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Evaluation of Horizontal Gene Transfer Between Genetically Engineered Cyanobacteria and Gram-Negative Bacteria

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EVALUATION OF HORIZONTAL GENE TRANSFER BETWEEN GENETICALLY ENGINEERED CYANOBACTERIA AND GRAM-NEGATIVE BACTERIA

by

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ABSTRACT

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Old Dominion University, 2021
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As the world population is increasing and societies become more technology driven, there is an imperative to develop 'green energy' sources to protect our planet. Cyanobacteria that have been genetically engineered to produce organic compounds that may be burnt as fuels show great potential, as they are an environmentally friendly and self-renewable, net carbon-neutral option. However, there are potential risks in the development and use of genetically modified organisms (GMOs). We need to understand in advance the risks that GMOs may pose to our environment and to animal and human health. This will enable experimental procedures, containment strategies and policies to be developed to prevent accidents and eliminate potential harmful effects of GMOs in the environment such as sharing antibiotic genes to native microorganisms. My research seeks to assess the bio-risk posed by engineered cyanobacteria and their capability of transferring genes of antibiotic resistance to other bacteria, they may encounter in their environment outside of laboratory conditions. Here genetically engineered (GE) cyanobacteria that contain antibiotic resistance genes commonly used as selective markers, are being studied and their ability to horizontally transfer to wild-type bacterial strains. In aim one, the plasmid vector that carries the transgenes to confer antibiotic resistance and to theoretically produce biofuel was introduced into the cyanobacteria, *Thermosynechococcus elongatus* BP1 and select other gram-negative bacterial strains, *Escherichia coli* K12, *Escherichia coli* DH5α,

and *Pseudomonas putida* KT2440, to assess their ability to carry the plasmid and obtain antibiotic resistance. In the second aim, the ability for the plasmid to undergo horizontal gene transfer (HGT), from GE cyanobacteria to two different strains of *E. coli* was determined. In the third aim, the ability of *P. putida* KT2440 to uptake the plasmid via HGT from GE cyanobacteria was studied. This research also examines the fundamental mechanisms of horizontal gene transfer, which is foundational to microbial life and not completely understood. The results reveal that HGT occurs between our model cyanobacteria and *E. coli* strains but not *Pseudomonas*. This research provides foundational knowledge to help develop policies and safeguards for the safe design and use of GMOs.

This thesis is dedicated to my parents Maria Konstantinou and George Zouros, and my brother Stratos Zouros.

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NOMENCLATURE

GE	Genetically Engineered
GMOs	Genetically Modified Organisms
HGT	Horizontal Gene Transfer
MDR	Multiple Drug Resistant
2-DOS	2-deoxystreptamnine
PBP	Penicillin binding proteins
PMI	Phosphomannose Isomerase
WT	Wildtype
CFUs	Colony Forming Units

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CHAPTER I

INTRODUCTION

Genetic Engineering with Synthetic Biology

The recent advances in genetic engineering have made it a fascinating field to be working in today. Especially with the discovery and development of modern gene editing tools such as the gene editing CRISPR/ Cas9 system, for which Emmanuelle Charpentier and Jennifer Doudna were recently awarded the Nobel Prize,[1] the possibilities for providing solutions to modern world problems through genetic engineering are seemingly endless. Medicine, industry, and agriculture all stand to benefit from employing genetic editing techniques for solutions to problems. At the same time, as promising as gene editing may be, the implications of permanently altering an organism's genome, both for the organism as well as its environment, remain unknown. Therefore, it is imperative that more research is done towards investigating this parameter.

An area where genetic engineering techniques show great promise is in the modification of cyanobacteria to produce biofuel. This is due to their unique capability of utilizing inorganic carbon --in the form of CO_2-- from their environment to produce organic products, with fast doubling times, while requiring little maintenance. This feature, combined with environmental concerns over the excess amounts of on CO_2 currently found in the atmosphere, make genetically engineered (GE) cyanobacteria that have the ability to produce organic molecules to be used as fuel, a promising avenue to be explored.

GE Cyanobacteria for Biofuels

Currently, 85% of the world energy demand relies on the consumption of fossil fuels[2] It is to this day a profitable and expanding field that, according to the U.S. Energy Information Administration increased production by 12% from 2017 to 2018.[3] However, it is a finite resource and the scenario of having to compete for diminishing oil reserves is a cause for great concern. For this reason, alternative fuel sources from natural, renewable reserves have been explored as options for being ultimately integrated in our daily lives. Examples of such renewable resources include solar, wind, hydroelectric and biomass energy. Of these, biomass is one that has the ability to make use of local energy supply, without the need of transportation. For this reason, it has been used in developing, non-industrialized countries to fuel local energy demand.[4]

Biofuel is energy derived from biological sources. When considering its application and integration to the engines we use today, the ideal biofuel can be used without major engine modifications. To achieve this, biofuel has mostly been produced in the form of biodiesel or bioethanol, which require little modification to some the engines we already use.[2] However, this is where the challenges of biofuel come into play. It is desirable to produce biofuel with certain characteristics so as to compete with the quality and energy produced from the combustion of fossil fuels. Bioethanol is a fossil fuel alternative that is largely produced from microbial fermentation of sugar or starch-rich plant, such as corn or sugarcane. When supplemented with gasoline, up to 85% (v/v) bioethanol can be used in engines without requiring modification to vehicles.[5] Limitations arise from the need of arable land and water required for the plants used in the bioethanol production process to grow on. Proposed methods of making production of bioethanol more sustainable involve the addition of chemical pretreatment to aid with the decomposition of the plants' biomass and water reutilization of the evaporators.[6-8] However, the

fact that bioethanol has a low energy density makes essential the need to turn to other fuel alternatives. Longer carbon chains than the well-studied bioethanol make for higher biofuel quality, as C-C bonds have higher energy content. Furthermore, higher energy density allows for easier and more efficient energy transportation as more energy can be transported or stored at a given volume.[9] For this reason, biofuel engineering has shifted towards the production of molecules with longer chain hydrocarbons.

Around the world laboratories in both academia and industry are researching the production of high energy compounds for biofuel production. The main approach is genetic engineering of microorganisms. They do not require vast areas of land for their growth, have faster doubling times that plants, and their genomes are easier to manipulate. As with all major emerging fields, however, the ramifications to our environment, as well as animal and human health must be thoughtfully understood. This is the goal of USDA funded work, that has led to a collaborative project between Dr. Lee's and Dr. Greene's lab at Old Dominion University. A vital part of that project is evaluating the biosafety of the GE cyanobacteria with genes associated with biofuel production and antibiotic resistance. The engineered plasmid designed to be introduced into cyanobacteria includes a butanol dehydrogenase and a keto-acid decarboxylase gene intended to utilize the cyanobacteria's intrinsic metabolic pathways to the production of biofuel and also includes genes conferring antibiotic resistance to the transformed cyanobacteria as selection markers in the cloning process. The pUC57 vector, that is used for the construction of the plasmid to be transformed into the cyanobacteria, carries a gene for ampicillin resistance, while the constructed cassette contains a gene conferring kanamycin resistance. Thus, the transformed cyanobacteria can be selected based on their ability to grow on media supplemented with antibiotics. Conferring onto the cyanobacteria tolerance to antibiotics

can potentially lead to health concerns when taking into account that if these GE cyanobacteria are released in the environment, they may pass these genes to other microorganisms that they encounter. The horizontal gene transfer (HGT) of these genes upon interaction of the GE cyanobacteria with other bacterial types is the focus of this thesis.

Antibiotics

Antibiotics are naturally secreted substances by bacteria in small amounts, constituting for 1.6% among microbial products.[10] The modern era of antibiotics began in 1929 with the first report on the discovery of penicillin by Alexander Fleming.[11, 12] Synthetically made antibiotics have since been a standard method of treatment of bacterial infections. Penicillin proved to be effective in treating bacterial infections during World War II, yet it was not long before resistance to the antibiotic emerged. In fact, penicillin-resistant strains were already identified in the 1940s.[13] Since then, antibiotic research and development has come a long way, yet widespread antibiotic resistance is still a problem with no lasting solution even today. Moreover, multiple drug resistant (MDR) strains have been identified, which pose a considerable threat as they create severe clinical problems. Years of increased and widespread overuse of antibiotics are eliminating the more susceptible strains, while selecting for the more tolerant ones. Hence, those that survive are the antibiotic resistant ones. When considering how to treat infections caused by strains with multi-drug resistance, multiple drugs may be used.[14]

Antibiotics are able to kill bacteria by targeting either their ability to reproduce, in which case they are called bacteriostatic, or their ability to produce necessary enzymes for their various functions and thus killing them, in which case they are called bactericidal. There are some that are considered broad spectrum antibiotics, more commonly used by doctors due to their effectiveness

against a wider range of bacteria. Below some of the different types of antibiotics relevant to my research are presented.

Aminoglycosides

Aminoglycosides are bactericidal and work by preventing bacteria to produce proteins that are essential for their function. Their basic structure consists of several aminated sugars bonded to a dibasic cyclitol (aminocyclitol) through a glycosidic bond.[15] For many clinically relevant aminoglycosides that aminocyclitol is a 2-deoxystreptamnine (2-DOS) ring and is a characteristic structural feature.[16, 17] Amino and hydroxyl substitutions to the core alter the molecule's mode of action [18]. Aminoglycosides act by binding to the ribosomes' small ribosomal unit in the cell's cytosol and impairing the bacteria's ability to produce proteins.[19] This causes misreadings or inhibition of translocation and thus interferes with the process of translation.[17] The mistranslations result in a misfolded protein that, when inside the cell envelope, increases drug uptake and, in combination with the increased ribosome binding lead to cell death.[20] Kanamycin belongs to this category.

Tetracycline and its derivatives bind the 16S rRNA component of the 30S ribosome subunit and block access of aminoacyl tRNAs to the ribosome. They do not cause mistranslation but instead limit the bacteria to a stationary state of growth.[20, 21] An interesting note is that even bacteriostatic antibiotics can cause cell death depending on the bacterial species or the treatment type. This has been found to be due to the sequence differences in the ribosomal proteins and RNAs.[22] When used in high concentrations, it has been found that they are capable of binding to ribosomal units of mammalian cells as well, due to their lack of specificity in binding.[23]

Streptomycin was the first from this group of antibiotics to be used in 1944 and was derived from *Streptomyces griseus.* Waksman and his colleague, Schatz, received the Nobel prize for the discovery of this antibiotic and its antimicrobial properties against *Mycobacterium tuberculosis.* [24, 25] It was subsequently produced and applied for treatment in medical cases of tuberculosis.[26] In contrast to penicillin, which is effective against gram-positive bacteria, streptomycin is effective against a broad range of both gram-negative and gram-positive bacteria.[27] Following streptomycin several other members of this class of antibiotics were isolated and synthesized, including neomycin, gentamycin, and kanamycin.[18]

Due to aminoglycosides mode of action, they need to be found in the bacteria cell's cytoplasm to be effective. Kanamycin A at physiological pH levels is triply protonated and can interact with the negatively charged bacterial membrane, increasing its disorder and contributing to the damaging effects against bacteria [16]. Kanamycin and aminoglycosides in general are able to ultimately reach the cytoplasm of the cell through hydrophilic diffusion.[28] Aminoglycosides have been found to be most effective against gram-negative bacteria instead of gram-positive.[29]

β-lactams

This class of antibiotics includes penicillin, cephalosporins and carbapenems. A key structural feature for these is the highly reactive β-lactam ring which contains 3 carbons and 1 nitrogen.[30] These antibiotics target the bacteria's cell wall which is composed of peptidoglycan. The bacterial cell wall of gram-positive and gram-negative cells differs in composition, with the former's consisting of more layers of peptidoglycan, resulting in a thicker cell wall, while the latter's less layers, resulting in a thinner cell wall.[31, 32] This antibiotic class's mode of action is by covalently binding to penicillin binding proteins (PBPs), which are essential for hydrolyzing the

crosslinking of the peptidoglycan cell wall.[30] The cell wall starts to break down, ultimately resulting in cell wall rupture.[32] β-lactams are inhibited by β-lactamases, which are the main mode of antibiotic resistance against this class of antibiotics. Development of β-lactamase inhibitors to counteract the action of the β-lactamases has proven to restore the susceptibility to the antibiotic in some cases and make for a tool with great promise for combating MDR microbes.[33, 34]

Ampicillin is a β-lactam, penicillin derivative that is active against gram-positive and gram-negative bacteria. Ampicillin is the second antibiotic that is used in my research, as it is incorporated as a selection marker in the pUC57 plasmid vector being used as a building block for pKB. It is a broad-spectrum antibiotic and when used in combination to β-lactamase inhibitors, it has been known to be effective against MDR microbes by restoring their susceptibility to the antibiotics.[34]

The spread of genes of ampicillin resistance has revealed some interesting discoveries. The genes associated with the production of β-lactamase, known as *bla* genes which are mostly responsible for ampicillin resistance, have been found to be associated with mobile genetic elements, such as plasmids, that are capable of being transferred across bacteria.[35-38] Interestingly, in a study performed by Laskey and colleagues, investigating the capability of transfer of plasmid that carried genes of ampicillin resistance among three different bacterial strains in mice, it was found that it is possible from the gram negative *E. coli* to *Salmonella* as well as a different strain of *E. coli*.[39]

Antibiotic Resistance

The problem of widespread antibiotic resistance is a multi-faceted one, whose effects have a huge impact on society each year, with antibiotic resistant bacteria infecting more than

2.8 million people and causing 35,000 deaths annually.[40] The emergence of antibiotic resistant microbes is predicted to have an even greater cost in human lives over the next years, with estimates predicting that within the next 30 years infections from antibiotic resistant microbes will cause the death of more than 10 million people annually, exceeding death even from cancer.[40, 41] The strain and financial burden that antibiotic resistant infections put on both the healthcare system and individuals is great. At the same time, a decrease in innovation in antibiotic drug development has failed to produce novel antibiotics to keep up with the pace that microbes are developing resistance to treatments.[42, 43] The challenge itself of producing an antimicrobial drug to which bacteria will not soon develop resistance, large development costs, and low approval rates have been a great demotivator for large pharmaceutical companies to be part of the antibiotic production pipeline.[44]

To combat the issue of antibiotic resistant infections, a first very important step is to decrease and eliminate the overuse and abuse of antibiotics in healthcare and elsewhere. While over-prescription of antibiotics and incorrect consumption are a great part of the problem, 80% of the use of drugs in the United States is in agriculture.[45, 46] Antibiotics are used to enhance the growth of livestock more so than to fight off dangerous diseases, a stark sign of overuse.[45, 47, 48] It has been proven that overuse of antibiotics in agriculture also affects antibiotic resistance in clinical settings.[49]

Six pathogens that are notorious for nosocomial infections that are especially hard to treat due to their high pathogenicity and resistance to multiple drugs, make up the acronym ESKAPE. These are: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species.[50] These are bacteria that have

high mortality rates.[51] There are multiple ways these pathogens have been able to acquire antibiotic resistant genes.

Aside from the development of resistance mechanisms to certain drugs, transfer of genes of antibiotic resistance from one strain to another is achieved through HGT. The various mechanisms of HGT such as plasmid conjugation, transduction via bacteriophage and transformation by uptake of naked extracellular DNA, allow for genes to be shared by means other than vertical transfer from parent to daughter cell.[52] Especially in clinical settings, this has allowed for exchanges between *P. putida* with the pathogenic *P. aeruginosa*, the former of which has been characterized as a gene exchange platform, including for genes of antibiotic resistance, for the latter.[53] Similar is the case for *S. epidermis* and the pathogenic *S. aureus*.[54] These examples show how once antibiotic resistance is found in an environment, it is not only possible, but almost inevitable, for it to transfer between bacterial strains. Moving forward, it is vital that researchers, medical workers, policymakers, and those working in agriculture, coordinate towards the common goal of slowing antibiotic resistant infections caused by overuse of antibiotics in all fields and the development and implementation of new, more effective tools against them.

Horizontal Gene Transfer

Gene transfer is essential for the spread of antibiotic resistance among different bacterial strains. This is made possible through the help of mobile elements such as plasmids.[55] In contrast to vertical gene transfer, which is from parent to daughter cells, and achieved through replication (Figure 1A), HGT is achieved by transduction, carried through with the help of viral vectors, such

as bacteriophages, transformation and conjugation (Figure 1B). These represent the main mechanisms of horizontal or lateral gene transfer in bacteria.

Bacterial conjugation is achieved by a pilus or adhesins between the two bacteria that facilitate the DNA transfer from the donor cell to the recipient.[56, 57] This allows for the exchange of plasmid vectors that carry various genes. The drivers of this exchange may be environmental pressures. [58] In a study by Laskey and colleagues, it was demonstrated that only when the selective pressure of the mice being treated with ampicillin was present was there plasmid transfer from the strain that already was ampicillin resistant to recipient strains in the mice gut. In contrast, when the mice were not treated with ampicillin, there was no plasmid transfer observed as the only strains that were able to colonize the mice's gut were those that already had the plasmid carrying genes for ampicillin resistance.[39]

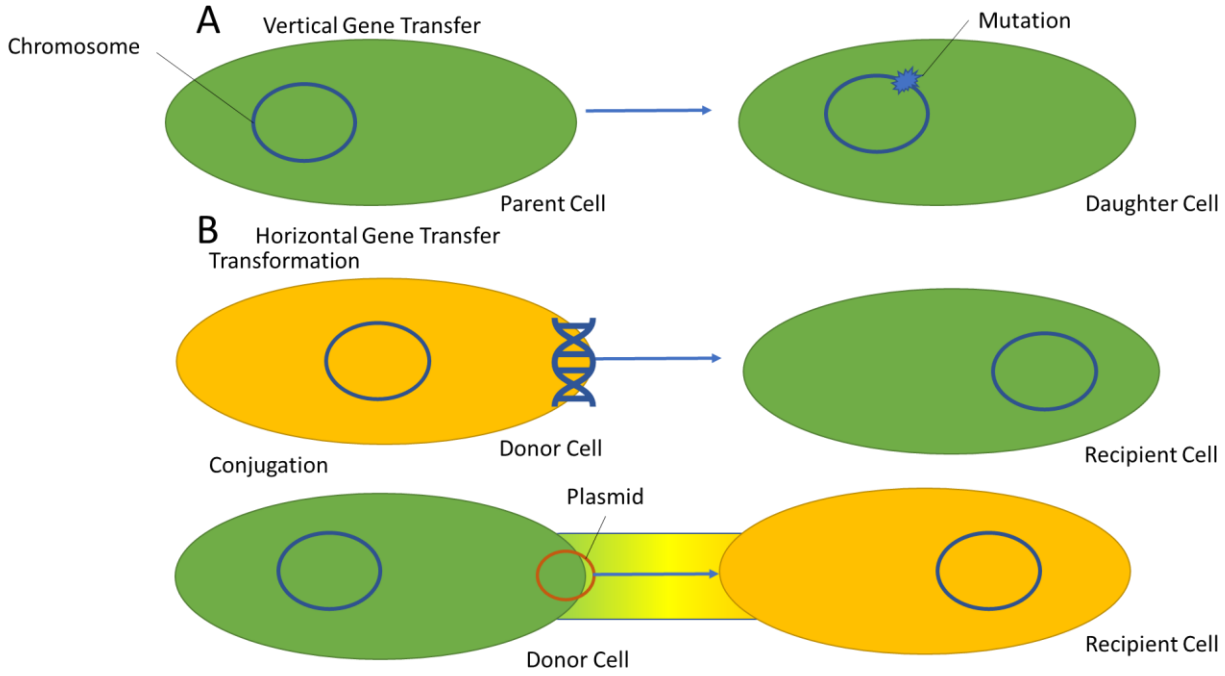

Figure 1. Mechanisms of gene transfer between bacteria. (A) Vertical gene transfer refers to the gene transfer from parent cell to daughter cell. Changes in the genetic material occur in the form of mutations. (B) Horizontal gene transfer refers to gene transfer from a non-vertical gene transfer from a donor bacterial cell to a recipient cell. Two main forms of gene transfer are transformation, during which the recipient cell uptakes DNA from the environment after it has been released by the donor cell, and conjugation, during which donor-cell to recipient-cell contact facilitates transfer of DNA, usually in the form of a mobile element, such as a plasmid. Figure created using MS Word.

The exact molecular mechanism of conjugation is complex and requires coordination on multiple fronts, as elements from the cytoplasm of the cell are not only being transferred outside the cell, but the bacterial cell is paired to another one. A membrane-associated secretion system is

employed for the purpose of DNA transfer, known as type IV secretion system (T4SS) (Figure 2).[56] As part of this system, DNA mobilization genes are used to mobilize DNA that will be transferred, while mating pair formation genes code for proteins that will be part of the macromolecular structure of T4SS. DNA transfer replication genes are responsible for binding to DNA at the origin of replication (oriT) of the donor cell plasmid and form a structure called a relaxosome, which itself consists of several proteins. The complex of proteins that are part of the relaxosome are capable of acting as a helicase by nicking the plasmid to be transferred within the oriT site and unwinding the DNA in a 5' to 3' direction.[59, 60]

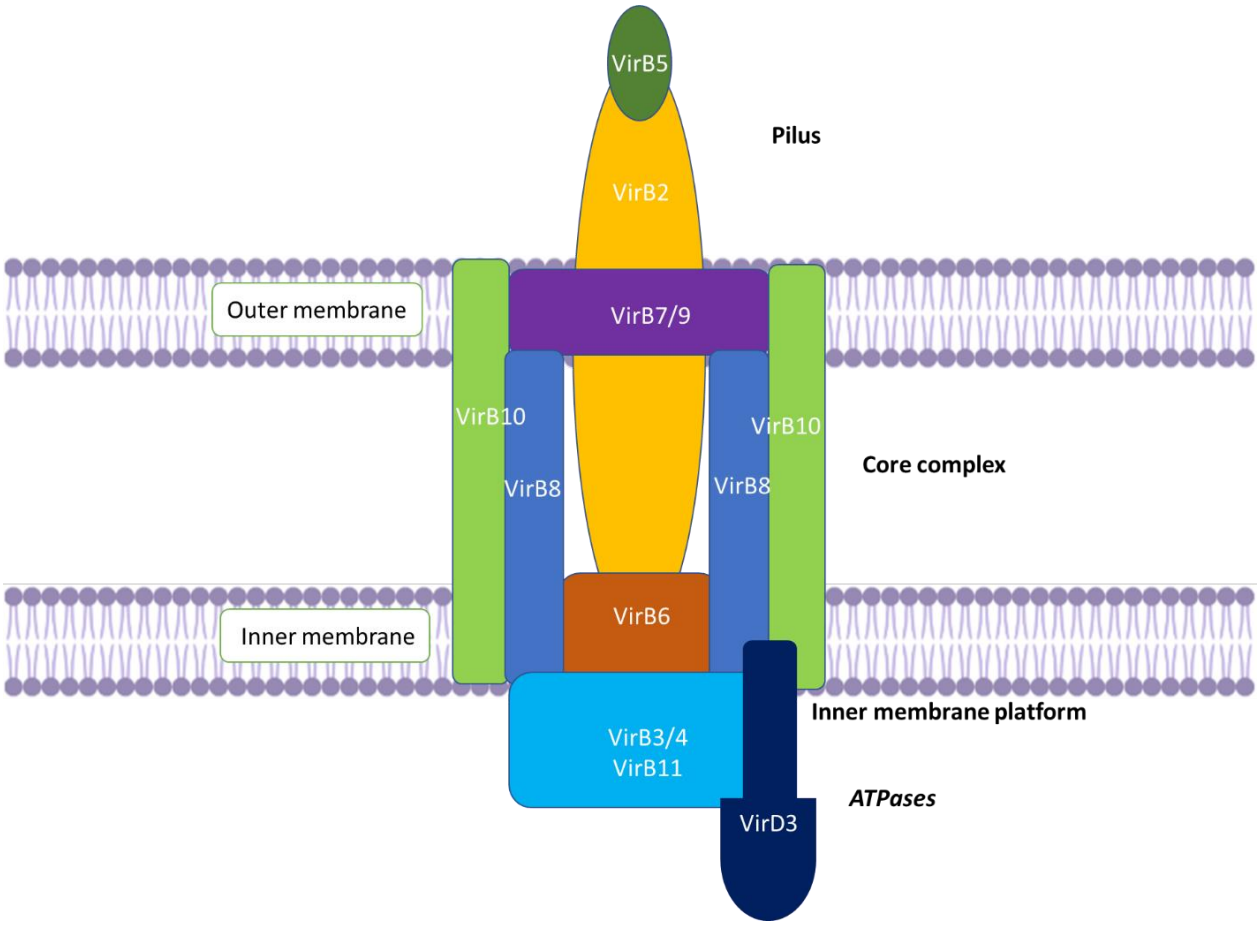

Figure 2. Schematic of T4SS. The protein complexes that are part of the pilus (VirB2 and VirB5), the core complex (VirB6-10), the inner membrane platform with the transmembrane domains of VirB3, VirB6, VirB8 and VirB10, and the ATPases VirB3, VirB6, and coupling protein VirD3. Created with Biorender.com and MS Word, based on data from the literature [56, 60]

Evolutionarily, T4SS has played an important role in contributing to the plasticity of bacterial genomes and allows them to acquire genes that grant lateral connections between bacterial lineages. There are multiple types of T4SS, whose variation in aspects such as gene content and order result in T4SSs with different component modules that are organized into a

single functional unit.[61] It is noteworthy that these systems are capable of transferring both nucleic acids and, in certain cases, proteins from gram negative and positive bacteria to prokaryotic and eukaryotic recipient cells.[62] Frequencies with which conjugation takes place among bacteria varies and differences in frequencies may be attributed to various factors. Generally, it has been found that decreased taxonomic relatedness between donor and recipient bacterial strains leads to decreased conjugation efficiencies.[61, 63, 64] Furthermore, there are differences in T4SS systems in gram-negative and gram-positive microorganisms. While T4SSs in gram negative organisms have been well studied and characterized, with the most well studied being that of *Agrobacterium tumefaciens*, gram positive ones are less characterized. From the knowledge we have on gram-positive conjugation so far, we know that it works better with self-transmissible or conjugative plasmids.[65] These are plasmids that incorporate sequences for their MPFs and thus are mobilizable on their own.

However, donor-recipient compatibility is not the only contributing factor to conjugation efficiencies. In experiments performed on mice, to investigate gene transfer through conjugation in the gut microbiota plasmids with different characteristics interfering with conjugation were used. Their conjugation efficiencies were measured using probed cells inside the gut microbiota. From this study, only one plasmid was able to be transferred to all probed cells, while the others has low or no transformation efficiencies.[66]

Lastly, it is known that T4SS contributes to the pathogenicity of bacteria, either by allowing them to uptake genes that increase their pathogenicity, or as it was more recently shown to secrete toxin effectors into other bacteria resulting in their destruction.[67] It was found that the plant pathogen *Xanthomonas citri* [68, 69] and human opportunistic pathogen *Strenotrophomonas maltophilia* [70] are capable of killing rival bacteria by secreting toxin effectors through T4SS [71].

In my thesis research, environmentally and clinically relevant strains were selected in order to investigate their interactions and HGT capabilities with those of the genetically engineered cyanobacteria, altered with the aim of producing biofuel. By investigating the presence of designer genes that were originally transformed into cyanobacteria in bacteria that they were in co-culture with, we are able to understand where HGT has occurred. We do this by employing genes conferring antibiotic resistance, which grant the bacteria that have uptaken them the ability to grow on media supplemented with antibiotics –kanamycin (40 μg/mL) or ampicillin (100 μg/mL).

Bacterial Strains

Thermosynechoccus Elongatus BP1

The genome of this bacteria was sequenced entirely in 2002 by Yasukazu et. al and it was found to be 2,593,857 base pairs long with no plasmid detected. It is a thermophilic bacterium that ideally lives in temperatures ranging from 45˚C to 55˚C and was originally isolated from hot springs in Japan.[72] Under the microscope it appears rod-shaped. As a photosynthetic microorganism, *T. elongatus* BP1 has the ability to uptake inorganic carbon from the atmosphere, yet it has also been found to be capable of assimilating organic carbon that was supplemented in the media.[73] The doubling time of this bacterium is 7-12 hours depending on how close to its ideal temperature it is being grown.[74, 75]

Escherichia coli

E. coli is a very well characterized bacterium, whose early success in first cloning experiments have made it the bacterium of choice for molecular cloning and continues to be used even today. It is a gram-negative, rod shaped bacterium, with a duplication time of

approximately 20 minutes. There are both pathogenic and non-pathogenic *E. coli* strains. Less than 1% of human gut bacteria constitute of *E. coli* or *E. coli* related bacteria, as they maintain a symbiotic relationship with the host organism by producing vitamin K12 as well as preventing gut colonization by pathogenic bacteria.[76, 77]

There are hundreds of *E. coli* strains currently known and being used in research laboratories. They are all derivatives of two main wildtype strains the K12 and B strain.[78] The derivatives of these strains that are commonly used in laboratory settings, such as the DH5α strain, have undergone various types of genetic modifications. The DH5α strain specifically has been modified to be negative for recA and endA genes. The endA gene codes for endonuclease I, which degrades double-stranded DNA, thus affecting the stability of the produced pDNA, while recA catalyzes DNA strand exchange reactions in ATP-dependent homologous recombination.[79] A mutation in its lac operon, lacZΔM15, makes it possible to perform blue-white screening of *E. coli* DH5α colonies to select for transformants when using plasmids such as pUC plasmids containing the lacZα sequence.[80, 81] These modifications make *E. coli* DH5α ideal for use in cloning methods and plasmid amplification.

Pseudomonas putida

Pseudomonas putida KT2440 is a gram-negative type of bacteria that is commonly found in soil and water. It is an opportunistic pathogen in nosocomial settings but otherwise is rarely the cause of diseases, a characteristic that makes it safe for use in industry for synthetic processes, along with its metabolic robustness.[82, 83] *P. putida* has the ability to degrade aromatic compounds found in its environment and can grow on such substrates, such as toluene and xylenes.[84] For this reason, is also used in chemical synthetic processes, with rapid growth and high biomass yield.[85]

Specifically, *P. putida* KT2440 has been found to have high tolerance to heavy metals and metalloids, whose abundance is increased in the rhizosphere, which make this strain appealing for potential industry application.[86] This has been characterized as an evolutionary trait that allows it to reside in depths of the soil where roots are found.

P. putida KT2440 is rarely identified as the cause of disease in cases not associated with an immunocompromised state, with the first such case of bacteremia reported in 2013.[82] *P. putida's* relative, *Pseudomonas aeruginosa* is the pathogen lying behind most nosocomial infections of this strain.[87] It is part of the group of pathogens collectively known and referred to as ESKAPE pathogens, which are commonly found in clinically acquired infections.[88] In certain cases of hospital acquired infections, *P. putida* bacteria were found along with *P. aeruginosa*. Genetic studies of these bacteria lead to the conclusion that *P. putida* served as an exchange platform of antibiotic resistance genes for *P. aeruginosa*.[53] This makes it especially dangerous for spread of antibiotic resistance in strains in hospitals and communities. In addition to their antibiotic resistance, both *P. aeruginosa* [89] and *P. putida* [83] are able to produce biofilm which enhances their ability to adhere on medical devices by forming clusters along with a self-produced adhesive matrix which includes polysaccharides, proteins and DNA.[90] In hospital settings, biofilm formation allows these bacteria to contaminate surfaces and medical devices as well as develop antibiotic resistance.[91]

Research Aims

The overall aim of this thesis is to provide further insight into the bio-risk that genetically modified organisms (GMOs) pose for the transfer and spread of foreign genes into the environments that they are introduced. An assessment is conducted of GE cyanobacteria's

capabilities to transfer genes of antibiotic resistance to bacterial strains they may encounter in their environment should they be found outside of contained laboratory conditions. The implications of this research are not only important for potential biofuel applications involving cyanobacteria, but for applications of GMOs in general. As we develop more sophisticated tools to manipulate genomes of microorganisms, their interaction with their environment and other bacteria is of particular interest from a biosafety perspective.

In aim one, the plasmid carrying the designer genes for biofuel production and antibiotic resistance to ampicillin and kanamycin, is introduced to the cyanobacteria, *T. elongatus* BP1 and three gram-negative strains to assess their capacity of carrying the plasmid. The three gram-negative bacterial strains transformed and studied along with the *T. elongatus* BP1 cyanobacteria are: *E. coli DH5α, E. coli K12,* and *P. putida*. The success of the transformation and the ability of a strain to carry the plasmid is assessed from the ability of the transformants to grow on media supplemented with antibiotic, as antibiotic resistant genes were included in the plasmid construct as selective markers. The strains were selected based on different criteria. *E. coli* DH5α is a commonly used laboratory strain that is often used for molecular cloning procedures and thus studying it alongside the GE cyanobacteria would provide us information about the horizontal gene transfer process with recipient cells optimized for uptake of foreign DNA. *E. coli* K12 is the parent strain to *E. coli* DH5α and has not undergone genetic modifications to prime it for such processes, thus it is more of a wild-type *E. coli* strain. Lastly, *P. putida* is a soil bacterium that could be found in the same environment as the cyanobacteria should the latter escape laboratory containment and is also a relative of the pathogenic *P. aeruginosa* that is notorious for causing infections in clinical settings.

In the second aim we study the ability of the cyanobacteria to transfer plasmid encoded genes conferring antibiotic resistance to two model *E. coli* strains, *E. coli* K12 and *E. coli* DH5*α*, while in the third aim we study the capability of HGT from the cyanobacteria to *P. putida* KT2440. These two aims were designed to provide insight into the mechanisms of HGT and the fate of genes conferring antibiotic resistance between the model cyanobacteria and the three selected bacterial strains. By demonstrating that HGT is possible from GE cyanobacteria to these strains that may interact with outside of laboratory containment, it is possible to formulate containment strategies for large scale applications of GE cyanobacteria for biofuel production to effect escape and understand the ramifications should this occur.

Experimental Methodology

The schematic in Figure 3 portrays the combination and overlap of biochemistry, genetics, microbiology, and bioinformatics techniques used to develop my experimental strategy and to conduct the HGT experiments. Once the constructed plasmid carrying genes aimed at biofuel production is transformed into cyanobacteria, the ability of it to be transferred to other types is assessed by growing them in co-culture with the GE cyanobacteria. The cells are then plated on selective media supplemented with antibiotics, to see if they have gained tolerance to it. If they have not, we may have a viable biofuel source safe to the environment and humans. However, if the bacteria gain antibiotic tolerance, they are a biohazard and containment methods for future applications should be proposed.

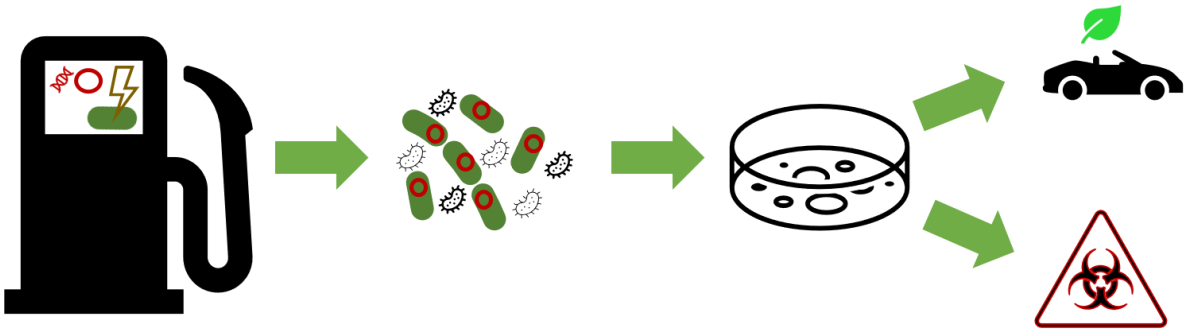

Figure 3. Graphical representation of the HGT experimental strategy for biohazard assessment of GE cyanobacteria. It involved transforming the designer pUC-57 based plasmid vector into *T. elongatus* BP1. Co-cultures of GE *T. elongatus* BP1 with wildtype *E. coli* K12, *E. coli* DH5α, and *P. putida* were set up, and after plating on selective media, bacterial colonies were screened for presence of the designer plasmid genes.

Bioinformatics

The pKB DNA construct was designed using DNAstar bioinformatics software tools and synthesized in collaboration with GenScript.com. The pKB DNA construct comprises PnirA - nirA promoter, KDC - keto-acid-decarboxylase, BDH - butanol dehydrogenase, PslpA - slpA promoter, KanR – kanamycin resistance gene, and Term-rubisco terminator. Up (RSI) and downstream (RSII) of the cassette are homologous recombination site sequences. Primers for PCR amplifications of certain genes of the plasmid and genes of the bacterial strains' genome were designed using CLC Genomics Workbench v.12 software (Qiagen). Primers for distinguishing among the selected bacterial strains during experiments (Appendix A) and sequencing data acquired from the HGT experiments were also designed using this software.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a common molecular biology technique that has recently gained much attention due to real time PCR (RT-PCR) being the method of choice for diagnosing patients with COVID-19.[92] By creating multiple copies of a target DNA template, we are able to amplify that DNA fragment and detect it using gel electrophoresis. This is a technique based on the separation of DNA fragments by size within an agarose gel using an electric field. Each DNA fragment produced by PCR has a specific size which can then be detected on the gel and measured using a DNA ladder.

Electroporation

To introduce the plasmid DNA into the wildtype cyanobacteria and assess the bacteria's ability to carry the plasmid, the pKB plasmid was transformed into these microorganisms using electroporation. The bacteria are inserted into a cuvette that is placed in to the electroporator (BIO-RAD, GenePulser Xcell). Optimized parameters for voltage, capacitance, and resistance for each bacterial strain are programmed into the electroporator to produce the appropriate pulse. The pulse allows for the plasmid to be inserted in the bacterial cells through pores formed as the pulse is discharged in the suspension where the cells and plasmid are present.[93]

Cell cultures

Co-cultures of the GE *T. elongatus* BP1 cyanobacteria along with *E. coli* K12, E. coli DH5α, and *P. putida* KT2440 were grown and cells were plated on selective media to determine which had taken up genes conferring antibiotic resistance. Monocultures of the different species are used for the appropriate control cultures.

Significance of Research

The need for green energy and the focus on GMOs creates both possibilities and problems. The latter is the concern over the unintended consequences of using GMOs. This is especially crucial as the emergence of antibiotic resistant strains is a major health threat that we are currently facing. Widespread antibiotic resistance among strains that are commonly found in clinical settings are the cause of more than 2.8 million antibiotic resistant infections and more than 35,000 deaths in the US, as reported by the CDC 2019 report.[94] The significance of this work lies in both the value of assessing the biohazard that GE cyanobacteria may pose when considered for biofuel production and the transfer capabilities of genes of antibiotic resistance to *E. coli* K12, *E. coli* DH5α and *P. putida* KT2440.

CHAPTER II

MATERIALS AND METHODS

Strains and Culture Conditions

T. elongatus BP1 is a photoautotrophic, thermophilic strain grown at 45˚C under constant light conditions under fluorescent lamps. The media used for the cyanobacteria in these studies were BG-11 and BG-11_0SA, the latter being a modified version of the former, as 4mM of $(NH_4)_2SO_4$ is the nitrogen source instead of KNO_3. This has been used as such by Thu et al. in studies using the same type of cyanobacterial transformants [95] and has been described previously.[96] Additionally, the BG-11_0SA growth medium for the *T. elongatus* BP1 transformants was supplemented with kanamycin (40 μg/mL). *E. coli* K12, *E. coli* DH5α, and *P. putida* KT2440 were grown in Luria Bertani (LB) medium at 37˚C. Transformed *E. coli* K12 and *E. coli* DH5α were grown in LB medium supplemented with either kanamycin (40 μg/mL) or ampicillin (100 μL/mL). *P. putida* 2440 transformants were grown in LB medium with kanamycin (40 μg/mL).

Plasmids

pKB plasmid

The plasmid that was used in this research has been used to demonstrate HGT from transformed *T. elongatus* BP1 to *E. coli* DH5α.[95] It is based on the pUC-57 plasmid vector, which carries genes of ampicillin resistance, and a pmb1 origin of replication (Figure 4). The designer genes, which aim to confer the ability to the cyanobacteria to produce butanol, keto-acid decarboxylase and butanol dehydrogenase, are found between two recombination sites, RSI and

RSII. They are preceded by an inducible promoter (nirA), which is induced by nitrate presence in the media [97, 98] and repressed by ammonium.[99, 100] The KanR gene conferring kanamycin resistance is preceded by two continuous promoters, the promoter for the cpc gene from *T. elongatus* BP1 and the promoter for the slpA gene of *Thermus thermophilus*. Finally, the cassette contains a rubisco terminator. The total size of pKB is 9,007bp.

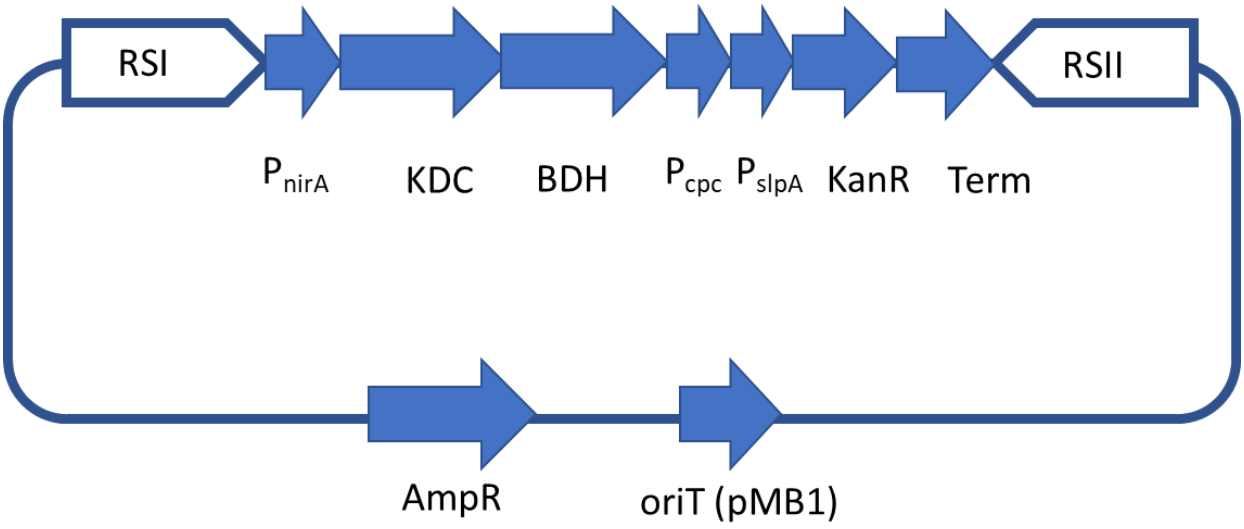

Figure 4. Schematic of the pKB plasmid design. The plasmid was constructed to carry the annotated set of genes: PnirA - nirA promoter, KDC - keto-acid-decarboxylase, BDH - butanol dehydrogenase, PslpA - slpA promoter, KanR – kanamycin resistance gene, and Term-rubisco terminator. Up (RSI) and downstream (RSII) of the cassette are homologous recombination site sequences aimed at guiding the integration of the cassette, into the cyanobacteria's genome. The origin of replication, oriT, and the ampicillin resistance gene, AmpR, are part of the pUC57 vector used to construct the cassette. Adapted from Nguyen et. al.[95]

pUC19

A minimal plasmid, without the cassette carrying the alcohol producing genes, was transformed into the *E. coli* K12 and *E. coli* DH5a for comparison of the transformation results with those of electroporation with the pKB plasmid. pUC19 is related to pUC57, which is used for the construction of pKB, and contains the same ampicillin resistance gene and origin of replication.[101-104] Its size is 2,686 bp. The pMB1 origin of replication ensures high copy numbers.[105] pUC19 also contains a fragment from the N-terminal of the β-galactosidase gene, that is part of the well-characterized lac operon, and offers another selection system for pUC19 transformants containing a lacZΔM15 mutation, such as *E. coli* DH5α Top10.[80]

Genetic Transformation of pKB into *T. elongatus* BP1

Wild-type *T. elongatus* BP1 cells were grown in liquid BG-11_0SA medium to reach OD_{730}=20.0. They were pelleted down by centrifugation and resuspended in 400 μL of water. 1μg of the pKB plasmid was added to the cell suspension. Electroporation was done at 5 kV/cm in a 2 mm cuvette using BioRad PowerPac Basic. A single pulse was provided for each transformation. Immediately after, the cells were transferred into flasks with 5mL of BG-11_0SA medium pre-warmed at 45˚C. The cells were then incubated overnight at 45˚C, under constant light conditions prior to being spread on agar BG-11_0SA medium supplemented with kanamycin (40 μg/mL). After 10-14 days of incubation at 45˚C and under constant light conditions, colonies of the transformed cells carrying the pKB with the kanamycin-resistance genes used as a selective marker appeared on the agar plates containing antibiotic. The colonies were streaked onto new plates and liquid cultures with BG-11_0SA medium with kanamycin (40 μg/mL) were

prepared as well, thus establishing a line of GE cyanobacteria to be used for the HGT experiments.

Verification of Cassette in *T. elongatus* BP1

Plasmid extraction was performed on the green colonies that grew on the BG-11_0SA plates with kanamycin (40μg/mL). PCR was performed to verify the presence of the pKB plasmid in the transformed cells. Two primers, Amp_fwd (TTACCAATGCTTAATCAGTGAGGCAC) and bdh0318_rev (CCTCCATTTCCTTTGCACCCT) were used, which were used to verify the plasmid as per Nguyen *et al.* with an expected band size of 4.5 kb.[95]

Horizontal Gene Transfer Experiments

Transformed *T. elongatus* BP1 cyanobacteria were grown in BG-11_0SA medium supplemented with kanamycin (40 μg/mL), at 45˚C, under constant illumination conditions, while their concentration was monitored by cell counting using a hemocytometer and an upright microscope (OLYMPUS LS). *E. coli* K12, *E. coli* DH5α, and *P. putida* K2440 overnight cultures were grown at 37˚C. Transformed *T. elongatus* BP1 cells were pelleted down by centrifugation, the supernatant was discarded, and the cells were suspended in fresh BG11_0SA medium without kanamycin to a final concentration of 10^7 cells/mL. 15 mL of the cyanobacterial suspension were then mixed with 15 mL of cells from each of the other strains, *E. coli* K12, *E. coli* DH5α, and *P. putida* KT2440 respectively, each at a concentration of 10^7 cells/mL to create the co-cultures. The resulting liquid co-cultures contained 15mL of LB medium and 15mL of BG-11_0SA for a total of 30mL. These co-cultures were incubated in a shaking incubator at 37˚C, under constant

light conditions provided by daylight fluorescent lamps. The controls for this experimental set-up were 30 mL cultures containing 15 mL of LB medium and 15 mL of BG-11_0SA medium with only one bacterial strain. Experiments were performed in triplicates.

Control Studies

Testing the Ability of E. coli DH5α, E. coli K12, and P. putida to Uptake DNA from the Media

The ability of *E. coli* K12, *E. coli* DH5α, and *P. putida* KT2440 to uptake the pKB plasmid from their media was evaluated by setting up 30 mL liquid cultures containing 1:1 LB to BG-11_0SA ratio. Cell suspensions of *E. coli* K12, *E. coli* DH5α, and *P. putida* KT2440 at a concentration of 10^7 cells/mL each, in addition to the 1μg of the plasmid, were added to the 30 mL liquid media. The cultures were incubated under constant light conditions and at 37˚C for 2 days before being plated on selective media. This was a critical control experiment aimed to demonstrate that any HGT observed from co-cultures of the bacteria was not an artifact of the DNA uptake from the supernatant.

Genetic Transformation of pKB into E. coli DH5α, E. coli K12, and P. putida KT2440

To assess the ability of the different bacterial strains to carry the plasmid, the plasmid was initially transformed directly into the cells. Prior to the transformation, the cells were made competent using the following protocol. Cells from 10mL volumes of overnight cultures at a concentration of 10^9 cells/mL as determined by cell counting were centrifuged down to a pellet. The supernatant was discarded. Of particular importance was to discard the supernatant in its entirety even at the expense of a few cells, so that no arc would be detected during the pulsing step of the electroporation due to the salts of the culture growth medium. A mixture of 10% glycerol

was prepared using filtered glycerol and Millipore water. For each of the different types of bacteria, 3 washes with 2mL, 1mL and 0.5mL of cold glycerol were performed respectively, centrifuging the cells for 3 minutes at maximum speed for each wash. During the washes, the cells were placed on ice and the temperature of the centrifuge was kept at 4℃.

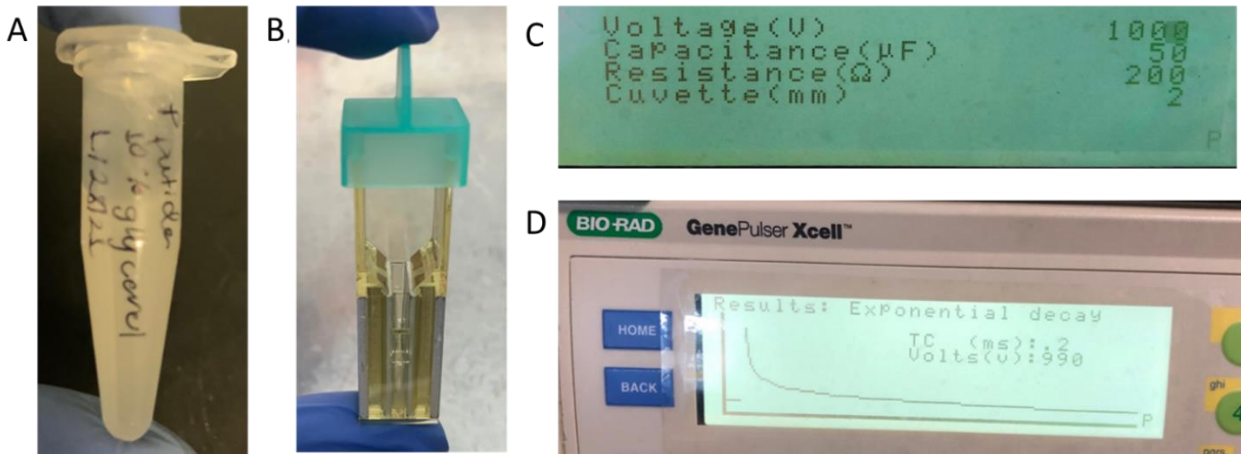

Figure 5. Transformation via electroporation. (A) Cells were made competent and suspended in 10% glycerol. (B) The cell and plasmid suspension were placed inside a pre-chilled 2mm cuvette. (C) The electroporation parameters were set on the electroporator. (D) The time constant was recorded for each electroporation.

Figure 5 shows snapshots of the process leading up to the pulsing of the bacteria for transformation of the plasmids. A 40-100μL volume of the electrocompetent cells was inserted in a microcentrifuge tube along with 0.5-1.0 μg of plasmid DNA and incubated on ice for 1minute. Plasmid DNA and cell mixture were transferred to pre-chilled 2mm cuvettes (Figure 5B) and were placed in the electroporation chamber of a Bio-Rad Gene Pulser Xcell microbial system that was

used for electroporation of the cells, following exponential decay protocols (Figure 5D). The cells were pulsed according to the parameters that were provided for each bacterial strain by the electroporator manufacturer, however, they were optimized for our cells to yield better transformation efficiencies. These parameters are summarized in Table 1. The cells were pulsed once and immediately transferred to a microcentrifuge tube with 1 mL of room temperature LB medium. The electroporated cells were incubated for 1 hr at 37℃, under shaking conditions of 150 rpm, before being plated on selective media. Transformed *E. coli* K12 and *E. coli* DH5α were plated on LB agar, LB with Kanamycin (40 μg/mL) and LB with Ampicillin (100 μg/mL), while *P. putida* KT2440 was plated on LB and LB with Kanamycin only, as it is naturally resistant to the Ampicillin. The plates were incubated at 37˚C for the next 4 days. Colonies that were able to grow on antibiotic following electroporation typically appeared after 2-3 days and were then screened for the presence of transgenes from the plasmid using colony PCR. Experiments were performed in triplicates.

Table 1. Summary of Electroporation Conditions for the Bacterial Strains

Bacterial Strain	Cuvette (mm)	Voltage (V)	Capacitance (μF)	Resistance (Ω)
T. elongatus BP1	2	1000	25	100
E. coli K12	2	2500	25	200
E. coli DH5α	2	2500	25	200
P. putida KT2440	2	1000	25	200

PCR Analysis and DNA Sequencing

To prepare the DNA for the colony PCR, with the tip of a cell spreader cells in a colony from the plate were put into 30 μL of Millipore water and incubated for 5 min. at 95°C. The mixture was then centrifuged for 3 minutes at maximum speed to collect cell debris on the bottom of the tube while keeping the DNA in the supernatant. For colony PCR, 2 μL of DNA template were used from these prepared samples. Apart from that, for the PCR mixtures for each of the samples, 10 μL of Phusion high fidelity PCR master mix (Thermo Fisher Scientific) were used, along with 0.5 μM of forward and reverse primers respectively, all of which add up to a total of 20μL of reaction mixture.

The transfer of the genes from the GE cyanobacteria to the various bacterial strains was confirmed with the detection of the foreign genes in bacteria from the co-culture with the transformed *T. elongatus* BP1 using PCR and DNA sequencing. Amplification was achieved with the primers cpcPKan_fwd (TAATAGGCGTTTCCCTTCGTTGCC) to KanN_rev (CAAAATGGTATGCGTTTTGACACATCC). Sequencing of the PCR product of the amplified 1kb kanamycin resistance gene was done by the Eastern Virginia Medical School Molecular Core. Alignments of the sequenced region were made using CLC Genomics Workbench v.12 (Qiagen) software.

CHAPTER III

RESULTS AND DISCUSSION

Transformation Experiments

Transformation of T. Elongatus BP1

Transformation of *T. elongatus* BP1 resulted in cyanobacteria transformants with the ability to express kanamycin resistance and grow on media supplemented with kanamycin (40 μg/mL) (Figure 6A). Colonies were obtained from transformation via electroporation after 14 days and were restreaked and grown in liquid media supplemented with kanamycin (40 μg/ mL) (Figure 6B). Amplifying a DNA region from plasmid extraction of the transformed cyanobacteria both part of the pUC57 vector and the cassette containing the genes aimed at the production of biofuel using primers Amp_fwd (TTACCAATGCTTAATCAGTGAGGCAC) and bdh038_rev (CCTCCATTTCCTTTGCACCCT), produced the results of Figure 6C. pKB is detected in sample 3 at 4.5 kb, at the same size as the positive control (Figure 6C). Primers cpcp_Kan_fwd (TAATAGGCGTTTCCCTTCGTTGCC) and KanN_rev (CAAAATGGTATGCGTTTTGACACATCC) were used to amplify the region containing the kanamycin resistance gene. The gene was detected at the expected band size of 1kb in Figure 6D) for the plasmid “P”.

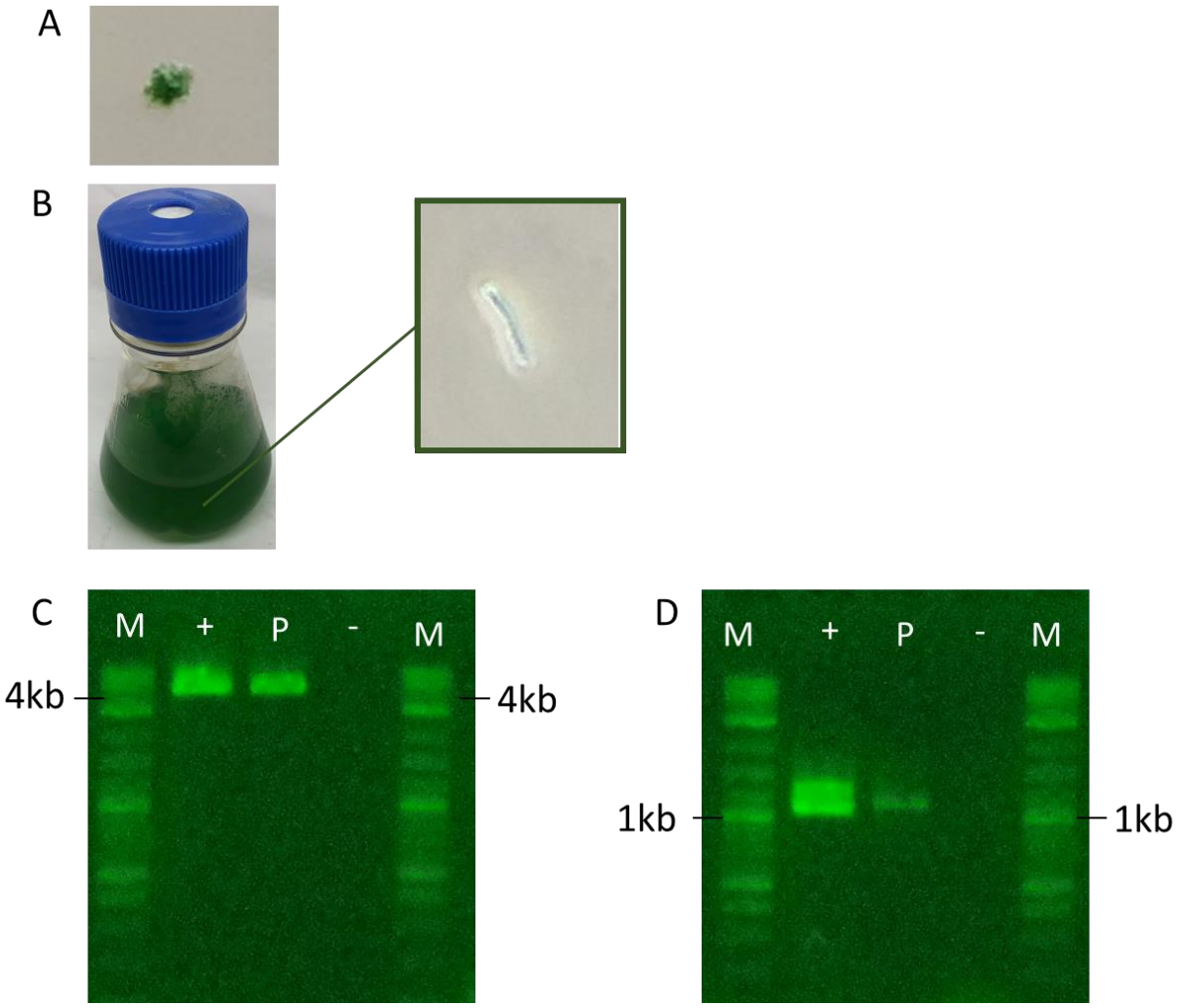

Figure 6. Transformation of pKB into *T. elongatus* BP1. (A) Colony growth on BG-11_0SA agar plate after 7 days of incubation under constant light conditions at 45˚C. (B) Cells were grown in liquid culture BG-11_0SA media supplemented with kanamycin 40 µg/mL. PCR on DNA from plasmid extraction from transformed *T. elongatus* BP1 using primers (C) cpcp_Kan_fwd and KanN_rev. and D. bdh_fwd and amp_rev. In panels C. and D., "M" denotes the molecular weight marker. pKB was used as the positive control, denoted with "+", while WT *T. elongatus* BP1 were used as the negative control, denoted with "- ". Plasmid extracted from transformed *T. elongatus* BP1 is denoted with "P".

Transformation of E. coli K12

The results of the transformation of *E. coli* K12 are shown in figure 3. Following plating of the electroporated cells, no colonies were observed after 3 days of incubation on the plates containing antibiotic. Transformation was repeated 4 times, altering electroporation parameters with the same results. This was perplexing as, in contrast, the *E. coli* DH5α had growth of individual colonies on ampicillin, as is depicted in Figure 5A. Suspecting that the reason for the unsuccessful transformation of *E. coli* K12, the true wildtype *E. coli* strain is the size of the plasmid in a bacterial strain which, unlike *E. coli* DH5α is not primed for cloning procedures, a different plasmid was transformed into *E. coli* K12. pUC19 is a minimal plasmid, it contains no cassette with genes of antibiotic resistance, but contains the same ampicillin resistance gene as pUC57 that was used for the construction of pKB and the same pMB1 origin of replication (Figure 7B).[101-103] Therefore, by transforming *E. coli* K12 with pUC19, we were able to see that while pKB was not transformed and expressed in *E. coli* K12, a plasmid with similar characteristics but smaller in size, 2,686bp versus 9,007bp, was able to be successfully transformed into the bacteria. The plates where electroporated cells were plated and incubated were covered with growth (Figure 7C).

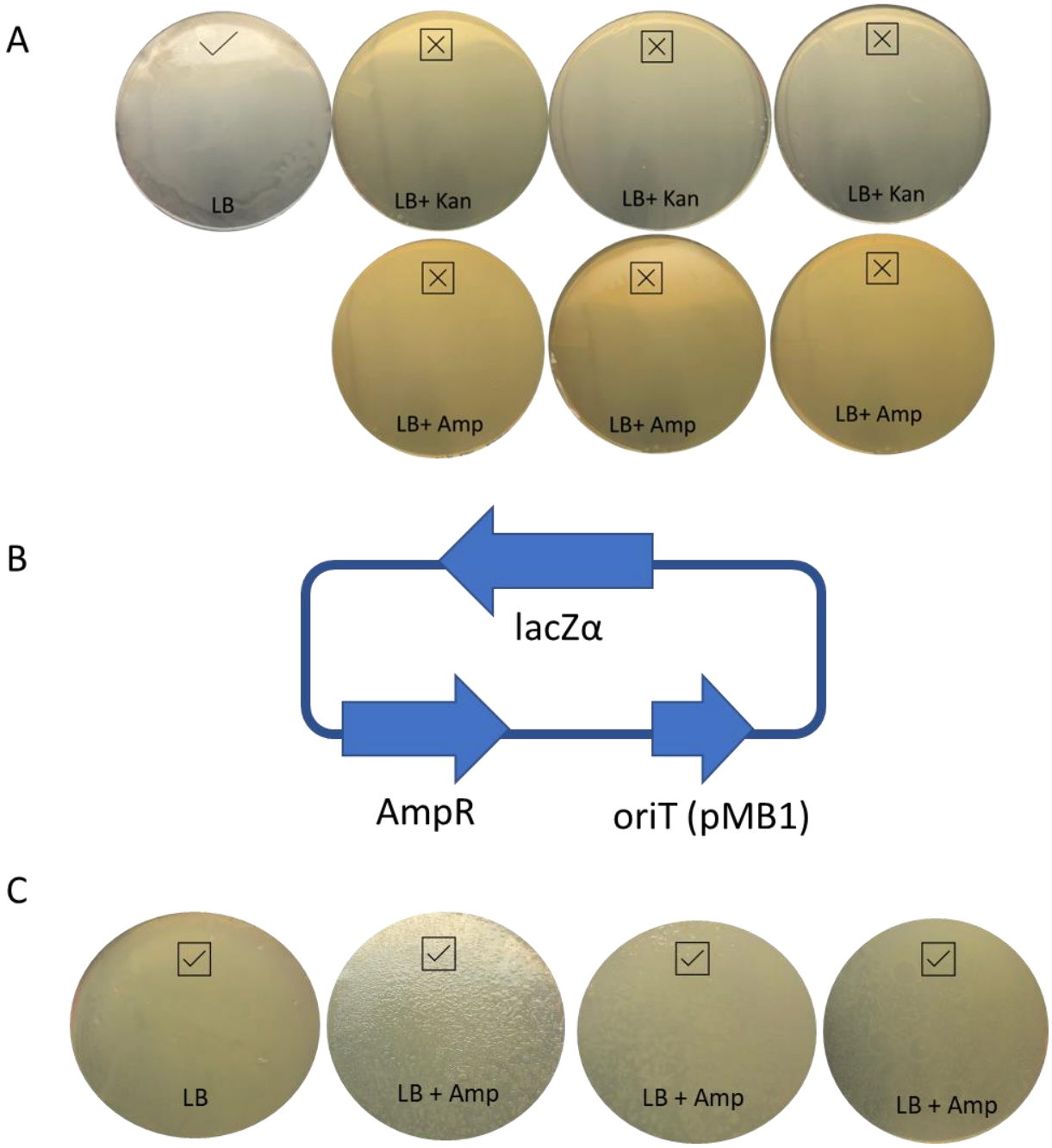

Figure 7. Transformation of *E. coli* K12. (A) The agar plates onto which pulsed *E. coli* K12 cells were plated on after electroporation with pKB. Check marks indicate presence of growth, while x's indicate no growth on the plate. (B) Schematic of the pUC19 plasmid vector. (C) The agar plates onto which pulsed *E. coli* K12 cells were plated after electroporation with pUC19 and 1 day incubation at 37˚C.

Transformation of E. coli DH5α

There were 16 colony forming units (CFUs) that were counted on the ampicillin plates where pulsed *E. coli* DH5α were plated (Figure 8A). They were screened with primers pmb1_fwd (CCCCCCTGACGAGCATCAC) and AmpR_rev (ATGAGTATTCAACATTTCCGTGTCG).

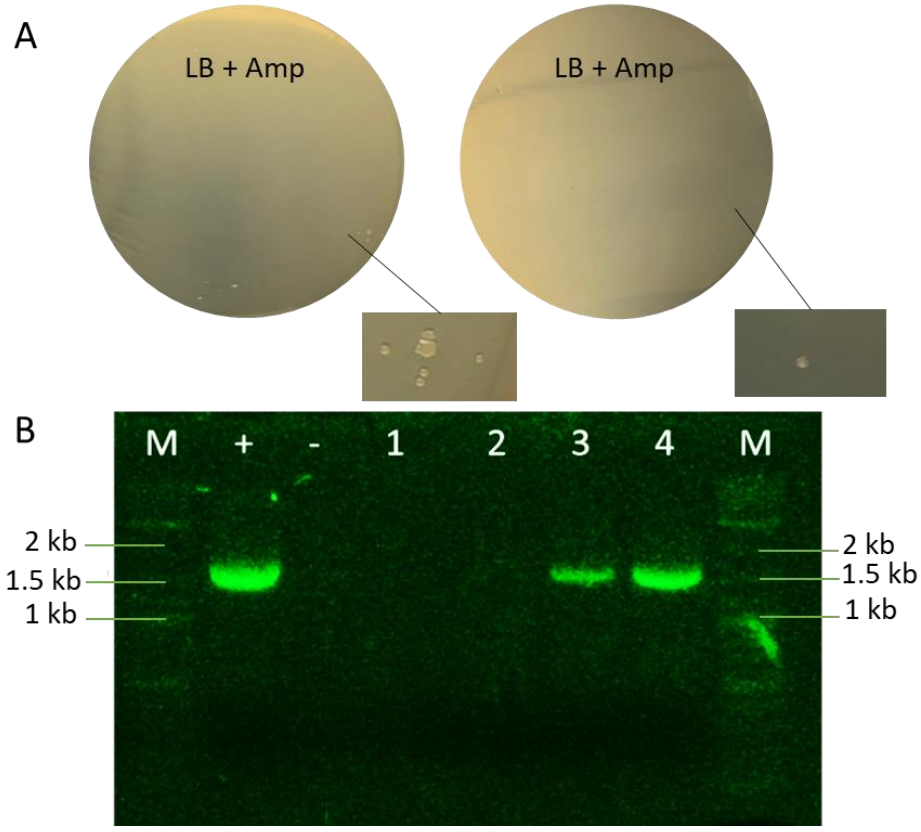

Figure 8. Transformation of pKB into *E. coli* DH5α. **(**A) The plates onto which pulsed *E. coli* DH5α was plated on. (B) PCR amplifying the region of the plasmid containing the origin of replication and the gene conferring ampicillin resistance. The band matches the expected size of 1.6 kb. The lanes are denoted by: M (molecular weight marker), + (positive control), - (negative control), lanes 1-4 (DNA sample from transformed *E. coli* DH5α colonies.

This difference between the two strains is intriguing particularly because of the close relation between these two strains. Since the cells on the LB plate had grown to confluency after one day of incubation of the plates, the parameters chosen for electroporation of the bacteria were not lethal to them, otherwise there would be no growth observed. Having excluded the possibility that the voltage with which the bacteria were pulsed could have been too high and thus detrimental to them, other possible explanations are explored for why the *E. coli* K12 were unsuccessful in taking up the plasmid and expressing the genes that would allow them to grow on either agar plates with ampicillin or agar plates with kanamycin, when the same parameters were used for the *E. coli* DH5α and were successful in producing transformants. When considering the differences between the two strains, it is notable that DH5α is a K12 derivative whose mutations have been designed to give it an advantage in cloning procedures. With *recA* and *endA* mutations, *E. coli* DH5α is designed for maximum transformation efficiency as these mutations grant the bacteria the ability to uptake foreign DNA without making alterations to it that would affect its expression. Given the size of the plasmids used in these transformation experiments which are over 10kb, it is possible that *E. coli* K12 enzymes prevent the expression of the genes present in the plasmid, thus not allowing for kanamycin or ampicillin resistance. The recA protein participates in DNA repair and recombination.[106] Bacterial strains that lack the *endA1* mutation could destabilize any vector inserts that they are transformed with by general recombination taking place in the host.[107] E*ndA* codes for endonuclease I which cuts pure plasmid DNA.[108] Thus, absence of mutations on these genes, allows for DNA degradation.

What is more, as counted with the cell counter plugin of ImageJ,[109] there were 16 CFUs of *E. coli* DH5α present on the plates that the transformants were plated on in this study. 150 μl of culture were plated on each agar plate. Given that the plasmid was extracted from *E. coli* DH5α to

begin with, the low amount of *E. coli* DH5α transformants obtained is less likely due to the strain's inability to carry or express the plasmid. But rather the low efficiency of transformation obtained by the protocol followed to make the cells competent, which involved using washes of 10% glycerol while keeping the cells on ice. Either of these explanations or a combination of both could account for the differences in the results we see for the transformation with electroporation of *E. coli* K12 and *E. coli* DH5α.

Transformation of P. putida KT2440

It is known that *P. putida* KT2440 is capable of growing on ampicillin prior to undergoing transformation and for this reason the plating of this strain on selective media was done on agar with kanamycin. The results are shown in Figure 5. 91 CFUs were counted. Both the verification of the kanamycin resistance genes and the part of the vector containing the origin of replication and the ampicillin resistance were achieved with PCR using primers cpcp_Kan_fwd (TAATAGGCGTTTCCCTTCGTTGCC)with Kan_rev (CAAAATGGTATGCGTTTTGACACATCC), with a band size for all of the colonies screened at the expected size of 0.8kb and primers pmb1_fwd (CCCCCCTGACGAGCATCAC)with AmpR_rev (ATGAGTATTCAACATTTCCGTGTCG) with a band size corresponding to the expected size of 1.6 kb for three of the five samples screened, as seen in Figure 5.

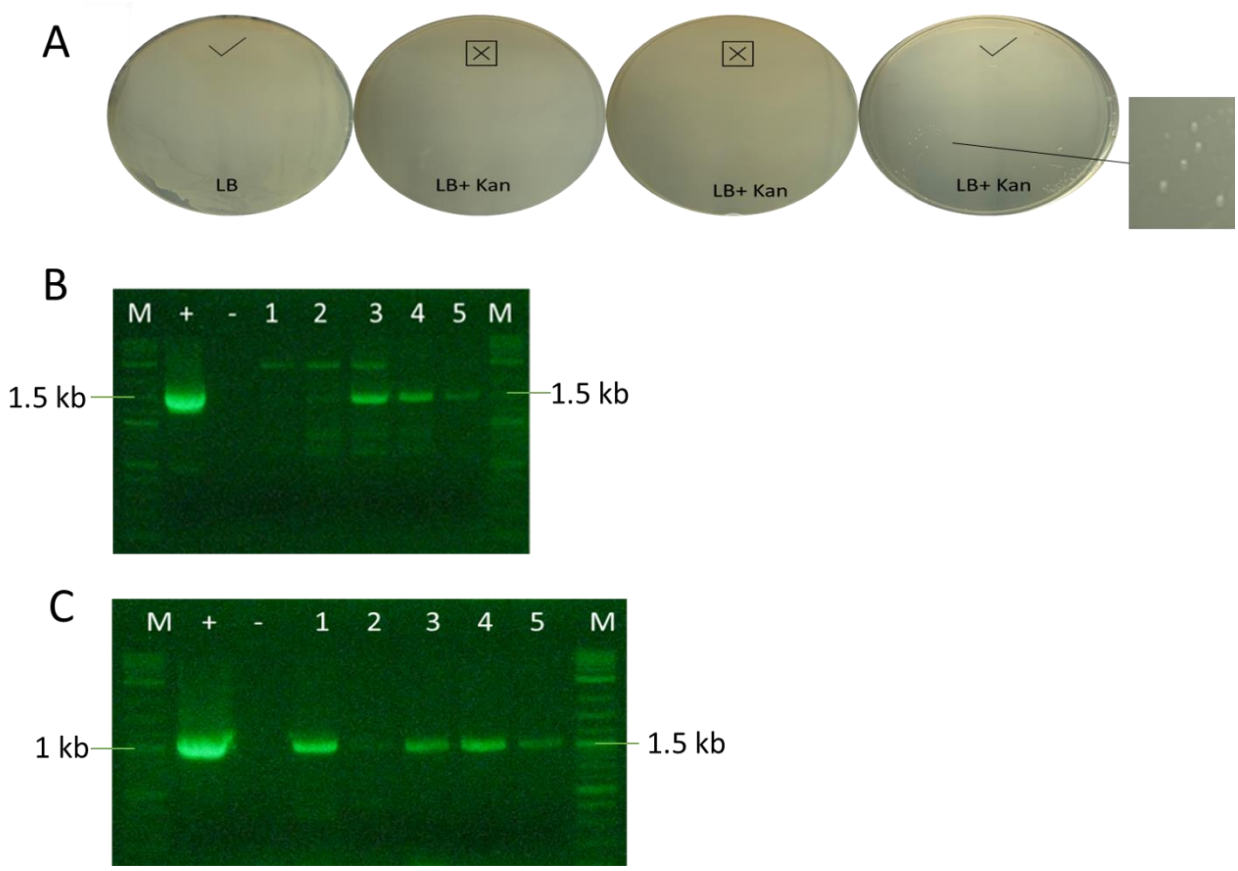

Figure 9. Transformation of *P. putida* KT 2440. (A) Colonies were detected on the plates with kanamycin where *P. putida* was plated. Select clusters are circled to indicate their positions in various locations. The cells on the LB plate grew to 100% confluency. (B) Colony PCR amplifying the region of the plasmid containing the origin of replication, using the primers pmb1_fwd and AmpR_rev. The lanes in (B) and (C) are denoted by: M (molecular weight marker), + (positive control, the pKB plasmid), - (negative control, non-transformed *P. putida*), lanes 1-5 (DNA sample from transformed *P. putida* KT2440 colonies). (C) Colony PCR amplifying the region of the plasmid containing the kanamycin resistance genes, using primers cpcp_Kan_fwd and Kan_rev. The DNA sample from them that was used is from the same colonies that were screened in the PCR in (B).

Together with the results of electroporating *E. coli* K12, *E. coli* DH5α, it is concluded that these bacterial strains are capable of carrying and replicating the pKB plasmid when transformed directly into them using electroporation. The low transformation rates are most likely due to the glycerol protocol followed to make the cells competent, as well as the size of the plasmid. To confirm the former, the pKB plasmid was transformed into competent *E. coli* Top10 cells. To confirm the latter, a control pUC 19 plasmid, with the same origin of replication as the pKB were transformed into the *E. coli* K12 and *E. coli* DH5α cells. pUC19 contains genes of ampicillin resistance so transformants of *E. coli* K12 and *E. coli* DH5α were able to grow on ampicillin plates.

HGT Experiments

The flask setup for the HGT experiments is shown in Figure 7. Flasks with only one type of bacterial strain are used as controls for each type of bacteria used. Co-cultures of the transformed cyanobacteria along with each of the *E. coli* K12, *E. coli* DH5α, and the *P. putida* KT2440 were also set up. All flasks contained a 1:1 ratio of LB to BG-11_0SA medium that added up to a total of 30mL. Over the progression of the two days that these cultures were incubated at 37˚C and shaking, they were monitored, and cell counting was done.

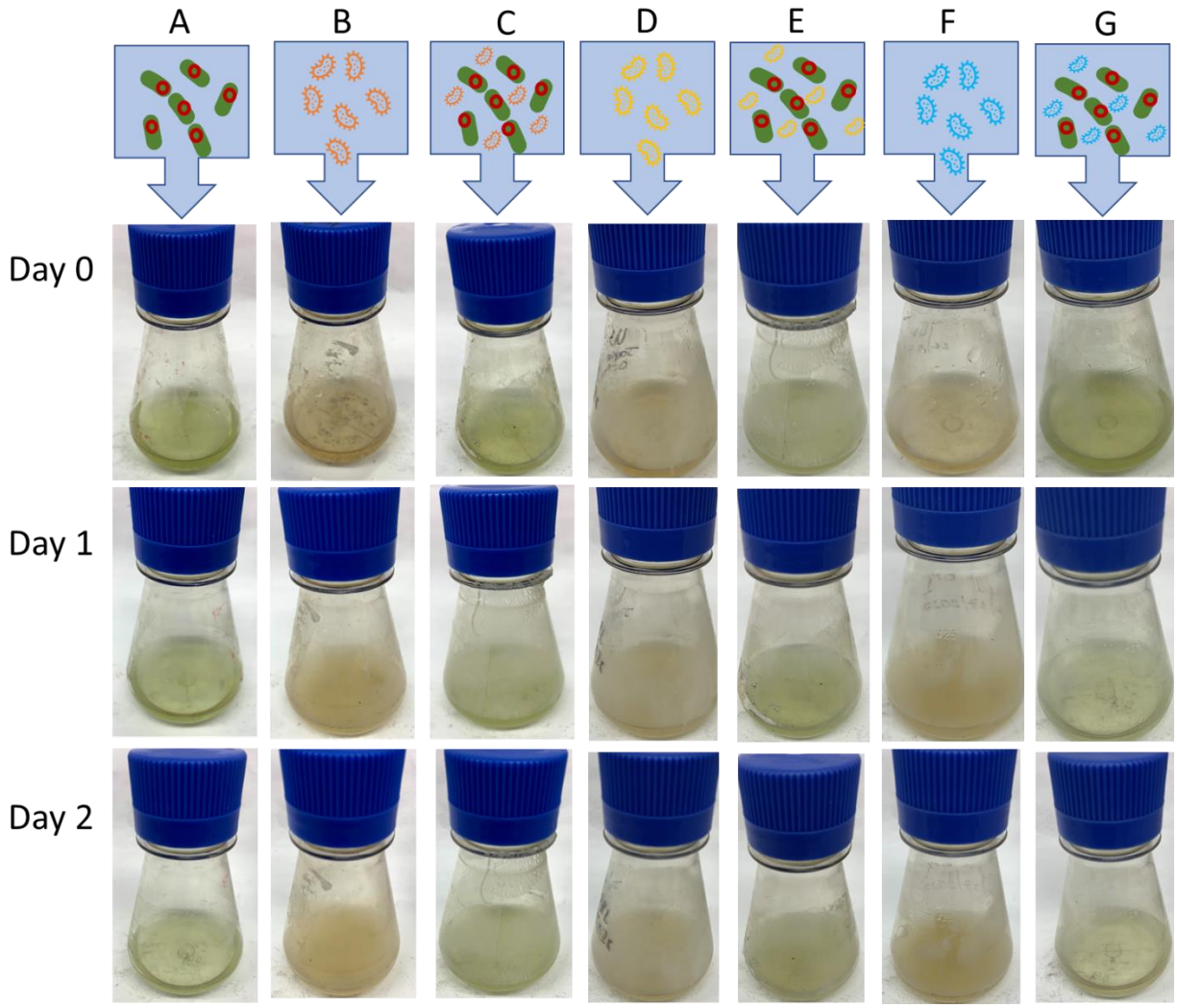

Figure 10. HGT experiments in liquid media. Flasks containing control monocultures, and co-cultures with both the cyanobacteria and each of other bacteria (*E. coli* K12, *E. coli* DH5α, and *P. putida* KT2440) strained were monitored and plated over the course of two days. (A) Monoculture of GE *T. elongatus* BP1. (B) Monoculture of *E. coli* K12. (C) Co-culture of GE *T. elongatus* BP1 and *E. coli* K12. (D) Monoculture of *E. coli* DH5α E. Co-culture of GE *T. elongatus* BP1 and *E. coli* DH5α. (F) Monoculture of *P. putida* KT2440 G. Co-culture of GE *T. elongatus* BP1 and *P. putida* KT2440.

It is observed that after day one the mono-culture and co-culture flasks containing *E. coli* K12, *E. coli* DH5α and the *P. putida* KT2440 monoculture obtain a cloudy appearance, and indication of bacterial cell growth. The mono-culture of GE *T. elongatus* BP1 and co-culture of the cyanobacteria with *P. putida* remain clear. The appearance of the cultures in the flasks is interesting to consider in correlation with cell counting data (Figure 11) that reveal that in the cloudier co-culture flasks there are more *E. coli* K12 (Figure 11A) and DH5α (Figure 11B) bacteria than the cyanobacteria. Conversely, in the cases when the co-cultures appear mostly clear, such as the *P. putida* and transformed BP1 co-culture in Figure 10G, cell counting revealed low *P. putida* KT2440 growth (Figure 11C).

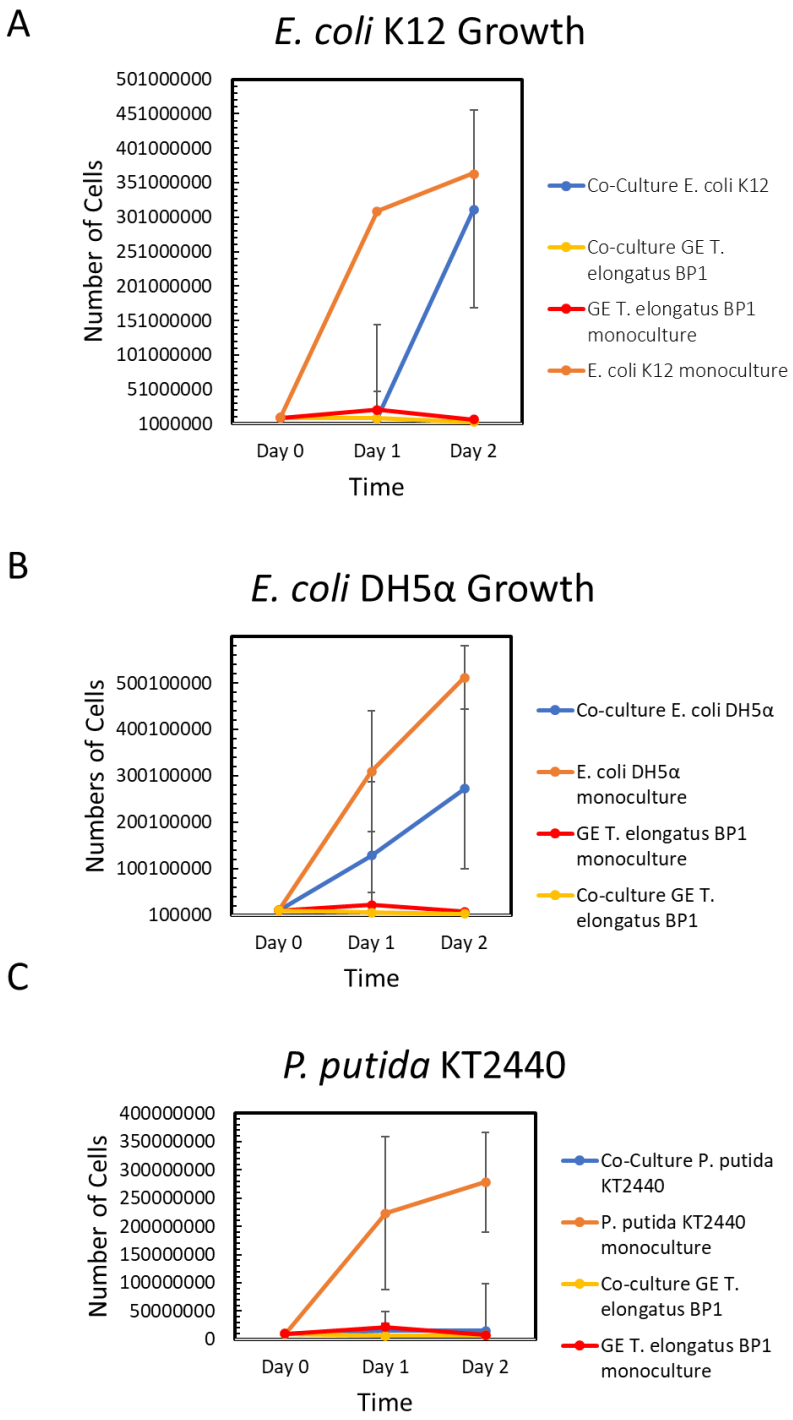

Figure 11. Bacteria growth curves for HGT experiments. Control monocultures of each cell type (orange), along with GE *T. elongatus* BP1 from monocultures (red) and co-cultures (yellow) are shown, while in blue is (A) Growth of *E. coli* K12 cells from co-cultures, (B) Growth of *E. coli* DH5α cells from co-cultures and (C) Growth of *P. putida* KT2440 cells from co-cultures.

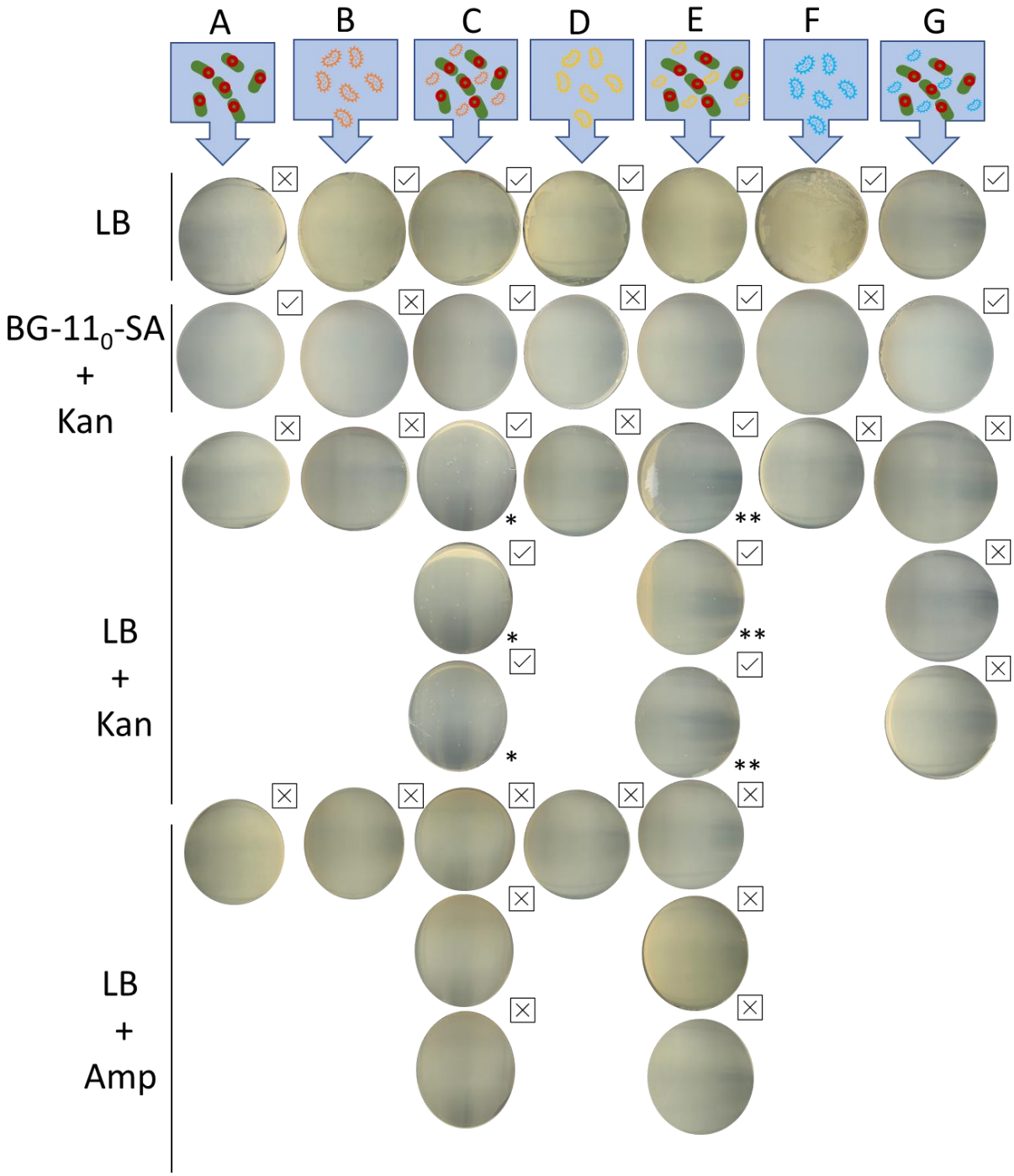

Figure 12. HGT experiments on plates using selective media. The check marks on the top right of each plate indicate that there was growth on the plate after 2 days incubation at 37 ˚C (with the exception of the BG-11$_0$-SA plates that took 7-10 days to present with growth) while the x's indicate that there was not growth on the plate. Growth of *E. coli* K12, the co-culture with

Figure 12 (Continued).

cyanobacteria and *E. coli* K12, *E. coli* DH5α, and the co-culture with cyanobacteria and *E. coli* DH5α was confluent on the LB plates. A-G labels indicate the culture flask from which cells were plated on each agar plate. A. Cells from the monoculture of GE *T. elongatus* BP1. (B) Cells from monoculture of *E. coli* K12. (C) Cells from co-culture of GE *T. elongatus* BP1 and *E. coli* K12. (D) Cells from monoculture of *E. coli* DH5α. (E) Cells from co-culture of GE T. elongatus BP1 and *E. coli* DH5α. (F) Cells from monoculture of *P. putida* KT2440 (G) Cells from co-culture of GE *T. elongatus* BP1 and *P. putida* KT2440. Asterisks indicate plates are shown enlarged for more detail in Figure 12 (*) and Figure 13 (**).

From Figure 12 it is observed that there was no growth on the LB agar or LB agar supplemented with kanamycin (40 μg/mL) or ampicillin (100 μg/mL). The *E. coli* K12, *E. coli* DH5α, and *P. putida* KT2440 cells from the control flasks with the monocultures were only able to grow on LB medium, with no antibiotic, whereas *E. coli* K12 and *E. coli* DH5α that had been in co-culture with the transformed *T. elongatus* BP1 were able to grow on LB agar with kanamycin (40 μg/mL). There were no colonies of *E. coli* K12 or *E. coli* DH5α detected on LB agar with ampicillin (100 μg/mL) from cells from their respective co-culture flasks with cyanobacteria.

P. putida KT2440 grown in co-culture with the GE *T. elongatus* BP1 cyanobacteria did not grow as well as the *P. putida* KT2440 control, as indicated by their decreased numbers (Figure 11C) and the appearance of the co-culture flasks which remained clear even after incubation of 2 days (Figure 10G). In contrast, the control monoculture of *P. putida* KT2440

turned cloudy after day 1 and had increased *P. putida* growth. Lastly, the growth of *P. putida* KT2440 cells from the co-culture with the GE *T. elongatus* BP1 on the LB plates with no antibiotic was of individual colonies and not confluent as is the growth demonstrated by cells from the monoculture of *P. putida* KT2440 plated on LB agar medium.

In addition, no colonies appeared on the LB plates with kanamycin (40 μg/mL) where cells from the *P. putida* KT2440 co-cultures with the cyanobacteria where plated (Figure 12G). The LB plate where cells from the co-cultures was plated did not present with confluent growth of *P. putida* KT2440 but individual pseudomonas colonies after 2 days of incubation. The cells from the control monoculture of *P. putida* KT2440 plated on the LB agar medium were confluent after two days of incubation of the plates at 37˚C. This could be an indication that the cyanobacteria or their metabolic products are toxic to the *P. putida* KT2440, resulting in the latter's destruction when grown together in co-culture. *P. putida* KT2440 was not plated on LB agar with ampicillin because as it is naturally resistant to ampicillin, growth on that antibiotic would not necessarily be a sign of horizontal gene transfer from the GE cyanobacteria.

The BG-11_0SA plates required more incubation time to exhibit colony growth of green *T. elongatus* colonies and were kept for more than 2 days to ensure the cells were able to grow (Figure 12). Green cyanobacterial growth appears on the BG-11_0SA plates from where cells from transformed *T. elongatus* BP1 control monoculture and the co-cultures were plated, after 7-10 days of incubation.

Colony PCR that was performed on selected *E. coli* K12 (Figure 13) and *E. coli* DH5α (Figure 14) colonies from the LB plates with kanamycin 40 μg/mL, confirmed the presence of the genes of kanamycin resistance that were part of the construct transformed into the GE cyanobacteria that the *E. coli* K12 and DH5α were in co-culture with. The bands present in lanes

with DNA from colonies 2, 5, and 6 for *E. coli* K12 (Figure 13) and 2, 4, 5, and 7 for *E. coli* DH5α colonies (Figure 14) are of the expected 1kb band size for the amplified kanamycin resistance genes. The primers used were cpcP_Kan_fwd (TAATAGGCGTTTCCCTTCGTTGCC) and KanN_rev (CAAAATGGTATGCGTTTTGACACATCC).

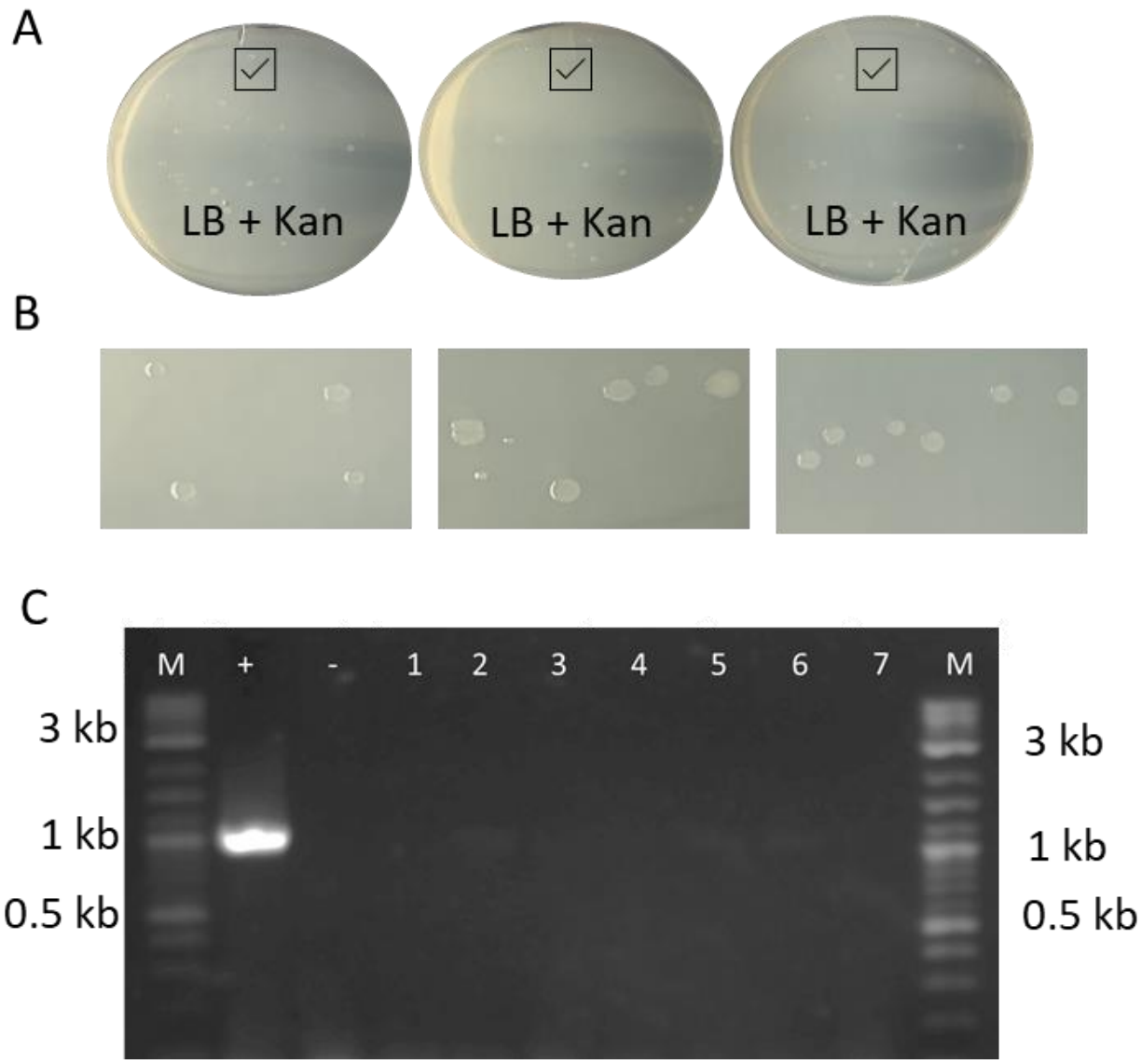

Figure 13. Confirmation of HGT from GE cyanobacteria to *E. coli* K12. (A) The LB plates with kanamycin 40 μg/mL onto which *E. coli* K12 colonies appeared, as indicated by the check marks. (B) A closer look at some of the *E. coli* K12 colonies on LB with kanamycin (40 μg/mL). (C) Colony PCR on the observed *E. coli* K12 colonies labeled 1-7, amplifying the kanamycin resistance gene, produced the expected band at 1kb for samples 2,5, and 6. "M" denotes the molecular weight marker, "+" indicates the positive control used (pKB), "- " indicates the negative control (*E. coli* K12).

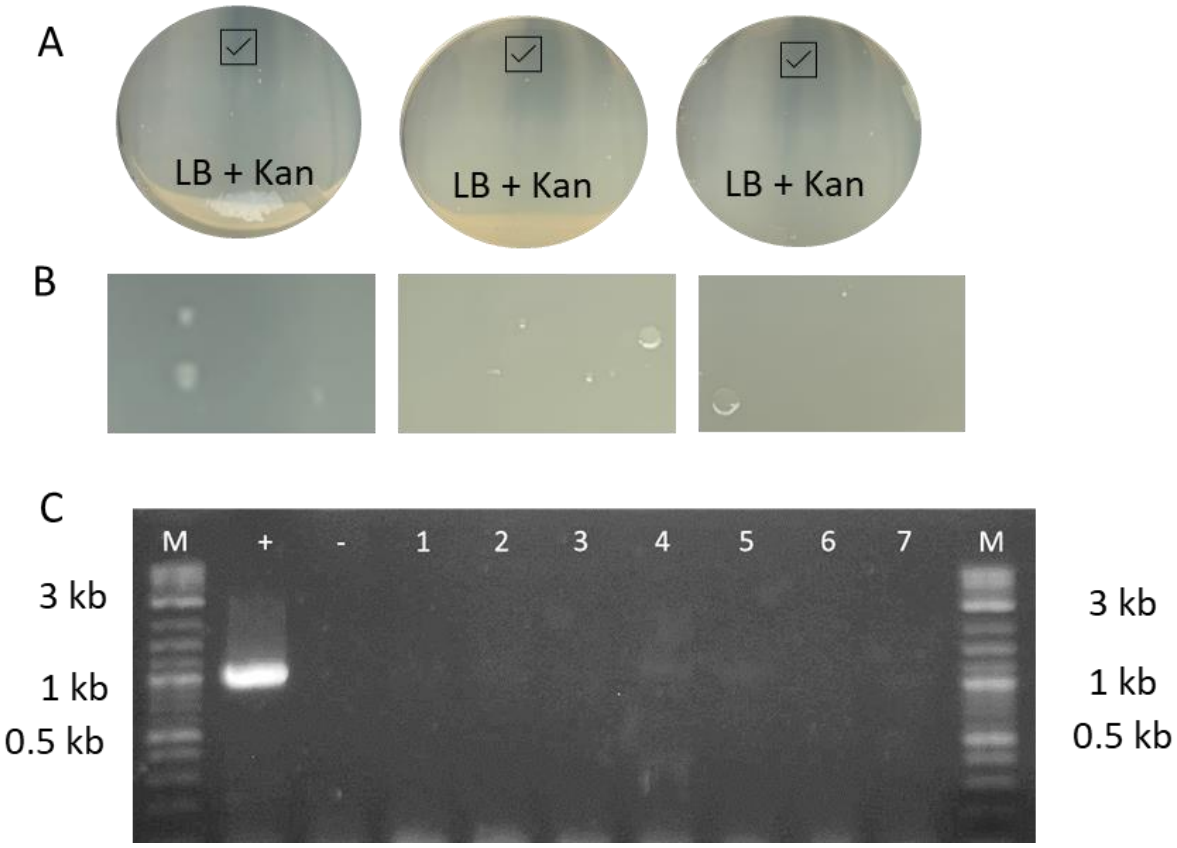

Figure 14. Confirmation of HGT from GE cyanobacteria to *E. coli* DH5α. (A) The LB plates with kanamycin 40 μg/mL onto which cells from co-culture of transformed *T. elongatus* BP1 and *E. coli* DH5α were plated and *E. coli* DH5α colonies appeared, as indicated by the check marks. (B) A closer look at some of the observed *E. coli* DH5α colonies on LB agar medium supplemented with kanamycin 40 μg/mL. (C) Colony PCR on the observed *E. coli* DH5α colonies labeled 1-7, amplifying the kanamycin resistance gene, using primer cpcp_Kan_fwd (TAATAGGCGTTTCCCTTCGTTGCC) and KanN_rev (CAAAATGGTATGCGTTTTGACACATCC), produced the expected band at 1kb for samples 2,4,5, and 7. "M" denotes the molecular weight marker, "+" is used to indicate the positive control used, which was the pKB plasmid, and "- " indicates the negative control used, which was DNA from *E. coli* DH5α cells not grown in co-culture with GE cyanobacteria.

To further validate the presence of the engineered construct in the colonies from the HGT experiments, overnight cultures from individual *E. coli* K12 and *E. coli* DH5α colonies that grew on LB agar medium supplemented with kanamycin (40 μg/mL) after incubation with the GE *T. elongatus* BP1 were grown, and plasmid was extracted from them. PCR was then run to confirm the presence of the gene conferring kanamycin resistance using primers cpcp_Kan_fwd (TAATAGGCGTTTCCCTTCGTTGCC) and KanN_rev (CAAAATGGTATGCGTTTTGACACATCC). The results (Figure 15) show that in 2 out of the 3 samples from plasmid extraction from *E. coli* K-12 (lanes 1-3), and 3 out of the 3 samples from plasmid extraction from *E. coli* DH5α (lanes 4-6) the band of the expected 1kb size was detected.

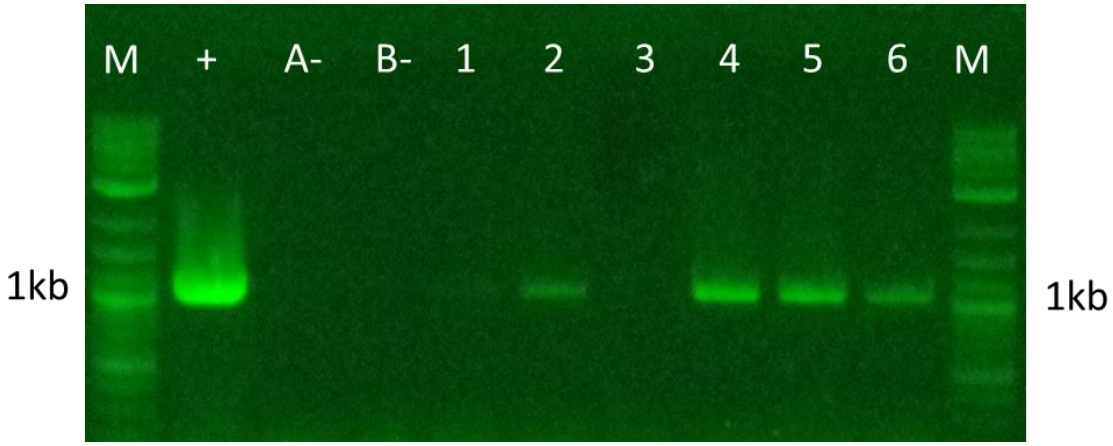

Figure 15. Confirmation of the presence of the engineered kanamycin resistance genes from plasmid extraction of *E. coli* K12 and *E. coli* DH5α. The expected bands appear at 1kb. "M" denotes the molecular weight marker; "+" denotes the positive control (PKB); "A-" denotes the *E. coli* K12 negative control; "B-" denotes *E. coli* DH5α negative control; lanes 1-3 contain DNA sample from plasmid extraction on *E. coli* K12; lanes 4-6 contain DNA sample from plasmid extraction on *E. coli* DH5α.

These findings demonstrate that *E. coli* K12 and *E. coli* DH5α are able to grow in co-culture with GE *T. elongatus* BP1 transformed with the pKB and uptake genes that allow it to be resistant to kanamycin and grow on LB agar supplemented kanamycin (40 μg/mL). Lastly, proof of HGT from the GE cyanobacteria to the *E. coli* K12 and *E. coli* DH5α strains are the sequencing results of the kanamycin resistance sequence from the transformed *T. elongatus* BP1 and the *E. coli* cells that were able to obtain kanamycin resistance after growing in co-culture with the GE cyanobacteria. In Figure 15, the trace data from the sequenced kanamycin resistance gene from the three bacterial strains shows that there is 100% conservation over the region shown.

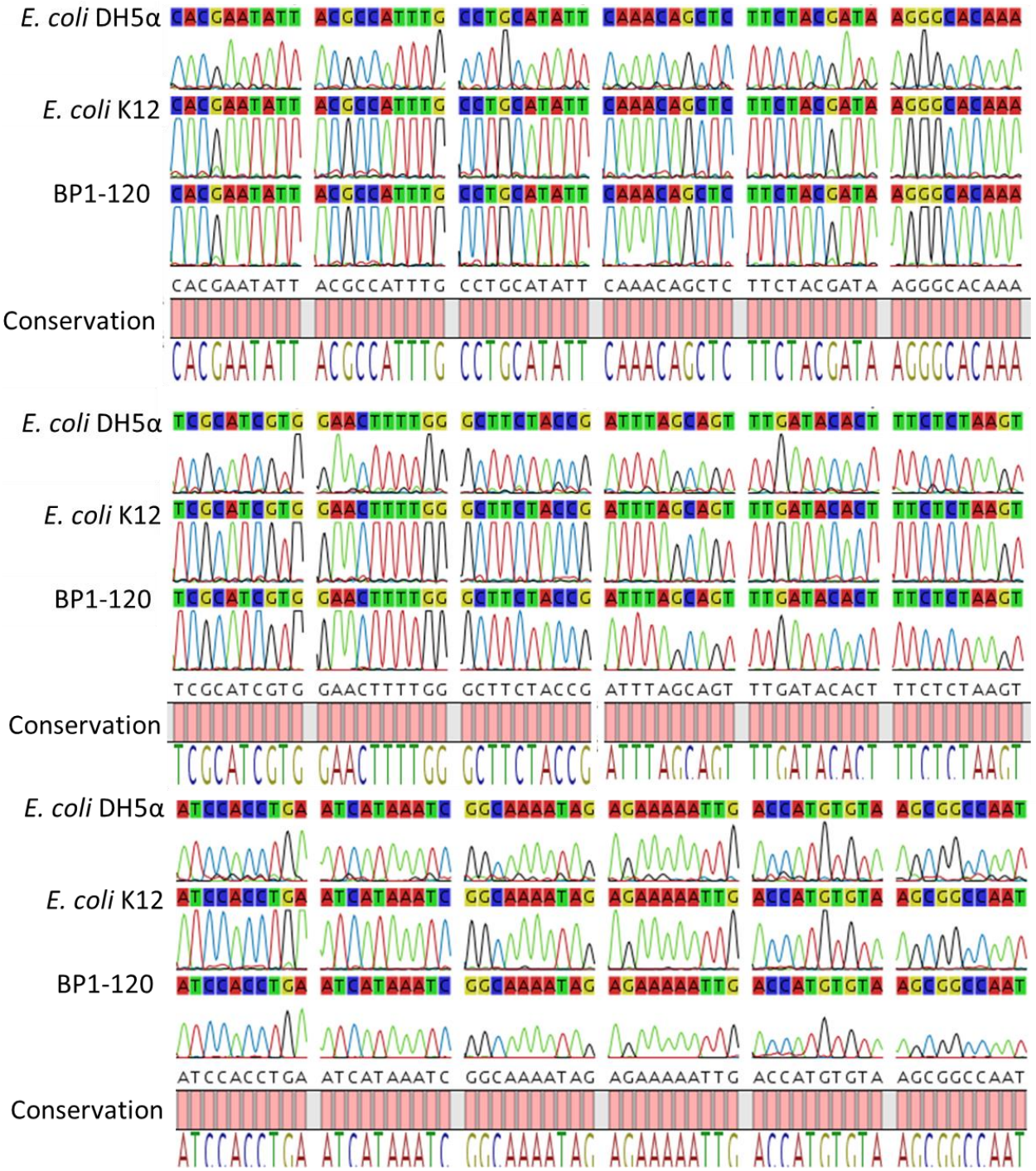

Figure 16. Sequencing of kanamycin resistance gene from transformed *T. elongatus* BP1 (BP1-120) and *E. coli* K12 and *E. coli* DH5α. In this figure, which includes trace date from sequencing, a 270 kb region that is part of the kanamycin resistance gene appears to be conserved among the three types of strains.

Control Studies

The capability of each of the bacterial strains used in the horizontal gene transfer experiments to be naturally transformed by the plasmid in liquid media were assessed. The liquid cultures in which the bacteria were incubated with the 1μg of plasmid were followed over the course of 2 days and each day cells from each culture flask were plated on selective media, containing either kanamycin (40 μg/mL) or ampicillin (100μg/mL), as pictured in Figure 11. *P. putida* KT2440 was not plated on LB agar supplemented with ampicillin (100μg/mL) as it is known that it is tolerant to it. With this setup, the conditions of the HGT experiments were closely mimicked to control for DNA uptake of these bacterial strains from the supernatant.

The bacteria were incubated at 37˚C, with the same concentration of plasmid that the cyanobacteria are transformed with. Cells were plated on selective media to assess their capability of growing on media supplemented with antibiotics. *E. coli* K12 and *E. coli* DH5α were plated on agar media supplemented with kanamycin (40 μg/mL) as well as ampicillin (100 μg/mL). After 2 days of incubation of the plates at 37˚C, the only growth observed was on the LB agar plates, where the cells grew to confluency (Figure 18). No colonies appeared on the agar plates supplemented with antibiotics indicating that the cells incubated with the plasmid did not uptake it from the media.

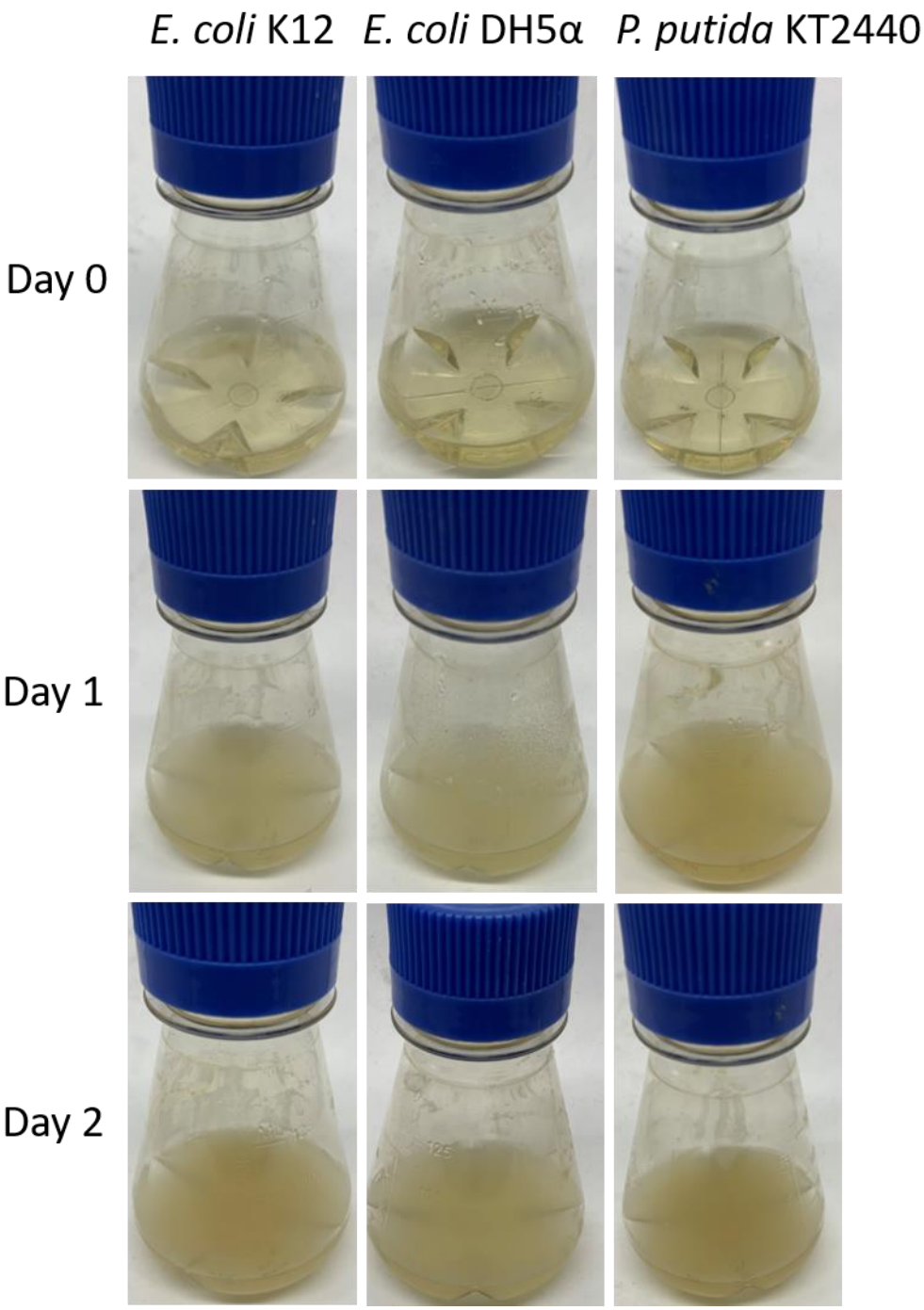

Figure 17. Assessment of bacteria to uptake pKB from liquid media. Cells of each type of strain, at a concentration of 10^7 cells/mL, were incubated at 37˚C for 2 days, in 30mL of 1:1 LB to $BG_0$11-SA media along 1000 ng of the pKB plasmid.

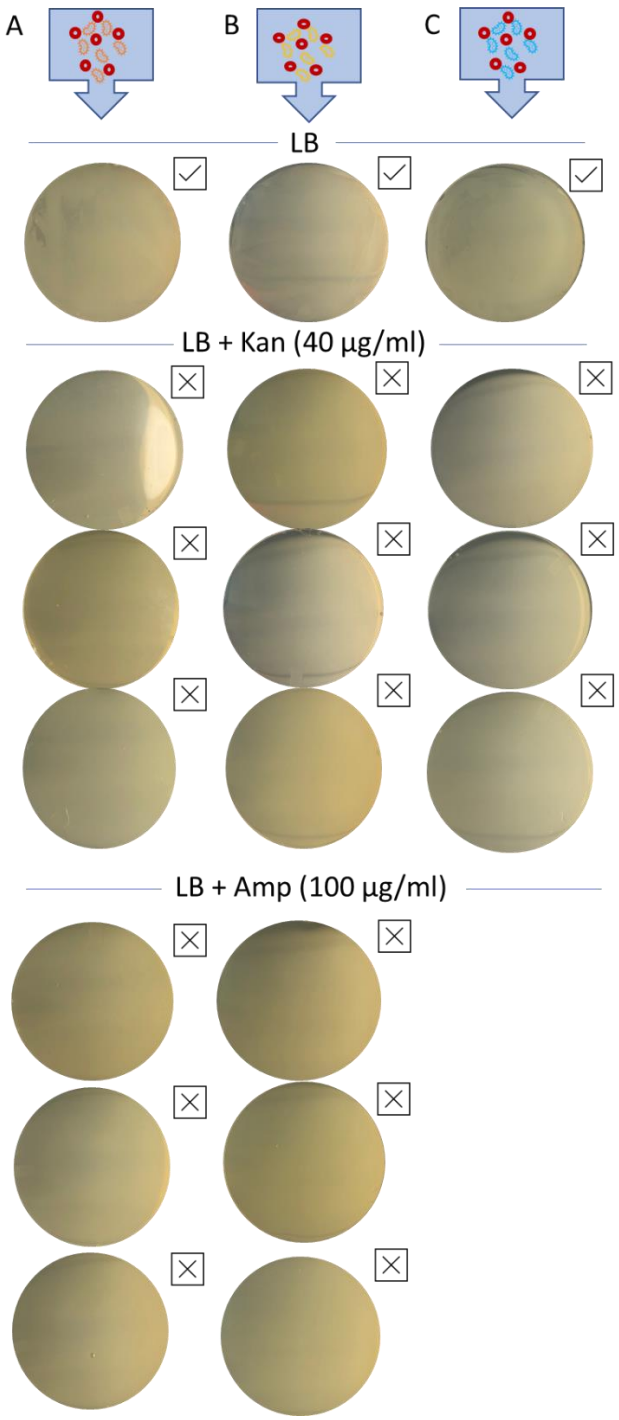

Figure 18. Plating of cells on selective media to assess natural transformation with pKB. (A) *E. coli K12*, (B) *E. coli* DH5α, and (C) *P. putida* KT2440 cells that were incubated with 1000ng of pKB were plated on selective media. The check marks on the top right of each plate indicate that there was growth observed, whereas the X's indicates there was no growth observed.

This is not to suggest that these bacterial strains are not naturally transformable, as there are reports of natural transformation of *E. coli* for instance.[110] However, natural transformation of *E. coli K12*, *E. coli* DH5α, and *P. putida* KT2440, specifically with pKB, was not observed when these experiments were performed in triplicates. Thus, this eliminates the possibility that the HGT observed is an artifact of uptake of naked DNA from the growth media. It is therefore concluded that the *E. coli* K12 colonies (Figure 12A) and *E. coli* DH5α (Figure 13A) colonies that grew on kanamycin (40 μg/mL) are a result of gene transfer from the GE *T. elongatus* BP1 to the bacteria. Verification with PCR further confirms these results (Figure 12C) and Figure 13C).

CHAPTER IV

CONCLUSIONS AND FUTURE WORK

Curious Case of *P. putida* KT 2440 and *T. elongatus* BP1

In summary, this work suggests that HGT of genes conferring antibiotic resistance is possible from GE cyanobacteria to *E. coli* K12 and *E. coli* DH5α. The results for E. coli DH5α are consistent with those reported by Nguyen et. al.[95] However, that is not the case with *P. putida* KT2440, in which case no HGT was observed. One factor that this could be due to is that expression of the plasmids with some of the same characteristics as pKB that was used here has been found to be unpredictable in *P. putida* KT2440.[111] However, since with direct transformation into *P. putida*, the kanamycin resistant genes were able to be expressed, it could be a mechanism of HGT that is not activated in this case. The abnormal growth on the LB agar plate were *P. putida* KT2440 that had grown in co-culture with the GE cyanobacteria were plated, is a perplexing phenomenon, indicating that the *P. putida* KT2440 is not tolerating the cyanobacteria very well. It could be possible that metabolic products secreted by the GE *T. elongatus* BP1 are deleterious to the *P. putida* KT2440. To evaluate whether the GE cyanobacteria are responsible for excreting metabolic products in their media that prevent the normal growth of *P. putida* KT2440 the supernatant of the GE cyanobacteria could be used to culture *Pseudomonas* cells in while monitoring their growth.

The control experiment where the uptake of the pKB plasmid by the bacterial strains from the media is assessed, indicates that it is not possible to occur. This indicates that the HGT via conjugation that is observed is not an artifact of DNA uptake from the cells by the media.

Conjugation is a guided process that requires coordination of the donor cyanobacterial cells and the recipient *E. coli* cells in the cases where it is observed.

We also observed that in cases when transformation via electroporation was possible, HGT was not observed and the opposite. For *E. coli* K12 in particular, while direct transformation via electroporation with the pKB plasmid was not successful, when grown in co-culture with the transformed *T. elongatus* BP1 cyanobacteria, the cells were able to uptake the kanamycin resistance genes. It is reported that the transformation of *E. coli* K12 may be prevented by the DNase activity that other *E. coli* strains, such as *E. coli* DH5a do not present due to the genetic modification they carry.[112] Therefore, HGT of the kanamycin resistance genes indicates that via conjugation *E. coli* K12 bacteria are able to uptake genes via conjugation that could not be directly transformed into them through electroporation. On the other hand, the *Pseudomonas* strain is a case where HGT is not observed, despite direct transformation of the pKB plasmid in the *P. putida* KT2440 cells having been verified.

Limitations

Limitations of this work arise from our inability to completely mimic environmental conditions that the cyanobacteria may be found in, having escaped laboratory containment. However, by lowering the temperature for the growth conditions, in the Percival they are grown at 45˚C whereas they are incubated at 37˚C for the HGT experiments and incubating them in media composition that is different from their preferred media we are testing their ability to survive and transfer genes to other bacterial strains under non-ideal conditions. This type of thermophilic bacterial strain is not capable of growing at temperatures below 30˚C, therefore

there is little concern for spreading genes of antibiotic resistance under those conditions. This way, the thermophilic quality of *T. elongatus* BP1 serves as a biohazard safeguard.

Future Work

The results of this research make it imperative to design strict biohazard containment policies and experimental designs, not only when working with GE cyanobacteria that have been modified for biofuel production in general but for GMO applications and experiments in general. There should also be laboratory containment policies in place put forth by government agencies such as the USDA oversighting GMO research that safeguard against the escape of GE bacteria, as they may pose a threat to human health when genes of antibiotic resistance are part of the engineering process.

HGT experiments from the *E. coli* K12 and DH5α cells that were able to uptake the kanamycin resistance gene from the GE *T. elongatus* BP1 cyanobacteria to other bacteria would reveal if secondary HGT events may be observed. Future work also involves the evaluation of the HGT capabilities of the genes conferring antibiotic resistance from the GE cyanobacteria to more bacterial strains. Specifically, gram-positive strains will be studied in order to acquire a deeper understanding of HGT towards different types of bacteria, as well as how these strains interact with the GE cyanobacteria. Gram-positive bacteria are composed of a thick peptidoglycan wall and their conjugation processes are different than gram-negative.[113] Preliminary experiments are underway with bacterial strains *B. licheniformis* ATCC 14580 and *S. epidermis* ATCC12228. Primers for the identification of the bacterial strains used in the lab have been designed (Appendix A). This research would shed more light on differences between HGT among gram-negative and gram-positive bacteria.

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APPENDIX

PRIMER DESIGN FOR IDENTIFICATION OF BACTERIA *P. PUTIDA* KT2440, *S. EPIDERMIS* ATCC 12228, AND *B. LICHENIFORMIS* ATCC14580

With the introduction of more bacterial strains in the lab, it was fundamental to be able to distinguish between them using a genetic technique. Primers were designed for *P. putida* KT2440 that was used in the HGT experiments as well as *Staphylococcus epidermis* ATCC 12228 and *Bacillus licheniformis* ATCC14580, that are intended for future experiments. Genes were selected based on the characterization previously done on them in the literature. For *P. putida*, primers were designed for the gyrase B gene, which in studies by Yamamoto and Harayama was amplified, sequenced and used for identification of *P. putida* against *E. coli* and *Bacillus Subtilis.*[114] GyrB was selected as the gene to be amplified for the identification of S. epidermis, as it has been found in its more pathogenic *S. aureus* relative to be associated with resistance to antibiotics.[115] For *B. licheniformis* the recF gene was selected that codes for the recF protein and has been characterized in its relative strain *B. subtilis.*[116]

Each of the primer sets (forward and reverse for each bacterial strain) were run on DNA samples from all the bacterial strains used in the lab to ensure that they amplify the target genes only in the strains they are expected to positively identify. Thus, the primer for *P. putida's* gyrB resulted in the band at 2.4 kb only for the sample containing DNA from *P. putida* KT2440, while it was negative for all other bacterial strains (Figure A1A). Similarly, when the primers designed for *S. epidermis's* gyrB and *B. licheniformis's* recF genes, and run through DNA from all the other bacterial strains, they resulted in a 1.9 kb (Figure A1B) and a 1.1 kb (Figure A1C) band for

the identification of *S. epidermis* ATCC 12228 and *B. licheniformis* ATCC 14580 respectively. The off-target band in lane D of Figure A1C) is where the primers designed for *B. licheniformis* amplified a sequence from *S. epidermis*. However, because the band in lane D is at 1.2 kb based on the MW marker, these primers result in positive identification of *B. licheniformis* ATCC 14580. These results show that the designed primers can be used for the positive identification of each of the bacterial strains.

Table A1. Primers designed for bacteria strain identification

Bacterial Strain	*P. putida* KT2440	*S. epidermis* ATCC 12228	*B. licheniformis* ATCC 14580
Gene	GyrB	GyrB	RecF
Forward Primer Sequence	ATGAGCGAAAATCAAACGTACGACTCC	CAGATAATTATGGTGCTGGACAGATACAAG	TATCGTCTTACCGTAATTATGAGCG
Reverse Primer Sequence	GACAGCGCGTTACTTTCGATGAAGT	AGAAATCTAGGTTGGCATAAACTGC	TCAGTCCGATAGCGTGCCATTTTCA
Expected Band Size (kb)	2.4	1.9	1.1

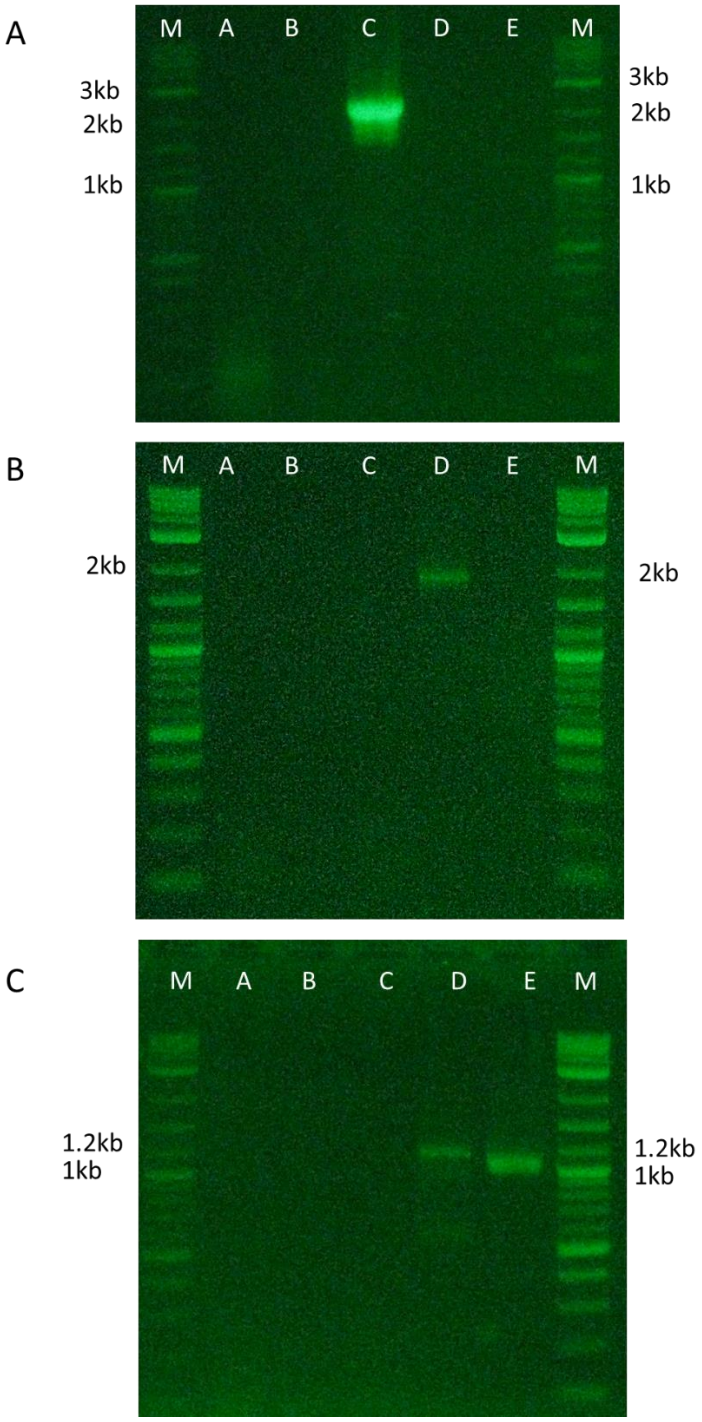

Figure A1. Identification of *P. putida* KT2440, *S. epidermis* and *B. licheniformis* with PCR. Primers designed for each strain in (A), (B), and (C) were run through DNA from all bacterial strains used in the lab (A-E): *E. coli* K12 (samples A), *E. coli* DH5α (samples B), *P. putida* KT2440 (samples C), *S. epidermis* (samples D), and *B. licheniformis* (samples E).

VITA

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