EXAMINING *ESCHERICHIA COLI* PLASMID GENE EXPRESSION IN *VIBRIO* SPECIES B-18

By

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ABSTRACT

The research project was divided into two chapters with the unifying theme of examining gene expression in *Vibrio* species B-18 using plasmid vectors from *Escherichia coli*. The experiments in the first part were performed with the primary objective of determining the optimum temperature to induce *lacZ* gene expression in *Vibrio* species B-18 carrying the runaway replication vector pJM9487, and also to compare the β-galactosidase levels in cells that were induced chemically, thermally or both. In the first experiment, β-galactosidase activity was measured in cells that were grown at room temperature (21°C), 35°C, 37°C, and 39°C. In the second experiment, β-galactosidase activity was measured in cells that were grown at room temperature (20°C), and 39°C in the presence and absence of the inducing chemical, 3-β-indoleacrylic acid (IAA). The results of the first experiment suggest that the optimum temperature to induce *lacZ* gene expression in B-18 carrying the runaway replication vector pJM9487 was 39°C. The second experiment initially found that the best induction was seen by temperature alone. However, the enzyme levels were much lower than that seen in the first experiment suggesting that more samples should have been taken. In both cases, fluctuating low levels of activity in uninduced cells were seen and these fluctuations were greatly exaggerated when the specific activity was considered. This may reflect some problems with the protein assay and also warrants further investigation. For both experiments, the results seen with B-18 were compared to an earlier study performed in *Escherichia coli* containing the same plasmid, pJM9487. The second part of this project consisted of experiments with the purpose of examining gene expression in B-18 carrying the plasmid pBBR1MCS. Initial experiments involved confirming that B-18 containing the plasmid was in pure culture as the strains were constructed by conjugation with *E. coli*. Serial subculturing of bioluminescent colonies on medium containing chloramphenicol followed by indole tests revealed that the B-18 isolates containing the plasmid were in pure culture. These pure cultures of B-18 containing pBBR1MCS were then examined for total β-galactosidase activity and for the capability of blue-white screening. Finally, plasmids were isolated from the pure cultures and subjected to restriction endonuclease digestion to confirm that the cells actually contained pBBR1MCS. Although the B-18 isolates were in pure culture, no β-galactosidase activity was seen and the growth on medium containing X-Gal showed no difference to the B-18 control. This indicates that there was no *lacZ* gene expression. Finally pBBR1MCS could not be isolated from either of the two chloramphenicol resistant B-18 strains (64.2, 65.2). Further research is required to confirm if the B-18 strains contain the plasmid pBBR1MCS in low levels or if the chloramphenicol resistance gene became integrated into the bacterial chromosome.

CHAPTER 1

Using Lower Temperatures to Induce Runaway Replication for the Expression of the β-galactosidase Gene in *Vibrio* species B-18

Roberto Ramirez, Biology 492, Spring 2014

Introduction and Purpose. Overexpression of cloned genes in desired organisms can be done by a variety of methods including runaway replication (3). This method involves an increase of gene quantity by the use of runaway replication vectors such as plasmid pRA96, which was used as a building block to generate plasmid pJM9487; the primary plasmid used for this experiment. One of the advantages of runaway replication is that the plasmid copy number, as well as the expression of cloned genes, is controlled by the incubation temperature. This prevents some of the disadvantages of other methods used to overexpress cloned genes. For example, a typical multicopy plasmid can be lost from cells in the absence of stability genes unless the appropriate antibiotic is present. Additionally, multicopy plasmids pose problems if the gene product is toxic for the cells. Also mutations could result to alter the amount and/or activity of the plasmid gene product over time in host cells that are metabolically stressed (3). Plasmid pJM9487 contains the *lacZ* gene, which encodes the β-galactosidase enzyme, and this plasmid uses runaway replication for the expression of this specific gene. At lower temperatures, the plasmid copy is low and the genes are not expressed. At higher temperatures, the plasmids vigorously replicate forcing the cell to preferentially transcribe and translate plasmid genes.

Vibrio species B-18 is a remarkable bioluminescent bacterium capable of naturally producing polyhydroxyalkanoates (PHA), which can be used for the manufacture of biodegradable plastics. This bioluminescent marine bacterium was isolated from Buckroe Beach, Virginia and temperature and antibiotic sensitivity profiles were determined by previous experiments (2, 5, 7, 8, 9). Those experiments determined that *Vibrio* species B-18 is very sensitive to low concentrations of the antibiotic chloramphenicol, and high concentrations of the antibiotic kanamycin (5, 9). Its temperature profile also demonstrated that B-18 is able to grow in temperatures that range from 17°C to 39°C with an optimum growth temperature at about 30°C. At temperatures above 39°C, B-18 cannot grow (2, 7, 8). The cardinal temperature range of B-18 is lower than that of *E. coli*, which was incubated at 41°C to induce runaway replication (3). It is also naturally lactose negative (4), making it an ideal candidate to explore runaway replication using lower induction temperatures. Using conjugation, runaway replication plasmids pJM9485 and pJM9487 were introduced into B-18 (9).

Recent experiments focused on one of these runaway replication vectors, pJM9485 (1, 10). Those experiments determined that the optimum induction temperature using B-18 (pJM9485) was 37°C, but there was noticeable β-galactosidase activity at 39°C. Those conclusions suggested that lower temperatures could be used to induce runaway replication in B-18 containing this plasmid.

The goal of this experiment was to determine if lower temperatures could serve to induce runaway replication for the expression of the *lacZ* gene in *Vibrio* species B-18 carrying a different runaway replication vector, pJM9487. One experiment compares β-galactosidase activity in cells incubated at 35, 37, and 39°C while the second experiment examines the enzyme levels for cells incubated at either a low temperature or a high temperature in the presence and absence of the chemical inducer used for the promoter (p*trp)* that controls the expression of the *lacZ* gene on this plasmid.

Materials and Methods.

Bacterial strains and plasmids. The marine bacterium *Vibrio* species B-18 isolated from Buckroe Beach, Virginia was used in this experiment (4). Plasmid pJM9487 was generated from the parent plasmid pRA96, which has a basal copy number of one. Plasmid pJM9487 contains the *lacZ* gene under control of the *trp* promoter (3). This plasmid was introduced into B-18 via conjugation (9).

Media and Culture Conditions. For routine culture maintenance, cells were grown in Sea Water Complete (SWC) medium supplemented with chloramphenicol (Cm) to a final concentration of 12.5 µg/ml. SWC medium contains 3.0 g yeast extract, 5.0 g peptone, 3.0 ml glycerol per Liter of artificial seawater. For agar plates, 15 g/L of agar was also added.

For the induction studies, room temperature (20-21°C) was used as the non-inducing temperature. An overnight culture grown at room temperature with shaking at 125 rpm was used to inoculate 200 ml of broth medium containing chloramphenicol in a 500-ml flask to an optical density at 600 nm wavelength (OD^{600}) of 0.1. At this time, a 2-ml aliquot was taken. The cells were harvested by centrifugation and the resulting cell pellet was stored at -20°C. The inoculated flask was incubated at the non-inducing temperature with shaking at 125 rpm. At regular intervals, 3-ml aliquots were withdrawn. One ml was used to measure the optical density, while cells from the remaining 2-ml sample were harvested and frozen at -20°C. This continued until the OD^{600} reached 0.2. At this time, larger aliquots (25 ml) were placed into pre-warmed sterile 250-ml flasks. As appropriate, these pre-warmed flasks contained the chemical

inducer indole acrylic acid (IAA) at a final concentration of 25 μg/ml. Cells were incubated at the new temperature with shaking at 125 rpm. Sample collection continued as described at regular timed intervals. All cell pellets were stored frozen at -20°C.

Enzyme Assays. Frozen cell pellets were thawed on ice and resuspended in an equivalent volume of sterile distilled water and kept on ice. β-galactosidase activity was measured according to the procedure by Miller (6). In this procedure, the cell suspensions were diluted in Z buffer and the cells were chemically lysed by the addition of chloroform and 0.1% SDS followed by vortexing. The cell lysates were equilibrated in a 28°C water bath prior to the addition of the ONPG substrate. Once a significant yellow color was seen or fifteen minutes had elapsed, the reaction was stopped by the addition of 1 M Na_2CO_3. The OD^{420} and the OD^{550} was recorded for each sample. Units of β-galactosidase were calculated from this data. The total protein content of lysed cells was measured using the Bio-Rad protein assay. This assay involved mixing aliquots of broken cells with the Bio-Rad dye reagent concentrate and measuring the OD^{595}. This assay was performed on aliquots of 10 mg/ml bovine serum albumin and the resulting data was used to construct a standard curve, seen in Figure 1, that was used to determine the protein concentration in the B-18 cell lysates.

Results.

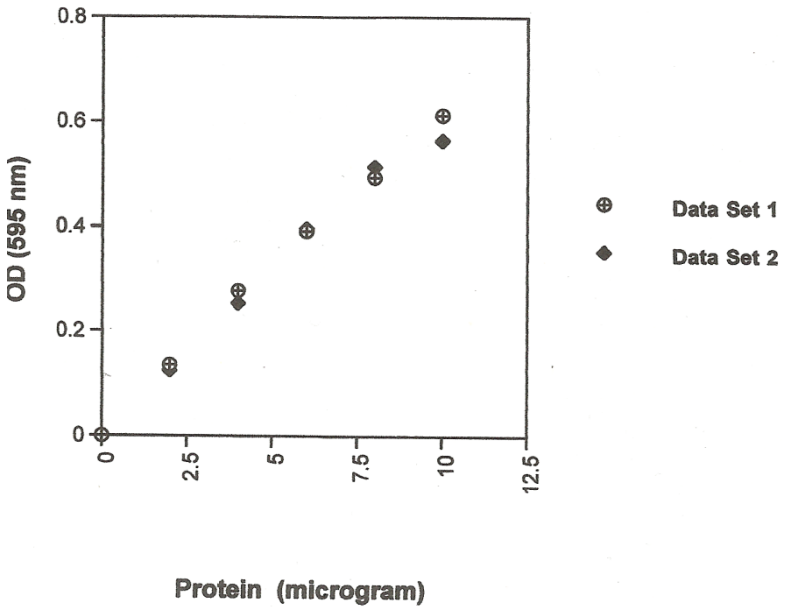

Figure 1. Protein Standard Curve. This graph was used to determine the protein concentration in the cell lysates for each sample collected using the Bio-Rad Protein Assay Kit.

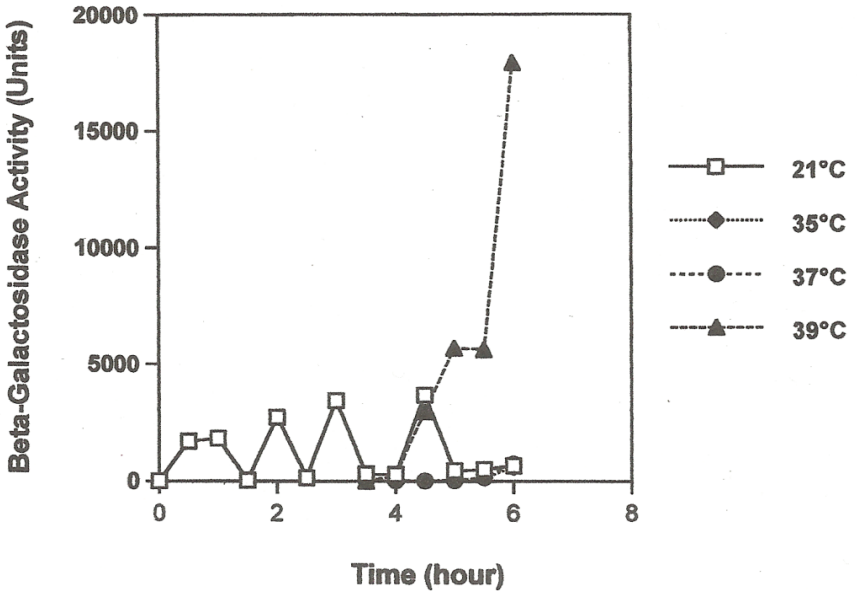

Figure 2. Total β-galactosidase activity in B-18 (pJM9487) at 21°C, 35°C, 37°C, and 39°C. Samples were taken at regular intervals as described in the Materials and Methods section and assayed for β-galactosidase activity using the procedure developed by Miller (6).

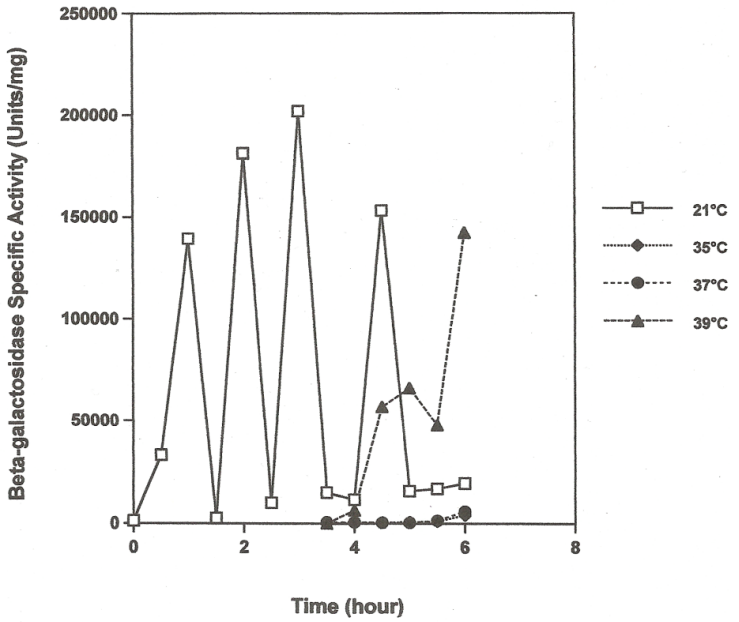

Figure 3. β-galactosidase Specific Activity in B-18 (pJM9487) at 21°C, 35°C, 37°C, and 39°C. Specific activity was calculated by dividing the β-galactosidase activity values from the previous figure by the total protein in the cell lysate.

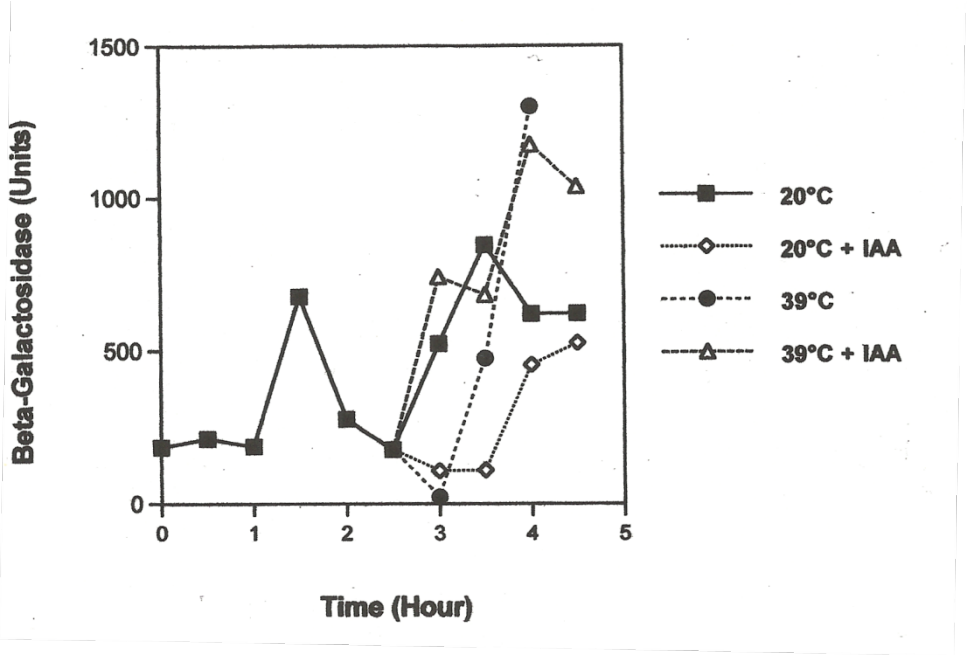

Figure 4. Comparison of Induction Conditions in B-18 (pJM9487). The cultures were incubated as described in the Materials and Methods section and samples were collected at regular intervals and assayed for β-galactosidase activity using the procedure developed by Miller (6).

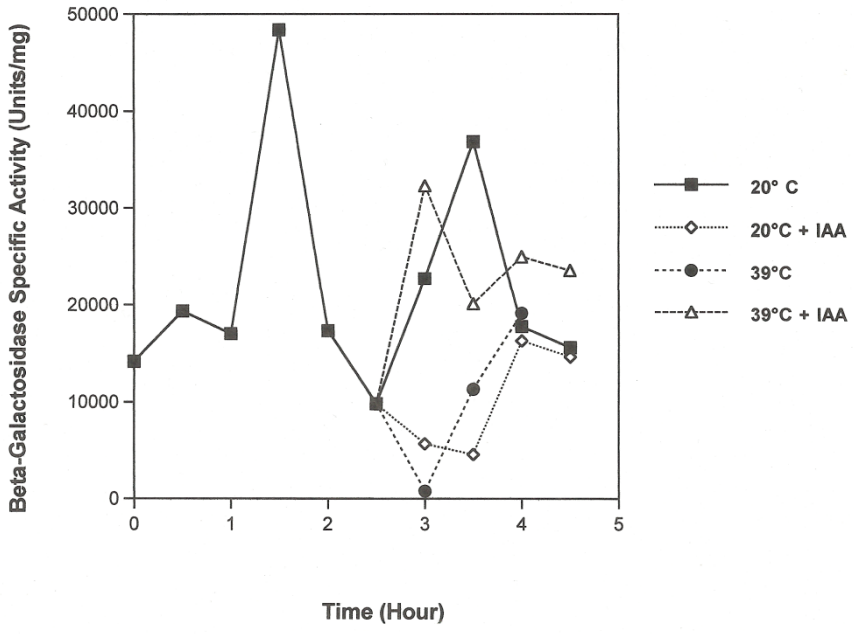

Figure 5. Comparison of Induction Conditions in B-18 (pJM9487) using Specific Activity. The β-galactosidase values used to generate the figure above were divided by the protein concentration of the cell lysates to show specifc β-galactosidase activity.

Discussion and Conclusions.

Figure 1 is the protein standard curve used to measure the protein concentration in all of the samples collected in the experiment so that specific activity (enzyme units per mg protein) could be calculated. The protein assay uses a dye that turns blue when protein is present in the sample. The amount of blue color was measured at a wavelength of 595 nm. The protein concentration in the samples was determined by extrapolation from the standard curve using the absorbance readings.

Figures 2 and 3 show the induction of the *lacZ* gene of runaway replication vector, pJM9487, in *Vibrio* species B-18. Cells were grown at room temperature until the OD^{600} was approximately 0.2. The culture was then placed into prewarmed flasks and cell samples were taken at regular intervals. These samples were then assayed for β-galactosidase activity and plotted against the incubation time in order to determine the optimum temperature at which the gene produces the highest levels of the enzyme. As it can be seen in figure 2, the best induction temperature was at 39°C, which produced the highest total β-galactosidase levels in B-18 (pJM9487). Figure 3 is a graph similar to the one in figure 2 with the exception that the graph in figure 3 measured β-galactosidase activity based on the amount of enzyme per total protein. Figure 2 seems to indicate that 39°C was the best temperature for induction. However, it does show slight fluctuations in activity at the non-inducing temperature. These fluctuations are much more pronounced when specific activity was considered (Figure 3). One possible explanation for this is that the technique used to lyse the cells was somehow incompatible with the protein assay. This is something that should be examined further. The results from Figure 2 could be compared to a similar study in *E. coli* performed by Kidwell et al in 1996. Figure 2 from this study shows that the optimum inducing temperature in this experiment was 39°C which produced total β-galactosidase levels of approximately 17,000 units. On the other hand, the maximum β-galactosidase levels seen in the Kidwell paper when the vector was only thermally induced at 41°C was around 7,000 units, or less than half of the levels in this experiment. One difference between the studies is that Kidwell paper began the induction at OD^{660} ~0.4-0.6 (3), while thermal induction in this experiment began at OD^{600} of about 0.2. Another major difference between the two graphs is that samples were collected in the Kidwell experiment for over 8 hours while this experiment was only performed for only 6 hours. This additional time may provide a much clearer insight as to the levels of enzyme induction.

Figure 4 compares the chemical induction with IAA to thermal induction at 39°C in B-18 (pJM9487) in an effort to determine the optimal induction conditions. In this experiment, the result for the last sample taken at 39°C had zero β-galactosidase activity. Fearing that the cell pellet was somehow lost during the procedures, this data point was not included in Figures 4

and 5. The graph illustrated by figure 4 suggests that thermal induction at 39°C produced the highest β-galactosidase levels. As seen earlier in Figures 2 and 3, the pre-induced samples grown at room temperature showed fluctuations in activity. Based on these results, it would appear that the temperature alone is capable of inducing *lacZ* gene expression in B-18 carrying the runaway replication vector, pJM9487. In *E. coli,* Kidwell et al concluded that both thermal and chemical induction produced higher levels of β-galactosidase for cells carrying this plasmid (3). Another important aspect to consider about figure 4 is that the maximum β-galactosidase levels produced at 39°C were very low, in the range of 1000-1500 units, while figure 2 shows that the maximum β-galactosidase levels produced were high, in the range of 15,000-20,000 units. This could reflect the need to take more samples during this second experiment; therefore further experiments are required. Finally, figure 5 compares the chemical induction with IAA to thermal induction at 39°C in B-18 (pJM9487) by taking into consideration the total protein levels. In this case, *lacZ* expression was best seen when the cells were grown at 39°C in the presence of IAA to produce the highest β-galactosidase levels at about 25,000 units/mg. This supports the data seen in Kidwell's experiments, which concluded that the optimum induction of *lacZ* expression was obtained at high temperatures in the presence of an inducing chemical. However, in a similar fashion seen in Figure 3, the fluctuations for the non-induced samples were more pronounced in Figure 5, indicating a need to reexamine the protein assay procedure.

This experiment concludes that the optimum temperature to induce *lacZ* gene expression in *Vibrio* species B-18 carrying the runaway replication vector pJM9487 is 39°C, producing total β-galactosidase levels of about 17,000 units. This leads to the conclusion that lower temperatures can be used to induce *lacZ* expression without the addition of an inducing chemical. Even though this experiment suggests that the best induction temperature is 39°C, it is not safe to assume that temperature alone is the best condition to induce the *lacZ* expression. Further experiments that collect more samples are required to support the data presented in this project. Based on the conclusions made by the Kidwell researchers using *E. coli* carrying plasmid pJM9487 (3), and suggested by figure 5 of this experiment, it can be expected that the best conditions to induce *lacZ* expression in *Vibrio* species B-18 carrying pJM9487 is a combination of both thermal and chemical induction.

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CHAPTER 2

Using Plasmid pBBR1MCS to Induce *lacZ* Gene Expression in *Vibrio* Species B-18

Roberto Ramirez, Biology 492, Fall 2014

Introduction/Purpose. Gene expression in microorganisms can be studied by genetic engineering in broad-host-range vectors, which can be used to transfer genes from one organism to another. Unfortunately many broad-host-range vectors are large (up to 10 kbp), contain a small number of cloning sites, and identifying organisms with recombinant DNA in these vectors is not easy to determine. To overcome these limitations, Kovach et. al developed the broad-host-range vector pBBRIMCS, which is relatively small (4717 bp), and experiments have shown that this plasmid is stably maintained in a variety of gram negative bacteria including *Bordetella, Brucella, Escherichia, Pseudomonas, Rhizobium, and Vibrio.* Plasmid pBBRIMCS also contains a chloramphenicol resistance gene, which is one of the methods that can be used to select for bacteria that contain recombinant DNA. More importantly, pBBRIMCS contains the *lacZ* gene, which encodes the enzyme β-galactosidase that allows the bacterium to utilize lactose. A multiple cloning site (MCS) containing 16 unique sites for restriction nucleases can be found near the beginning of the *lacZ* gene (4). This is important because using medium containing 5-bromo-4-chrolo-3-indodyl-β-D-galactopyranoside (X-Gal), bacteria containing recombinant DNA cloned into the MCS can be separated from bacteria that lack a cloned insert using the blue-white screening method. A map of pBBRIMCS is shown in figure 1.

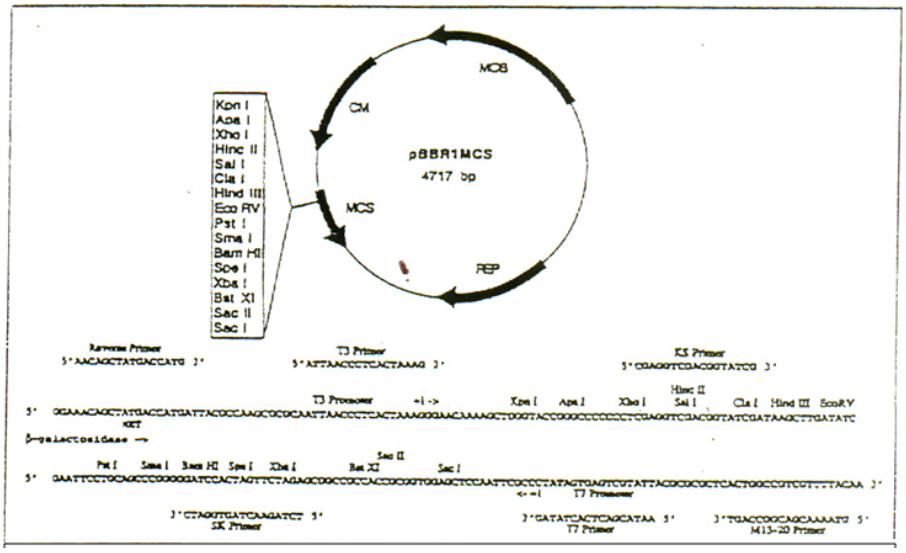

Figure 1. Map of pBBR1MCS. MCS: multiple cloning site found within the *lacZ* gene. CM: chloramphenicol resistance gene. (Reprinted from Reference 4).

Blue white screening is a method used to isolate bacteria containing cloned DNA. This method is based on the expression of β-galactosidase, which is encoded by the *lacZ* gene under control of the inducible tac promoter in pBBRIMCS (4). β-galactosidase is an enzyme that catalyzes the formation of glucose and galactose from lactose. Bacteria that have not successfully integrated the plasmid will have an intact *lacZ* gene and therefore produce β-galactosidase that hydrolyzes X-gal into 5-bromo-4-chrolo-3-indodyl which quickly becomes 5, 5'-dibromo-4,4'-dichloro-indigo to turn the colonies blue. On the other hand, recombinant bacteria that have the plasmid integrated into the MCS will have a disrupted *lacZ* gene and therefore, they will be unable to produce β-galactosidase. Without any β-galactosidase present, the recombinant bacteria colonies will be unable to hydrolyze X-gal and therefore they will grow as clear or white colonies (6). This technique is relatively simple and provides very accurate results.

Vibrio species B-18 is a marine bioluminescent bacterium that was isolated from Buckroe Beach, Virginia (3). A fatty acid profile analysis identified it as *Vibrio parahaemolyticus*, while a genotypic method identified it as *Vibrio alginolyticus.* It is known only as *Vibrio* species B-18. One of unique qualities of this marine bacterium is that can accumulate polyhydroxyalkanoates (PHA). These linear polyesters are usually produced by some types of bacteria to store carbon and energy, and can be used in the manufacture of biodegradable plastics. Another important feature of *Vibrio* species B-18 is that it is negative for both lactose and indole and positive for DNAse. These features make this bacterium an excellent choice for examining plasmid-driven *lacZ* gene expression and also aid in the purification from other bacteria, such as *E. coli,* which are not bioluminescent and are positive for indole and negative for extracellular DNAse.

Previous studies have determined the temperature and antibiotic resistance profile for *Vibrio* species B-18 (2, 5, 7, 8, 10). The data from these previous experiments suggest that B-18 is very sensitive to the antibiotic chloramphenicol (5, 10). The data also suggest that its optimum temperature for grown is about 30°C, which is lower than the optimum growth temperature for *E. coli* (2, 7, 8). B-18 has also been used in a series of experiments where runaway replication vectors such as pJM9485 and pJM9487 were introduced into B-18 to determine if *lacZ* gene expression could be induced at different temperatures (1, 9, 11). For this experiment, pBBR1MCS was integrated into *Vibrio* species B-18 via conjugation (10) with the primary objective to determine if this broad-host-range vector can be used to induce *lacZ* gene expression in B-18. Figure 2 shows the expected fragment sizes of various plasmids digested with the restriction enzyme *BamHI* and shows pBBR1MCS was initially present in the B-18 isolates.

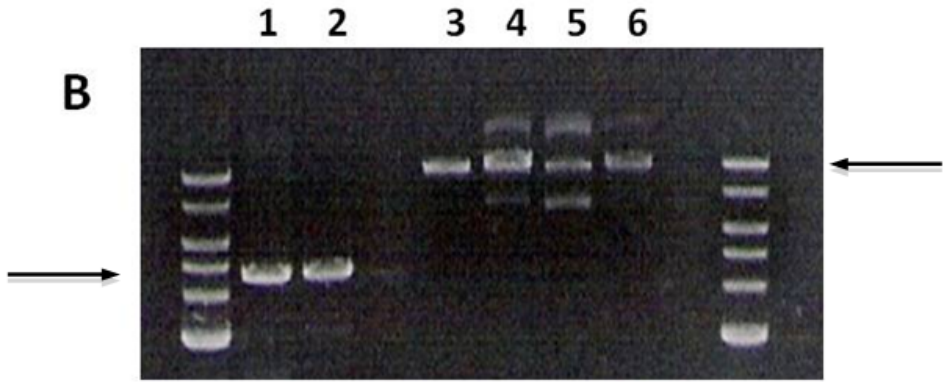

Figure 2. *Bam*H1 digests of plasmids. Samples were digested with *Bam*HI prior to electrophoretic separation in 1% agarose-TAE. A 1-kb molecular weight ladder flanked sample lanes in each gel and the following bands can be seen from the bottom as follows: 3 kb, 4 kb, 5 kb, 6 kb, 8 kb, and 10 kb. **B**] Plasmid pBBRMCS1 purified from *E. coli* S17 (lane 1) and B-18 (lane 2) and plasmid pJM9485 purified from *E. coli* (lane 3) and B-18 (lanes 4-6). Expected sizes for the linear plasmids are as followed: pBBR1MCS, 4.707 kb; pJM9485, 9.8 kb. The arrows indicate the location of the linear plasmids. (Reprinted from Reference 10).

Materials and Methods.

Table 1. Bacterial Strains and Plasmids Used in This Study

Name	Features	Reference
***Vibrio* species B-18**	Indole negative, chloramphenicol sensitive, lactose negative	3
***Vibrio* species B-18 (pBBR1MCS)**	Indole negative, chloramphenicol resistant, lactose negative	10
E. coli	Indole positive, chloramphenicol sensitive, lactose positive	Bio 348 Stock
***E. coli* XL1-Blue(pBBR1MCS)**	Indole positive, chloramphenicol resistant, lactose positive	3

Culture Conditions and Media.

For routine culture maintenance, B-18 was grown in Sea Water Complete (SWC) medium, which contains 3 g yeast extract, 5 g peptone, 3 ml glycerol per Liter of artificial seawater. B-18 containing a plasmid was grown in SWC supplemented with chloramphenicol (Cm) to a final concentration of 12.5 µg/ml. *E. coli* was grown on Luria-Bertani (LB) medium (Difco) supplemented as needed with Cm to a final concentration of 25 µg/ml. For agar plates, 15 g/L of agar was also added.

To determine if the B-18 (pBBR1MCS) cultures are contaminated with *E. coli*, cultures were streaked onto SWC-Cm and incubated at 25°C. Plates were observed in the dark for

bioluminescence and SIM deeps (Remel) were inoculated from the 1^{st}-2^{nd} sectors of the streak plates, where the heavier non-isolated growth is found. These were incubated at 25°C for 2 days. Kovacs reagent was added and a cherry red color indicates a positive result for indole. Isolated colonies were used to streak new SWC-Cm plates. This was repeated twice until bioluminescent cultures that were indole negative were produced. These cultures were then made into new freezer stocks and were used for the rest of the experiments.

X-Gal plates were prepared by adding 800 μg each of XGal and IPTG to the surface of agar plates. Ten isolated colonies of B-18 with and without pBBR1MCS were patched onto the surface of the plates, which were incubated at 30°C for 24 hours. Blue growth indicates that the organism could use lactose, while white growth indicates that the organism could not use lactose.

For the *lacZ* expression studies, an overnight culture of B-18 (pBBR1MCS) was grown at 30°C and was used to inoculate 200 ml of broth medium containing chloramphenicol in a 500-ml flask to an optical density at 600 nm wavelength (OD^{600}) of 0.1. At this time, a 2-ml aliquot was taken. The cells were harvested by centrifugation and the resulting cell pellet was stored frozen at -20°C. The inoculated flask was incubated at 30°C with shaking at 125 rpm. At regular intervals, 3-ml aliquots were withdrawn. One ml was used to measure the optical density, while cells from the remaining 2-ml sample were harvested and frozen at -20°C. This continued until the OD^{600} reached 0.2. At this time, larger aliquots (25 ml) were placed into pre-warmed sterile 250-ml flasks and IPTG was added as appropriate to a final concentration of 1 mM. Incubation continued at 30°C with shaking at 125 rpm and samples were taken at regular intervals for an additional 3 hours. All cell pellets were stored frozen at -20°C.

Enzyme Assays.

Frozen cell pellets were thawed on ice and resuspended in an equivalent volume of sterile distilled water and kept on ice. β-galactosidase activity was measured according to the procedure by Miller (6). In this procedure, the cell suspensions were diluted in Z buffer and the cells were chemically lysed by the addition of chloroform and 0.1% SDS followed by vortexing. The cell lysates were equilibrated in a 28°C water bath prior to the addition of the ONPG substrate. Once a significant yellow color was seen or fifteen minutes had elapsed, the reaction was stopped by the addition of 1 M Na_2CO_3. The OD^{420} and the OD^{550} were recorded for each sample. Units of β-galactosidase were calculated from this data.

Plasmid Purification and Analysis.

Plasmid was purified from *E. coli* and B-18 using the alkaline lysis method and concentrated using Qiafilter spin columns (Qiagen Corporation). The plasmid preparations were subjected to digestion with *Bam*HI and these digests were analyzed by electrophoresis in 1% agarose-TAE gels. The gel was observed using a UV transilluminator linked to a digital camera.

Results

Table 2. Indole Assays

Tube Number	**Date**	**Indole**	**Second Sample**	**Date**	**Indole**	**Third Sample**	**Date**	**Indole**
B-18 (Control)	9-18-14	Negative						
***E. coli* (Control)**	9-18-14	Positive						
64	9-18-14	Negative	64.1	9-19-14	Negative	64.2	9-21-14	Negative
65	9-18-14	Negative	65.1	9-19-14	Negative	65.2	9-21-14	Negative
66	9-18-14	Positive	66.1	9-19-14	Negative	66.2	9-21-14	Negative
67	9-18-14	Positive						
68	9-18-14	Positive	68.1	9-19-14	Negative	68.2	9-21-14	Negative
69	9-18-14	Positive						
70	9-18-14	Negative	70.1	9-19-14	Negative	70.2	9-21-14	Negative
71	9-18-14	Negative						

Indole tests were used to determine if B-18 strains containing pBBR1MCS (isolates #64-71) were in pure culture and not contaminated with *E.coli* so that they could be used in further experiments. B-18 is known to be indole negative while *E. coli* is known to be indole positive, which is why they were used as controls. Isolated bioluminescent colonies were streaked onto new plates (second and third sample) and were tested to further confirm the purity of the B-18 isolates.

Table 3. Total β-galactosidase activity in B-18 (pBBR1MCS)

Sample	Volume (ml)	Time (min)	OD^{420}	OD^{550}	OD^{600}	Units β-gal
A1	0.2	15	.019	.012	.114	0
A2	0.2	15	.022	.015	.132	0
A3	0.2	15	.025	.016	.159	0
A4	0.2	15	.027	.018	.187	0
A5	0.2	15	.025	.017	.219	0
A6	0.2	15	.029	.019	.263	0
A7	0.2	15	.043	.032	.332	0
A8	0.2	15	.066	.052	.437	0
A9	0.2	15	.084	.060	.588	0
A10	0.2	15	.118	.089	.771	0
A11	0.2	15	.099	.072	1.012	0
A12	0.2	15	.104	.065	1.258	0
C1	0.2	15	.028	.019	.333	0
C2	0.2	15	.040	.025	.441	0
C3	0.2	15	.048	.032	.589	0
C4	0.2	15	.047	.029	.774	0
C5	0.2	15	.044	.028	1.001	0
C6	0.2	15	.062	.036	1.27	0
D1	0.2	15	.026	.016	.064	0
D2	0.2	15	.027	.016	.334	0
D3	0.2	15	.036	.024	.453	0
D4	0.2	15	.033	.022	.591	0
D5	0.2	15	.042	.026	.738	0
D6	0.2	15	.050	.036	.104	0

Samples were collected from cells that were grown at 30°C in the presence (C, D) or the absence (A) of the chemical inducer, IPTG. Total -galactosidase activity was determined using the procedure developed by Miller (6). The reaction was stopped using a stop solution (Na_2CO_3) at 15 minutes in all sample tubes and -galactosidase activity was calculated from this data.

Table 4. Growth and Color of B-18 +/- pBBR1MCS on X-Gal Medium

Sample	Medium	Growth/Color of 10 patches
B-18	SWC –X gal – IPTG	10/10 Blue
64.2	SWC – Cm –X gal – IPTG	10/10 Blue
64.5	SWC – Cm –X gal – IPTG	10/10 Blue
B18	SWC – X gal – NO IPTG	8/10 Blue
64.2	SWC – Cm –X gal – NO IPTG	7/10 Blue
64.5	SWC – Cm –X gal – NO IPTG	10/10 Blue
B-18	SWC – Cm –X gal – NO IPTG	No growth

Ten individual samples were patched onto SWC containing X-gal with and without IPTG and incubated at 30°C for 2 hours. The plates were observed for growth and the color of each bacterial patch. B-18 lacking a plasmid was used as a control.

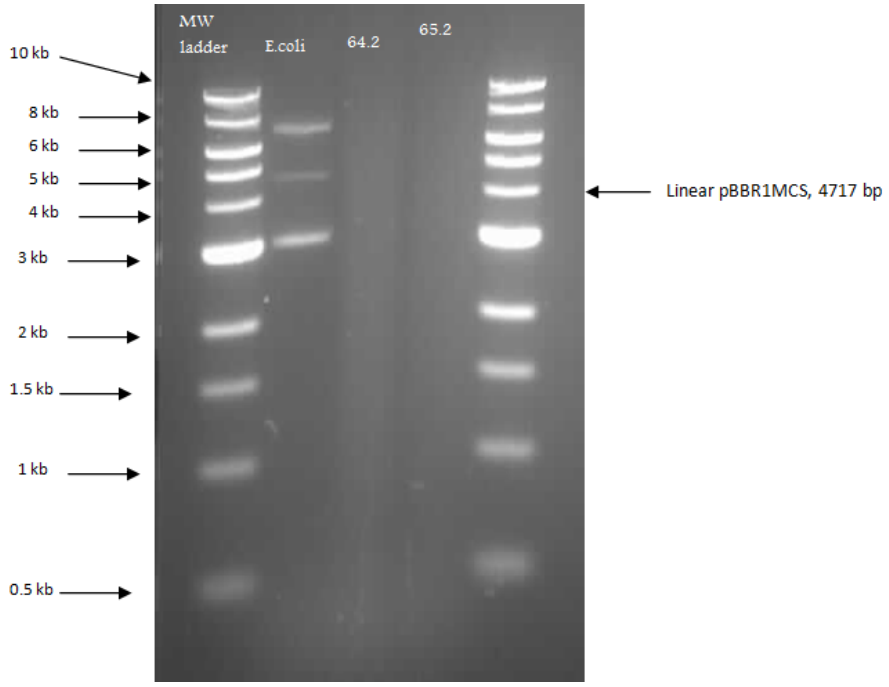

Figure 3. Agarose gel showing the fragment sizes produced when plasmids isolated from *E. coli* (pBBR1MCS) and both clones of B-18 (pBBR1MCS) were digested with the restriction endonuclease *BamH1*. A molecular weight ladder flanks the sample lanes and the sizes of the fragments are indicated in kilobases (kb). The gel was stained with ethidium bromide and visualized under a UV light.

Discussion.

Because *Vibrio* species B-18 produces an extracellular DNAse, it was not possible to use electroporation for the introduction of plasmid DNA inside these cells. Instead, conjugation was used (10). In this technique, *E. coli* carrying pBBR1MCS is placed on top of B-18 on agar plates, which are incubated for several days to allow for the plasmid to be transferred from *E. coli* to B-18. The cells are then plated onto selective media. In this case, bioluminescent colonies that can grow on agar containing chloramphenicol were selected for further study. The eight B-18 (pBBR1MCS) candidates were grown on SWC-Cm and all were bioluminescent. Table 2 shows the results of indole tests. *E. coli* is indole positive, where B-18 is indole negative. Although four of the eight stocks were initially contaminated with *E. coli,* sub-culturing isolated bioluminescent colonies on SWC-Cm resulted in growth that was indole negative, suggesting that these strains are in pure culture. Two of these strains, 64.2 and 65.2 were selected for further study.

Table 3 shows the levels of total β-galactosidase activity in B-18 (pBBR1MCS) for cultures that were incubated in the presence and absence of IPTG. The samples were grown at 30°C until the OD^{600} was approximately 0.2. Strains 64.2 and 65.2 were induced using IPTG two and half hours after the start of the experiment. β-galactosidase activity was then measured from these cell samples. As it can be seen from table 3, all samples demonstrated zero activity. These findings suggest that *lacZ* gene expression in *Vibrio* species B-18 was not successfully induced using the broad host range vector pBBR1MCS.

Due to the unexpected results from the β-galactosidase activity data, it was imperative to further test if the B-18 strains actually contained the pBBR1MCS vector. This plasmid was designed as a broad-host range cloning vector with blue-white screening capability (4). Perhaps the *lacZ* gene expression in these cells was too low to be detected by the β-galactosidase enzyme assays, but is sufficient for blue-white screening. Table 4 shows the results when B18 +/- pBBR1MCS were patched onto SWC medium containing X-Gal. The two B-18 clones could grow in media containing chloramphenicol, but B-18 alone did not grow in the presence of the antibiotic. This suggests that the cells did contain the plasmid. However, there was no significant difference between the number of blue colonies in the two B-18 clones that contained the plasmid versus the B-18 control. Since B-18 containing the plasmid should have produced blue colonies on X-Gal and the B-18 control should have been white, it would appear again that the *lacZ* gene was not expressed.

The next step was to determine if the B-18 clones still contained plasmid pBBR1MCS. A large-scale alkaline lysis procedure was used to purify the plasmids from both *E. coli*

(pBBR1MCS) and from B-18 strains 64.2 and 65.2. In this procedure, cells were broken in a buffer containing sodium dodecyl sulfate and sodium hydroxide and large cell debris was removed by precipitation with sodium acetate followed by centrifugation. The supernatant containing plasmid DNA was then placed in cold ethanol to precipitate out the plasmid DNA, which was then collected by centrifugation. The preparations were then further purified and concentrated using Qiafilter spin columns. These plasmid preparations were then digested overnight with the restriction enzyme *Bam*H1 and the resulting fragments were separated via gel electrophoresis. The results are shown in figure 3. These results were compared to results from similar experiments performed shortly after completion of the conjugation experiments (Figure 2). The *E. coli* lane in figure 3 shows incomplete digestion of the sample. The band at about 5 kb is at the predicted size for the linear plasmid while the bands above and below represent undigested plasmid DNA (supercoiled and nicked circular). A similar result was obtained when the experiment was repeated with more enzyme (data not shown), suggesting that there was an excess of plasmid DNA in the *E. coli* sample. The lanes for B-18 (64.2 and 65.2) do not show any bands. The slight smears visible around 1 kb are most likely due to RNA. Taken together, it would seem that either pBBR1MCS is maintained at a very low copy number in B-18 clones 64.2 and 65.2 or that the chloramphenicol resistance gene from pBBR1MCS became incorporated into the chromosome and the remainder of the plasmid was lost. Both scenarios would explain the fact that these two isolates are chloramphenicol resistant, have zero β-galactosidase activity, and have no detectable plasmid.

In conclusion, pBBR1MCS is not a useful candidate for the induction of *lacZ* gene expression in *Vibrio* species B-18 and is also not useful for blue-white screening. This is supported by the zero levels of total β-galactosidase activity seen and also by the results of the experiments involving the growth of B-18 containing the plasmid in media containing X-gal. It could not be confirmed that the B-18 samples still contained pBBR1MCS. Therefore further experimentation is warranted. Perhaps a southern blot analysis is required to compare the genomic DNA from B-18 and samples 64.2 and 65.2 using a probe based on DNA sequence of the chloramphenicol resistance gene. If hybridization is seen in samples 64.2 and 65.2 but not in B-18 alone, it would imply that the chloramphenicol resistant gene became incorporated into the B-18 genome.

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