistance mechanism within a 6.8 kb plasmid, p1471. The plasmid p1471 contains a *qnrS2 gene*. It is likely that the chromosomal mutations give JG1471 an elevated Cp resistance, but that the plasmid-associated *qnrS2* gene boosts Cp^R to high levels (>32 µg/mL) above typical therapeutic concentrations (1- 4 µg/mL Cp). Neither the mechanisms of *qnrS2* nor the contribution to Cp^R is well characterized at present. A common approach to the study of different genes and their effects is to attach a gene of interest to a vector or other genetic tool that can allow expression of the gene in a new strain to observe phenotypic differences.

One way to do this is to assemble a plasmid that ligates the gene of interest to a fragment that allows for replication and selection of the newly assembled plasmid. A Gibson Assembly (GA) is a procedure that is commonly used to accomplish this goal. It is a reaction that includes exonuclease, DNA polymerase, and DNA ligase activity in one buffer, making it a simple and effective strategy to introduce

new DNA sequences. Primers used for the GA must have an overhang where the two pieces of DNA can overlap for the purpose of ligation. The exonuclease chews back the 5' ends of the DNA fragments, the fragments anneal where the primers overhang, DNA polymerase extends the 3' ends, and DNA ligase seals the nicks to leave a fully assembled plasmid containing the original fragments. Figure 1 shows a diagram of how the GA works, while figure 2 shows the plasmids used for this research and the experimental plan for the GA. The newly assembled plasmid should have factors such as novel antibiotic resistance genes to provide selectivity, an origin of replication, and a multiple cloning site to allow future options for gene exploration.

In order to explore the Cp^R coded by *qnrS2*, p1471 was isolated from JG1471 and ligated to a pKAS46 fragment to allow for replication in *E. coli* and its selection yielding pEL1. The goal was to combine p1471 with a pKAS46 fragment. The pKAS46 fragment contained an R6K origin of replication so that the plasmid could use an *E. coli* system to replicate, a kanamycin (Km) resistance gene to confer antibiotic resistance for selection of transformants, and a multiple cloning site (MCS) to allow for future use of the assembled plasmid as a shuttle vector in experiments.

Upon transformation of the plasmid containing the resistance factors, pEL1, into *E. coli* and Cp susceptible (Cp^S) Hm21 *Aeromonas veronii,* the MIC of the recipient strain should rise substantially due to the presence of *qnrS2* in the previously Cp susceptible (Cp^S) bacteria. If the pEL1 plasmid containing *qnrS2* is transformed into *gyrA* and *parC* mutants of Hm21RS (a Cp^S strain of *A. veronii*), the MIC of the resultant cells should be higher than that of Hm21 *A. veronii* which does not contain any chromosomal mutations to increase Cp^R in the QRDR. The increased MIC of the two mutants with and without the presence of *qnrS2* from pEL1 should provide a fuller picture of Cp^R in *Aeromonas veronii* as a whole.

III. Methods

3.1 JG1471 isolation and genome sequencing

JG1471 was isolated from a Leeches USA shipment in February 2013 using a technique previously described[28]. Briefly, leeches were anaesthetized in 70% alcohol and a dorsal incision was made to withdraw intra-lumenal fluid (ILF) from the crop, a digestive compartment analogous to the stomach. The ILF was cultured on media containing Cp at a clinically high concentration of 6 μg/mL from which JG1471 was isolated, subcultured, and frozen in glycerol stock for long-term storage. Genomic DNA was extracted using MasterPure™ DNA and RNA Purification Kit and the NexteraXT libraries were prepared for Illumina® MiSeq 2 x 250bp sequencing. Reads were combined and assembled using CLC Genomics Workbench from Qiagen.

3.2 Strains, growth and storage conditions

All strains are kept in cryovials in a -80°C freezer. *Aeromonas* strains were incubated at 30°C and *E. coli* strains were incubated at 37°C. Strains used from the collection are listed below in Table 1.

Strain Name	Use for Strain	Plasmid of interest	Species	Phenotype of interest
JG1471	isolate CpR plasmid for Gibson Assembly	p1471	*A. hydrophila*	CpR
DH5α-λpir	Isolate KmR plasmid for Gibson Assembly	pKAS46	*E. coli*	KmR
Hm21R	Isolate plasmid for transformation positive control	pMMB207	*A. veronii*	CmR
Hm21	CpS recipient strain for pEL1 transformation	N/A	*A. veronii*	CpS, CmS, KmS
Hm21RS	Wild type strain for *gyrA* and *parC* mutants	N/A	*A. veronii*	CmS, KmS
Hm21RS *gyrA* mutant	CpS recipient strain for pEL1 transformation	N/A	*A. veronii*	Unknown level of CpR (MIC), CmS, KmS
Hm21RS *parC* mutant	CpS recipient strain for pEL1 transformation	N/A	*A. veronii*	Unknown level of CpR (MIC), CmS, KmS

Table 1: Strains used for the GA and transformation. Cp^R=Resistant to >32 μg/mL Cp, Km^R=resistant to 100 μg/mL kanamycin, Cm^R=resistant to 1μg/mL chloramphenicol

3.3 Media preparation

LB broth was made by adding 10 g of Bacto™ Tryptone (Becton, Dickinson and Co (BD) and Sparks, MD), 5 g of Bacto™ Yeast Extract (BD and Sparks, MD), and 10 g of NaCl into 1 L of distilled water, and autoclaved for 20 minutes at 121°C after which antibiotics were added when necessary. LB agar was made by addition of 1.5% Bacto™ agar (BD and Sparks, MD) before sterilization. Blood agar for E-testing was prepared by the addition of 44 g of Columbia Blood Agar Base (Acumedia Inc. and Lansing, MI) to 1 L of dH_2O and sterilization at 121°C for 20 minutes. Mueller Hinton agar for E-testing was prepared by addition of 38 g of Mueller Hinton™ (BD and Sparks, MD) agar to 1 L of dH_2O and sterilization.

3.4 Growth curves

Strain Hm21 and JG1471 from frozen stock were streaked for isolation on LB plates and LB with 4 µg/mL Cp plates, respectively, and grown 16 hours at 30°C. Overnight cultures were made with 5 mL of LB and 4 µg/mL Cp and grown in 30°C shaker at 200 rpm for 16 hours. 50 µL of each culture was incubated in 5 mL of LB broth without antibiotics with shaking at 200 rpm at 30°C until the OD_{600} of the culture was between 0.3 and 0.4. Once the subcultures reached that range, they were placed on ice. The volume of the subculture required to start the growth curve with an OD of 0.005 and a 2 mL total volume was calculated.

Absorbance measurements were taken in duplicate of the following bacterial cultures in respective media: Hm21 + LB, Hm21 + 0.007 µg/mL Cp LB, Hm21 + 0.01 µg/mL Cp LB, Hm21 + 0.05 µg/mL Cp LB, Hm21 + 1 µg/mL Cp LB, and Hm21 + 4 µg/mL Cp LB. JG1471 was grown and measured at the same Cp concentrations listed for Hm21. 200 µL of each mix was added to a 48-well plate. A set of control wells were measured for absorbance containing media but no bacterial culture. These wells were used to measure background absorbance and this value was subtracted from the absorbance measurements of each bacterial culture in media containing the respective Cp concentration. For example, if X absorbance units were measured in LB with no Cp added, then X was subtracted from the average of triplicate

measurements of bacterial culture + LB no Cp. The plate was measured using a Biotek® Microplate reader at 30°C over 24 hours and readings were taken every 10 minutes.

3.5 Plasmid isolation and purification

The strains containing plasmids p1471 and pKAS46 were streaked from frozen stocks for isolation on 4 µg/mL Cp LB plates and 100 µg/mL Km plates respectively. JG1471 was grown at 30°C for 16 hours. *E. coli* + pKAS46 was grown at 37°C for 16 hours. Overnighters were then made with 10 mL LB and the required antibiotic and incubated for 16 hours at 225 rpm. Plasmid purification was performed using the Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI). To confirm isolation of the plasmid, purified plasmids were run on an 0.8% SeaKem® LE Agarose gel (Lonza, Hopkinton, MA) for 40 minutes at 85V.

3.6 Gibson Assembly and Transformation into *E. coli.*

To ligate p1471 to a fragment of pKAS46, the first step of the GA was isolation of the plasmid as described above. Then, the 6.8kb p1471 and 2 kb pKAS46 fragments were PCR amplified. The primers in Table 3 were designed for a Phusion PCR reaction. All tubes included 2.5 µL of both the forward and reverse primer, 25 µL of Phusion master mix, the amount of plasmid DNA required for a target of 5-10 ng in the reaction tube, and the required volume of PCR H_2O for a total volume of 50 µL. For the p1471 reaction, the annealing temperature was 65°C. The following cycling conditions were applied: i) 98°C for 30 seconds, ii) 98°C for 10 seconds, 65°C for 30 seconds, and 72°C for 2 minutes, iii) Step ii repeats 30 times, iv) 72°C for 10 minutes, and v) held at 10°C indefinitely. In the pKAS46 reaction, the annealing temperature of the primers is 62°C. The protocol requires that the reaction mix be heated at i) 98°C for 30 seconds, ii) 98°C for 10 seconds, 62°C for 30 seconds, and 72°C for 1 minutes and 10 seconds, iii) repeat step ii 30 times, iv) 72°C for 10 minutes and v) 10°C indefinitely.

The template pKAS46 plasmid was prevented from being transformed in forthcoming steps by a *Dpn*I restriction digest. The pKAS46 plasmid was isolated and purified directly from bacterial cells, and is

methylated. *Dpn*I is a restriction enzyme that binds and digests at G-methylated A-T-C in both the forward and reverse direction[29]. Since *Dpn*I only digests methylated DNA, any remaining pKAS46 template was be digested but the amplified 1.9 kb fragment remained uncut as the PCR product was not methylated[29]. 5µL of Cutsmart Buffer, 1 µL of *Dpn*I enzyme, the volume required for 1 µg of pKAS46 and the volume of PCR H_2O required for a 50 µL total volume were combined in a PCR tube and placed at 37^oC for 16 hours, 80^oC for 20 minutes for enzyme inactivation, and then held at 10^oC indefinitely.

Next, the GA was performed to ligate the insert and vector fragments. A 1:1 (p1471 to pKAS46) GA ratio, a pUC19 positive control, and a negative control with neither p1471 or pKAS46 present were used. The total volume in the GA tube was 10 µL. 5 µL was made up of the GA master mix. The amount of p1471 should be 50 ng total. In order to have a 1:1 GA ratio, the concentration of pKAS46 DNA required was 0.011 pmoles, and the total amount (ng) of pKAS46 required was 29 ng. The DNA was added to the GA tube with the appropriate amount of dH_2O for a total concentration of 10 µL. The negative control contained 5 µL of GA master mix and 5 µL of dH_2O. The three GA reaction tubes were incubated for 30 minutes at 50^oC and then placed on ice until used for transformation.

The three GA reactions were transformed into competent DH5α-λpir *E. coli* Hanahan cells[30]. The transformation reactions contained the following: the 1:1 GA reaction, the GA reaction without DNA, and pUC19 as a positive control. The Gibson assembly was diluted by adding 30 µL of distilled water to the 10µL GA. Next, 2 µL of this dilution was added to a tube on ice and 50 µL of Hanahan competent cells were added and mixed. 1 µL of pUC19 was added to the 50 µL of competent cells as a positive control. The solutions were placed on ice for 30 minutes. They were heat-shocked in a 42^oC water bath for 1:15 minutes, removed and placed on ice for 3 minutes. Next, 950 µL of SOC media was added to each tube. The samples were incubated with shaking (200 rpm) at 37^oC for 30 minutes.

After the transformation, the cells were plated to select for growth. The 1:1 ratio and negative control GA mixes were plated on LB and LB + 100 µg/mL Km for selection of transformants: a 10/90

cell/SOC dilution on LB and LB + 100 µg/mL Km, and 100 µL directly spread on LB and LB + Km. The leftover culture was centrifuged at 8000 rpm for 2 minutes, the supernatant was decanted, and the culture was resuspended and spread on a Km plate. The positive controls were plated on LB+ 100 µg/mL ampicillin (Amp) plates: 100 µL directly spread, a 10-fold dilution of 100 µL culture in 900 µL SOC (plating 100 µL total from the mix), and a centrifuged culture as mentioned above for the Amp plates. All plates were incubated for 16 hours overnight at 37^{o}C and growth was observed.

3.7 Confirmatory tests

Three colonies from the transformed pEL1 Km plate (from cells grown on the centrifuged GA 1:1 ratio of p1471 to pKAS46 Km plates) were streaked on 100 µg/mL Km plates. The plasmid pEL1 assembled with the Gibson assembly was then repurified from the *E. coli* cells and run on a gel using the specifications mentioned in step 3.5 above. The next steps were diagnostic tests to confirm that the plasmid isolated from the transformed cells was indeed pEL1. First, a diagnostic PCR was performed using primers designed to detect *qnrS2.* Six plasmid purifications total were performed from the subcultured isolated colonies, and they were tested with the *qnrS2* primers. The sequences of the *qnrS2* forward primer is GCAAGTTCATTGAACAGGGT, and the sequence of the *qnrS2* reverse primer is TCTAAACCGTCGAGTTCGGCG. A p1471 sample was used as the positive control for the PCR, with pKAS46 as the negative control. The reaction tubes include 12.5 µL of *Taq* polymerase, 0.25 µL of both the forward and reverse primer, and 20-30 ng of template DNA (with PCR H_2O to bring the tube volume to 25 µL total). The PCR tubes were put in the PCR machine and were heated to 94^{o}C for 2 minutes then 94^{o}C for 30 s, 50^{o}C for 30 seconds, then 72^{o}C for 30 seconds. The previous three steps repeated 30 times, and then the samples were heated to 72^{o}C for 10 minutes and held at 10^{o}C until removed from the thermocycler. The PCR products were run for 45 minutes at 85 Volts on a 1.2% SeaKem® LE Agarose gel to visualize the bands.

The next diagnostic test consisted of PCR using the original primers that linearized the fragments for the GA (listed in Table 3). If both the p1471 and pKAS46 fragments were amplified by the GA primers, the identity of the isolated plasmid as pEL1 could be confirmed. The same six isolated plasmid samples used for the *qnrS2* diagnostic PCR mentioned above were used for the GA primer PCR. The GA PCR step followed the same PCR instructions as were mentioned above in section 3.6. The PCR products were then run on a 0.8% SeaKem® LE Agarose gel for 42 minutes at 85V.

3.8 Sanger Sequencing

pEL1 was sequenced from the left inverted repeat region of *qnrS2* through both sites joining pKAS46 and p1471. Based on the sequences of p1471 and pKAS46 previously determined through sequencing with PacBio and Illumina, eight primers were designed for every 500-600 nucleotides. The annealing temperature for the primers was between 55 and 65^{o}C, 18-25 nucleotides long, with a 1 or 2 nucleotide GC clamp on the 3' end of the primer. The first primer began 50 nucleotides upstream of the inverted repeat region of *qnrS2* and the next primer was designed for 550 nucleotides downstream from there. The primers used for sequencing pEL1 are shown in Table 4. Each primer's sequencing container contained 1.5 µL of 2.5x BigDye v1.1, 3 µL of 5x BigDye Buffer, 200 ng of plasmid template, and the amount of dH_2O for a total volume of 20 µL. The primer tubes were placed in a thermocycler and went through these steps: i: an initial denature stage of 96^{o}C for 2 minutes, a denature stage at 96^{o}C for 20 seconds, an annealing phase of 10 seconds at 50^{o}C, an extension phase at 60^{o}C for 4 minutes. The three previous steps were then repeated 32 times, and the tubes were held in the thermocycler at 12^{o}C until removed.

Next, 5 µL of EDTA solution at pH8 and 125 mM was added to each of the primer reactions. 70 µL of ice cold 95% ethanol was added to the solutions and they were mixed by pipetting. The samples precipitated overnight at -20^{o}C. The samples were spun for 10 minutes at 4^{o}C at max speed and the supernatant was removed. 600 µL of 75% ice-cold ethanol was added to the tubes which were briefly

vortexed and centrifuged at max speed for 10 minutes at 4^{o}C again. The supernatant ethanol was decanted. This ethanol step was repeated twice more. The excess ethanol was left to evaporate off the tubes by leaving the lids of the MCF tubes open for 20-30 minutes. The samples were sequenced by the DNA Biotech facility and the returned sequences were analyzed using SnapGene.

3.9 E-testing

E-testing was performed to quantify whether or not the addition of pEL1 provides *E. coli* cells with an increased resistance to Cp. E-testing measures the minimum inhibitory concentration (MIC) of a certain bacteria. MIC is the lowest concentration of the desired antimicrobial agent that is required to prevent visible growth of a bacteria. First, strains are streaked for isolation on blood agar. The strains tested were DH5α-λ *pir E. coli* without pEL1 and DH5α-λ *pir E. coli* with pEL1 present. For the *Aeromonas* E-testing, the strains tested were Hm21, Hm21+pEL1, Hm21RS, *gyrA* mutants of Hm21RS with and without pEL1, and *parC* mutants of Hm21RS with and without pEL1 present. The quality control (*E. coli* ATCC 25922) was also streaked on blood agar. The plates were placed in the 35^{o}C incubator for 18-20 hours. 4 mL of autoclaved 0.85% NaCl was pipetted aseptically into test tubes. All of the tested strains had a single test tube. For each, isolated colonies were aseptically added from the blood agar plate until the solution matched the turbidity of the 0.5 McFarland Standard. Autoclaved cotton swabs were placed in the solution, allowed to sit for a minute, excess moisture was removed, and they streaked on Mueller Hinton agar three times. Excess moisture was absorbed by letting the plate sit for 20 minutes. Forceps were used to aseptically pick up the Etest® Cp strips (bioMérieux and Durham, NC), and place on the agar with writing face up. The plates were incubated for 18-20 hours at 35^{o}C and the Etest® strip was read by observing the place on the strip where the apex of the zone of inhibition met the bacterial growth.

3.10 Transformation into Cp^S *Aeromonas veronii*

Preparation of competent *Aeromonas* cells[31]:

Cp^S *A. veronii* was inoculated in LB broth. It was incubated with shaking at 30^{o}C overnight. 0.5 mL of the overnight culture was added to 50 mL of SOB broth in a 250 mL Erlenmeyer flask. The bacteria grew at 30^{o}C until the OD_{600} = 0.5. The cells were transferred to chilled centrifuge tubes. The culture was centrifuged for 8 minutes at 6000 rpm at 4^{o}C. The supernatant was poured off without disturbing the culture. 1 mL of ice cold 10% glycerol was added and cells were resuspended by pipetting. 5 mL of ice cold 10% glycerol was added to each tube and mixed gently. The centrifugation was run for 8 minutes, 6000 rpm at 4^{o}C. The supernatant was decanted and the above steps were repeated from the 1 mL resuspension three more times. After the wash steps, the supernatant was removed without pellet loss, the remainder was transferred into MCF tubes, and centrifuged on a tabletop centrifuge for 8 minutes at 60000 rpm at 4^{o}C. The supernatant fluid was poured and the cells were resuspended in 150 µL of ice-cold 10% glycerol. 55 µL of the competent cells were used for each electroporation.

Electroporation of cells:

Electrocompetent *Aeromonas* cells were electroporated at 12kV/cm with 50 ng of purified pEL1 and incubated in SOC medium for recovery at 35^{o}C for one hour with gentle shaking as previously described[32].Transformation of *Aeromonas* with pMMB207 was conducted as a positive control. 10 ng and 50 ng of pMMB207 DNA was added. 100 µL of the electroporated solution was plated on 1 µg/mL chloramphenicol (Cm) plates, and 100 µL on LB agar. The rest of the solution was centrifuged for 2 minutes at 8000 rpm at room temperature, resuspended after all but 100 µL of the supernatant fluid was poured off, and the cells were then resuspended and plated.

50 ng of pEL1 was used for the transformation, and 100 µL of the solution was plated on 100 µg/mL Km plates, 100 µL on LB plates, and spun down and plated on 100 µg/mL Km plates as described for the positive control.

In order to explore *qnrS2* and the p1471 plasmid further, 10, 50, and 100 ng of each p1471 and an IncU plasmid were transformed into Hm21. The p1471 transformations were spun and plated on 1 µg/mL Cp plates and the IncU transformations were spun and plated on 4 µg/mL Cp plates.

The two plasmids were each included in the experiment for different reasons. The IncU plasmid also contains the *qnrS2* gene, but is present only as a single-copy plasmid in the bacterial cell, where pEL1 and p1471 are high copy plasmids. Observing a difference in MIC between one copy and multiple copies of *qnrS2* could help provide more information on the gene and its mechanisms. To determine whether the native plasmid could be transformed using the same methods, p1471 was included in the transformation procedure and it was observed to see if there would be any phenotypic differences between the p1471 and pEL1.

The procedure for competent *Aeromonas* cells and electroporation was repeated using *gyrA*-mutant Hm21RS and *parC*-mutant Hm21RS to transform pEL1 into the two strains. Both were taken from frozen stocks and streaked on 100 µg/mL streptomycin (Sm) plates. Colonies were inoculated from streptomycin plate into 5 mL LB broth containing 100 µg/mL Sm and the cells were made competent using the protocol above. When the transformed media was plated, the 100 µL direct volume to the Km plates was not included in order to make sure there were enough transformed colonies in the plate that included the results from the spun-down centrifuge step.

No plasmid was transformed in the negative controls, and 100 µL of the solution was plated on 100µg/mL Km plates, 1 µg/mL Cm plates, LB plates, and spun down and plated on 100 µg/mL Km agar. The plates were placed at 30^{o}C for 16-18 hours and results were recorded. The transformed colonies were taken from the Km plates and streaked onto new Km plates for isolation, future overnight cultures, creation of frozen stock, and isolation of plasmid using the protocol in 3.5. Plasmid identity was then confirmed with the steps in 3.7.

3.11 Oxidase testing

Oxidase testing was performed to support that the strains with pEL1 transformed were oxidase positive. Colonies were scraped on a sterile Kim wipe that had been saturated with the oxidase test solution (0.048 g of N',N',N'-Tetramethyl-p-phenylenediamine dihydrochloride in 8 mL of nanopure water). A positive oxidase test result was the appearance of a blue color upon addition of the colonies to the saturated Kim wipe.

IV. Results

4.1 Growth curve

It may be hypothesized that the increased energy needed to replicate p1471 as a high copy plasmid would lead to some defect in the growth rate in LB when compared to Hm21 in the absence of antibiotics. The growth rates of JG1471 and Cp^S control *A. veronii* strain Hm21 during the exponential phase were determined in varying levels of Cp (Figure 3). In LB without Cp, Hm21 had a growth rate of 0.046 hr^{-1} while JG1471 had a slower growth rate of 0.007 hr^{-1}. Though the growth rate with just LB was higher for Hm21, the growth rate of JG1471 in the presence of Cp was consistently higher than that of Hm21, as can be seen in Table 5. At 0.05, 1, and 4 μg/ml Cp, Hm21 had a growth rate of zero. While the growth rate of JG1471 in LB was lower than that of Hm21 in LB, the growth yield of JG1471 was larger in the stationary phase, with a higher optical density based on cell growth. JG1471 had the highest growth yield when grown in the presence of LB + 4 μg/mL Cp. The ability of JG1471 to grow at 4 μg/mL Cp is a result of Cp resistance mechanisms.

4.2 Bioinformatic Analysis of p1471

The Cp^R of JG1471 is likely a result of the mutations in *gyrA* and *parC,* as well as the presence of *qnrS2* in p1471. The Cp^R plasmid p1471 contains *qnrS2,* which may play a role in its high MIC, but *qnrS2* is not the only gene present on the plasmid. It also contains a gene coding for the NspV endonuclease type II restriction enzyme that could be involved in ensuring the maintenance of the plasmid. Downstream from this is an *nspV* modification methylase gene to protect its own DNA against the cleavage action of the NspV restriction enzyme[33]. This is a common mechanism used by bacteria and archaea to disrupt the DNA of potential pathogens while still protecting the host DNA but it has also been proposed as a mechanism to ensure plasmid stability[33]. p1471 also contains a *repB* gene, which codes for a plasmid replication protein that may help regulate replication of the plasmid[34]. Another gene on p1471 is an addiction module antitoxin. While toxin/antitoxin systems have not been well characterized, it has been

hypothesized to be similar to the *nspV* endonuclease and methylase system, with toxic actions counteracted by the antitoxin encoded on its own plasmid[35]. The combination of the toxin/antitoxin and *nspV* restriction enzyme system could provide JG1471 with an increased protection against foreign microbes or pathogens. The important genetic marker for Cp^R on the plasmid is the aforementioned *qnrS2.* In order to study the resistance that the presence of *qnrS2* provides, the p1471 plasmid was ligated to the pKAS46 1.99 kb fragment using the GA and transformed into Cp^S bacteria.

4.3 Plasmid isolation and purification

The 1.99kb pKAS46 fragment was chosen for several reasons. An origin of replication was included (R6K) to allow for replication of the plasmid after transformation into *E. coli.* The addition of the R6K origin of replication allowed for this replication in DH5α-λpir cells but shout not change replication or the copy number in *Aeromonas* as plasmids with the R6K ori are suicide vectors in *Aeromonas*[36] . The Km-resistance marker present in the pKAS46 fragment allowed for selection of transformed colonies after the GA and transformation. This extra selective marker was helpful because the Cp resistance provided by the presence of *qnrS2* in the plasmid was not quantified, so 100 μg/mL Km plates were used to select for successfully transformed cells. The multiple cloning site in the pKAS46 fragment allows for future use of the plasmid as a shuttle vector in experiments. Transformation into *Aeromonas* has been problematic in the past, so a well-characterized shuttle vector such as pEL1 that transformed successfully would be a valuable tool for future use.

In order to isolate the p1471 and pKAS46 plasmids for the GA protocol, a Wizard® Plus SV Minipreps DNA Purification System was used. In Figure 4, a 0.8% SeaKem® LE Agarose gel shows the successful isolation of the two plasmids. p1471 shows a band at 6.8 kb and pKAS46 shows a band at 5.9 kb. After purification of the two plasmids, the GA was performed to ligated the p1471 and pKAS46 fragments.

4.4 Gibson Assembly and Transformation

In order to continue with the GA, p1471 and the 1.99kb fragment of pKAS46 were amplified by PCR for a linear product. In Figure 5, a 0.8% SeaKem® LE Agarose gel suggests that the PCR amplification was successful; both p1471 and the pKAS46 fragment show the expected band size. Figure 2 shows a diagram of the fragments and ligated construct of pEL1.

After amplification of p1471 and the fragment from pKAS46, a *Dpn*I digest was performed to digest the methylated template DNA from the pKAS46 PCR tube. In previous experiments without this *Dpn*I step, pKAS46, rather than pEL1, was re-isolated at the end of the transformation procedure because the template DNA from the PCR out-competed the assembled plasmid. Figure 6 shows the difference between a confirmatory PCR with *qnrS2* with and without the addition of the *Dpn*I digest to the protocol. In A, no *qnrS2* was detected from the transformed plasmid because pKAS46 was re-purified rather than p1471. In B, a *Dpn*I digest was performed before the GA protocol, the pKAS46 template DNA was not transformed, and the pEL1 plasmid annealed properly.

After the fragments annealed, the newly assembled pEL1 plasmid was transformed into Hanahan cells. The marker for a successful transformation was the presence of colony growth on the Km plates from the GA transformants. The 1:1 p1471 to pKAS46 GA ratio provided 6 distinct colonies on the 100 µg/mL Km plate from the centrifuged and concentrated cells. Of these six distinct colonies, three were streaked on Km plates for isolation, and confirmatory assays were performed to make sure that the transformation products did contain pEL1.

4.5 pEL1 confirmatory assays

In order to confirm that the transformed colonies from the Km plate actually received their Km resistance from pEL1 rather than pKAS46, several confirmatory assays were performed. First was the PCR reaction with *qnrS2* primers mentioned in part 4.2. The gene *qnrS2* was part of the pEL1 assembled construct due to the addition of the p1471 fragment to the assembly. Because the *qnrS2* primers did

show amplification of the *qnrS2* gene from the plasmid in part B of figure 5, there was evidence that p1471 was present in the final construct.

The next confirmatory assay was a PCR using the original primers designed to linearize the fragments for the GA. If the fragments assembled correctly to make pEL1, both p1471 and the 1.99kb pKAS46 fragment should have been amplified when pEL1 was tested. The confirmatory PCR with the GA primers was a success. Samples B-F in Figure 7 show a band near 6.8kb, exactly where the p1471 linear fragment is expected to be. Sample A seems to have some form of truncated version of the fragment, so this particular pair of the *E. coli* and plasmid were excluded from future experiments. Samples A-F all show the expected 2kb band of the amplified pKAS46 fragment. The diagnostic assays confirmed the identity of the transformed plasmid to be pEL1 and frozen stocks were made of *E. coli* with the plasmid for use in future experiments.

4.6 Sanger Sequencing

After confirmation of successful p1471 and pKAS46 assembly, sequencing confirmed that no mutations or ligation mistakes had occurred during the GA process. If a mutation had occurred in *qnrS2* or part of the pKAS46 fragment, future tests could be impacted. Eight primers were designed to amplify the assembled region. Because *qnrS2* is the gene of interest in the p1471 plasmid, primers were designed to begin at the left of the inverted repeat region of the gene and continue through *qnrS2,* its right inverted repeat region, and pass all the way through the pKAS46 fragment. The eighth primer did not bind or amplify the DNA, but primers 1-7 did sequence the desired region, as seen in Figure 8. The seven primers sequenced the pEL1 area of interest and showed definitively that there were no mutations in the sequence of the plasmid as compared to the original components. The plasmid pEL1 was successfully assembled and transformed into *E. coli* without mutations.

4.7 E testing of *E. coli* + pEL1

The presence of the pEL1 plasmid allowed for a test to determine the phenotypic changes associated with the presence of the plasmid in *E. coli*. In order to determine what level of Cp^R was conferred to the susceptible strain upon addition of the pEL1 plasmid, E-testing was performed to determine the MIC of Cp for the bacteria. ATCC25922 *E. coli* was used as a quality control for the E-testing and showed an MIC within the expected bounds.

DH5α-λ *pir E. coli* showed a marked increase in MIC upon addition of pEL1 (Table 6). The MIC increased from 0.032 µg/mL without plasmid to 0.25 µg/mL when pEL1 was present in the bacteria. Because this *E. coli* strain does not have any of the chromosomal mutations that lead to Cp^R, the change in resistance of this strain is likely due to pEL1 and the presence of the gene *qnrS2.*

4.8 Transformation of pEL1 into *A. veronii*

In order to study the effect of the presence of *qnrS2* in Cp^S *Aeromonas,* pEL1 was transformed into *A. veronii.* After transformation of five reactions containing 50 ng of pEL1 added to 55 µL competent *A. veronii,* a total of 13 isolated colonies grew on the spun-down plates across the five reactants. Five of the colonies were streaked for isolation, inoculated in overnighter cultures, and saved as frozen stocks. The positive controls showed growth of many colonies containing pMMB207 growing on 1 µg/mL Cm plates, and the negative control showed a lack of growth on all plates except LB. p1471 and an IncU plasmid containing *qnrS2* were also transformed in Hm21 but neither yielded any transformants on the selective Cp media. pEL1 was purified from frozen stocks #1 and #2 of Hm21 pEL1. From there, *qnrS2* and GA primers were used to confirm the identity of the transformed plasmid as pEL1. The presence of PCR products using the *qnrS2* and GA primers (Fig. 9), confirms that pEL1 was present in Hm21 + pEL1.

pEL1 was transformed into *gyrA* and *parC* mutants of Hm21RS *A. veronii. gyrA* and *parC* Hm21RS mutants are both hypothesized to have an increased Cp^R due to the genomic mutation, so transformation of pEL1 to each allowed further exploration of the MIC conferred by the different

resistance factors. There was a lawn of growth on the 100 µg/mL Km plates (transformed with 10, 50, and 100 ng of pEL1). Fig. 10 shows the presence of *qnrS2* in all but two of the Hm21RS *gyrA* and Hm21RS *parC* strains after colony PCR of the transformed cells. Oxidase tests were performed on all transformed colonies to support that *Aeromonas* was the recipient bacteria. The oxidase tests were positive as expected for *Aeromonas.* The next step was to observe the MIC change in *Aeromonas* as a result of pEL1.

4.8 E-Test of *Aeromonas* + pEL1

Transformation of pEL1 into strains of Cp^S *A. veronii* was hypothesized to lead to an increased MIC for each strain as a result of the presence of *qnrS2*. After transformation of the *qnrS2* containing pEL1 plasmid into the Cp^S *A. veronii* bacteria in the first trial, the MIC increased to 6 µg/mL from its starting point of 0.002 µg/mL. The addition of the pEL1 plasmid led to a dramatic increase in the MIC and changed a Cp^S strain of *Aeromonas* to a Cp^R one. Table 7 shows the MIC results for the 1st and 2nd E-test trials.

After the second trial of E-testing, the MIC results of Hm21 *A. veronii* + pEL1 showed a different MIC value; instead of the 6 µg/mL MIC determined in trial 1, the MIC was 0.032 µg/mL. While there is a large difference between the MIC values from the two trials, both values support that addition of *qnrS2* to a Cp^S strain of *Aeromonas* increases the MIC dramatically. The *gyrA* Hm21RS mutant showed a phenotype MIC of 0.094 µg/mL before transformation of the plasmid due to its genomic mutation, and 0.125 µg/mL after addition of pEL1. The *parC* Hm21RS mutant did not contain inherent resistance as a result of the genomic mutation, but increased from 0.003 µg/mL to 0.032 µg/mL after transformation of pEL1, just as the second Hm21 + pEL1 trial did.

V. Discussion

Bacteria with elevated resistance to Cp are increasingly being isolated in hospitals, and a greater understanding of the factors which contribute to Cp resistance and how they are acquired is needed to combat this growing problem. *Aeromonas* species can be found in water sources such as hospital wastewater, drinking water in cities, and natural aquatic environments in addition to the leech gut[37]. It has been suggested that horizontal transfer in such settings plays a role in the acquisition of antibiotic resistance determinants later found in clinical and environmental isolates[37]. This factor combined with the widespread overuse of antibiotics leads to a buildup of Cp in the environment and selection of resistant bacteria[37,38]. Selection for resistance factors due to the presence of low-level Cp in the environment may be the mechanism by which leech symbionts acquire resistance factors. JG1471 may have adapted to grow slightly better in the presence of Cp if the bactericidal agent is found, as has been suggested, in many water and waste sources[3]>. The high Cp resistance found in JG1471 is likely a result of *gyrA* and *parC* mutations in the genome and the *qnrS2* gene present on the p1471 plasmid.

The gene *qnrS2* is a resistance factor that has not been well characterized but is observed in *Aeromonas* strains isolated from the environment and clinical sources[24,40]. This resistance gene has been discovered on IncU and IncQ-like plasmids that take part in conjugative transfer and is a likely mechanism by which *qnrS2* and other *qnr* genes have disseminated into environmental isolates[24,41]. In order to study the *qnrS2* gene present in p1471, p1471 was ligated the 2kb fragment of pKAS46 to form the pEL1 plasmid. The addition of the R6K origin of replication allowed the pEL1 plasmid to replicate in DH5α-λpir *E. coli* but did not affect the copy number of pEL1 in *Aeromonas* because of the role of pKAS46 as a suicide vector in *Aeromonas*[36].

When *qnrS2* was transformed into the Cp^S DH5α-λ *pir E. coli* cells, there was an MIC increase from 0.032 µg/mL to 0.25 µg/mL. These results were consistent with those in previous literature about *qnrS2* addition to *E. coli* (Table 2). When plasmids containing *qnrS2* were conjugated from *Aeromonas*

species into various strains of *E. coli* in other studies, the MIC values were similar. One *E. coli* transconjugant experienced a change from 0.015 µg/mL to 0.25 µg/mL upon addition of the gene[42]. Two separate *qnrS2* isolated plasmids conferred an increase from <0.01 µg/mL to 0.25 µg/mL in both *E. coli* TOP10 transconjugants[24]. One *qnrS2* plasmid found in a clinical sample changed the MIC of *E. coli* from 0.023 µg/mL to 0.5 µg/mL, while another environmental *qnrS2* plasmid changed the *E. coli* MIC from <0.02 µg/mL to 0.15 µg/mL[4>,43]. While not all the MIC values for *E. coli* + *qnrS2* are exactly the same, all are low when compared to the 4 µg/mL clinical cut off for resistance. This increase in MIC in *E. coli* continues to support the hypothesis that the presence of *qnrS2* increases Cp^R, but it does not suggest if MIC values are comparable when pEL1 is present in a Cp^S *Aeromonas* strain

Because the R6K origin of replication does not allow for replication in *Aeromonas* strains, the origin of replication coded by the p1471 plasmid is used, which suggests that the copy number of pEL1 in the *A. veronii* recipient strain is comparable to the copy number from its original host, JG1471. The original plasmid p1471 is a very high copy plasmid in *A. hydrophila*. The successful assembly and transformation of pEL1 into *E. coli* and *A. veronii* provided an opportunity to evaluate the level of Cp resistance conferred by pEL1 to both bacteria, as determined by E-testing. While addition of pEL1 to *E. coli* (DH5α λ-pir) caused a ~8 fold increase in the observed MIC (from 0.032 µg/mL to 0.25 µg/mL), the transformation of pEL1 into a Cp^S *A. veronii* led to an unexpected 10.5 to 3,000 fold MIC increase (0.002 µg/mL to 0.032 and 6 µg/mL in two independent experiments). Although not determined, it is likely that *qnrS2* is expressed in the recipient strains and is responsible for the observed MIC increase, though the exact mechanism of *qnrS2* is unknown. High copy levels of *qnrS2* present in the cell may lead to a higher level of Cp^R. While *qnrS2* gene conjugation in previous studies has shown an MIC increase in *E. coli*, as seen in Table 2, the resultant bacteria were still Cp^S with MIC values closer to 0.25 or 1. In cases where the *qnrS2* gene was present in the bacteria without other resistance factors, both *Aeromonas* and *Salmonella* strains isolated from the environment and clinical samples showed a Cp^S phenotype[4>,44]. The

addition of the pEL1 plasmid to Hm21 *A. veronii* led to a dramatic increase in the MIC and changed a Cp^S strain of *Aeromonas* to a Cp^R one. Further study of how this plasmid increases the MIC of Cp in *Aeromonas* strains when genomic substitutions in the QRDR are already present will help determine how various combinations of these genetic determinants alter overall Cp^R.

The addition of the *qnrS2* gene to *gyrA* and *parC* Hm21RS mutants also showed an increase in MIC in Table 7 after E-testing. The *gyrA* mutant had an MIC of 0.94 µg/mL before transformation, likely due solely to the mutation in the QRDR of *gyrA.* After transformation with pEL1, this MIC increased to 0.125 µg/mL. This showed an MIC increase that was likely based on the combination of the *gyrA* genomic mutation in the QRDR and the plasmid-mediated *qnrS2* Cp^R gene. The *parC* Hm21RS mutant itself did not show any inherent Cp^R despite its genomic mutation, with an MIC of 0.003 µg/mL. But after addition of pEL1 and *qnrS2,* the MIC of the *parC* Hm21RS mutant increased to 0.032 µg/mL. A second trial of Hm21 + pEL1 led to an MIC increase of 0.003 µg/mL to 0.032 µg/mL. This ~10.5-fold increase in MIC is in stark contrast to the 3,000-fold MIC increase shown in the first E-test of Cp^S Hm21 + pEL1. Future research will explore the reason for the difference in MIC measured from the same transformed bacteria. It is possible that the copy number of the pEL1 plasmid is subject to the stage of cell growth; determination of plasmid copy number in the literature has been shown to be variable based on the stage of growth of some bacteria, whether they are in lag, exponential growth, or the stationary period[45]. It is possible that there was a lower copy number of pEL1 due to a change in bacterial growth that led to a decrease in MIC after the second trial of E-testing. The next steps will be E-testing of the Hm21 + pEL1 bacteria after being grown to different growth points to explore whether this results in a change in MIC that could account for the observed discrepancy. While the MIC values are different between the two trials of Cp^S Hm21 pEL1, the MIC of every strain tested was increased after transformation with pEL1. It is likely that the addition of *qnrS2* was responsible for the increase in MIC observed in the transformed strains.

Future research will focus on further understanding the role *qnrS2* has in Cp resistance. Because pEL1 is a high copy plasmid in *A. veronii,* it is likely that increased copies of *qnrS2* lead to the 6 µg/mL MIC observed. In order to study this hypothesis, a conjugative transfer will take place with an IncU plasmid, previously purified from a Cp^R *Aeromonas* leech symbiont, that contains a copy of the *qnrS2* gene. IncU is a single-copy plasmid, which means that *qnrS2* would only be present as a single copy. It is expected that with this single copy of *qnrS2* in the susceptible strain, the MIC will be lower than that determined after transformation of pEL1 with its likely high copy number. Further research will be performed to determine the specific copy number of the plasmid in each host strain so that exact comparisons can be made. Efforts will also continue to transform the native plasmid, p1471, into the Cp^S *A. veronii* strain to determine whether the addition of the 2kb pKAS46 fragment to the p1471 plasmid caused any phenotypic differences in MIC.

One area of future study will be determining if growth defects are caused by the presence of various Cp^R determinants such as genomic mutations in the QRDR or plasmid-mediated factors like *qnrS2*. This will be accomplished by performing growth curves of *A. veronii* strains with and without the resistance factors to compare rates. The growth rate of Cp^R JG1471 was lower than that of Cp^S Hm21 in LB without Cp (table 5), but JG1471 with and without Cp showed a higher growth yield. One hypothesis is that the strains with the highest Cp^R will have a decreased growth rate like JG1471 because the cell is expending so much energy on producing compounds to resist Cp-mediated cell death. While the research of levels of Cp^R and their effects are clinically relevant, they are not the only applications of JG1471 and *qnrS2.*

The MCS of pEL1 provides the option of its use as a shuttle vector for future studies in *Aeromonas.* A shuttle vector is a valuable biological tool that allows for addition of a gene of interest or DNA recombination[46]. Shuttle vectors are widely used in the biological sciences, with uses including genetic engineering and gene cloning in yeast and bacteria[47]. These tools are often used to express a

protein of interest in a bacteria or host that was not native to it and observe the phenotypic changes[47]. Since pEL1 is transformable into *A. veronii,* this plasmid may be used to express other genes or factors of interest using the MCS. There is some evidence that, after a gene deletion, complementation via plasmid may produce unstable results or false negatives due to plasmid instability or the copy number of the plasmid in the cell[48]. The potentially variable copy number of pEL1 due to the presence of p1471 in *Aeromonas* cells could be a factor in effectiveness of this plasmid as a shuttle vector.

If the shuttle vector is successful, however, the MCS could be used to insert other resistance factors into the plasmid and study their effects. Addition of a *qepA* or *oqxAB* gene that codes for an efflux pump and the subsequent change in resistance could provide an interesting future direction, especially if the *qnrS2* gene is removed from the plasmid to study the effect of the efflux pumps without outside resistance factors. New Cp^R factors are continually being discovered due to increasing Cp^R in *Aeromonas* and other bacteria, and this field of study will likely continue to expand and increase in importance as bacterial antibiotic resistance becomes more prevalent.

VI. Conclusion

The antibiotic resistance plasmid pEL1 was assembled correctly using a Gibson Assembly; confirmatory PCRs and Sanger sequencing confirmed a successful assembly and a complete sequence match to what was expected. This pEL1 plasmid could prove a valuable tool as an *Aeromonas* shuttle vector in future research. The presence of the gene *qnrS2* is an important resistance factor of pEL1. The presence of *qnrS2* provides a significant increase in MIC in susceptible strains. In *E. coli,* the presence of the gene brought the MIC from 0.016 µg/mL to 0.25 µg/mL. In Cp^S *A. veronii,* the MIC changed from 0.002 µg/mL in the susceptible strain to 6 µg/mL in trial 1 and 0.003 µg/mL to 0.032 µg/mL upon addition of the plasmid in trial 2. *qnrS2* can also combine with genomic resistance factors to increase MIC. The *gyrA* Hm21RS mutant had an inherent MIC of 0.094 µg/mL due to the mutation in the QRDR and increased to 0.125 µg/mL in the presence of *qnrS2.* The MIC differences shown in trial 1 and trial 2 may be a result of the copy number of pEL1 varying depending on cell growth stages and leading to a range of Cp^R phenotypes due to the different number of copies of the *qnrS2* gene.

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VII. Figures

Table 2: Examples of *qnrS2* and its effect on the MIC of different bacteria in the literature

Reference	Year	Gene present	Increase in MIC of Cp	Chromosomal mutations	Bacteria	Plasmid	Type of Isolate
Mobilizable IncQ-Related Plasmid Carrying a New Quinolone Resistance Gene, qnrS2, Isolated from the Bacterial Community of a Wastewater Treatment Plant	2006	*qnrS2*	Not specified	N/A	Activated sludge bacteria	pGNB2 (IncQ type)	Environmental
		qnrS2	from <0.02 µg/mL to 0.15 µg/mL	N/A	*E. coli* KAM3	pGNB2 (IncQ type)	
Plasmid-mediated QnrS2 determinant from a clinical Aeromonas veronii isolate	2008	*qnrS2*	8 µg/mL	*gyrA* and *parC*	*A. veronii*	pA272	Clinical
		qnrs2	from 0.023 µg/mL to 0.5 µg/mL	*none*	*E. coli* transconjugant	pA272	
Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental Aeromonas spp.	2008	*qnrS2*	4 µg/mL	one amino acid substitution in Ser83Ile in GyrA	*A. punctata*	p37 (IncU type)	Environmental
		qnrS2	from <0.01 µg/mL to 0.25 µg/mL	N/A	*E. coli* TOP10 transconjugant	p37 (IncU type)	
		qnrS2	>32 µg/mL	two amino acid substitutions in Ser83Ile in GyrA and Ser80Ile in ParC	*A. media*	p32 (IncU type)	Environmental
		qnrS2	from <0.01 µg/mL to 0.25 µg/mL	N/A	*E. coli* TOP10 transconjugant	p32 (IncU type)	
Plasmid-mediated quinolone resistance in Aeromonas allosaccharophila recovered from a Swiss lake	2008	*qnrS2*	0.12 µg/mL	none in QRDR	*A. allosaccharophila*	p34 (IncU type)	Environmental
		qnrS2	from <0.01 µg/mL to 0.25 µg/mL	N/A	*E. coli TOP10 transconjugant*	p34 (IncU type)	
Coprevalence of plasmid-mediated quinolone resistance determinants QepA, Qnr, and AAC(60)-Ib-cr among 16S rRNA methylase RmtB-producing Escherichia coli isolates from pigs	2008	*qnrS2*	not specified	N/A	rmtB-positive E. coli	plasmid type not specified	Veterinary
		qnrS2	from 0.015 µg/mL to 0.06 µg/mL	N/A	*rmtB*- positive *E. coli* transconjugant	plasmid type not specified	
Plasmid-Mediated Quinolone Resistance in Salmonella Isolated from Patients with Overseas Travelers' Diarrhea in Japan	2009	*qnrS2*	0.5-1 µg/mL (2 samples)	none	*Salmonella Braenderup*	plasmid type not specified	Clinical
		qnrS2	0.5 µg/mL	none	*Salmonella Agona*	plasmid type not specified	
		qnrS2	0.5 µg/mL	none	*Salmonella Alachua*	plasmid type not specified	
Plasmid-mediated QnrS2 determinant in an Aeromonas caviae isolate recovered from a patient with diarrhoea	2010	*qnrS2*	not specified	not specified	*A. caviae*	plasmid type not specified	Clinical
		qnrS2	0.75 µg/mL to 1.5 µg/mL	not specified	transconjugant	plasmid type not specified	
First description of the qnrS-like (qnrS5) gene and analysis of quinolone resistance-determining regions in motile Aeromonas spp. from diseased fish and water.	2012	*qnrS2*	16 µg/mL	amino acid substitutions in *gyrA* and *parC*	*A. sobria*	plasmid type not specified	Diseased Fish
		qnrS2	256 µg/mL	amino acid substitutions in *gyrA* and *parC*	*A. hydrophila*	plasmid type not specified	Diseased Fish
		qnrS2	4 µg/mL	amino acid substitutions in *gyrA* and *parC*	*A. hydrophila*	plasmid type not specified	Diseased Fish
		qnrS2	0.012 µg/mL	none	*A. hydrophila*	plasmid type not specified	Diseased Fish
Ornamental fish as a source of plasmid-mediated quinolone resistance genes and antibiotic resistance plasmids	2014	*qnrS2*	8 µg/mL	unknown	*A. hydrophila*	plasmid type not specified	Koi Carp
		qnrS2	4 µg/mL	unknown	*A. hyrdophila*	plasmid type not specified	Koi Carp
Prevalence and characterisation of quinolone resistance mechanisms in Salmonella spp.	2014	*qnrS2*	2 µg/mL	*gyrA* substitution	*Salmonella enteritidis*	plasmid type not specified	Animals, food, and feed
Quinolone resistant Aeromonas spp. as carriers and potential tracers of acquired antibiotic resistance in hospital and municipal wastewater	2016	*qnrS2*	from 0.015 µg/mL to 0.25 µg/mL	No	*Escherichia coli* transconjugant	IncU	*Aeromonas* donor from clinical setting

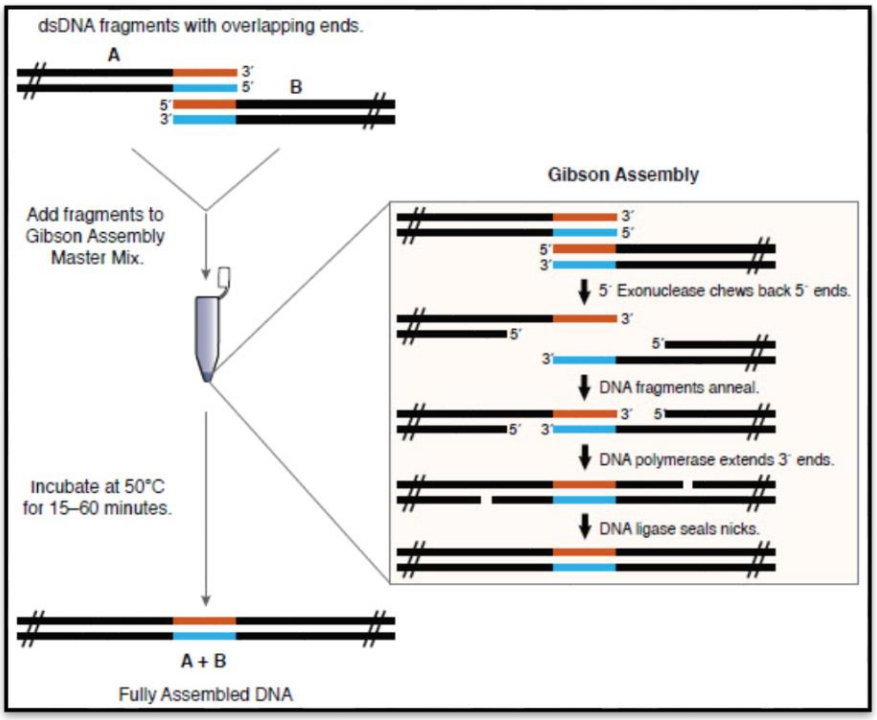


Figure 1: After primers are designed to overlap each fragment, the GA assembles the DNA. The 5' ends are chewed back by exonuclease, DNA polymerase extends the 3' ends of the DNA after it has annealed, and DNA ligase seals the nicks in DNA for a fully assembled plasmid[49].

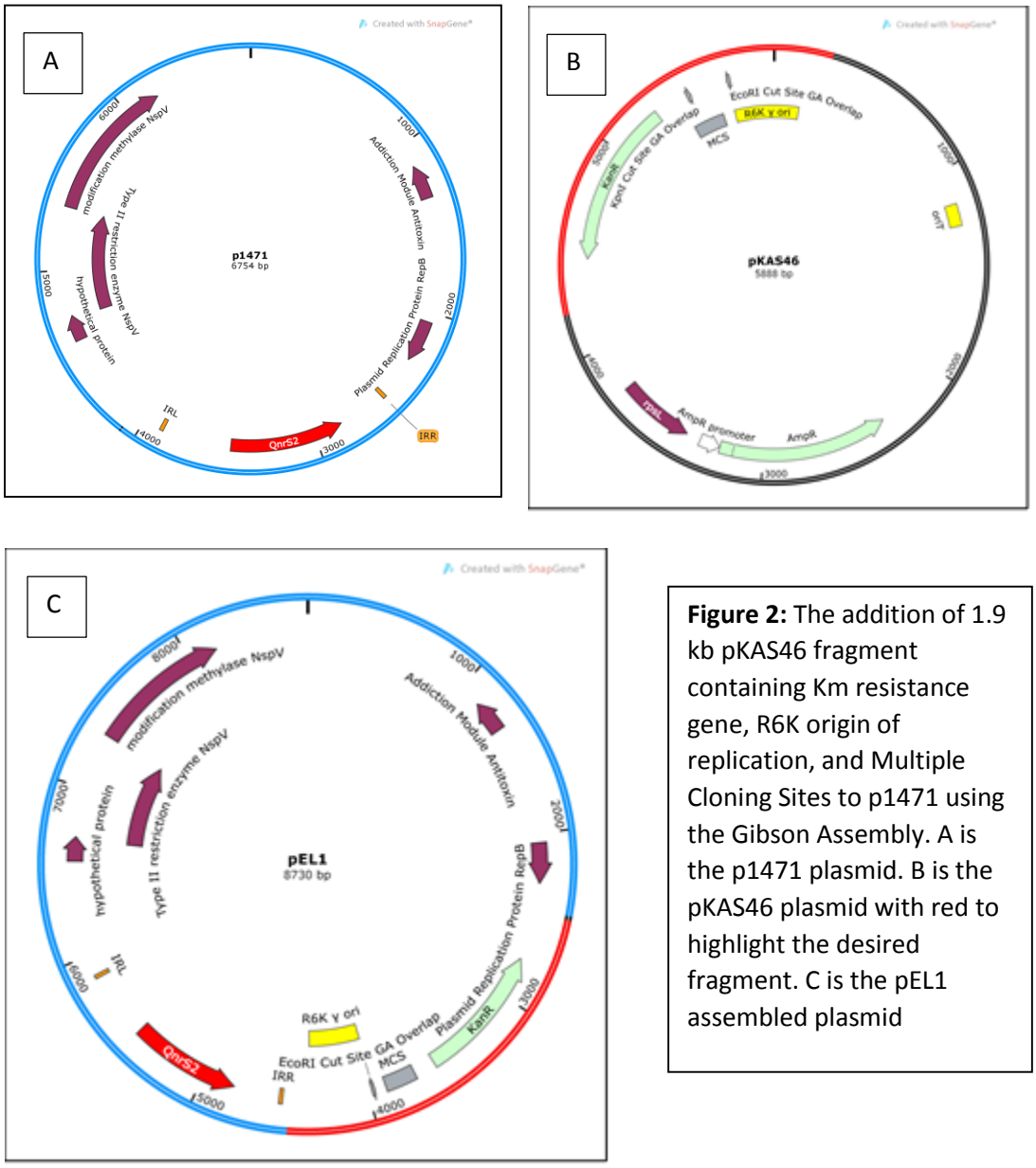


Figure 2: The addition of 1.9 kb pKAS46 fragment containing Km resistance gene, R6K origin of replication, and Multiple Cloning Sites to p1471 using the Gibson Assembly. A is the p1471 plasmid. B is the pKAS46 plasmid with red to highlight the desired fragment. C is the pEL1 assembled plasmid

Plasmid	Direction	Primer sequence
p1471	Forward	CCCAAGAGGAACAAATCGCTC
p1471	Reverse	GGGGCAACCATAAAAACAGC
pKAS46	Forward	GCTGTTTTTATGGTTGCCCCgagcgtgacaatcacgaaac
pKAS46	Reverse	AGCGATTTGTTCCTCTTGGGgtgttgctgactcataccag

Table 3: The primers designed using SnapGene to linearize the p1471 plasmid and the 2kb fragment of pKAS46 and allow ligation during the Gibson Assembly

Primer Name	5'-3' orientation	Annealed correctly?
E1	CGTTTCGACACACAAGGCAG	Yes
E2	TGGCAGCGATCAGAGTACAC	Yes
E3	TTCCAACAATGCCAGCTTGC	Yes
E4	AAACGTGTTTGAATGGGGCC	Yes
E5	TCACGTACTAAGCTCTCATGTTTG	Yes
E6	GTAATACAAGGGGTGTTATGAGCC	Yes
E7	GCTTTTGCCATTCTCACCGG	Yes
E8	CCCCAAGAGGAACAAATCGC	No sequence

Table 4: The primers designed using SnapGene to sequence the area of interest in the pEL1 plasmid. Primer E1 begins on the left of the inverted repeat region of *qnrS2,* and Primer 7 ends just after the ligation site between pKAS46 and pEL1. The entire *qnrS2* gene and pKAS46 fragment are sequenced.

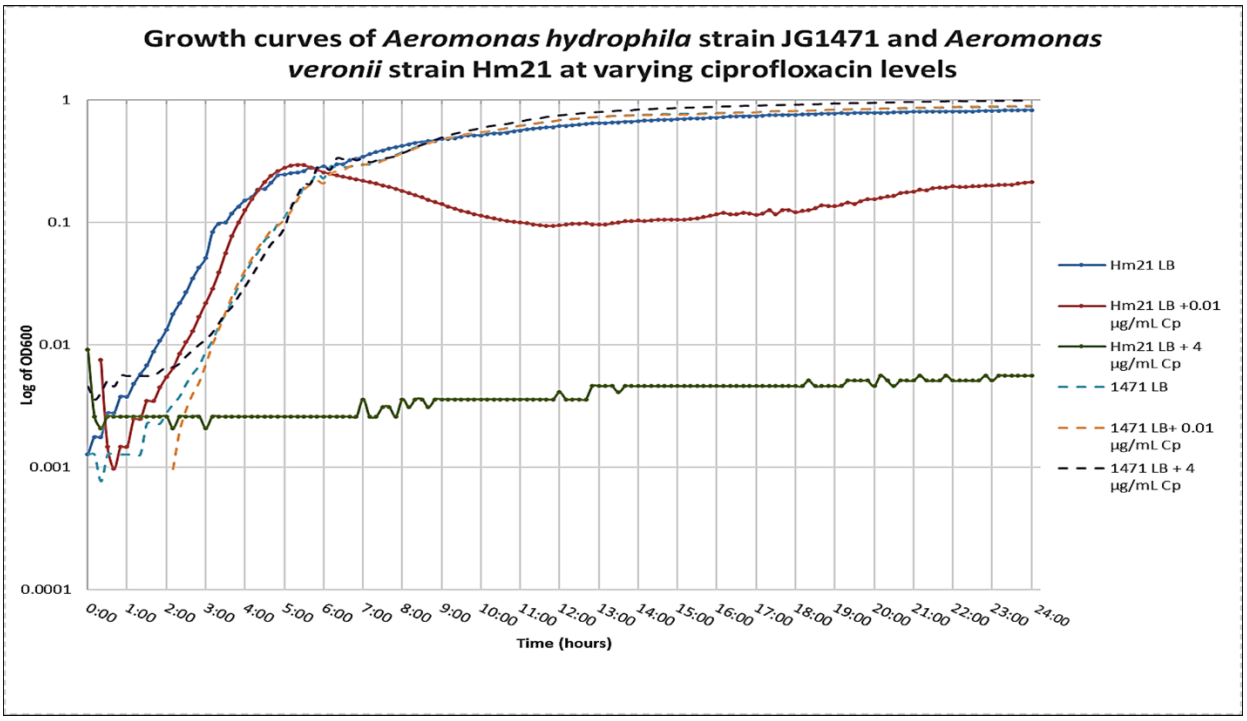


Figure 3: Increased Cp leads to growth defect in Hm21, which is CpS . The presence of p1471 may lead to a growth defect in LB, but provides a growth advantage in JG1471 when 4 µg/mL Cp is present in the LB growth medium.

	LB	LB+0.007 µg/mL Cp	LB + 0.01 µg/mL Cp	LB + 0.05 µg/mL Cp	LB + 1 µg/mL Cp	LB + 4 µg/mL Cp
Hm21 growth rate (hr^-1)	0.046	0.027	0.034	0	0	0
JG1471 growth rate (hr^-1)	0.007	0.04	0.044	0.042	0.032	0.03

Table 5: The specific growth rates for the Hm21 and JG1471 growth curves in LB + Cp

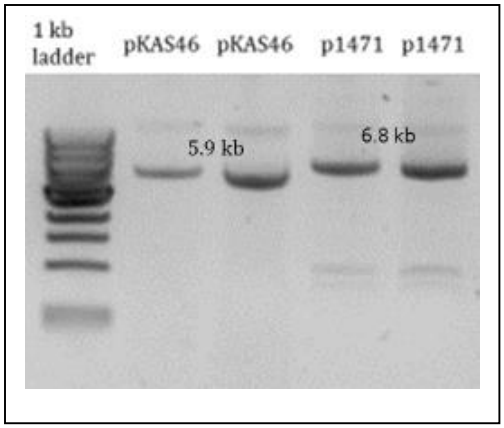


Figure 4: The 6.8kb p1471 and 5.9kb pKAS46 were both successfully isolated by using the Promega Wizard miniprep kit.

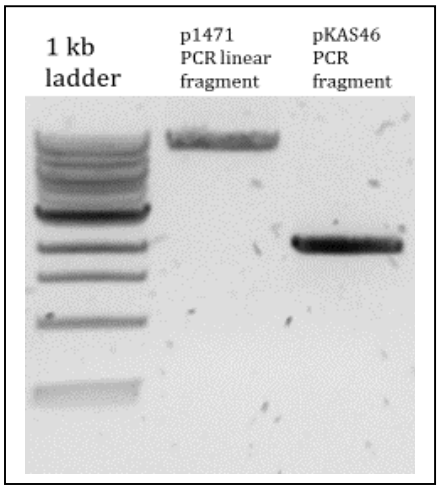


Figure 5: The linear fragments amplified by Gibson Assembly primers for p1471 and pKAS46: p1471 is a linear 6.8 kb, while pKAS46 primers solely amplified a 1.99kb fragment containing the R6K, Km^R, and MCS.

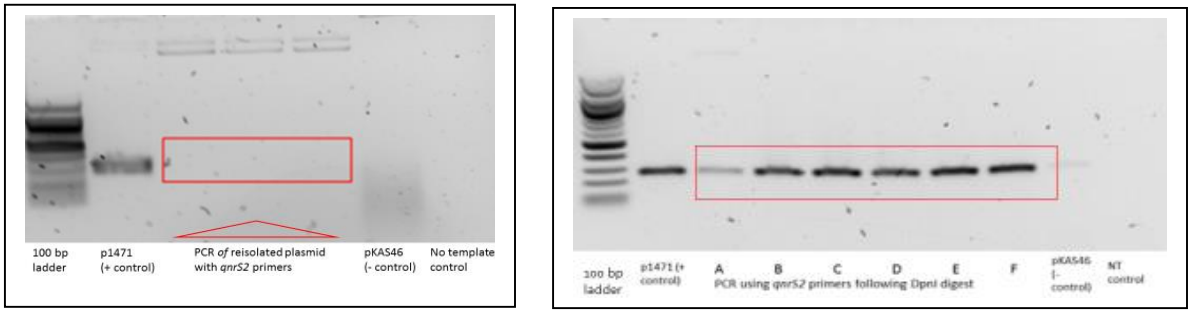


Figure 6: The importance of the *Dpn*I digest to prevent transformation of template plasmids such as pKAS46. A shows the ultimate results of the transformation and plasmid purification if the *Dpn*I digest does not cut up the methylated DNA. B shows the confirmatory testing of *qnrS2* PCR when the *Dpn*I digest is performed to digest pKAS46. Without the *Dpn*I digest, pEL1 was not successfully transformed because it was out-competed by the template pKAS46 used for PCR.

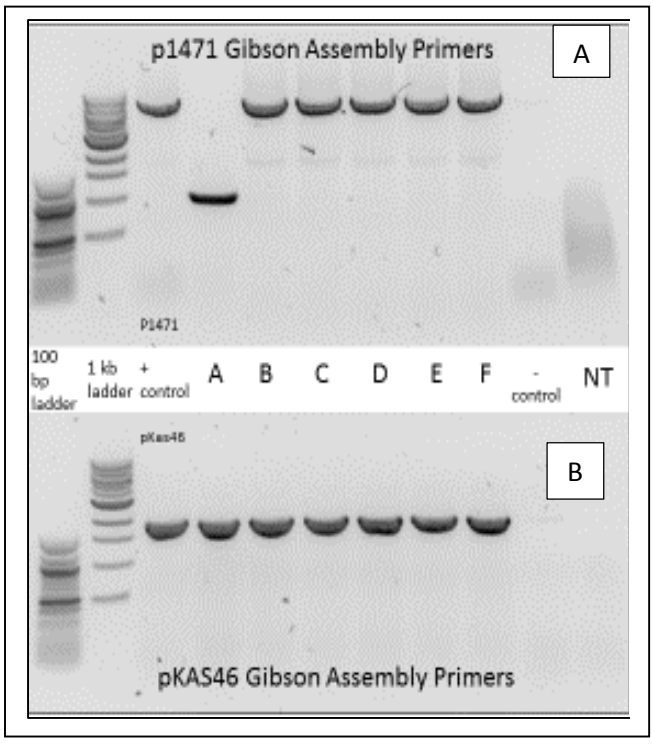


Figure 7: In A, the transformed and purified plasmid is tested using PCR using the original p1471 GA primers. Isolated plasmids B-F all show the expected 6.8kb band for p1471In B, the plasmids are tested using PCR using the pKAS46 GA primers to produce a 2kb fragment. Samples A-F all show a band at the 2kb length where the samples should be. In samples B-F, both fragments were observed after purification form the transformed plasmids.

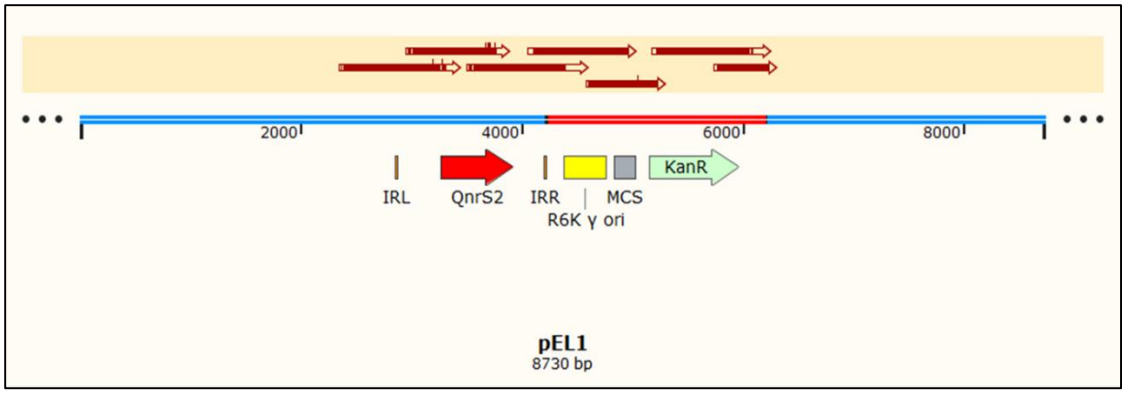
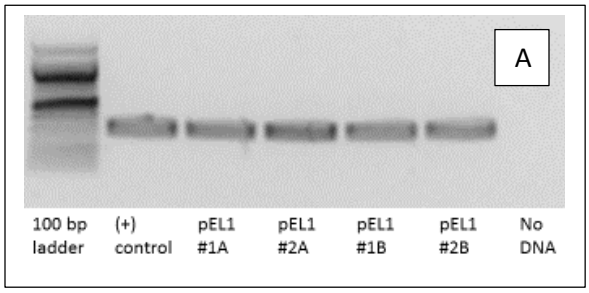


Figure 8: Seven primers designed to amplify the area of ligation of the pEL1 plasmid showed successful assembly of the plasmids. No point mutations or changes of any kind were found in the sequence.

Strain	plasmid	MIC (µg/mL)
E. coli	none	0.032
E. coli	pEL1	0.25
E. coli	pEL1	0.25

Table 6: MIC results from transformation of pEL1 into DH5αλpir *E. coli* cells.



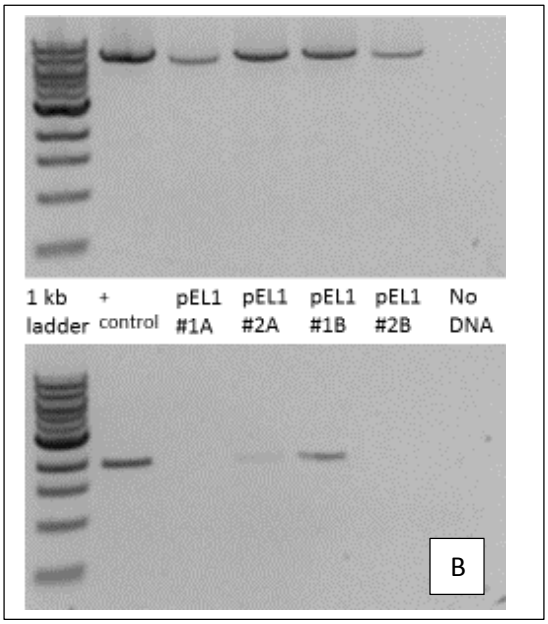


Figure 9: A shows the results of a PCR using *qnrS2* primers. It confirms that the isolated plasmids contain *qnrS2.* Figure B shows the results of a PCR using the GA primers, with the p1471 fragment above and the pKAS46 fragment below. pEL1 #2A was used in further transformation into the mutant strains of *A. veronii.*

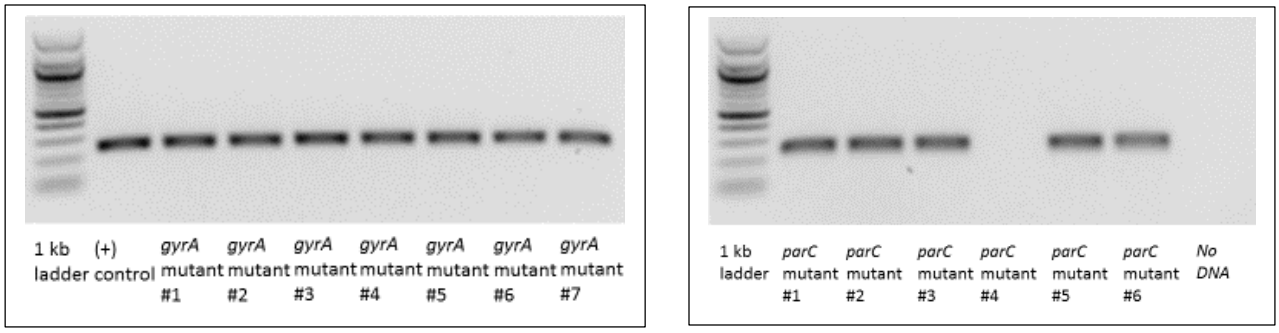


Figure 10: *qnrS2* colony PCR of *gyrA* and *parC* Hm21RS mutants after transformation of the pEL1 plasmid. Moving forward, *gyrA* mutant #1 and #2 were used, as were *parC* mutants #1 and #2 because they both had confirmed pEL1 presence using *qnrS2* PCR.

		MIC (µg/mL)	
Strain	**Plasmid**	**Trial 1**	**Trial 2**
Hm21	none	0.002	0.003
Hm21	pEL1	6	0.032
Hm21RS	none	N/A	0.003
***gyrA* mutant Hm21RS**	none	N/A	0.094
gyrA mutant Hm21RS	pEL1	N/A	0.125
***parC* mutant Hm21RS**	none	N/A	0.003
parC mutant Hm21RS	pEL1	N/A	0.032

Table 7: MIC values determined with and without the presence of pEL1 in recipient *Aeromonas* strains

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