

12-2010

Development of a Genetic Modification System in *Clostridium scatologenes* ATCC 25775 for Generation of Mutants

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DEVELOPMENT OF A GENETIC MODIFICATION SYSTEM IN
Clostridium scatologenes ATCC 25775 FOR GENERATION OF MUTANTS

A Thesis

Presented to

The Faculty of the Department of Biology

Western Kentucky University

Bowling Green, Kentucky

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science

By

Prasanna Tamarapu Parthasarathy

December 2010

DEVELOPMENT OF A GENETIC MODIFICATION SYSTEM IN
Clostridium scatologenes ATCC 25775 FOR GENERATION OF MUTANTS

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ACKNOWLEDGEMENTS

It is a pleasure to thank those who made this thesis possible. I am indebted to my supervisor Dr. Kinchel Doerner who took great efforts to explain things simply and made me work with the Coy chamber. It is difficult to overstate my gratitude to my committee members Dr. Rodney King, Dr. Claire Rinehart, and my lab co-worker Donna Kridelbaugh for their ideas and input in designing experiments and technical advice related to the project. I also thank Dr. John Andersland for his patience and cooperation in use of the EM facility.

My heartfelt thanks to Chandrika, Krupali, Katie, Clinton, Ali, Shravya, Jahnavi, Laksmi Reddy, Shoba, Sri, Sailaja, Kishore, Jennifer, Kiran and other student colleagues and seniors for helping me get through the difficult times, for all the emotional support and entertainment they provided. I would also like to thank the staff in the Department of Biology, Biotech Center and Bioinformatics Center for their wonderful help and support with regards to various official matters.

Finally I would also like to thank all my family members, my twin, Syam, Siva, Ranga, Ajay, Arun and Pranay who provided me encouragement and good company more than anyone else.

The authors express thanks to the WKU Biology department, WKU Biotechnology Center, and the USDA-ARS [USDA#: 58-6406-1-017] and NIH Grant Number 2 P20 RR-16481 for providing financial support.

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DEVELOPMENT OF A GENETIC MODIFICATION SYSTEM IN
***Clostridium scatologenes* ATCC 25775 FOR GENERATION OF MUTANTS**

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December 2010

43 Pages

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3-Methyl indole (3-MI) is a malodorous in food and animal waste and *Clostridium scatologenes* ATCC 25775 is the model organism for the study of 3-MI production.

3-MI is an anaerobic degradation product of L-tryptophan and can cause pulmonary disorders and death in cattle and goats. To elucidate the 3-MI biosynthesis pathway and the underlying genes, it is necessary to develop a system to allow genetic modification in *Clostridium scatologenes* ATCC 25775. Bacteriophages and transposons are useful tools to achieve this goal. Isolation of *Clostridium scatologenes* ATCC 25775 bacteriophage was attempted by prophage induction and enrichments using environmental sources. To induce prophages, cultures of *Clostridium scatologenes* ATCC 25775 were exposed to an effective concentration of mitomycin C at 2 µg/ml and 5 µg/ml. Induction with temperature was performed at 42°C and 55°C. Bacteriophage liberation, determined by a decrease in optical density was not observed in response to mitomycin C or by different growth temperatures. Nineteen environmental samples were tested for the presence of a bacteriophage that could infect *Clostridium scatologenes* ATCC 25775. The first cycle of

enrichments suggested a decrease in cell density, consistent with the presence of a bacteriophage but this was not observed in further iterations. Plaque assays were performed to confirm the presence of phage, but no plaques were observed. Although, different experimental conditions were tested, a transducing bacteriophage capable of infecting *Clostridium scatologenes* ATCC 25775 was not isolated. Transposons have been successfully used to generate mutants in *Clostridium difficile*. Therefore, we attempted to introduce transposons Tn5 and Tn916 into *Clostridium scatologenes* ATCC 25775 using electroporation. Transposon mutagenesis using Tn916 did not yield antibiotic resistant colonies. In contrast, commercially available transposon Tn5 gave antibiotic resistant colonies. However, further screening of the colonies using transposon specific primers in PCR reactions, did not yield any PCR product. We were unsuccessful in developing a genetic modification system in *Clostridium scatologenes* ATCC 25775 using bacteriophage or transposons.

INTRODUCTION

The genus “Clostridium” includes gram positive, spore forming human pathogens such as *Clostridium difficile*, *Clostridium perfringens* and non-pathogenic species such as *Clostridium scatologenes*, *Clostridium acetobutylicum* and many other environmental species that are responsible for production of compounds responsible for malodors in food and animal waste. Members of the clostridia display a wide range of fermentative metabolisms resulting in the production of a wide diversity of end products. These include p-cresol, indole, 3-methyl indole (3-MI, skatole) and p-ethylphenol which are products of the anaerobic metabolism of aromatic amino acids and plant material (2). 3-MI, a known malodorant, is an anaerobic degradation product of L-tryptophan and is also harmful to cattle and goats by causing pulmonary disorders and possibly death. In cattle, absorption and systemic circulation of 3-MI is followed by cytochrome P450 mediated bioactivation, which generates multiple reactive intermediates (10). 3-MI has also been shown to cause damage to human bronchial epithelial cells (20).

Clostridium scatologenes ATCC 25775 (*C. scatologenes*) is the model system for 3-MI production (1), but the genes responsible for the production and regulation of 3-MI are unknown. To understand the genes involved for the conversion of tryptophan to 3-MI, it is necessary to develop a system, that will allow genetic modification in *C. scatologenes*. The system should allow the generation of mutants that are defective in the synthesis of 3-MI.

Members of the genus clostridia are not naturally competent (10). To date, genetic tools for targeted inactivation of genes have been reported for clostridium and in only a few

strains including *C. acetobutylicum*, *C. perfringens*, and most recently, in *C. difficile* (6). In selected species of clostridia, gene transfer by protoplast transformation, electroporation, conjugation or shuttle vectors containing replicons from clostridial and streptococcal plasmids has been accomplished (16). We attempted to develop a system to allow genetic modification of *C. scatologenes* using bacteriophages, and transposons to generate mutants that are defective in 3-MI production.

Bacteriophages are ubiquitous in nature. Many bacteriophages have the potential to infect a cell and integrate the phage genome into the bacterial chromosome. In *E. coli*, bacteriophage lambda is a powerful tool to study gene structure and expression and it has also been used to construct genetic maps. There are no reports suggesting the presence of a bacteriophage capable of infecting *C. scatologenes*. A transducing phage that can infect *C. scatologenes* would be useful to characterize the 3-MI biosynthesis pathway. The mutants thus generated could then be isolated and sequenced.

Genetic manipulation of clostridia has been achieved by using commercially available transposon-transposase complexes, shuttle vectors and transposons such as Tn916 and Tn531 (19). Transposons are mobile DNA elements that can move from a vector and integrate randomly into the host chromosome. Since, *C. scatologenes* is not naturally competent; methods such as chemical transformation or electroporation may be used to deliver DNA containing the transposon of interest into the cells.

This work attempted to generate mutants in *C. scatologenes* by using transposon mutagenesis. We also attempted to isolate a transducing phage of *C. scatologenes* that would allow the genetic information to be moved between strains.

MATERIALS AND METHODS

Media preparation: Defined medium (11) for *C. scatologenes* was prepared using 500 mM L-glutamic acid (per liter), 1 ml of Sigma Chemical vitamin mixture (R7256), 1 µl of vitamin K₁ (Sigma), 5% v/v of mineral solutions 1 and 2, Pfenning trace mineral solution (per liter: 0.1 g, ZnSO₄ H₂O; 0.03 g, MnCl₂ H₂O; 0.3 g, H₃BO₃; 0.2 g, CoCl₂ 6H₂O; 0.01 g, CuCl₂ 2H₂O; 0.2 g, NiCl₂ 6H₂O; 0.03 g, Na₂MoO₄; 1.5 g, FeCl₂ 4H₂O; 0.01 g, Na₂SeO₃), 0.5% w/v ammonium sulfate; 0.4ml resazurin; 0.1% w/v hemin solution and 0.3 g of sodium bicarbonate. The mixture was boiled for 5 min under CO₂. After cooling, 0.1 g of L-cysteine HCl monohydrate was added and the pH of the solution was adjusted to 7.00 using NaOH while bubbling CO₂ through the medium. The flask was stoppered and transferred to a Coy anaerobic chamber (atmospheric conditions: 95% CO₂ and 5% H₂). Ten millimolar final concentration of sterile anaerobic glucose was added to the media, and dispensed in 10 ml aliquots into Balch tubes and autoclaved.

Tryptone yeast extract (TY) media was prepared (per 100 ml) by dissolving 2 g of tryptone; 1 g of yeast extract; 5 ml of mineral solution 1; 5 ml mineral solution 2; 0.4 ml 0.4% v/v resazurin; 0.1 ml 1% hemin solution; 0.5 µl 1µg/ml of vitamin K₁. TY was prepared in a round bottomed flask (2000ml) and allowed to boil for 10 min with bubbling CO₂, after which the flask was removed from the flame and cooled to room temperature on ice. The pH was adjusted to 7.00 using 5M NaOH while passing CO₂ and the medium was then transferred to the Coy anaerobic chamber. Mineral solution 1(per liter), 6 g of K₂HPO₄ was dissolved in water and autoclaved. To prepare mineral solution 2 (per liter); 12 g of NaCl; 12 g (NH₄)₂ SO₄; 6 g KH₂PO₄; 1.2 g CaCl₂ 2H₂O; 2.5 g MgSO₄ 7H₂O were dissolved in water and autoclaved.

Cultures of *C. scatologenes* were maintained using TY or in defined medium (11) by transferring 1ml of culture in TY into 10 ml of the medium and incubated at 37°C. Transfer of cells was performed at least every four days.

Growth curves: Defined medium was used for growth curve experiments. Midlog cultures of *C. scatologenes* were used for experiments that involved prophage induction and enrichment of bacteriophage. A Shimadzu Biomini spectrophotometer was used to record the absorbance at 600 nm fitted to house Balch tubes.

Plaque assays: To determine if bacteriophage were released during prophage induction or by enrichment, plaque assays were performed using a spot test and agar overlay methods. For performing the spot test, 100µl of mid log culture of *C. scatologenes* were plated onto TY plates in 0.7% top agar (0.7 g agar was dissolved in 100 ml TY broth, autoclaved and stored at 55°C). The top agar was allowed to solidify and 3 µl of test samples and a control (SM buffer only) were spotted onto the plate. The plates were incubated anaerobically at 37°C. Observations were recorded after 24-48 hours and putative plaques were aseptically picked using a sterile pipette tip and suspended in 50 µl of SM buffer for further analysis. For the agar overlay method, 100 µl of mid log culture of *C. scatologenes* was mixed with 100 µl of test samples (obtained from either enrichment or prophage induction) in microcentrifuge tubes and incubated for 20 min inside the anaerobic chamber. Following incubation, 5ml of 0.7% top agar was added and plated on TY plates. The plates were incubated for 24-48 hours at 37°C anaerobically. Putative plaques were picked aseptically into SM buffer (50 µl) and re-assayed by spot test for the presence of phage.

Prophage induction: Stocks of Mitomycin C at 200 µg/ml and 500 µg/ml in methanol were used to induce prophage in *C. scatologenes*. Hundred microliters was introduced into 10ml of *C. scatologenes* when the cultures reached an optical density of 0.1 at 600nm such that, the final effective concentrations of mitomycin C would be 2 µg/ml and 5 µg/ml. All experiments were carried out in triplicates. Mean values of the optical density reading were plotted against time. Methanol (50%) acted as a negative control. Absorbance was recorded every two to four hours until the optical density value for controls reached 1.00. To collect any bacteriophage released during the treatment, the cultures were centrifuged at 16,000 rpm and the supernatant filter sterilized through 0.2 micron filter. The filtrate was analyzed using the spot test.

Prophage induction using elevated temperature was carried out using 10ml of mid log cultures of *C. scatologenes* incubated initially at 37°C. Once, the cultures reached an OD of 0.1 at 600nm, the samples were shifted to two different temperatures (42°C and 55°C) and incubated for 3 hours. Cultures at 37°C were used as control. The supernatant obtained after centrifugation at 16,000 rpm was filtered (0.2 µm filter) and subjected to spot test.

Sample collection for enrichment of bacteriophage: Nineteen samples were collected in sterile 50ml polyethylene tubes from rivers, parks, soil, compost and other environmental sources as shown in Table 1. Samples were transported to the laboratory on ice and stored at 4°C until further processing. On the day of collection, enrichment experiments (cycle1) were carried out for all 20 samples using mid log cultures of *C. scatologenes*.

Table 1: List of environmental samples collected for enrichment of bacteriophage

Sample number	Sample	Collection site
1	Water	Barren River, Bowling Green
2	Sediment	Barren River, Bowling Green
3	Sediment	Primary lagoon, KY
4	Water	Griffith Park, KY
5	Sediment	Griffith Park, KY
6	Sediment	Griffith Park, KY
7	Sediment	Griffith Park, KY
8	Sediment	Griffith Park, KY
9	Soil	Cow farm, KY
10	Manure (Moist)	Swine farm, KY
11	Manure (Dry)	Swine farm, KY
12	Water	Primary swine lagoon, KY
13	Water	Secondary swine lagoon, KY
14	Compost	WKU Farm, KY
15	Soil	Soy bean field, KY
16	Water	Pond, KY
17	Sediment	Pond, KY
18	Water	Spring water
19	Silage	WKU farm, KY

Enrichment: An enrichment method can increase the likelihood of finding a phage even if it is present in low numbers. Each sample (0.8g) was aseptically transferred into 10 ml of TY broth containing 1 ml of freshly inoculated mid log phase *C. scatologenes*. Tubes were incubated for 48 hours at 37°C. The mixture was then centrifuged at 16,000 rpm for 10min at 4°C and the supernatant was filtered through a 0.22 micron filter. The filtrate was analyzed using a plaque assay and also analyzed by electron microscopy. Further, the filtrate (100µl) was added to mid log cells of *C. scatologenes* and incubated at 37°C and the optical density was recorded for every 4 hrs. The cells were processed after the optical density values for controls (anaerobic water) reached 0.8 at 600 nm. Samples were centrifuged at 16,000 rpm for 10 min at 4°C. The resulting supernatant was filtered through a 0.22 micron filter and the filtrate (first round of enrichment; cycle 1) was added to mid log cells of *C. scatologenes* and incubated at 37°C. The optical density was recorded for every 4 hrs. The cells were processed after the optical density values for controls (anaerobic water) reached 0.8 at 600 nm. The samples were centrifuged at 16,000 rpm for 10 min at 4°C. The supernatant was filtered through a 0.22 micron filter. This filtrate obtained (round 2 of enrichment; Cycle 2) was used to infect mid log cells of *C. scatologenes* and subjected to the same procedure as explained above. The filtrates from cycles 1, 2 and 3 were analyzed by plaque assays and electron microscopy.

Electroporation using EZ - Tn5 transposome complex: The EZ Tn5 transposome complex was prepared according to the standard protocol (Epicenter). Two microliters of the EZ Tn5 transposon DNA (stock 100 µg/ml) was added to 4 µl of EZ Tn5 transposase and mixed thoroughly. Two microliters of 100% sterile glycerol was

then added and mixed thoroughly. The mixture was vortexed and incubated at room temperature for 30 min. The EZ Tn5 complex was then incubated at 4°C overnight and transferred to -20°C after overnight incubation. A fresh overnight culture of *C. scatologenes* (1.5 ml) was transferred into sterile 60 ml TY broth. The cells were allowed to grow at 37°C until they reach an OD of 1.0-1.4 at 600 nm. In an anaerobic chamber, 30 ml of the above culture was transferred into falcon tubes and sealed. The cells were centrifuged (16,000 rpm) for 10min at 4°C and were immediately returned to the anaerobic chamber. The supernatant was discarded and the cells were resuspended in 10 ml of ice cold electroporation buffer (EP). The lids were resealed and the tubes were spun for 10 min at 4°C and 16,000 rpm. The pellet was washed again in 2.3ml of ice cold EP buffer and finally resuspended in 800µl of EP buffer. One microliter of the EZ Tn5 transposome complex (stored at -20°C) and 580 µl of freshly prepared cells were added to a 0.4 cm electroporation cuvette. The cuvette was chilled on ice for 2- 5 min and placed in the electroporation chamber (Gene pulser Xcell, Biorad) and subjected to electrical pulses at different voltages using exponential conditions with 25µF capacitance and resistance at 300 Ω. Pre warmed TY (800µl) was added and the cell suspension was transferred into a 1.5ml micro centrifuge tube. The samples were incubated for 3 hours at 37°C. Two hundred microliters of the cell suspension was then plated onto TY agar plates supplemented with kanamycin (250 µg/ml). The plates were incubated at 37°C for 4 days inside the coy chamber. Polymerase chain reaction was carried out to confirm the presence of Tn5 transposon in kanamycin resistant colonies of *C. scatologenes*. The primers specific for the Tn5 transposon are listed in Table 3. The PCR reactions contained 25µl of Econotaq master mix, 1 µl of Tn5 forward primer (25 nM), 1µl of Tn5

reverse primer (25 nM), 22 μ l of sterile nanopure water and mixing was done on ice. One microliter of cell suspension was added to forty nine micro liters of the reaction mixture. In addition, 16S rRNA primers, 27f and 1492r were used as a positive control.

Table 2: Primer sequences used to confirm the presence of Tn5 transposon

Primers	Sequence	Reference
Tn5 Forward	5' AGATGTAGGTGTTCCACAGGGTAG 3'	This study (Epicenter)
Tn5 Reverse	5' GGTTGATGAGAGCTTTGTTGTAGGT 3'	This study (Epicenter)
16S RNA (27f)	5' ACCATGCGGTCCAAGTTGTTATGC 3'	Lane D.J (12)
16S RNA (1492r)	5' GCCTACATGAAGTCGGAATCGCTA3'	Lane D.J (12)

Electroporation using Tn916 transposon: pAM 120 (Figure 1) is a shuttle vector containing the Tn916 transposon. The pAM 120 plasmid was isolated from *E.coli* CG120 cells using a standard protocol as described in the Ultra clean plasmid isolation kit (MoBio Lab. Inc., Carlsbad, CA). The concentration of the plasmid was measured with a Nanodrop. For the electroporation experiments, defined medium was used to obtain early log phase cells of *C. scatologenes*. Mid log cells (1.5 ml) were harvested using centrifugation inside the anaerobic chamber. The supernatant was discarded and the pellet was resuspended in 800 μ l of chilled sterile EB buffer. The buffer (EB) contained 272 mM sucrose, 7 mM NaPO₄, 1mM MgCl₂, with pH adjusted to 7.4. The above steps were repeated twice and the cells were used for electroporation. Processed cells (800 μ l) and 5 μ l of prepared pAM120 were added to a pre chilled 0.4 cm electroporation cuvette. The mixture was incubated on ice for 10 min. The cells were then pulsed at different voltages using exponential conditions with 50 μ F capacitance and resistance at 200 Ω . Duplicate electroporations were done at voltages 1000, 1500, 2000 and 2500. Pulsed cells were incubated on ice for 10 min and then 100 μ l of electroporated cells were transferred to 10 ml of sterile TY. The cells were incubated at 37°C for 3 hours and 100 μ l of the electroporated cells were plated onto TY plates containing 20 μ g/ml of tetracycline.

Minimum inhibitory concentration of *C. scatologenes* on TY plates:

Minimum inhibitory concentrations of kanamycin and tetracycline on the growth of wild type *C.scatologenes* were determined on TY plates. TY broth was prepared as described in the methods section. Stocks of kanamycin (10 mg/ml) and tetracycline (10 mg/ml) were prepared using sterile water. Antibiotic stocks were filter sterilized using 0.2 micron filter. Varying volumes of kanamycin and tetracycline stock were added to sterile TY

with 1.5% agar such that the final concentration of kanamycin on the plate was; 500 $\mu\text{g/ml}$, 250 $\mu\text{g/ml}$, 125 $\mu\text{g/ml}$, 62.5 $\mu\text{g/ml}$, 30 $\mu\text{g/ml}$, 20 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, 5 $\mu\text{g/ml}$. Tetracycline final concentrations were 50 $\mu\text{g/ml}$, 40 $\mu\text{g/ml}$, 30 $\mu\text{g/ml}$, 20 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$. Midlog phase cells (1.5ml) of *C. scatologenes* at an OD of 0.15 at 600nm were spun for 1 min inside the hood on a table top centrifuge and the supernatant was discarded. The cells were resuspended in sterile TY broth and 100 μl of these cells were plated on TY with different concentrations of kanamycin and tetracycline as mentioned above. All plates were incubated at 37°C inside the Coy chamber for 3 days after which, colonies were enumerated.

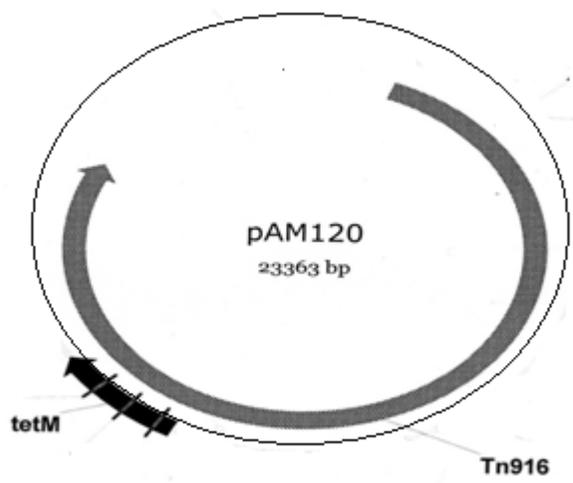


Figure 1: Plasmid pAM 120 with Tn916 transposon carrying a tetracycline marker.

Minimum inhibitory concentration of *C. scatologenes* in TY broth: The minimum inhibitory concentration of tetracycline against *C. scatologenes* cells was performed. Tetracycline final concentration was 50 µg/ml, 40 µg/ml, 30 µg/ml, 20 µg/ml, 10 µg/ml, 5 µg/ml and 0 µg/ml. One milliliter of midlog phase cells with an OD 0.15-0.2 were added to the TY tubes containing different concentrations of the antibiotic. Cultures were incubated at 37°C for 72 hours and the MIC was determined based on the absence of growth. Absorbance was recorded at 600nm using a spectrophotometer.

SM buffer: SM buffer (per liter) was prepared by dissolving 5.8 g of NaCl, 2 g of magnesium sulfate, 1M Tris. pH was adjusted to 7 using HCl. The buffer was autoclaved and stored at RT for future use.

Electron Microscopy of Phage preparations: The putative plaques obtained from spot tests and agar overlay methods were resuspended in SM buffer and analyzed by electron microscopy (JEM 100CX). Phage filtrates obtained from all cycles of enrichment were also subjected to microscopic analysis. Negative staining was performed, using 1% uranyl acetate. Formvar coated grids were used upon which 10 µl of the phage preparation was added and allowed to adsorb for 2 min. The grids were washed with 30µl of nanopure water for 2 min and were floated on 30µl droplets of 1% uranyl acetate for 2 min. Excess liquid was removed using filter paper wedges. The grids were viewed at a magnification of 20,000X with eucentric centering. Undiluted and 1:5 diluted samples were examined for the presence of phage.

RESULTS

Prophage induction using Mitomycin: A hypothetical representation of the prophage induction is illustrated in the Figure 2. It is expected that, a sudden drop in optical density will occur because of the induction of a bacteriophage that leads to lysis of host cells. Growth curve analysis of controls without mitomycin C and test samples with final concentrations of mitomycin C at 2 μ g/ml and 5 μ g/ml are shown in Figures 3 and 4 respectively. A sharp decrease in the optical density was not observed in the test samples with either concentrations of mitomycin C.

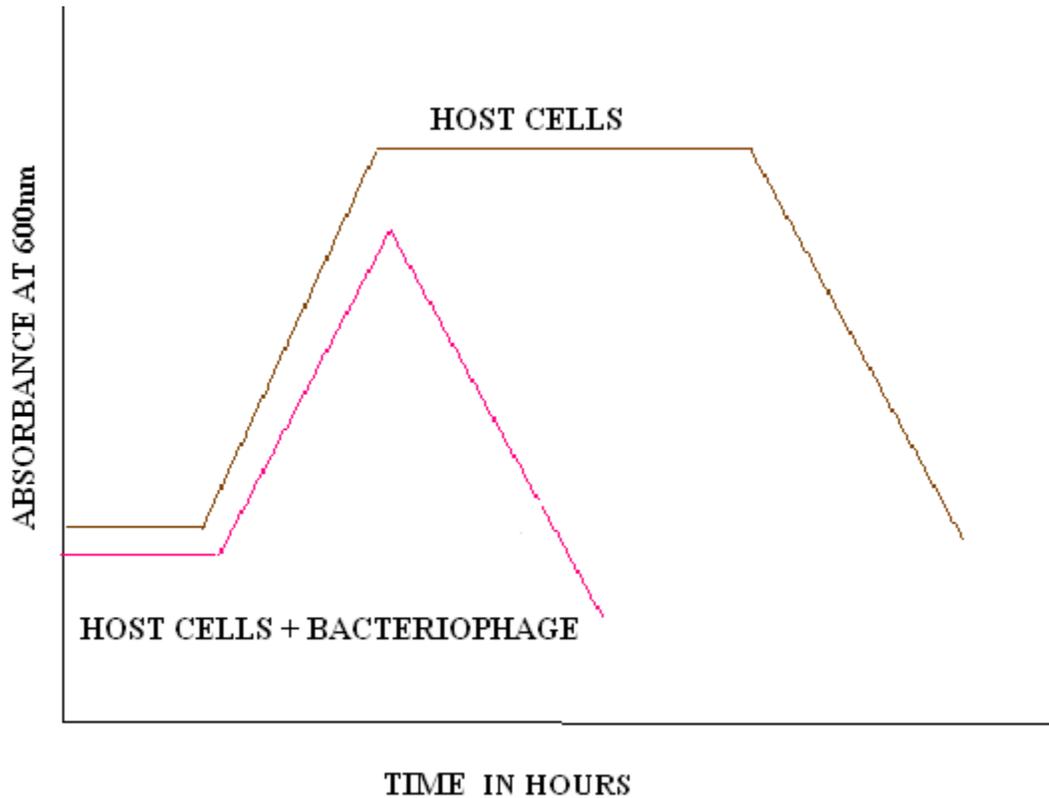


Figure 2: Illustration of bacteriophage induction. A hypothetical growth curve of a bacterial culture experiencing prophage induction. A sharp decrease in absorbance after induction is seen due to phage induced lysis of host cells when compared with control. The drop in the absorbance in the control after stationary phase is due to the autolysis of the host cells (*Clostridium scatologenes* ATCC 25775).

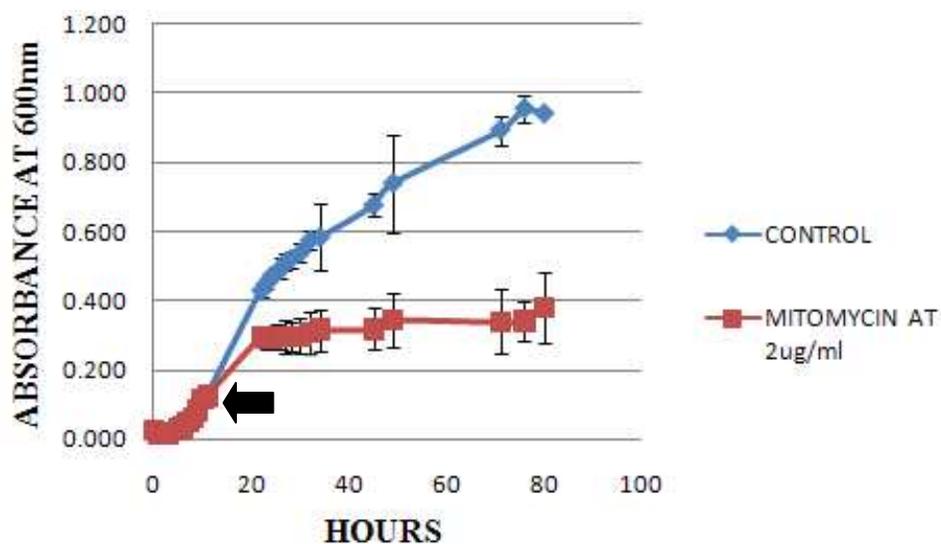


Figure 3: Induction of prophage in *Clostridium scatologenes* ATCC 25775 with mitomycin C at 2 μ g/ml. The experiment was performed in triplicate using defined medium with glucose. The arrow indicates absorbance at which mitomycin C (100 μ l of 200 μ g/ml) was added to test samples. The error bars indicate standard deviation between the 3 samples.

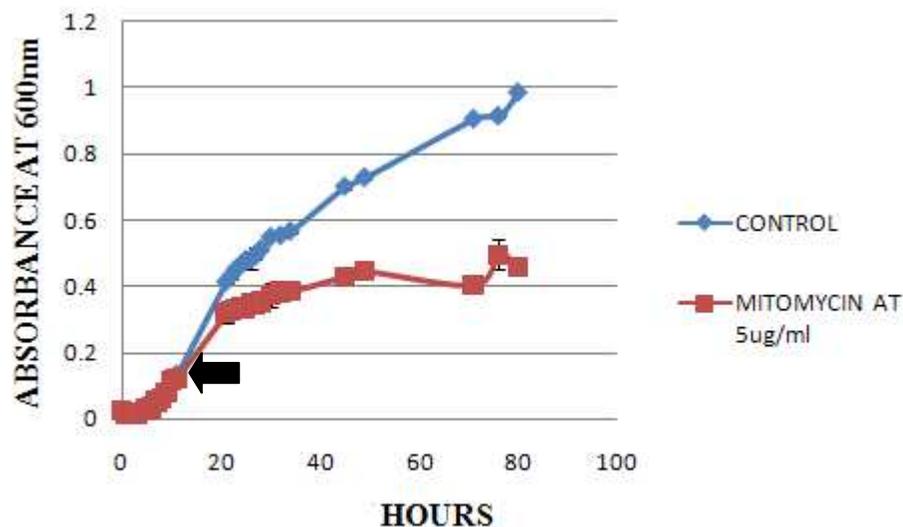


Figure 4: Induction of prophage in *Clostridium scatologenes* ATCC 25775 with mitomycin C at 5 μ g/ml. The experiment was performed in triplicate using defined medium with glucose. The error bars indicate standard deviation between the 3 samples. Arrow indicates the absorbance at which, mitomycin C (100 μ l of 500 μ g/ml) was introduced into the test samples (mid log phase). Prophage induction using 5 μ g/ml did not induce a prophage and resulted in decrease in absorbance as seen in the growth curve with mitomycin C at 2 μ g/ml.

Prophage induction using temperature: Prophage induction using temperatures at 42°C and 55°C did not yield bacteriophage from *C. scatologenes*. The tubes were observed for clearance by visual inspection. Turbidity was observed in all the tubes including the controls which were incubated at 37°C. This result suggests that no prophage was induced using two different temperatures. The filtrates obtained were analyzed using plaque assays (spot test and agar overlay method). Both the assays were negative for the presence of a bacteriophage that can infect *C. scatologenes*.

Phage isolation using enrichment: Enrichment experiments using 20 environmental samples failed to yield a bacteriophage capable of infecting *C. scatologenes*. Three rounds of enrichment procedures (cycle 1, cycle 2 and cycle 3) were performed for each sample that showed a significant drop in optical density in cycle 1 of enrichment. These samples include lagoon sediment (Fig 5), lagoon top water (Fig 6) and silage (Fig 7). During cycle 2 from each of these samples, the optical density was not observed to decrease when compared to control (data not shown). This might be due to the chemical composition of the initial filtrates that tend to inhibit the growth of the host cells. However, from cycle 2, the concentration of these chemicals that inhibit growth decrease and hence there is no drop in the absorbance from round 2 of enrichment.

Figure 8 shows enrichment using higher concentrations of phage filtrate from silage.

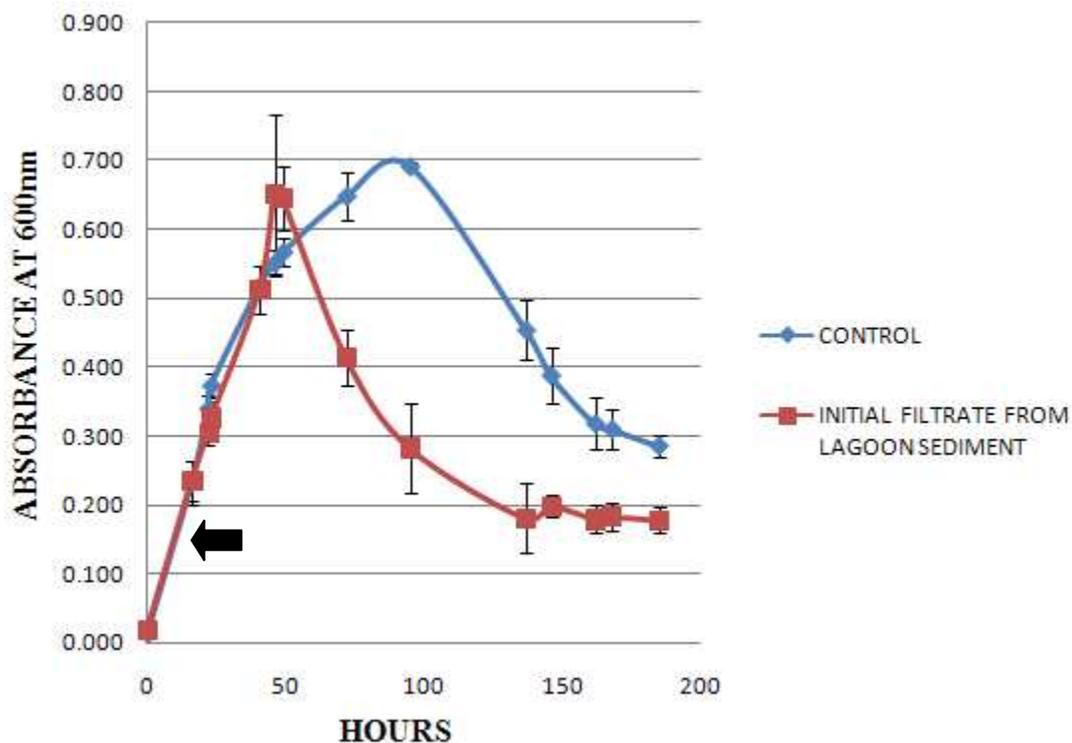


Figure 5: Growth curve analysis of *Clostridium scatologenes* ATCC 25775 with initial enrichment using filtrate from lagoon sediment. The experiment was performed in triplicate using defined medium with glucose. The error bars indicate standard deviation between the 3 samples. The arrow indicates the absorbance at which filtrate (100 μ l) from initial lagoon sediment was introduced into the test samples.

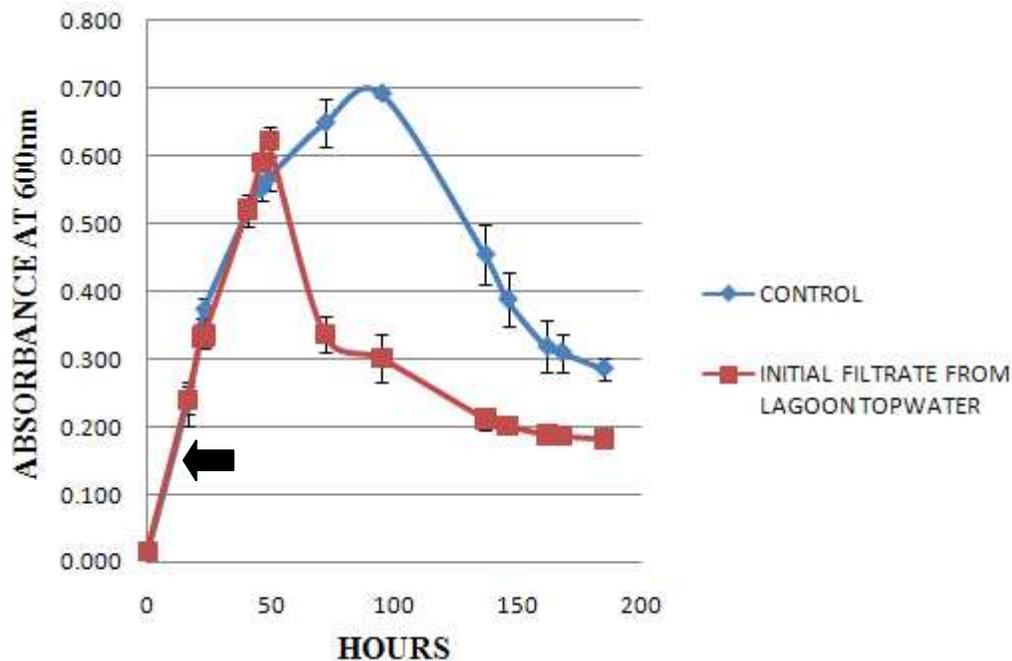


Figure 6: Growth curve analysis of *Clostridium scatologenes* ATCC 25775 with initial enrichment using filtrate from lagoon top water. The experiment was performed in triplicate using defined medium with glucose. The error bars indicate standard deviation between the 3 samples. The arrow indicates the absorbance at which filtrate (100 μ l) from lagoon top water was introduced into the test samples. A steep drop in absorbance is observed in the test sample indicating the presence of a bacteriophage in the initial filtrate obtained from lagoon top water.

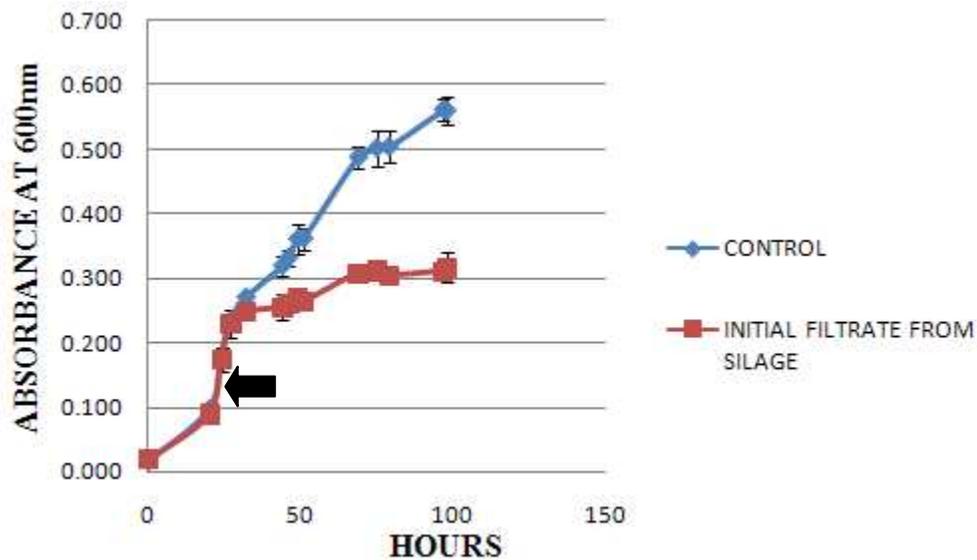


Figure 7: Growth curve analysis of *Clostridium scatologenes* ATCC 25775 with initial enrichment using filtrate from silage. The experiment was performed in triplicate using defined medium with glucose. The error bars indicate standard deviation between the 3 samples. The arrow indicates the absorbance at which filtrate (100 μ l) from silage was introduced into the test samples.

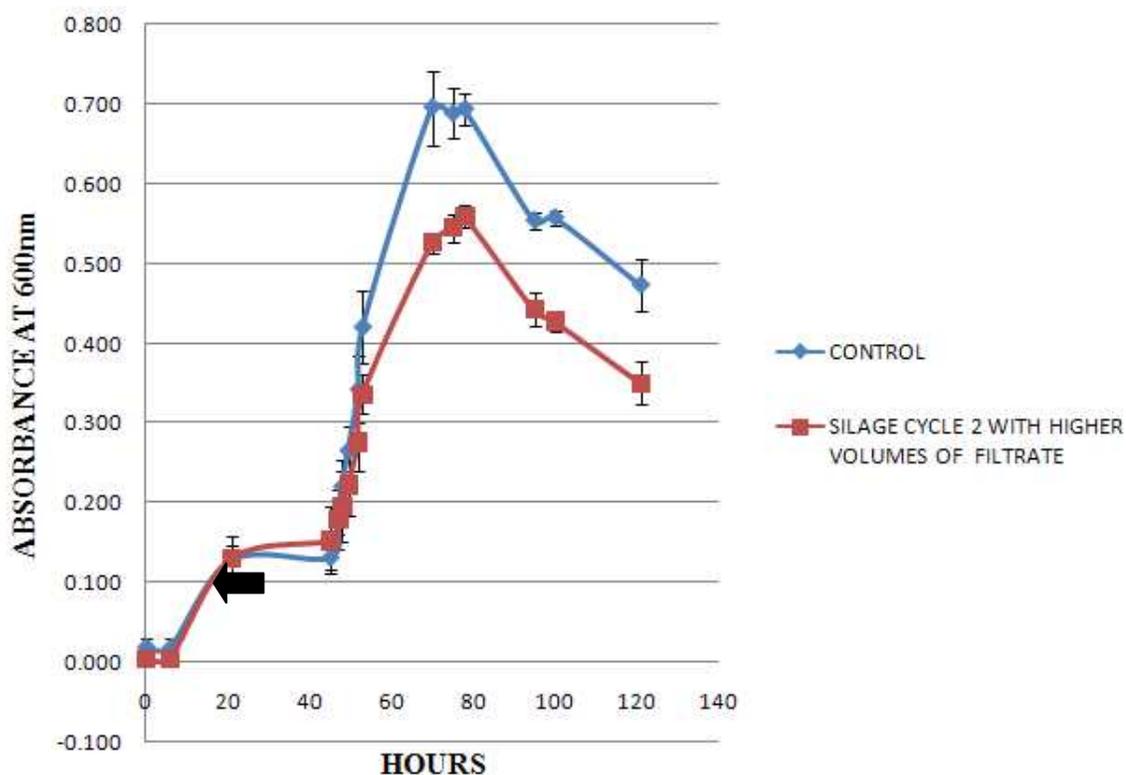


Figure 8: Growth curve analysis of *Clostridium scatologenes* ATCC 25775 with initial enrichment using higher volumes from silage. The experiment was performed in triplicate using defined medium with glucose. The error bars indicate standard deviation between the 3 samples. The arrow indicates the absorbance at which higher volumes of filtrate (2000 μ l) from silage were introduced into the test samples.

Minimum inhibitory concentration of kanamycin and tetracycline towards

C. scatologenes: Kanamycin (500 and 250 µg/ml) inhibited the growth of *C. scatologenes* in TY broth and TY plates. Results were recorded after 24 hours; but cultures which did not exhibit any growth were re- incubated for 4 days. Growth was observed at kanamycin concentrations of 5, 10, 20, 30, 62.5 and 125 µg/ml in both TY broth and plates. Tetracycline concentrations of 20, 30, 40 and 50 µg/ml inhibited the growth of *C. scatologenes* in TY broth and TY plates. Tetracycline at 10 and 5 µg/ml did not inhibit any growth. Based on these results kanamycin at 250 µg/ml and tetracycline at 20 µg/ml were used to select cells that received Tn5 or Tn916 transposon respectively.

Generation of *C. scatologenes* mutants using Tn5 transposome: Tn5 is a commercially available composite transposon which can randomly integrate into a bacterial chromosome and has been used to generate mutants in *C.perfringens*. To generate mutants in *C. scatologenes*, different electroporation conditions were attempted (Table 3) using a Tn5 transposome complex. Tn5 mutagenesis resulted in kanamycin resistant colonies on TY plates. Although the number of colonies formed varied with voltage and time constants, all colonies were picked, grown in TY broth with kanamycin (250µg/ml) and screened for the presence of Tn5. Different voltage conditions were tried to optimize transformation efficiency. PCR analysis of colonies obtained at 1500 volts (Table 4) did not indicate the presence of an amplified fragment (Figure 9). A positive control was amplified with the Tn5 specific primers. All other samples resulted in PCR products that migrated differently from the positive control. These products may be due to primer dimer formation. A negative control showed the same non specific amplification products. Similar results were obtained with electroporation at 2000 volts.

Though kanamycin resistant colonies were formed following shocking at 2000 volts (Table 5), PCR based screening was negative for the presence of Tn5 (Figure 10).

Control experiments indicated the Tn5 primers were specific for Tn5 and the conditions allowed the successful amplification of the DNA fragments from whole cells. No colonies were recovered whose cells were electroporated at 2500 volts.

Table 3: Electroporation conditions used for Tn5 transposon mutagenesis

Condition	Voltage set (volts)	Time constant ^a observed (ms)
1	1500	7.4
2	2000	7.6
3	2500	7.5

^a Time constants obtained when *Clostridium scatologenes* ATCC 25775 was electroporated with Tn5 transposon using exponential conditions at 25 μ F capacitance and resistance at 300 Ω .

Table 4: Results of electroporation with Tn5 at 1500 volts

Plate	Concentration of kanamycin	Time of expression (hrs)	Number
Control at 10 volts	500µg/ml	6	0
Control at 1500 volts	250µg/ml	6	0
PE1 ^a at 1500 volts	500µg/ml	6	0
PE1 at 1500 volts	250µg/ml	6	0
PE2 ^b at 1500 volts	500µg/ml	6	0
PE2 at 1500 volts	250µg/ml	6	1
PE1 at 1500 volts	500µg/ml	3	0
PE1 at 1500 volts	250µg/ml	3	2
PE2 at 1500 volts	500µg/ml	3	0
PE2 at 1500 volts	250µg/ml	3	3

^aPE1 corresponds to plate electroporation 1 and ^bPE2 corresponds to its duplicate.

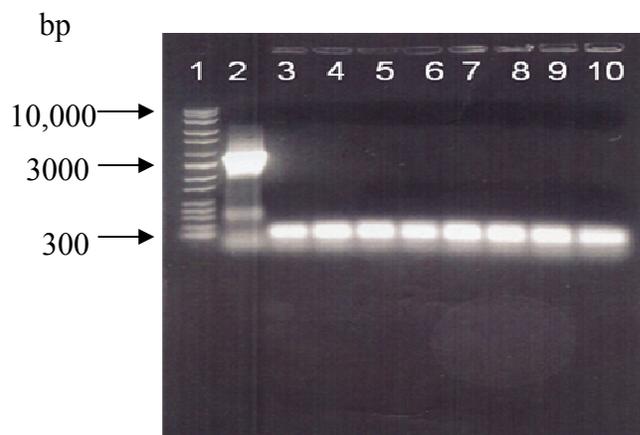


Figure 9: Agarose gel analysis of whole colony PCR products from transposon candidates recovered after electroporation at 1500 volts. Lane 1 is a 1kb ladder; Lane 2 represents positive control for Tn5; Lanes 3 to 9 are the combination of four kanamycin resistant colonies formed at 1500 volts; and Lane 10 is wild type *Clostridium scatologenes* ATCC25775. Band relative to positive control (3000 bp) as shown in Lane 2 was absent in all the test samples.

Table 5: Results of electroporation with Tn5 at 2000 volts

Plate	Concentration of kanamycin	Time of expression (hrs)	Number
Control at 10volts	500µg/ml	6	0
Control at 2000 volts	250µg/ml	6	0
^a PE1 at 2000Volts	500µg/ml	6	0
PE1 at 2000Volts	250µg/ml	3	4
^b PE2 at 2000Volts	500µg/ml	6	0
PE2 at 2000Volts	250µg/ml	6	1
PE1 at 2000Volts	500µg/ml	3	0
PE1 at 2000Volts	250µg/ml	6	2
PE2 at 2000Volts	500µg/ml	3	0
PE2 at 2000Volts	250µg/ml	3	2

^a PE1 corresponds to plate electroporation 1 and ^bPE2 corresponds to its duplicate.

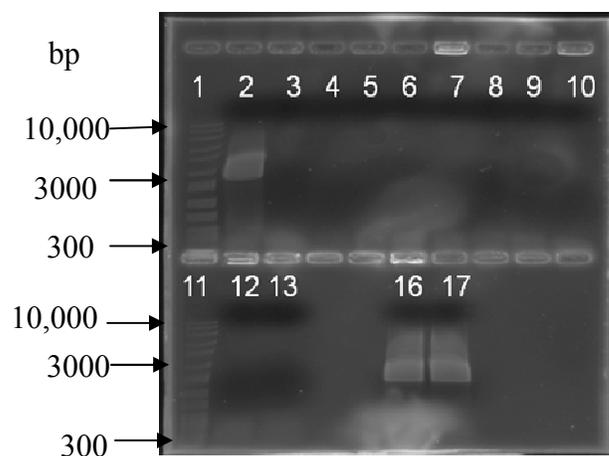


Figure 10: Agarose gel analysis of whole colony PCR products from transposon candidates formed at 2000 volts. Lane 1 and Lane 11 correspond to 1Kb ladder; Lane 2 is Tn5 positive control; and Lanes 3 to 10 and 12, 13 are kanamycin resistance colonies recovered after electroporation at 2000 volts. Lane 16 corresponds to 16S rRNA control with wild type *Clostridium scatologenes* ATCC25775. Lane 17 includes 16S rRNA analysis with kanamycin resistant colony formed on TY plate. The PCR products in positive control (3000bp) and 16S rRNA analysis indicate that the primers were specific to Tn5 and the protocol to amplify DNA fragments from whole cells worked efficiently. No bands similar to the positive control were observed in the test samples.

Generation of mutants of using Tn916 transposon containing pAM120:

Tn916 containing pAM 120 plasmid was isolated by standard plasmid isolation procedure. The concentration of the plasmid was 58 ng/μl by Nanodrop. Five microliters (290 ng/μl) of the plasmid was used to electroporate harvested cells of *C. scatologenes*. Different voltages were used to optimize the electroporation conditions at 50 μF capacitance and resistance at 200Ω (Table 6). The time constants obtained were almost the same at different voltages tested. Electroporation using the Tn916 transposon containing pAM120 plasmid did not yield any tetracycline resistant colonies. However, 1,856 colonies were obtained on TY plate without tetracycline when pulsed at 1000volts. Electroporation at 10 volts gave 2860 colonies on TY plate.

Electron microscopy: Phage filtrates from cycle 1 of the enrichment samples, putative plaques, filtrates from chemical and temperature induction did not exhibit any structure resembling a bacteriophage when viewed under TEM.

Table 6: Results of electroporation using pAM 120 plasmid

Condition	Voltage set (volts)	Time constant ^a observed (ms)
1	1000	5.3
2	1500	5.6
3	2000	5.5
4	2500	5.6

^a Time constants obtained when *Clostridium scatologenes* ATCC 25775 was electroporated with pAM 120 plasmid using exponential conditions at 50 μ F capacitance and resistance at 200 Ω .

Plaque assays: Filtrates obtained from silage and lagoon sediment gave putative plaques (<10) that were round and clear when assayed by using spot test or by agar overlay methods (Table 9). Filtrate from cycle2 of silage resulted in putative plaque formation by both spot test and agar overlay methods. However, a re-assay of these putative plaques by spot test and analysis by electron microscopy failed to show the presence of a bacteriophage.

Table 9: List of samples analyzed by plaque assays

Sample number	Plaque assay	Sample
19	Spot test	Silage
3	Spot test	Lagoon Sediment
19	Spot test	Silage cycle 2 filtrate
19	Agar overlay	Silage cycle 2 filtrate

DISCUSSION

The main goal of this project was to develop a genetic system in *C. scatologenes* which could be used to identify the genes involved in 3-MI biosynthesis pathway. Bacteriophages are useful tools for genetic analysis, thus the initial experiments were designed to isolate a bacteriophage. Generalized transduction using a transducing bacteriophage can be used for genetic manipulation in host cells such as construction of mutant strains. Fragments of the host genome are packaged into the capsids instead of the phage DNA into the capsids. This results in a functional phage particle, which can attach and deliver the packaged DNA into specific bacteria. But, due to the absence of the phage DNA, the infection does not lead to lysis of the bacterium. Instead, the injected foreign bacterial DNA can be incorporated into the genome and can generate mutants. Transposons are useful for generating random mutants. Commercially available transposons like Tn5 were previously used to generate random mutants efficiently in *C. perfringens* (9). We used Tn916 and a commercially available transposon, Tn5, to generate mutants in *C. scatologenes*.

A bacteriophage specific to *C. scatologenes* can be obtained by either of the two methods; prophage induction or by enrichment of environmental samples. Bacteriophages co-exist in the same environment where their hosts show active reproduction. *C. scatologenes* was isolated from food during the 20th century (13). *C. drakei* was isolated from sediments of acidic coal mines and *C. carboxidivorans* from agricultural settling lagoon (8). Potential anaerobic environments to search for a bacteriophage include lagoons, swamps, compost piles and acidic coal mines where oxygen levels are quite low. We collected 19 environmental samples and used enrichment methods to increase the

likely hood of finding a phage. The virulence of certain strains of *C. botulinum* and *C. difficile* is attributed to the presence of prophage and several temperate phages of clostridia have been induced using chemicals such as mitomycin C (14). There is a possibility that *C. scatologenes* is a lysogen. Therefore, we tried conditions known to induce a prophage by; mitomycin C or temperature. However, enrichment and prophage induction did not yield a bacteriophage specific to *C. scatologenes*.

There are several possible explanations for our results. A bacteriophage specifically infecting *C. scatologenes* is not present in the environmental samples we screened or there was no prophage in the strain we used for phage isolation. No further increase in absorbance was observed for the test samples, during chemical induction with mitomycin C. This may be due to the effect of mitomycin C on cells and not due to the induction of the prophage in the cells. Another possibility might be the presence of a restriction modification system which is more common in the genus clostridia because of which we failed to isolate a transducing bacteriophage that can infect *C. scatologenes* from the environment. A steep decrease in absorbance was observed in growth curves using filtrates from lagoon sediment and lagoon top water by enrichment. Such a significant drop was not observed in consecutive experiments using filtrates from cycle 2 and cycle 3. The initial drop may be attributed to the composition of the filtrate. The initial filtrate might likely contain inhibitors or chemicals that can induce lysis of the host cells. These inhibitor concentrations get diluted out with further iterations consistent with the idea that, a steep drop is not observed from cycle 2. Also, spot tests and agar overlay analysis confirmed the absence of a bacteriophage.

The second approach to generate mutants was to use a Tn5 transposon. The approach did yield kanamycin resistant colonies. However, the colonies obtained from the Tn5 mutagenesis did not show the presence of a transposon inside the cells, as determined by PCR. Control plates without kanamycin showed growth after two days, whereas the electroporated cells took four days at 37°C to show initial growth. The absence of a transposon and growth on high concentrations of kanamycin can be attributed to the decrease in the effective concentration of kanamycin in the plates over time or degradation of transposon due to the presence of a restriction modification system inside the cells. The restriction enzymes like Csp45I, Cac81, and CfoI were isolated from *C. sporogenes*, *C. acetobutylicum* and *C. formicoaceticum* respectively. Another possibility might be because of the spontaneous mutations that may have occurred in the wild type cells which allow for growth in high levels of antibiotics.

Future experiments include the optimization of plaque assays by using different stages of cells (early log phase) instead of mid log phase cells. Prophage induction by UV can be another method of inducing a prophage from *C. scatologenes*. UV irradiation has been shown to induce lytic cycle by a Rec A mediated mechanism. Confirmation of the presence of a restriction modification system in the bacterium by using a known template DNA and cell lysate of *C. scatologenes* could be useful to methylate DNA before electroporation.

Screening samples such as lagoon samples at different time points, sewage, acid coal mine sediments, anaerobic animal wastes, poultry waste and enrichment of these samples might lead to discovery of a bacteriophage specific for *C. scatologenes*. Another approach is to perform transposon mutagenesis using commercially available tetracycline

resistance carrying Tn5 transposon since *C. scatologenes* is known to be tetracycline sensitive at 20 µg/ml. Previous studies in *C. difficile* show that a Tn916 shuttle conjugative transposon can also be used to generate mutants in the bacterium (17). Recent methods for generating mutants include ClosTron, a group II intron which was used to knock out genes for the first time in *C. botulinum* and *C. sporogenes* (4).

Using late log phase cells which have a weakened cell wall may facilitate the uptake of transposon DNA more efficiently than a mid log phase cell with an intact cell wall. Also, an increase in the concentration of DNA used for electroporating the competent cells may help increase the transformation efficiency. Another approach to develop a genetic modification system is the use of conjugation instead of electroporation. A mariner based transposon system that involves Himar 1 element constructed on a pseudo suicide vector (18) could be used instead a Tn5 transposome complex. Himar 1 is a member of Tc1/ mariner super family transposon that requires no factors for transposition other than their self-encoded transposase. This makes them ideal candidates for development into generalized genetic tools. These mariner based transposon systems were effectively used to generate mutants in clostridia with low G+C content.

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