International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2016): 79.57 | Impact Factor (2015): 6.391

Characterization of a Cryptic Plasmid from the Deep Subsurface Bacteria, *Pseudomonas*spp.B0623

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Abstract: ***Cryptic plasmids from bacteria which had been isolated from the deep terrestrial subsurface of the Savannah River Site (SRS), Aiken, South Carolina were characterized. This study examined two strains of bacteria;Comamonas sp. B0669 and Pseudomonas spp. B0623. The aim of the study was to characterize twocryptic plasmids and verify previous microarray analysis by usingDNA sequencing to find genes that encode heavy metals resistance. During the project the focus of the study changed from Comamonas sp. B0669 to Pseudomonas spp. B0623 because the yields of purified DNA were too low and the technical difficulties occurred with the very large plasmid from Comamonas sp. B0669. The plasmid of Pseudomonas spp. B0623 was purified and characterized using restriction digest enzyme analysis, followed by DNA ligationwith a vector (pUC19) and transformation into competent cells (E.coliXl1-Blue). This study has potential applicationsin bioremediation to treat sites contaminated with heavy metals, and can provide information on the biology of microorganisms living in the deep subsurface.***

Keywords: Plasmid, Subsurface bacteria, *Pseudomonas spp*. B0623, Heavy metals

1. Introduction

Bacteria living in soil are impacted by human pollution. Soil contaminated by heavy metals, antibiotics, and organic compounds can be toxic to bacteria or select for certain genotypes in the microbial population (Sevgi et al., 2010).Antibiotics and organic compounds are carbon-based, and so can be transformed or degraded, although some persist in the environment long-term (Mitchell &Gu, 2010). Heavy metals cannot be degraded or destroyed, and they are stable and persistent environmental contaminants; thus, they can be accumulated in soils and sediments over time (Chopra et al., 2009).

In the 1980s, a group of microbiologists in the U.S. studied terrestrial subsurface environments from three geographic locations. Sediment cores were collected from as deep as 2.7 km below land surface and at temperatures up to 60^0C. The U.S. Department of Energy (DOE) funded the collection and identification over 10,000 strains of bacteria that were isolated from the three terrestrial subsurface locations. These strains of microorganisms are mostly bacteria--gram-negative bacteria such as *Comamonas* and *Pseudomonas* and gram-positive bacteria such as *Bacillus* and *Streptococcus.* These 10.000 strains collectively are known as the Subsurface Microbial Culture Collection (SMCC). (Balkwill et al., 1997)

One of the three sites is the Savannah River Site (SRS). This is a location where acidic low-level radioactive waste solutions were discharged into three unlined seepage basins at a site named the F-Area, from 1955 through about 1989. Because of nuclear weapon production during the Cold War, the groundwater is still acidic and contaminated with significant levels of U(VI), radionuclides (Bea et al., 2013), metals, anions and organic solvents(Benyehuda et al., 2003). Bacteria from this site will be the focus of this thesis.

Heavy metals

Several studies have showed that the high levels of heavy metals adversely influence the qualitative and the quantitative composition of microbial communities by affecting growth, morphology, and biochemical activities; resulting in a decrease in biomass and diversity. Nevertheless, microorganisms have developed resistance mechanisms to deal with metal toxicity. Studies have shown that bacteria isolated from industrial soil are significantly more resistant to several different heavy metals Hg, Cd, Cu, Cr, Pb, Zn and Ni than bacteria from agricultural soil(Abdul Malik et al., 2002).Some heavy metals are needed as trace elements in low quantities. However, these elements can become strongly inhibitory for susceptible microorganisms at comparatively low concentrations (Sevgi et al., 2010).

Metal resistant bacteria have several mechanisms that allow them to resist the toxicity of heavy metal ions, allowing them to survive and persist under metal-stressed conditions. These mechanisms fall into three main categories; the efflux of metal ions to the outside of the cell, the accumulation and complexation of the metal ions inside the cell, and the reduction of the heavy metal ions to a less toxic state. The adaptation of bacteria to heavy metals has involved the acquisition of resistance systems through an assortment of chromosomal-, transposon-, and plasmid-mediated routes. (Sevgi et al.,2010)

Most contamination sites around the world are the result of human activities such as discharge of industrial wastes into natural waterways, various metallurgical industries, accidental spills or mining. Hazardous wastes can include heavy metals such as lead, cadmium, mercury, silver and chromium. These metals are not beneficial to bacterial cells, and they are toxic even at low concentrations (Trajanovska et al., 1997).However, resistance to a range of heavy metals has been found widely among bacteria isolated from the deep terrestrial subsurface (Benyehuda et al., 2003).

The *merB*-1 gene is part of the *mer* operon (Mathema et al., 2011). It is part of an operon that confers resistance to mercury in *Staphylococcus aureus* (Soge et al., 2008), and the gene encodes for an organomercurylyase, which cleaves the carbon-mercury bond in organomercurial compounds, detoxifying them (Mathema et al., 2011). Subsequently, volatilization of mercury is accomplished by the reduction of the resulting Hg^{2+} to volatile Hg^{0} by the mercuric reductase encoded by *merA*. (Sone et al., 2013). The genes that encode mercury resistance are often found on plasmids and transposons (Trajanovska et al., 1997).

The *cnr* gene confers resistance to nickel and cobalt in *Cupriavidus spp*. (formerly *Ralstonia spp.*) strain CH34. In *Cupriavidus spp*. strain CH34, the *cnr* gene is located on plasmid pMOL28. Resistance to nickel and cobalt is based on cation efflux (Grass et al., 2000), which is driven by a chemo-osmotic proton-antiporter system (Taghavi et al., 2001). The resistance-encoding genes are often located on plasmids and probably spread by horizontal transfer (Trajanovska et al., 1997).
Horizontal gene transfer:

Horizontal gene transfer (HGT) has been described as a major force for genetic innovation among bacterial species (Wisecaver et al.,2013).HGT is the movement of DNA between organisms of different lineages. HGT is important because of its implication in the spread of antibiotic resistance, heavy metal resistance, and other forms of resistance and novel metabolic function (Park et al.,2003). HGT has been investigated in surface soils, natural waters, and biofilm communities, but little is known about the occurrence of HGT in deep subsurface bacteria. Nevertheless, a few studies have shown that HGT is evident among gamma and beta proteobacteria isolates from the deep subsurface layers of the Savannah River Site. Based on this evidence, it appears that HGT has played a distinct role in the evolution of metal resistance in deep subsurface bacteria (Coombs and Barkay, 2004; Martinez, 2006).

At the end of the1950s, classification of plasmids became important after the discovery of resistance plasmids and their wide distribution. Plasmids are circular pieces of extra-chromosomal DNA, and contain genes that ensure stable inheritance, as well as genes which are useful to the host cell. Transposons are mobile genetic elements that can excise themselves from one part of a host's genome and insert themselves into other parts of the genome. Transposons have created a great deal of variation and flexibility in plasmids(Couturier et al., 1988). In spite of the widespread existence of plasmid DNA in bacterial isolates from aquatic and marine environments little is known of the incidence and function of plasmid DNA in isolates from subsurface environments and most plasmids remain cryptic. Nevertheless, most of these plasmids of unknown function are believed to be beneficial, conveying an advantage to the host in the form of resistance to antibiotics and/or metals, metabolism of organic compounds, orthe ability to fix nitrogen (Fredrickson et al., 1988).

Bioremediation
Bioremediation is a technique of using microorganisms to eliminate or treat contaminated sites in soils, sediments, water and air (Hazen & Henry, 2005). Bioremediation depends on transforming or degrading contaminants to chemicals that are non-hazardous or less hazardous. Bioremediation can occur through biotransformation, a word that refers to any change of the molecular structure of a compound by living organisms, andit can also occur through biodegradation, which is the breaking down of organic components to smaller organic or inorganic components by organisms (Tabak et al., 2005). Bioremediation is one of the most cost-effective approaches for the treatment of contaminated environments. The aim of the bioremediation approach is to immobilize metals and radio nuclides as insoluble complexes in aquifers and the vadose zone, and also to degrade organic chelating agents to prevent or slow mobilization. However, metabolically active microbial communities are required for bioremediation in the subsurface (Benyehuda et al., 2003).

***Pseudomonas spp.* B0623:**
The bacterium *Pseudomonasspp.* B0623 has been isolated from a sediment core extracted from 244 to 259 meters below land surface at the U.S Department of Energy Savannah River Site (SRS) in Aiken, South Carolina, USA(Brown&Balkwill, 2009). *Pseudomonasspp.* B0623 is one of the SMCC strains that has previously been characterized by phylogenetic analysis of its 16S ribosomal RNA (rRNA) gene sequence(Balkwill et al., 1997). The genus *Pseudomonas*contains gram-negative, aerobic, motile, short rod-shaped cells. The classification of *Pseudomonas* has been redone many times on the basis of phenotypic features, DNA–DNA hybridization, 16S rRNA gene sequence similarity and chemotaxonomic data.Many studies have shown that *Pseudomonas* strains can degrade pollutants which are caused by human activities (Kumar Gupta et al., 2008) such aspesticides, industrial byproducts that are discharged to water and air resources, household chemicals and pharmaceuticals that are used in animal feeding operations(Kolpin et al., 2002). Because*Pseudomonas* strains have been shown to degrade pollutants, they are often used in the bioremediation of contaminated sites (Kumar Gupta et al., 2008).*Pseudomonas* strains are useful for genetic manipulation due to the presence of resistance markers and ease of culture and use (Taghavi et al., 2001).

The metabolic versatility of *Pseudomonas* enables this genus to be ubiquitous in the nature (Kumar Gupta et al., 2008). The genus *Pseudomonas* includes many species of environmental, clinical, agricultural, and biotechnological interest, and*Pseudomonas* species are considered dominant in the rhizosphere. The genus *Pseudomonas*has 118 species at the current time, and these species can be organized into nine groups including the four major *P. aeruginosa*, *P. putida*, *P. syringae*, and *P. fluorescens* groups. Figure. 1 shows the taxonomy of *Pseudomonasspp.* The largest group of *Pseudomonas* is the *P. fluorescens* group, this group has 20% of the known *Pseudomonas* species (Bodilis et al., 2011).

The purpose of the present study is to confirm data from previous microarray studies by using sequence analysisto demonstrate the presence of plasmid-borne metal resistance genes. Initially strain B0669 was used, however there were technical difficulties with the very large plasmid from this

strain, thus the focus of this study has changed to another plasmid which is from strain B0623. The original microarray studies indicated that *Pseudomonas* spp. B0623 has the potential to carry metals resistance genes on its plasmids. In particular the plasmid that is ~2.0 kbis believed to carry genes for mercury and cobalt resistance. Our hypothesis is that sequences encoding these heavy metal resistances are present on our cryptic plasmid, and will match with the findings of previous microarray studies.

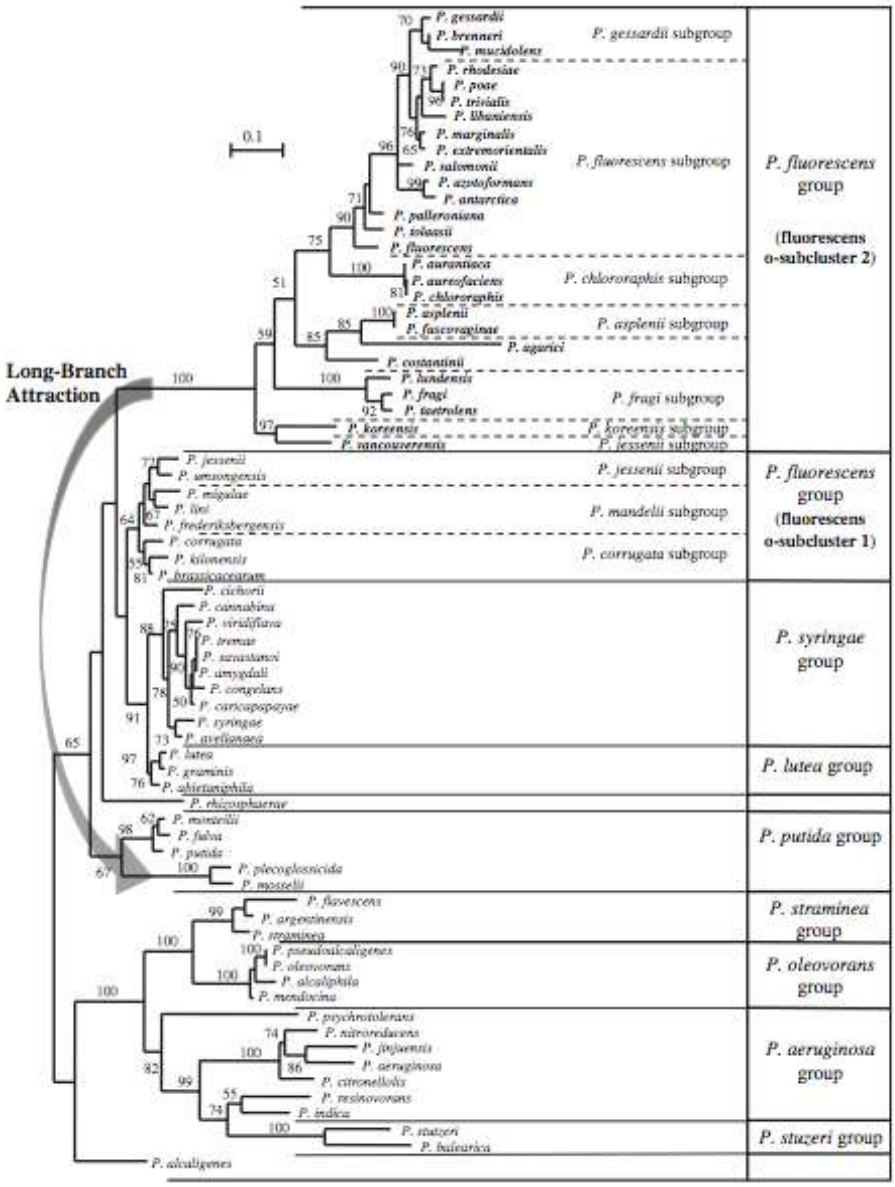

Figure 1: The taxonomy of *Pseudomonas spp.*118 species in *Pseudomonas* can be grouped into nine groups including the four major *P. aeruginosa, P. putida, P. syringae, and P. fluorescens* groups. (Bodilis et al., 2011)

2. Material and Methods

Bacterial strains and culture conditions

The SRS sediment sample containing *Pseudomonasspp.* B0623 and *Comamonassp.* B0669 was previously obtained from 244 meters below the soil surface (Brown & Balkwill, 2009).Both strains were stored in 40% glycerol stocks at -80°C. Both strains were routinely cultured on nutrient-rich media, Luria-Bertani (LB) broth(10g Bacto-tryptone, 5g yeast extract, 10g Nacl in 1L H_2O) or agar(10g Bacto-tryptone, 5g yeast extract, 10g Nacl, 15g agar in 1L H_2O).Plates and broth cultures were routinely incubated at 28°C overnight.

DNA Purification

Plasmid was isolated from *Pseudomonasspp.*B0623 and *Comamonassp.* B0669 by using both the Qiagen Plasmid Mini kit(Qiagen Inc., Valencia CA) and Qiagen Plasmid Maxi prep kit(Qiagen Inc., Valencia CA). For the Qiagen Plasmid Mini prep kit, 1 ml of bacterial culture was pelleted by centrifugation at a speed of 8000 rpm(6800x*g*)for 3 minutes, and resuspended in P1 buffer. Then, P2 buffer was added and mixed completely by inverting the tube 4-6 times. N3 buffer was added and mixed immediately. After that, the sample was centrifuged at 13000 rpm (~17900*xg*) for 10 minutes. The supernatant was applied to a QIA prep spin column and centrifuged for 60 seconds. PB buffer was added to wash the QIAprep spin column, and the sample was subsequently centrifuged for 60 seconds. PE buffer was

added to wash the QIAprep spin column, and then the column was centrifuged for an additional 1 minute to remove any PE buffer. Finally, the column was transferred to a clean eppendorf tube and EB buffer was added in the center of the column to elute the DNA. The column was centrifuged for 1 minute. The plasmid DNA sample was stored at -20°C.

For the Qiagen Plasmid Maxi prep kit, 1 liter of *Pseudomonasspp.* B0623 culturewas centrifuged at 6000*xg* for 15 minutes. The bacterial pellet was resuspended in P1 buffer. P2 buffer was added, mixed completely by inverting the bottles 4-6 times, and the sample was incubated at room temperature for 5 minutes. Then, pre-chilled P3 buffer was added and the samples were incubated on ice for 15-20 minutes. After incubation the bacterial cells were centrifuged at 14,000*xg* for 10 minutes at 4°C. A QIAgen filter tip was equilibrated by adding QBT buffer. The B0623 supernatant was then applied to the Qiagen-tip, and filtered by gravity flow.QC buffer was added to QIAgen tip in order to wash away any contaminants in the plasmid DNA preparation. To elute DNA, QF buffer was added and the flow-through was collected. Isopropanol was added to precipitate the DNA, and the samples were centrifuged at 11,000*xg* for 30 minutes at 4°C. The isopropanol was discarded and 70% ethanol was added to wash the pellet at room temperature. The samples were centrifuged at 11,000*xg* for 10 minutes. The pellets were air-driedfor 10 minutes and then TE buffer was added to re-dissolve the DNA. The purified DNA was stored at -20°C.

Gel Electrophoresis

Plasmid DNA samples were electrophoresed at 80 volts for 60 minutes by using Owl™EasyCast™ B1A Mini Gel system. Agarose gels were prepared by adding 0.3g of agarose powder (MIDSCI, St. Louis, MO) into 50ml 1xTAE buffer(40nM Tris, 20mM acetic Acid, 1mM EDTA). The gel was heated until the agarose melted completely. Liquid gel was poured into the casting tray and allowed to solidify. The DNA marker used was Lambda DNA/ *EcoRI + HindIII* (Thermo Scientific, Pittsburg, PA). After the samples were electrophoresed, the gel was stained with ethidium bromide (40 µl/L) for 10 or 15 minutes, and then de-stained with water for 10 minutes. Photographs were taken using the FOTO/Analyst Investigator (Fotodyne, Inc, Hartland, WI) and visualized using ImageJ (Abramoff et al., 2004).

Gel Purification:

The Qiaquick Gel Extraction Kit (Qiagen Inc., Valencia CA)was used in order to purify the plasmid DNA of *Comamonassp.* B0669 and *Pseudomonas spp*. B0623 from agarose gels. After cutting the DNA fragments from the gel using UV illumination, 3 volumes of QG buffer were added to 1 volume of gel. Then, the samples were incubated at 50°C for 10 minutes, inverting intermittently for 2-3 minutes in order to help dissolve gel slices. Isopropanol was added to the samples and they were mixed. The samples were transferred to QIA quick spin columns and centrifuged for 1 minute. QG buffer was added to the spin column, and the samples were centrifuged for 1 minute. PE buffer was added to wash the QIAquick spin columns, and then the columns were centrifuged for an additional 1minute to remove any PE buffer. After transferring the columnsinto new eppendorf tubes, EB buffer was added. The samples were centrifuged for 1 minute to elute the DNA. Purified DNA was stored at -20°C.

Restriction Enzyme Digests

Pseudomonas spp. B0623 plasmid was digested with *Sau3AI, EcoRI, and BamHI* (New England Biolabs, Ipswich, MA)enzymes. The reaction mix contained B0623 plasmid (28 µl), 10X BSA (4 µl), enzyme (0.5 µl) and buffer that corresponded to each specific enzyme (4 µl). In order to make diluted BSA, 10 µlof 100X BSA was added into 90 µl sterilization water. The samples were incubated for one hour for *EcoRI* and *BamHI* and for 15minutes for *SAU3AI* at 37°C.*Sau3AI* enzyme was subsequently inactivated by incubating the sample at 65°C for 20 minutes.

In order to digest the vector pUC19 DNA(Thermo Scientific, Pittsburg, PA) before ligation to*Pseudomonas spp.* B0623 DNA (see ligation section below), *BamHI* enzyme was used to cut the pUC19 by adding pUC19 (38 µl), BSA (5 µl), *BamHI* enzyme (2 µl) and its corresponding buffer (5 µl). The sample was incubated at 37°C for 1 hour. After digestion, thepUC19 sample was loaded onto an agarose gel. Gel purification was performed to purify pUC19 DNA from the gel, as described above.

Ligation of DNA

The Fast Link Ligation Kit and Ligation Protocol with T4 DNA Ligase (Epicentre Technologies, Inc., Madison, WI)wereperformed to ligate *Pseudomonas spp.* B0623 DNA with the cloning vector pUC19.

For ligation of DNA, 1 µl of purified *BamHI*-digested pUC19,10 µlof *Sau3AI*-digestedB0623 plasmid, ATP (1.5 µl), Fast-link ligase (1 µl) and its corresponding buffer (1.5 µl). The sample was incubated at room temperature for 15 minutes. Then heat inactivation of the ligase was performed by exposing the sample to heat at 70°C for 15 minutes. A second ligation protocol, using T4 DNA Ligase(New England Biolabs, Inc, Ipswich. MA)was also used. This ligation was set up as follows: T4 DNA ligase buffer (2 µl), T4 DNA ligase (1 µl), pUC 19 with *BamHI* enzyme (1 µl), B0623 plasmid with *SAU3AI* enzyme (5 µl), water (11 µl). The mix was incubated at room temperature for 15 minutes.

Transformation

Ligation product was transformed into *E. coli* (Xl1-Blue). 5 µl of the ligation mix was added to 50 µl of XL1-Blue cells,and the mixture was incubated for 30 minutes on ice. After incubation, a 2 minute heat-shock at 42°C was performed, followed by a 2 minute incubation on ice. Then 1 ml from LB broth was added to the mixture and the culture was incubated at 37°C for 1 hour with shaking. Uncut/unligatedpUC19 was used as a transformation control following the same procedure as above except that 1 µl of pUC19 was added to to 50 µ l of XL1-Blue cells. Transformants were plated on blue/white screening agar containing 100 µ M ampicillin, 0.02 mg/ml X-gal, and 0.1 *m* MIPTG, then the plates were incubated at 37°C overnight. From the plates, white colonies were selected for screening.

3. Results

The aim of this study was to isolate cryptic plasmids from *Comamonassp.* B0669 and *Pseudomonas spp*. B0623 and characterize these plasmids using bioinformatics. Micro array analysis of some of the plasmids from these two strains indicate that they may encode genes for resistance to mercury, nickel, cobalt and cadmium. The preparation of *Comamonassp.* B0669produceda chromosomal DNA band and one plasmid band that was visible at approximately 10 kb, as shown in figure 2.Many attempts were made to gel purify plasmid DNA from *Comamonassp.* B0669, and the yields were too low to continue with for this strain(data not shown). Therefore, the focus of this study has changed from *Comamonassp.* B0669 to *Pseudomonas spp*. B0623because of the technical difficulties with the very large plasmid of *Comamonassp.* B0669.

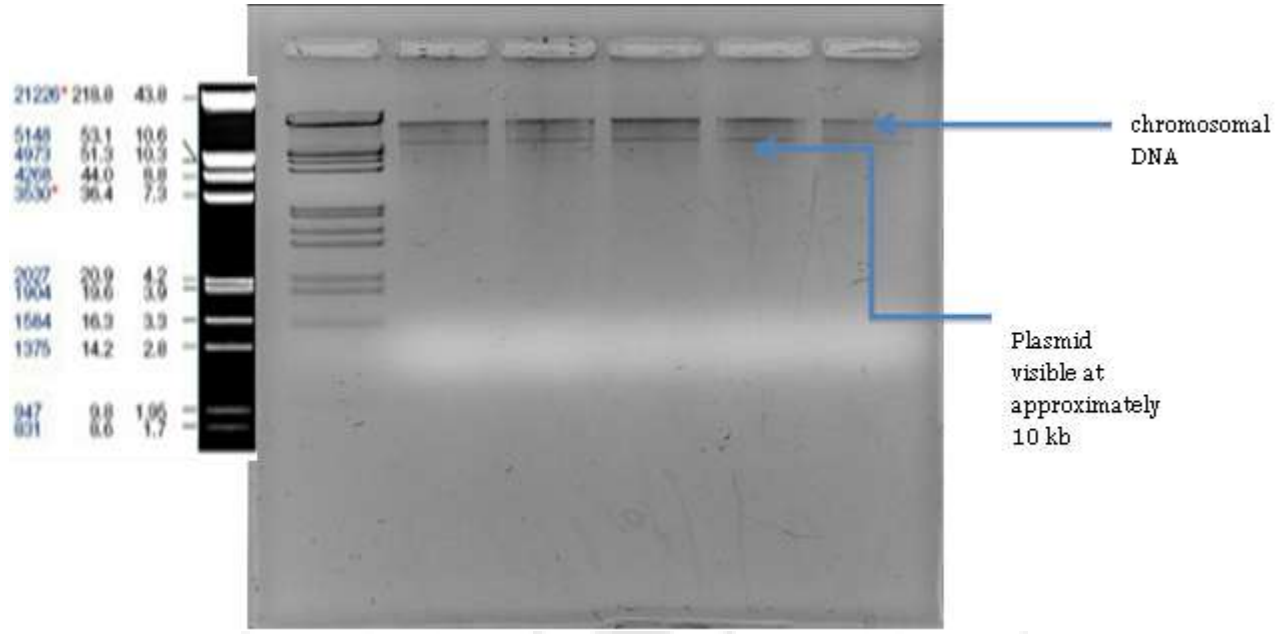

Figure 2: B0699 maxiprep DNA electrophoresed on an agarose gel. In lane 1 is the Lambda DNA/ EcoRI + HindIII marker. Lanes 2-5 containB0669 genomic DNA. The top band is chromosomal DNA, and the bottom one is the plasmid, visible at approximately 10 kb

Genomic DNA from *Pseudomonas spp*. B0623was purified from cells using both the Qiagen plasmid mini prep kit and the Qiagen plasmid maxi prep kit. As shown in figure 3, purification using the mini prep kit for *Pseudomonas spp*. B0623 resulted in one band which was visible at approximately 23 kb. The yields of all plasmid bands from this strain were too low to proceed to gel purification. Therefore, we decided to use the maxi prep kit for purification. As shown in figure 4, purification using the maxi prep kit for *Pseudomonas spp*. B0623 resulted in chromosomal DNA and five plasmid bands that were visible from 1.9 kb to 23 kb. The plasmid with size ~2.0 kb was excised from the gel and purified using the Qiaquick gel extraction kit.

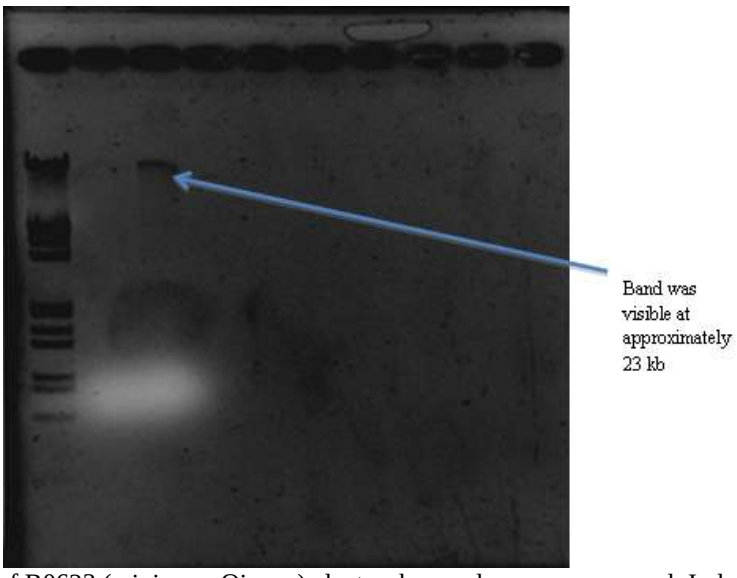

Figure 3: Plasmid prep of B0623 (miniprep, Qiagen) electrophoresed on an agarose gel. In lane 1 is the Lambda DNA/ EcoRI + HindIII marker. Lane 3, only one band was visible at approximately 23 kb.

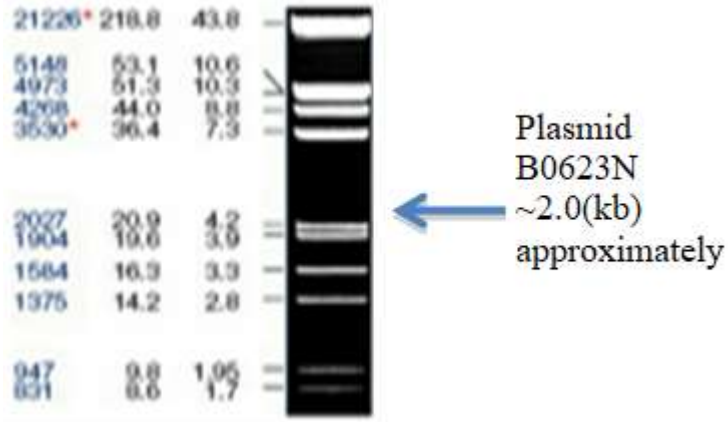

Figure 4: Plasmid prep of B0623 (maxiprep, Qiagen) electrophoresed on an agarose gel. In lane 1 is the Lambda DNA/ EcoRI + HindIII marker. Lanes 2&3 contains genomic DNA from B0623. The top band in lanes 2 and 3 is chromosomal DNA, and the five bottom bands are plasmids of varying size, from 1.9 kb to 23 kb approximately.

Subsequently, the isolated plasmid B0623N (~2.0 kb) was tested with three different restriction enzymes *EcoRI*, *BamHI* and *Sau3A*. As shown in figure 5, the plasmid was not cut with *EcoRI* and *BamHI*. This indicates that the plasmid didn't contain any restriction enzyme recognition sites for *EcoRI*or*BamHI*.

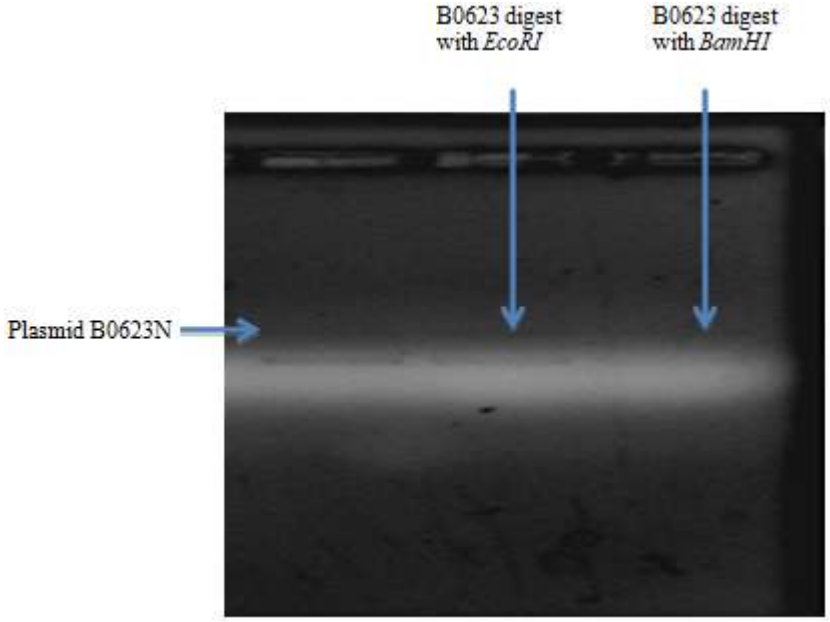

Figure 5: Restriction digest of plasmid B0623N. Lane 1 is the uncut control. Lane 2 is plasmid digested with *EcoRI* . Lane 3 is plasmid digested with *BamHI*.

The plasmid B0623N was incubated with 4 μ l of the restriction enzyme *Sau3A* for 1 hour. The plasmid does not appear to have been cut by the enzyme, since the bands from the digest sample were identical to the uncut plasmid sample, as shown in figure 6. This is likely because the enzyme was expired. Thus, we decided to repeat the restriction digest of plasmid B0623N withfresh*Sau3A* in various amounts (1μl & 0.5μl) and times (15 min& 30 min), as shown in figure 7,8. The enzyme cut the plasmid, as demonstrated by the disappearance of the band at 2.0 kb, and the appearance of a very small band at ~500 bp.

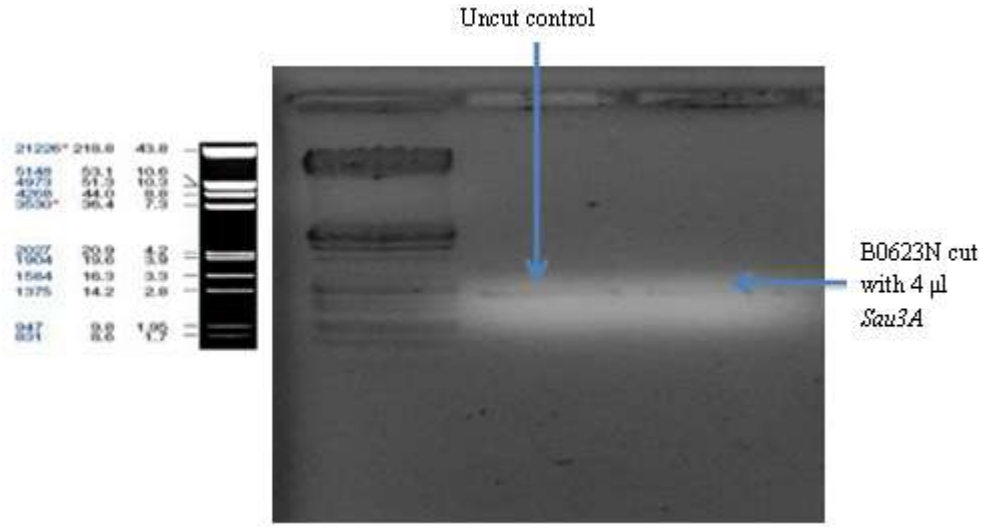

Figure 6: Restriction digest of plasmid B0623N with 4 µl *Sau3A*. Lane 1 is the Lambda DNA/ EcoRI + HindIII marker. Lanes 2 uncut control. Lane 3 digested plasmid

A restriction digest of 0.5 µl *Sau3A*for 15 minutes of the plasmid B0623Nproduceda very small band that was low yield. Due to the low yield and small size, it did not seem that this DNA would be a good candidate for sequencing. Consequently, we decide to test the plasmid with six additional restriction enzymes. The restriction enzymes were *PstI*, *SalI*, *SacI*, *Xbal*, *SmaI* and *KpnI*. Figure 9 shows the restriction digest with these six enzymes. However, none of the new enzymes appeared to cut B0623N. Thus, we decided to resume using*Sau3A*to cut the plasmid B0623N.

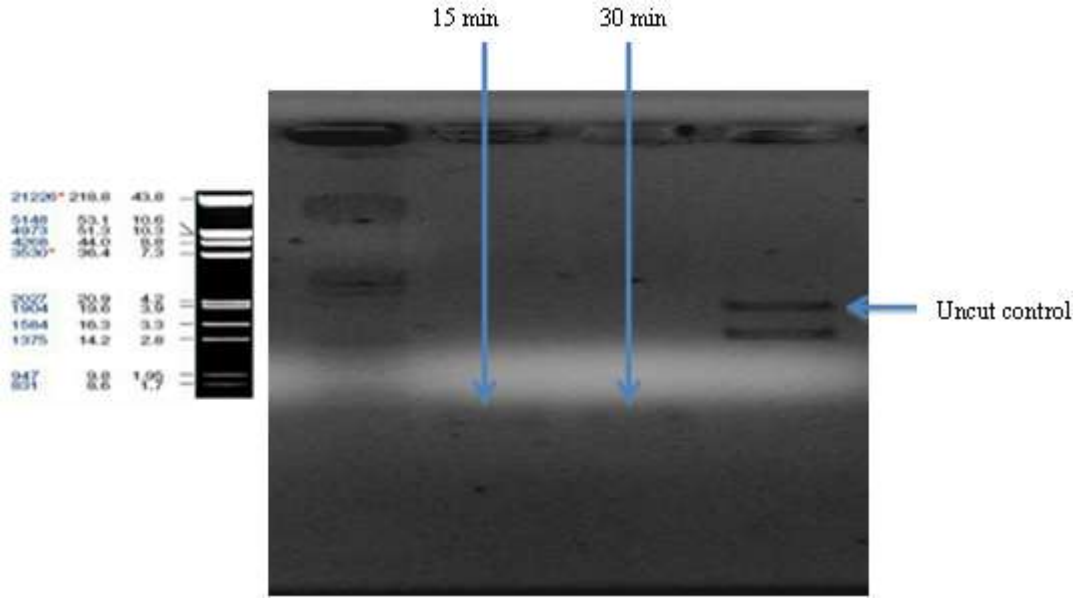

Figure 7: Restriction digest of plasmid B0623N with 1µl*Sau3A*. Lane 1 is the Lambda DNA/ EcoRI + HindIII marker. Lanes 2 is B0623N plasmid digested for 15 min. Lane 3 is B0623 plasmid digested for 30 min. Lane 4 is an uncut control.

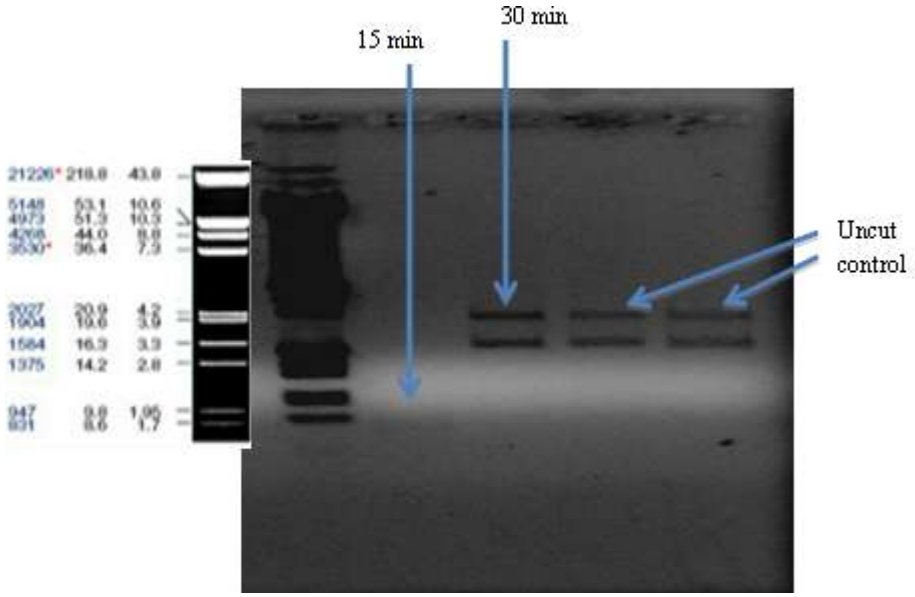

Figure 8: Restriction digest of plasmid B0623N with 0.5μl*Sau3A*. Lane 1 is the Lambda DNA/ EcoRI + HindIII marker. Lane 2 is digested B0623N plasmid (15 min.). Lane 3 is digested B0623N plasmid digested for30 min. Lane 4 is an uncut control, incubated at 37ºC for 15 min. Lane 5 is an uncut control incubated at 37ºC for 30 min.

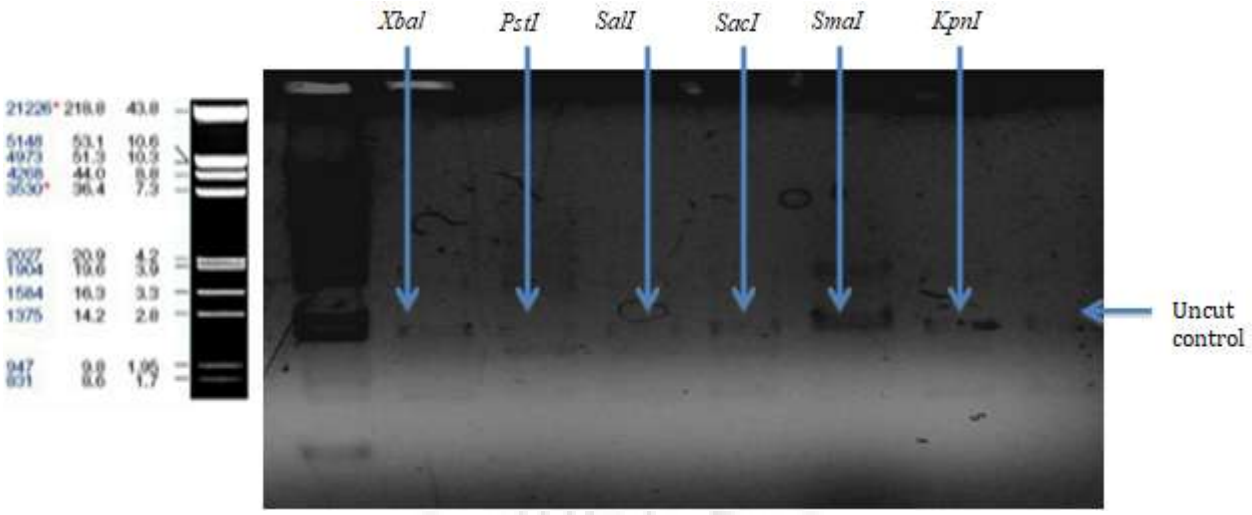

Figure 9: Restriction digest of plasmid B0623N with six enzymes

The ligation of DNA is the covalent linking of two DNA molecules, using either cohesive ("sticky") or blunt ends. This procedure is performed using DNA ligase, which catalyzes the formation of a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another. For this project the ligation was performed using pUC19 as a vector, and ligating it to the cut pieces of plasmid B0623N.Fast-Link DNA ligase functioned to bind the sugar phosphate backbones of the two fragments together to create recombinant DNA. In order to digest the vector pUC19, *BamHI* was used. The cut DNA was purified from a gel before the ligation step with *Pseudomonas spp.* B0623 DNA. Figure 10 shows the digest of pUC19.

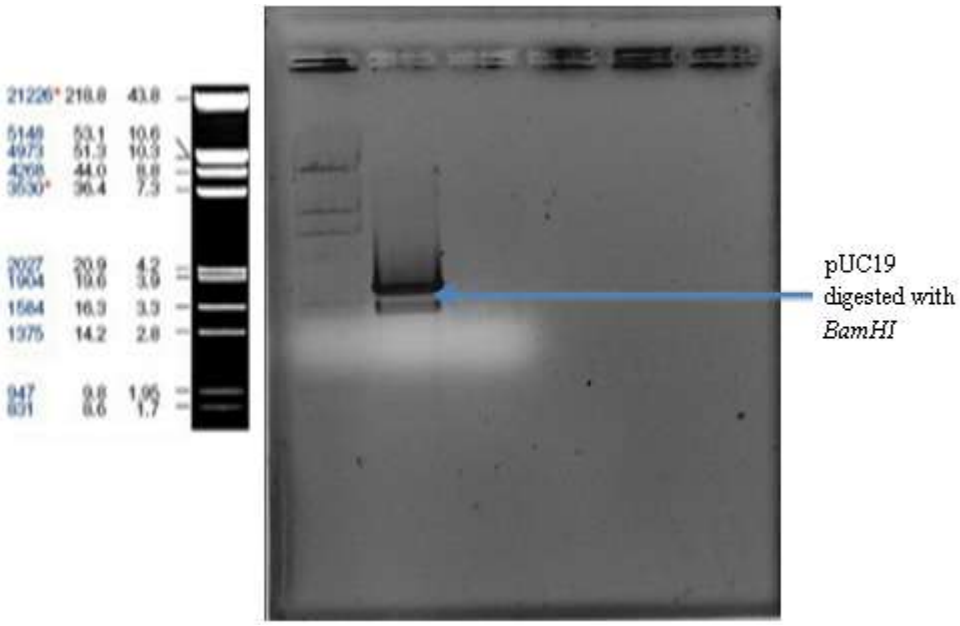

Figure 10: Restriction digest of pUC19 DNA with *BamHI.*Lane 1 is the Lambda DNA/ EcoRI + HindIII marker. Lanes 2 is digested pUC19. Lane 3 uncut control

The recombinant DNA of the ligation mixture of pUC19and B0623Nwas transformed into *E. coli* (Xl1-Blue), which is a host strain for routine cloning applications. Then the transformants were plated on blue white screening agar containing ampicillin, X-gal, and IPTG. IPTG is an inducer of the *lacZ* gene. X-gal is a chromogenic indicator—if cells produce intact LacZ enzyme, the enzyme will hydrolyze the X-gal and produce a blue colored product. Therefore, if the cell has pUC19 plus B0623N (which is inserted into the multiple cloning site of pUC19, disrupting the *lacZ* gene), the cell will produce white colonies, while if the cell has only pUC19 without B0623N, it will produce blue colonies. Ampicillin antibiotic is a selection for successful transformation of cells, indicating if they have plasmid or not. The plasmid pUC19 contains a gene encoding beta-lactamase, an enzyme that breaks down the antibiotic ampicillin. In our experiments, the transformant plates of XL1-Blue cells didn't produce any colonies either white or blue. A separate control transformation with just pUC19 plasmid produced only blue colonies, as expected. The trials were repeated several times and the same results obtained.

The results of this study were inconclusive, because we were unable to confirm that any of the*E. coli* (Xl1-Blue) transformants contained the desired plasmid insert, and thus we were not able to continue on to the DNA sequencing part of the project. The problem likely is due to the ligation step, since the transformation controls were successful. A follow-up experiment in which pUC19 plasmid is cut and then re-ligated to itself without insert would provide information as to whether the ligation protocol is working properly. In the future, characterization of the B0623N plasmid will be possible provided that a suitable restriction enzyme is found, and the ligation can be optimized.

4. Discussion

The purpose of this study was to characterize a cryptic plasmid from a deep subsurface bacterium. Based on DNA microarray analysis, the plasmid is believed to contain genes encoding beneficial phenotypes. The isolates *Comamonassp.* B0669 and *Pseudomonas spp*. B0623 have been isolated from the deep terrestrial subsurface from 244 to 259meters below land surface at the Savannah River Site (SRS) (Brown & Balkwill, 2009).Initially we examined the large plasmid from *Comamonassp.* B0669. Although we were able to purify DNA from this strain, we were unable to separate the plasmid from the genomic DNA at high yield. Therefore, we chose to focus on a smaller plasmid from *Pseudomonas spp*. B0623 to determine if it possessed metal resistance genes. Most plasmids from the deep subsurface are large enough to carry multiple genes encoding useful functions; such as conjugative transfer, catabolism of organic compounds or heavy metals resistance. However, the plasmids in the terrestrial subsurface have not been characterized (Fredrickson et al., 1988).Previous studieswith *Pseudomonas spp*. B0623showed that this bacterial strain appeared to contain genes encoding resistance to heavy metals. More specifically, *Pseudomonas spp*. B0623 contains *merB-1* and *Cnr-1* genes, which encode resistance to the mercury and cobalt. *Sphingomonas spp.* LH128 was isolated from polycyclic aromatic hydrocarbons (PAH) contaminated soil. This organism has been identified to contain two genes phnA1f and phnA2f, that encode a dioxygenase complex that is able to degrade and oxidize low-molecular-weight PAHs and the high-molecular- weight PAHs (Schuler et al, 2009). These genes were identified because some one took the time to characterize an unknown plasmid from an environmental strain.

In this study, plasmid DNA purified using the Qiagen mini prep kit resulted in inconsistent recovery of DNA and low yield. Therefore, we decided to use the Qiagen maxi prep kit so that it would be possible to obtain DNA from larger volumes of culture. This approach has been very successful

in increasing DNA yield, and we have been able to successfully gel purify a single plasmid band of about 2.0 kb that we refer to as B0623N.

Although the purification of B0623N has gone well, there have been several setbacks that have prevented us from achieving our goal of characterizing this cryptic plasmid. One of the major problems in this study was finding a suitable restriction enzyme that would cut B0623N. Many of the enzymes that were tested did not appear to cut the plasmid DNA. The most successful enzyme used was *Sau3A*. This restriction digest enzyme has a 4 base pair recognition site. Most enzymes used for this type of work recognize 6 base pair sequencemotifs. Because of this *Sau3A* cuts more often then other enzymes and so cuts the DNA into smaller pieces. We tried to solve the problem of over-digestion by performing partial digests for 15 minutes or 30 minutes rather than 1 hour. In addition, the copy number of the plasmid was very low, which posed some difficulties during the purification steps.

The most significant barrier to sequencing of the cryptic plasmid appeared to be the ligation step. This was unsuccessful due to the lack ofeither white or blue colonies on the white blue screening agar. In this study, we tried two protocols for ligation; the Epicentre Fast Link Ligation kit and the ligation Protocol with T4 DNA Ligase. In both cases, however, no transformants were obtained, except for the uncut pUC19 control, which produced blue colonies on the agar plates. These protocols were repeated and the same results were obtained. To resolve this issue, we would repeat the ligation, first with cut and then re-ligated pUC19 to test the ligation conditions, and when that was successful, we would re-try the ligation with B0623 plasmid and pUC19.

Although technical issues prevented us from obtaining DNA sequence for bioinformatics analysis, it is clear that this work has potential, and that in the future it will be possible to sequence the cryptic plasmid from *Pseudomonas spp*. B0623 and find genes that encode heavy metals resistance. The LI-COR 4300 DNA Analyzer can be used to sequence the plasmid DNA, using sequencing primer binding sites (M13R and M13F) that are present on pUC19. The DNA analyzer works by using lasers to scan samples and using two photodiodes to detect fluorescence from the fluorescently labeled primers that become incorporated into the DNA sequence.

This study has implications for bioremediation in the deep terrestrial subsurface; if the expected heavy metal resistance genes are present, it indicates that *Pseudomonas spp*. B0623 will be able to resist heavy metal pollutants, and may also degrade organic compounds in sites contaminated with mixed wastes site with high concentrations of metals. Furthermore, this study has the potential to uncover the role of *Pseudomonas* plasmids in the acquisition of resistance genes in the deep subsurface. Further research should be conducted in order to further characterize the plasmid, and identify whether the plasmid contains gene encoding.

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