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# A Tale of Two Investigations in Molecular Biology: the Use of PCR Technology to Identify Bacteria Containing Genes for PHA Synthesis and the Antibiotic Sensitivity Profile of Vibrio Species B-18

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# A TALE OF TWO INVESTIGATIONS IN MOLECULAR BIOLOGY: THE USE OF PCR TECHNOLOGY TO IDENTIFY BACTERIA CONTAINING GENES FOR PHA SYNTHESIS AND THE ANTIBIOTIC SENSITIVITY PROFILE OF *VIBRIO* SPECIES B-18

By

Diana B. Lizarazo

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in

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The School of Arts and Sciences

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2008

**ABSTRACT**

Polyhydroxyalkanoic acid or PHA is a bacterial storage material with application as a biodegradable plastic. The enzyme that produces this polymer from alkanoic acid monomers is called PHA synthase, which is encoded by the *phaC* gene. The composition of PHA is determined by the substrate specificity of the enzyme as well as the substrates available in the cell. A bioluminescent *Vibrio* species, named B-18, has been shown to produce PHA of an unusual composition and may contain a novel PHA synthase.

The goal of the first phase of this project was to determine if PCR technology could be employed to find other bacteria that produce PHA of a similar composition to that of B-18. PCR is a technique where DNA sequences located between two primers are amplified, provided that the template DNA contains sequences that the primers can bind. Three different *Vibrio* species were examined using four different primer sets that flank the *phaC* gene. Unfortunately, only the control DNA was amplified indicating that this method or at least these primer sets are not that useful for identifying bacteria that contain PHA genes.

The goal of the second phase of the project was to determine if it is possible to introduce *Escherichia coli* plasmids into B-18. If successful, this would allow for mutational analyses of B-18 and other studies in the future. First, an antibiotic sensitivity profile of B-18 was determined using ampicillin, chloramphenicol, kanamycin, and tetracycline. This determined if B-18 was naturally sensitive to the antibiotics as well as the best concentration to use. Attempts were then made to introduce *E. coli* plasmids into B-18 via electroporation. This technique uses short electrical pulses to create transient pores so that small pieces of DNA, like plasmids, can enter and leave cells.

Finally, a summer research internship was performed at Brown University through the Leadership Alliance. The gene expression of the ADAR enzyme and its potential targets in *Hydra magnipapillata* were examined. The results of this third phase will not be presented in this thesis. Rather, they will be presented in poster sessions during Honor’s week and at the Sigma Xi spring banquet.

# CHAPTER 1

## Screening for Bacteria that Synthesize PHA through PCR Analysis

Diana Lizarazo, Biology 491, Spring 2007

**Introduction and Purpose.** Polyhydroxyalkanoates (PHAs) are polyesters that are synthesized by bacteria when carbon sources are plentiful and one other nutrient, such as nitrogen, is limiting. In the absence of carbon sources, PHA is utilized as a source of carbon and energy. PHAs have received much attention since they show similar properties to petroleum-based plastics, yet PHA is both biodegradable and biocompatible. Several practical applications are being considered including biodegradable containers and medical devices. As a polymer, PHA is composed of hydroxyalkanoic acid monomers. The size of the monomers imparts different properties for the plastic. For example, PHA comprised of hydroxybutyric acid is very brittle, while that composed of hydroxyoctanoic acid is more elastic or rubbery. PHA synthase is the enzyme responsible for placing the hydroxyalkonic acid monomers together to form the polymer.

Polymerase Chain Reaction (PCR) is a technique used to amplify DNA between two primers, provided that the template DNA contains sequences homologous to the oligonucleotide primers. Several primer sets that can amplify DNA containing the PHA synthase gene (*pha*C) have been developed and are shown in Table 1 below.

**Table 1.** PCR Primer Sets that can amplify DNA sequences containing *phaC*.

| Forward Primer | Reverse Primer | Sequence Homology | Fragment Size (bp) |
|---|---|---|---|
| phaC.F | phaC.R | within B-18 *phaC* | 337 |
| phaCAe.f | phaCAe.r | within *R. eutropha phaC* | 565 |
| delprimer3 | phaCVp.R | entire B-18 *phaC* | 1903 |
| delprimer2 | phaCVp.R | entire *phaC* & *orf2* | 2297 |

Some of the primer sets were developed with sequence data from a 2.9-kb *Bam*HI-*Sal*I fragment of bioluminescent bacterium (B-18), whereas one set of primers was constructed from the sequence information from the *phaC* gene from *Ralstonia eutropha*, which was formerly known as *Alcaligenes eutrophus*.

The purpose of this project was to see if these primer sets could be used to screen for bacteria that contain the *phaC* gene. To answer this question, genomic DNA from the following bacteria will be included in this analysis. *Escherichia coli* was used as the negative control in this the study since the bacterium is not known to accumulate PHA. B-18 was used as the positive control since the sequence of the *phaC* gene from this bacterium was used to devise virtually all of the primer sets (Table 1). The *phaC* gene from B-18 has been cloned and sequenced, and shows strong homology with *pha*C genes from other Vibrios, such as *V. cholerae* and *V. parahaemolyticus*. Genomic DNA from *V.cholerae*, *V. vulnificus*, and *V. parahaemolyticus* was included in all further experiments.

**Procedure.**

**Isolation of Genomic DNA.** Standard molecular techniques were used in the isolation of genomic DNA. A 1.5-ml aliquot of an overnight bacterial culture was microcentrifuged and the resulting pellet was resuspended in TE buffer. SDS and proteinase K were added and the mixture was incubated at 37ºC for one hour to lyse the cells and digest all proteins, including nucleases. A CTAB/NaCl solution was added and the mixture was incubated at 65°C for 10 minutes. The mixture was extracted with chloroform:isoamyl alcohol and the aqueous (top) portion was removed, being careful to avoid the white precipitate. The aqueous phase containing the genomic DNA was extracted with phenol/chloroform/isoamyl alcohol and the aqueous phase was collected and precipitated with isopropanol. After centrifugation, the pellet was washed with 70% ethanol, dried, and resuspended in TE buffer. The concentration and purity of the genomic DNA preps were determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer and by electrophoresis in 1% agarose-TAE gels. The gels were stained with ethidium bromide, visualized with an UV-transilluminator, and photographed with a Fotodyne camera containing Polaroid film.

**PCR.** For each reaction, 0.25 mg genomic DNA was subjected to PCR using one of the primer sets (Table 1) and using the conditions described in Table 2. The PCR products

and the appropriate DNA size standards were subjected to electrophoresis in agarose-TAE gels. These were stained with ethidium bromide and the DNA fragments were visualized on a UV transilluminator and photographed using a Fotodyne camera containing Polaroid film.

**Table 2.** PCR Conditions.

| **Program** | **“PHA-PCR”** | **“Stringent –ToxR”** | **“JMU-PCR”** |
|---|---|---|---|
| Initial Melting | 94ºC, 3 minutes | --- | 94ºC, 2 minutes |
| Cycles<br>• Melting<br>• Annealing<br>• Extension | 30 Cycles:<br>94ºC, 1 minute<br>50ºC, 2 minutes<br>72ºC, 1.5 minutes | 20 Cycles:<br>94ºC, 1 minute<br>63ºC, 1.5 minutes<br>72ºC, 1.5 minutes | 35 Cycles:<br>94ºC, 1 minute<br>50ºC, 2 minutes<br>72ºC, 3 minutes |
| Final Extension | 72ºC, 7 minutes | --- | 72ºC, 10 minutes |
| Hold | 4ºC | 4ºC | 4ºC |

**Results.**

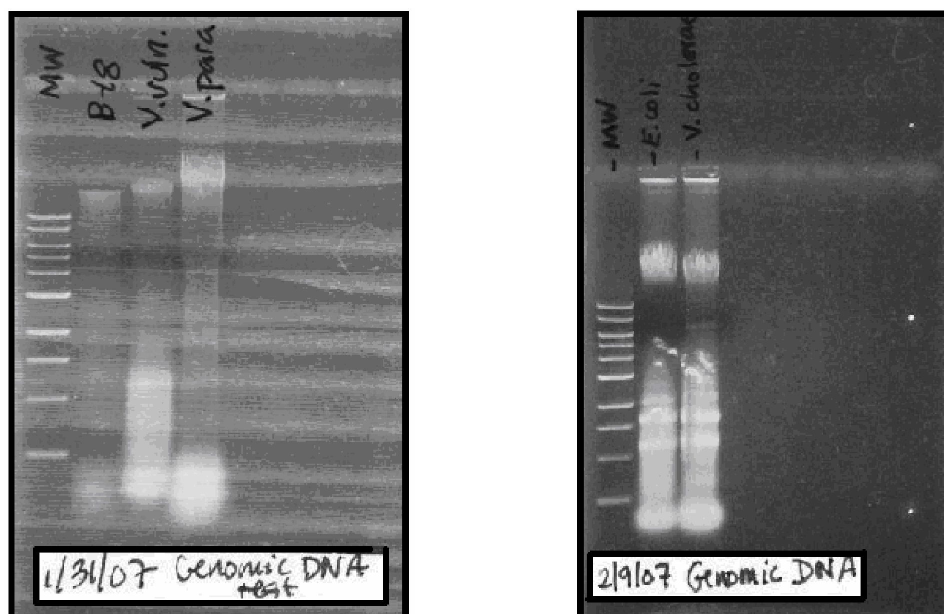


**Figure 1**. Genomic DNA preparation. MW: DNA size standards, B-18: bioluminescent *Vibrio* species, V. vuln.: *Vibrio vulnificus*, V. para: *V. parahaemolyticus,* E. coli: *Escherichia coli,* V. cholerae: *Vibrio cholerae.*

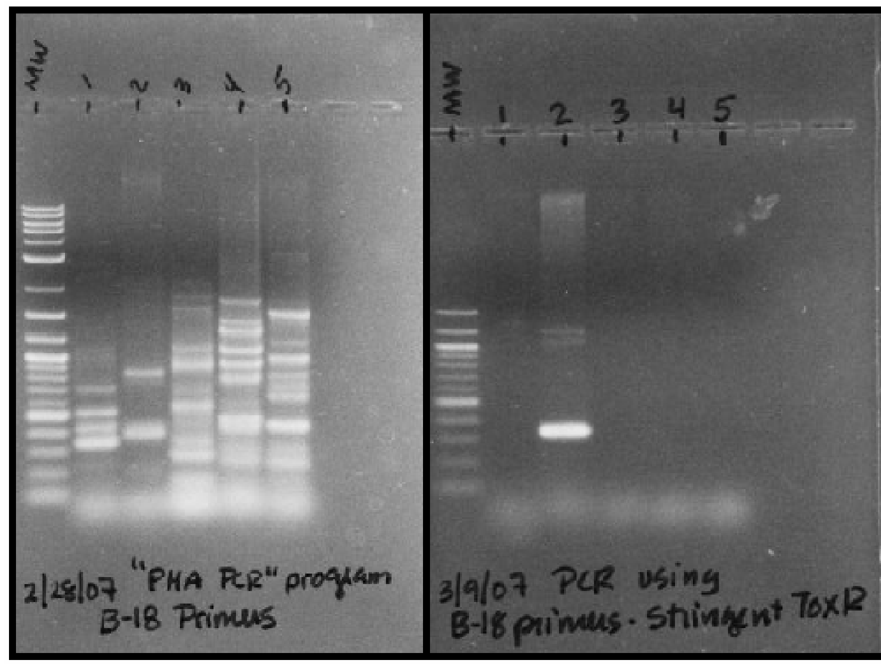


**Figure 2**. PCR using the B-18 primer set (phaC.R/phaC.F)- comparing "PHA-PCR" program and the "stringent ToxR" program. Lanes: MW= DNA size standards; 1= *E. coli*; 2= B-18; 3=*V. cholerae*; 4= *V. parahaemolyticus*; 5= *V. vulnificus*.

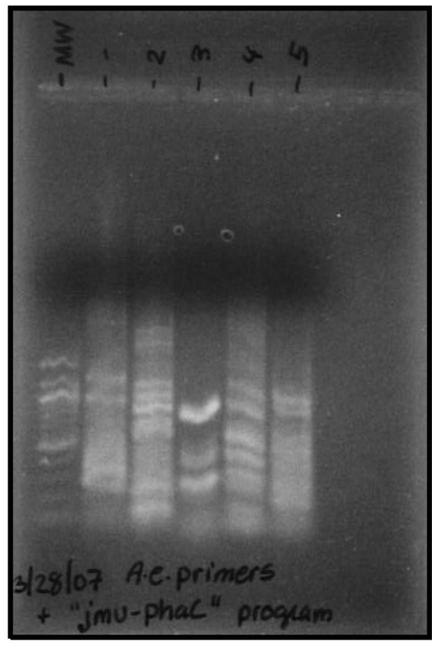


**Figure 3**. PCR using the Ae primers and the "JMU-phaC" program. Lanes: MW= DNA size standards; 1= *E. coli*; 2= B-18; 3=*V. cholerae*; 4= *V. parahaemolyticus*; 5= *V. vulnificus*.

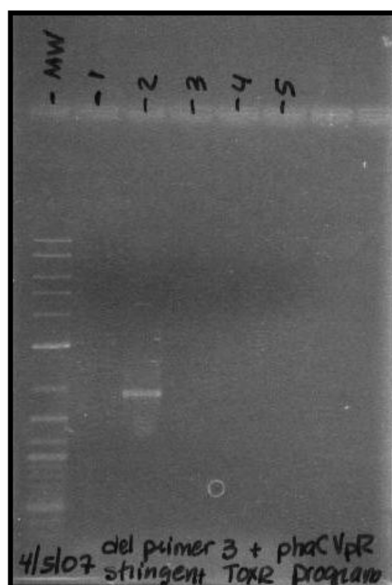


**Figure 4**. PCR using del primer 3 and phaCVp.R and the "stringent ToxR" program. Lanes: MW= DNA size standards; 1= *E. coli*; 2= B-18; 3=*V. cholerae*; 4= *V. parahaemolyticus*; 5= *V. vulnificus*.

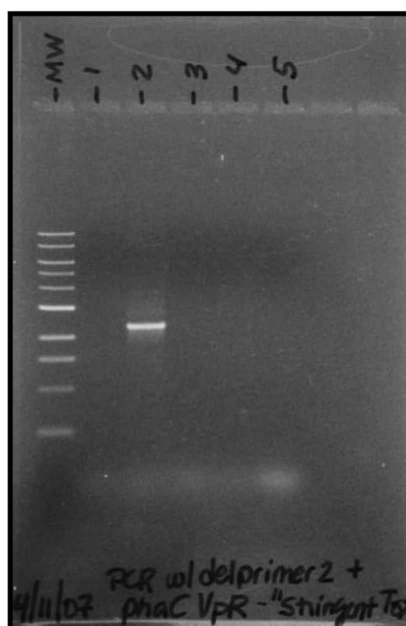


**Figure 5.** PCR using del primer 2 and phaCVp.R and the "stringent ToxR" program. Lanes: MW= DNA size standards; 1= *E. coli*; 2= B-18; 3=*V. cholerae*; 4= *V. parahaemolyticus*; 5= *V. vulnificus*.

**Discussion.** The objective of the experiment was to determine if the primer sets described in Table 1 could be utilized to screen bacteria for the *pha*C gene. Genomic DNA was isolated (Figure 1) and subjected to PCR using one of the programs listed in Table 2.

In the first experiment, primers with homology to sequences internal to the B-18 *phaC* gene were employed. Figure 2 compares results obtained with two thermocycler programs. “Stringent-ToxR” uses conditions that are much more stringent than “PHA-PCR”. When the samples were amplified using “PHA-PCR”, several bands were produced in the B-18 control. When the samples were subjected to amplification with the more stringent conditions, only the 337-bp fragment resulted. The use of more stringent conditions such as higher annealing temperature and fewer cycles resulted in fewer bands and gave cleaner results for the B-18 control. However, the more stringent conditions eliminated all of the bands in the other samples.

In the second experiment, primers corresponding to homologous sequences found within the *phaC* gene from *R. eutropha* were used. If successful, PCR should result in the amplification of a 565-bp fragment. Unfortunately, multiple bands were seen with every DNA sample (Figure 3).

In the third experiment, primers corresponding to sequences flanking the B-18 *phaC* gene were employed to amplify a 1903-bp fragment using stringent conditions. Figure 4 shows that only the positive control contained a DNA fragment.

In the last experiment, primers corresponding to sequences flanking the B-18 *phaC* gene and an upstream gene of unknown function, *orf2*, were used in a PCR experiment. Again, stringent conditions were employed. Figure 5 shows that the only sample that contained the expected 2297-bp fragment was the positive control.

In conclusion, none of the primers could be used to screen bacteria for the presence of the *phaC* gene using the PCR conditions described in this paper. The experiments could be improved in several ways. Amplification with the Ae primer sets should have included *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) genomic DNA as a control. Also, perhaps more stringent conditions could have been employed for PCR with the Ae primer sets. Finally, to confirm if the experimentals contained the *phaC* gene, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* should have been plated onto nitrogen-limiting minimal medium containing hydroxybutyrate. After incubation for several days, the colonies would have been examined microscopically for PHA inclusions.

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# CHAPTER 2

## Antibiotic Sensitivity Profile and Plasmid Uptake of a Bioluminescent *Vibrio* Species

Diana Lizarazo, Biology 492, Spring 2008

**Introduction and Purpose.** The focus of this study was a bioluminescent gram-negative marine bacterium isolated from the shoreline of Buckroe Beach, Virginia. This strain was subjected to a fatty acid profile analysis, which showed a strong alignment with *Vibrio parahaemolyticus*, while a DNA-sequence-based identification showed a strong alignment with *Vibrio alginolyticus*. This isolate has been designated as *Vibrio* species B-18, and has been shown to accumulate polyhydroxyalkanoic acid, otherwise known as PHA. Some of the genes involved in PHA synthesis have been cloned into *Escherichia coli* and studies of gene expression are ongoing (3). The goal of this project was to determine if *E. coli* plasmids commonly used in cloning could be introduced into and maintained within the marine organism so that future molecular studies may be performed in the marine organism.

**Materials and Methods.**

**Table 1.** Bacterial strains and plasmids used in this study.

| Plasmid | Resistance | Other Features* |
|---|---|---|
| pBluescriptSKII+ | Ap | MCS, *lac*Z gene |
| pACYC177 | Ap, Km | |
| pACYC184 | Cm, Tc | |
| pBBRMCS1 | Cm | MCS, *lac*Z gene, broad host range |
| pBBRMCS2 | Km | MCS, *lac*Z gene, broad host range |
| pJM9484 | Cm | p*tac*:*lac*Z, runaway replication |
| pJM9485 | Cm | p*tac*:*lac*Z, runaway replication |
| pJM9486 | Cm | pt*rp*:*lac*Z, runaway replication |
| pJM9487 | Cm | pt*rp*:*lac*Z, runaway replication |

*MCS: multiple cloning site; *lacZ*: gene for lactose utilization; broad host range plasmids: may be carried by many different kinds of bacteria; and runaway replication: temperature-dependent plasmid replication to control the plasmid copy number.

Plasmids were isolated from *E. coli* using the alkaline lysis procedure (1). The concentration and relative purity was determined by spectrophotometry at 260 and 280 nm.

**Media Preparation.** Marine media (Difco) containing 1.5% agar supplemented with the following antibiotics was prepared. Antibiotic concentrations were based upon that commonly used for *E. coli*. Media lacking antibiotics was used as a control in the experiments.

**Table 2.** Antibiotic Concentration.

| Antibiotic | Relative *E. coli* Dosage | Final Concentration (µg/ml) |
|---|---|---|
| Ampicillin (Ap) | 1x<br>2x | 100<br>200 |
| Chloramphenicol (Cm) | 0.25x<br>0.5x<br>1x<br>2x | 6.25<br>12.5<br>25<br>50 |
| Kanamycin (Km) | 0.5x<br>1x<br>2x<br>4x | 25<br>50<br>100<br>200 |
| Tetracyclin (Tc) | 1x<br>2x | 10<br>20 |

**Antibiotic Sensitivity Profile.** A flask containing 50 ml of marine broth was aseptically inoculated with B-18 and allowed to grow overnight at room temperature (22-26ºC) with shaking at 90 rpm. This culture was diluted one-hundred-fold followed by six serial ten-fold dilutions in sterile water. An aliquot of 0.1 ml was removed from each of the ten-fold dilutions and plated onto marine media containing antibiotic at concentrations described above. Marine media lacking antibiotics were included as controls. Each dilution was plated onto two plates. Subsequently, the plates were incubated overnight at room temperature. The next day the colonies were counted on each plate and this information

was used to determine the viable count (number of colonies forming units/ml) for each concentration of antibiotic.

**Electroporation Procedure.**

**Preparation of competent cells.** Electrocompetent B-18 was prepared using the protocol devised for *E. coli* (2). Briefly, an early log phase culture was chilled on ice for at least 15 minutes prior to centrifugation at 2500 rpm, 4ºC, 10 minutes in a Centra CL3R refrigerated clinical centrifuge. The cell pellet was resuspended and centrifuged twice in decreasing volumes of 1mM Hepes buffer, pH 7.0. The pellet was resuspended and centrifuged once in 10% glycerol before resuspending in 10% glycerol and aliquoted into microcentrifuge tubes. Care was taken to keep the cells cold throughout the procedure. These aliquots were stored frozen at -70ºC until used.

**Electroporation.** Electrocompetent cells were thawed on ice prior to use. Aliquots of 10 µl of plasmid DNA were placed in a chilled microcentrifuge tube followed by 150 µl of thawed competent cells. Electroporation was performed in 0.2 cm gap cuvettes using a MicroPulser electroporator (BioRad). Immediately after the pulse was applied, 1.0 ml of growth medium was added and the cells were allowed to incubate at room temperature prior to plating onto agar plates. The plates were incubated at room temperature for several days and examined for growth.

**Results.**

**Table 3A/B**. Antibiotic Sensitivity Profile of B-18. These experiments were performed as described on two different days.

| Relative Cm Concentration | $10^{-4}$ | $10^{-5}$ | Number of Colonies<br>$10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | # cfu/ml |
|---|---|---|---|---|---|---|---|
| No antibiotic | $200^{+}$<br>$200^{+}$ | $200^{+}$<br>$200^{+}$ | 148<br>236 | 16<br>11 | 3<br>8 | 1<br>0 | $1.92 \times 10^{8}$ |
| 0.25x | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0 |
| 0.5x | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0 |
| 1x | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0 |
| 2x | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0 |

| Relative Antibiotic Concentration | $10^{-4}$ | $10^{-5}$ | Number of Colonies<br>$10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | # cfu/ml |
|---|---|---|---|---|---|---|---|
| No antibiotic | TM<br>TM | TM<br>TM | 365<br>258 | 30<br>48 | 17<br>12 | 3<br>2 | $3.90 \times 10^{8}$ |
| Ap - 1x | TM<br>TM | TM<br>TM | $300^{+}$<br>$300^{+}$ | 99<br>99 | 8<br>19 | 2<br>0 | $9.9 \times 10^{8}$ |
| Ap - 2x | TM<br>TM | TM<br>TM | $300^{+}$<br>$300^{+}$ | 154<br>100 | 17<br>17 | 1<br>1 | $1.27 \times 10^{9}$ |
| Km - 0.5x | TM<br>TM | TM<br>TM | $300^{+}$<br>$300^{+}$ | 130<br>135 | 7<br>14 | 2<br>1 | $1.33 \times 10^{9}$ |
| Km - 1x | TM<br>TM | TM<br>TM | $300^{+}$<br>$300^{+}$ | 115<br>134 | 9<br>22 | 1<br>6 | $1.25 \times 10^{9}$ |
| Km - 2x | TM<br>TM | TM<br>TM | $300^{+}$<br>$300^{+}$ | 138<br>106 | 7<br>13 | 3<br>1 | $1.22 \times 10^{9}$ |
| Km - 4x | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0 |
| Tc - 1x | TM<br>TM | TM<br>TM | $200^{+}$<br>$200^{+}$ | 52<br>51 | 3<br>0 | 1<br>0 | $5.15 \times 10^{8}$ |
| Tc - 2x | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0<br>0 | 0 |

Ap: ampicillin, Cm: chloramphenicol, Km: kanamycin, Tc: tetracycline, and TM: too many colonies to count. The #cfu/ml was calculated by multiplying the average colony number between 20-300 by the inverse of the dilution on the plate.

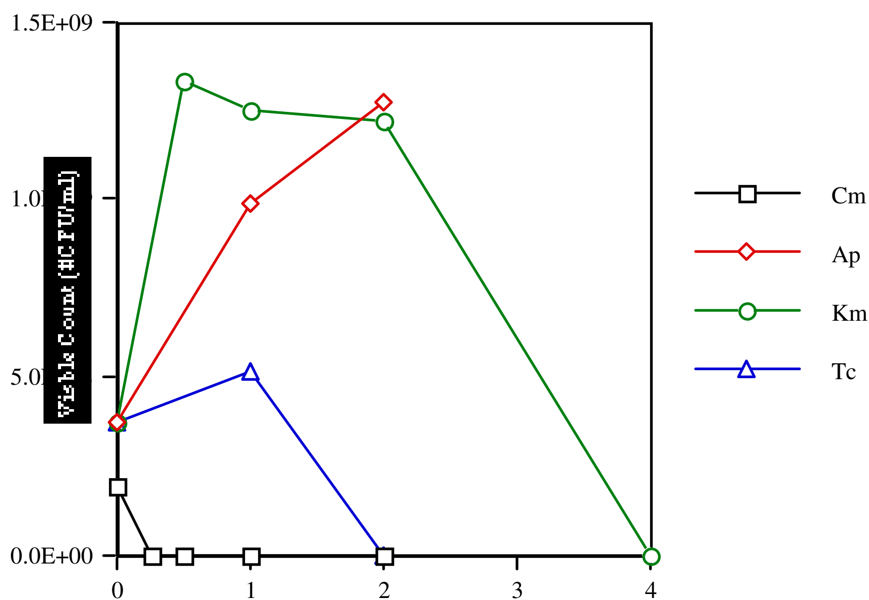


**Figure 1.** Antibiotic Sensitivity Profile of *Vibrio* species B-18. Ap: ampicillin, Cm: chloramphenicol, Km: kanamycin, Tc: tetracycline. Relative antibiotic concentrations were according to Table 2.

**Table 4**. Initial Electroporation Attempt using 10-day-old Competent B-18

| **Plasmid** | **0 Cm** | **0.25x Cm** | **1x Cm** |
|---|---|---|---|
| No plasmid | 0 | 0 | 0 |
| pACYC184 | | 0 | 0 |
| pJMU9486 | | 0 | 0 |
| pBBRMCS1 | | 0 | 0 |
| pJMU9484 | | 0 | 0 |
| pJMU9485 | | 0 | 0 |
| pJMU9487 | | 0 | 0 |

**Table 5**. Troubleshooting the Procedure for Competent Cell Preparation

| Plate | Growth |
|---|---|
| Step 1 | + |
| Step 3 | + |
| Step 6 | + |
| Step 8 | + |
| Step 9 | + |
| Step 10 | + |
| Step 11 | + |

The steps refer to the procedure for competent cell preparation described in (3). Competent B-18 were prepared as described and a small aliquot (0.1 ml) was removed at intervals and plated onto marine medium. The plates were incubated at room temperature for several days and examined for growth. (+): Growth; (-): No Growth.

**Table 6**. Second Electroporation Attempt using One-day-old Competent B-18

| Plasmid | No Antibiotic | 6.25 µg/ml Cm | 25 µg/ml Cm |
|---|---|---|---|
| pACYC184 | + | - | - |
| pJMU9486 | + | - | - |
| pBBRMCS1 | + | - | - |
| pJMU9484 | + | - | - |
| pJMU9485 | + | - | - |
| pJMU9487 | + | - | - |

(+): Growth; (- ): No Growth

**Discussion.** An antibiotic sensitivity profile (Figure 1) revealed that B-18 is sensitive to the following antibiotics. B-18 is sensitive to chloramphenicol at all levels tested, sensitive to tetracycline at a concentration of 20 µg/ml or 2 times the *E. coli* dosage, and sensitive to kanamycin at a concentration of 200 µg/ml or 4 times the *E. coli* dosage. B-18 was found to be resistant to all concentrations of ampicillin used in this study. Because it is not known if *E. coli* plasmids could replicate in B-18, electroporation with plasmids conferring chloramphenicol resistance was selected for further study.

Electrocompetent B-18 cells were made using a procedure used to prepare electrocompetent *E. coli*. Cells were stored at -70ºC for ten days prior to electroporation with one of six plasmids conferring chloramphenicol resistance (Table 1). Time constants of approximately 5.0 milliseconds, indicating ideal reaction conditions, were obtained in

every case. For the first attempt, cells were incubated in SOC at room temperature prior to plating onto marine agar containing either 6.25 or 25 µg/ml chloramphenicol. Competent cells only were plated onto marine agar containing 0, 6.25, and 25 µg/ml chloramphenicol. No growth was detected after four days at room temperature (Table 4), suggesting that something was wrong with the competent cells.

Using a fresh culture of B-18, competent cells were prepared and aliquots were plated onto marine agar at several intervals, in order to determine when the cells died during the procedure. Aliquots were taken from the original culture, after every buffer change, and after the prepared cells were stored frozen at -70ºC overnight. Growth was seen on every plate (Table 3) indicating that B-18 cells cannot withstand storage at -70ºC for extended periods of time. For comparison, competent *E. coli* can last for several months.

Electroporation with the six plasmids conferring chloramphenicol resistance was repeated using one-day-old competent cells. Once again, the time constants were ideal. Outgrowth was performed in marine media, not on SOC. In addition, outgrowth was allowed to occur for 150 minutes instead of 45 minutes at room temperature. As in the previous electroporation experiment, the electroporation mixtures were plated onto marine media containing 0, 6.25, and 25 µg/ml chloramphenicol. The plates were incubated for two days at room temperature. Growth was observed only on plate that did not contain antibiotics (Table 6). This observation indicates that the cells survived the electroporation procedure and outgrowth. It is still unclear whether the plasmid entered the cells, and if it did, whether the plasmid was able to replicate in B-18.

To examine if SOC medium should be used for the outgrowth after electroporation, competent B-18 and B-18 from marine agar were inoculated into 5 ml of SOC broth and incubated for several days with aeration at room temperature. In both cases, B-18 failed to grow in SOC broth. The observation indicates that this medium is not suitable for growth. SOC is a rich medium (containing 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4{\cdot}7H2O$, and 20 mM glucose) used for the outgrowth of *E. coli* after electroporation.

Given the hurdles that need to be overcome, electroporation does not seem like the best method to introduce plasmids into B-18. An alternative method, conjugation or mating with *E. coli* S-17, should be attempted next. However, it is still unknown if these plasmids will be able to replicate within B-18. At least one of these plasmids, pBBRMCS1, is a broad range vector that can be carried by a wide variety of bacteria including *Bordetella*, *Vibrio*, *Escherichia*, *Pseudomonas*, *Rhizobium*, *Brucella*, *Ralstonia* (4, 5). The vector should be able to be maintained within B-18, which is a *Vibrio* species.

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