Antibiotic Resistance Screening in Metagenomics Libraries Generated from Cave Soil of Puerto Rico.

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Abstract

After more than 70 years of their discovery, abuse and misuse of antibiotic substances, has selected for pathogens the abilities to confer antibiotic resistances, threatening modern medicine and becoming one of the main health concerns of the 21st century. The development of novel technology to help solve and understand the current trends in antibiotic resistance is a must. By the use of techniques such as metagenomics, the detection and analysis of functional microbial activity in the environment has been made possible through culture independent approaches. In our research a metagenomic library (ML) from cave Ventana's soil in Puerto Rico was generated using the direct DNA extraction method. The cave environment was selected due to the low amount of studies on antibiotic resistance in caves. The ML was produced using the fosmid vectors delivered into *Escherichia coli* Epi300, averaging 40,000 clones. The ML was screened for resistance for some of the first and most broadly used antibiotics. After determining Minimal Inhibitory Concentration for the antibiotics; gentamicin (MIC 8. 0µg/ml), kanamycin (MIC 12.0µg/ml) and tetracycline (MIC 1.0µg/ml), the ML was spread on LB plates containing different antibiotic concentrations. Activity was found for kanamycin and tetracycline. In addition, the presence of fosmid insert in clones was confirmed through an enzyme restriction analysis. The ML showed one clone resistant to tetracycline (MIC 10.0µg/ml) and one clone highly resistant to kanamycin (MIC>1200.0µg/ml). Target genes were inactivated using Tn5 transposon mutagenesis, sequenced and bioinformatics analysis predicted an efflux like mechanism conferring the resistance for tetracycline. More data is needed to determine resistance mechanisms to kanamycin, literature suggests it could be related to aminoglycoside modifying enzymes. This is the first metagenomic library generated from caves in Puerto Rico and the first antibiotic resistance functional study done from caves in the island.

Resumen

Luego de más de 70 años del descubrimiento de los antibióticos, el abuso y mal uso ha seleccionado para que la resistencia a antibióticos emerja entre patógenos microbianos. Esto amenaza la medicina moderna y se ha convertido en uno de los problemas de salud pública en el siglo 21. El desarrollo de tecnologías noveles para resolver y entender la resistencia a antibióticos es crucial. Con el uso de técnicas independientes de cultivo como la metagenómica, la detección y análisis de actividades funcional de genes de resistencia a antibióticos de comunidades microbiana en el ambiente es posible. Se produjo una biblioteca metagenómica (BM) de cueva Ventana en Puerto Rico, la misma fue generada usando el método directo de extracción. El ambiente de la cueva fue escogido debido a la poca cantidad de estudios no cultivables que existen de resistencia a antibióticos en cueva. La BM se produjo utilizando fósmidos como vectores y *Escherichia coli* Epi300 como huésped, resultando en un aproximado de 40,000 clones. Luego de determinar la concentración mínima de inhibición para los antibióticos gentamicina (MIC 8. 0µg/ml), kanamicina (MIC 12.0µg/ml) y tetraciclina (MIC 1.0µg/ml) la BM se esparció en platos con LB con diferentes concentraciones de antibiótico. Se estudió la biblioteca para algunos de los primeros antibióticos que más han sido utilizados a nivel mundial. Se encontró actividad para kanamicina y tetraciclina. En adición, la presencia de fósmidos se confirmó mediante un análisis de restricción. La BM mostro un clon con resistencia a tetraciclina (MIC 10.0µg/ml) y un clon altamente resistente a kanamicina (MIC>1200.0µg/ml). Los genes fueron inactivados mediante mutagenización con el transposón Tn5, secuenciados y un análisis bioinformático predijo que un mecanismo de excreción "efflux" causa la resistencia para tetraciclina. Para kanamicina se necesita más data para poder determinar el mecanismo causando la resistencia, la literatura sugiere que el mismo puede estar relacionado a encimas modificadoras de aminoglucósidos. Este es el primer

estudio donde se genera una biblioteca metagenómica de cuevas en Puerto Rico y el primer estudio de resistencia a antibiótico funcional hecho en cuevas en la isla.

Dedication

I'd like to start dedicating this work to life itself, it is her who sets the stage to lets us dream and make dreams possible.

First, a especial dedication to Marta González Santiago (tití Marta). There was a time in life that I had no guidance, I was lost and there were no role models that could help me out reach for. You saw this and took me as one of your own without the need of doing so, without asking for anything in exchange. You showed me the ways and helped me be the person I am today. Without you I would have never made it. I deeply thank you in my heart as if I were one of your sons. You only dreamed of me having a bachelor's degree to defend myself in life, but this is a step further and not the last one I am to take. Thanks for everything, I will always be grateful and I promise that the teachings you gave me will be transmitted to everyone that comes in my path. Te amo y estoy eternamente agradecido, gracias.

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Jackson W. Foster said in 1964, *never underestimate the power of the microbe…*

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CHAPTER 1

Introduction and Literature Review

1.1 Introduction

Before the discovery of antibiotics humans relied on conventional treatments to cure microbial diseases. At the time, some care options included serum therapies, amputations, rest and cleaning of wounds with abrasive agents. Such accessible treatments were not successful in the cure of infections and the highest mortality rates were caused by microbes (Cohen *et al.,* 1992). In 1937, scientist began unraveling the first commercial group of drugs capable of inhibiting growth of bacteria, the sulfonamides (Davies *and* Davies, 2011) and recognized molecules capable of inhibiting growth of microorganisms as antibiotics. This event in conjunction with the discovery of penicillin and streptomycin, historically produced the base for what is known today as the golden age of antibiotics, where most of today's antimicrobial drugs were discovered. In the 1940's the commercialization of antibiotics provided innovative tools for the cure of microbial diseases, helping lower risks and eradicating certain types of infectious diseases from hospitals. The use of common antibiotics available to combat pathogenic bacteria is no longer of use to health services, due to the emergence and expansion of bacteria resistant to antibiotics (Yoshikawa, 2002; Levy *and* Marshall, 2004; Aminov *and* Mackie, 2007). Growing rates of such resistance has caused a rise in the costs of both public and private health services around the world. This phenomenon not only affects the economy but also raises health complications and death rates. The clinical stage has become a more complicated scenario due to emerging multi drug resistance strains. Additionally the problem expands to other economic fields such as food and farming (IFT report, 2006) where antibiotics are commonly used in low levels as growth promoters for cattle (Lowy et al., 2003; Drlica and Perlin, 2011), pigs, poultry (Lowy et al., 2003; Bager et al., 1997), plants (Liu et al., 2008) and aquaculture (Hirsch et al.,1999; Cabello, 2006). Another aspect to be taken in

consideration is international traveling (Carlet *et al.,* 2012) allowing rapid distribution of antibiotic resistant pathogens.

Most antibiotics came from soil cultivable sources (White et al., 2005); a small effort has been done to produce new antibiotics in the industrial world. Moreover, it's not a cost effective strategy, and requires too much time in order to isolate new prospects for drug development (Projan, 2003). In microbiological sciences, one of the greatest challenges is the cultivation of fastidious species (Kamagata and Tamaki, 2005). In 1985, *Staley and Konopka* noticed under a microscope that cultured dependent approaches did not represent the diversity present in environmental samples; such phenomena was known as *the great plate anomaly*. Further, using PCR techniques that amplified *16SrDNA* regions in both cultivated samples and environment samples demonstrated under representation of microbial diversity (Hugenholz, 1998). Such observations provided the bases for what it is known today as the capability of cultivating microbial species. As of today, the use of culture based methods provides the capacity to recover 0.1-1.0% of the microorganism present in the environment. Pace et al., (1985*)* proposed the idea to isolate and clone environmental DNA from soil samples, however it was not until 1991 when the first report of an environmental fragment cloned inside a surrogated host was attained. Likewise, Handelsman et al. (1998) described the technique and for the first time the term Metagenomics was used. Approaches in molecular biology such as metagenomics provide access to the uncultivable majority 99% of microorganisms in the environment. Functional metagenomics is the study of genomic DNA obtained from the environment, ligated to a specialized vector transferred into a surrogate host. Moreover, the collection of the genomes present in the environment stored inside the isogenic host is known as metagenomic libraries. Metagenomics provides a tool for the understanding of uncultivable microbes and processes in the environment.

Such molecular approach is a tool for studying environmental genome fragments instead of studying individual organisms, allowing up to a 100% capability of screening and selecting for activities present in the environment. With the use of metagenomic DNA's functional screening we seek to understand antibiotic resistant mechanisms available from cave Ventana in the municipality of Arecibo, Puerto Rico. There is little or no information available on antibiotic resistance studies done in caves in the island of Puerto Rico. The study of cave Ventana will provide the first metagenomic library generated from soil in caves of Puerto Rico and the first functional metagenomics study of antibiotic resistance in caves in the island.

1.2 Literature Review

Antibiotic Resistance

In 1973 the World Health Organization defined antimicrobial resistance as "*the ability of a parasite [microbe] strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject"*. Microbial resistance includes a broad arrangement of microbes such as bacteria, fungi, protozoa, yeast and viruses; this study focuses on antimicrobial resistance in bacteria.

Since the antimicrobial drug era started more than 80 years ago, humans have gained the capacity to fight microbial diseases. The production and distribution of novel drug therapies provided successful treatments against the most common diseases in hospitals. After a driven success by the golden age of antibiotics most hospital admissions were related to cancer, diabetes and other non-pathogenic diseases (Cohen, 1992). Subsequently, after production and distribution of antimicrobial drugs, abuse and misuse of therapies favored microorganisms adaptability against drug treatments (NIAIDS, 2012; FDA, 2012; WHO, 2012), this success complicated management of treatments and resulted in emerging resistant strains.

In a medical environment antimicrobial resistance is the condition when a patient is given an antimicrobial drug and it fails to respond to treatment (Drlica and Perlin, 2011). This has brought a number of different consequences to human health that will be discussed further on. The origins of antibiotic resistance remote to the ecology of the microbial communities present in different environments. At first, it was believed antimicrobial resistance was caused by antibiotic producing microorganisms carrying resistance genes to protect themselves from secondary metabolite products (Benveniste and Davies, 1973). Still, after advances in research it has been found that antibiotic resistance is a complex panorama were determinants such as microbial

metabolism, structure and microbial signaling are involved (Martinez, 2009). Resistance can be caused by different horizontal gene transfer "HGT" elements (Thomas and Nielsen, 2005) such as; integrons, transposons, and plasmids. Depending on the type of element conferring the antibiotic resistance these can be mobilized and transferred between bacteria of the same or different species (Allen et al., 2010). The scenario gets even more complex as microbial environments constitute communities with molecules that could present a hormesis effect. Working as antibiotics in a high concentration level and like intracellular signaling, metabolism expression or quorum sensing peptides at lower concentrations (Davies et al., 2006; Martinez, 2009). Microbes compete in different niches in the environment, the diversity of cellular mechanisms present in microbial communities could be so diverse that antibiotic resistance genes could have other functions in nature (Allen et al., 2010).

Overall, antimicrobial resistance can be divided into three forms; acquired, transmitted and intrinsic. Acquired resistance is when a microbial pathogen gains resistance genes from a point mutation in its genome or from lateral gene transfer (Tenover, 2006). Transmitted or disseminated resistance is when a pathogen is already resistant before being exposed to antibiotics (Davies, 1994; O'Brien, 1997). The third type, intrinsic resistance is when pathogens are naturally resistant to a given drug without the need of a specific gene that would confer the resistance, for example; Gram-negative bacteria lack uptake of Vancomycin due to its inability to penetrate the outer cell membrane (Giguere *et al.,* 2006). Antibiotic resistance has at minimum doubled treatment time for resistant strains, raised health-care costs and consequently mortality rates (Andersson, 2010; Nugent, *et al.*, 2010; WHO, 2012). If this problem is not taken care of, the rate of microbial diseases could increase into one of the main causes of hospitalization globally.

In a medical aspect current systems have been developed to monitor the most frequent antimicrobial resistant pathogens such as Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Mycobacterium tuberculosis* (Nugent *et al.,* 2010). Still, there are multiple organizations nationwide and internationally recognized such as; the Alliance for the Prudent Use of Antibiotics, Center for Disease Control of the United States, Center for Global Development, European Center for Disease Control, Food and Drug Administration of the United States, National Institute of Health of the United States, United Kingdom Health Protection Agency and World Health Organization that recognize antimicrobial resistant diseases as a primary worldwide health concern and are working to fight this epidemic.

It has been shown that a 12 month exposure to antibiotics leads to generation of resistant strains (Costello, 2010). Moreover, the Center for Disease Control (2009) estimated health care associated multiple drug resistant organisms in US hospitals to cause 1.7 million infections; from this an approximate of 99,000 deaths occurred (Zell *et al.,* 2007). Bacterial strains such as *Escherichia coli* (Dyar *et al.,* 2012), *Pseudomonas aeruginosa (*Drenkard *et al.,* 2003*), Staphylococcus aureus* (Hiramatsu *et al.,* 1997; Herold *et al.,* 1998; Bergerbachi, 2002; Naimi *et al*., 2003; Klevens *et al., 2007), Streptococcus pneumonia (*Appelbaum*, 1992;* Doerm *et al*., 2012), *Mycobacterium tuberculosis* (Ramaswamy, 1998; Lowy *et al.,* 2003), *Klebsiella pneumonia, Neisseria gonorrhoeae, Salmonella enterica* (Drlica and Perlin, 2011), and *Enterococcus sp.* (Cohen, 1992; Arias, 2012) are some of the most frequently found examples of bacteria that gained resistance.

Economic consequences involve higher costs for treatments of antimicrobial resistant diseases. Treatments are divided into first, second and third line treatments, first of being the most commonly used, followed by more expensive and complex treatments that are named second and

third line treatments. First line treatments for drugs are no longer being effective against resistant strains, forcing to consider or taking into account the higher cost second and third lines of treatments. For diseases such as Tuberculosis (TB) caused by the bacterium *Mycobacterium tuberculosis*, first line treatments range in an average of US $20.00 per patient, while infections with Multiple Drug Resistant Tuberculosis (MDR-TB) requiring second line treatments range in $3,500 per patient (Nugent *et al.,* 2010) reflecting a 175 time fold increase in costs. Moving to a broader spectrum including all bacterial resistant diseases, data reflects that hospital impatiens costs for resistant infections in the US for 2009 reflected an incremental cost of $18,588 - $29,069 per patient, resulting in $16.6 to $26.6 billion increment nationwide (Roberts *et al.,* 2009), while there was a $8.1 billion increment for hospital acquired sepsis and pneumonia infections (Eber *et al.,* 2010). Further on in the United States "from 1974 to 2004 Methicillin Resistant *Staphylococcus aureus* (MRSA) prevalence increased from 2% to more than 50%, resulting in tens of thousands of deaths" (Nugent *et al.*, 2010). Such scenario is not only visible for the United States, antimicrobial diseases have been reported worldwide.

In Puerto Rico, multiple cases of antibiotic resistance bacteria (ARB) have emerged in hospitals. As stated by the government of Puerto Rico in 2004 health informative bulletin; 100% of *Staphylococcus aureus*, 12% of *Enterococcus faecalis* and 56.3% of *Enterococcus faecium* strains isolated in hospitals were Vancomycin resistant. Moreover, 47% of isolated strains of *Streptococcus pneumoniae* were resistant to penicillin. Other previous studies in 1996 and 1998 done in different geographical zones around the island reported antimicrobial resistance for Tuberculosis targeting antibiotics, while in 1998, 88.8% of cases of TB were resistant to antibiotics used (pr.gov, 2004). Likewise previous studies conducted in Gram-negative bacilli in hospitals around the island found isolates to have a significantly higher resistance to beta-lactamic and

amikacin antibiotics (Vazquez *et al.,* 2003). While, in the in the municipality of Ponce a newspaper reported a *MRSA* (La Perla del Sur, 2012) was obtained from the local hospital. Furthermore, the US-News reported a total of 37 states, Washington DC and Puerto Rico to contain antibiotic resistant bacteria capable of resisting more than 15 types of antibiotics (Koebler, 2012). Moving on to additional data from a surveillance program in 1996 to 2003, 328,837 bacterial isolates were collected from 11 hospitals throughout Puerto Rico in conjunction with isolates in 358 U.S. Institutions, targets were studied for antibiotics resistance (Gums *et al.,* 2007). Strains from Puerto Rico were found to be different from one another, while nationwide strains were found to be similar. Antibiotic resistance is present in the island and in some areas resistance has been found to be higher than other countries such as the United States.

Antibiotics

Antimicrobial resistance mechanisms are dependent on the antibiotics mode of action. Antibiotics are defined as natural or chemotherapeutic agents capable of killing or inhibiting growth of microorganisms. There are two classes of antibiotics; bactericidal and bacteriostatic (Madigan *et al.,* 2009). Bactericidal antibiotics are those that kill bacteria, while bacteriostatic antibiotics restrain bacterial growth. Major classes include; aminoglycosides, beta lactams, lincosamides, macrolides, quinolones, sulfonamides and tetracycline antibiotics. *Aminoglycosides* are used mostly to treat infections of Gram-negative bacteria, it's mode of action involves binding to the 30S ribosomal subunit, this causes the peptidyl-tRNA to translocate from the A-site to the P-site on the ribosome causing misreading on the DNA coding sequences and inhibiting protein biosynthesis (Kotra *et al.,* 2000; Madigan *et al.,* 2009). Examples of aminoglycosides include kanamycin (Kang *et al.,* 2012), gentamicin and streptomycin (Hancock, 1981). Beta-lactams mode of action consist in inactivating enzymes in the bacterial cell membrane involved in the cell wall

biosynthesis. The drugs bind to trans-peptidase enzymes that are responsible for the process of trans-peptidation, that results in crosslinking of glycan linked peptide chains (Waxman and Strominger, 1983; Madigan *et al.,* 2009) and examples include penicillins, cephalosporins (Waxman and Strominger, 1983) monobactams and carbapenems (Tenover, 2006). *Lincosamide* class of antibiotics are used to fight Gram-positive bacteria (Rezanka *et al.,* 2007), examples include Lincomycin that is known to be a bacteriostatic antibiotic, they bind to the 23S unit of the 50S ribosomal subunit of bacteria and cause peptidyl-tRNA dissociation (Rezanka *et al.,* 2007; Tenson *et al.,* 2003). *Macrolides* work by binding to the 50S ribosomal subunit of the bacterial ribosome, this inhibits the translocation of the peptidyl tRNA (Kirst, 2002; Gaynor and Mankin, 2005), examples include azithromycin and erythromycin (Scholar and Pratt, 2000). *Quinolones* are a group of broad spectrum antibiotics that interfere with DNA replication and transcription, inhibiting the DNA gyrase and the topoisomerase II enzymes (Madigan *et al.,* 2009). Quinolones and derivatives fluoroquinones are cytotoxic antibiotics (Elsea *et al.,* 1992). *Sulfonamides* are a group of widespectrum antibiotics, the first antibiotic produced Sulfamidocrisoidina (commercial name Prontosil) belonged to this group. They work by metabolic antagonism; they compete with para-aminobenzoic acid (PABA) for the incorporation into folic acid, once sulfonamides are substituded with PABA, folic acid cannot be biosynthesized (Hitchings, 1973; Madigan *et al*., 2009), examples of sulfonamides include sulfacetamide (Onset Therapeutics, 2012) and trimethoprim (Hitchings, 1973). *Tetracyclines* are also a group of broad spectrum antibiotics; they are named for having four (tretra) hydro-carbon rings (Madigan *et al*., 2009). There are proteins that synthesizes inhibitors that bind to the 16SrRNA part of the 30SrRNA ribosomal subunit and prevent amino-acyl tRNA from binding to the A site of the ribosome (Roberts, 2006), examples of tetracyclines include; tetracycline and doxycycline (Ruhe *et al.,* 2005). Antibiotic mechanisms

are diverse between the different classes, at the same time antimicrobial resistance mechanisms depend on the way antibiotics work in order to counter a response.

Antibiotic Microbial Resistance Mechanisms

Microbial resistant organisms take multiple pathways in order to survive exposure to antibiotic treatments. As antibiotics are capable of targeting specific regions in the cells, antibiotic resistance mechanisms are capable of targeting different drugs utilizing different mechanisms. Microbial resistant mechanisms such as efflux pumps (Levy, 1992; Livermore, 2002) help lower antibiotic concentrations in the cell affecting drugs like macrolides (Woods, 2006), cephalosphorines, flouroquinolones (Giraud *et al.,* 2000), penicillin (Livermore, 2002), and tetracyclines (Li *et al.,* 1994). Moreover, other mechanisms bacteria use to obtaining resistance is through the production of microbial polyketide synthases type I (*PKS*) (Hutchinson, 1999), examples of PKS include methylases (Woods, 2006), penicillin binding proteins (PBP) (Lowy, 2003) and beta-lactamases (Berger-Bachi, 2002). These mechanisms inhibit antibiotics in different ways, for example *Erm*-type methyltransferases a type of methylase inhibits macrolide antibiotics by dimethylation of the site A2058 nucleotide in the 23SrRNA (Gaynor and Mankin, 2005), this decreases drug affinity, making it ineffective. Moreover beta lactams mode of action works by inhibiting cell wall biosynthesis of peptidoglycan, PBP's work by binding to beta lactam antibiotics (Nguyen-Distèche *et al.,* 1982) due to their similar structure with peptidoglycans, this is an irreversible reaction that inactivates the beta-lactamic antibiotics enzyme. Further on, beta-lactamases such as penicillase inactivates penicillin a beta-lactamic antibiotic by hydrolyzing its beta-lactamic ring (Pollock et al., 1955). It is known that methylases are capable of inactivating antibiotics such as macrolides and lincosamides, while PBP's are known for their capabilities to inactivating methicillins and penicillins (Waxman and Strominger, 1983), further on beta-

lactamases, can inactivate antibiotics belonging to the families of penicillins (Ghuysen, 1994) and the first classes of cephalosphorins (Livermore, 1995) through the process of hydrolysis. The beta-lactamases break the beta-lactam ring open inactivating the antibiotic molecule. Other types of resistance include chromosomal point mutations, these modify enzyme regions or domains according and or interfere with the antibiotic known to affect DNA synthesis mechanisms such as the topoisomerase IV and DNA-gyrase (Lowy, 2003). Point mutations affect the mode of action of antibiotics that inhibit bacterial replication processes such as quinolones and fluoroquinolones. For example this antimicrobial resistance in quinolones works by mutating the coding sequence of the gene *parC* that encodes the topoisomerase IV structure (Khodursky and Cozzarelli, 1998). This mutation affects the assembly of the enzyme and the antibiotic prevents the binding of the topoisomerase IV to inhibit the bacterial processes. Further on, DNA molecules such as plasmids (Roberts, 2011; Zhang *et al.,* 2011) and mobile genetic elements; integrons (Roberts, 2011; Segal *et al.,* 2003; Zhang *et al.,* 2004) transposons (Roberts, 2011; Kobayashi *et al.,* 2012) horizontal gene transfer mechanisms such as transformation (Barlow, 2009; Hawkey and Jones, 2009) and transduction (Barlow, 2009) have been mechanisms used to transfer resistance genes from one bacterium to another. It is known, that there is a high variability in microbial resistance mechanisms; different mechanisms could cause resistance to the same type of antibiotic. These events produce a highly complex scenario were multiple pathways must be studied to understand resistance to a single type of antibiotic.

Tetracyclines Resistance

Tetracycline forms a complex with Mg^{2+} in the cytoplasm, binding to the 16S ribosomal RNA near the A acceptor site. The antibiotic affects protein synthesis as it elongates the production step; as a result unfolded proteins are produced inhibiting microbial growth. Four

general mechanisms have been found to emerge against Tetracycline; efflux, inactivation of the tetracycline molecules, rRNA mutations and ribosomal protection.

Active efflux of tetracycline (efflux pumps)

Efflux pumps can be divided into 6 groups, which further divide into 21 classes (White et al., 2005). Tetracycline enters the cells through porous channels into the cytoplasm. The mechanisms of resistance by efflux pumps are an active channel that exports the drug out of the cytoplasm by inhibiting intracellular accumulation, moreover making the drug ineffective. Such efflux pumps work in a similar way to Ca^+ and Na^+ sodium pumps, efflux channels are energized by the downhill entry of one proton. Moreover, it is known substrates of Na^+, K^+ and the tetracycline molecule can interchange by external K+ ions.

Degradative inactivation of the tetracycline molecules is one of the most uncommon mechanisms found for tetracycline resistance. Three degrading proteins are known from bacteria; TetX, Tet37 and an unnamed one found in *Bacteroides fragilis* and *Pseudomonas aeruginosa*. The most known is TetX that confers resistance in aerobic microorganisms through a FAD-containing monooxygenase (Yang et al., 2004).

The *rRNA mutations* were discovered in 1998 in *Propionibacterium acnes.* It involved a single base G-C mutation in the 1058 16SrRNA base in *Escherichia coli* JM109 (Ross et al., 2001). Nucleotide 1058 is located in the helix 34 of the 16SrRNA, leading to a disturbance in the base pairing of helix 34 that interferes with tetracycline binding at the site (Bauer et al., 2004 ,Sanchez-Pescador et al., 1998) .

Ribosomal protection. In general there are eleven ribosomal protection genes with similarity to the ribosomal elongation factors EF-G and EF-Tu that encode for the protection of the ribosomes from tetracycline both in-vitro and in-vivo (White et al., 2005). The most studied

ribosomal protection determinants are Tet(O) and Tet(M), originally isolated from *Campylobacter jejuni* and *Streptococcus spp.* It is believed this proteins affect the assembling of tetracycline to the Tet-1 site in the 30S subunit of the ribosome (Connell, 2003). They also hydrolase GTP to exit the ribosome after removing the antibiotic (Connell et al., 2003).

Aminoglycosides Resistance

Aminoglycosides bind to the major grove of the 16SrRNA were they make contact with intermediary water molecules, this in turn displaces the proofreading bases A1492 and A1493 (White et al., 2005). This leads to cell lysis due to a compromise in cellular integrity by misfolded or mistranslated membrane proteins (Davies, 1987). Aminoglycosides have three resistance mechanisms; reduced uptake or decreased cell permeability, alterations in the ribosomal binding site and enzymatic modifications.

Reduced uptake or decreased cell permeability is the effect of aminoglycoside efflux pumps. The Gram-negative bacteria contain a region known as the resistance nodulation division (RND), this is known as the efflux system relevant. The RND will work in conjunction with a membrane fusion protein (MFP) and an outer membrane factor (OMF) to pump aminoglycosides out of the cell. Different variations of the efflux genes will be present in different microorganisms. The most known three models are the *Burkholderia pseudomallei* (Moore et al., 1999), *Escherichia coli* (Rosenberg et al., 2000) and *Pseudomonas aeruginosa* (Aires et al., 1999). They consist of efflux mechanisms paired with the RND, MFP and OMF components.

Enzymatic modifications are the most common type of resistance for aminoglycosides. Some involve: O-phosphotransferases (APH), N-acetyltransferases (ACC), and O-adenyltransferases (ANT).

Aminoglycoside phosphotransferases will catalyze ATP dependent phosphorylation of the hydroxyl group. Aminoglycoside phosphotransferases differ regiospecifity of phosphate transfer to the aminoglycoside structure I, where 7 different sites for detoxification by phosphorylation have been identified: APH(3'), APH(2"), APH(3"), APH(6'), APH(9), APH(4) and APH(7"). The most common are APH(3')-IIIa and APH(2")-Ia (White et al., 2005).

Aminoglycoside acetyltransferases are the largest group of aminoglycoside inactivating enzymes; it includes more than 50 unique enzymes that affect Gram-negative and Gram-positive bacteria. They are enzymes that catalyze acetyl-CoA dependent acetylation of an amino group (Mingeot-Leclerco, 1999). They are divided into four classes, based on the regiospecificity of the acetyl transfer, the main groups are: ACC(1), ACC(3), ACC(2') and ACC(6') (White et al., 2005).

Aminoglycoside adenyltransferases catalyze an ATP-dependent adenylation of the hydroxyl group. There are four classes of aminoglycoside nucleotidyltransferases; ANT(6) "Gram-positive", ANT(4'), ANT(3") and ANT(2") "Gram-negative" (White et al, 2005) .

Screening for resistance

Traditional methods to fight antibiotic resistance included searching for new drugs to match off emerging resistant microorganisms. Still a problem arises; most of the known antimicrobial natural products were obtained from cultivable microorganisms. Being one of the main reasons microbial resistances is arising due to the lack of new treatments. Most treatments available have been around for more than 50 years (Nugent et al., 2010), giving a chance for bacteria to evolve into resistant strains. Cultivable drug screening has its limits, as only 1% of known microorganisms are cultivable (Staley and Konopka, 1985; Pace et al., 1991). Further on at this point research on antibiotics has been undertaken solely on academia as pharmaceutical industries have abandoned the search for new antibiotics due to the lack of income obtained in

comparison to life quality drugs (Drlica and Perlin, 2011). The uses of culture independent strategies provide access to a higher percentage of genomic DNA available from uncultivable microorganisms. Culture free approaches such as metagenomics provides tools to unravel new antibiotic resistant mechanisms that can lead to further understand AR and to generate novel antimicrobial drugs. Examples of such mechanisms will be described further on.

Metagenomics

In 1985 *Staley and Konopka* noticed under a microscope that cultured dependent approaches did not represent the diversity present in environmental samples, such phenomena was named "the great plate count anomaly". Further on PCR techniques that amplified *16SrDNA* regions in both cultivated samples and environment samples (Pace *et al*., 1991) demonstrated microbial diversity was underrepresented. Such data provided the base to establish what was known and unknown about our microbial environments. As of today it is known only 0.1-1.0% of microorganism have been cultivated using classic methods in microbiology. Still, only 1.0% of the microbiota represented using cultivable media novel enzymes, drugs and further natural products such as antibiotics have provided advances in today's society. Approaches such as optimizing isolation and cultivation of less cultivable microorganisms could provide access to part of the unrepresented 99% of microorganisms. Low effort is focused on such processes as they required numerous time and labor consuming research. Some examples of classic methods to isolate resistant organisms include cultivation of known pathogenic species such as *Escherichia coli* (Dogan *et al.,* 2012), *Helicobacter sp.* (Shabestari *et al.*, 2012) and *Staphylococcus aureus* (Zmantar *et al.*, 2012), for study of its resistance genes. Other types of cultivable approaches include studying different environments such as manure (Korhonen et al., 2012; Wang *et al*.,

2012), human microbiota (Lacroix and Walker, 2012; Ready *et al*., 2012) and agricultural soils (Onan and LaPara, 2012) for resistant strains.

Approaches in molecular biology such as the field of "Metagenomics" provide an entry to the underrepresented microorganisms in the environment. Functional metagenomics is the field that takes environmental genomic DNA from a source; ligates that DNA into a specialized vector (fosmid) and then places the vector into an isogenic cell to find functional and sequencing aspects, providing an approach to study uncultivable microbes. Since its ability to deliver access to the DNA available in the surroundings, metagenomics provides a prevailing tool for the understanding of understated microbes and processes in the environment. In 1985 Pace and colleagues proposed the idea to isolate and clone environmental DNA from soil samples. It was not until 1991 when the first report of an environmental fragment cloned inside a surrogated vector was attained (Pace et al., 1991). Moreover, in 1998 Handelsman and colleagues described the technique and for the first time the term metagenomics was used (Handelsman *et al.,* 1998). While searching soil environments allows for the potential discovery of novel drugs, due to the need of natural defense mechanisms between bacteria, studying soil communities can provide novel and unknown antimicrobial resistant mechanisms (Donato *et al.,* 2011). Also it is known that soil environments contain the vast majority of resistance mechanisms (Hopwood, 2007).

Direct DNA Extraction Method

Previous studies of species richness estimate soil to contain from 8.3 million to 2,000 cells per gram (Gans et al., 2005, Scholls and Handelsman, 2006). Moreover up to 1% of total bacteria are estimated capable of growing in laboratory conditions (Amann et al., 1995). The remaining 99% of bacteria are out of reach with the use of cultivable approaches. The use of molecular techniques such as metagenomics allows access to DNA from uncultivable populations.

Metagenomics fascilitates the expression of genes with the use of a surrogated host (Liles et al., 2007), allowing selection of novel enzymes related to antibiotic resistance.

In order to obtain a representative metagenomic library from an environment, access to high molecular weight DNA is imperative (Delmont et al., 2011). There are different ways that DNA can be obtained from soil, two consistently used methods are the direct and indirect DNA extraction methods. Both methods provide a high quality molecular weight DNA. Still, they consist on different approaches that select for certain microorganisms in the environment. The most regularly used strategy the direct method (Delmont et al., 2011) consists on extracting DNA directly from the soil matrix (Bertrand et al., 2005). The major advance in the direct DNA extraction methods is that it provides a higher yield in quantity of DNA with low DNA size, the indirect method provides a lower yield in DNA with high DNA size (Delmont et al., 2011). Further on, its major drawback is that the DNA is sheared in the extraction process (Jacobsen and Rasmussen, 1992, Bertrand et al., 2005). Another drawback of the direct method is that when compared to the indirect method the purity of the DNA will be compromised by the presence of contaminants such as humic and fulvic acids (Berry et al, 2003). Still, the method is good in order to obtain a high molecular weight DNA for functional antibiotic resistance screening.

Metagenomics Antibiotic Resistance Studies

Metagenomics has been previously used to identify antimicrobial resistance genes, mechanisms and communities with much success. The ability to functionally express environmental genomic DNA inserts in surrogated hosts, allows for screening novel resistance genes. Previous studies involve isolation of antibiotic resistance genes and resistant related secondary metabolites from multiple source environments on a vast variety of antibiotics such as aminoglycosides (Riesenfeld *et al.,* 2004, Donato *et al.,* 2011, Torres-Zapata *et al.,* 2012),

tetracycline's (Martiny *et al.,* 2011), beta lactams (Allen *et al*., 2009; Donato *et al.,* 2011) and trimethoprim (Torres-Cortes *et al.,* 2011). Not only genes have been discovered, resistance mobile elements such as plasmids (Szczepanowski *et al*., 2008), transposons and insertion sequences (Zhang *et al.,* 2012) have come across using metagenomics. Plasmid examples include; pST2 which contains beta-lactam resistance and pST10 which contains transposase ISPps1 (Zhang *et al.,* 2012).

Metagenomics in Caves

A cave is a natural space formed below the surface by weathering of rocks and sediment (Eleanor, 2002). Caves form by a process of erosion, were water combines with limestone slowly forming the structure in a process that can take millions of years. In Puerto Rico there is a northern and a southern region covered by limestone where caves and karts topography prevail (Peck, 1974). These regions are abundant with caves. After an extensive research, data implies there are no cave studies of microbial sources using metagenomics or antibiotic resistance in the island. Furthermore, general studies of metagenomics in caves are scarce (Lee et al., 2014). The most relevant include the study on acid cave biofilms, where metagenomics, rRNA, culture-dependent and lipid analyses were used to study the microbiota in the Frasassi cave system in Italy (Jones *et al.,* 2012). Other metagenomic studies include identification of ammonia-oxidizing archaea in the radioactive thermal caves of the Australian Central Alps (Bartossek *et al.,* 2012). More recent approaches include studying energy dynamics in carbonate caves (Ortiz et al., 2014). Previous cultivable approaches have studied antibiotic resistance in caves (Bhullar et al., 2012), confirming the presence of antibiotic resistance in an un-impacted source, isolating resistant determinants for 26 antibiotics (Kirandeep Bhullar, 2011).

Figure 1.1 *Map of cave Ventana.* Located in the sector Hato Viejo in the municipality of Arecibo, Puerto Rico. Image shows highlighted in red the municipality of Arecibo in the map of Puerto Rico. Red star indicates location of the cave.

A metagenomic library was completed from samples collected from soil in cave *Ventana* (CV) located at the municipality of Arecibo, Puerto Rico. The temperature at the sample site ranged from 25 to 28ºC, pH at 8.10 and GPS coordinates were 18º22'15"N; 66º41'27"W. *Ventana* is one of the 212 registered caves located in the Karst region of the island, containing 19% of the terrestrial surface and extending 1,760 km^2 (680 mi^2) through the northern part of the island. The cave provides an environment with a mineral composition of mostly limestone with stable parameters throughout the year consisting of high humidity, constant high temperatures and low light sources. Moreover, soil samples were collected surrounding a stalagmite formation. The resulting library was used for functional screening of microbial antibiotic resistance to three antibiotics.

Figure 1.2 *Soil sampling site at cave Ventana.* Samples were taken 2 inches apart from the formation of the stalagmite. Samples were taken 2.0cm from the surface, until 60.0g of soil were collected. White arrow indicates sampling site.

Chapter 2

Construction of Large Insert Metagenomic DNA Libraries from Cave Soil

2.1 Introduction

Microorganisms represent the most abundant genera in the phylogenetic tree, they can be found present in almost every environment, impacting locations, their organisms and inorganic resources at a daily basis. Non-cultivable approaches such as metagenomics provide a tool to study and characterize microbial communities. Functional metagenomics consists on expressing genes contained within genomic DNA from the environment. Such process is done by utilizing a surrogated host such as *Escherichia coli* (Warnecke et al., 2007), *Streptomyces lividans* (*G.Y.S.* Wang et al., 2000, McMahon et al., 2012) *or Bacillus subtilis* (Biver et al., 2013). These hosts provide the machinery necessary to read foreigner DNA into functional proteins. The generation of functional metagenomic libraries allows us to screen for target functions such as antibiotic resistance (Allen et al., 2010; Donato et al., 2010).

A metagenomic library was constructed from high molecular weight DNA from cave Ventana in Arecibo, Puerto Rico using the direct DNA extraction method.

2.2 Methods

Soil Sampling Site

Soil Samples (60.0g) were collected from cave Ventana in Arecibo, Puerto Rico. Samples were obtained from soil surface to a depth of 2.0cm. All soil samples were collected using sterile spatulas and placed inside sterile Whirl pack® bags. After collection samples were transported to the laboratory, kept on ice; this process took approximately 6 hours. The location of the samples was determined with a Global Positioning System (GPS) to obtain exact coordinates of sampling site. Once in the laboratory, dilutions from 10^{-3}-10^{-6} in 0.85% NaCl were cultivated at 37ºC and 25ºC on LB Agar Petri dishes, this was done to determine colony forming units per gram of soil. The remaining soil samples were placed at -80ºC, to preserve cells in the soil matrix. The necessary documentation and the permits required for the soil collection were sent to the Department of Natural Resources of Puerto Rico.

Generation of Metagenomic Libraries

DNA Extraction from soil

The process consists in isolating high weight molecular DNA from soil. Afterwards, the DNA is cleaned and prepared for insertion in a surrogated host. Once the DNA is inside the cells it can be studied for functional activities.

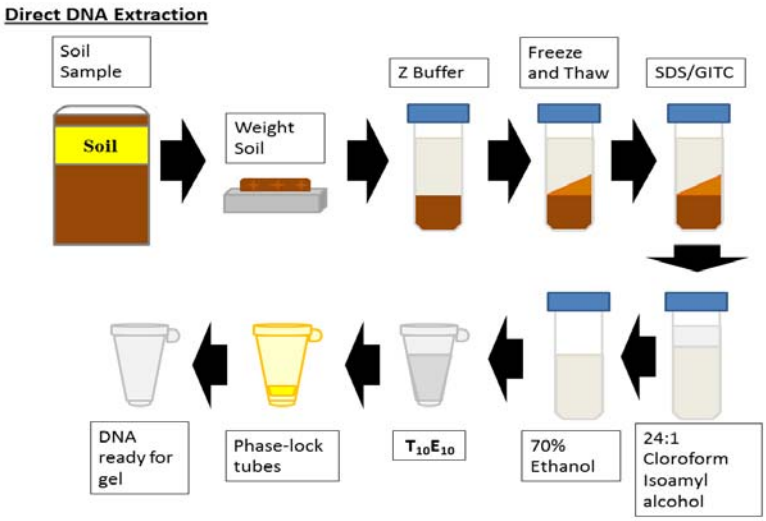


Figure 2.1 *DNA Extraction Method.* Flowchart for extraction of DNA using the direct method. First a soil sample is collected, soil is weighted, washed and DNA is lysed directly from the matrix. The extracted DNA is transformed inside a surrogate hosted by a specialized vector.

The soil was passed through a sterile sieve (mesh size 2.0 mm), allowing all no-soil materials such as rocks and plant material to be removed from the sample. Then 30.0g of soil were washed in Z buffer (100mM Tris-HCl pH 8.0, 100mM sodium phosphate solution, 100mM EDTA, pH 8.0, 1.5M NaCl, 1.0% CTAB [hexadecyltrimethyl ammonium bromide]), the solution was gently mixed in order to allow homogenization, the buffer helps to break the cells un-stabilizing the cell wall, 250 ml centrifuge bottles were used to mix soil and Z buffer. Optional 1.0mg/ml lysozyme was added and incubated at 37ºC for 15 min, this helped to break the cell walls. Then they were freeze-thawed (3 times) using about 2.0lb of crushed dry ice, adding isopropanol or

ethanol, this provided a ~ -80ºC temperature and allowing the cells to freeze for 40 minutes. Later on, soil/Z buffer solution was thawed in a 65ºC water bath (40 min). Further on 9.0ml of 20% SDS and 4.5ml of 5.0M GITC were added (mixed gently). Samples were incubated at 65.0ºC for 2 hours with occasional gentle mixing and centrifuge for 20min, 15,000 x g, at 10.0ºC. Supernatant were transferred into a clean centrifuge bottle. A DNA extraction was done using chloroform:isoamyl alcohol (24:1) gently mixing for 10 min. Samples were centrifuged, 15,000 x g, for 20 min at 10ºC, supernatant containing DNA was passed into sterile centrifuge bottles. Then DNA was gently mixed for 5 minutes with a 70% volume of isopropanol. Sample was left undisturbed for 20 min at room temperature, allowing remaining particles from soil to settle at the bottom of the bottle. Supernatant was recovered and samples were centrifuged at 15,000 x g, 40 min at 10.0ºC and supernatant was carefully discarded, this time the pellet contained the DNA. Pellet was re-suspended with 2.0ml of $T_{10}E_{10}$ (pH 8.0, 10.0mM Tris-HCl, 10.0mM EDTA). Using a widebore pipette tips, liquid was pipette in microcentrifuge tubes. The use of these tips helped diminish shearing of DNA. Furthermore, DNA was extracted with an equal volume of Tris buffered phenol-chloroform, inverted a few times and centrifuge maximum speed at room temp for 10 min in Phase-lock tubes, allowing the removal of residual amounts of lipids and proteins. Aqueous solution on top layer containing DNA was removed and extract once with chloroform:isoamyl alcohol, once again using Phase-lock centrifuge tubes. Then 2.0µl were removed from solution containing DNA and the concentration was measured using Eppendorf Biophotometer. Then DNA was stored at this phase, long term -80ºC and short term 4ºC. Soil remains were inactivated using sodium hypochlorite, all tools used for the procedures that could be autoclaved were autoclaved to eliminate possible pathogens present in soil.

DNA Sizing Gel

A pulse field gel electrophoresis (PFG) was prepared and BioRad PFG machine field with 2.0L of 0.5x TAE. Samples were placed in a low melting point (LMP) agarose gel and sealed with LMP agarose. A mid-range yeast marker and control DNA (~40kB) were used as DNA size selection guides. The PFG gel ran for 4 hours at 14ºC, 120º angle. After the gel run, a selection of 25-40kB DNA was done through a sizing gel. The sides and center parts of the gel were removed and stained; they were measured with a cm ruler to determine location of DNA, control DNA and yeast ladder marker were used as guides. The gel containing target DNA was cut using a sterile sharp blade in a rectangular form, these called "noodles".

Noodle Electroelution

Spectra/Por® molecular porous dialysis membrane tubing with a 45.0mm width and 29.0mm diameter were prepared by boiling in sterile water (SMQ) for 10 minutes, then membranes were rinsed three times in cold 0.5M TAE. Afterwards, noodles were soaked in 4ºC sterile 0.5M TAE, positioned on a 150x15mm Petri dish. Clips were positioned at one end of the membrane and filled with sterile 0.5M TAE solution, the gel piece were then loaded in the bag. All air was removed from bags, making sure no air bubbles were present as to not interfere with the electro elution process. Afterwards, the other side of the membrane was clipped and placed inside an electrophoresis chamber were it was electroeluted overnight to remove DNA from agarose into liquid, 49V, 4ºC in 0.5M TAE. After the overnight gel run, current was inverted for one minute to remove DNA adhered to the dialysis bag. Liquid containing DNA was removed from the bag using a wide bore pipette tip and an average of 750µl was recovered. After sample concentrations were measured DNA was precipitated.

DNA Precipitation

Samples were measured using the Qubit® 1.0 Fluorometric Quantitation machine to determine DNA concentration; afterwards a sodium acetate/ethanol precipitation was done. Then, 3.0M sodium acetate pH 5.2 was used in combination with ethanol to dissociate the charge in the phosphate bonds of the DNA and to change the electrical coefficient of the solution in order to provide a safer environment for the DNA. No more than 350µl of DNA were used per sample treatment as this would have top out the solution in the microtube, 1/10 sodium acetate and 2.5 volumes of ethanol 100% were used to treat the sample. The samples were placed in -80ºC for 30 minutes and then centrifuged at 15,000g, 4ºC for 20 minutes, afterwards supernatant was dumped from the microtube using a micropipette tip to not disturb the pellet. After removal of alcohol the pellet was air dried for 5 minutes inverted on a wipe paper and then DNA resuspended in $T_{10}E_1$. The pellet was left five minutes at room temperature for it to mix with the $T_{10}E_1$ solution. After this phase DNA was ready to be modified and transducted into the surrogate host *Escherichia coli* Epi300 using the Epicentre Fosmid Library Kit. After DNA was extracted it was processed with Epicentre's CopyControl Fosmid library production kit.

Epicentre DNA End Repair

Directions were done following *Epicentre's CopyControl Fosmid library production kit*. A reaction was prepared with: end repair buffer, nucleotide mix, ATP, environmental DNA, repair enzymes. Such reaction helps to convert uneven ends on DNA inserts into blunt ends, this is required for the fosmid DNA to attach to the environmental DNA. The reaction was incubated at room temperature for 45 minutes and later on end repair enzymes were inactivated using the 70ºC dry bath for 10 minutes.

Fosmid Construction Precipitation

A precipitation was done to reduce the volume of liquid present in the environmental DNA sample. To achieve this, sodium acetate 3.0M, pH 5.2 and 1.0 vol. of cold isopropanol were added. Then DNA was left on ice for 20 minutes for precipitation to occur, then centrifuged at max speed for 20 minutes and supernatant discarded. Soon after the pellet was washed with 70% ethanol to remove remaining impurities of isopropanol and once again they were centrifuged for 5 minutes at max speed and the supernatant discarded. The pellet was air dried placing the microtube upside down on a wipe for approximately 15 minutes. Finally, the pellet was resuspend in $T_{10}E_1$ and flicked in order for it to mix with the solution. DNA concentrations were measured using the Qubit® 1.0 Fluorometric Quantitation machine

Fosmid Construction Ligation

A 10.0 µl ligation reaction required the addition of 250ng of environmental DNA for optimal results. A mix containing sterile water, 10X Fast-link ligation buffer, 10 mM ATP, CopyControl vector (0.5µg/µl), insert DNA and Fast-Link DNA ligase was done. Once all reactants were mixed they were left in an overnight reaction for the fosmid to ligate with the inserts. Afterwards, ligase was inactivated at 70ºC for 15 minutes.

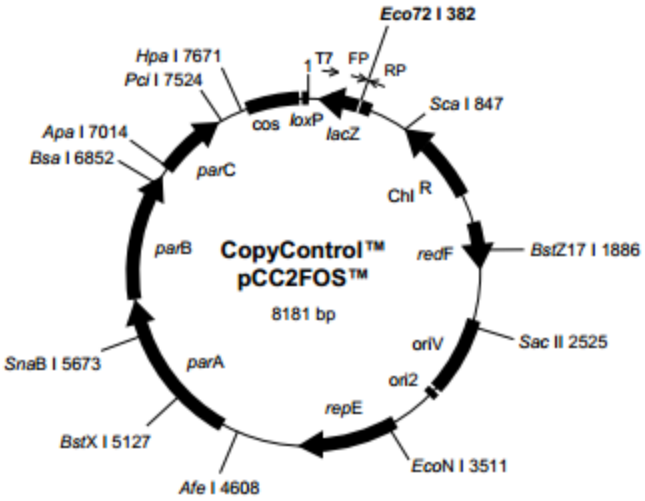


Figure 2.2 *The pCC2FOS vector map.* Source: taken from Epicentre's CopyControl Fosmid Library Kit protocol.

Preparing the Escherichia coli Epi300

A 50.0 ml culture of *Escherichia coli* Epi300 by Epicentre was grown in LB culture media containing $MgSO_4$ and maltose. The addition of maltose allowed for the formation of Lambda terminals on the cells outer surface allowing the phage to recognize its host. At the same time $MgSO_4$ provided stability to the cell. The culture was left to grow overnight and the next day a culture was prepared and grown for approximately 2 hours for it to be used in the phage infection.

Packing and Infection of Fosmid

A tube of MaxPlax Lambda Phage by Epicentre was thawed to completion on ice. Then half of the reaction (25µl) of the phage were transferred carefully to the fosmid containing the insert. After the addition, solution was mixed slowly avoiding bubbles as this could have affected the efficiency of the reaction. Moreover, it was incubated at 30°C and after 2 hour of incubation time the rest of the phage was added to the reaction, 2 more hours of incubation were done until the complete assembly of the phage. In this step the phage was assembled and the fosmid packed

inside the phage for infection of the host cell. Later the reaction volume was taken to 500µl with phage dilution buffer and 25µl of chloroform was added to remove remaining impurities that could affect the infection. Phage solution was gently mixed and 40µl added to the 400µl *E. coli* culture. Phage infection in bacterial culture was left at 37ºC without shaking for 1 hour; this allowed the phages to infect the host cell with the insert DNA containing vector. After completion of infection, culture was plated in LB plates containing a concentration of 20µg/ml chloramphenicol. One plate was prepared with the addition of 12.5µg/ml X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) to provide a vector with insert ratio of clone colonies. After 24 hours of incubation at 37ºC of the Petri plate colonies were visible. The remaining culture of infected cells was plated in order to continue with the metagenomic libraries construction. After confirmation of colonies from preliminary plating the resulting LB containing infected colonies plated and left to incubate for 24 hours prior to storage at -80ºC. To determine the number of clones present, Petri plates were counted with the use of a colony counter.

Fosmid Extraction

A modified version of QIAGEN fosmid miniprep and standard DNA extraction/precipitation protocol by Lynn Williams and Heather Allen in (2004) was used to extract fosmids from clones. This process was done to confirm the fosmid presence our metagenomic libraries. Different solutions provided by the company QIAGEN; P1, P2, N3 and other solutions and reactants available in the lab; chloroform, 3.0M sodium acetate, pH 5.2, isopropanol, 70% EtOH and $T_{10}E_1$ were used.

The fosmid extraction process consisted in growing individual selected clones in an overnight culture (16-20 hours). The cultures were centrifuged and a pellet was formed by spinning at 9,280g for 3 minutes; this allowed the removal of cultivable media from cells. Then, P1 solution

was added to resuspend cell pellets. The P1 solution contained RNAse to degrade any RNA present. Afterwards, buffer P2 (lysis buffer) was used to lyse the cells, the buffer contains NaOH and SDS detergents that help break the cells membrane. Later on, the use of N3 buffer neutralize the previous reactions. Afterwards, chloroform was added to the samples in order to separate organic and acquose phases. The DNA would be present in the acquose phase, separated from all cell debris and contaminants that would be in the organic phase. After isolating the acqueous phase sodium acetate and cold isopropanol (-20^{o}C) were added to our sample for a period of 10-30 minutes to precipitate the DNA. Afterwards, the DNA was centrifuged at 16,168g for 25 minutes to form a pellet. Supernatant was discarded, the pellet contained the DNA. Further on, the pellet was washed with EtOH 70% to purify DNA and once again it was centrifuged at 16,168g for 5 minutes. The remaining alcohol was removed from solution by pipetting and the pellet was air dried close to a flame. After the pellet dried and no traces of ethanol were left, $T_{10}E_1$ was added to resuspend DNA in desired volume.

Fosmid digestion

Once fosmid DNA was extracted from the clones, DNA was digested with a restriction enzyme to verify the presence of the fosmid DNA and metagenomic DNA insert. The process consisted in mixing 1.0µl of reaction buffer, 1.0µg of DNA, 10 units of restriction enzyme and sterile deionized water to a 10.0µl volume, incubated at 37^{o}C for 8 hours. Two restriction enzymes were used independently to study the fosmid DNA, this were *Not*I and *Pst*I. The fosmid size is 8,181bp with an insertion site at 382bp. The *Not*I enzyme has a two bands restriction pattern; nucleotides 2 and 643 in the fosmid are the restriction sites recognized and cut by the enzyme providing two bands in the agarose gel, one of 7,801bp and one of 380bp. The *Pst*I restriction enzyme has a four band restriction pattern; the nucleotides 429, 4067 and 5608 in the fosmid are

recognized and cut by the enzyme providing three bands in the agarose gel at 3642bp, 2577bp and a 1545bp, 429b.

After digestion with the enzyme, the reaction was inactivated at 70ºC for 10 minutes and the DNA was run in an agarose gel 1.0%, 40V overnight (16 hours). The presence of the fosmid band and different metagenomic DNA insert patterns were observed in the agarose gel.

Library Storage

Libraries were stored in Sub Pools and Master Pools of clones. Cells were kept on ice until stored. Then 600.0µl LB with chloramphenicol 20.0µg/ml were pipetted onto a Petri plate containing the clones. Clones were collected using a flamed glass hockey stick and the LB/cell mixture was pipette to a 15.0ml falcon tube. Process was repeated until a subpool of 5,000 clones was reached, then pools were transferred to cryovials were they were mixed with glycerol at an 80% cell, 20% glycerol ratio for long term storage. The cells were pipetted into 15.0ml tube using wide bore tips and placed on ice; the process was repeated until every colony was stored. Then 10.0µl from each tube was placed in to a cryogenic tube, this tube called the master pool contains cells from all the pools. Further on vials were stored at -80ºC for long term storage.

Characterization of metagenomic libraries

Libraries were characterized in the following manner; first off percentage of clones per insert was recorded, this was done in 2 ways and can only be done before storing the library for the first time. First LB plates containing X-gal substrate were used to grow clones from the metagenomic library, any blue colonies obtained represent a putative empty clone, and white colonies represent cells with inserts. The Epi300 cells are alpha complementary and the *lacZ* gene will be disrupted if an insert is present in the fosmid. Second off, random white colonies were selected to extract their fosmid, 10 colonies were selected and a ratio of number of colonies per

insert was calculated. The controls used for this test were: (1) positive control *Escherichia coli* Epi300 with empty vector, (2) negative control *Escherichia coli* Epi300. This two measures of X-gal LB plates and fosmid verification provide us with an average number of inserts per clones stored in the libraries. Colonies differing in a macro level from normal *Escherichia coli* Epi300 surrogated host were also stored and selected for future analysis. Library clones were counted to determine number of clones present in each library. Moreover, a restriction analysis was done to the fosmid DNA in order to determine differences between the DNA restriction patterns.

2.3 Results

DNA Extraction

DNA was extracted directly from the soil matrix. A high molecular weight DNA >40kbp was obtained, the white square marks the area removed for the generation of the noodles, the red circle marks the 40kb control DNA (Figure 2.4). The chef gel for sizing of metagenomics DNA was prepared in collaboration of Ashley Shade, Fabienne Wichman and the Jo Handelsman research team at Yale University, CT as part of the Mastering Metagenomics Summer Workshop (https://sites.google.com/site/masteringmetagenomics/home).

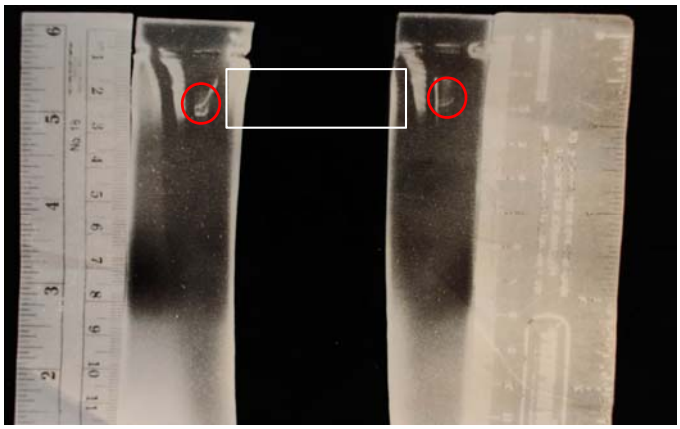


Figure 2.3 *Sizing gel from DNA cave Ventana.* Image shows sizing gel used to select DNA of 40kb for generation of metagenomic libraries noodles. Red circle represents 40kb control DNA, white square represents the region selected to make the DNA noodles.

Generation and characterization of large-insert metagenomic library

One large insert metagenomic library was generated from DNA of cave Ventana. The library was characterized by determining the number of clones, percentage of fosmids with insert and insert size. The library consisted of approximately 40,000 clones. Initial characterization indicated that around 1% of the clones did not contain insert (blue colonies) through a Petri plate X-gal assay. Ten clones were randomly selected to be grown in LB broth with chloramphenicol, grew indicated they contain the fosmid (Figures 2.5 and 2.6). Moreover fosmid DNA was extracted from clones ran through an agarose gel (Figure 2.7) and restriction digested to see differences in insert patterns. All 10 clones presented inserts and 9 of them displayed different restriction patterns (Figure 2.8).

Figure 2.4 *Clones from metagenomic library.* This is a 10^{-4} dilution of the metagenomic library. LBA Petri plate with 12.5µg/ml of Cm.

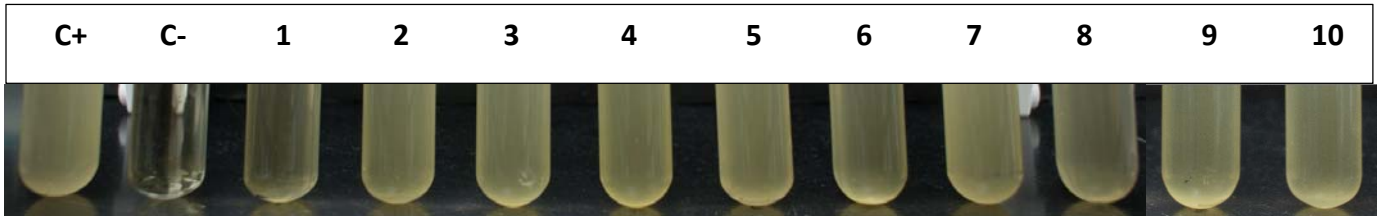


Figure 2.5 *Clones grown in LB broth with Cm.* Ten clones randomly selected were grown in LB broth with 12.5µg/ml Cm + 2.5µl Epicentre induction solution. Positive control (C+), negative control (-), samples 1-10.

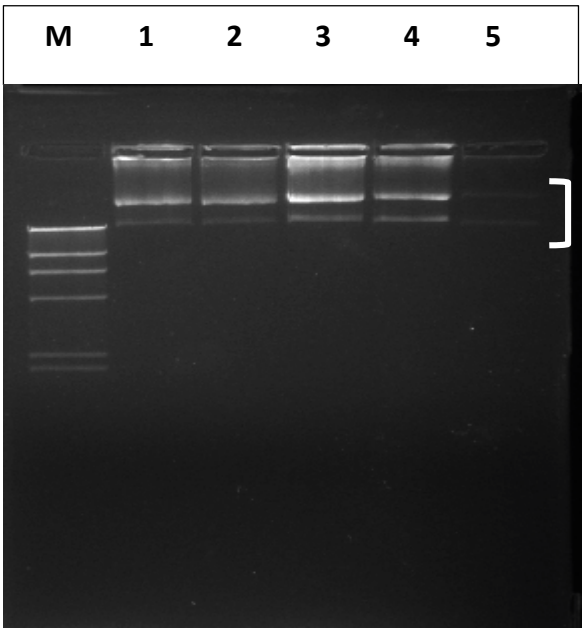


Figure 2.6 *Agarose electrophoresis gel of library clone fosmids DNA.* Electrophoresis agarose gel run of library clones, 1% agarose gel. M- Lambda/*Hind*III marker, lanes 1 to 5 contain fosmid DNA extracted from clones. White bracket indicates fosmid DNA.

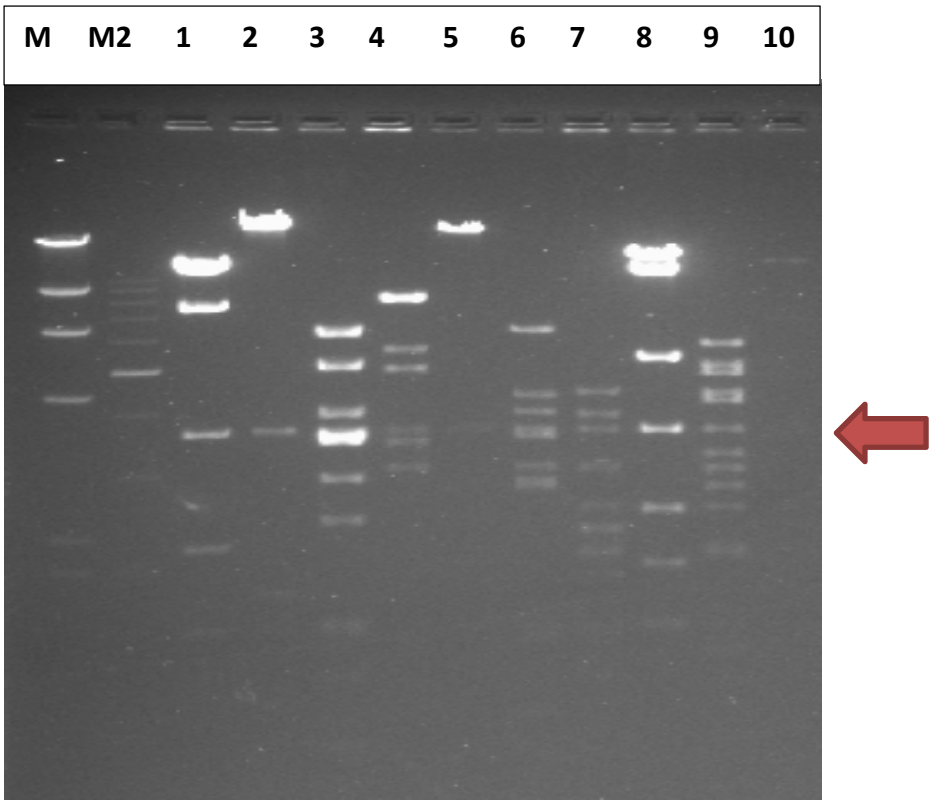


Figure 2.7 *Electrophoresis agarose gel run of library clones digested with PstI.* Electrophoresis 1% agarose gel of selected clones cut with *Pst*I. M- Lambda/*Hind*III marker, M2- 1kB marker, samples from lanes 1 to 10. Red arrow indicates vector band.

2.4 Discussion of Results

A direct DNA extraction methods was used to isolate DNA fragments of approximately 40Kbp for the generation of the library. During the sizing analysis of the direct DNA extraction showed a smear in the sizing gel starting from approximately 60Kbp downwards with a high yield concentration of DNA (Figure 2.4). Other studies have demonstrated construction of libraries with direct DNA extraction methods that show a DNA size from 1.8 to 45kbp (Gillespie et al., 2005).

This study generated the first large insert metagenomic libraries from cave soil in Puerto Rico, emphasizing from cave Ventana samples. The restriction analysis of clones suggests a library with diverse high molecular weight fragments of 40kB. The library overall consisted of approximately 40,000 clones with a 99% insert vector. There are several studies in caves using metagenomics. For example, Ortiz et al., (2014) identified the communities present in Kartchner caverns in Arizona, using swabs to sample the cave soil surface. The communities were analyzed by pyrosequencing the metagenomic DNA without the generation of a fosmid library. In our study, the generation of metagenomic libraries will allow us to understand functional analysis such as antibiotic resistance. In another cave metagenomic study developed by Jones et al., (2013), they researched and analyzed the cave biofilm in a highly acidic cavern by pyrosequencing the metagenomic isolated DNA. While cave metagenomic studies are scarce, metagenomic libraries have been generated in a vast number of environments. For example Donato et al., (2010) sampled apple orchard soil in Wisconsin and constructed metagenomic libraries using the indirect method. The indirect method provides a higher purity DNA but at the same time 100 times fold less yield of DNA that could affect diversity present in the metagenomic library studied (Delmont et al., 2011). In contrast to our research their insert size was of approximately 30kb and contained approximately 446,000 clones (Donato et al., 2010). Further on, other soil libraries generated by

Brennerova et al., 2009 produced 87,000 and 50,000 clones from jet fueled contaminated soils. Both Donato et al., (2010) and Brennerova et al., (2009) produced different amount of clones, the number of clones obtained are dependent on (1) the environment, (2) the bacterial groups present in the environment and (3) the capacity of the DNA extraction method to isolate the metagenomic DNA (Delmont et al., 2011). In other non-soil environments such as microbial mats studied by Torres et al., (2010), the indirect extraction method was used. This allowed the generation metagenomic DNA inserts larger than 20kb in libraries of 1,200 and 30,000 clones from two different type of microbial mats. Other marine studies include Strittmatter et al., (2007) where they constructed fosmid libraries from 3,000m deep of Mediterranean plankton, obtaining approximately 5,000 fosmids. Once again suggesting the amount of clones obtained in a metagenomic library is related to the type of environment studied (Delmont et al., 2011).

Chapter 3

Screening Functional Antibiotic Resistance in Metagenomic Libraries from Soil of Cave Ventana

3.1 Introduction

The main objective of this research was to monitor metagenomic libraries in order to screen for functional antibiotic resistance. The metagenomic library generated from cave Ventana provide an environment that has not been studied in the island and it is believed it could contain new types of antibiotic resistance. This assumption is based on previous cultivable studies of caves that have shown the presence of antibiotic resistance. For example Bhullar et al., (2012) isolated 93 bacterial strains and >50% resulted in some type of antibiotic resistance. Also, D'Costa et al., (2011) described antibiotic resistance as an ancient phenomenon embedded in microbial communities, originating and diversifying in the environment long before antibiotics were discovered (Aminov et al., 2007). This suggests there is a possibility of finding antibiotic resistance genes present in a diverse variety of environments, including caves.

3.2 Methodology

Minimum inhibitory concentration

The minimum inhibitory concentrations (MIC's) of kanamycin, gentamicin and tetracycline, were determined for TransforMax™ EPI300™ Electrocompetent *Escherichia coli* with empty vector pCC2FOS (Figure 3.1). The control cells were washed three times with 0.85% NaCl physiological saline solution to remove LB with Chloramphenicol (12.5 μg/ml) and any traces of cellular debris. Concentrations of 0.1-100.0 μg/ml were used to determine MIC's for host cell. The MIC was also performed on antibiotic resistant clones. The antibiotic stock concentrations were done measuring every antibiotic and diluting in the corresponding solute (gentamicin: 20.0 mg/ml Gentamicin sulfate salt, sterile water; kanamycin: 50.0 mg/ml of Kanamycin sulfate salt, sterile water; Tetracycline: 30.0 mg/ml Tetracycline hydrochloride, 95% ethanol). The positive control for the test were metagenomic clones from other studies previously

known to confer resistance to the antibiotics tested: kanamycin, tetracycline and gentamicin. The negative control used was *Escherichia coli* Epi300.

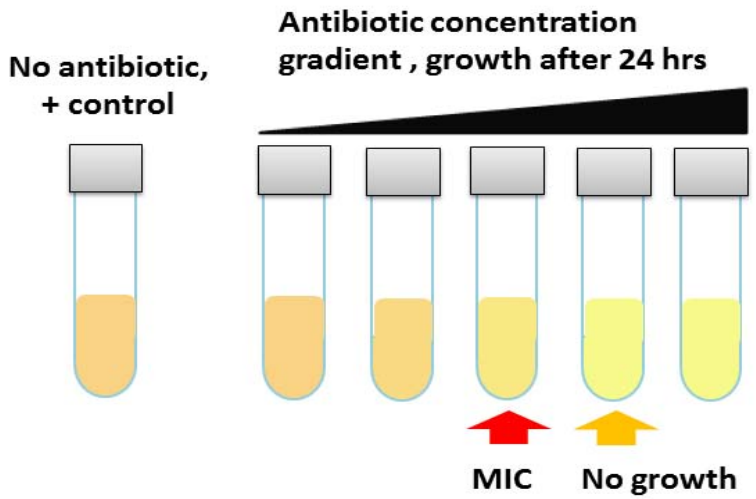


Figure 3.1 *Minimum Inhibitory Concentration for Eschericha coli Epi300.* The Minimum inhibitory concentration (MIC) was determined on surrogated host for the different antibiotics using an *Escherichia coli* Epi300 with empty vector. A gradient of antibiotic concentration (increments of 1.0 µg/ml for kanamycin and gentamicin, 0.5 µg/ml for tetracycline) was done for every antibiotic to determine the MIC.

Screening for antimicrobial resistance clones

Microbial resistance screening was done on metagenomic libraries generated using individually a multiple arrangement of antibiotics. Minimal Inhibitory Concentration (MIC) were established on *E. coli* Epi300 surrogated host with empty vector (control MIC) to determine concentrations used for antimicrobial resistance screening. For the different antibiotics the MIC was: gentamicin 5.0 µg/ml, kanamycin 12.0µg/ml and tetracycline 1.0 µg/ml. Test gradients were done from 1X MIC until no clones were capable of growing nX MIC, were n is the number of MIC's performed. First LBA plates were made of a 4.0mm thickness, these plates contained different concentrations of the chosen antibiotics. Screening was done at a higher concentration than the MIC for the *E. coli* Epi300 surrogated host with empty vector. The antibiotic resistance

screening was done using the metagenomic library generated from the cave. Metagenomic libraries were grown in 5.0ml LB with 12.5 µg/ml Cm until they reached a cell density of 0.2 OD600. In microcentrifuge tubes 1.0mL of culture was added and centrifuged, further on supernatant was removed from the microtube after a pellet was formed. Afterwards, 1.0mL of saline solution (0.85% NaCl) was added to wash the cells. These were then poured on Petri Plates, 100µL of saline solution with cells were spread plated to every Petri plate. After 24 hours resulting resistant clone colonies could be seen. Those colonies that conferred resistance were selected, stored and analyzed for mechanism conferring activity. The positive control for the test were metagenomic clones previously known to confer resistance to the antibiotics tested: kanamycin, tetracycline and gentamicin. These antibiotics were selected as they were some of the first and mostly used antibiotics. The negative control used was *Escherichia coli* Epi300.

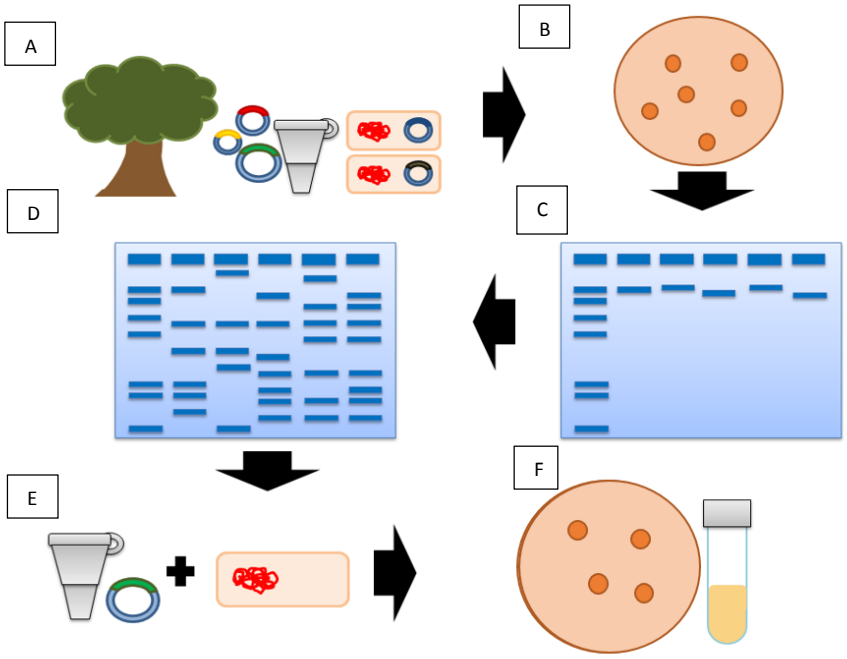


Figure 3.2 *Methodology for functional screening antibiotic resistance.* This method was used for screening different antibiotics to isolated antibiotic resistance clones. **A.** Construction of metagenomic libraries. **B.** Plating of clones in antibiotic containing plates, a gradient is done parting on the MIC concentration of the antibiotic. **C.** Verification of fosmid present in putative

resistant clones. **D.** Verification of inserts inside fosmid clones and variation between different putative antibiotic resistant candidates. **E**. Re-insertion of vector inside surrogated host. **F.** Confirmation of antibiotic resistance caused by metagenomic inserts and characterization of antibiotic resistance.

Inactivation and sequencing of antimicrobial resistance genes

Clones that contained resistant gene inserts were studied to characterize and identify genes conferring the antibiotic resistance (Figure 3.3). This was done via *in-vitro* transposon mutagenesis using the EZ-Tn5^{TM} kit by Epicentre. Reactants provided in the kit were mixed as stated by the manufacturer in order to prepare the transposon. Once the reaction was prepared: 1.0µl EZ-Tn5 10X Reaction Buffer, 0.2ng fosmid DNA, DNA 10X molar equivalent of EZ-Tn5 <Not I/KAN-3> transposon, 1.0 unit of EZ-Tn5 Transposase, sterile water and volume to a 10µl reaction, the components were mixed together and incubated for 2 hours at 37^{o}C (this is the optimal temperature for the transposase to work). After two hours, the stop solution provided by the kit was added, mixed and heated at 70^{o}C for 10 minutes to inactivate the transposase. Afterwards, 1.0µl of transposition fosmid DNA was mixed with 50.0µl *E. coli* Epi300 electro competent cells, followed by an electroporation to transform the ligated vector with transposon inside isogenic cells. After electroporation, 950µl of LB culture media were added to the cells and inoculated at 37^{o}C for 1 hour. Then, cells were plated in Petri dishes containing Tn5 selection and chloramphenicol antibiotic as a selectable markers for the fosmid. The colonies obtained were replica plated in sets of three Petri plates, these contained Tn5 selection antibiotics and current antibiotic resistance of the DNA metagenomic insert. Colonies that were not capable of growing in the antimicrobial resistance media were selected for studying. Further on, DNA was extracted and sequenced in duplicates. This was done to reduce the percentage of error and any possible mismatches done by a DNA analyzer. Samples were sent to a sequence provider Macrogen USA for sequencing (http://www.macrogenusa.com/).

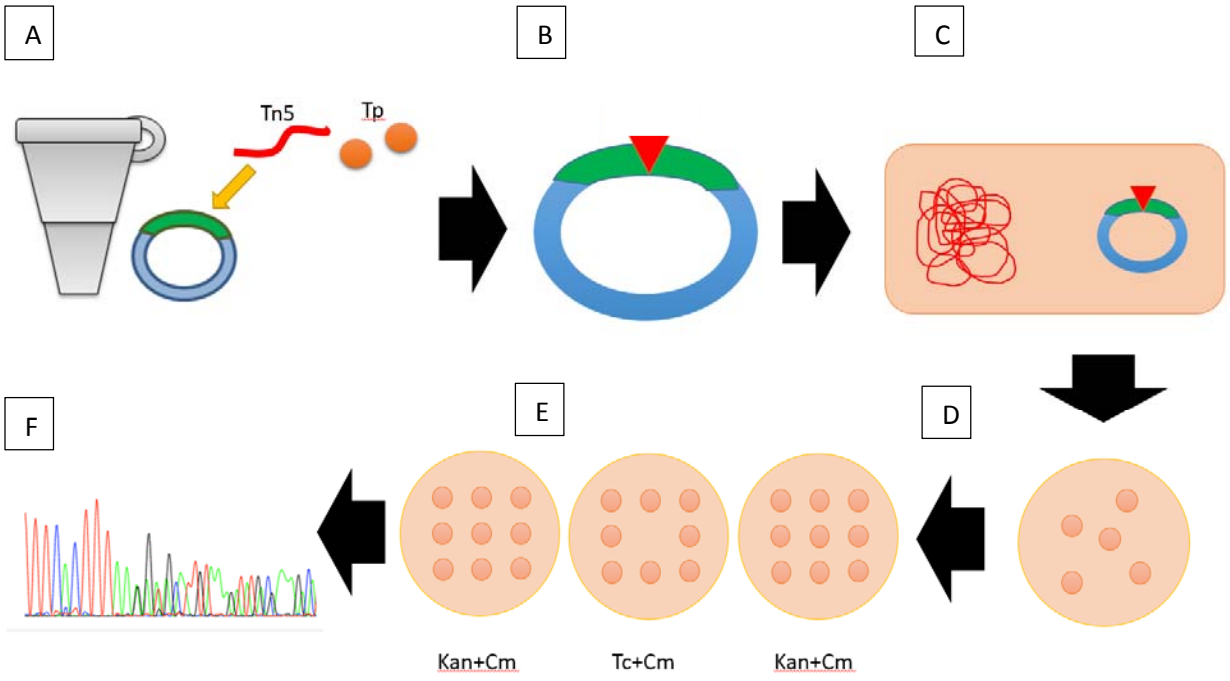


Figure 3.3 *Methodology to identify the predicted antibiotic resistance genes.* The method used to determine sequence of predicted antibiotic resistance genes open reading frames. **A.** In-vitro mutagenization of metagenomic DNA inserts. **B.** Fosmid with Tn5 in DNA insert. **C.** Re-insertion of mutagenized metagenomic inserts in surrogated host. **D.** Growth of mutant candidates in LBA plates with 12.5 µg/ml Cm. **E.** Patching technique used to determine which of the mutants lost the antibiotic resistance activity. **F.** *In-silico* analysis to determine sequence of DNA inserts.

Bioinformatics Analysis

Samples containing at least 500ng in 10µl of fosmid DNA were sent to be sequenced using the primers KAN FP-1 (forward) and KAN RP-1 (reverse) for tetracycline resistant clones. The mutants were sequenced from inside the transposon and out to the recombinant fosmid sequence (Figure 3.4). After arrival of DNA sequences CLC Main Work Bench 7.6 (http://www.clcbio.com/) software was used to assemble the data.

Figure 3.4 *Ez-Tn5 Transposon Insertion Site Junction.* Image by Epicentre, 2012.

Sanger sequences arrived in sets of duplicates of both forward and reverse primer amplicons, each set contained approximately 2,000bp (1,000bp forward, 1,000bp reverse). The sequences were trimmed to maintain a Phred of 20 or higher, then pairwise aligned. Reverse primer sequences were reverse complemented and joined with the forward primer sequence. Further on the Tn5 mosaic end sequences and one of the 9bp repeats were removed to align both primers, at the same time the transposon insertion region was highlighted. The open reading frames were determined by searching for frames with 150 straight codons uninterrupted by a stop codon. For mutant 5-6, open reading frames were predicted at 100 codons, to allow determination of partial sequences at the extremes of the sequence. Once the predicted open reading frames were identified, further bioinformatics analysis proceeded.

A data assembly was generated using the programs blastn and blastx by NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), further data from the mutagenized inserts was obtained by the Antibiotic Resistance Database (http://ardb.cbcb.umd.edu/) for blastn and blastx protein analysis of antibiotic resistance. Model assembly was based on, previous literature, ORF obtained by the CLC analysis, highest percentages of query coverage, identity and the lowest e-value. Data was also analyzed using EMBL-EBI's Pfam 27.0 (http://pfam.xfam.org). Mutant protein sequences were analyzed and aligned with Pfam's database A.

3.3 Results

Clones were obtained with resistance to kanamycin (60) and tetracycline (8). No metagenomic clones were isolated with gentamicin resistance in this study.

Minimum Inhibitory Concentration for the antibiotics

The minimal inhibitory concentrations (MIC) for kanamycin, tetracycline and gentamicin are shown in Figure 3.5, 3.6 and 3.7 respectively. The selected MIC values for each antibiotic are summarized in Table 3.1.

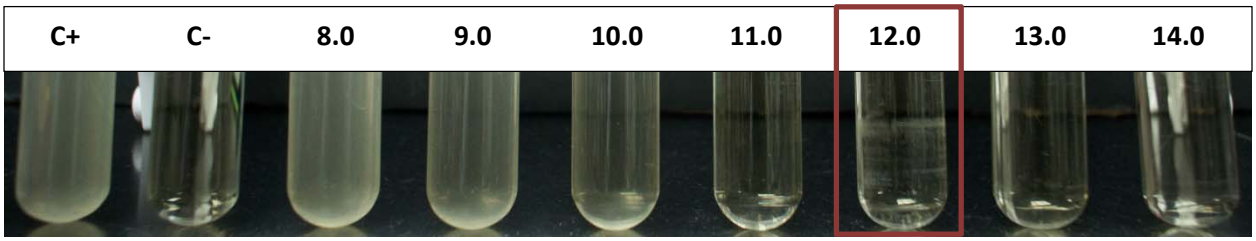


Figure 3.5 *Minimum inhibitory concentration (MIC) of kanamycin for Escherichia coli Epi300 strain with a fosmid with no insert*. Positive control (C+) Kanamycin resistant clone, Negative control (C-) *Escherichia coli* Epi300 with no insert. The MIC for Epi300 shown in the red rectangle. Concentrations are in ug/ml.

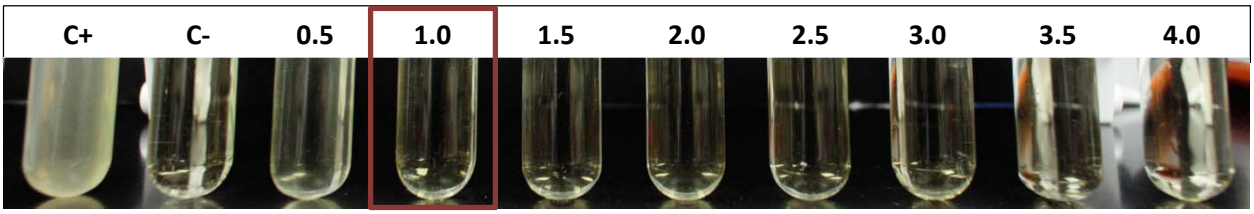


Figure 3.6 *Minimum inhibitory concentration (MIC) of tetracycline for Escherichia coli Epi300 strain with a fosmid with no insert*. Positive control (C+) tetracycline resistant clone, Negative control (C-) *Escherichia coli* Epi300 with no insert. The MIC for Epi300 shown in the red rectangle. Concentrations are in ug/ml.

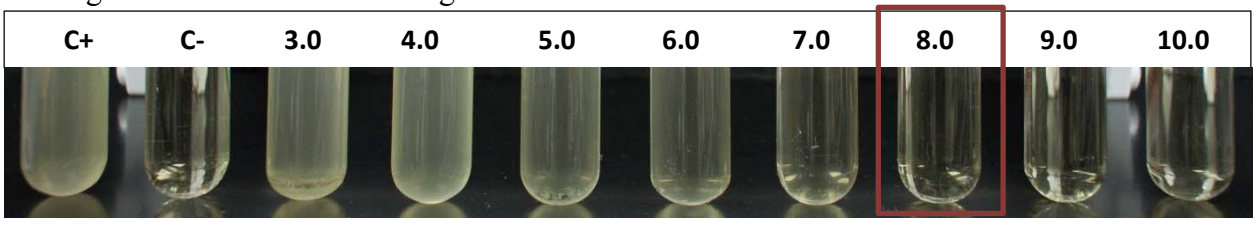


Figure 3.7 *Minimum inhibitory concentration (MIC) of gentamicin for Escherichia coli Epi300 strain with a fosmid with no insert*. Positive control (C+) gentamicin resistant clone, Negative control (C-) *Escherichia coli* Epi300 with no insert. The MIC for Epi300 shown in the red rectangle. Concentrations are in ug/ml.

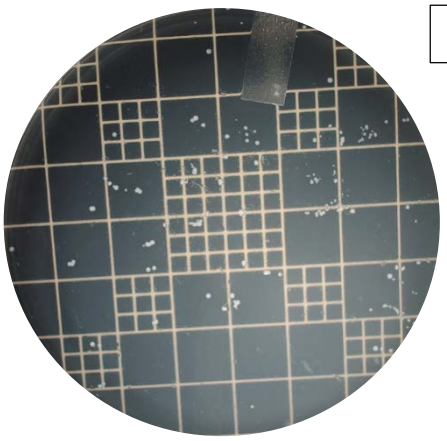
Table 3.1 Minimum Inhibitory Concentrations for *Escherichia coli* Epi300 with empty vector.

Antibiotic	MIC value for Epi300
Kanamycin	12.0µg/ml
Tetracycline	1.0µg/ml
Gentamicin	8.0µg/ml

Kanamycin resistance

Kanamycin clones (Figure 3.8 A) were isolated from cave Ventana library. Approximately 60 clones were obtained from the metagenomic library from 2X-10X MIC (Figure 3.8 B). Clones resistance was confirmed in an LB broth test where all the clones were capable of growing (Figure 3.9). The fosmid extracted from the clones showed high molecular weight DNA (Figure 3.10) and the restriction analysis of 8 random clones (Figure 3.11) showed all have the same restriction pattern. The MIC determined the specific clone capable to grow at 1200.0 µg/ml (Figure 3.12). The extracted fosmid from the clone was transformed in to a surrogated host *Escherichia coli* Epi300 and maintained the resistance activity.

A



B

Kanamycin μg/ml	**Number of clones**
50	64.3
100	62.33
200	60.1
500	58.33
1000	56.33

Figure 3.8 *Kanamycin resistant clones.* A. Kanamycin resistant clones isolated from cave Ventana's metagenomic library. Petri plates contain LBA, 12.5 μg/ml Cm and 1000 μg/ml Kan. B. Table shows average number of clones obtained after a selection of kanamycin LBA plates.

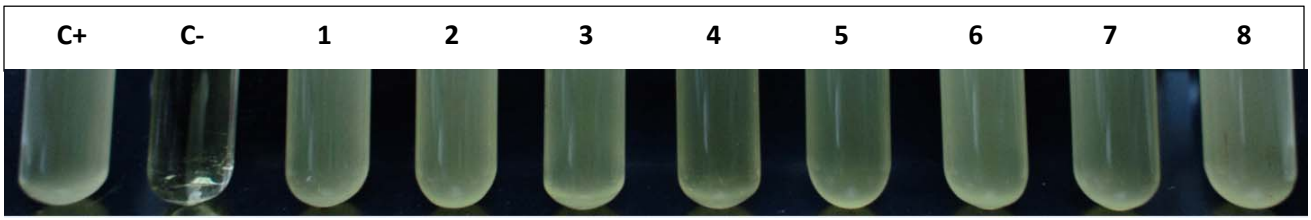


Figure 3.9 *Kanamycin resistant clones confirmed in LB broth.* Kanamycin resistant clones were isolated from LBA plates and grown in LB broth. From the clones isolated, 10 were capable of growing in LB broth with 12.5 μg/ml Cm + 50.0 μg/ml Kan + 2.5μl Epicentre Induction Solution. Clones capable of growing contain the fosmid as it has a selection marker for chloramphenicol (Cm) resistance. Positive control (C+) Kanamycin resistant clone, Negative control (C-) *Escherichia coli* Epi300 with empty vector.

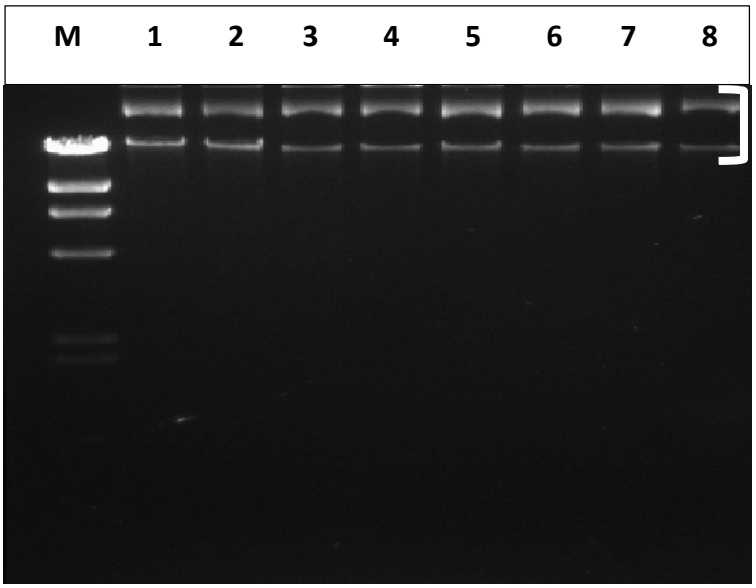


Figure 3.10 *Kanamycin resistant clones fosmids agarose gel.* **E**lectrophoresis run in 1% agarose gel shows kanamycin resistant clones extracted fosmid DNA. M- Lambda/*Hind*III marker, 1-8 fosmid samples. White bracket shows the location of fosmid DNA.

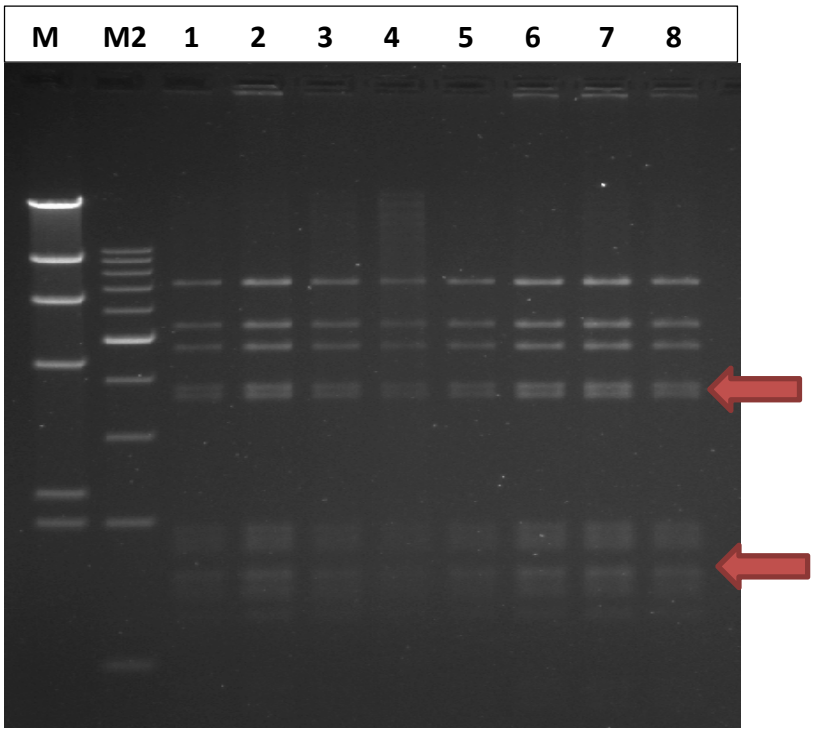


Figure 3.11 *Kanamycin resistant clones digested with PstI. The electrophoresis in 1% agarose gel shows k*anamycin resistant clones digested with *Pst*I restriction enzyme. M- Lambda/*Hind*III marker, M2- 1kB marker, 1-8 digested kanamycin resistant clones. Red arrows indicate vector bands.

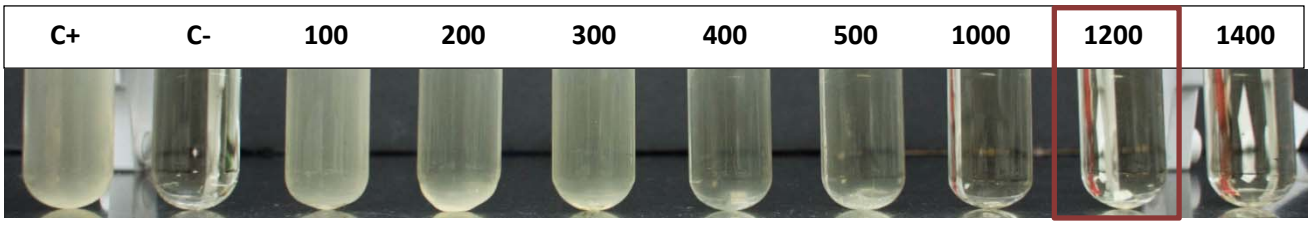


Figure 3.12 *Minimum inhibitory concentration (MIC) of kanamycin for metagenomic clones with kanamycin resistance.* Specific clone showed to have a MIC of 1200.0µg/ml for kanamicin. Positive control (C+) Kanamycin resistant clone, Negative control (C-) *Escherichia coli* Epi300 with empty vector. Red rectangle indicates MIC.

Transposon mutagenesis of kanamycin resistant clones

One specific kanamycin resistant clone was mutagenized and 114 mutants were obtained and patched after performing the Tn5 mutagenesis (Figure 3.13). None of the mutants presented the desired phenotype ($Cm^{+}Kan^{-}$), Figure 3.13.

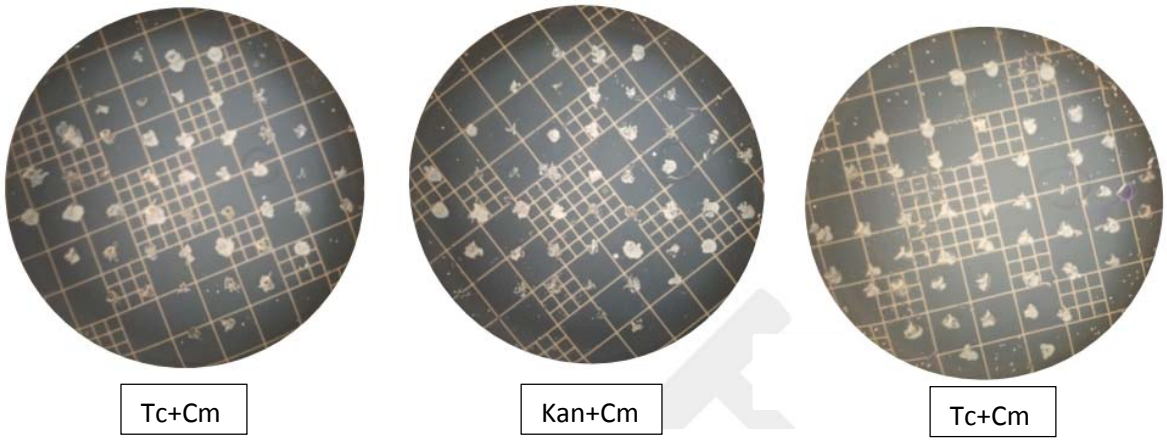


Figure 3.13 *Transposon mutagenesis of kanamycin resistance inactivation test.* A total of 114 kanamycin resistant mutants were patched to confirm phenotype ($Cm^{+}Kan^{-}$). Tc – tetracycline, Kan – kanamycin, Cm – chloramphenicol.

Tetracycline Resistance

The antibiotic functional assay performed showed that these are tetracycline clones in the cave Ventana library. Initially, 8 clones were obtained from the metagenomic library at 3X MIC (Figure 3.14 A). A second screening at 5.0 µg/ml showed only two colonies were capable of growing, both of them presented the same size and morphology (Figure 3.14 B). Clones resistance was confirmed in an LB broth test, only one of the clones was capable of growing (Figure 3.15). The clones fosmid was extracted (Figure 3.16) and the restriction analysis was done (Figure 3.17) showing 4 distinct restriction patterns for the first 8 clones obtained. The MIC was determined for one of the tetracycline clones at 10.0 µg/ml (Figure 3.19). The fosmid extracted from the clone was transformed in a surrogated host *Escherichia coli* Epi300, maintaining the resistance activity.

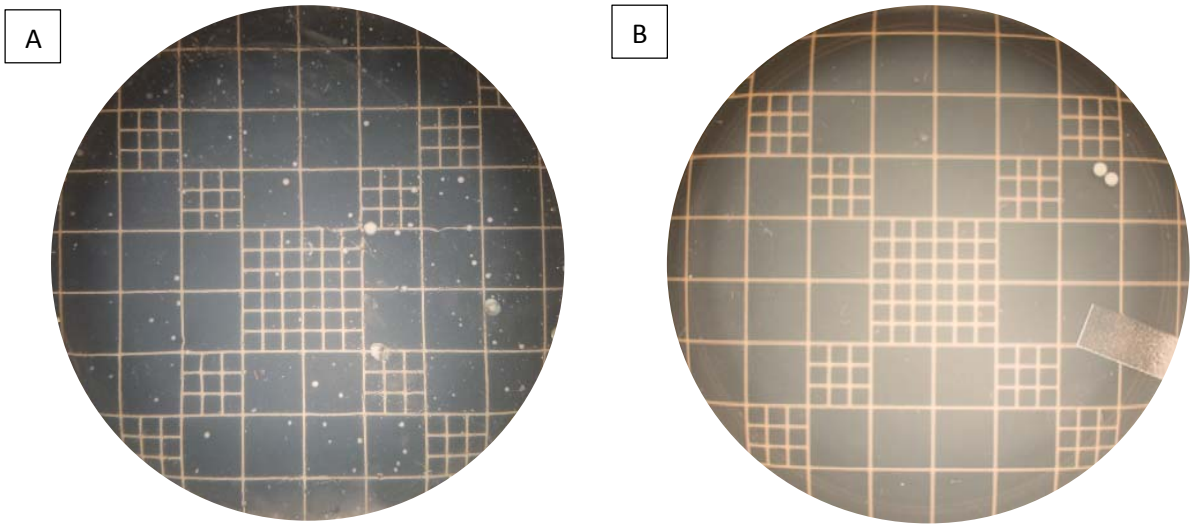


Figure 3.14 *Tetracycline resistance clones.* Tetracycline functional assay shows resistant clones isolated of cave Ventana's metagenomic library. A. From tetracycline 3.0 µg/ml 8 clones were isolated. B. From tetracycline 5.0 µg/ml Petri plates 2 clones were isolated. LBA + 12.5 µg/ml Cm + 5.0 µg/ml Tet.

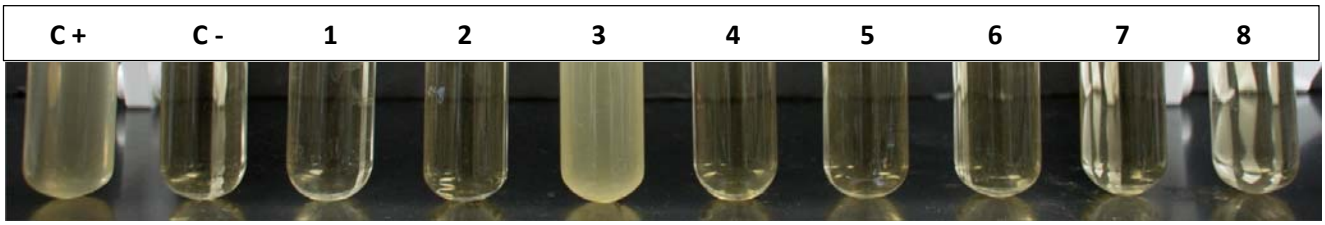


Figure 3.15 *Tetracycline resistant clones confirmed in LB broth.* Tetracycline resistant clones were isolated from LBA plates and grown in LB broth. From the clones isolated, 1 was capable of growing in LB broth with 12.5 µg/ml Cm + 5.0 µg/ml Tc + 2.5µl Epicentre Induction Solution. Positive control (C+) tetracycline resistant clone, negative control (C-) *Escherichia coli* Epi300 with empty vector.

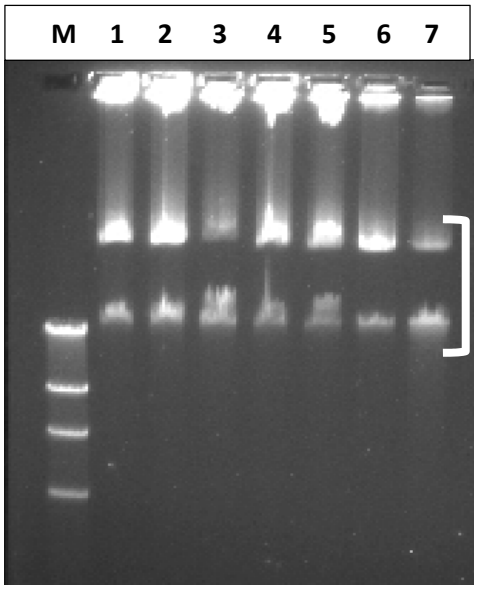


Figure 3.16 *Tetracycline resistant clones fosmids agarose gel.* The electrophoresis 1% agarose gel shows tetracycline resistant clones fosmid DNA. M - Lambda/*Hind*III marker, lanes 1 to 7 tetracycline resistant fosmids. White bracket indicates fosmid DNA.

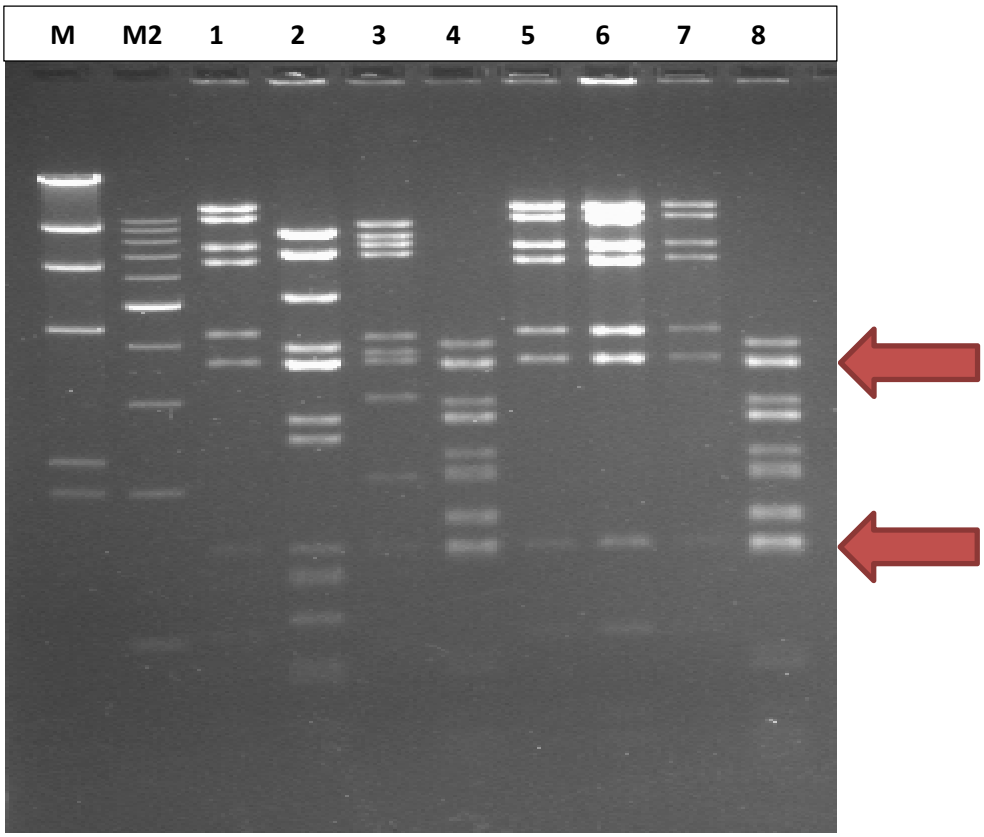


Figure 3.17 *Tetracycline resistant clones digested with PstI.* Electrophoresis 1% agarose gel of tetracycline resistant clones digested with *Pst*I. M – Lambda/*Hind*III marker, M2- 1kB marker, 1-8 digested tetracycline resistance fosmids. Red arrow indicates vector bands.

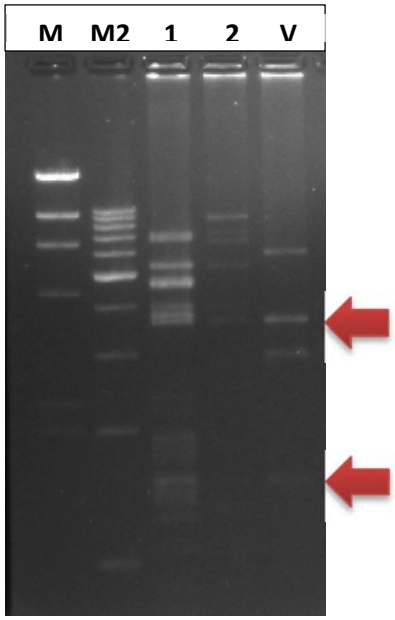


Figure 3.18 *Vector bands agarose gel.* M- Lambda/HindIII, M2- 1kb, 1- KanR clone, 2- TetR clone, 3- pCC1FOS. The pCC1FOS vector bands are marked with red arrows represent the same bands present in the pCC2FOS vector. Both vectors share a 3,642 and 1,545bp bands.

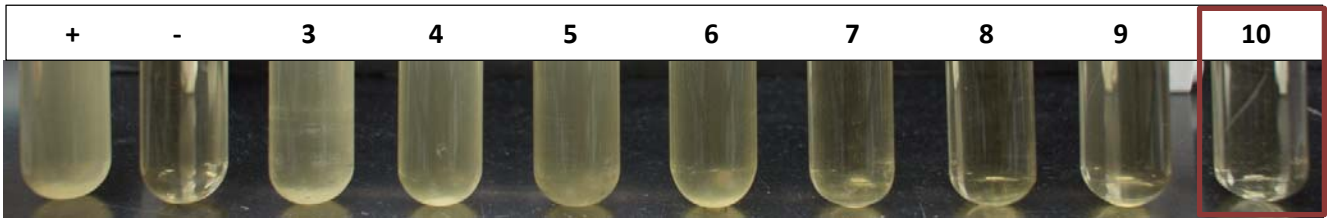


Figure 3.19 *Minimum inhibitory concentration (MIC) of tetracycline for metagenomic clones with tetracycline resistance.* This clone (#3, Figure 3.17) showed to have a MIC of 10.0µg/ml for tetracycline. Positive control (C+) tetracycline resistant clone, negative control (C-) *Escherichia coli* Epi300 with empty vector. Red rectangle indicates MIC.

Transposon Mutagenesis of Tetracycline Resistant Clones

After performing the transposon mutagenesis to the tetracycline resistant clone. From all the mutants 24 out of 25 mutants showed the desired mutant phenotype ($Cm^{+}Tc^{-}$) (Figure 3.19). Fosmids were extracted (Figure 3.21) and a restriction analysis was done with their DNA (Figure 3.22).

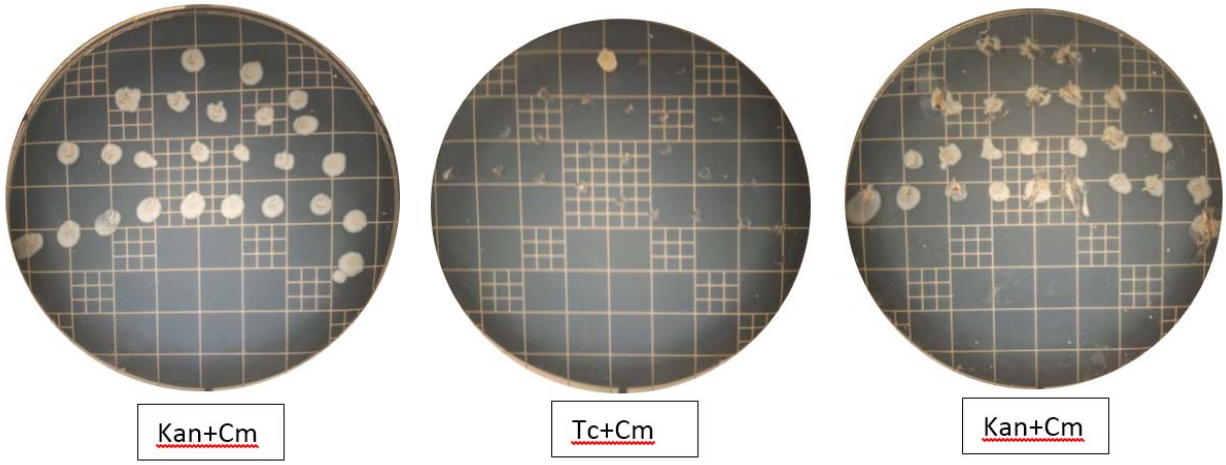


Figure 3.20 *Transposon mutagenesis of tetracycline resistance inactivation test.* A total of 25 tetracycline resistant mutants were patched to confirm phenotype (Cm+Tc-).

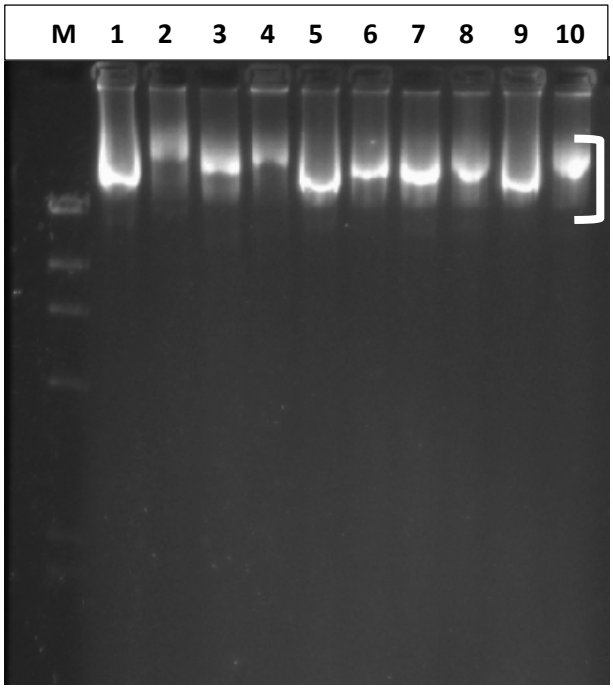


Figure 3.21 *Tetracycline mutant fosmids.* Electrophoresis 1% agarose gel run of tetracycline transposon mutagenization fosmids. M- Lambda/*Hind*III, 1-10 – fosmids with Tn5 transposon. White bracket shows fosmid DNA.

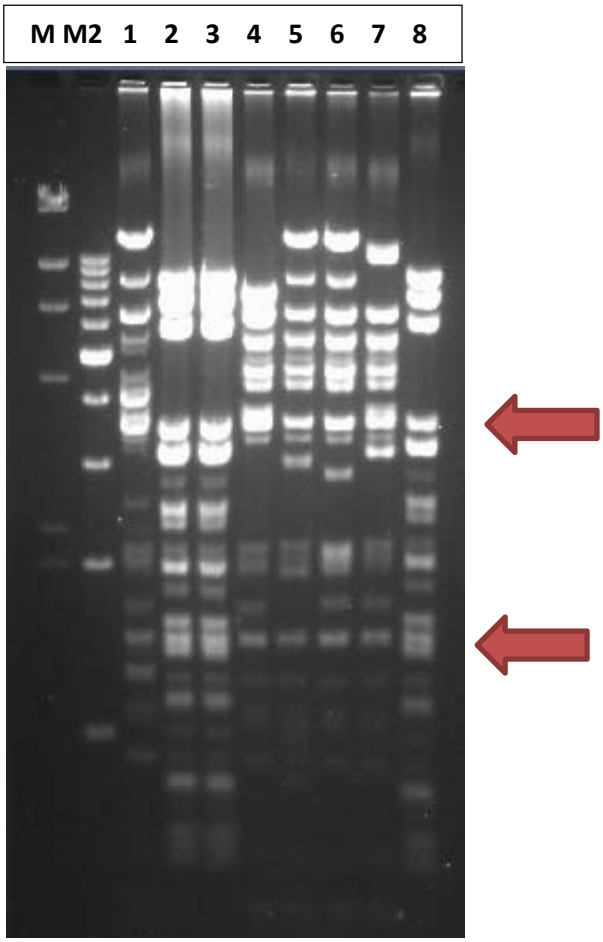


Figure 3.22 *Tetracycline mutagenized fosmids digested with Pst*I. Tetracycline transposon mutagenesis fosmids extracted, digested and ran through 1% agarose gel. M- Lambda/*Hind*III, M2- 1kb, 1-10 – fosmids with Tn5 insertion digested with *Pst*I. Sample 4 shows some DNA degradation. Red arrow represents vector bands.

Bioinformatic Analysis of the Tetracycline Mutated Clones

From a total of 10 Tet-3 generated mutants sent to be sequenced, 5 (mut-2, mut-3, mut-4, mut-5 and mut-6) out of 10 sequences analyzed and predicted models are shown in Figures 3.23, 3.25, 3.26. For mutant 2 (Figure 3.23 A) 3 *orfs* and 5 promoters were obtained were the Tn5 transposon inserted in 2 of the predicted *orfs*. Mutant 3 (Figure 3.23 B) obtained 2 predicted *orfs* and 3 promoters were the Tn5 transposon inserted in both overlapping ORFs. Combined mutants 2 and 3 Tn5 transposon aligned in the same region (Figure 3.23 C) and contained 3 *orfs* and 5 promoters. For mutant 4 (Figure 3.25) 3 ORFs were obtained were the Tn5 transposon inserted in 2 of the

orfs. For mutant 5 (Figure 3.26 A) 2 *orfs* were obtained where the predicted transposon inserted at 1 of the *orfs*. For mutant 6 (Figure 3.26 B) 4 *orfs* were obtained and the predicted Tn5 transposon inserted in 2 of the *orfs*. Allignment of mutants 5 and 6 was possible (Figure 3.26 C) 5 *orfs* were predicted, Tn5 transposons inserted in different regions affecting 3 *orfs*. The predicted antibiotic resistance genes of the different mutants; hydrolase domains, Glycosyltransferase GTB type superfamily (Figure 3.23), HAD family hydrolases (Figure 3.25) and the PAP2 haloperoxidase family (Figure 3.26). Target analyses for the mutants are shown in Tables 3.2 and 3.8. Antibiotic resistance protein analysis related the closest mechanism to be an efflux pump like resistance mechanism with capability of multidrug resistance, shown in Table 3.9.

Tetracycline Mutagenesis Analysis of Tn5 insertions

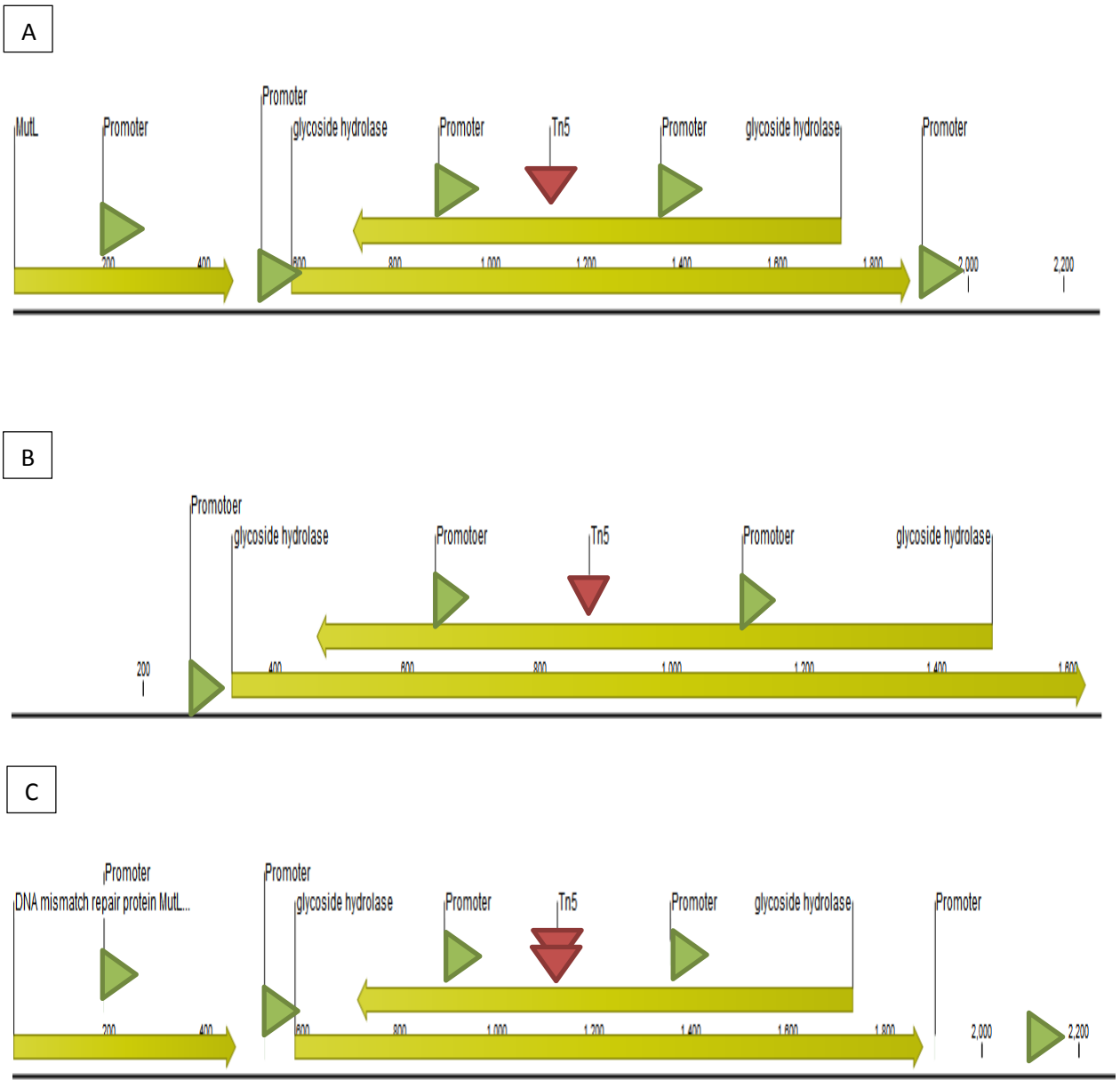


Figure 3.23 *Bioinformatics analysis of Mut-2, Mut-3 and Mut-2-3.* A. Mutant 2 open reading frames (*orfs*). B. Mutant 3 *orfs*. C. Combined mutant 2 and 3 *orfs*. Red arrow indicates transposon insertion site. Yellow arrows indicate mutant possible orf targets. Green arrows indicate promoter regions.

Family	Description	Entry type	Clan	Envelope		Alignment		HMM	
				Start	End	Start	End	From	To
Glycos_transf_1	Glycosyl transferases group 1	Family	CL0113	215	379	221	377	14	170

```
#HMM      kkkiilsvgrlekeKgvdllikafallkeqenpnlklvivGdgeeekelkklaeelelsdnvifvgevpredleelyrnadifvlpsrsEgfGivllEAmaaglpviasnvggpaeivkdgvtGllvkpkdaealaeaiekllkde.elrerlgenar
#MATCH    + + ++++grl+ +Kg+ +l++a+++l+++ ++ ++ ++ Gdg+ + e ++ +e+++l + v++ g+ ++ d++ ++ +a ++vlps  Eg++++++E +a+g p+++s v g++e+v++g++G+lv+++da+al++a+++ll+ +  + +++g+++
#PP       5667899*******************997.****************************************************************************************************999999999886
#SEQ      DSRNLVCIGRLVGQKGQLVLLEAARRLRDA-GERFNIHLLGDGPLRGEIENFIEQHRLAECVKLHGFASNADVRRHIEQARAMVLPSFAEGLPVAIMESLALGRPCVTSYVAGIPELVVPGQSGNLVPAGDADALVAAVRELLATPvSKLTEMGRRGM
```

HMM length	Bit score	E-value	Predicted active sites
172	111.1	3.7e-32	n/a

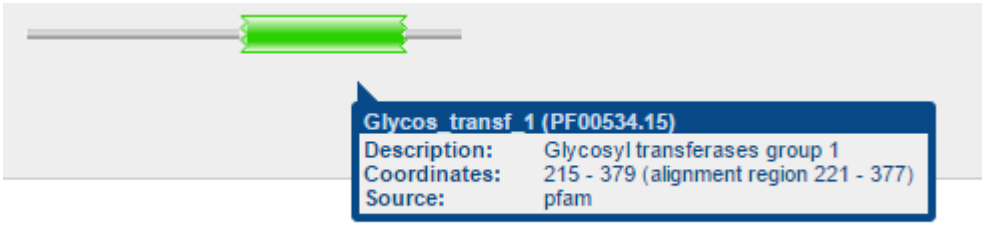


Figure 3.24 *Pfam analysis of mutant 2-3.* Predicted open reading frame match on Pfam A database.

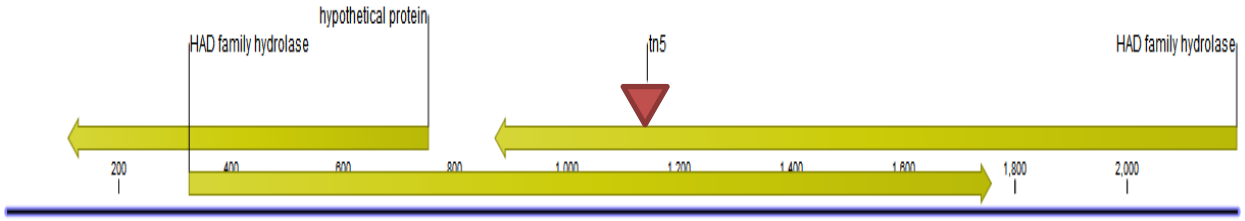
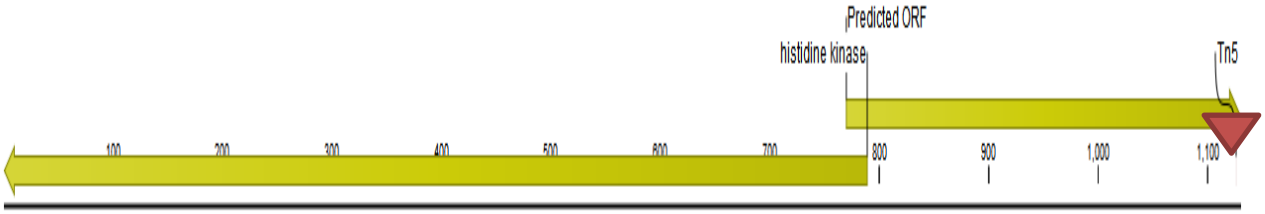
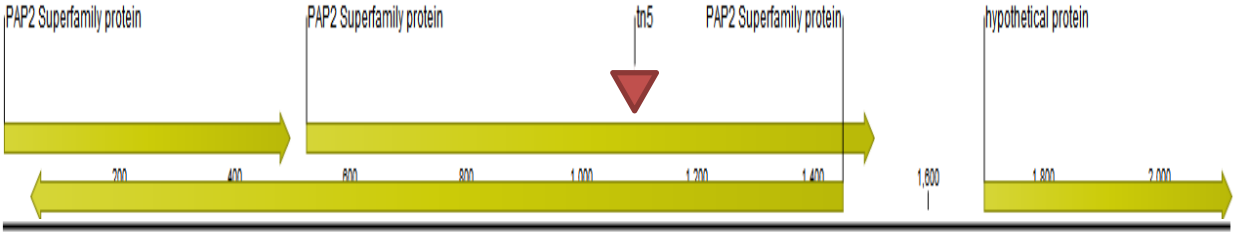


Figure 3.25 *Bioinformatics analysis of Mut-4.* Open reading frames obtained from mutant 4 data. Red arrow indicates transposon insertion site. Yellow arrows indicate mutant possible orf targets.

A



B



C

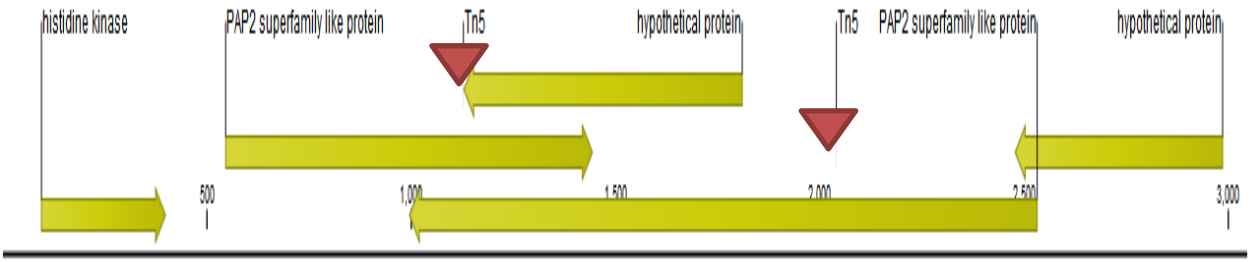


Figure 3.26 *Bioinformatics analysis of Mut-5, Mut-6 and Mut-5-6.* A. Mutant 5 open reading frames (*orfs*). B. Mutant 6 *orfs*. C. Combined mutant 5 and 6 *orfs*. Red arrow indicates transposon insertion site. Yellow arrows indicate mutant possible orf targets.

Table 3.2 Target Analysis for Predicted Tetracycline Resistance of Mutant-2.

Name	Size (bp)	Position on insert	Organism related	Function	NCBI accession #	Query coverage	e-value	Identitty
DNA mismatch repair protein MutL	459	3-461	*Singulisphaera acidiphila* DSM 18658	DNA mismatch repair protein	WP_015246086.1	98%	1e-25	45%
glycoside hydrolase	1296	583-1878	*Crocosphaera watsonii*	Glycoside hydrolase	WP_007310213.1	92%	1e-146	52%
glycoside hydrolase	1023	712-1734	*Crocosphaera watsonii*	Glycoside hydrolase	WP_007310213.1	100%	1e-117	51%

Table 3.3 Target Analysis for Predicted Tetracycline Resistant Mutant-3.

Name	Size (bp)	Position on insert	Organism related	Function	NCBI accession #	Query coverage	e-value	Identitty
glycoside hydrolase	1253	334-1626	*Crocosphaera watsonii*	glycoside hydrolase	WP_007310213.1	93%	9e-146	52%
glycoside hydrolase	1023	463-1485	*Crocosphaera watsonii*	Glycoside hydrolase	WP_007310213.1	100%	1e-117	51%

Table 3.4 Target Analysis for Predicted Tetracycline Resistant Mutant-4.

Name	Size (bp)	Position on insert	Organism related	Function	NCBI accession #	Query coverage	e-value	Identitty
hypothetical protein	645	109-753	*Halonatronum saccharophilum*	Hypothetical protein	WP_027340074.1	99%	4e-38	37%
HAD family hydrolase	1434	325-1758	*Tuberibacillus calidus*	HAD family hydrolase	WP_027726331.1	53%	1e-36	39%
HAD family hydrolase			*Tuberibacillus calidus*	HAD family hydrolase			1	

Table 3.5 Target Analysis for Predicted Tetracycline Resistant Mutant-5.

Name	Size (bp)	Position on insert	Organism related	Function	NCBI accession #	Query coverage	e-value	Identitty
histidine kinase	789	1-789	[Dactylosporangium aurantiacum]	histidine kinase	WP_033363419.1	84%	4e-61	57%
Predicted ORF with conserved transport ATP binding protein, Interval- 90-242	360	770-1129	*n/a*	Transport ATP binding protein	TIGR02857	n/a	4.58e-03	n/a

Table 3.6 Target Analysis for Predicted Tetracycline Resistant Mutant-6.

Name	Size (bp)	Position on insert	Organism related	Function	NCBI accession #	Query coverage	e-value	Identitty
PAP2 superfamily protein	496	1-496	Uncultured bacterium	PAP2 membrane protein superfamily	AHZ45595.1	56%	2e-86	49%
PAP2 superfamily protein	1407	47-1453	Uncultured bacterium	PAP 2 membrane protein superfamily	AHZ45595.1	86%	4e-80	46%
PAP2 superfamily protein	984	524-1507	Uncultured bacterium	PAP2 membrane protein family	AHZ45595.1	75%	5e-28	45%
hypothetical protein	430	1697-2126	*Sporichthya polymorpha*	Hypothetical protein	WP_019874820.1	41%	0/039	39%

Table 3.7 Target Analysis for Predicted Tetracycline Resistant Mutant-2-3.

Name	Size (bp)	Position on insert	Organism related	Function	NCBI accession #	Query coverage	e-value	Identitty
DNA mismatch repair protein MutL	459	1-461	*Singulisphaera acidiphila* DSM 18658	Mismatch repair protein	YP_007202629.1	98%	4e-21	42%
glycoside hydrolase	1296	583-1878	*Crocosphaera watsonii*	glycoside hydrolase	WP_007310213.1	92%	1e-145	52%
glycoside hydrolase	1023	712-1794	*Crocosphaera watsonii*	glycoside hydrolase	WP_007310213.1	100%	2e-117	51%

Table 3.8 Target Analysis for Predicted Tetracycline Resistant Mutant-5-6.

Name	Size (bp)	Position on insert	Organism related	Function	NCBI accession #	Query coverage	e-value	Identitty
histidine kinase	306	94-399	*Dactylosporangium aurantiacum*	Histidine kinase	WP_033363419.1	98%	2e-18	60%
PAP2 superfamily protein	900	545-1444	uncultured bacterium	PAP2 superfamily protein	AHZ45595.1	49%	1e-34	48%
hypothetical protein, partial	684	1128-1811	*Sphingomonas sp*	Hypothetical protein	WP_029728856.1	99%	1e-55	47%
PAP2 superfamily protein	1539	995-2533	*Uncultured bacterium*	PAP2 superfamily protein	AHZ45595.1	78%	6e-80	46%
hypothetical protein	510	2478-2987	*Sporichthya polymorpha*	Hypothetical protein	WP_019874820.1 |	38%	0.054	38%

Table 3.9 Antibiotic Resistance Genes Database Mutant Analysis for Tetracycline Mutants.

Mutant	Gene Type	Average Similarity	Resistance	Description	PubMed ID
Mut-2	*srmb, erme*	56%, 50%	Lincosamide, streptogramin, bmacrolide	*srmb*: ABC transporter system, Macrolide-Lincosamide-Streptogramin B efflux pump. *erme*: rRNA adenine N-6-methyltransferase, which can methylate adenine at position 2058 of 23S rRNA, conferring resistance to erythromycin.	1508047, 3934045
Mut-3	*srmb*	56%	Lincosamide, streptogramin, bmacrolide	ABC transporter system, Macrolide-Lincosamide-Streptogramin B efflux pump.	1508047
Mut 2-3	*srmb*	56%	Lincosamide, streptogramin, bmacrolide	ABC transporter system, Macrolide-Lincosamide-Streptogramin B efflux pump.	1508047
Mut- 4	*aac2i, mexi, teta, otrb*	58.33%, 48.72%, 46.67%, 42.22%	Netilmicintobramycin 6 n netilmicindibekacingentamacin, multiple drug resistance, tetracycline, tetracycline	Aminoglycoside N-acetyltransferase, which modifies aminoglycosides by acetylation. , Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump. Major facilitator superfamily transporter, tetracycline efflux pumps.	11083623, 137398, 11381101, 11381101
Mut- 5	*mexy, oprj, otrb, tet*	60%, 56.25%, 52.38%, 45.24%	Aminoglycosideglycyline, glycylcylinefluoroquinolonerox ithromycinerythromycin, tetracycline, tetracycline	*mexy* and *oprj*: Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pumps. *otrb* and *tet*: Major facilitator superfamily transporter, tetracycline efflux pumps.	
Mut- 6	*bl1_sm, mexx, mexa, mexy*	57.42%, 55.17%. 46.67%, 44.89%	Cephalosporin, aminoglycosideglycylcycline, multiple drug resistant, multiple drug resistant	*bl1_sm*: Class C beta-lactamase. This enzyme breaks the beta-lactam antibiotic ring open and deactivates the molecule's antibacterial properites. *mexx*, *mexa*, *mexy:* Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	2227364, 8226684, 8226684, 9925549
Mut 5-6	*mexx, mexb, mexy, mexw*	55.17%, 44.89%, 43.59%, 42.74%	Aminoglycosideglycylcycline, aminoglycosidetigecylineflouro quinolonebeta_lactamtetracycli ne, aminoglycosideglycylcline, multiple drug resistant	*mexx, mexh, mexy, mezw*: Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	9925549, 8226684, 9925549, 10984043

*Analysis was done at a $1x10^{-4}$ e-value.

3.4 Discussion of results

The study consisted in monitoring metagenomic libraries for the isolation of antibiotic resistance genes. The antibiotics used in the study were kanamycin, gentamicin and tetracycline. They were selected as they are some of the most commonly used antibiotics worldwide, due to their high efficacy and low cost (Forge and Schacht, 2000, Chopra and Roberts, 2001). The functional screening done on the library produced for the isolation of resistance for two classes of antibiotics; kanamycin (Figure 3.8) and tetracycline (Figure 3.14). A restriction analysis done to 8 of the kanamycin clones showed they all have the same restriction pattern (Figure 3.11). Two possibilities could be assumed; (1) all the clones were siblings from the same cell or (2) the pattern of DNA is characteristic of a group of bacteria dominant in the cave's environment. In turn, for tetracycline resistance of the eight clones isolated, four had different restriction patterns (Figure 3.17). Further on, after a resistance confirmation, only one clone was capable of growing in LB broth with tetracycline MIC 3.0 µg/ml. This test was repeated for a MIC 5.0 µg/ml and only two colonies were capable of growing (Figure 3.14 B), suggesting an MIC 3X higher than the value obtained in the surrogated host with empty vector is needed to perform the test. This has not been confirmed in literature but studies by Donato et al., 2010 characterized MIC's for tetracycline at >0.5 µg/ml and screened for resistance starting at MIC >10.0 µg/ml.

To date, there are no known published studies on functional screening of antibiotic resistance in caves. However, functional metagenomic studies have shown the presence of antibiotic resistance in various environments (Pehrsson et al., 2013) including; apple orchard soils (Donato et al., 2010), sea gulls (Martiny et al., 2011), Alaskan soils (Lang et al., 2010), wastewater treatment plants (Wang et al., 2013), food fermenting microbiota (Devirgillis et al., 2014) and the human intestine (Moore et al., 2011). Moreover, kanamycin and tetracycline resistance have been

shown by functional metagenomics. For example Donato et al., (2010) and Martiny et al., (2011) isolated functional resistance activity to kanamycin and tetracycline. Moreover, Cheng et al., (2012) isolated all known ribosomal protection type of resistance genes; *tet*(o), *tet*(w) and *tet*(32) by functional metagenomic studies of the human gut.

To determine antibiotic resistance in our clones we first had to determine Minimum Inhibitory Concentration (MIC) values (Table 3.1, Figures 3.5, 3.6 and 3.7). Other studies by Torres et al., (2012) and Donato et al., (2010) have generated MIC's with pCCFOS vectors and *Escherichia coli* Epi300 cells for metagenomic libraries. Torres et al., (2012) obtained MIC values of 2.0 µg/ml for gentamicin and 4.0 µg/ml for kanamycin. They did not study tetracycline MIC's. Meanwhile, Donato et al., (2010) obtained 2.0 µg/ml kanamycin and 0.5 µg/ml for tetracycline. They did not study gentamicin. MIC values for both authors are far off from the values obtained in this study (Figures 3.5, 3.6 and 3.7). The differences present in the MIC values could be due to different fluctuations in the protocols and materials used. Studies by Donato et al., (2010) and Torres et al., (2012) do not indicate the use of chloramphenicol (Cm) or the cell density used to determine the MIC values, such components could be the cause of the differences. Moreover, MIC for the functional clones suggests a 1,200.0 µg/ml resistance for the kanamycin and a 10.0 µg/ml resistance for the tetracycline clone. Due to the MIC obtained with the control strain, the kanamycin resistant clones have been rated as highly resistant containing a MIC higher than > 160 µg/ml (Elisha and Steyn, 1994). High level kanamycin resistance has been previously associated with antibiotic modifying enzymes (White et al., 2005), this could give us an idea of what mechanism could be associated to such resistance.

In order to characterize the genes causing the antibiotic resistance transposon mutagenesis is done using Epicentres EZ-Tn5 ™ Insertion Kits. Mutagenesis was successful for both targets,

still no inactivation of kanamycin resistance was obtained in the kanamycin clone tested (Figure 3.13). This could be due to a multiple resistance gene arrangement (Aires et al., 1999) or a DNA structural conformation that allows the Tn5 transposon to bias against the target resistance genes (Ason and Reznikoff, 2003). The Tet-3 clone inactivation of tetracycline resistance was successful on 24 out of 25 mutants (Figure 3.20) and the mutant DNA was sent to be amplified and sequenced by Sanger sequencing. The analysis of Tet-3 mutants included three putative conserved domains: a Glycosyltransferase_GTB_type superfamily (Figure 3.23), a PAP2 haloperoxidase family (Figures 3.25) and a HAD family hydrolase (Figure 3.26). Out of the ten sequences sent in duplicates, four were successfully analyzed. The six low quality sequences were believed to be caused by multiple transposon insertions in the metagenomic insert, a phenomenom known to occur in Tn5 transposons (Goryshin and Reznikoff, 1998), if multiple transposon are present in the mutant, there will be multiple regions that could be recognized by the Tn5 primers. Further studies could be done to confirm multiple transposon insertions by analyzing fosmid DNA in Pulse Field Gel Electrophoresis. The transposon will add 1.2kb to the size of the fosmid DNA, if more than one transposon is present inside the target, it should have 1.2kb more for every transposon present in the insert. Electrophoresis by agarose gel does not have the resolution needed to confirm this differences in high molecular weight DNA. Another method that could help in identifying multiple transposon insertions is by digesting mutant DNA with a restriction enzymes and comparing pattern differences caused by multiple transposon insertions.

The analysis yield 3 predicted models for tetracycline resistance that work in conjunction in 1 mechanisms. Transposon insertions were able to generate two side by side mutants; 2,3 and 5,6, (Figures 3.23 and 3.26). The mechanisms for mutant 2,3 consist on a glycoside hydrolase with open reading frames in both directions. Both transposons in mutant 2 and mutant 3 inactivated

the same region and produced loss of function. The region predicted ORFs most similar results code for 2 glycoside hydrolases and 1 DNA mismatch repair protein MutL (Table 3.7) at protein level. MutL is part of the DNA repair system, DNA repair system is associated to the composite active sites of ABC ATPases (Junop et al., 2001). Glycoside hydrolases have played their role in antibiotic resistance such as NagZ glycoside hydrolases confer resistance to cephalosporins by catalizing the formation of the AmpC inducer molecule (Asgarali et al., 2009) and some are associated to vancomycin and aminoglycoside resistance genes (Panesso et al., 2010). Further analysis was performed using the Antiboitic Resistance Database (ARDB) revealing homology with the *srm*b gene (Table 3.9) that codes for an ABC transporter system and a Macrolide-Lincosamide-Streptogramin B efflux pump (Geistlich et al., 1992). The mechanism present in this mutant, resemble similarity to those implicated in active efflux mechanisms present in gram positive and negative bacteria involved in efflux tetracycline resistance (Levy, 1992). Another analysis was done through Pfam's database A were homology with a Glycosyl transferase group 1 protein was obtained (Figure 3.24). Such proteins have been found to confer low levels of resistance to macrolide antibiotics by glycosylation (Gourmelen et al., 1998).

In case of the mutants 5-6, the predicted ORFs resulted in proteins related to a histidine kinase, PAP2 family like proteins and an unknown ORF (Figure 3.26). Previous studies have related histidine kinases to antibiotic resistance as they have been found to regulate expression in AdeABC efflux pumps of multiple drug resistance mechanisms (Marchand et al., 2004). This mechanism has been known to produce resistance to tetracycline and a vast arrangement of multiple antibiotics (Wieczorek et al., 2008). The PAP2 family proteins are phosphatidic acid phosphatase type 2/haloperoxidases. These enzymes are known to catalyze desphosphorilation reactions (Carman et al., 2006). Moreover, the PAP2 proteins have been associated to work in

bacitracin resistance in *Bacillus subtillis* strains by competing with bacitracin for dephosphorylation of C55-isoprenyl pyrophosphate. These mechanisms are found in strains containing ABC transporter efflux pumps (Bernard et al., 2003), suggesting the genes found in mutant 5-6 can be related with efflux pump like mechanisms. Further bioinformatics analysis by the ARDB, suggest homology for Mut 5-6 (Table 3.9) with *mexx, mexh, mexy, mezw* genes at protein level. These genes work in multidrug resistance-nodulation-cell division transporter systems (RND protein family) and multidrug resistance efflux pumps (Poole et al., 1993; Mine et al., 1999). No matching data was obtained through the Pfam analysis.

Finally, last predicted ORFs for mutant 4 contained HAD family hydrolases and a hypothetical protein. This mutant with loss activity did not pair with any of the other mutants. The HAD family like domains are a superfamily of haloacid dehalogenases involved in a variety of processes such as amino acid biosynthesis and detoxification (Koonin and Tatusov, 1994). Haloacid dehalogenases have not been related directly to antibiotic resistance mechanisms, studies have shown the HAD are underexpressed in presence of antibiotics such as vancomycin (Awad et al., 2013). Furthermore the mutant sequence that was analyzed in the ARDB showed sequence similarity (Table 3.9) with *aac2i, mexi, teta,* and *otrb* genes at the protein level. The *aac2i* gene is associated with an Aminoglycoside N-acetyltransferase that modifies aminoglycosides by acetylation (Kotra et al., 2000). The *mexi* gene is related to bacterial efflux transporters of the resistance-nodulation-cell division transporter system family (Andreenko and Suvorov, 1976) and the *tetA* and *otrB* genes are related to multidrug resistance efflux pump and major facilitator superfamily transporters and tetracycline efflux pump (Chopra and Roberts, 2001). The data present in the mutant 4 suggests it could be related to cell transporters and efflux pumps. No matching data was obtained through a Pfam analysis.

None of the sequences in any of the mutants were able to match at the nucleotide level, when analyzed through the databases. This suggests that the sequences analyzed could be of novel biological system yet to be described.

All the data together proposes a possible efflux like mechanism regulated by a histidine kinase is causing the resistance to tetracycline. Moreover, data suggests there is a possibility of this being a multiple drug resistance mechanism. Still, the observations made in this study are not enough to confirm that such type of mechanism is causing the functional tetracycline resistance. Further experimentation is recommended in order to characterize the entire mechanism. Due to the complex structure of efflux mechanisms, full sequencing of the metagenomic insert could provide a broader panorama of all the genes or most of the genes involved in such resistance. Once the predicted genes are completed and finished, a complementation assay is necessary to determine which of the genes in the metagenomics insert are reported for the resistance. The process will consist in generating primers to amplify the wild type version of the active predicted mutant ORFs throughout the bioinformatics analysis or if inserts are too large to be amplified by PCR, DNA fragments could be restriction digested to reduce size of fragments. Then target DNA would be cloned into a vector and transferred to a surrogated host that contains the mutant fosmid with insert and loss of activity. The predicted functional genes could be *trans* complemented in order to rescue the phenotype. This could help us determine the essential genes in the functional tetracycline resistance activity.

Chapter 4

Summary, Conclusions, Recommendations, Bibliography and Literature Cited

Summary, conclusions and recommendations

- A high molecular weight cave metagenomic library containing 40,000 clones with 99% insert was generated.
- A highly resistant clone (MIC>1200.0 µg/ml) was isolated for kanamycin.
- A tetracycline resistant clone (MIC 10.0 µg/ml) was isolated.
- Bioinformatics analysis on the tetracycline resistant clone suggest the resistance could be caused by a membrane transport system such as an efflux pump.
- This is the first cave metagenomic library generated in Puerto Rico. It is also the first functional metagenomics antibiotic resistance study in caves in the island, were different types of antibiotic resistance were found.
- The use of the indirect extraction method to extract biased DNA by the direct method will allow for a more complete study on the antibiotic resistant genes present in the environment.
- Bioinformatics analysis suggests multiple drug resistance capabilities on the Tet3 clone, the generation of multiple antibiotic essays to confirm multiple antibiotic resistance is highly advised.
- The use of more classes of antibiotics will provide a broader spectrum of the entire Resistome of the cave.
- Sampling and constructing libraries of different regions of the cave will provide a wider assessment of the antibiotic resistance present.
- Complementation assays of the predicted genes will provide a complete characterization of the resistance conferring genes.

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