

**Characterization of the *Pseudomonas aeruginosa* Bacteriophage B3:  
Investigations into the Host Range and Insertion Site**

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## Abstract

The purpose of this study was to further characterize the *Pseudomonas aeruginosa* phage B3 with regards to its host range and the insertion of the phage into its host. Attempts to infect several soil isolates, previously identified as pseudomonads, were unsuccessful. *Pseudomonas aeruginosa* strain PAO1 was then infected with B3 and ten lysogens were isolated. Lysogeny was verified by polymerase chain reaction (PCR) using primers specific for B3. Direct genome sequencing to identify the insertion site of B3 was unsuccessful. The genomic DNA from the lysogens was digested with the restriction enzyme *BtsI* and analyzed by southern blot with a probe targeted at the end of the B3 genome. Of the ten lysogens, three sets of lysogens each had similar insertion positions indicating a random method of insertion into the host genome with a potential preference for a certain sequence of DNA (semi-random insertion). Further investigations should include sequencing of the insertion sites of B3 into the *P. aeruginosa* genome.

## TABLE OF CONTENTS

	Pages
List of Tables and Figures	3
Introduction	5
Materials and Methods	7-9
B3 Host Range	7
Generating PAO1/B3 Lysogens	7
Identification of Lysogen Colonies via PCR Amplification	7-8
DNA Extraction from Lysogens	8
Direct Genome Sequencing	8
Southern Blot	9
Results	10
Host Range	10
Direct Genome Sequencing	10
Southern Blot	10
Discussion	12-13
Further Investigation	13

## LIST OF TABLES AND FIGURES

	Page
Table	
1. Approximate Size of the probed fragment of PAO1/B3 lysogens	12
Figure	
1. Southern Blot #14	11
2. Southern Blot #15	11
3. Southern Blot #18	11

## Introduction

Recent interest in bioremediation has prompted the need for innovative methods by which petroleum contaminated soils can be cleaned. Since hydrocarbons are naturally present in soil due to the decomposition of organic materials (Kok et al, 1989) several species of organisms within the soil community already possess hydrocarbon degradation capabilities; for example, the alkane catabolic pathway (Whyte et al, 1997). Therefore, some studies have focused on the utilization of the indigenous microbial community by enhancing their pre-existing abilities to bioremediate (Radwan et al, 1995). Another approach at bioremediation may be accomplished by genetically engineering a plasmid to contain oil degrading genes. This plasmid can then be introduced into the microbial community via a host-vector system.

Among genera such as *Bacillus* and *Streptomyces*, *Pseudomonas* is not only known to be abundant in soils but its ability to utilize oil is well documented (Radwan et al, 1995; Krylov et al, 1980a). *Pseudomonas aeruginosa*, more specifically, has been found to have a high propensity for lysogeny (Holloway et al, 1960). These characteristics, and the fact that the genome of *P. aeruginosa* strain PAO1 (PAO1) has been sequenced (Stover et al, 2000), make PAO1 a potential host for genetic engineering.

Among the numerous bacteriophages of which *P. aeruginosa* is a host, B3 presents itself as a potential phage vector. B3 requires a solid media or a highly viscous liquid media in order to adsorb to PAO1 (Roncero et al, 1990). This environment is provided by the soil particles where bioremediation will hopefully occur. Like PAO1, sequencing and annotation of the B3 genome was also finished in 2000 (Braid, 2000), making both PAO1 and B3 good candidates for a potential host-vector bioremediation system. However, further characterization of B3 is necessary to evaluate the possibility of altering the B3 genome before infection into PAO1.

B3 is a transducible, temperate phage (Howe, 1987) with a polygonal head and a flexible, non-contractile tail (Slayter et al, 1964). Although the tail regions differ, the head and the DNA of B3 (Krylov et al, 1980b) display striking similarity to the *E. coli* phage Mu (Braid, 2000). For this reason, Mu can be used as a model for B3. Mu requires the proteins coded by genes A and B for integration to occur. Gene A codes for the transposase protein (Ulyczynj et al, 1994; Howe, 1987) which attaches to the integration site on the host chromosome and introduces nicks allowing Mu to integrate its DNA into its host's DNA. The role of the gene B product is the subject of some contention, but it functions to increase the degree of integration and transposition (Howe, 1987).

B3 is assumed to integrate into the PAO1 genome at random locations by a method similar to Mu (Braid, 2000). It has been shown that B3 has an unspecified amount of non-phage DNA at either end of its genome (Krylov et al, 1980a). As with Mu, it is assumed that B3 must also package its DNA in headful amounts (Howe, 1987). This means that the head of B3 is capable of holding a larger volume of DNA than is present in the B3 genome (Slayter et al, 1964). Therefore, when the phage DNA is being packaged into the head, extra host DNA is taken along with the phage DNA to fill the head. This residual host DNA results in variable end lengths on the B3 genome.

It is obvious that further characterization of B3 is necessary before its capabilities as a vector can be utilized. The focus of this study was to survey the host range of B3 on a number of *Pseudomonas* soil isolates and to characterize the insertion of B3 into the PAO1 genome. Direct genome sequencing was used to survey the insertion sites on the PAO1 genome and southern blot was used to analyze the insertion sites of several PAO1/B3 lysogens. If the integration of B3 is in fact random, the southern blots will reveal probed regions of different lengths.

## **Materials and Methods**

### **B3 Host Range**

Soil isolates were confirmed to be pseudomonads by Fatty Acid Methyl Ester (F.A.M.E.) analysis (Hamrick, 2001). Each isolate was grown overnight in Trypticase Soy Broth (TSB) at 37° C. In a 15 ml screwcap tube, 300 µl of PAO1 culture was mixed with 100 µl of a 10<sup>-7</sup> dilution of B3 phage lysate and incubated for 30 minutes at 37° C. To each tube, 5 mls of phage top agar was added. The tubes were inverted gently, and poured over a Trypticase Soy Agar (TSA) plate. Phage top agar is composed of tryptone (8 g/L), NaCl (5 g/L), and agar (7.5 g/L). These plates were incubated at 37° C for approximately 24 hours. Each plate was examined for the presence of plaques indicative of B3 infection. The following soil isolates were examined for the ability to propagate B3: L3RO1, L4RO3, L2PS1B, L4MD11, DEAH 32, DEAH 33, DEAH 39, DEAH 40, DEAH 42, DEAH 43, and DEAH 45. PAO1 was used as a positive control.

### **Generating PAO1/B3 Lysogens**

A culture of PAO1 was grown in TSB at 37° C overnight. PAO1 was plated with a 10<sup>-9</sup> dilution of B3 as was described above. After overnight incubation, sterile toothpicks were used to pick individual plaques. Each toothpick was incubated in TSB at 37° C overnight to allow any uninfected PAO1 cells to become infected with B3. The TSB cultures were streaked for isolation on TSA and incubated overnight at 37° C.

### **Identification of Lysogen Colonies via PCR Amplification**

PCR was performed directly on individual colonies from the TSA plates using G5L (5' GACTCACCCACCAACCGCAAGAAA 3') and G6R (5' CGAACCGGTCAAC-

GACATGGACGAACTCA 3') primers. Primers G5L and G6R are specific for B3 DNA, extending from base pair 5088 to base pair 6397, resulting in an amplicon of approximately 1310 base pairs in length. Final concentrations of reagents in the PCR reactions were as follows: 10 mM Tris-HCl, 2.5 mM MgCl<sub>2</sub>, 0.2 uM dNTP, 5 nM of each primer, and 2.5 U of Taq Polymerase (Fisher Scientific). Cycling parameters were: 96° C for 2 min, forty cycles of 94° C for 1 min, 60° C for 1 min, 72° C for 2 min, and one cycle at 72° C for 7 min. PCR products were run on a 1% agarose gel for visualization.

### **DNA Extraction from Lysogens**

Colonies that produced 1310 bp amplicons were grown on TSA. DNA was extracted from overnight cultures using the Fast Prep DNA Kit (Bio 101). DNA was visualized on a 1% agarose gel, quantified by spectrophotometry, and PCR'd again (as above) to verify the presence of B3 DNA within the PAO1 genome.

### **Direct Genome Sequencing**

Sequencing reactions were performed on the lysogen DNA using Big Dye Terminator (Applied Biosystems) and the primers G1LEND (5' CGGTACGATGGCGCGAATGCTGACAAAT 3') and G23REND (5' GCCTGGCCTAAGCGTTTTGATAAT 3') specific for B3 DNA. The primers G1LEND and G23REND extend outward from base pair 257 and base pair 38039 respectively. These reactions should cover the DNA sequences at both ends of the B3 genome and out into the residual host DNA thereby revealing the insertion sequence on the PAO1 genome. Reactions were run on an ABI 377 and analyzed by ABI Prism software.



## Southern Blot

Lysogen DNA was digested using the restriction enzyme *BtsI*. *BtsI* has 24 cut sites within the 38,440 bp B3 genome with the last cut site occurring at base pair position 35461. Approximately 5 µg of each DNA sample was digested with 1 U of *BtsI*. PCR was performed on B3 DNA using the primers G22L (5' CGTCCGGCCTGCAGATGAACTA 3') and G22R (5' CGTTGATCAATAGGCCGGTGTTAG 3') in order to generate a 892 base pair amplicon.

Final concentrations of the PCR reaction were as follows: 10mM Tris-HCl, 2 mM MgCl<sub>2</sub>, 0.4 uM dNTP, 20 ug/ml BSA, 1.5 U Taq Polymerase (Fisher Scientific). Cycling was performed as follows: 96° C for 2 min, forty cycles of 93° C for 30 sec, 50° C for 30 sec, 72° C for 2 min, and 72° C for 7 min. The amplicon was cleaned using the Microcon YM-100 (Millipore) and was quantified by spectrophotometry. The PCR product was labeled using the BioNick Labeling System (GibcoBRL) and cleaned up using the MoBio PCR Cleanup Kit. The subsequent probe should anneal between bp 37147 and bp 38039 on the B3 genome. Blotting was performed using a 0.5% agarose gel of the lysogen DNA digests. The gel was blotted on an Immobolin-N (Millipore) membrane using alkaline capillary transfer with 0.4 N NaOH. Total blotting time was 3 hours. The membrane was incubated with the probe at 68° C for approximately 24 hours. Final hybridization mixture was 6X SSC, 5X Denhardt's, 0.5% SDS, 100 ng/mL probe, and 100 µg/mL of sheared salmon sperm DNA. The BluGene® Nonradioactive Nucleic Acid Detection System (GibcoBRL) was used for visualization of the Southern Blot.

## **Results**

### **Host Range**

All attempts at infecting a pseudomonad soil isolate with B3 were unsuccessful. None of the samples tested produced an identifiable plaque upon incubation.

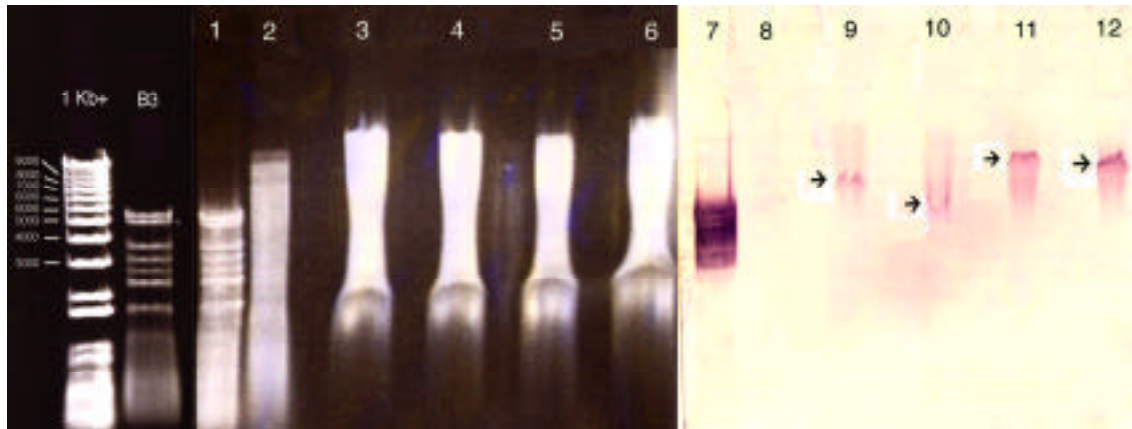
### **Direct Genome Sequencing**

Attempts at direct genome sequencing were equally unsuccessful. The electropherograms revealed high peaks at the beginning of the run which were most likely the primers. The rest of the electropherogram appeared to contain a mixture of DNA and no clear sequence was discernable.

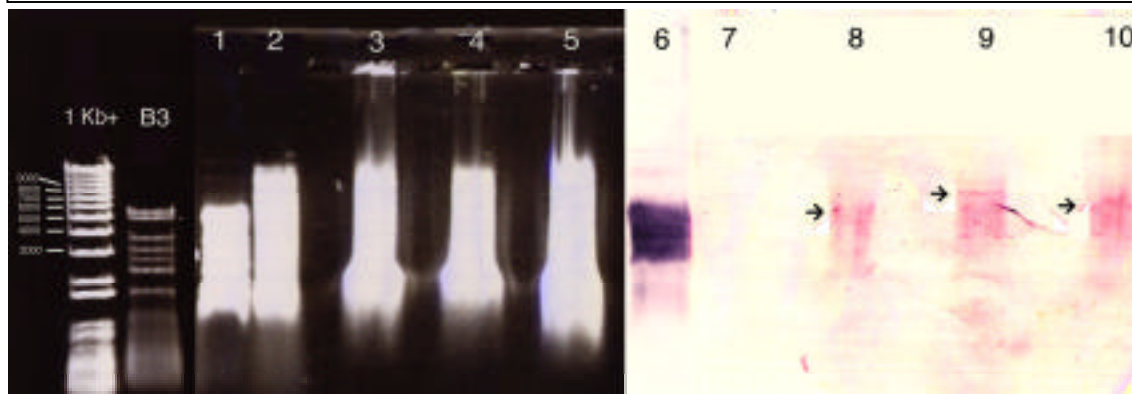
### **Southern Blot**

Upon isolation of suspected PAO1 lysogens, PCR was used to verify the presence of the B3 prophage. Ten isolates that produced an expected amplicon of approximately 1310 bp were deemed positive for lysogeny. DNA from the lysogens was digested and analyzed by southern blot to determine the approximate position of the B3 prophage within the PAO1 genome.

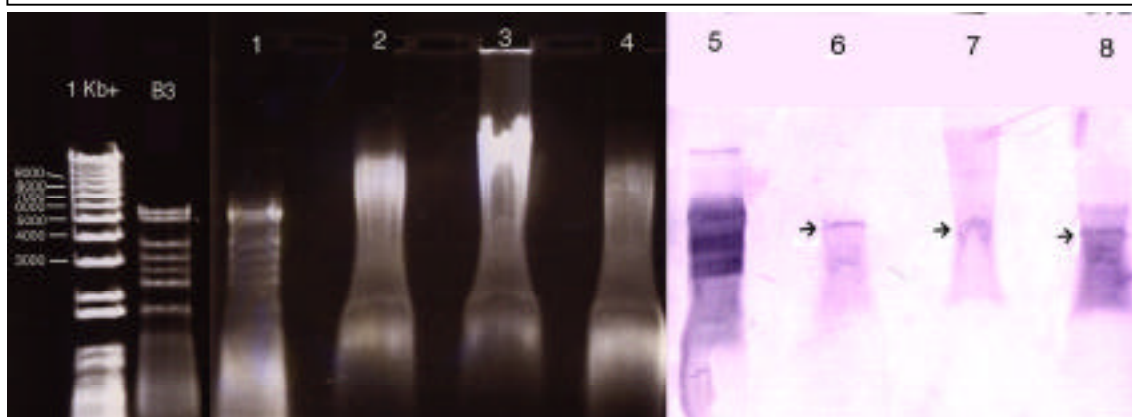
The blots are shown in Figures 1, 2, and 3. Isolated and digested B3 DNA was included on each blot as a positive control for the hybridization of the probe. Although the probe should only bind to one region on the B3 genome, thereby producing a single band, several bands are apparent due to the variable end lengths of the B3 genome.



**Figure 1.** Southern Blot #14 for the detection of the B3 prophage within the *P. aeruginosa* genome. Each lane holds 5 ug of DNA digested with BtsI. Lanes 1-6 illustrate the 0.5% agarose gel electrophoresis from which the membrane (lanes 7-12) was blotted. Lanes: 1&7, B3 (positive control); 2&8, *P. aeruginosa* (negative control); 3&9, Lysogen 5; 4&10, Lysogen 10B; 5&11, Lysogen 13; 6&12, Lysogen 17.



**Figure 2.** Southern Blot #15 for the detection of the B3 prophage within the *P. aeruginosa* genome. Each lane holds 5 ug of DNA digested with BtsI. Lanes 1-5 illustrate the 0.5% agarose gel electrophoresis from which the membrane (lanes 6-10) was blotted. Lanes: 1&6, B3 (positive control); 2&7, *P. aeruginosa* (negative control); 3&8, Lysogen 19; 4&9, Lysogen 31; 5&10, Lysogen 39.



**Figure 3.** Southern Blot #18 for the detection of the B3 prophage within the *P. aeruginosa* genome. Each lane holds 5 ug of DNA digested with BtsI. Lanes 1-4 illustrate the 0.5% agarose gel electrophoresis from which the membrane (lanes 5-8) was blotted. Lanes: 1&5, B3 (positive control); 2&6, Lysogen 10; 3&7, Lysogen 18; 4&8, Lysogen 27.

**Table 1:** Approximate size of the probed fragment of PAO1/B3 lysogens

<b>Lysogen Strain #</b>	<b>Size of Hybridizing DNA (bp)</b>
27	3600
18	3800
10	4000
19	5000
10B	6000
39	6000
5	8000
31	8000
17	9000
13	10,000

The relative locations of the B3 prophage within each lysogen, as compared to isolated B3 DNA and a 1Kb+ ladder (Gibco-BRL), are listed in Table 1. Note that the identified size of the bands is a rough approximation as compared to B3 DNA. Because some of the bands are less distinct and more smeared, an estimated error of approximately 1000 base pairs can be assumed for each identified probed region. Therefore, even though one band is identified at bp 3600, and another at bp 4000, both lysogens may in fact contain B3 DNA at the same site.

## **Discussion**

The host range of B3 has been investigated to an extent. Of the 11 soil isolates tested, no evidence of B3 infection was apparent. This is most likely because B3 requires the presence of a particular pillus in order to adsorb to a host (Roncero et al, 1990) and this pillus may only be present on the surface of PAO1. This warrants further investigation into the prevalence of PAO in soil and its candidacy as bioremediation host.

As predicted, the Southern Blots revealed the B3 prophage in a variety of positions within the PAO1 genome. Several groups of lysogens revealed bands within 1000 base pairs of one another which is within the estimated error. These results may indicate a preference for a particular region or sequence of DNA even though the integration of B3 is assumed to be random. This theme of semi-random insertion is consistent with the predictions of Phage Mu integration (Howe, 1987). It is also important to note that if there is a plasmid present in PAO1 it is possible for B3 to insert into this plasmid thereby skewing the results (Plotnikova et al, 1984).

### **Further investigation**

It is known that B3, like most temperate phages, carries extra host DNA on the ends of its genome. Attempts at direct genomic sequencing off of the end of B3 and into the host DNA were unsuccessful. Future experiments using a polishing and ligation reaction to circularize the B3 genome may meet with more success. PCR can be used to amplify the region between the two ends of B3, using primers specific for B3 DNA, which should theoretically be PAO1 DNA. The PCR product can then be cloned and sequenced and the insertion sites of several B3 genomes can be analyzed. Polishing and ligation were attempted in this study using *Pfu* Polymerase (Stratagene) and T4 Ligase (Invitrogen) but PCR was unsuccessful. This method of characterizing the insertion sequence should be investigated further. Preliminary ligation reactions were successful therefore indicating the possibility of success with PCR if different methods are tested and optimized.

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