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DNA transfer in the soil bacterium Rhodococcus

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DNA transfer in the soil bacterium *Rhodococcus*

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Abstract

Gene transfer plays an important role in bacterial evolution. Especially in an under explored species like Rhodococcus, a type of bacteria found in the soil. Rhodococcus has several applications in the pharmaceutical industry and in the production of antibiotics. Rhodococcus possess several unique sets of properties which makes it beneficial to have a reliable method of producing mutants of Rhodococcus. The goal of the experiment was to find an efficient way of forming Rhodococcus colonies with kanamycin resistant genes. The project began from an unexpected observation from an earlier experiment with Rhodococcus strain MTM3W5.2. where I attempted to transform this strain with a transposon via electro-transformation. The colonies that grew/ appeared transformants were screened to confirm the presence of kanamycin gene, however there was no amplified DNA seen on the PCR gel (i.e. absence of the kanamycin gene). The electro-transformant colonies were selected on LB plates containing different higher concentrations of kanamycin. Then the appeared transformants were again screened via disk diffusion assay and were classified into 3 different kanamycin resistant phenotypes. Majority of the "C" phenotypic colonies (i.e., high level resistance to kanamycin) appear to contain the kanamycin gene, but these colonies were less in numbers. This led us to try another method of gene transfer which is conjugation. Conjugation was carried on a double selection antibiotic plate containing both chloramphenicol (30 μg) and kanamycin (100 μg). The transconjugate colonies that appeared on the double selection plates were also screened by PCR, but none of the colonies had amplified DNA suggesting absence of the kanamycin gene. The colonies seen on the double selection plate were possibly due to spontaneous mutation or some type of unknown phenotypic variation. However, in the future, double selection plates with higher concentrations of antibiotics can possibly give us transconjugants with kanamycin genes.

Acknowledgements

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Introduction

Rhodococcus

Rhodococci are Gram positive, non-motile, aerobic soil bacteria that belong to the Actinobacteria phylum. It was first discovered by Zopf in 1891. The bacteria from this genus are commonly found in soil and aquatic environments, as well as in animal dung and in insects and plants. They contain mycolic acids in their cell envelope and are "nocardioform" bacteria (i.e. they lack defined morphology) (Bell et all., 1998). About 23,000 antibiotics have been discovered, from bacteria, out of which 10,000 of them were found from bacterial taxon, *Actinomycetes*. These organisms tend to produce bioactive metabolites such as antibiotics which are important in the current world (Borisova, 2011). *Rhodococcus* is a member of the actinobacteria and is a relative of *Streptomyces,* a species that also produces a lot of antibiotics. The genus *Rhodococcus* has a lot of species, and each species produces different metabolites which are each equally important in the current world of antibiotic resistance. The different species produce different colonies and have different pigments which can easily be seen in Figure 1.

Figure 1:

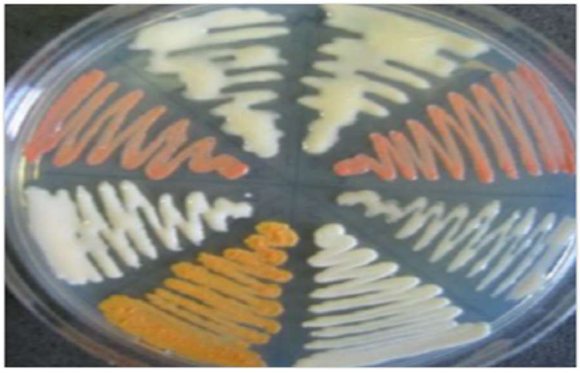


Figure 1: Different species of *Rhodococcus* containing different pigments.

Uses of *Rhodococcus*:

Rhodococcus is one of the most important bacteria present around us. It is used in various industries and is characterized by fast growth and a simple life cycle. *Rhodococcus* has also played a great role in the environmental remediation industry through the degradation of pollutants and desulphurization of the products of fossil fuel combustion. (Elsayed et all., 2017). In a 2002 article detailing the experiments done to establish *Rhodococcus'* desulfurization pathways, *Rhodococcus* strains were found to have the ability to desulfurize plates of benzothiophene and dibenzothiophene. An additional study revealed the strain's ability to degrade the alkyl derivatives from benzothiophene and dibenzothiophene (Tanaka et all., 2002). This ability to desulfurize these compounds found in fossil fuels becomes especially pertinent due to the increasing levels of pollutants like sulfur dioxide released as a result of combustion of fossil fuel-based energy sources. In a 2001 article by Smith and his co-authors, the trends of increased sulfur dioxide are analyzed. The article demonstrated the increasing sulfur dioxide emissions over time and commented on the nature of sulfur dioxide as a greenhouse gas (Smith et all., 2001). In the chemical industry, *Rhodococcus* has been used in the production process of Nicotinamide - an essential B vitamin for both humans and animals. As a part of the final process in producing nicotinamide, *Rhodococcus* is employed to convert the intermediate product of 3-cyanopyridine into the final nicotinamide ("PROCESS FOR PREPARING NICOTINAMIDE", 1998).

Rhodococcus is used for these specific purposes due to its unique properties which lend it the ability to perform the specific functions. In a study which mapped the *Rhodococcus* genome for the strain RHA1, the genetic basis for *Rhodococcus'* unique properties were revealed. Interestingly, *Rhodococcus* possess a large genome which is arranged into a linear chromosome

and 3 plasmids. Additionally, *Rhodococcus*' favorable qualities which make it pertinent in the industrial purposes are related to its life processes. In comparison to other bacterial species, *Rhodococcus* has a simpler life cycles and faster growth rates. These properties make the genus more favorable in laboratory settings as well as industrial settings (Mcleod, et al., 2006).

Rhodococcus offers great possibilities in the pharmaceutical industry through the production of secondary metabolites, the catabolism of steroids, and the use as a biocatalyst. Though *Rhodococcus* and the produced secondary metabolites are primarily associated with use in industrial scenarios, a 1999 study discovered antibiotic activity. A study on the *Rhodococcus* species Mer-N1033 discovered five tetrapeptides producing antifungal effects on *Candida albicans* and *Cryptococcus neoforms* species (Chiba et al., 1999). Furthermore, a study by Masato Iwatsuki discovered the presence of two lasso-structured anti-mycobacterial peptides from a culture broth of *Rhodococcus* jostii K01-B0171 (Iwatsuki et al., 2007). These peptides – entitled lariatins A and B – displayed significant inhibition to *M. smegmatis*. In addition to this, Lariatin A displayed inhibition of *M. tuberculosis*, while using the liquid micro-dilution method (Iwatsuki et al, 2007). In a 2006 article by Mcleod et.al. (2006) the proposed steroid catabolic properties in *Rhodococcus* were detailed. The article bases its findings off of the insights proposed by the mapping and study of the complete genome of the RHA1 *Rhodococcus* species. This study discovered the presence of four pathways for steroid catabolism as well as numerous genes attributed to steroid metabolism within the RHA1 species (Mcleod et al., 2006). Finally, *Rhodococcus* species (strain) MTM3W5.2, which was discovered here at ETSU, produces an inhibitory compound that works like a bacteriocin and kills related *Rhodococcus* bacteria including the veterinary pathogen *R. equi* (Ward et al., 2018).

Gene transfer

Horizontal gene transfer is sharing of genetic material between organisms that are genetically related, that is, they do not have the parent-offspring relationship. This type of gene transfer is widely seen in archaea and bacteria (Sun, 2018). Horizontal gene transfer plays an important role in bacterial evolution. The horizontal transfer of genes among unrelated bacteria is a major cause of the emergence of antibiotic resistance in most bacterial pathogens. This ever-rising resistance has limited the options for treatment of bacterial infections. In an ecosystem, bacteria can transfer a gene to another bacterium in one of 3 ways: transformation, transduction, or conjugation (von Wintersdorff, 2016).

1. **Transformation**

Transformation is the free up take of the DNA from the environment by the bacterial cell. Natural transformation was first discovered by Fred Griffith in 1992 by using Pneumococcal strains. (Griffith, 1928). Natural transformation systems require some special proteins and the cell which expresses these proteins is called competent. The mechanism of DNA transfer is well conserved in both Gram-positive and Gram -negative bacteria. Artificial transformation is also carried out in labs by making the bacterial cells competent and capable of taking in the free DNA via various treatments of the bacterial cells. (Chen & Dubnau, 2004).

2. **Conjugation**

Bacterial conjugation was first discovered in *Escherichia coli* species. Conjugation involves the physical contact between a donor and a recipient cell. During this process the DNA is pushed from donor to the recipient. This process is more efficient than transformation. The process of conjugation typically involves a special plasmid called the self-transmissible plasmid. (Lederberg & Tatum, 1946). A 2017 publication from the American Chemical Society discusses an experiment pertaining to the conjugation of certain plasmids from a *Rhodococcus* p52 species

to a *Pseudomonas aeruginosa* bacterium. The experiment determined that the p52 species could undergo conjugation with recipient *Pseudomonas aeruginosa,* causing the recipient to inherit the desired traits contained in the transferred plasmid. The p52 strain contains two plasmids (pDF01 and pDF02) both of which are self-transmissible plasmids containing genes like, Ori T (origin of transfer i.e. a point at which the transfer starts), relaxases, type IV secretion system and coupling protein (virD4) needed for the plasmid mobilization during conjugation. All these genes are known as *tra* genes and encodes for all the necessary function required for conjugation. The pDF02 plasmid specifically had 3 relaxase genes (tra A) but 2 of them were incomplete and were not translated because of absence of start codon or a premature stop codon but complete Tra A shared 35 % identity with plasmid present in pDF01 indicating requirement of this gene for conjugation. Both the plasmids encode genes for a type IV secretion system which forms a pore and allows a plasmid DNA to move from donor to recipient. The conjugative transfer of the plasmid was tracked for upwards of 50 generations and the plasmid resilience throughout generations was noted. The transfer was crucial in establishing genetic bioremediation properties in the recipient species. (Sun et al., 2017).

3. **Transduction**

Bacteriophages play a very important role in transduction. These bacteriophages are viruses and act as vectors to carry out gene transfer. The bacteriophages carry the genetic material through generalized transduction or specialized transduction. (Modi et al., 2013)

Transformation in *Rhodococcus* via electroporation

Electroporation is a very common technique used in many labs for transformation. In this technique the cells are passed through electric pulses (intensity in kilovolts per centimeter) for

microsecond to milliseconds which temporarily disrupts the semi permeability of the plasma membrane resulting in leakage of cellular metabolites and increased uptake of drugs, molecular probes and DNA surrounding the cell (Tsong, 1991).The Transformation in *Rhodococcus* via electroporation requires an optimal initial electric field. The optimal electric field strength of the discharge pulse denoted as E^0 is very important. E^0 for bacterial electro transformation varies from 7KV/cm to 12 kv/cm. This is very crucial because if the electric field is higher than the upper limit, the field will start damaging the cells and the DNA inside it. In a similar way if the electric field is lower than the lower limit, the cell membrane is not porous enough to uptake the genetic particle around it. (Miller et al., 1988). It is important to utilize an optimal field strength to ensure appropriate transfer of the genetic material. The medium of electroporation is also very important and impacts the efficiency of transformants. Usually Demineralized water is used as a medium but this increase discharge current which impacts the survival rate of the cell. Due to this, a PEG buffer is used to maintain the discharge current and increase the survival rate of the cells (Mahillon, et al., 1989). In electroporation, the cell suspension of *Rhodococcus* is exposed to a high voltage exponential decay discharge for a brief amount of time in the presence of plasmid DNA making the plasma membrane of the cell permeable to take up the plasmid DNA (Desomer, et al., 1990).

Transposon

A transposon can be best described as a jumping gene. It moves from one DNA molecule to another, inserting a foreign DNA molecule into the host genome. It is one of the most common mutagens used in genetics for insertional mutation. It is a useful tool in studying antibiotic resistance, pathogenesis, gene therapy, and analyzing regulatory genes. (Muñoz-López, et al., 2010). The DNA transposon moves from one DNA to another through the cut and paste

transposition mechanism. Essentially, transposon proteins scan the host DNA and bind to the inverted repeat sequence in the DNA forming a synaptic complex and bringing the two transposon ends together which are then excised. Consequently, the complex recognizes the target site and inserts the DNA molecule. (Skipper, et al., 2013).

pTNR

Transposons are important tools in genetic engineering. One specific transposon is found on the plasmid, pTNR. This transposon is notable for being active within *Rhodococcus erythropolis* and other species of Rhodococcus. pTNR is a non-replicative transposon which indicates a "cut-and-paste" mechanism of transposition by two terminal inverted repeats (Muñoz-López & Garcia-Perez, 2010).

The pTNR transposon contains a kanamycin resistance gene. pTNR's random insertions give the recipient species the ability to resist kanamycin. pTNR, being ~5473 bp, contains multiple genes. Important genes include the *istA* and *istB*—necessary for the activity of IS1415, an insertion sequence; the *kan*^{r} gene which encodes resistance to kanamycin; two inverted regions, IR1 and IR2—IR1 is upstream of *istA* and IR2 is upstream of *istB*; and an origin of replication for the plasmid DNA so that it can be propagated in *E. coli* (Sallam,et al., 2006). See Fig. 2)

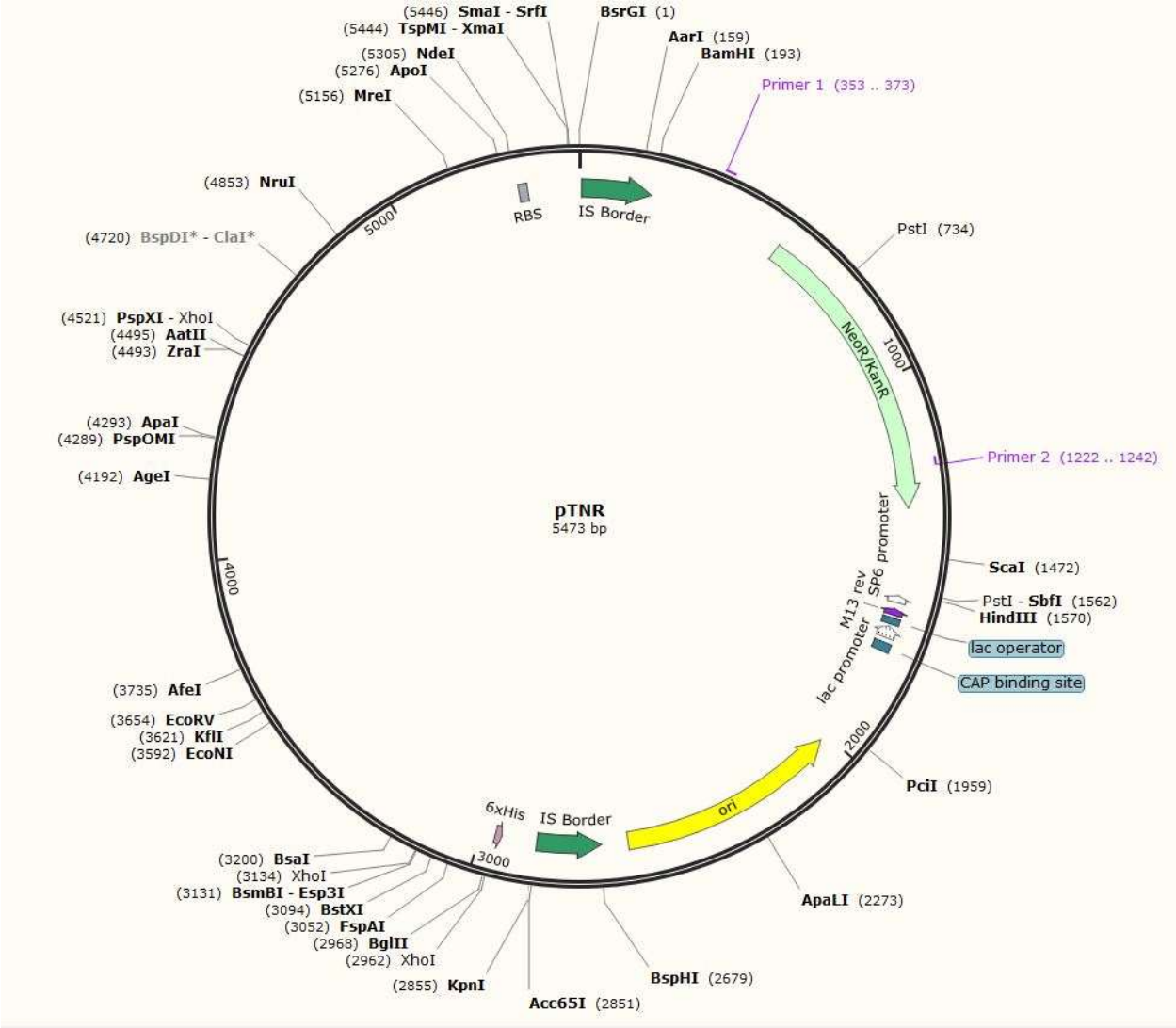


Figure 2: Map of the plasmid/transposon pTNR

Via electroporation, pTNR may enter *Rhodococcus* cells, and a single copy of the transposable element consisting of kanamycin resistance, IS borders, and the replication origin for *E. coli* can be inserted into the recipient's chromosome. Electroporation allows pTNR to enter the membrane of recipients because of the increased permeability of the cell membrane (Tsong, 1991).

pTNR is a very important tool to generate random mutant libraries of recipient strains. It may also assist in determination of gene function because of the phenotypic alterations due to disruption of the gene's "reading frame".

Antibiotic resistance

One of the biggest crises impeding the current health care systems is antibiotic resistance. Antibiotic resistance is dreadful and dangerous. Day by day, increasing numbers of pathogens are forming resistance towards known antibiotics (Ventola, 2015).

Figure 3 shows the historic progression of the formation of antibiotic resistance alongside the discovery of antibiotics.

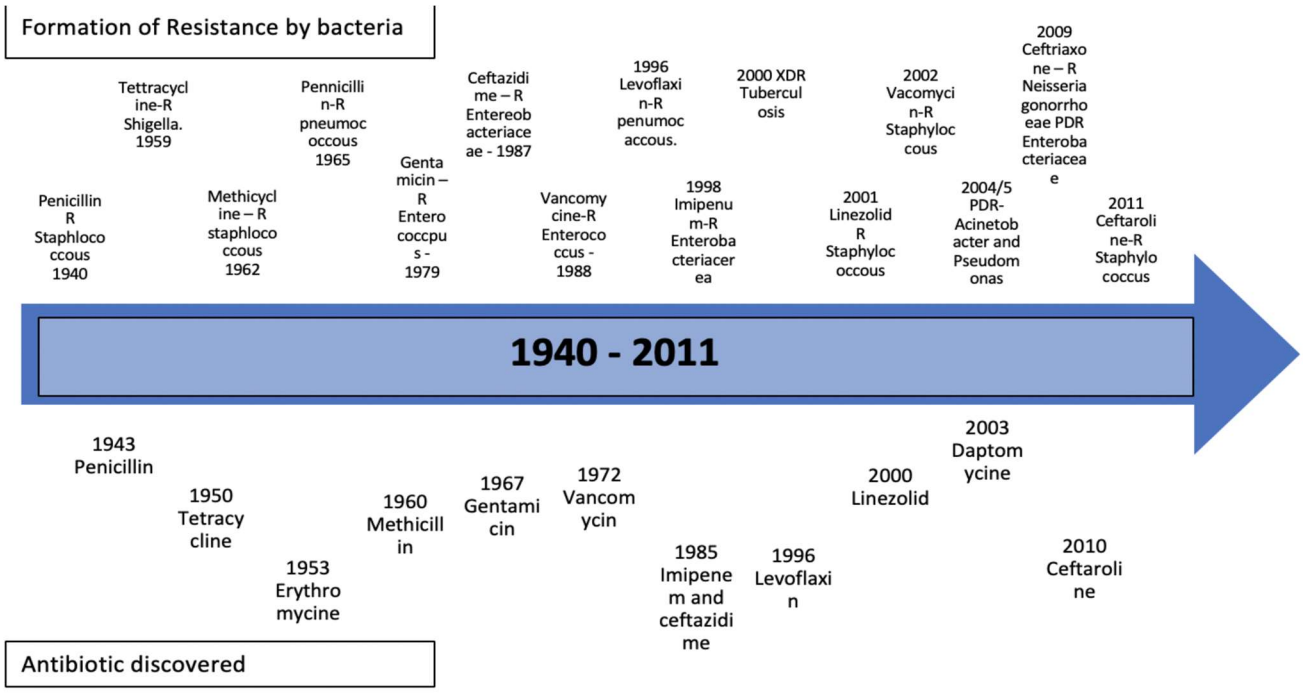


Figure 3: History of antibiotic resistance

However, in the modern era, the usage of antibiotics has become incredibly widespread, bringing the concerns of antibiotic-resistant bacteria. Increased excess prescription, overuse of

veterinary drugs, poor hygiene practices in hospitals, and poor personal hygiene have all led to rapid diagnosis of antibiotic resistance infections. Over prescription has become a major reason for antibiotic resistance. In a study in 2015, CDC (Center for Disease Control) researchers analyzed 2010-2011 National Ambulatory Medical Care Survey (NAMCS) and National Hospital Ambulatory Medical Care Survey (NHAMCS) to determine the number of outpatient visits resulting in antibiotic prescriptions by age, region, and diagnosis in the United States from 2010-2011 and found out that approximately 154 million prescriptions for antibiotics were written by doctors' offices and emergency departments each year, out of which 30 percent were unnecessary. This is a clear indication of a dangerous overuse of the "magic bullet" used to cure a multitude of bacterial diseases ("CDC: 1 in 3 antibiotic prescriptions unnecessary", 2016)

Current work

In this project I have described experiments to improve the detection and recovery of true transformed *Rhodococcus* cells with the pTNR transposon DNA. In addition, this project began from an unexpected observation from an earlier experiment with *Rhodococcus* strain MTM3W5.2. Initially I attempted to transform (i.e. move a small DNA molecule into *Rhodococcus*) this strain with a transposon to produce random insertional mutation in the chromosome of this bacterium. However, what appeared to be transformed bacteria (i.e the bacterial cell that inherited the transposon DNA) were not actual transformed cells but rather some phenotypic variants of the parental cells. I hypothesize that in-vitro conjugation of *Rhodococcus* species MTM3W5.2 maybe more effective in creating mutants through the pTNR transposon system than electro-transformation.

Methods and Procedures

Preparation of bacterial seeds culture

A bacterial seed culture was prepared by inoculating a single *Rhodococcus* MTM3W5.2 colony into 2 mL of RM broth. The inserted colony must be broken to allow optimal growth. If this step is not performed properly, optimal turbidity may not be achieved efficiently. Also, the culture must be shaken for an equal dispersion of bacteria throughout the medium. The inoculated broth was placed in a 27°C water bath with shaking until the optimal turbidity was observed (usually 24 hours). These bacterial seed cultures were used to inoculate plates or larger broth cultures.

Bacterial strains and growth conditions

Rhodococcus sp. MTM3W5.2 is the wild-type strain isolated and purified here at ETSU that produces an antibiotic inhibitory compound (Ward et al., 2018). *Rhodococcus* sp. MTM3W5.2 mutants were grown in RM broth at 19°C to allow production of the inhibitory compound which is only produced at temperature below 21°C, so special care was necessary to maintain the temperature between this range. The culture was grown for 2 weeks. The inhibitory compound was produced from only bacteria that formed at the top of the RM broth as a biofilm. Prior to the extraction of the compound, the broth culture was checked for contaminants by inoculating the broth culture on a RM plate.

Preparation of the electrocompetent cells

A seed culture was prepared by inoculating a colony of the MTM3W5.2 into 2 mL of RM broth and incubated with shaking at 28°C for 24 hours in a water bath shaker. A 250-mL flask with 50 mL of RM broth was inoculated with 1 mL of the overnight seed culture. An initial

optical density was measured. The culture was incubated at 28°C by shaking in a water bath shaker until a Klett reading of 85-90 was observed. A sterile loop of the culture was streaked onto a RM agar plate to test for possible contamination. The agar plate was incubated at 28°C for four days. If there was no contamination observed, the electro-competent cells could be used. The remaining culture was transferred to a 250 mL centrifuge bottle and placed on ice for 5 minutes. Then, the culture was centrifuged in a large rotor (FiberliteTM F14-6 x 250LE Fixed-Angle Rotor, Thermo Scientific) at 4℃ and 6,000 RPM for 7 minutes. The supernatant was discarded, and the cells were resuspended in 30 mL of ice-cold 10% glycerol. The cells were kept on ice while the re-suspension was being carried out and iced for 10 minutes. The centrifugation was repeated under the same conditions, and the supernatant was discarded. Kept on ice, the cells were resuspended in a 15-mL bottle with 10% glycerol. Then, the cells were transferred to Oakridge tubes and iced for 10 minutes to repeat the centrifugation under the same conditions. The supernatant was transferred in a bottle to be discarded. Finally, the resuspended cells were transferred into a small 0.5-mL microfuge tubes, and the electro-competent cells were stored in the -80°C freezer.

Transformation of electrocompetent cells by electroporation

Electro-competent *Rhodococcus* cells stored at -80°C were thawed on ice for 10 minutes. The cells must thaw before using them. The electroporation cuvettes were sterilized by UV sterilization for 4 hours. These sterilized cuvettes were place on ice to chill. 1-2 µL of DNA was added to the tube of 100 µl of thawed competent cells and mixed gently. The competent cells and DNA mixture was transferred to a 1-mL sterilized cuvette. The pipette tip was placed on the side of the cuvette to let the mixture run down the side to avoid the formation of bubbles. The outside of the cuvette was dried before progressing further. After drying, these were placed in an

electroporator set at 2500 V. The “pulse button” was pressed twice, and a beep sounded once the pulse finished. The pulse-time was recorded. A pulse-time between 3-5 seconds is desirable; however, a pulse-time closer to 5 seconds is preferred. If the pulse-time elapsed for less than 3 seconds or a pop was heard, it should be repeated. The cuvette was removed from the electroporator and 500 µl of Super Optimal broth with Catabolite repression (SOC broth) was added to the cells. The cells and SOC broth were transferred to a test tube. The test tube with transformed *Rhodococcus* cells was incubated for 4-5 hours at 28°C. Prior to spreading cells out on agar plates, the transformed cells were diluted 10-fold (10^{-1}), 100-fold (10^{-2}), 1,000-fold (10^{-3}), and 10,000-fold (10^{-4}).

Preparation of mutant wheel plates

After transformation, each colony was transferred into an LB plate—making a wheel structure. Each mutant colony was labeled with specific mutant number and stored at 4°C. Each wheel had eight mutants streaked onto the RM plate. Each mutant was identified by the specific number. These plates were made to keep a stock of the MTM3W5.2 mutants.

Disk diffusion assay

Disk diffusion assay was used to screen the mutants of the parent strain for their level of resistance to kanamycin. This method detected the sensitivity of the mutants to this antibiotic used to select transformants. For this method, paper disks were made from a heavy gauge Whatman blotting Paper GB004. The blotting paper was punched with a hole puncher, and the disks were collected in a petri dish. The petri dish was autoclaved and stored.

The sterile paper disks were then soaked with different concentrations of kanamycin. The paper disk was dried completely and stored. Then, the mutant seed culture was prepared by

inoculating a single colony into 2 mL of LB broth. The inoculated broth was placed in a 27°C-water bath shaker overnight or until the optimal growth density was observed. The mutants were inoculated on LB plates and were screened with a disk containing the inhibitory substance. The sensitivity or resistance of the mutants against the inhibitory compound was noted.

Preparation of Plasmid DNA

An overnight seed culture of *E. coli* pEX18Km 3964 AD was made by inoculating 2-2.5 mL of LB broth containing kanamycin. Then, the culture broth was poured into a 1-mL microfuge tube. The tube was centrifuged on top speed for at least 50 seconds. All broth was aspirated off, and the cell pellet was saved. At 37°C, 100 mL of solution A containing fresh lysozyme (2 mg/mL) was added to the cell pellet. The tube was vortexed to mix the solution (the tube was kept on ice for 10 minutes after being vortexed). 200 mL of fresh solution B (H20 2.1 mL+ 1N NaOH 600 mL, 10% SDS 300 mL) was added to the epi tube and was vortexed at low speed with an open lid. The tube sat at room temperature for 5 minutes. 150 mL of solution C was added to the tube and was mixed by inverting the tube several times to produce a white flocculant precipitate. The tube was let to sit on ice for 15-20 minutes.

Again, centrifuge the tubes for at least 10 minutes at topmost speed. Then, pour off supernatant and remove all liquid to a new 1.5-mL microfuge tube. The white flocculant was removed from the new tube (it was important to not allow any white flocculent in the liquid). 1 mL of cold EtOH was added and mixed by inverting the tube. It sat on ice for 5 minutes. For 10 minutes, the tubes spun and all EtOH was aspirated from the tube. 400 mL of solution D (for mini preps) was added and DNA pellets were dissolved by gently vertexing it. Again, 1 mL of cold EtOH was added to the tube and mixed by inverting the tube. It sat on ice for 5- 10 minutes.

The tube spun for another 10 minutes, all the EtOH was aspirated off, and was allowed to air dry. All the DNA was re-dissolved in 30 μL of 0.1 TE buffer plus RNase to make sure all the DNA was taken from the wall of the tube.

Gel electrophoresis

A 0.75% agarose gel was first made within a 11 x 16 cm casting tray. A gel comb was set to create 8 wells. The agarose was allowed to solidify for about 10 minutes. The solidified gel was stored in a refrigerator until needed. Then 6 μL of the marker, λ/HindIII, was pipetted into a microwell plate. 4 μL of water was added with the marker into the microwell. 1 μL of loading dye (containing glycerol) was mixed with the prior components, and these contents were mixed inside the microwell. Plasmids isolated from transformed *E. coli* cells, J1 – J6, were also added into separate microwells. 2 μL of J1 plasmid, 7 μL of water, and 1 μL of loading dye (glycerol) was added to a microwell. This was repeated for J2, J3, J4, J5, and J6 in separate wells. These solutions were mixed with a micropipette separately in their designated wells. Once all the samples were mixed, the agarose gel was taken out of the refrigerator and put inside the gel tank. The samples were loaded into the wells.

1X TBE buffer was placed inside the gel tank with the agarose gel. The anode was placed on the opposite end of the gel to where the DNA was travelling—to travel toward the positive charge due to the negatively charged phosphates in the backbone of DNA. The cathode was placed on the end of the gel where the DNA started. The gel electrophoresis was set to 120 V for 90 minutes. The voltage was verified to stay constant throughout the electrophoresis of the DNA. The purple portion of the loading dye was made sure to not migrate past three-fourths the length of the agarose gel. As soon as the gel was taken off voltage, the gel was drained from the 1X

TBE buffer. Gloves were worn throughout these next few steps due to the carcinogen, ethidium bromide (EtBr) being used. A solution consisting of 100 μL of EtBr and 100 mL of distilled water (dH2O) was mixed inside a plastic tub. The agarose gel was carefully placed in the EtBr solution, and the gel was stained for 45 minutes. The EtBr-dH2O solution was drained into a designated tub for EtBr. The gel was rinsed with tap water and de-stained in a new tub filled with 200 mL of water. The plastic tub filled with water was transported to the photographing machine. The gel was put onto a clear tray, situated beside a ruler (1-cm mark is next to the wells of the gel), and placed inside the machine. The machine was set to photograph for EtBr staining. Once completed, the photographs were saved onto a flash drive and exported onto a computer.

Conjugation of *Rhodococcus* with *E. coli* cells

Escherichia coli derives its relevance as a host due to its unique properties relating to its established genetics and good transformation competence. Commonly, the S17 strain, or its analogue SM10, is employed in experiments relating to the conjugative transfer of genetic material. This specific strain contains the RP4 plasmid inserted in the chromosome, which provides essential components necessary for the conjugative transfer like the tra genes (Strand et al., 2014). To mobilize a plasmid in this donor strain to a recipient bacterial cell, only an origin of transfer (*ori* T) site on the plasmid is required.

Recipient *Rhodococcus* CM1 is a chloramphenicol resistant variant of the wild strain MTM3W5.2. It was grown in LB broth containing 30 ug/ml of chloramphenicol. The donor *E coli* S17 – 1 contains the plasmid pEX18km3964AD and was grown in LB broth containing kanamycin (50ug/ml). Using swabs for transfer, a seed broth was used to inoculate corresponding separate plates with the same antibiotics. After growth on a plates (for the E. coli

donor this was at 37°C overnight and for the *Rhocococcus* recipient this was at room temperature for 2-3 days), 1-2 mL of LB broth without antibiotics was used to wash off the bacteria from the plates. A glass spreader was used to wash each plate separately. The bacterial cells were collected in a separate glass tube. 700 µL of collected cells of the donor plus 700 µl of the recipient were added to a microfuge tube. This tube was centrifuged for 30 seconds at room temperature. Following this, the supernatant was removed using a glass pipet. The mixed donor and recipient cell pellet were resuspended in ~ 1 mL of LB with no antibiotics. On an LB plate, 200-250 mL of the mixed donor and recipient cells from the LB broth was spread on LB plates (no antibiotic) and allowed to grow overnight at ~30°C (or room temperature) overnight. On the following day, 2 mL of LB broth with no antibiotics were used to wash the mixed cells off these plates. This wash and cell mixture should be collected in a test tube. The collected mixed cells were then spread (100 uL) on LB double selection plates (30 µg /ml chloramphenicol plus 100 ug/ml kanamycin) and incubated at 22°C or room temperature for 3-7 days.

Lane	Plasmid	DNA (µL)	H_2O (µL)	Dye (µL)
1	HindIIII Marker	5	5	1.2

Single Colony PCR

#	Reagent	Volume (µL)
1	PCR – H_2O (Fridge)	30
2	5x PCR buffer (GoTaq® Flexi)	10
3	10x DMSO (37 °C)	1.5
4	25 mM $MgCl_2$ (Promega™)	3
5	10 mM dNTP mix	1
6	20 mM forward primer (left)	1.25
7	20 mM reverse primer (right)	1.25
8	DNA template: single bacterial colony	1
9	Taq polymerase (GoTaq® Flexi)	0.75
-	TOTAL	50

Suspend one colony no older than one week old in 10 µL (100ul for a large colony) PCR-H_2O. Use the program, Tom1, for about two hours and forty minutes. The parameters of the program Tom1 are as follows:

1) 95°C 5 minutes

2) 95°C 1 minute

3) 55°C 1 minute

4) 72°C 2 minutes

5) Steps 2-4 repeated (x29)

6) 72°C 5 minutes

7) 4°C Hold

The primers used are shown in Fig. 2 (primer 1 and primer 2) and will amplify the kanamycin resistance gene from both pTNR and pEX18Km3964AD.

Plasmids for PCR:

1. pTNR
2. pEX18kmAD396

Results and Discussion:

Electro-transformation with pTNR plasmid

Initially *Rhodococcus* (wild) strain MTM3W5.2 was transformed with the plasmid pTNR (which contains a transposon) to generate random insertional mutations. However, transformation of *Rhodococcus* proved to be unexpectedly difficult due to unpredictable results. The time constant after electroporation was always within the acceptable range which ensured that adequate number of cells were left to continue the procedure. Measures were taken such that

only fresh competent cells were used. Despite this, the number of colonies always varied. "Transformants" were selected by spreading electroporated cells on different concentrations of kanamycin (50-200 µg/mL) but the yield of colonies was not predictable.

Nine random "transformant" colonies selected on LB plates containing kanamycin (100ug/ml) were screened for the presence of the kanamycin resistance gene by a colony PCR method. No amplified DNA was detected in any of these nine colonies (see Fig. 4). This suggested that not all colonies present were due to transformation of the pTNR plasmid (i.e., that the cells did not take up the plasmid DNA), but rather appeared through a spontaneous mutation or some kind of unknown phenotypic variation.

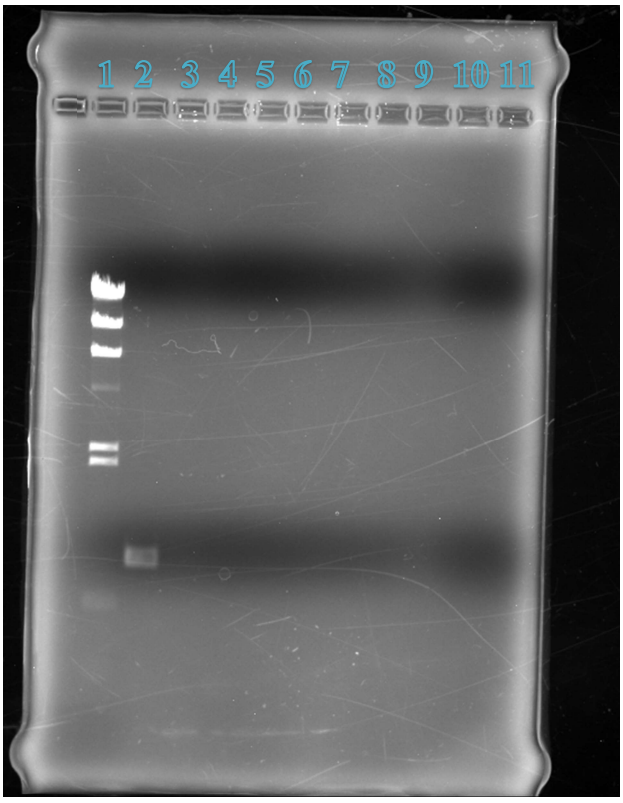


Figure 4. Colony PCR to amplify the kanamycin gene. Lane 1, the molecular size marker (lambda DNA cut with HindIII). Lane 2, the PCR positive control colony. Lane 3, The electro-transformant colony 4794. Lane 4 through lane 11 contain the PCR reactions for colonies 4795, 4796, 4798, 4853, 4849, 4955, 4997, and 4959 respectively. Only lane 2 contains an amplified DNA band indicating the presence of the kanamycin gene.

Disk diffusion assays

In other species of bacteria, it is well known that inheritance of the kanamycin gene will impart resistance to high levels of the antibiotic (i.e., high concentrations of kanamycin). Since screening colonies by PCR is expensive and time consuming, it may be possible to screen colonies by a disk diffusion assay to detect "true" transformant colonies (i.e., have picked up the kanamycin gene). I propose that true transformant colonies will be resistant to high levels of

kanamycin, while those colonies that do not have the kanamycin gene will not be resistant to high concentrations of the antibiotic. To assess the validity of the possible pTNR transformant colonies, paper disks containing 800 and 400 µg of kanamycin were place onto plates inoculated with individual colonies to test and see the zones of inhibition of the colonies. The purpose of testing these zones were to see if the numbered colonies have taken up the kanamycin resistance gene. For organisms that have taken up this gene, there won't be any zone of inhibition due to the higher resistance to this antibiotic—the opposite is said for organisms that have not taken up the kanamycin resistance gene.

Electro-transformant colonies were also selected (spread onto) on LB plates containing ever higher concentrations of kanamycin: 250, 300, 350 and 400 µg/ml of the antibiotic. Roughly 25 colonies from each selection plate were then screened by the disk diffusion assay described above. Results from this disk diffusion assay indicated that all colonies screened by this assay fall into one of three different phenotypes labeled phenotype "A", "B" and "C" (see Table 2). Only the "C" phenotype appears to be resistant to a high level of kanamycin.

TABLE 2. Colony phenotypes for the disk diffusion assay.

COLONY PHENOTYPE	ZONE[A] FOR 400UG DISK	ZONE FOR 800 UG DISK
"A"	+	+
"B"	-	+
"C"	-	-

[A] zone refers to the zone of no bacterial growth around the paper disk containing the antibiotic indicating a sensitive phenotype

Colonies from each of the four selection plates that were screened by the disk diffusion assay and are listed in tables (in the appendix.). Table 3 below lists a summation of all the colonies that fall into each phenotype and from each selection plate.

Table 3. Number of colonies of each of three resistance phenotypes.

SELECTION PLATE[A]	PHENOTYPE "A"	PHENOTYPE "B"	PHENOTYPE "C"
250 µg kanamycin	15	2	6
300 µg	11	9	5
350 µg	4	3	0
400 µg	1	1	3

[A] Values are in micrograms of antibiotic per ml of LB agar. Note that the plates with the higher concentrations of antibiotic had very few transformant colonies.

Colony PCR to detect the kanamycin gene.

Several colonies from each of the three resistance phenotypes were screened by colony PCR for the presence of the kanamycin gene. Table 4 below lists the results.

Table 4. PCR amplification of the kanamycin gene for each resistance phenotype.

Colony number	Phenotype	PCR Amplicon
4078	"A"	-
4082	"B"	-
4050	"B"	-
4057	"B"	-

4062	"B"	-
4063	"B"	-
4048	"C"	-
4055	"C"	+
4056	"C"	+
4058	"C"	+
4061	"C"	+
4072	"C"	+

It is noted that most of the colonies with the "C" phenotype (i.e., high level resistance to kanamycin) appear to contain the kanamycin gene (Figure 5) and are thus the only true transformed (transformant) colonies. Those colonies with the "A" and "B" phenotype do not appear to contain the kanamycin gene based on the PCR experiment and are thus not true transformants.

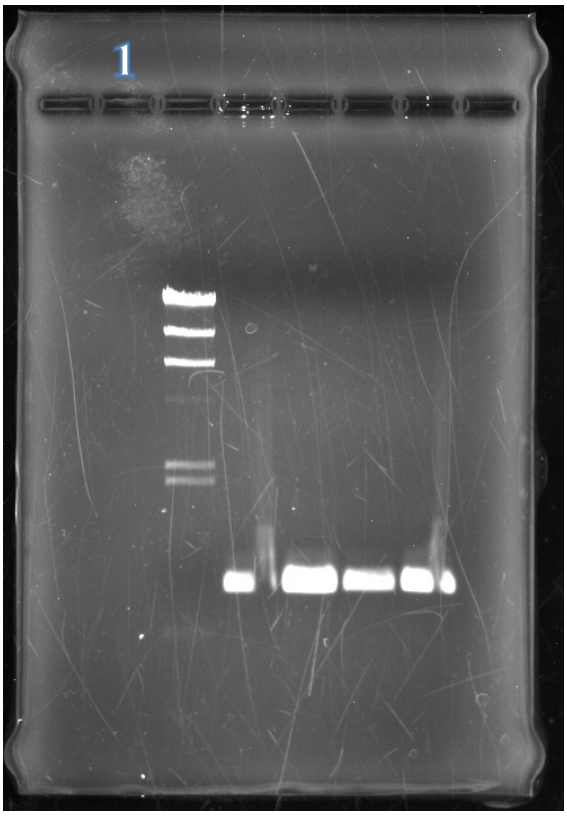


Figure 5. Colony PCR to amplify the kanamycin gene. Lane 1, the size marker (λ DNA cut with HindIII). Lane 2, colony 4055 ("C" phenotype). Lane 3, colony 4056 ("C" phenotype). Lane 4, colony 4058 ("C" phenotype). Lane 5, colony 4061 ("C" phenotype).

Conjugation between *Escherichia coli* and *Rhodococcus*

In addition to transformation, a gene can be transferred between bacterial cells by other means. One of these is by conjugation. Other scientists have reported that *Rhodococcus* can carry out conjugation with other

Rhodococcus bacteria and with *E. coli* (Strand et al., 2014). To attempt a conjugation experiment between a *Rhodococcus* recipient cell and an *E. coli* donor cell, the donor cell must have a plasmid with an origin of transfer site (*ori* T) in the plasmid DNA. The pTNR plasmid/transposon does not have an ori T site, so a different plasmid was used for these experiments. That plasmid is pEX18Km3964AD (see Figure 6).

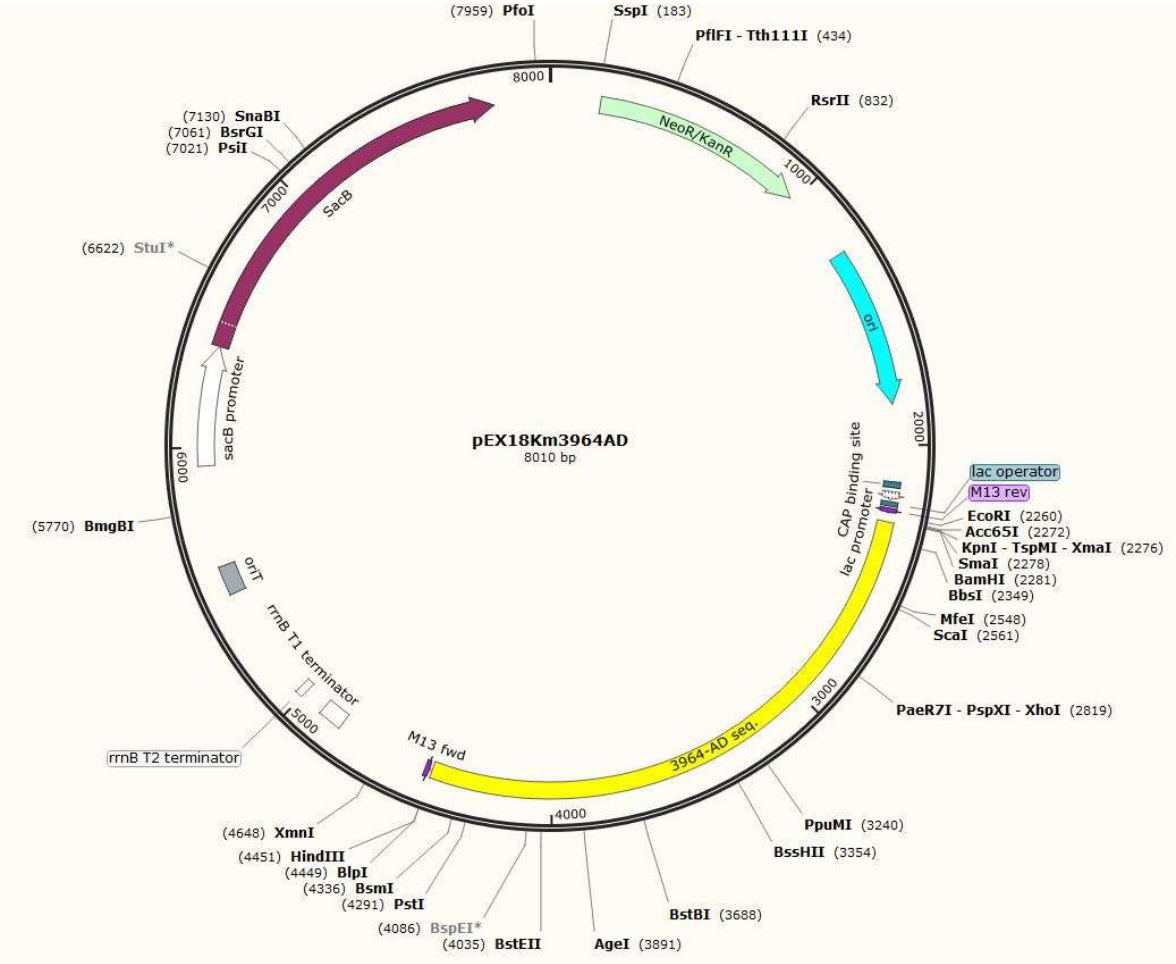


Figure 6. Map of plasmid pEX18Km3964AD

This plasmid was moved (by transformation) into the *E. coli* strain S17-1 to create the donor strain to be used in conjugations with *Rhodococcus.* A plasmid prep was done to ensure that this *E. coli* donor strain has the plasmid pEX18Km3964AD (see Figure 7).

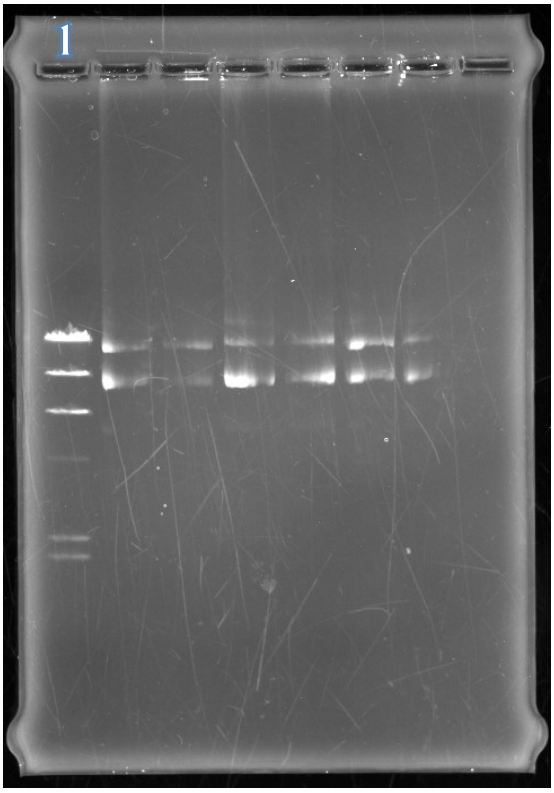


Figure 7. Plasmid preparations from colonies of the donor strain S7-1 containing the plasmid pEX18Km3964AD. Lane 1, the size marker Lambda DNA cut with HindIII. Lane 2, plasmid DNA from colony J1.

A conjugation experiment was carried out between the above *E. coli* donor strain (J1) and the *Rhodococcus* recipient strain Cm1 (as described in the methods section). "Transconjugate" colonies were selected on double antibiotic LB plates containing both chloramphenicol (30 ug/ml) and kanamycin (100 ug/ml) such that only colonies of *Rhodococcus* that have obtained the pEX18Km3964AD plasmid from the donor *E. coli* will grow. Plates were incubated at about 30^{o}C for one week or longer. No colonies appeared on any of the test plates until day seven of incubation (see table 5 and figure 8).

Figure 8: Colonies seen on the seventh day

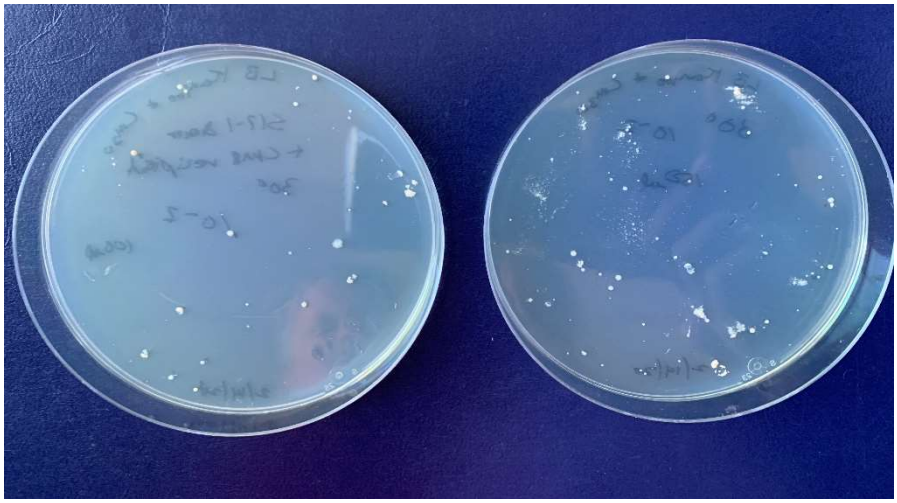


Table 5. Number of transconjugate *Rhodococcus* colonies on double selection plates.

Cell mix[a]	Day colonies appeared	Dilution	No. of colonies
A	7	1 / 10	~80
	7	1 /100	~18
	13	1 / 100	~27
B	7	1 / 100	~140

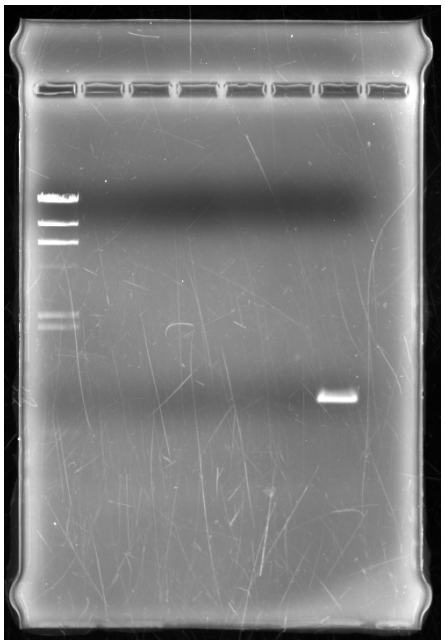
[a] Cell mix A is as described in the methods section. For cell mix B, the cell pellet in the microfuge tube was allowed to incubate at room temperature overnight, then dilutions were spread on double selection plates.

Colonies on the double selection plates (Table 5) were tested to see if they were true "trans-conjugates". That is, a true transconjugate should have the kanamycin gene in their genome. To test for this, colony PCR was done on 10 random colonies from the plates listed in table 5. None of these 10 colonies showed any amplified DNA, thus they do not contain the kanamycin gene and are not true trans-conjugates (see table 6 and Figure 9)

Table 6. PCR amplification of the kanamycin gene in transconjugate colonies.

Colony number	PCR Amplified DNA
308	No
309	No
311	No
312	No
313	No
318	No
319	No
321	No
322	No
323	No
RMP2.31 (+ control)	Yes

Figure 9



Conclusion:

Bacterial conjugation is believed to be an efficient way of gene transfer. Transferring a gene between *Rhodococcus* and *E. coli* via conjugation did not produce colonies with kanamycin gene but produced a colony with a kanamycin resistance phenotype which suddenly appeared on the seventh day after inoculation. This was confirmed by screening the appeared colonies on a PCR gel, as no amplicons were seen on the gel. The colonies which appeared on the seventh day were possibly due to random mutation in the chromosome. Thus, the experiment did not produce true trans-conjugants but in the future double selection plates with higher concentrations may possibly provide better results for producing trans-conjugate colonies with kanamycin gene.

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Appendix

Preparation of LB plates

Ingredients	Amount
Distilled water (d H_2O)	500 mL
Sodium Chloride	5 g
Tryptone	5 g
Yeast Extract	2.5 g

Agar	7.5 g

This medium was prepared by combining the following components:

The ingredients were mixed properly together with help of a magnetic stirrer and autoclaved at 121°C. The medium was poured into sterile Petri dishes (100 mm x 15 mm polystyrene, Fisher brand) and left on the benchtop to cool and solidify overnight.

Preparation of Rich Medium plates/ Broth

Rich Medium (RM) plates were made by properly mixing the ingredients from Table 2 with the help of a magnetic stirrer. The mixture was boiled to mix the ingredients, and then, it was autoclaved. After autoclaving it, the medium was cooled to 55°C in a water bath and poured into sterile Petri dishes that were left on the benchtop to cool and later stored in the refrigerator. To make broth, agar was left out.

Ingredients	Amount
Distilled water (d H_2O)	500mL
Dextrose	5 g
Nutrient Broth	4 g
Yeast Extract	0.25 g
Bacto Agar	7.5 g

Table 2: Recipe for MH Plates

Preparation of LB-Kanamycin plates

These plates are made by following the below protocol for LB plates except kanamycin is added to the solution before pouring the plates. Similarly, LB-chloramphenicol plates are also made but instead of kanamycin, chloramphenicol is added.

Ingredients	Amount
Distilled water (d H_2O)	500 mL
Sodium Chloride	5 g
Tryptone	5 g
Yeast Extract	2.5 g

Agar	7.5 g
Kanamycin	2 mL

Table 3: Recipe for LB-Kanamycin Plates

A stock solution of the kanamycin is already made containing 50 µg/µL. 2 mL of stock is needed per 500 mL of medium.

Tables for result section

25 µL of undiluted *Rhodococcus* cells on kanamycin 250 mg/mL plates

Strain Number	Inhibition zone for 800 mg/mL disk in (mm)	Inhibition zone for 400 mg/mL disk in (mm)
4083	2.4	1.0
4082	2.2	0.9
4081	2.5	1.9
4080	2.4	1.9
4079	0.0	0.0
4078	2.8	2.2
4077	3.1	1.7
4076	0.0	0.0
4067	2.3	0.9
4066	2.3	1.0
4065	0.0	0.0
4064	0.0	0.0
4063	1.9	1.1
4062	2.0	1.0
4061	0.0	0.0
4060	2.5	1.3
4051	1.6	1.0
4050	2.0	1.0
4049	Bad Colony	Bad Colony
4048	0.0	0.0
4047	1.7	0.0
4046	2.4	1.1
4045	1.5	0.0
4044	1.6	1.2

Strains that have likely acquired the kanamycin resistance gene are the following: 4079, 4076, 4065, 4064, 4061, and 4048. Out of these six strains, they showed full resistance of the kanamycin disks regardless of the concentration. Even if a higher concentration of 800 mg/mL

disks are used on these strains, they will resist the kanamycin and grow regardless of this high concentration. Eighteen of the strains either produced a bad colony or a major zone of inhibition. These colonies did survive slightly better with the lower concentration of kanamycin; however, the kanamycin still killed them regardless of the 800 or 400 mg/mL disks. Colonies that could not survive from the 800 mg/mL disk but could the 400 mg/mL disk include 4047 and 4045. These two colonies do not resist the kanamycin at higher concentrations, but they do exhibit resistance of kanamycin at lower concentrations.

75 µL of 1/10 diluted *Rhodococcus* cells on kanamycin 250 mg/mL plates

Strain Number	Inhibition zone for 800 mg/mL disk in (mm)	Inhibition zone for 400 mg/mL disk in (mm)
4035	0.0	0.0
4034	Dots	0.0
4033	2.0	1.0
4032	0.0	0.0
4031	Dots	1.0
4030	1.1	0.0
4029	1.3	0.0
4028	0.0	0.0

Strains 4035, 4032, and 4028 exhibited full resistance of kanamycin regardless of concentration. Strains that did not show full resistance include 4030 and 4029.

25 µL of undiluted *Rhodococcus* cells on kanamycin 300 mg/mL plates

Strain Number	Inhibition zone for 800 mg/mL disk in (mm)	Inhibition zone for 400 mg/mL disk in (mm)
4075	0.0	0.0
4074	1.8	0.0
4073	2.0	0.0
4072	0.0	0.0
4071	1.9	0.8
4070	1.6	0.9
4069	1.7	0.8
4068	1.8	0.0
4091	1.6	1.2
4090	2.3	1.4
4089	1.6	1.2

4088	2.0	0.0
4087	Dots	0.0
4086	1.9	1.4
4085	1.9	0.0
4084	0.0	0.0
4099	1.7	0.7
4098	Dots	0.0
4097	1.1	0.0
4095	1.8	1.2
4094	1.6	1.2
4093	2.0	1.5
4096	1.1	0.0
4092	1.2	0.0

Strains that show full resistance of kanamycin in the 25 µL undiluted *Rhodococcus* cells on kanamycin 300 mg/mL plates include 4084, 4075, and 4072. A large number of strains showed partial resistance of kanamycin; these include 4097, 4096, 4092, 4088, 4085, 4074, 4073, and 4068.

75 µL of 1/10 diluted *Rhodococcus* cells on kanamycin 300 mg/mL plates

Strain Number	Inhibition zone for 800 mg/mL disk in (mm)	Inhibition zone for 400 mg/mL disk in (mm)
4059	0.0	0.0
4052	0.0	0.0

Both strains in this category likely took the kanamycin resistance gene. Unfortunately, the sample size in this category is very low because very few colonies were present after the dilution of these *Rhodococcus* cells.

25 µL of undiluted *Rhodococcus* cells on kanamycin 350 mg/mL plates

Strain Number	Inhibition zone for 800 mg/mL disk in (mm)	Inhibition zone for 400 mg/mL disk in (mm)
4043	1.1	1.0
4042	1.7	0.0
4041	1.5	0.9
4040	1.6	0.0
4039	1.3	0.0
4038	Bad colony	Bad colony
4037	1.3	1.1

4036	1.4	0.9

None of the colonies within this category represented a full resistance of kanamycin; though, partial resistance was shown in 4042, 4040, and 4039. Because the concentration is much higher in this group, these colonies are likely to show resistance of kanamycin at lower concentrations of kanamycin plates.

25 µL of undiluted *Rhodococcus* cells on kanamycin 400 mg/mL plates

Strain Number	Inhibition zone for 800 mg/mL disk in (mm)	Inhibition zone for 400 mg/mL disk in (mm)
4058	0.0	0.0
4057	1.6	0.9
4056	0.0	0.0
4055	0.0	0.0
4054	1.2	0.0
4053	Bad colony	Bad colony

These colonies were highly resistant to kanamycin because very few showed large zones of inhibition. Full resistance was observed by strains 4058, 4056, and 4055. 4054 showed resistance on the 400 mg/mL disks, and 4057 showed a sensitivity of kanamycin with a low relatively small zone of inhibition.