Identification of receptors for two novel *Escherichia coli* O157:H7 bacteriophage

A Thesis

Presented to

The Faculty of Graduate Studies

Of

Carleton University

By

Adam Moss

In Partial fulfillment of requirements

For the degree of

Master of Science

December 2008

Your file Votre référence
ISBN: 978-0-494-81687-5
Our file Notre référence
ISBN: 978-0-494-81687-5

Abstract

Identification of receptors for two novel *Escherichia coli* O157:H7 bacteriophage

E. coli O157:H7 is a major human pathogen transmitted to humans primarily through contaminated meat products. Reducing the pathogen load in food animals with the use of natural bacteriophage can improve food safety. The objective of this thesis was to characterize the receptor sites of two novel bacteriophage that form part of an effective cocktail product under development at Gangagen Life Sciences Inc. This was accomplished by developing and characterizing mutants resistant to R26, a T1-like phage and P39, a T4-like phage. Most mutants resistant to P39 were sensitive to R26 and vice versa suggesting that the two phage target different receptor molecules. Lipopolysaccharide (LPS) was identified as a potential receptor for phage R26 as indicated by differences in LPS profiles of the R26-resistant mutants. Sequence analysis of the *rfaF* gene in Ec4BIMR26R12, an R26-resistant mutant, revealed a mutation that would give rise to a truncated non-functional enzyme, resulting in truncated LPS. OmpC was shown to be required for P39 binding using PCR, Southern blot analysis and genome walking. A deletion in the region of the *ompC* gene resulting in a non-functional OmpC protein was identified. This was confirmed by sequencing and complementation studies by expressing OmpC from *E. coli* O157:H7 in a P39-resistant mutant, Ec4BIMP39R3.

Acknowledgments

I would like to thank my co-supervisors Dr. Kishore Murthy and Dr. Myron Smith for all of the guidance and support they have given me over the years. Thanks to GLSI and Kishore for giving me the opportunity to pursue my Masters degree while continuing my career at GLSI. I would also like to thank GLSI for their financial support for the duration of this project. Thank you to Myron for agreeing to take me on as a student under these circumstances and always being there when I needed help. I would also like to thank Dr. Koji Hiratsuka and Dr. Battouli Said-Salim for their help and feedback throughout the duration of my research. I would also like to acknowledge the other members of the GLSI team. It was a pleasure working with you and thank you for all your help along the way. I would like to thank Dr. Yasunori Tanji from the Tokyo Institute of Technology, Japan for his generous donation of the vectors used for complementation assays in this research. Thank you to my loving wife Cara and my beautiful little girl Clare. You have been loving and supportive throughout the duration of my studies and I most certainly could not have done it without you by my side. I would also like to thank my family and Cara's family for their support and understanding over the last few years. Finally I would like to thank my friends for supporting me in my absence. I have often missed out on important events, but you have all been there for me whenever I needed you.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

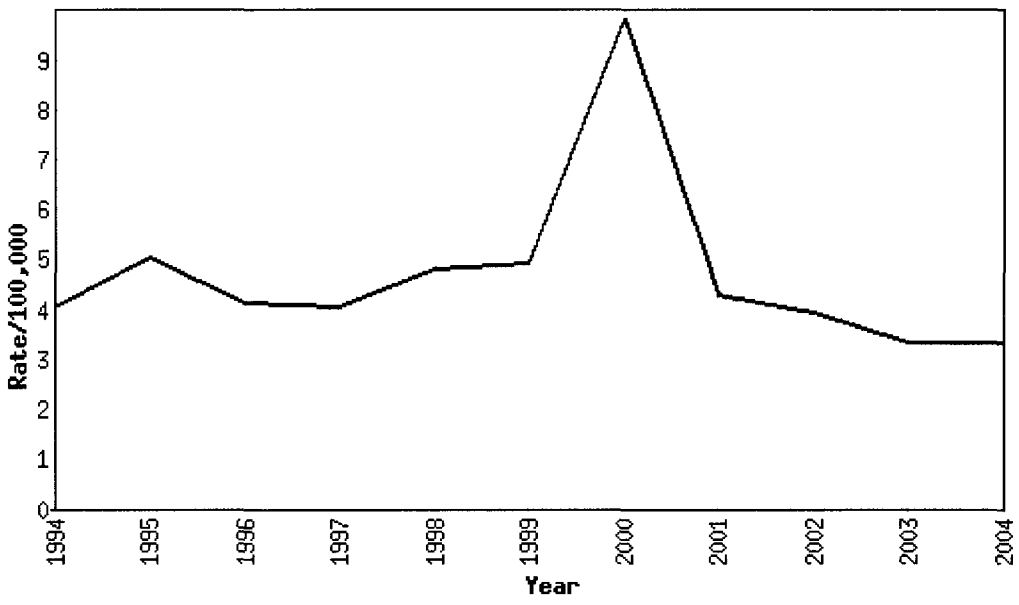
AP	alkaline phosphatase
ATP	adenosine triphosphate
BCIP	5-Bromo-4-chloro-3- indolyl phosphate
DIG	digoxigenin
dH_2O	distilled water
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
F	phenylalanine
Fig	Figure
FSIS	Food Safety and Inspection Service
G	glycine
Gal	galactose
Glc	glucose
GlcNAc	N-acetylglucosamine
GLSI	Gangagen Life Sciences Inc.
HC	haemorrhagic colitis
HCl	hydrochloric acid
Hep	L-glycero-D-manno-heptose
HUS	hemolytic uremic syndrome
IPTG	isopropyl-beta-D-thiogalactopyranoside
KCl	potassium chloride
Kdo	3-deoxy-D-manno-oct-2-ulosonic acid
L	leucine
LB	Luria-Bertani
LPS	lipopolysaccharide
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MOI	multiplicity of infection
NaOH	sodium hydroxide
$Na_3C_6H_5O_7 \cdot 2H_2O$	sodium citrate
NaCl	sodium chloride
NBT	nitro blue tetrazolium
$NH_4OH \cdot H_2O$	ammonium hydroxide
NIG	National Institute of Genetics
OmpC	outer membrane protein C
OD	optical density
Oligo	oligonucleotide
P	phosphoryl
PCR	polymerase chain reaction
PEtN	phosphoethanolamine
pfu	plaque forming units
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

SSC	saline sodium citrate
TBE	tris-HCl/boric acid/EDTA
TE	tris/EDTA buffer
tris-HCl	tris-hydrochloride
UV	ultraviolet
W	tryptophan
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

1. Introduction

Escherichia coli (E. coli) O157:H7 was first identified as a human pathogen in 1982 with two outbreaks of haemorrhagic colitis (HC) in Oregon and Michigan that were linked to hamburgers from the same fast food chain. It is however possible that the strain did exist for some time before 1982, but was never identified due to the lack of suitable detection systems (Riley, 1987; Law, 2000). Since its discovery, *E. coli* O157:H7 has been identified as a major cause of food and waterborne illnesses in the developed world (Armstrong *et al.*, 1996). The incidence of outbreaks has since increased dramatically and the origin of the infection has varied, with water and food sources being the most common causes of infections. In Canada the levels of *E. coli* O157:H7 infection has remained relatively stable with values of ~3.3/100,000 (1,048 cases) reported in 2006 (Public Health Agency of Canada, 2006; Statistics Canada, 2007). **Fig. 1** illustrates the incidence rates in Canada between 1994 and 2004. Aside from a spike in rates in the year 2000, which is the year of the Walkerton tragedy (Ontario, Canada), the levels of incidence of *E. coli* O157:H7 infection have remained relatively stable. In the United States, the levels of *E. coli* O157:H7 infection are 1.31 cases/100,000 and have been stable at that level for the last decade (Centers for Disease Control and Prevention, 2007). It should be noted in both cases that these rates reflect only confirmed cases of *E. coli* O157:H7 infection based on stool samples. It is believed that the actual rate of infection is ten times higher due to unreported cases. Many people will not go to the doctor if they have mild symptoms and therefore it is impossible to get an accurate estimation of the incidence of *E. coli* O157:H7 infection (Public Health Agency of Canada, 2003).

Figure 1: Rates of verified cases of *E. coli* O157:H7 in Canada per 100,000 people from 1994 – 2004.



	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004
Rate/100,000	4.05	5.05	4.14	4.08	4.84	4.96	9.81	4.30	3.96	3.35	3.36

(Generated by Centre for Infectious Disease Prevention and Control, Public Health Agency of Canada, 2005)

Outbreaks of *E. coli* O157:H7 are typically linked to contaminated meat products and to some extent to lettuce and other salad greens grown using manure from livestock as fertilizer. *E. coli* O157:H7 contamination of meat products is the leading cause of recalls in the food industry, sometimes with devastating consequences to the business. A recent example was in the fall of 2007 when Topps Meat Company, one of the United States of America's largest manufacturers of frozen hamburgers, recalled more than 21.7 million pounds of ground beef products in one of the largest meat recalls in recent years. The size of the recall caused the company to go out of business since meat processing was their main business (Belson and Fahim, 2007). The Food Safety and Inspection Service (FSIS) has reported that in 2004 only 0.17 % of samples tested in the USA were positive for *E. coli* O157:H7 which marks a significant decline from a rate of 0.86 % in 2000 (Becker, 2006). A possible explanation for the reduction in meat contaminations rates is due to the ability of meat producers to use the contaminated meat for the manufacture of fully cooked meat products of lower quality prepared in their factories prior to being sold to the consumer. Since proper cooking kills the pathogen, this is an acceptable practice and the producers do not have to report it to the regulators.

One reason *E. coli* O157:H7 is a major concern is that a relatively low number of cells are required for infection and the effects are relatively harsh. The dose of *E. coli* O157:H7 required to cause infection is known to be well below 100 organisms (Karch *et al.*, 2005; Chart, 2000). An *E. coli* O157:H7 infection generally causes diarrhea and cramps that leads to bloody diarrhea in 70-90 % of cases. This could further develop into HC which is characterized by severe inflammation and oedema of the colonic mucosa (Karch *et al.*, 2005; Chart, 2000). At this point, it is possible for the infection to develop

into a more serious condition, hemolytic uremic syndrome (HUS). HUS usually develops anywhere from five to thirteen days after the appearance of first symptoms and has been seen in about 15 % of infections involving children under the age of ten. This age group is most severely affected, with the elderly a close second (Karch *et al.*, 2005). Overall it has been shown that 10 % of patients develop HUS with 5 % developing chronic renal failure and 5 % ultimately dying (Chart, 2000).

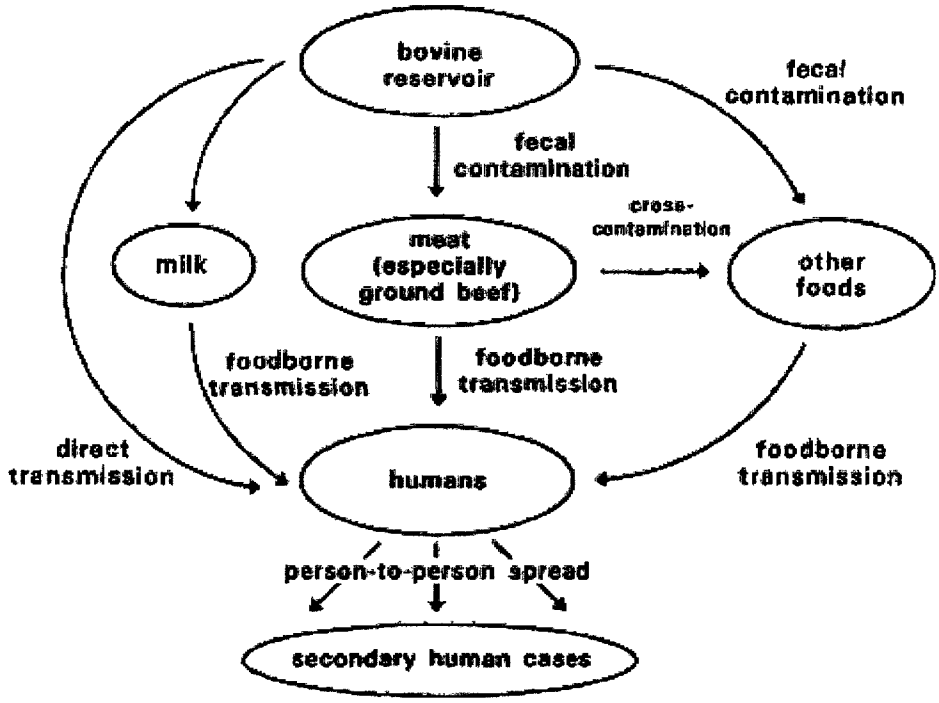
A key factor in understanding why this organism is such a major pathogen is its prevalence in nature. Most infections can be traced back to an animal source. *E. coli* O157:H7 is commonly found in the feces of healthy cattle (Armstrong *et al.*, 1996), and other animals such as sheep and pigs. The levels of *E. coli* O157:H7 in feces are highest in the summer; on the other hand, levels on animal hides peak from spring through fall (Barkocy-Gallagher *et al.*, 2003). The most important figures to consider for food safety are the incidence rates at the time of slaughter, where numbers as high as 27.8 % of animals infected with *E. coli* O157:H7 have been reported in the summer months (Hussein and Bollinger, 2005). This incidence frequency has to be viewed in the context of the total beef cattle slaughtered in North America alone, which was about 40 million head, of which Canada accounted for about 3 million head (2007 figures) (USDA NASS, 2008; Agriculture and Agri-Food Canada, 2008). The contamination potential of bovine fecal *E. coli* O157:H7-incidence alone is staggering.

Though contaminated meat is believed to be the largest source of *E. coli* O157:H7 contamination in the food supply, other animal sources include unpasteurized milk and cheese. Feces from infected animals can not only lead to contaminated meats and milk products, but can also cause contamination of vegetables due to the use of manure as

fertilizer. Feces also contaminate waterways and other surface water sources through runoffs from manure fertilizer used in the fields, which subsequently contaminates vegetables through irrigation. The infection risk is further magnified since many vegetables and fruits are consumed un-cooked (Armstrong *et al.*, 1996). Surprisingly, apple cider, which is also often unpasteurized, has been linked to outbreaks of *E. coli* O157:H7 in recent years (Armstrong *et al.*, 1996; Buchanan *et al.*, 1998). **Fig. 2** illustrates several possible modes of infection of *E. coli* O157:H7.

Some methods currently used to reduce *E. coli* O157:H7 contamination during processing include spot carcass decontamination (remove visible contaminants to meet FSIS zero-tolerance performance standards), thermal decontamination (high-pressure, hot-water rinse or pressurized steam), and irradiation of case-ready product (National Cattlemen's Beef Association, 2006). There are many researchers who believe that the best method for reducing the number of *E. coli* O157:H7 infections is to treat the *E. coli* O157:H7 at the source, i.e. treat the animals carrying the pathogen when the animals are in the feedlot before being shipped to the slaughter plant. By treating the animals that are reservoirs of *E. coli* O157:H7 one may reduce the load in the food supply as well as reduce the load of *E. coli* O157:H7 in the environment. Bacteriophage therapy has been one of the proposed methods for treating animals that may contain *E. coli* O157:H7 in their digestive tracts, potentially in combination with vaccines and antibiotic treatments (National Cattleman's Beef Association, 2006). Bacteriophage therapy is one of the preferred methods of treatment since phage are naturally abundant in animals and in the environment making it an environmentally friendly alternative (Hendrix *et al.*, 1999).

Figure 2: Modes of infection of *E. coli* O157:H7.



(Modified from Armstrong *et al.*, 1996)

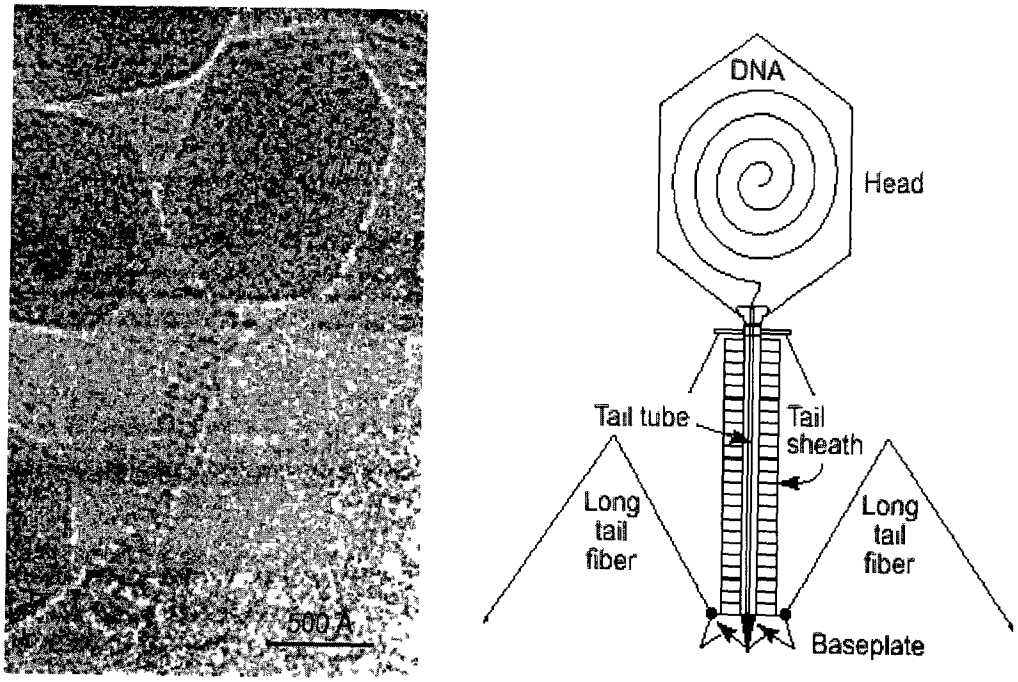
Some other benefits of bacteriophage, when compared to antibiotics, include the fact that bacteriophage self-replicate exponentially in the presence of the specific bacterium they target. They are also self-limiting. As the amount of bacteria in the system decreases, the number of phage in the system decreases. In addition to their inability to multiply in the absence of an appropriate host bacterium, environmental conditions play a major role in controlling phage numbers, with phage being sensitive to extreme heat, cold and UV radiation. Another advantage of phage-based therapeutics is that a number of different phage for any given pathogen can be found in nature making it a technology that can address the issue of development of resistance very effectively. Finally, unlike antibiotics which often cause bacterial imbalance as they do not target specific bacteria and frequently lead to secondary bacterial infections, bacteriophage target a species or strain of bacteria causing minimal disruption to the natural flora (Lorch, 1999). This last advantage means that it is possible to develop a phage product that will reduce the numbers of *E. coli* O157:H7 in the intestinal tract of the treated animal without affecting the natural flora of the intestinal tract and thus have few side effects on the treated animal (Babalova *et al.*, 1968; Mandeville *et al.*, 2003; Tanji *et al.*, 2004). An added benefit of this approach is that by reducing the pathogen load in the animal's feces, one would effectively reduce environmental contamination and ultimately human infection rates.

Hankin provided the first recorded evidence of heat labile, filterable particles with bactericidal properties in 1896. He noted that the waters of the Ganges River in India had antiseptic properties against many different organisms. At the time, he did not understand the concept of bacteriophage. He concluded that it was a heat labile chemical entity that gave the water its antiseptic properties (Hankin 1896). Bacteriophage were discovered

independently by Twort in 1915 and Felix d'Herelle in 1917 (Twort, 1915; d'Herelle, 1917). Bacteriophage are natural parasites of bacteria. They do not possess the required machinery to generate energy or produce proteins on their own. Instead, they contain the genetic material required to direct their own replication within an appropriate host. Bacteriophage typically target a subset of bacteria within a species; however, it is possible that several related bacterial species could be an appropriate host for a given phage strain.

Bacteriophage are found readily in nature and are one of the most abundant life forms. They are usually concentrated near their host but have been shown to maintain infectivity for decades in the absence of an appropriate host (Babalova *et al.*, 1968; Kutter and Sulakvelidxe, 2005). Bacteriophage have developed various approaches to infect host bacteria. Unlike many other viruses, bacteriophage remain attached to the cell surface throughout the duration of the infection process. Of the known bacteriophage, over 95% are tailed bacteriophage, representing the order Caudovirales. These bacteriophage are further divided into the families *Myoviridae*, *Siphoviridae*, and *Podoviridae*. Bacteriophage belonging to these three families have contractile, long non-contractile and short non-contractile tails, respectively. While bacteriophage tails from all the families are complicated structures, the *Myoviridae* family uses a contractile tail that is especially elaborate (Kutter and Sulakvelidxe, 2005; Rossmann *et al.*, 2004). **Fig. 3** illustrates a typical *Myoviridae* bacteriophage showing the tail structure. As illustrated in this figure, the tail is attached to a proteinaceous capsid that contains the phage genome. The tail is used for host recognition and attachment, and for delivery of the phage genome into the host cell.

Figure 3: Schematic representation of *Myoviridae* bacteriophage (right) based on electron micrograph (left).

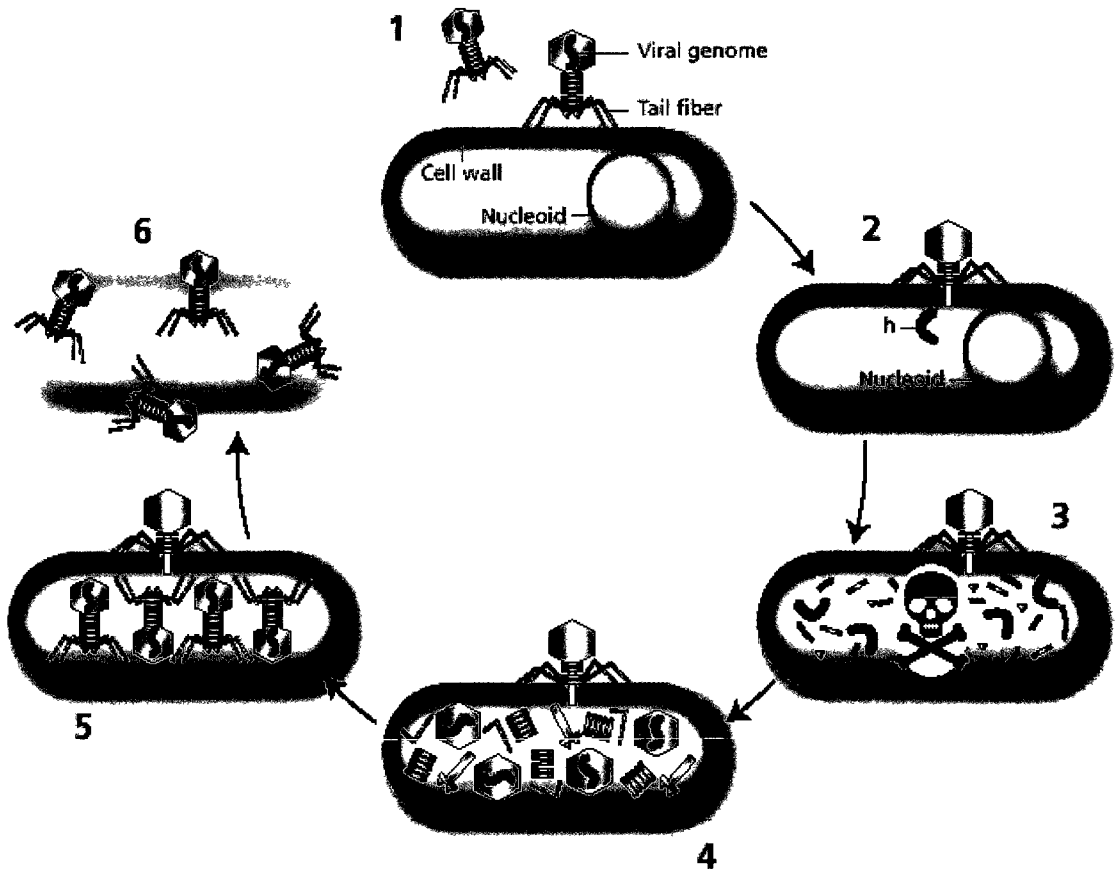


(Modified from Rossmann *et al.*, 2004)

The two types of bacteriophage studied in this research project were similar to T4 of the *Myoviridae* family and T1 of the *Siphoviridae* family. Bacteriophage T4 infects *E. coli* and is one of the most well studied bacteriophage. The diversity and success of the bacteriophage in the T4-like family can be traced back to its efficiency of infection and the fact that only half the genes present in the T4 genome are required for reproducing daughter bacteriophage in the *E. coli* host (Rossmann *et al.*, 2004; Leiman *et al.*, 2003). The capsid contains double-stranded DNA that has 274 open reading frames of which at least 40 encode structural proteins. During infection the long tail fibers attach the phage particle to the cell surface and undergo a change from the 'hexagonal' to the 'star' conformation. This conformational change causes the tail tube to penetrate through the cell envelope, which results in the transfer of the bacteriophage genome into the cell (Rossmann *et al.*, 2004; Leiman *et al.*, 2003). Unlike other viruses, bacteriophage T4 is extremely efficient at infecting host cells. Only a single T4 particle is required to infect a bacterial cell. Upon infection, the bacteriophage interferes with host-specific functions such as nucleic acid and protein synthesis. This ensures that all host resources will be diverted to the production of phage components, finally resulting in the production of as many as 100 bacteriophage progeny within a single host cell. Following the production of phage particles, the host cell is lysed and the new phage are released ready to infect other host cells. In T4 DNA, cytosine residues are replaced with 5-hydroxymethylcytosine. This modification protects the phage DNA from many host restriction enzymes during the infection process (Rossmann *et al.*, 2004; Leiman *et al.*, 2003; Calender, 1998). **Fig. 4** illustrates the typical life cycle of a lytic bacteriophage such as T4.

Figure 4: *Myoviridae* bacteriophage life cycle.

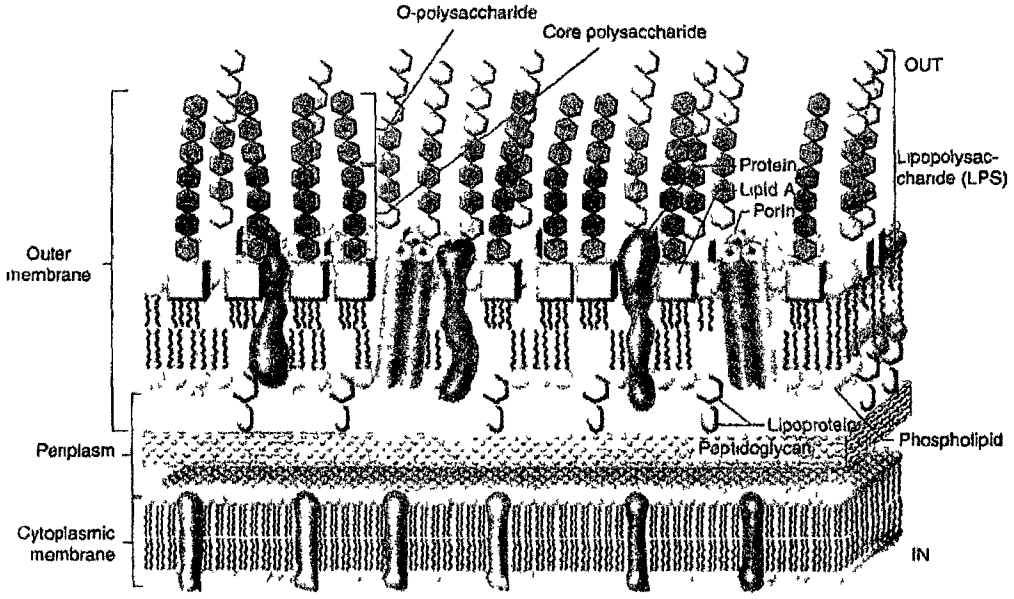
(**1**) Phage attaches to a specific host bacterium and (**2**) injects its DNA, (**3**) disrupting the bacterial genome and killing the bacterium, and (**4**) taking over the bacterial DNA and protein synthesis machinery to make phage parts. (**5**) The process culminates with the assembly of new phage and (**6**) the lysis of the bacterial cell wall to release a hundred new copies of the input phage into the environment.



(Modified from Thiel, 2004)

In contrast to T4, bacteriophage T1 is the least understood of the T bacteriophage. Delbruck first characterized T1 in 1942. It carries double stranded DNA in a polyhedral head and has a noncontractile flexible tail. Phage T1 adsorbs to the host receptor in two stages. The first stage involves a reversible binding, followed by an energy requiring irreversible binding. The DNA is then passed into the host cell through the tail fiber. T1 does not have modified DNA and is sensitive to many host restriction enzymes (Calender, 1988). **Fig. 5** illustrates the membrane structure of gram-negative bacteria with components that can be used as receptor sites for bacteriophage. These include the lipopolysaccharide (LPS), which is broken down into the core polysaccharide and the O-polysaccharide and cell surface proteins, such as porins, that are often associated with bacteriophage receptor sites. LPS and outer membrane protein C (OmpC) have been identified as receptor sites on *E. coli* K-12 for bacteriophage T4. It is believed that the presence of both LPS and OmpC are required for bacteriophage T4 binding. The two component nature of this phage-receptor system confounds the receptor site and is an example of why it is difficult to determine the receptor sites in the more complex phage-receptor systems. When a receptor site is identified, it is unclear whether this is the only receptor site and what the exact role of the identified molecule plays in phage infection (Yu and Mizushima 1982; Henning and Jann, 1979). For bacteriophage T1 of the *Siphoviridae* family, FhuA, an outer membrane protein required for the uptake of ferrichrome into the cell, has been identified as a receptor site on *E. coli* K-12. TonB is an internal membrane protein of *E. coli* that interacts with FhuA to open the ferrichrome channel. Both proteins are required for T1 infection of *E. coli* K-12 (Killmann *et al.*, 2001; Langenscheid *et al.*, 2004).

Figure 5: Membrane structure of gram-negative bacteria.



(Modified from Kutter and Sulakvelidxe, 2005)

Gangagen Life Sciences Inc. (GLSI), a biotechnology company in Ottawa, was established to develop phage-based food safety products. The approach taken by this company was to isolate and characterize natural bacteriophage in order to develop a product to reduce human pathogens entering the food chain by treating animals at source prior to slaughter (Thiel, 2004). A bacteriophage product under development was shown to be effective at reducing the *E. coli* O157:H7 load in beef cattle with an oral dose of the preparation (data unpublished). The selected phage were also shown to have a different host range profile using *E. coli* O157:H7 field isolates, suggesting that they targeted different receptor sites on the bacteria. As exemplified by antibiotic resistance in pathogenic bacteria, mutations in the bacterial genome are a concern in any anti-bacterial treatment strategy, and therefore it is generally held that a phage cocktail is required to effectively combat *E. coli* O157:H7 and other bacterial pathogens (Mandeville *et al.*, 2003; Tanji *et al.*, 2004; Hermos *et al.*, 2007). Knowledge of the receptor sites will help develop the most effective phage cocktail for reducing *E. coli* O157:H7, a major pathogen in the food supply.

In this thesis the hypothesis that the phage cocktail developed at GLSI as an anti *E. coli* O157:H7 product contained phage that recognize different receptor sites on the surface of *E. coli* O157:H7 bacteria is tested. First, naturally occurring *E. coli* O157:H7 mutants that were resistant to infection by R26 (a T1-like phage) and P39 (a T4-like phage) were isolated. These *E. coli* mutants were further characterized by examining the ability of different bacteriophage in a library to adsorb to these strains. All bacterial isolates used in this study were drawn from the above mentioned panel of resistors. OmpC has been identified as a potential receptor site on *E. coli* for T4 bacteriophage.

Since one of the bacteriophage was similar to T4 as determined by sequence analysis of the phage genome, the *ompC* gene in resistors was analyzed for potential mutations. This was achieved by PCR and Southern blot analysis followed by confirmation of the mutations by DNA sequencing. Complementation studies were carried out to confirm that OmpC was required for binding by the T4-like phage. LPS profiles were also used to characterize the mutants. To determine if changes in the LPS profiles were due to mutations that altered the function of enzymes in the LPS production pathway appropriate genes from selected isolates were amplified by PCR and evaluated by DNA sequence analysis.

2. Materials and Methods

Experimental protocols, bacterial strains, bacteriophage and oligonucleotide primer sequences were obtained from GLSI unless otherwise noted.

Materials:

All chemicals used in these studies were of analytical grade and obtained from Fisher Scientific (Ottawa, Ontario), VWR (Mississauga, Ontario) or Sigma-Aldrich (Mississauga, Ontario). All oligonucleotide primers were synthesized by Sigma-Aldrich (Mississauga, Ontario).

2.1 Bacterial Strains and Bacteriophage

E. coli O157:H7 strains and bacteriophage used for these studies are listed in **Table 1**. Bacterial strains were maintained on Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl). LB agar plates were prepared by adding agar to LB broth to a final concentration of 1.5%. Bacterial cultures were routinely grown at 37°C overnight. Bacteriophage were propagated by infecting the host bacteria at room temperature followed by incubation at 37°C. All microbial manipulations were done inside a BK2 Biological Safety Cabinet from Microzone.

2.2 Generation of Bacteriophage Resistant Mutants

A culture of the desired host bacterial strain was prepared by inoculating 3 mL of LB broth with an isolated bacterial colony and incubated with shaking at 200 rpm and 37°C for 3 hrs. For generating P39 (a T4 like phage)-resistant mutants, 500 µL of a 3 hour culture (OD_{600}=1.0) of the desired bacterial strain was mixed with the target bacteriophage at a multiplicity of infection (MOI) of 1.0 and incubated in 10 mL of LB broth overnight at 37°C with shaking at 200 rpm.

Table 1: *E. coli* O157:H7 strains and bacteriophage used in this study.

Host Serotype	Name	Source
E. coli O157:H7	Ec2	GLSI
E. coli O157:H7	Ec4	GLSI
E. coli K-12	ATCC 31608	ATCC
E. coli K-12	RK4784	NIG Collection
E. coli O157:H7 Bacteriophage	P39	GLSI
E. coli O157:H7 Bacteriophage	R26	GLSI
E. coli K-12 Bacteriophage	T4	GLSI

The following day, the overnight culture was centrifuged at 2,000 g in a GH-3.8 swinging bucket rotor with an Allegra 6R centrifuge. The supernatant was discarded and the resulting pellet was streaked out to obtain isolated colonies of the surviving bacteria (representing possible resistor mutants). Ten streaks on LB agar plates using a 1 µl loop were prepared for each culture and incubated overnight at 37°C. Isolated colonies from the streaked plates were further tested for phage sensitivity.

2.3 Bacteriophage Sensitivity Assay

Sensitivity of the mutants generated as outlined above was tested both qualitatively (streak method) and quantitatively (plate method), as described below.

2.3.1 Qualitative Assay (Streak Method)

Five µL of phage with a titer of between 1 x 10^8 pfu/ml and 1 x 10^9 pfu/mL was applied to the surface of the agar near the top and bottom edges of the plate. A sterile 1 µL inoculating loop was used to spread phage evenly across the plate in a line bisecting the plate and the liquid was allowed to absorb into the agar. A single bacterial colony was suspended in 100 µL of LB broth in a 1.5 mL microfuge tube and using a 1 µL loop, the suspended cells was streaked perpendicular to the dried phage in a single stroke. The plates were incubated at 37°C overnight. Sensitive cultures show an interruption of bacterial growth where the phage streak intersects the bacterial streak whereas resistant cultures exhibit continuous growth with no interruptions.

2.3.2 Quantitative Assay (Plate Method)

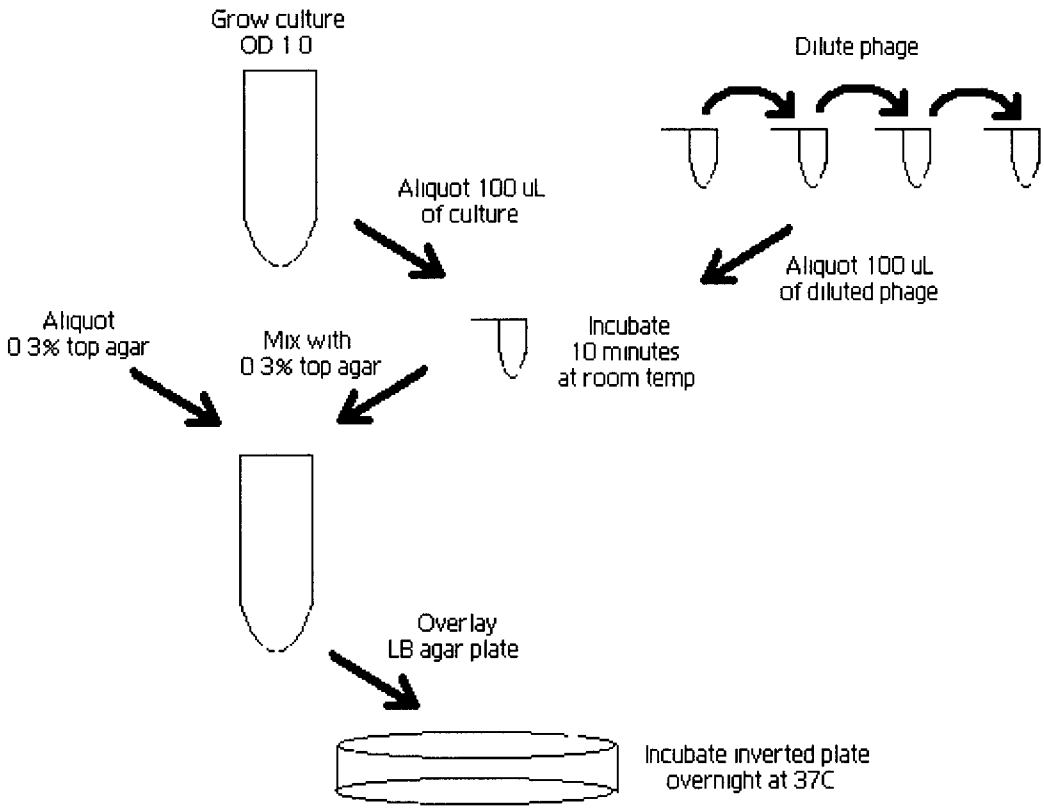
A 3 hour culture (OD_{600} ~1.0) was prepared by inoculating 3 ml LB broth with an isolated bacterial colony and incubating at 37°C with shaking at 200 rpm. Inside the biological safely cabinet, 100 µL of the bacterial culture was aliquoted into pre-labelled

microfuge tubes. One hundred μL of LB broth was added to one tube as a negative control. Phage of interest were serially diluted in a range that would give countable number of plaques when plated on the host bacterium and stored at room temperature until use. One hundred μL of each phage dilution was added to bacterial suspensions in microfuge tubes and incubated at room temperature for 10 minutes. The bacteria/phage mixture was then added to 2.5 ml top agarose (0.3% agarose in LB broth) at 50 °C, thoroughly mixed, and spread on agar plates, immediately rotating the plates gently to give a uniform overlay. The plates were left at room temperature on a level surface to ensure the agar had set and then inverted, and incubated overnight at 37°C. This protocol is illustrated in **Fig. 6**. The following day, plaque forming units (pfu) on the plates were counted and recorded. The average concentration of phage in the original stock was calculated by multiplying the pfu by the dilution factor and then dividing by the volume plated to obtain pfu/mL.

2.4 Serology

Serological confirmation of the host bacterial strains was done using a latex agglutination kit from Oxoid (DR0620) following the manufacturer's protocols. In brief, latex beads coated with antibodies specific to the O157 antigen were mixed with bacterial suspensions for one minute. If the specific O antigen is present on the bacterial cell surface, latex bead mediated agglutination of cells occurs giving a precipitate (Oxoid, Catalog # DR0620).

Figure 6: Quantitative assay (Plate method).



2.5 Polymerase Chain Reaction (PCR)

Bacterial genomic DNA for PCR was isolated using the Bio-Rad InstaGene Matrix Kit following the manufacturer's protocols (Bio-Rad, LIT544 Rev G). The primer sequences used in this study are presented in **Table 2**. PCR reactions were performed in an Applied Biosystems Gene Amp PCR System 2700 or Eppendorf Master Cycler EP using Taq DNA polymerase (New England BioLabs) according to established protocols. The following PCR conditions were used: initial denaturation for 5 minutes at 94°C followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at the desired temperature for 30 seconds and extension for 1 minute/kb product at 72°C followed by a final extension of 10 min at 72°C. The PCR reaction was mixed with DNA loading dye [0.025% (w/v) bromophenol blue in 0.5X TBE, 50% (v/v) glycerol], loaded on an agarose gel, and subjected to electrophoresis. The PCR product was visualized by staining the gel with ethidium bromide and viewing at 254 nm over a UV light box.

Table 2: List of oligonucleotide primers used for PCR amplification and sequencing.

Oligo Name	Sequence (5' to 3')	Bases	Target Gene
O157F	CGGACATCCATGTGATATGG	20	*rfbE*$_{O157}$ (1)
O157R	TTGCCTATGTACAGCTAATCC	21	*rfbE*$_{O157}$ (1)
Stx1F	ATAAATCGCCATTCGTTGACTAC	23	*stx*$_1$ (1)
Stx1R	AGAACGCCCACTGAGATCATC	21	*stx*$_1$ (1)
Stx2F	GCAATGTGCTTCCGGAGTATC	21	*stx*$_2$ (2)
Stx2R	CCTGTCGCCAGTTATCTGAC	20	*stx*$_2$ (2)
HlyAF	GCATCATCAAGCGTACGTTCC	21	*hlyA* (1)
HlyAR	AATGAGCCAAGCTGGTTAAGCT	22	*hlyA* (1)
EaeAF	GACCCGGCACAAGCATAAGC	20	*eaeA* (1)
EaeAR	CCACCTGCAGCAACAAGAGG	20	*eaeA* (1)
Flic H7F	GCGCTGTCGAGTTCTATCGAGC	22	*fliC*$_{H7}$ (3)
Flic H7R	CAACGGTGACTTTATCGCCATTCC	24	*fliC*$_{H7}$ (3)
Oc5end	AAAAGGATCCATGAAAGTTAAAGTACTGTCC	32	*ompC* (4)
Oc3end	TTAGAACTGGTAAACCAGACCCAG	24	*ompC* (4)
OMPCintF	TTACGACCATTGATGACACCC	21	*ompC* (2)
OMPCintR	AGGTTACTGACCAGCTGACC	20	*ompC* (2)
OMPCextF	GAACGGTAACTTCAGTAGCG	20	*ompC* (2)
OMPCextR	GATGTTAGGTGCTTATTTCGC	21	*ompC* (2)
OMPCext2F	GGTGCAATCACCGTCACGG	19	*ompC* (2)
OMPCext2R	CAATGTTGTCGTAGATCTGCC	21	*ompC* (2)
OMPCR1F	CACCACCGAATTCTGGCAG	19	*ompC* (2)
OMPCR1R	CTGCCAGAATTCGGTGGTG	19	*ompC* (2)
OMPCIntFR	CTGGGTGTCATCAATGGTCG	20	*ompC* (2)
OMPCintRF	GGTCAGCTGGTCAGTAACC	19	*ompC* (2)
Oc5endF	ACTTTAACTTTCATGTTATTAACC	24	*ompC* (2)
Oc3endR	GGTTTACCAGTTCTAATCTCG	21	*ompC* (2)
OMPCext3R	CTCAAGGCGGAAACTGTCC	19	*ompC* (2)
OMPCext4R	GCGGATCTCATACAGCTCG	19	*ompC* (2)
OMPCext5R	ATTGCGTTGAGGTCACGGC	19	*ompC* (2)
NewExt2SeqF	GGGTGTAATGGTACAAAGCG	20	*ompC* (2)
NewExt2SeqR	GCCAAAGATTAACGCTTCCG	20	*ompC* (2)
NewExt4SeqR	TCTCATACTGACGCTGCGC	19	*ompC* (2)

Oligo Name	Sequence (5' to 3')	Bases	Target Gene
rfaFF	GCGACGCATAAGAGCTCTGC	20	*rfaF* (2)
rfaFR	GCAACGTATGGAGAACATCGC	21	*rfaF* (2)
rfaCF	GCTATTGTTACAAGAGGAAGCC	22	*rfaC* (2)
rfaCR	TGTTGAGGTCATCTTATCTCCG	22	*rfaC* (2)
waaYF	CTGGTAGTTCTCATCAATACCC	22	*waaY* (2)
waaYR	GCAATCAGTATCGGTATTGTGC	22	*waaY* (2)
waaPF	GGTTACAACGATTTTCGAATCG	22	*waaP* (2)
waaPR	TACAGTCTGCCAGAGAAAGCG	21	*waaP* (2)
kdtAF	ATGCTCGAATTGCTTTACACCG	22	*kdtA* (2)
kdtAR	AACAACCTCAATGCGTTTTCGG	22	*kdtA* (2)

(1) Primers were based on Paton and Paton, 1998
(2) Designed as part of this research from sequence by Makino *et al.*, 1999
(3) Primers were based on Gannon *et al.*, 1997
(4) Primers were based on Yu *et al.*, 1998
(5) Primers were based on Morita *et al.*, 2002

2.6 Lipopolysaccharide Profile

The LPS profile was obtained by the protocol of Chart *et al.*, 1989 with slight modifications as follows. From an overnight culture of the desired bacterial culture (OD_{600} ~ 2.0), 100 µl was centrifuged for 5 minutes at 13,000 g. The resulting pellet was resuspended in 100 µL of sample buffer [0.625 M tris-HCl (pH 6.8), 25% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, 5% (v/v) ß-mercaptoethanol]. This preparation was boiled for 10 minutes. The solution was then centrifuged for 30 seconds at 13,000 g. One hundred µL of fresh Proteinase K [0.1% (w/v) in 0.05 M tris-HCl] was added and the preparation incubated for 1 hour at 60°C. A 10 µL aliquot of the digest was subjected to discontinuous SDS-PAGE using 7.5 % stacking and 12 % separating gels, and followed by visualization using silver stain, as described below.

The following solutions were used for staining:

1. Fixing solution: 42.5% (v/v) 95% ethanol, 5% (v/v) acetic acid.
2. Oxidizing solution: 42.5% (v/v) 95% ethanol, 5% (v/v) acetic acid, 0.7% (w/v) periodic acid.
3. Developing solution: 0.063% (v/v) 37% formaldehyde (prepared fresh daily) and, 0.0063% (w/v) citric acid.
4. Staining solution was prepared fresh daily as follows: In 10 ml of dH_2O, 280 µL of 0.5 M NaOH and 1 mL of NH_4OH were mixed. Silver nitrate (0.5 g) was dissolved in 2.5 mL of dH_2O in a separate container and added dropwise to the $NaOH/NH_4OH$ solution. The volume was then brought up to 75 ml with dH_2O.

Once the dye front ran out of the SDS-PAGE gel, the LPS bands were stained as follows. The gel was incubated overnight in fixing solution with gentle shaking. The next morning

the gel was soaked in oxidizing solution for 5 minutes with shaking and then washed 3 times in distilled water for 15 minutes each, with shaking. The LPS bands were then stained by gently agitating the gel in silver staining solution for 10 minutes. The unbound silver was removed from the gel by washing 3 times in distilled water for 10 minutes each with shaking. Silver bound to the LPS was detected by gently agitating the gel in developer solution until the bands could be visualized, usually within 5 minutes. The gel was then washed 3 times with distilled water to stop the reaction, soaked in 10% glycerol for 30 min, placed in a plastic frame between cellophane sheets, dried overnight in air and stored at RT.

2.7 Southern Blot Procedures

2.7.1 DNA Isolation and Electrophoresis

The required bacterial cultures were grown overnight in 3 mL LB broth at 200 rpm to an $OD_{600} \sim 2.0$. From the overnight culture, 2 mL was centrifuged for 2 minutes at 13,000 g to pellet the cells. The supernatant was removed and the cells were resuspended in 467 μL of TE buffer (50 mM tris-HCl (pH 8.0), 1 mM EDTA). Next, 30 μL of 10 % SDS and 3 μL of 20 mg/mL proteinase K were mixed with the samples and incubated for one hour at 56°C. One volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the sample and mixed gently. The sample was then centrifuged for 3 minutes at 13,000 g using a microcentrifuge. The aqueous layer was transferred to a new tube and extraction with phenol:chloroform:isoamyl alcohol was repeated one more time. The aqueous layers from the two extractions were mixed together, 1 volume of 24:1 chloroform:isoamyl alcohol was added and mixed gently. The sample was then centrifuged for three minutes at 13,000 g using a microcentrifuge. The aqueous layer was

transferred to a new tube and the extraction process repeated with another volume of 24:1 chloroform:isoamyl alcohol. The aqueous layers from the two extractions were mixed together before the addition of 1/10 volume of 3 M sodium acetate (pH 5.2) and then 0.6 volumes of isopropanol were added and mixed gently until the DNA began to precipitate. The DNA was spooled with a pipette tip, washed with 1 mL of ice cold 70% ethanol for 30 seconds to remove residual salts and briefly air dried. Finally the DNA was resuspended in 200 µL of TE and stored at –20°C. DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Five µg of DNA was digested with *Sal*I, *Eco*RI and *Hin*dIII according to manufacturer's protocols (New England BioLabs) and DNA was separated by electrophoresis on a 0.7% agarose gel. DNA fragments were visualized by staining with ethidium bromide.

2.7.2 Blotting

After agarose gel electrophoresis, the DNA was transferred to Immobilon-Ny+ membrane following the manufacturer's protocol (Millipore, 2005). In brief, the gel was soaked in depurination solution (0.25 N HCl) for 15 minutes and rinsed briefly in Milli-Q water. The gel was then soaked in denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 30 minutes and again rinsed with Milli-Q water. The gel was then incubated in neutralizing solution (1 M tris-HCl (pH 8.0), 1.5 M NaCl) for 30 minutes. A capillary blotting stack was prepared as outlined in the user guide. Briefly, a tray was filled with 20X SSC (3 M NaCl, 0.3 M Sodium citrate brought up to pH 7.0) and a raised support for the gel was placed in the middle. Two sheets of Whatman 3MM filter paper were soaked in 20X SSC and laid across the support touching the liquid in the tray. The gel was then placed onto the support with the wells facing up. Strips of parafilm were then placed around the gel to

ensure the flow of buffer did not go around the gel. The gel was then covered with the Immobilon-Ny+ Membrane. Three sheets of Whatman 3MM filter paper were soaked in 20X SSC and laid onto the membrane. Air bubbles were removed at each step of the assembly. A 10 cm stack of cut paper towels was placed onto of the stack. A piece of glass was placed on top of the paper towels followed by a 300 g weight, ensuring an even weight distribution across the membrane. The DNA was then allowed to transfer overnight. The next morning the stack was disassembled and the wells were marked on the back of the blot for orientation. The blot was then rinsed in 6X SSC to clean the blot and then placed on a sheet of filter paper to dry. Once dried, the blot was exposed to low wavelength (254 nm) UV light for one minute to fix the DNA to the membrane for subsequent detection.

2.7.3 Detection

The DNA fragment that was to be used as a probe was labeled using the Digoxigenin non-radioactive labeling system from Roche Diagnostics (Roche, 2004). Briefly, the *ompC* gene was amplified by PCR using Ec4 as template and Oc5end and Oc3end primers. The PCR product was gel purified using the QIAquick Gel Extraction Kit from Qiagen according to manufacturer's protocol (Qiagen, 2002). The purified PCR product was labeled using the Roche DIG DNA labeling and detection kit. The protocol outlined in the Roche kit was used for both labeling and detection. The labeling reaction was allowed to proceed overnight. Genomic DNA fragments on the blot were allowed to hybridize with the DIG labeled probe as per the instructions in the manual (Roche, 2004). Briefly, the membranes were pre-hybridized for thirty minutes using DIG Easy Hyb solution preheated to 50°C. The probe prepared as outlined earlier was denatured by

heating in boiled water for 5 minutes and mixed into pre-heated hybridization solution. The prehybridizing solution was removed and the hybridization solution containing the probe was added to the membrane and allowed to hybridize overnight. The next day the membrane was washed twice for five minutes each at room temperature in 2 X SSC, 0.1% SDS with agitation, followed by two washes of 15 minutes each with prewarmed 0.5X SSC, 0.1% SDS at 65 °C with agitation. After washing, the bound probe was detected using Anti-Digoxigenin antibody conjugated to alkaline phosphatase according to the protocol outlined in the manual (Roche, 2004). The detection procedure involved blocking the membrane with Roche blocking solution followed by detecting the labeled probe using Roche Anti-Digoxigenin-AP and NBT/BCIP as substrate. The reaction was stopped by washing for five minutes with distilled water. The membrane was dried for storage (Roche, 2004).

2.8 Sequencing

DNA fragments generated by PCR were purified using the Qiagen PCR purification kit following the manufacturer's protocols (Qiagen, 2002). These fragments were sequenced using appropriate *ompC*-specific primers which are listed in **Table 2**. Sequencing was done at the Ontario Genomics Innovation Centre (Ottawa, ON) using an ABI automated sequencer and Big Dye terminator chemistry.

2.9 Adsorption Assay

The adsorption assay was performed by the protocol of Morita *et al.*, 2002 with slight modifications as follows. An adsorption assay was used to evaluate the possibility that bacteriophage could bind to the bacterial isolates even though they were not able to lyse select strains of bacteria. The adsorption assay was accomplished by growing the

target strain in LB broth to OD_{600} ~1.0 and mixing 2 mL of bacteria with bacteriophage and the volume brought up to 10 mL with LB broth. For bacteriophage P39, $1X10^8$ pfu were used and for bacteriophage R26, $1X10^7$ pfu were used. The phage and bacteria were mixed and a 1 mL sample was taken and filtered immediately. The preparation was mixed and samples were taken and filtered at 5, 10, 15 and 20 minutes. All filtrates were diluted in LB broth and titered against Ec4 as described earlier in the phage sensitivity (2.3.2 quantitative assay).

2.10 Complementation Assays

2.10.1 Preparation of Electrocompetent Cells

Electrocompetant cells were prepared as outlined in the instruction manual for Bio-Rad Micro Pulser Electroporation apparatus (Bio-Rad, Catalogue # 165-2100). In brief, 100 mL of LB broth was inoculated with 1/100 volume of a fresh overnight culture of *E. coli* DH5α. The cells were grown at 37°C with shaking at 200 rpm to an OD_{600} of 0.5-0.7. The cells were chilled on ice for 20 minutes. All subsequent steps were carried out on ice. The cells were centrifuged at 4,000 g for 15 minutes at 4°C using a pre-cooled centrifuge bottle. The supernatant was discarded and the cells were gently resuspended in 100 mL of ice-cold 10% glycerol and centrifuged at 4,000 g for 15 minutes at 4°C. The supernatant was discarded and the cells were gently resuspended in 50 mL of ice-cold 10% glycerol and centrifuged at 4,000 g for 15 minutes at 4°C. The supernatant was discarded and the cells were gently resuspended in 5 mL of ice-cold 10% glycerol and then centrifuged at 4,000 g for 15 minutes at 4°C. The supernatant was discarded and the cells were now gently resuspended in 500 µL of ice-cold 10% glycerol. The cells were then diluted to 1-3 x 10^{10}cells/mL in 10% ice-cold glycerol and aliquoted in 50 µL

aliquots and stored frozen at -80 °C until used. The same procedure was used for preparing electrocompetent cells of RK4784 and Ec4P39-R3. The constructs used for complementation studies were generously provided by Dr. Yasunori Tanji (Tokyo Institute of Technology, Japan). The two constructs used here contained the *ompC* genes from *E. coli* O157:H7 and *E. coli* K-12 inserted into the pTV118N vector from Takara Bio Inc. (Shiga, Japan) under the control of the *lac* operon (Morita *et al.*, 2002). These plasmid constructs were amplified and purified using the QIAGEN Plasmid DNA Purification Midi Kit following the protocol outlined in the QIAGEN plasmid purification handbook (Qiagen, 2005). The purified constructs were quantified and verified by restriction digest to confirm the identity of the vector as well as the identity and orientation of the *ompC* insert.

2.10.2 Electroporation

Electroporation of electrocompetant cells was carried out using the Micro Pulser Electroporation System from Bio-Rad (Bio-Rad, Catalogue # 165-2100). The manufacturer suggested protocol was used. In brief, 50 µL of electrocompetant cells prepared as outlined above were thawed on ice, and mixed with 1 µL of DNA at 100 ng/µL and incubated on ice for 1 minute. The mixture was transferred to a chilled 0.2 mm cuvette and tapped to remove any air bubbles. The cuvette was then inserted into the chamber slide and electroporated for ~5.8 ms at 3.0 kV. The cuvette was removed and 1 mL of SOC (2% bacto tryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added immediately. The cells were gently resuspended by pipetting up and down, transferred to a 13 ml snap cap tube and incubated at 37°C for 1 hour with shaking at 200 rpm. Samples were then diluted and

plated on LB agar plates containing 100 μg/mL ampicillin. For ease of detecting clones carrying the desired vector, 100 μL of 100 mM IPTG and 20 μL of 50 mg/mL X-gal was spread on top of the agar plates before plating the electroporated cells. The plates were incubated overnight at 37°C. The next morning, the numbers of blue and white colonies were recorded and white colonies were screened by PCR for the presence of the vector.

2.10.3 Bacteriophage Sensitivity (Plate Method)

To evaluate the ability of the resistant clones to bind to the phage of interest in the presence of the OmpC vector, the bacteriophage sensitivity assay was carried out. The strains of bacteria with and without the *ompC* carrying constructs were tested against the two bacteriophage to determine if the addition of the *ompC* gene from *E. coli* K-12 or *E. coli* O157:H7 had any effect on the efficiency of plating for the two bacteriophage. A control with the vector alone was also included. The only changes to the plating method described earlier (section 2.3.2) were that the cultures containing the constructs or the vectors were grown in LB broth containing 100 μg/mL ampicillin and also plated on LB agar containing 100 μg/mL ampicillin. IPTG (1mM) was added to all cultures to induce transcription from the vector insert and ensure the *ompC* gene was expressed (Morita *et al.*, 2002).

3. Results

To develop an effective phage product and reduce the possibility of the development of resistance by the host it is important to characterize the receptors that candidate bacteriophage recognize on the bacterial surface and develop a cocktail using phage that recognize different receptor molecules. To determine potential receptor sites for two candidate bacteriophage to be used in the phage cocktail product, the approach used was to develop mutants resistant to each of the bacteriophage in the formulation and characterize them. Comparing the properties of the mutants with the native strain helped define the receptor molecule.

3.1 Development of Resistors

E. coli O157:H7 strain Ec4 was infected with either P39, a T4-like bacteriophage, or R26, a T1-like bacteriophage, and incubated overnight at 37°C with shaking. These overnight cultures were streaked on to LB plates to obtain isolated colonies.

3.1.1 Bacteriophage Sensitivity

Isolated mutants were initially tested qualitatively to determine if the strains were resistant to the target bacteriophage. To keep better track of the mutants generated, the isolated resistant strains were named as follows: parent strain, the phage used for generation of the mutant, and the isolate number. Example: Ec4P39-R1 would be a resistant isolate generated from *E. coli* O157:H7 strain Ec4 using phage P39. R at the end of the name represents the mutant number, in this case R1 representing isolate 1. The results obtained with mutants selected for this study are summarized in **Table 3**.

Table 3: Qualitative assay (Streak method) results for isolated mutants.

Ec4 mutants selected as resistant to phage P39 (MOI = 1.0)			**Ec4 mutants selected as resistant to phage R26 (MOI = 1.0)**		
	P39	**R26**		**P39**	**R26**
Ec4P39-R1	-	+	Ec4R26-R1	+	-
Ec4P39-R2	-	+	Ec4R26-R 2	+	-
Ec4P39-R3	-	+	Ec4R26-R 3	+	-
Ec4P39-R4	-	+	Ec4R26-R 4	+	-
Ec4P39-R5	-	+	Ec4R26-R 5	+	-
Ec4P39-R6	-	+	Ec4R26-R 6	+	-
Ec4P39-R7	-	+	Ec4R26-R 7	+	-
Ec4P39-R8	-	+	Ec4R26-R 8	+	-
Ec4P39-R9	-	+	Ec4R26-R 9	+	-
Ec4P39-R10	-	+	Ec4R26-R 10	+	-
Controls			Ec4R26-R 11	-	-
Ec4	+	+	Ec4R26-R 12	-	-
Ec2	+	-	**Controls**		
			Ec4	+	+
			Ec2	+	-

"+" Strain was sensitive to phage
"-" Strain was resistant to phage

For each phage and bacteria combination, 10 bacterial isolates that were resistant to the target phage and sensitive to the other phage were selected for further characterization to determine changes to key surface molecules. It should be noted that for the R26 and Ec4 combination, two additional colonies that were resistant to both R26 and P39 were selected for further characterization. **Fig. 7** illustrates an example of the qualitative assay. The top two plates show the streak test performed with phage P39 and ten bacterial isolates, Ec4P39-R1-R10. Isolate Ec4P39-R10 did not grow well and the streak test was replated in this case. P39 was streaked down the middle of the plate and the mutants were streaked perpendicular to assess their susceptibility to the phage. Ec2 and Ec4 were used as controls. Both Ec2 and Ec4 were sensitive to P39 and showed a disruption in the bacterial steak, while all the resistor isolates were resistant to P39 as indicated by a continuous streak. The bottom two plates represent a repeat of this assay with R26. In this case, Ec2 is resistant to R26 and Ec4 is sensitive along with all of the resistor isolates. Results obtained with the qualitative streak method were confirmed using the quantitative assay (plate method). The plate method helps quantify the interaction between the phage and the bacterial mutants. Results on the plating efficiency of bacteriophage P39 and R26 on Ec4 mutants are presented in **Figs. 8** and **9** respectively. As seen in **Fig. 8**, all 10 Ec4P39 mutants were resistant to phage P39 while Ec4R26 mutants 1-10 were sensitive to phage P39.The plating efficiencies of phage P39 with Ec4R26 mutants were comparable to that of the parent strain Ec4. However, in agreement with the streak assay results, Ec4R26-R11 and -R12 were resistant to phage P39. As shown in **Fig. 9**, phage R26 does not infect Ec4R26 mutants.

Figure 7: Example of qualitative assay (Streak method).

P39 or R26 phage were streaked onto the agar surface along the vertical line bisecting the plate. Subsequently, wildtype and P39-resistor mutants were streaked across plate from right to left. Interrupted growth of bacteria at and left of the line indicates susceptibility. Ec2 and Ec4 were the control samples. Mutants were labelled Ec4P39-R1-R10.

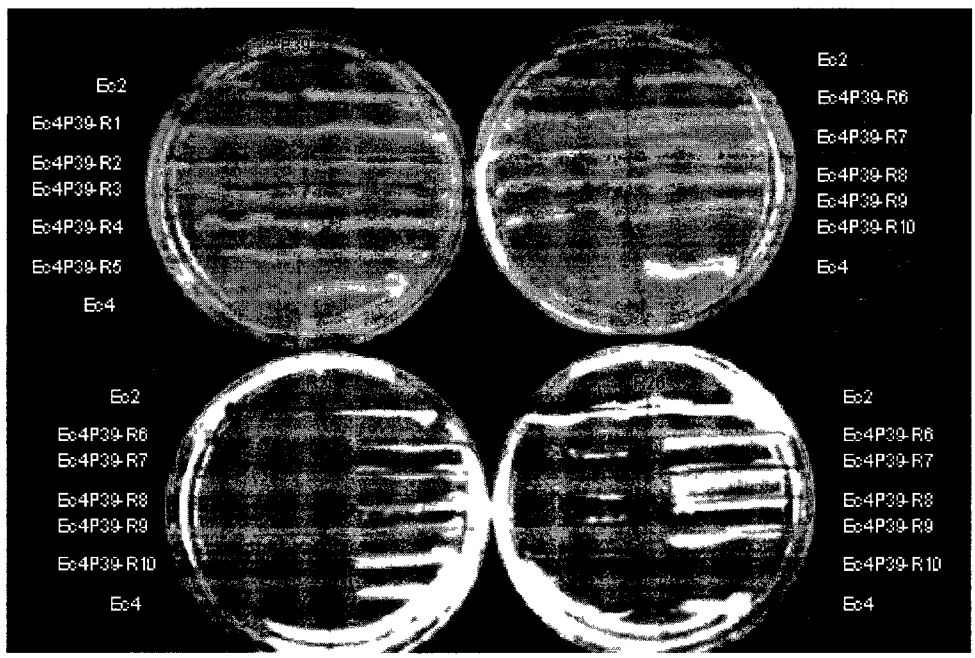


Figure 8: Quantitative assay of plating efficiency of isolated mutants with bacteriophage P39.

Error bars represent standard deviation (n=6). Two groups of isolates were observed, those that were resistant to P39 had a pfu/mL < 10 and those that were sensitive to P39, including the parent strain Ec4, had a pfu/mL > 1.8×10^9. The resistant strains include Ec4R26-R11, R12 and Ec4P39-R1-R10 and had significantly lower pfu/mL values than for the Ec4 (control) strain ($P < 4 \times 10^{-7}$ based on student t-test).

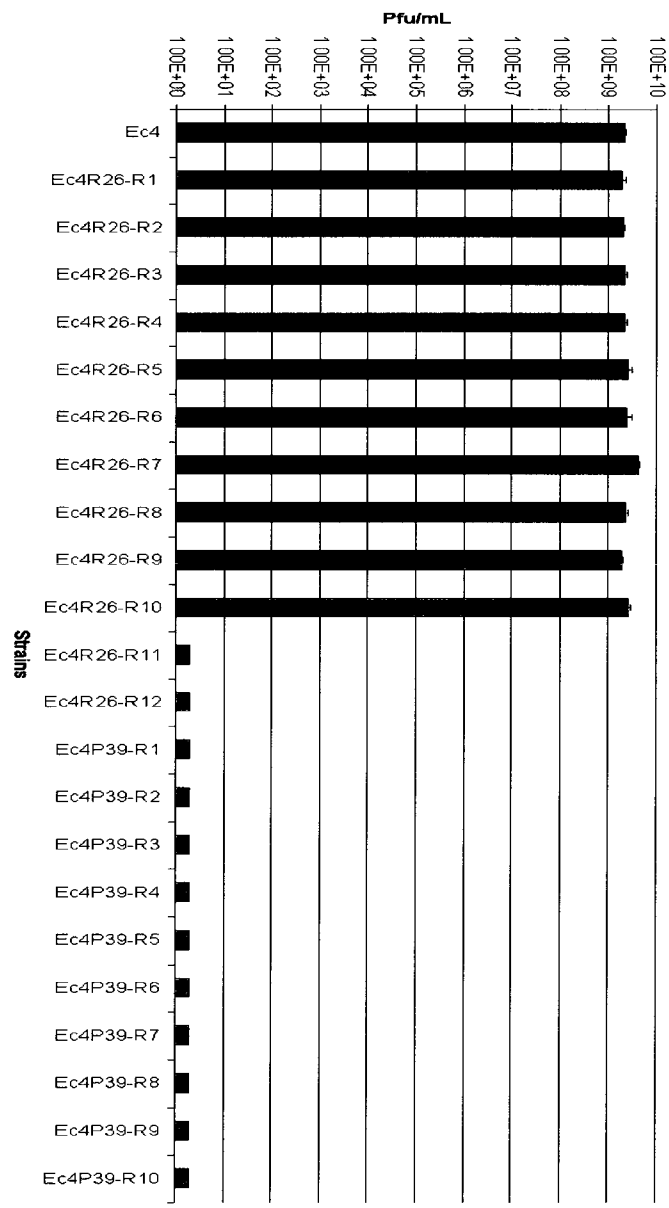
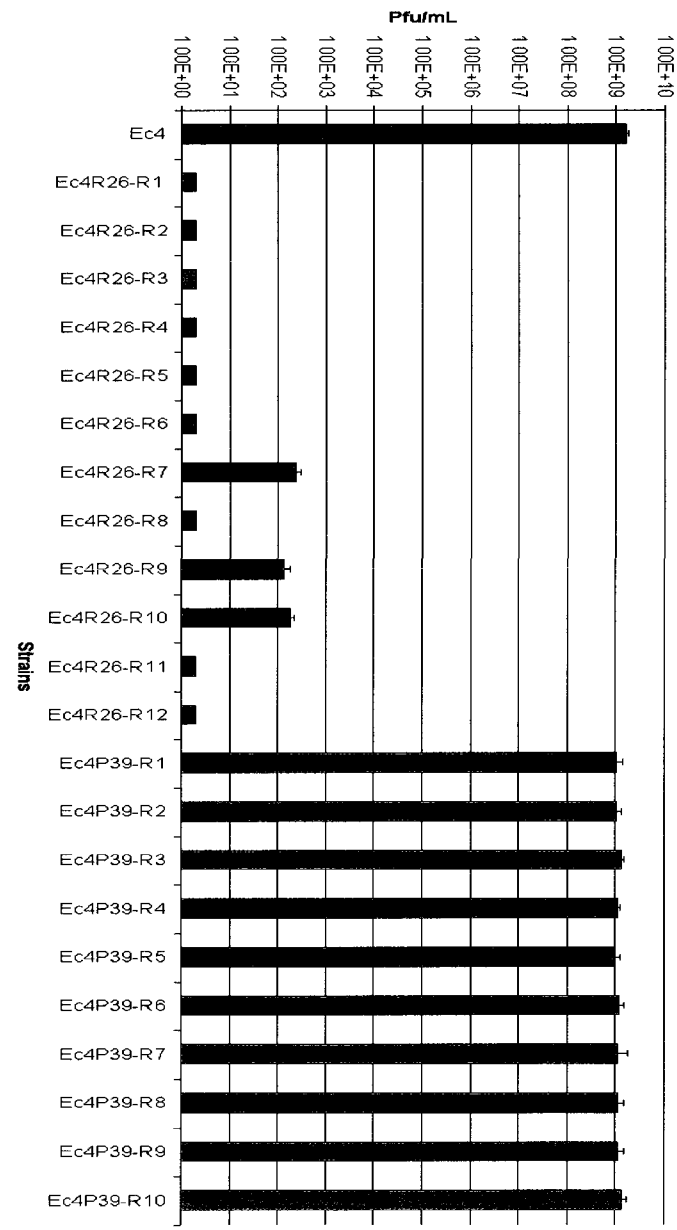


Figure 9: Quantitative assay of plating efficiency of isolated mutants with bacteriophage R26.

Error bars represent standard deviation (n=6). Two groups of isolates were observed, those that were resistant to R26 had a pfu/mL < 10^2 and those that were sensitive to R26, including the parent strain Ec4, had a pfu/mL > 1.0 x 10^8. The resistant strains include Ec4R26-R1-R12 and had significantly lower pfu/mL values than for the Ec4 (control) strain ($P < 8.2 \times 10^{-6}$ based on student t-test).



Three of the mutants (Ec4R26-R7, -R9 and -R10) yielded approximately 100 pfu/mL of R26 phage but are considered as resistant since these values are more than 7 logs lower than values with parent strain Ec4. The difference in plating efficiency of these three resistant strains as compared to the parent strain was shown to be significant using the student t-test ($P<8.2x10^{-6}$, in all cases). All 10 Ec4P39 mutants were sensitive to phage R26 at levels comparable to the Ec4 parental strain.

3.1.2 Confirmation that the Resistors were *E. coli* O157:H7

In order to ensure that the resistant strains were in fact *E. coli* O157:H7 and not contaminants, they were checked by serology and PCR. This was especially important for the two isolates, Ec4R26-R11 and -R12, which were resistant to both P39 and R26 phage used in the study. The presence of the O157 antigen was confirmed by agglutination assay with a monoclonal antibody to the O antigen bound to latex beads. A commercially available latex agglutination kit from Oxoid was used in these studies. Results obtained with this kit confirmed that 20 of the mutant strains listed in **Table 3** were positive for the O antigen. The two strains that did not react with either phage (Ec4R26-R11 and -R12) were however negative using this serology kit.

These results were further tested by PCR analysis. In addition to the *E. coli* O157:H7 gene, the presence of five additional virulence marker genes were also evaluated to support that the resistant strains contained the same virulence markers as their parent strain Ec4. The six virulence markers that were screened included genes for $rfbE_{O157}$, stx_1, stx_2, *hylA*, $fliC_{H7}$, and *eaeA*. Amplicons of the expected size were detected for all 22 mutants along with Ec2 and the Ec4 parental strain, indicating the presence of all six virulence markers in the mutants. The expected size PCR fragments for the genes,

based on published sequence, are summarized in **Table 4**. PCR results for all 22 strains tested and the controls with the above primers in two separate multiplex PCR and run on a 2% agarose gel are presented in **Fig. 10**. These PCR results indicate that all 22 mutants listed in **Table 3**, including Ec4R26-R11 and -R12, were indeed derived from Ec4 since these virulence genes are characteristic of *E. coli* O157. The lack of agglutination signal in serological tests with Ec4R26-R11 and -R12 indicates these strains may have severely altered cell surface composition.

3.1.3 Adsorption Assay

Once the sensitivity of the mutants to phage P39 and R26 was determined, an adsorption assay was done to test whether resistance to phage infection was due to lack of phage binding. A lack of binding would suggest that the phage binding site on the surface of a given bacterial strain was not intact or accessible (Kutter and Slakvelidze, 2005). The adsorption assay measured the number of plaque forming units in a filtered sample of a bacterial strain and phage that had been allowed to interact for 0, 5, 10, 15 and 20 minutes. The level of phage binding was expressed as a percentage of binding at time 0. If the phage adsorbs to the bacterial strain subsequent pfu values, measured with a susceptible host, would be reduced. If the phage does not adsorb to the bacterial strain, pfu in the filtrate would be similar to those of time 0.

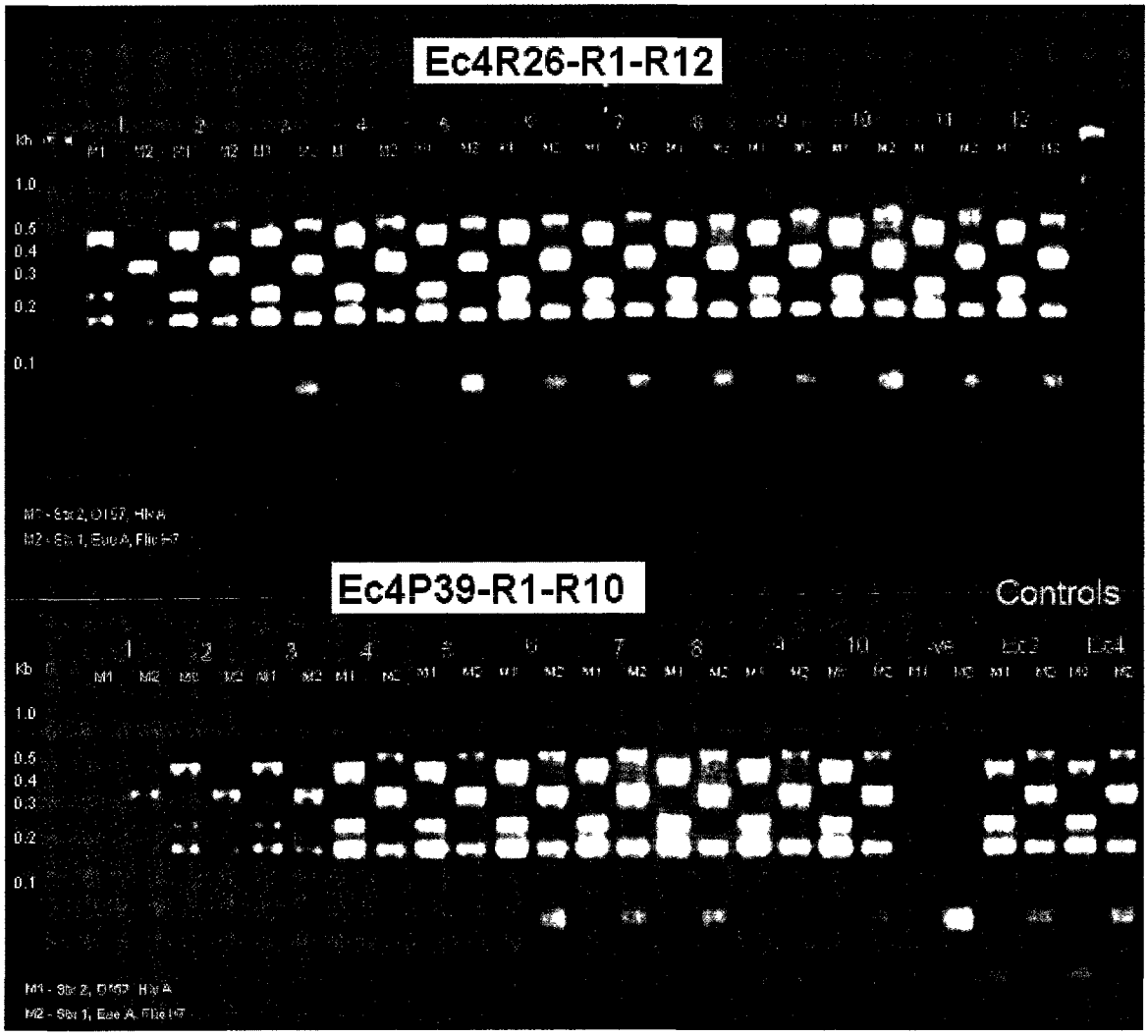
Seven resistant mutant strains were selected at random for the adsorption assays. The number of isolates screened at this stage was limited to identify trends in the groups due to the large resource demands for this assay.

Table 4: PCR product sizes for selected virulence markers.

Virulence Marker	Primers	Expected Fragment (bp)
rfbE$_{O157}$	O157F/O157R	259
stx$_1$	Stx1F/Stx1R	180
stx$_2$	Stx2F/ Stx2R	181
hylA	HlyAF/HlyAR	534
fliC$_{H7}$	Flic H7F/Flic H7R	625
eaeA	EaeAF/EaeAR	384

Figure 10: PCR results for mutants.

Ec4R26-R1 to –R12 and Ec4P39-R1 to –R10 mutants were assayed in two PCR reactions. Lanes are labelled according to the reaction – M1 = multiplex 1 (stx_2, $rfbE_{O157}$, and *hylA*), M2 = multiplex 2 (stx_1, *eaeA*, and $fliC_{H7}$), and the respective phage resistant mutant 1, 2, 3, etc. indicate -R1, -R2, -R3. Molecular size marker in kb is at the left in each panel.



These included three strains (Ec4P39-R1, -R3 and -R7) that were resistant to P39 and sensitive to R26, three strains (Ec4R26-R1, -R7 and -R8) that were resistant to R26 and sensitive to P39, and one strain (Ec4R26-R11) that was resistant to both P39 and R26. The positive binding controls included in this assay were Ec2 and the parent strain Ec4 (for P39) and Ec4 (for R26). These were included to demonstrate that typical phage adsorption levels were greater than ~90%. Negative controls included in this assay were an *E. coli* K-12 strain (ATCC31608, a non-host strain of P39 and R26) and an LB control which contained no bacteria. These negative control samples showed that >90% of phage (compared to time 0) were present in the filtrate over the duration of the 20 minute assay for P39 and >60% of phage (compared to time 0) for R26, and indicated that the phage was not adsorbing non-specifically to surfaces, including non-host bacteria, during the experiment. **Fig. 11** and **Fig. 12** illustrate the relative binding efficiencies for P39 and R26, respectively, to each bacterial strain, and the no bacteria control, as a function of time. From **Fig. 11**, it can be seen that the isolates that were resistant to P39 in the sensitivity assays (Ec4P39-R1, -R3, -R7, and Ec4R26-R11) resulted in a modest or no decrease in phage titer in the adsorption assays, similar to the negative controls. In contrast, incubating P39 and any of the five strains that were sensitive to P39 (Ec2, Ec4, Ec4R26-R1, -R7, and -R8) resulted in a decrease of >90% in the P39 titers, indicating that P39 efficiently binds to these strains at similar levels as with the positive controls. Evident from Fig. **12** is that R26 binds effectively to Ec4P39-R1, -R3 and -R7, and, interestingly, to R26-resistant strains Ec4R26-R1, -R7 and Ec2.

Figure 11: Adsorption assays of selected bacterial strains with P39.

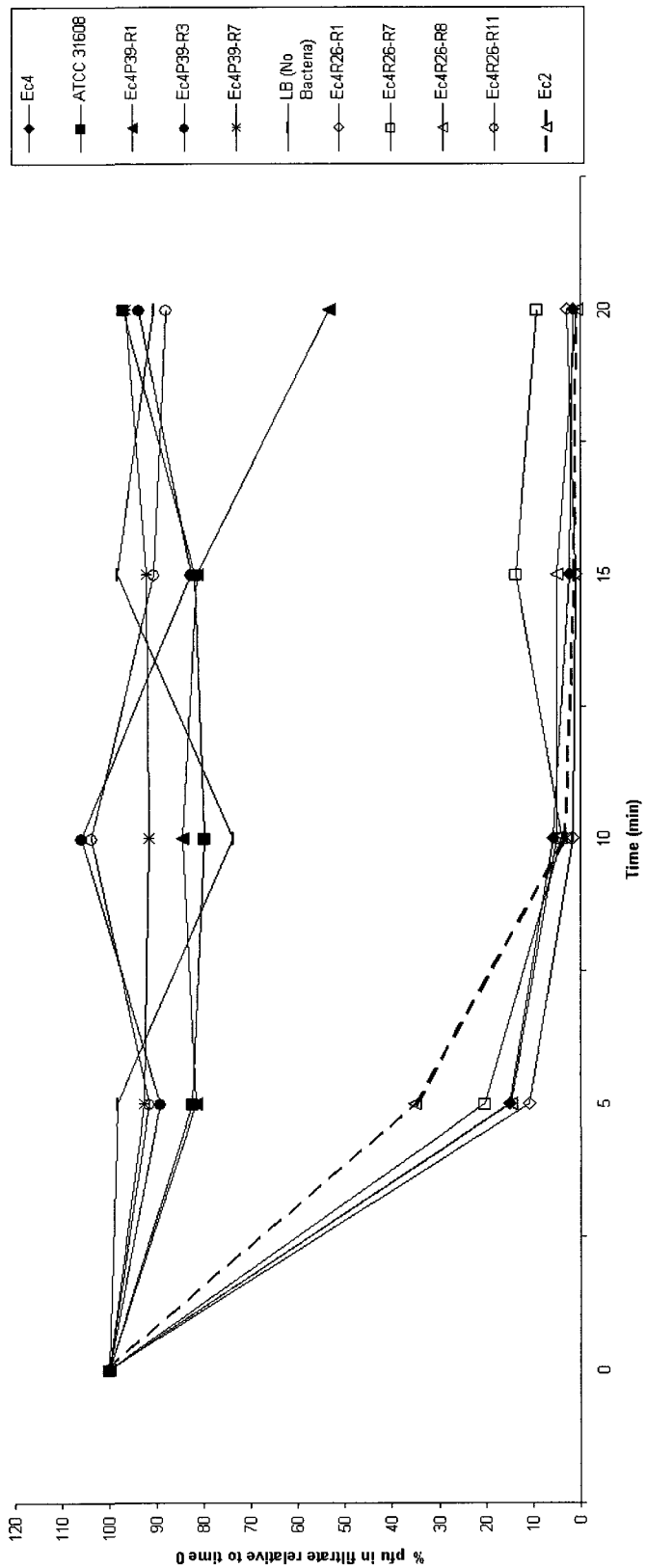
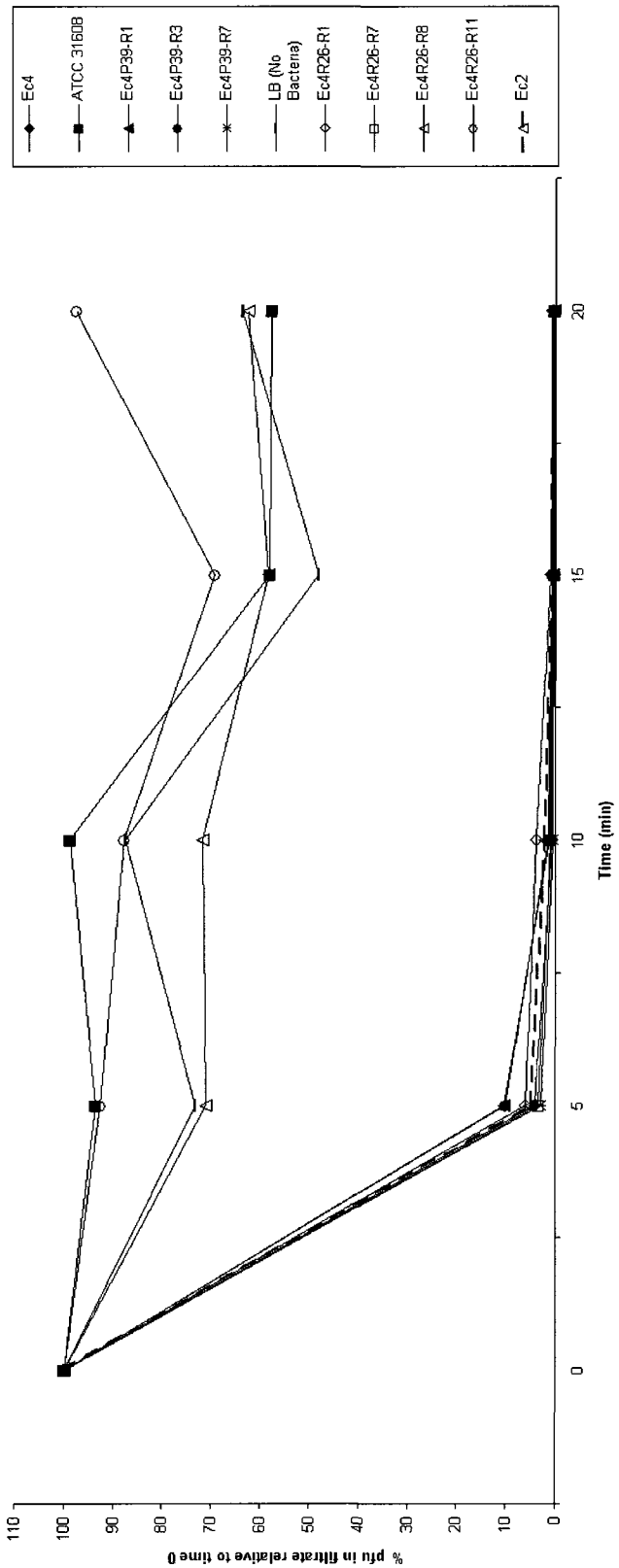


Figure 12: Adsorption assays of selected bacterial strains with R26.



The adsorption assay results with the above six bacterial strains were similar to those with the positive Ec4 control. Of the R26-resistant strains, only Ec4R26-R8 and -R11 did not adsorb R26 phage. The negative controls included in this assay were *E. coli* K-12 strain (ATCC31608), Ec2 (which has been shown to be non-susceptible to R26 infection, cf **Table 3**), and LB control containing no bacteria. *E. coli* K-12 and LB controls resulted in a modest or no decrease in phage titer over the 20 minutes of the assay. These samples were included for determining background adsorption levels and to set a baseline for samples where the phage was not being adsorbed. Interestingly, R26 does bind effectively to Ec2 as evident from the >99% reduction in R26 levels. Overall, the adsorption assays indicate that resistance to P39 of the Ec4P39-resistor strains is associated with a loss of a bacterial surface receptor or accessibility of the receptor to the target phage that results in reduced or no adsorption by the P39 phage. That R26 is able to bind to these Ec4 P39-resistor strains indicates that phage R26 recognizes a separate binding site than does P39. Nevertheless, some overlap in the P39 and R26 binding sites is evident since neither phage infects, nor adsorbs to, Ec4R26-R11. The situation with R26 resistance appears to be more complicated than for P39 resistance. The R26 adsorption assays suggest that at least two mechanisms are involved in resistance; one involves loss of phage binding and the other possibly involves the inability of the bacteriophage to infect the host cell. Similar to the P39-resistant mutants discussed above, Ec4R26-R8 and -R11 are resistant to, and do not allow binding of, phage R26. These two mutants presumably have altered surface receptors so that the phage does not recognize them as a suitable host. Ec4R26-R1 and -R7, along with the wildtype Ec2, are not susceptible to infection by R26 but still adsorb phage R26. This suggests that a

process downstream from adsorption might be altered in these bacterial strains. A possible explanation for this result would be if a surface protein were altered to prevent the phage from injecting its DNA. A similar observation was made for phage T1, where it was shown that proper interaction of T1 with *E. coli* K-12 was dependent on TonB. TonB is not the receptor site for the phage, but is required for the phage to properly infect the cell (Langenscheid et *al.*, 2004; Killmann *et al.*, 2001).

3.2 Characterization of R26 (T1 Like) Resistant Mutants

It is well known that bacteriophage infect bacterial cells by interacting with cell surface receptors. One potential receptor site is lipopolysaccharide (LPS). LPS has been identified as a receptor site for phage T4 as well as many other bacteriophage (Kutter and Slakvelidze, 2005; Yu and Mizushima, 1982). The LPS profile was obtained by initial digestion and release of the LPS from the membrane using Proteinase K. The LPS was then separated by SDS PAGE followed by visualization using silver stain. This procedure was followed for the R26-resistant mutants, the Ec4 parental strain used in this study and strain Ec2 as represented in **Fig. 13**. The bands across the top of the gels represent the O antigen and the lower bands represent the inner/outer core. Lanes 1 and 2 represent Ec2 and the parent strain, Ec4, to which the mutants were compared. Of the R26-resistant mutants only Ec4R26-R1, -R7, -R9 and -R10 matched the LPS patterns of the parent strain. The remaining mutants have truncated (varying lengths of O antigen missing) LPS patterns. In lanes 13 and 14, the two mutants Ec4R26-R11 and -R12 display an additional pattern representative of a deep rough mutant with only the Lipid A and possibly a few sugar residues from the inner core visible on the gel.

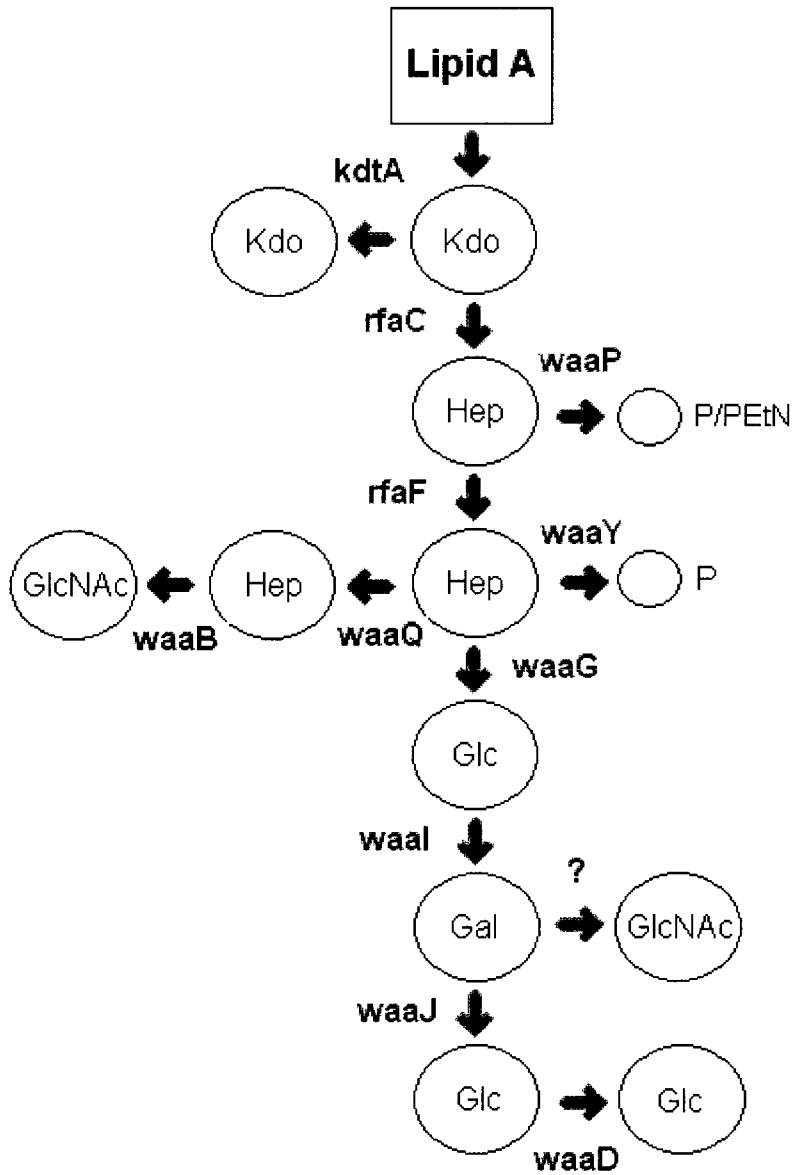
Figure 13: LPS patterns for *E. coli* O157:H7 R26-resistant mutants.

Lanes 1-2 contains controls Ec2, Ec4. Ec4R26-resistant strains -R1 to -R12 were loaded in order in lanes 3 – 14. Solid arrow indicates the O antigen and the open arrow indicates the inner/outer core components of the LPS.

Deep rough mutants typically contain mutations in the core LPS genes causing severely truncated LPS and have distinct morphology. Cells are slimy and do not form discrete colonies like typical *E. coli*. (Laird *et al.*, 1994). Based on the severely modified LPS patterns in Ec4R26-R11 and -R12, further investigations were carried out to identify mutation(s) in key enzymes involved in the inner core pathway. **Fig. 14** illustrates the structure of the inner/outer core for *E. coli* O157:H7 and the enzymes involved in the synthetic pathway. Of these, *kdtA, waaP, waaY, rfaC,* and *rfaF* (highlighted with red text in **Fig. 14**) were investigated based on their proximity to the lipid A fraction of the LPS. As per the published *E. coli* O157:H7 sequence, the expected sizes of PCR products for these genes are summarized in **Table 5**. Both Ec4R26-R11 and -R12 along with Ec4 had PCR fragments of the expected size for each of the five genes when resolved on an agarose gel (data not shown). The PCR products were digested with two restriction enzymes to determine if there were detectable mutations in the genes being investigated (data not shown). Of all the digests, only the PCR product for Ec4R26-R12 *rfaF* showed a shift when the PCR product was digested with *Hae*III. This result is shown in **Fig. 15**. The first four lanes show the uncut PCR products for Ec4, Ec4R26-R11, -R12 and the no template (negative) control. All the samples, except the negative control show amplification of the *rfaF* gene with the expected fragment size of 1125 bp. The next four lanes show the *rfaF* PCR product digested with *Mse*I. Based on published sequence, the expected size for the bands is 915 bp, 172 bp and 38 bp. Since the 38 bp fragment is too small to resolve on a 2% agarose gel, only 2 bands were visible. The parent strain, Ec4, and the two mutants, Ec4R26-R11 and -R12, had identical patterns suggesting that there were no mutations affecting the *Mse*I fragment patterns.

Figure 14: Inner core enzymes for the LPS pathway.

Enzymes screened by PCR are highlighted with red text. The following abbreviations were used: Kdo – 3-deoxy-D-manno-oct-2-ulosonic acid, Hep – L-glycero-D-manno-heptose, P/PEtN – phosphoethanolamine, P – phosphoryl, GlcNAc – N-acetylglucosamine, Glc – glucose, Gal – galactose, ? – unknown enzyme.



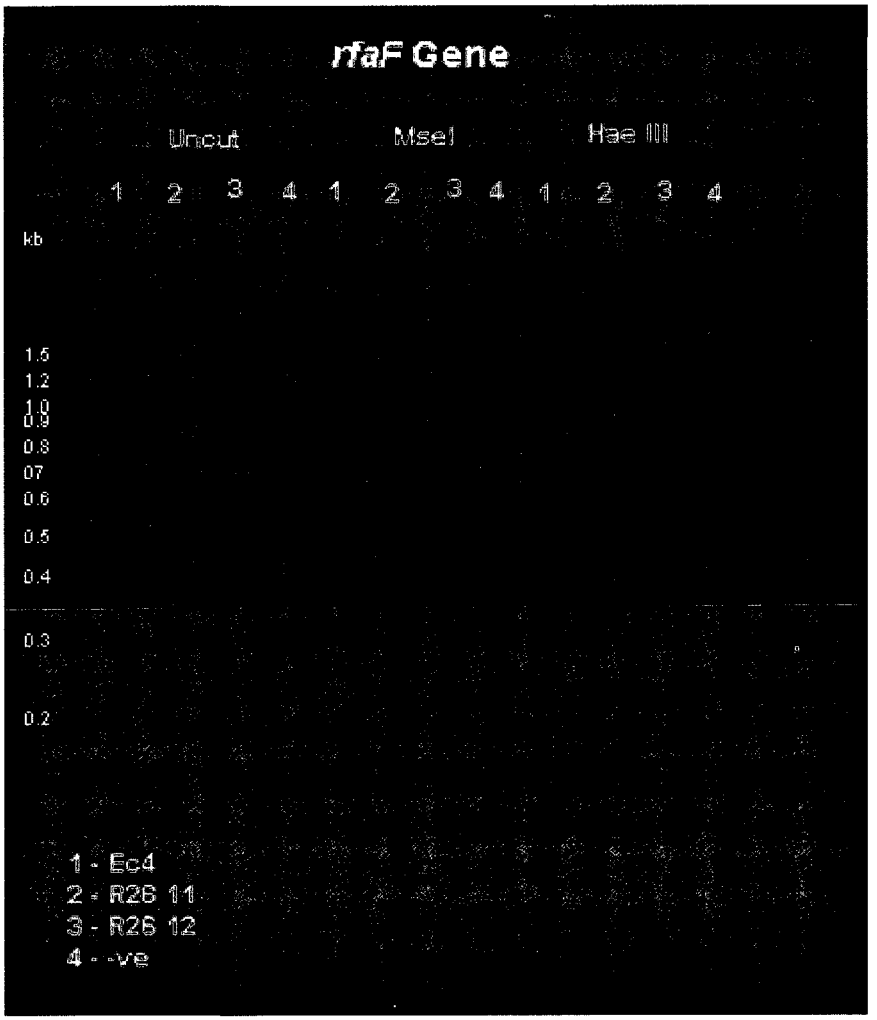
(Modified from Kaniuk *et al.* 2004)

Table 5: Expected PCR product sizes for selected LPS inner core genes.

LPS gene	Primers	Expected Fragment (bp)
kdtA	kdtAF/kdtAR	1285
waaP	waaPF/waaPR	846
waaY	waaYF/waaYR	975
rfaC	rfaCF/rfaCR	1029
rfaF	rfaFF/rfaFR	1125

Figure 15: Digested PCR products of *rfaF*.

The first four lanes show the uncut PCR products for Ec4, Ec4R26-R11, -R12 and the no template (negative) control. The next four lanes show the *rfaF* PCR product digested with *Mse*I. The final four lanes show the *rfaF* PCR product digested with *Hae*III. Molecular size marker in kb at left and right of gel.



For the *Hae*III digest, the following band sizes were expected based on the published sequence: 364, 293, 221, 120, 56, 40, and 31 bp. Again the four smaller bands were not resolved on this gel and only the largest three fragments were observed. For the parent strain, Ec4, and one of the mutants, Ec4R26-R11, the three largest fragments were of the expect size, however, for Ec4R26-R12, the largest fragment appeared to be larger than expected. Evidently, one of the *Hae*III sites was lost in this strain, resulting in a larger fragment. Due to the differences in the restriction patterns, the three PCR products for the *rfaF* gene were sequenced and a point mutation was detected in the Ec4R26-R12 sequence. **Fig. 16** shows the sequence alignment of a portion of the *rfaF* gene for Ec4R26-R12 and Ec4 (which is identical to the published sequence for this gene). Predicted amino acid sequence is given below the alignment. The Ec4R26-R11 sequence was 100% identical to Ec4 and is not shown in the figure. At base position 383 the mutant strain differed from the parent strain with an adenine substituted for a guanine. This difference is highlighted in yellow in **Fig. 16** and leads to the elimination of a *Hae*III restriction site (highlighted in red letters). The mutation alters the TGG (tryptophan, W) codon in the parent strain to a TAG (stop) in the mutant sequence (underlined and marked with an asterisk). This results in truncation of the *rfaF* gene product in mutant Ec4R26-R12. The predicted size of the truncated rfaF protein is 127 amino acids compared to 348 amino acids in the parent strain. Only the relevant sequence is shown in **Fig. 16**, as there was 100% identity in the remaining sequence. The mutation that caused the deep rough-like mutation in Ec4R26-R11 was not identified.

Figure 16: Alignment of region of *rfa*F gene with point mutation.

A base substitution (G > A, highlighted in yellow) in Ec4R26-R12 results in a premature stop in the *rfa*F gene and loss of a *Hae*III restriction site (GGCC, red letters in Ec4 sequence). The G > A substitution alters the TGG codon [codes for tryptophan (W)] into a nonsense TAG codon (underlined and designated with an asterisk). Only the relevant sequence is shown here, as there was 100% identity in remaining sequence.

```
>Ec4          ATGAAAATACTGGTGATCGGCCCGTCTTGGGTTGGCGACATGATGATGTCGCAAAGTCTC
                                                                          60
>Ec4R26-R12   ATGAAAATACTGGTGATCGGCCCGTCTTGGGTTGGCGACATGATGATGTCGCAAAGTCTC
               M  K  I  L  V  I  G  P  S  W  V  G  D  M  M  M  S  Q  S  L

>Ec4          TATCGCACGCTCCAGGCGCGCTATCCCCAGGCGATAATCGATGTGATGGCACCGGCATGG
                                                                         120
>Ec4R26-R12   TATCGCACGCTCCAGGCGCGCTATCCCCAGGCGATAATCGATGTGATGGCACCGGCATGG
               Q  A  R  Y  Y  R  T  L  P  Q  A  I  I  D  V  M  A  P  A  W

>Ec4          TGCCGTCCATTATTATCGCGGATGCCGGAAGTTAACGAAGCTATTCCTATGCCTCTCGGT
                                                                         180
>Ec4R26-R12   TGCCGTCCATTATTATCGCGGATGCCGGAAGTTAACGAAGCTATTCCTATGCCTCTCGGT
               C  R  P  L  L  S  R  M  P  E  V  N  E  A  I  P  M  P  L  G

>Ec4          CACGGAGCGCTGGAAATCGGCGAACGCCGCAAACTGGGTCATAGTCTGCGTGAAAAGCGC
                                                                         240
>Ec4R26-R12   CACGGAGCGCTGGAAATCGGCGAACGCCGCAAACTGGGTCATAGTCTGCGTGAAAAGCGC
               H  G  A  L  E  I  G  E  R  R  K  L  G  H  S  L  R  E  K  R

>Ec4          TACGACCGCGCCTACGTCTTACCTAACTCCTTCAAATCCGCATTAGTGCCTTTCTTCGCG
                                                                         300
>Ec4R26-R12   TACGACCGCGCCTACGTCTTACCTAACTCCTTCAAATCCGCATTAGTGCCTTTCTTCGCG
               Y  D  R  A  Y  V  L  P  N  S  F  K  S  A  L  V  P  F  F  A

>Ec4          GGTATTCCTCATCGCACCGGCTGGCGCGGCGAGATGCGCTACGGTTTACTCAACGATGTA
                                                                         360
>Ec4R26-R12   GGTATTCCTCATCGCACCGGCTGGCGCGGCGAGATGCGCTACGGTTTACTCAACGATGTA
               G  I  P  H  R  T  G  W  R  G  E  M  R  Y  G  L  L  N  D  V


>Ec4          CGCGTGCTCGATAAAGAAGCCTGGCCGCTAATGGTGGAACGCTATGTCGCGCTGGCCTAT
                                                                         420
>Ec4R26-R12   CGCGTGCTCGATAAAGAAGCCTAGCCGCTAATGGTGGAACGCTATGTCGCGCTGGCCTAT
               R  V  L  D  K  E  A  W/*  P  L  M  V  E  R  Y  V  A  L  A  Y

>Ec4          GACAAAGGCATTATGCGCACAGCACAAGATCTGCCGCAGCCATTGTTATGGCCGCAGTTG
                                                                         480
>Ec4R26-R12   GACAAAGGCATTATGCGCACAGCACAAGATCTGCCGCAGCCATTGTTATGGCCGCAGTTG
               D  K  G  I  M  R  T  A  Q  D  L  P  Q  P  L  L  W  P  Q  L

>Ec4          CAGGTGAGCGAAGGTGAAAAATCATATACCTGTAATCAATTTTCGCTTTCATCAGAACGT
                                                                         540
>Ec4R26-R12   CAGGTGAGCGAAGGTGAAAAATCATATACCTGTAATCAATTTTCGCTTTCATCAGAACGT
               Q  V  S  E  G  E  K  S  Y  T  C  N  Q  F  S  L  S  S  E  R

>Ec4          CCGATGATTGGTTTTTGCCCGGGTGCGGAGTTTGGTCCGGCAAAACGCTGGCCACACTAC
                                                                         600
>Ec4R26-R12   CCGATGATTGGTTTTTGCCCGGGTGCGGAGTTTGGTCCGGCAAAACGCTGGCCACACTAC
               P  M  I  G  F  C  P  G  A  E  F  G  P  A  K  R  W  P  H  Y
```

It is possible that a point mutation was present in one of the other four genes screened for Ec4R26-R11 and was not detected by restriction analysis of the PCR products since only a limited number of enzymes was used. It is also possible that a mutation may have occurred in another, uninvestigated gene in the LPS inner core pathway. Overall, the identification of altered LPS patterns and a mutation associated with altered LPS profiles in Ec4R26-R12 demonstrates that LPS plays an important role in R26 adsorption and is a potential receptor molecule for phage R26. The possibility that LPS is a secondary receptor cannot be ruled out at this time. Nor can we exclude the possibility that resistance to P39 by Ec4R26-R12 may be due to a loss of membrane structure/function that may be associated with LPS deficiencies.

3.3 Characterization of P39 (T4 Like) Resistant Mutants

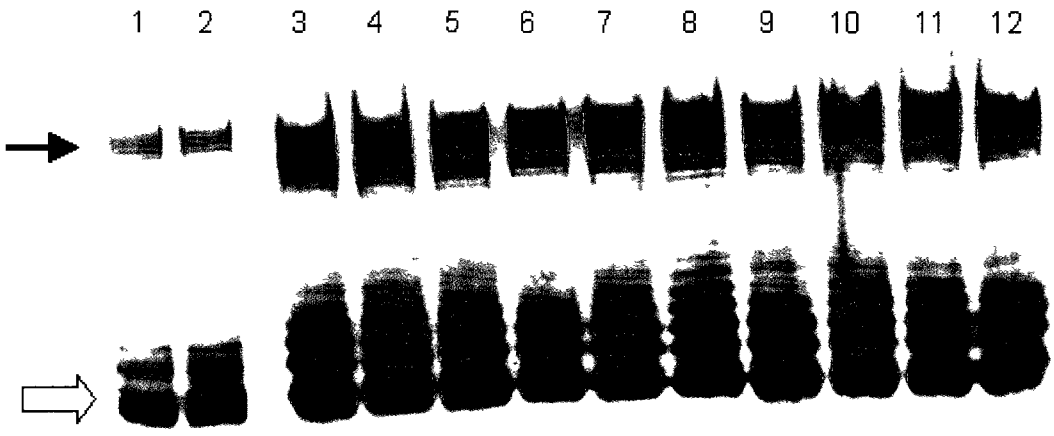
As discussed previously, LPS and OmpC have been identified as receptor sites for phage T4, as well as many other bacteriophage (Kutter and Slakvelidze, 2005; Yu and Mizushima, 1982). Since P39 is a T4-like phage, both these molecules were investigated as potential receptor sites in the P39-resistant mutants selected in this study. The results obtained from these studies are presented below.

LPS:

The LPS profiles were examined to determine if there were any differences between the parental Ec4 strain and the P39-resistant mutants (**Fig. 17**). As in **Figure 13**, the bands across the top of the gel represent the O antigen and the lower bands represent the inner/outer core LPS components. In this case, all 10 P39-resistant mutants had LPS patterns identical to the parental strain.

Figure 17: LPS patterns for *E. coli* O157:H7 P39-resistant mutants.

Lanes 1-2 contains controls Ec2, Ec4. Ec4P39-resistant strains -R1 to -R10 were loaded in order in lanes 3 – 12. Solid arrow indicates the O antigen and the open arrow indicates the inner/outer core components of the LPS.

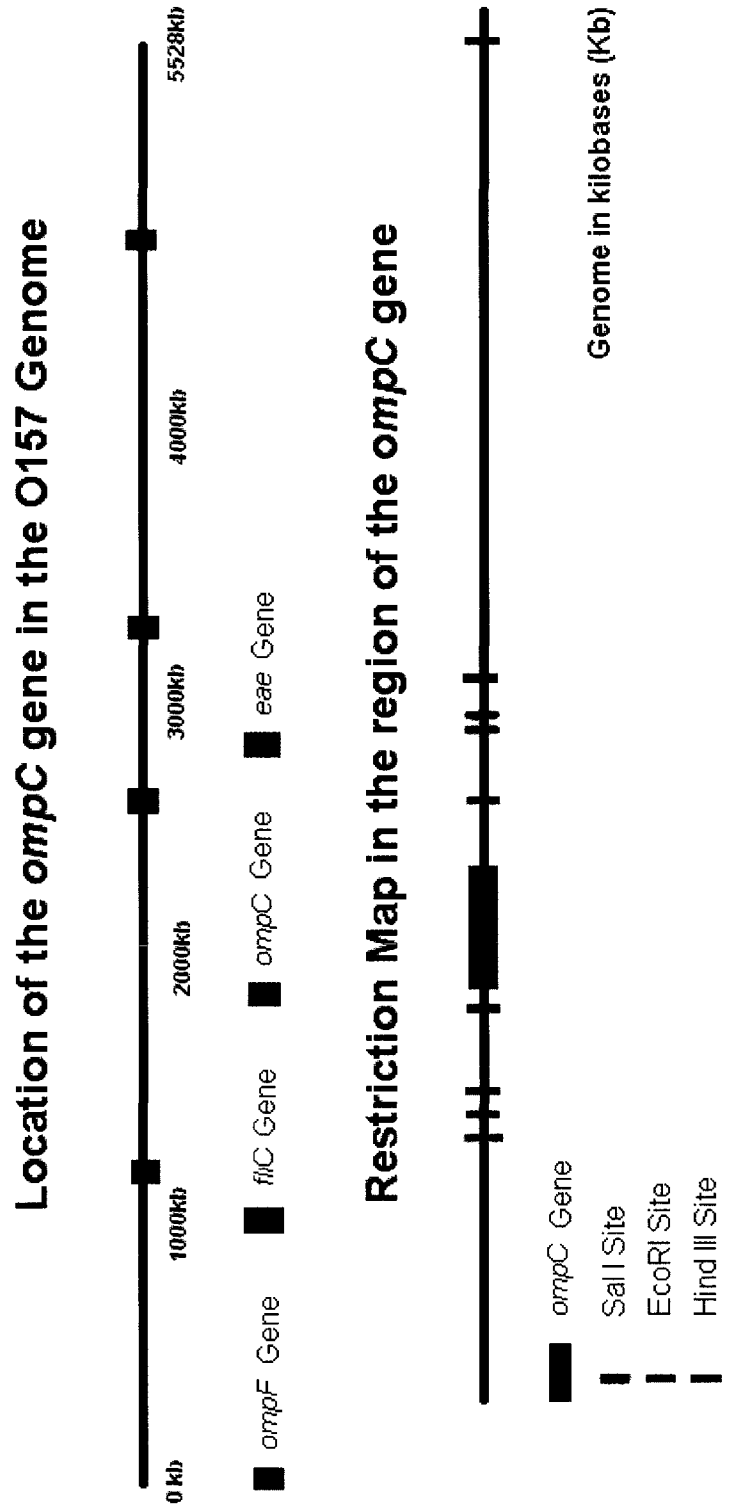


This suggests that there are no major differences in the LPS patterns of the mutants although minor differences may remain undetected by this assay. A comparison of the LPS profiles of the resistant mutants shown in **Fig. 13** to those of **Fig. 17** suggests that LPS is altered in R26-resistant mutants but not P39-resistant mutants.

OmpC:

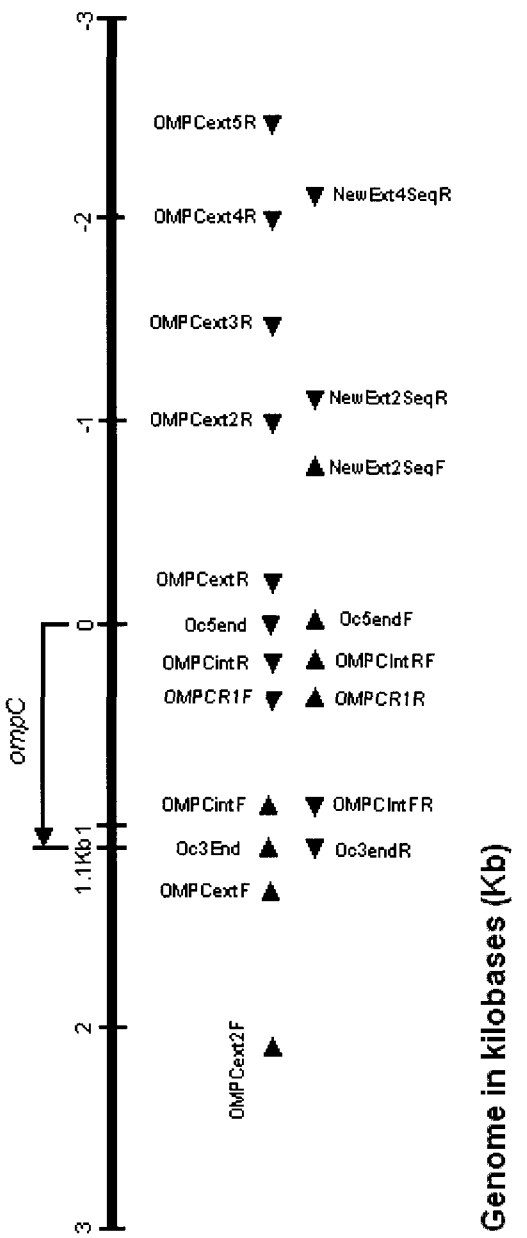
The *ompC* gene in *E. coli* O157:H7 is 1104 bp in length. It contains an internal *Eco*RI restriction site and is flanked by *Hin*dIII and *Sal*I sites making it useful for analysis by Southern blot. **Fig. 18** illustrates the position of the *ompC* gene relative to virulence genes *FliC, eae* and the outer membrane protein F (*ompF*) in the *E. coli* O157:H7 genome. A restriction map of the area surrounding the *ompC* gene is also included in this figure as it would be useful in further analysis. To determine if the *omp*C gene was mutated in the P39-resistant mutants, a PCR based approach was used with two representative strains resistant to P39. **Fig. 19** illustrates a primer map of all *ompC* primers used in this study. Initially the *ompC* gene from P39-resistant mutants was amplified using the following primer sets: Oc5end/Oc3end, OMPC IntF/OMPC IntR, and OMPC ExtF/OMPC ExtR. Primers were chosen to amplify the entire ompC gene as well as one fragment that was internal and one external to the gene with the intention of identifying the region of the mutation. The parent strain Ec4 generated fragments of the expected size: Oc5end/Oc3end – 1114 bp, OMPC IntF/OMPC IntR – 730 bp, and OMPC ExtF/OMPC ExtR – 1558 bp. However, no PCR products were generated for Ec4P39-R1 and -R5, the mutants screened in this study.

Figure 18: Map of the *ompC* and flanking genes in *E. coli* O157:H7.



(Based on Sequence Published by Makino *et al.*, 1999)

Figure 19: Primer map of the *ompC* gene for *E. coli* O157:H7.

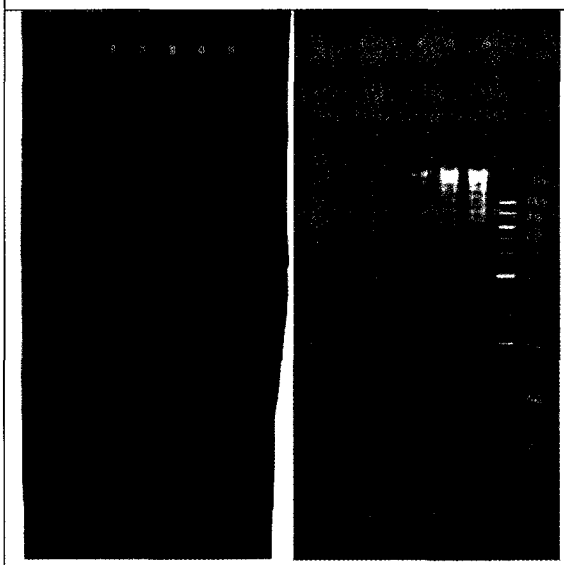
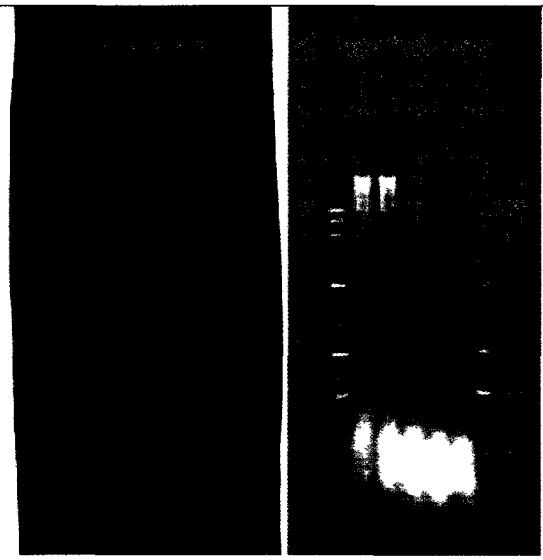
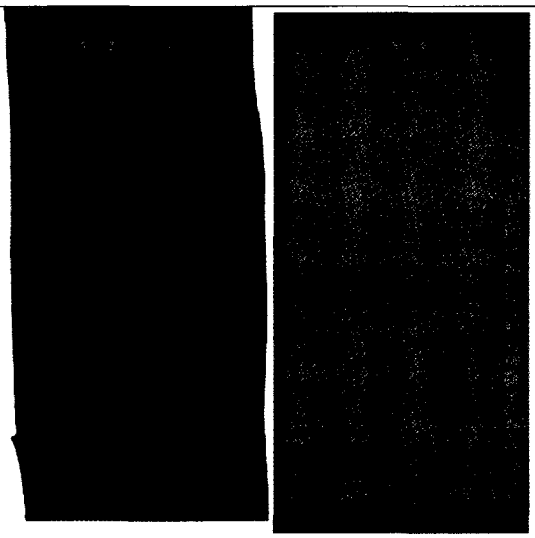


(Based on Sequence Published by Makino *et al.*, 1999)

Since the *ompC* gene could not be amplified in the mutants using the primers designed to amplify the entire coding region, it was important to confirm that (i) the gene was present in the mutants and (ii) if present, were there any major deletions/mutations in the gene. This was accomplished by Southern blot analysis of the genomic DNA isolated from the mutants. A PCR generated fragment of the full length *ompC* gene from the parent strain (Ec4) (1114bp) was used as a probe for Southern blot analysis. Genomic DNA from Ec4 (the parent strain), Ec4P39-R1, -R5 (P39-resistant mutants), Ec4R26-R11, -R12 (deep rough mutants) was digested with *Hin*dIII, *Sal*I and *Eco*RI in an effort to determine what region of the *ompC* gene was affected thus preventing the gene from being amplified by PCR. Based on the published genome sequence of *E. coli* O157:H7, the Southern blot of the *Hin*dIII digest using an *ompC* gene probe should give one band at ~7.8 kb (Makino *et al.*, 1999). A single band corresponding to the expected size was observed for the parent strain, Ec4, and the deep rough-like mutants Ec4R26-R11 and -R12 (**Fig. 20**). However, strains Ec4P39-R1 and -R5 (lanes 4 & 5) yielded a fragment that was approximately 2.0 kb smaller than expected. This could represent a 2.0 kb deletion between the two *Hin*dIII sites flanking the *ompC* gene shown in **Fig. 18**. The Southern blot of the *Eco*RI digest of *E. coli* O157:H7 genomic DNA should give two visible bands, one at 25.3 kb and the other at 2.0 kb. As seen in **Fig. 18**, one of the *Eco*RI restriction sites is located within the *ompC* gene. Two bands of the expected sizes were observed for the parent strain, Ec4, and the deep rough-like mutants, Ec4R26-R11 and -R12 (**Fig. 20**). The P39-resistant mutants, Ec4P39-R1, and -R5, gave only one band which was similar in size to the ~25.3 kb fragment observed in the parental strain. The smaller band, 2.0 kb, was not present for either of the P39-resistant mutants.

Figure 20: Southern Blot analysis of *ompC* gene.

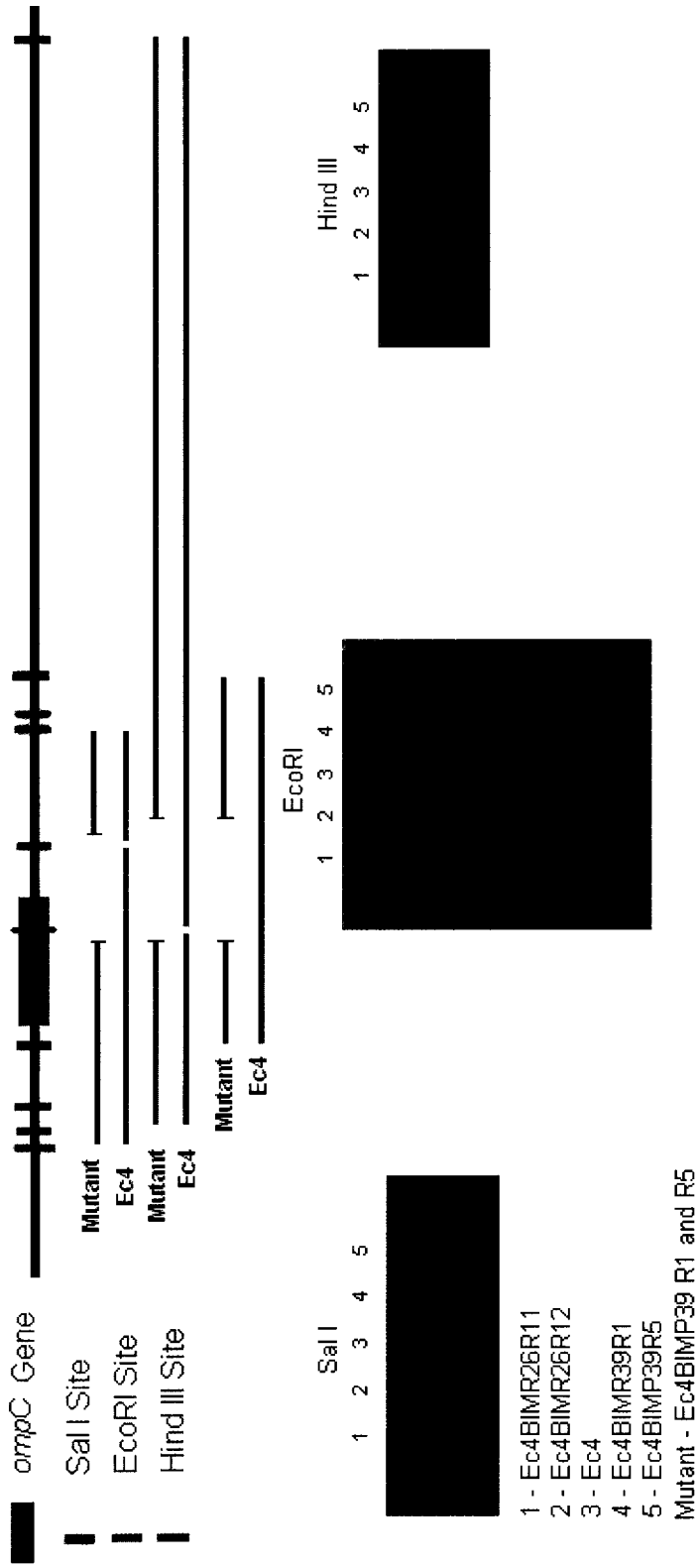
For each of the *Hin*dIII, *Eco*RI and *Sal*I panels, the ethidium bromide-stained agarose gel is shown at right at approximately the same scale as the Southern blot image shown at left. Size standards were loaded in the most right and left lanes on agarose gel images and sizes are given in kb at right of each panel.

***Hin*dIII Digest**	***Eco*RI Digest**
	
***Sal*I Digest**	**Legend**
	1 Ec4R26-R11 2 Ec4R26-R12 3 Ec4 4 Ec4P39-R1 5 Ec4R39-R5

Based on the restriction map of the region presented in **Fig. 18**, if the *Eco*RI site internal to the *ompC* gene was not present, then the observed band would be approximately 27.3 kb minus the size of the deletion of ~2.0 kb as suggested by the *Hin*dIII Southern blot presented above. Based on this data, the expected size of the band observed for the *Eco*RI digest would be about 25 kb which was the size of the observed fragment. In the Southern blot of the *Sal*I digest a single band at 3.5 kb is expected. This is what was observed for the parent strain, Ec4, and mutants Ec4R26-R11 and -R12 (**Fig. 20**). However in the P39-resistant mutants Ec4P39-R1, and -R5 (lanes 4 & 5) the band representing the *ompC* gene was approximately 5 kb, which is significantly larger than that seen with the parent (Ec4 – lane 3) strain. Based on the published sequence and as presented schematically in **Fig. 18**, if the first *Sal*I site 5' to the *ompC* gene was deleted, a fragment of 7.3 kb would be expected. If the deletion of ~2.0 kb, as suggested by the *Hin*dIII Southern blot is factored in, a fragment of ~5 kb would be expected. This corresponds well with the size of the bands observed for both the P39-resistant mutants Ec4P39-R1 and -R5 (lanes 4 & 5).

The results described in this section for Southern blot studies are summarized schematically in **Fig. 21** for Ec4P39-R1 and -R5. The *Hin*dIII digest suggested a deletion of approximately 2.0 kb between the restriction sites in the P39-resistant mutants Ec4P39-R1 and -R5 and included a major part of the *ompC* gene. This was further confirmed by Southern blots of *Sal*I and *Eco*RI digests which confirmed that one *Sal*I site external to the *ompC* gene and one *Eco*RI site internal to the *ompC* are included in the deleted ~2.0 kb region. As highlighted in **Fig. 21**, the Southern blot results would also explain the PCR results described previously.

Figure 21: Schematic of the Southern Blot results of the *ompC* gene.



PCR products for the *ompC* gene would not be amplified since some of the PCR primers used were located in the deletion at the 5' end of the gene.

In order to confirm the Southern blot results, new primers were designed flanking the 5' and 3' ends of the *ompC* gene and tested with Ec4, Ec4P39-R1 and -R5. The primers used for this portion of the study and their location with reference to the *ompC* gene are illustrated in **Fig. 19**. These primer sets and the expected amplicon sizes are summarized in **Table 6**. Amplicons of the expected size were produced with all primer pairs when Ec4 DNA was used as the template. Only primers that amplified regions of the 3' end of the gene produced amplicons with Ec4P39-R1 and -R5. Primer sets designed to amplify regions of the 5' end of the *ompC gene* did not produce amplicons with DNA template from the Ec4P39-R1 and -R5 mutants, confirming that these strains carry a deletion in this region of the gene. Information from these PCR studies was used to refine the map of the deletion based on Southern blots.

The OMPCIntF primer was used along with new primers that were designed for genome walking from the 5' end of the *ompC* gene. Primers were designed at approximately 500 bp from each other upstream of the *ompC* gene as given in **Table 7** along with the expected fragment sizes. These primer pairs were used to assess the *ompC* gene in Ec4P39-R1 through -R10 along with the parent strain Ec4 as illustrated in **Fig. 22**.

As evident from **Fig. 22**, the parent strain Ec4 and the P39-resistant mutant Ec4P39-R7 generated fragments that matched the expected sizes of 2.4 kb with the primer set OMPCIntF/OMPCext3R. P39-resistant mutants Ec4P39-R1, -R4, -R5, -R8, -R9, and -R10 gave PCR products that were approximately 600 bp, and no PCR products were amplified when DNA from mutants Ec4P39-R2, -R3, and -R6 was used.

Table 6: Primers and expected PCR product sizes for *ompC* gene mapping.

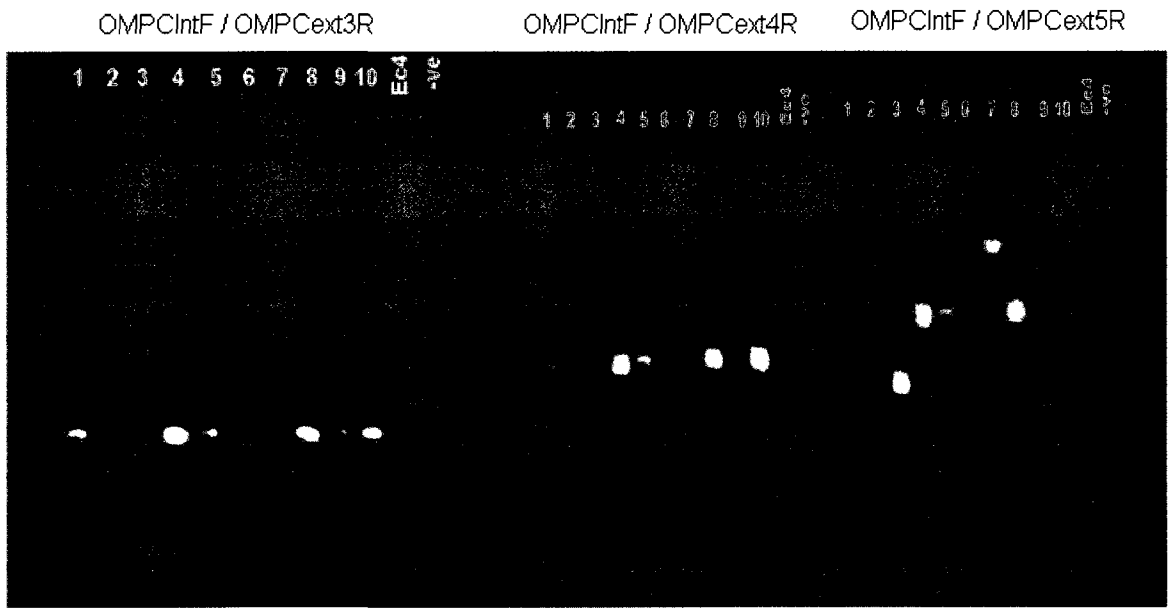
Region of *ompC* gene screened	Primers used to generate an amplicon	Expected fragment (bp)	Size of PCR product (bp)
3' end	Oc3end / OMPCIntFR	201	201
3' end	OMPCextF / OMPCIntFR	421	421
3' end	OMPCext2F OMPCIntFR	1190	1190
3' end	OMPCextF / Oc3endR	255	255
3' end	OMPCext2F / Oc3endR	987	987
Internal (*Eco*RI) to 3'	OMPCintF / OMPCR1R	543	N/A
Internal (*Eco*RI) to 3'	Oc3end / OMPCR1R	723	N/A
Internal (*Eco*RI) to 3'	OMPCextF / OMPCR1R	943	N/A
Internal (*Eco*RI) to 3'	OMPCext2F / OMPCR1R	1712	N/A
Internal (*Eco*RI) to 5'	OMPCR1F / OMPCintR	204	N/A
Internal (*Eco*RI) to 5'	OMPCR1F / Oc5end	410	N/A
Internal (*Eco*RI) to 5'	OMPCR1F / OMPCextR	633	N/A
Internal (*Eco*RI) to 5'	OMPCR1F / OMPCext2R	1373	N/A
5' end	OMPCintRF / Oc5end	216	N/A
5' end	OMPCintRF / OMPCextR	449	N/A
5' end	OMPCintRF / OMPCext2R	1189	N/A
5' end	Oc5endF / OMPCextR	236	N/A
5' end	Oc5endF / OMPCext2R	1005	N/A

Table 7: PCR primers and expected amplicon sizes for *ompC* deletion mapping.

Forward Primer	Reverse Primer	Expected Fragment (bp)
OMPCIntF	OMPCext3R	2417
OMPCIntF	OMPCext4R	2924
OMPCIntF	OMPCext5R	3423

Figure 22: PCR of the *ompC* region for the resistant mutants.

Ec4P39-R1 to –R10 were assayed in three PCR reactions. Gels are labelled according to the primers used (OMPCIntF / OMPCext3R, OMPCIntF / OMPCext4R, and OMPCIntF / OMPCext5R. Lanes are labelled with the respective phage resistant mutant 1, 2, 3, etc. indicate -R1, -R2, -R3, etc. and the controls Ec4 and –ve (no template control). Molecular size standard in kb are at the left and right in each panel.



With the second primer set, OMPCIntF/OMPCext4R, the parent strain Ec4 and the P39-resistant mutant Ec4P39-R7, again generated fragments that matched the expected fragment size of 2.9 kb. On the other hand, mutants Ec4P39-R1, -R4, -R5, -R8, -R9, and -R10 generated PCR products that were approximately 1.1 kb, and mutant Ec4P39-R3 gave a PCR product that was approximately 350 bp. No PCR products with these primers were produced from mutants Ec4P39-R2 and -R6.

For the third primer set, OMPCIntF/OMPCext5R, the parent strain Ec4 and the P39-resistant mutant Ec4P39-R7 again generated a fragment that matched the expected size of 3.4 kb. Mutants Ec4P39-R1, -R4, -R5, -R8, -R9, and -R10 gave PCR products that were approximately 1.6 kb and mutant Ec4P39-R3 gave a PCR product that was approximately 850 bp. Mutants Ec4P39-R2 and -R6 again did not yield PCR products with this primer pair. This suggested that the ten P39-resistant mutants could be classified into four groups as follows:

Group 1: Ec4P39-R7: has no deletion.

Group 2: Ec4P39-R1, -R4, -R5, -R8, -R9, and -R10: have a ~1.8 kb deletion between the OMPCIntF/OMPCext3R primers (based on the fact that a 600 bp PCR product was amplified where the expected PCR product was 2.4 kb).

Group 3: Ec4P39-R3: has a ~ 2.6 kb deletion between the OMPCIntF/OMPCext4R primers (based on the fact that a 350 bp PCR product was amplified whereas the expected PCR product was 2.9 kb).

Group 4: Ec4P39-R2 and -R6: have a $\geq$2.9 kb deletion that includes the OMPCext5R primer or some other rearrangement involving the 3' end of the *ompC* gene. For the scope

of this research, mutants in this group were not characterized further to determine the exact nature of the mutation.

At this point, all of the phage-resistant mutants were screened with the full length *ompC* gene primers to test whether deletion mutations are also common in the Ec4P39 group. Primer set, Oc5end/Oc3end (expected fragment size: 1.1 kb), was used to screen Ec2, the parent strain Ec4, along with the 10 Ec4P39 mutants and the 12 Ec4R26 mutants as shown in **Fig. 23**. As expected, the parent strain and all of the Ec4R26 mutants amplified PCR products that were of the expected size. Consistent with the previous PCR and Southern blot results with the Ec4P39 mutants, only Ec4P39-R7 generated a PCR product that matched the expected size. None of the remaining nine Ec4P39 mutants generated a PCR product. In order to determine if the isolate which was resistant to P39 but generated a PCR product for the *ompC* gene had a mutation within this gene, it was sequenced. An alignment of the sequence of the resistant mutant, Ec4P39-R7, with the parent strain, Ec4 is presented in **Fig. 24**. Only portions of the gene sequence where differences were observed are presented. The remainder of the *ompC* gene sequence of the mutants showed a 100% identity to the parent strain. The sequence obtained for Ec4 matched the published sequence (Makino *et al.,* 1999) whereas Ec4P39-R7 had a 46 bp insertion close to the start of *ompC*. The 46 bp insertion appears to have resulted from a local duplication within the *ompC* gene caused by slippage of DNA polymerase during replication. This insertion is predicted to result in a frameshift and a change in the amino acid starting at position 60 (highlighted in yellow). The insertion also introduced a stop codon, TGA, in the DNA sequence, highlighted in red. The start codon for all of the strains is highlighted in green in **Fig. 24**.

Figure 23: PCR of the full length *ompC* gene for the resistant mutants. Ec4P39-R1 to –R10 and Ec4R26-R1 to –R12 mutants along with control strains Ec2 and Ec4 were assayed. Lanes are labelled with the respective phage resistant mutant 1, 2, 3, etc. indicate -R1, -R2, -R3, etc. and the controls Ec2 and Ec4. Molecular size standard in kb are at the left and right in each panel.

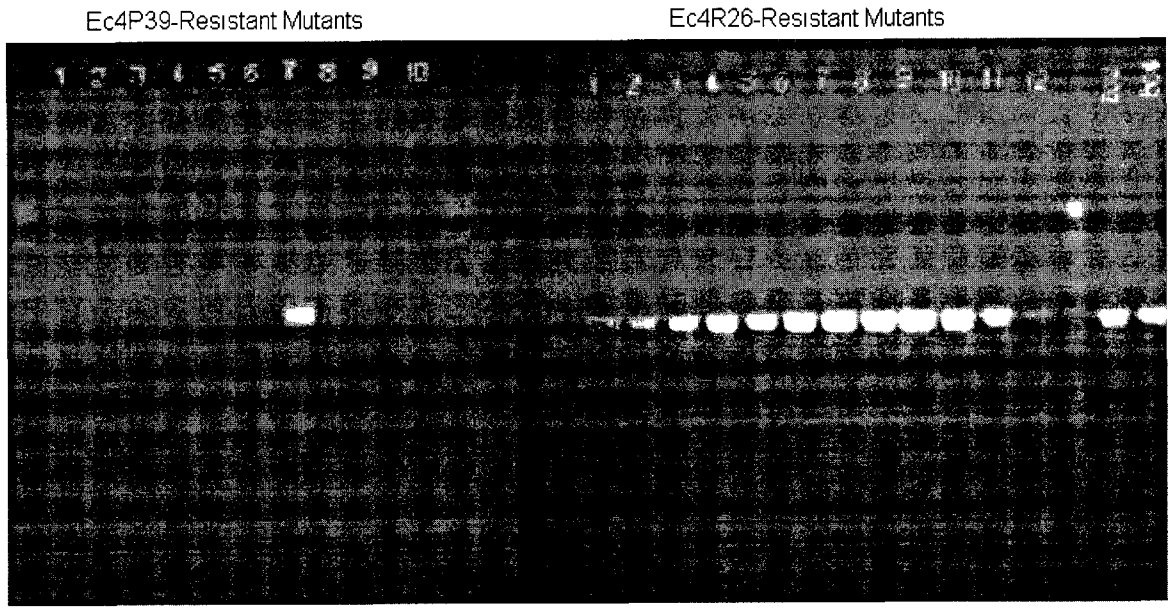
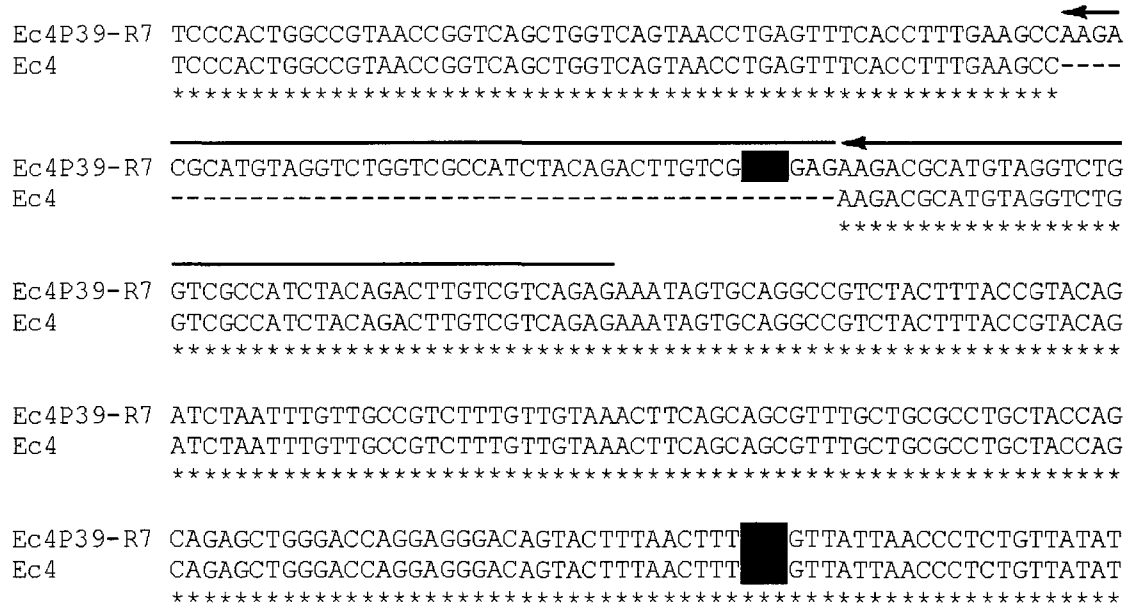


Figure 24: Alignment of sequence for *ompC* gene from Ec4 and Ec4P39-R7. There was 100% identity across all other regions of the gene except for the region displayed here. Start codon is highlighted in green. The duplication is noted with an arrow over the sequence. This insertion is predicted to result in a frameshift and a change in the amino acid starting at position 60 (highlighted in yellow) followed by the introduction of a stop (highlighted in red).



The translated amino acid sequence for the parental Ec4 strain, and Ec4P39-R7, the P39-resistant mutant that contains a 46 bp insertion in the *ompC* gene is illustrated in **Fig. 25**. As seen in the DNA sequence, the 46 bp insertion in Ec4P39-R7 causes a frameshift mutation and a change in amino acid 60 from Glycine (G) to Leucine (L), which is highlighted in yellow. In the Ec4P39-R7 *ompC* sequence this frameshift is followed immediately by a stop, which is highlighted in red in **Fig. 25**, truncating the *ompC* protein to 60 amino acids. The parent strain Ec4 on the other hand has a Phenylalanine (F) at position 61 and generates a 364 amino acid functional copy of the OmpC protein as illustrated in **Fig. 25**. As described previously, Ec4P39-R3 had the largest deletion that was confirmed by PCR analysis. In order to identify the exact location and size of the deletion in Ec4P39-R3, new PCR primers, Oc3end/NewExt4SeqR, were used to generate a PCR product for sequencing. A PCR product of approximately 600 bp was obtained from Ec4P39-R3, as expected. In the case of the parent strain Ec4, primer pairs Oc5endF/ NewExt2SeqR (expected fragment size 1097 bp) and NewExt2SeqF/NewExt4SeqR (expected fragment size 1268 bp) were used to generate PCR products for sequencing. PCR products of the expected size were obtained for Ec4. The aligned sequences for the relevant portion of *ompC* is summarized in **Fig. 26**. Deletion of a 2569 bp fragment was evident in the Ec4P39-R3 mutant. Otherwise, there was 100% identity between sequences from Ec4P39-R3 and Ec4 on both sides of the deletion (**Fig. 26**). The deletion included the promoter region and the 5' end of the *ompC* gene. Based on this result, it would not be possible for Ec4P39-R3 to express OmpC.

Figure 25: Alignment of protein sequence for *ompC* gene from Ec4 and Ec4P39-R7. The amino acid sequence of Ec4P39-R7 and Ec4 is shown below. An insertion in the DNA sequence of Ec4P39-R7 caused a frameshift, which resulted in a change in amino acid 60 from Glycine (G) to Leucine (L) and is highlighted in yellow. In the Ec4P39-R7 *ompC* sequence this frameshift is followed immediately by a stop, which is highlighted in red. The parent strain Ec4 has a Phenylalanine (F) at position 61 and generates a 364 amino acid functional copy of the OmpC protein.

```
Ec4P39-R7     MKVKVLSLLVPALLVAGAANAAEVYNKDGNKLDLYGKVDGLHYFSDDKSVDGDQTYMRLL
Ec4           MKVKVLSLLVPALLVAGAANAAEVYNKDGNKLDLYGKVDGLHYFSDDKSVDGDQTYMRLG 60
                 ************************************************************

Ec4P39-R7     L------------------------------------------------------------ 120
Ec4           KGETQVTDQLTGYGQWEYQIQGNSAENENNSWTRVAFAGLKFQDVGSFDYGRNYGVVYD

Ec4P39-R7     ------------------------------------------------------------ 180
Ec4           VTSWTDVLPEFGGDTYGSDNFMQQRGNGFATYRNTDFFGLVDGLNFAVQYQGKNGSVSGE

Ec4P39-R7     ------------------------------------------------------------ 240
Ec4           GMTNNGREALRQNGDGVGGSITYDYEGFGIGAAVSSSKRTDDQNSPLYIGNGDRAETYTG

Ec4P39-R7     ------------------------------------------------------------ 300
Ec4           GLKYDANNIYLAAQYTQTYNATRVGSLGWANKAQNFEAVAQYQFDFGLRPSLAYLQSKGK

Ec4P39-R7     ------------------------------------------------------------ 360
Ec4           NLGVINGRNYDDEDILKYVDVGATYYFNKNMSTYVDYKINLLDDNQFTRDAGINTDNIVA

Ec4P39-R7     ---- 364
Ec4           LGLV
```

Figure 26: Alignment of DNA sequences from Ec4 and Ec4P39-R3 *ompC* gene regions. There was a 2569 bp deletion in the *ompC* gene of Ec4P39-R3. Sequences on either side of this deletion showed 100% identity between Ec4 and Ec4P39-R3.

```
Ec4          328 ACCCAGAGCTACGATGTTATCAGTGTTGATGCCAGCGTCACGAGTGAACTGGTTGTCGTC 387
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ec4P39-R3    328 ACCCAGAGCTACGATGTTATCAGTGTTGATGCCAGCGTCACGAGTGAACTGGTTGTCGTC 387

Ec4          388 CAGCAGGTTGATTTTGTAGTCAACATAGGTGGACATGTTTTTGTTGAAGTAGTAGGTCGC 447
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ec4P39-R3    388 CAGCAGGTTGATTTTGTAGTCAACATAGGTGGACATGTTTTTGTTGAAGTAGTAGGTCGC 447

Ec4          448 GCCAACATCAACATATTTCAGGATATCTTCGTCGTCGTAGTTACGACCATTGATGACACC 507
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ec4P39-R3    448 GCCAACATCAACATATTTCAGGATATCTTCGTCGTCGTAGTTACGACCATTGATGACACC 507

Ec4          508 CAGGTTTTTACCTTTAGACTGCAGGTAAGCCAGGGACGGACGCAGACCGAAGTCGAACTG 567
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ec4P39-R3    508 CAGGTTTTTACCTTTAGACTGCAGGTAAGCCAGGGACGGACGCAGACCGAAGTCGAACTG 567

Ec4          568 GTACTGAGCAACAGCTTCGAAGTTCTGTGCTTTGTTCGCCCAACCCAGGGAACCTACGCG 627
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ec4P39-R3    568 GTACTGAGCAACAGCTTCGAAGTTCTGTGCTTTGTTCGCCCAACCCAGGGAACCTACGCG 627

Ec4          628 AGTTGCG  634  … 2569 bp DELETED REGION IN EC4P39-R3 …
                 |||||||
Ec4P39-R3    628 AGTTGCG  634

Ec4        3203 CATTTTACGGGCAATCAATGAAGAGATAGTCTCACTGCTGCCGCTCGGCCTGCTGGTTCA 3254
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ec4P39-R3  635 CATTTTACGGGCAATCAATGAAGAGATAGTCTCACTGCTGCCGCTCGGCCTGCTGGTTCA 694

Ec4        3255 CGATCAGGAATCGAACCGCACTGTCATAAGTAACAAAATTGCCGATCATTTGCTGCCGCA 3314
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ec4P39-R3  695 CGATCAGGAATCGAACCGCACTGTCATAAGTAACAAAATTGCCGATCATTTGCTGCCGCA 754

Ec4        3315 TTTGAATCTGCAAAACATCACCACCATGGCGGAACAGCATCAGGGGATTATTCAGGCGAC 3374
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ec4P39-R3  755 TTTGAATCTGCAAAACATCACCACCATGGCGGAACAGCATCAGGGGATTATTCAGGCGAC 814

Ec4        3375 GATCAATAACGAGCTGTATGAGATCCGCATGTTCCGCAGCCAGGTTGCGCCGCGCACACA 3434
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ec4P39-R3  815 GATCAATAACGAGCTGTATGAGATCCGCATGTTCCGCAGCCAGGTTGCGCCGCGCACACA 874

Ec4        3435 AATTTTCATTATTCGCGATCAGGATCGCGAAGTGCTGGTAAACAAGAAACTCAAGCAGGC 3494
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ec4P39-R3  875 AATTTTCATTATTCGCGATCAGGATCGCGAAGTGCTGGTAAACAAGAAACTCAAGCAGGC 934
```

Overall, the results provided evidence for different mutations present in the *ompC* gene of the Ec4P39-resistant mutants. In all cases, the sequence demonstrated that the OmpC protein would either be truncated or not synthesized. To further confirm this observation, complementation studies with OmpC protein were performed. Two plasmid constructs were obtained from Dr. Yasunori Tanji of the Tokyo Institute of Technology. One construct was for OmpC from *E. coli* O157:H7 while the second construct was for OmpC from *E. coli* K-12 (Morita *et al.,* 2002). In addition, an *E. coli* K-12 mutant with a deletion in the *ompC* gene (RK4784) was obtained from the NIG collection. The constructs were used to transform the P39-resistant mutant, Ec4P39-R3, and the *E. coli* K-12 mutant, RK4784 with a deletion in the *ompC* gene. Ec4 was used as a control to test the plating efficiency of P39 and *E. coli* K-12 (ATCC31608), was used as a control to test the plating efficiency of T4. Both the mutants were transformed with the vector alone (to determine background noise), as well as the vectors containing OmpC from *E. coli* O157:H7 and OmpC from *E. coli* K-12. The transformants were then tested with the Quantitative assay (Plate Method) to determine their plating efficiency with P39 and T4. The results of the plating efficiency with phage T4 are presented in **Fig. 27**. The control with ATCC31608 shows a plating efficiency of approximately $1x10^9$ pfu/mL while the second control strain, Ec4, did not show any plaques. This was expected because T4 does not infect *E. coli* O157:H7. T4 did plate on the *E. coli* K-12 $OmpC^-$ mutant, RK4784, with a plating efficiency of $\sim 3x10^6$ pfu/mL. The difference in plating efficiency was significant as determined by a student t-test (P value < 0.0011). T4 did not infect Ec4P39-R3 or any of the transformants even when complemented with OmpC from *E. coli* K-12. Ec4P39-R3 was similar to its parental strain, Ec4, in this respect.

Figure 27: Complementation studies: T4.

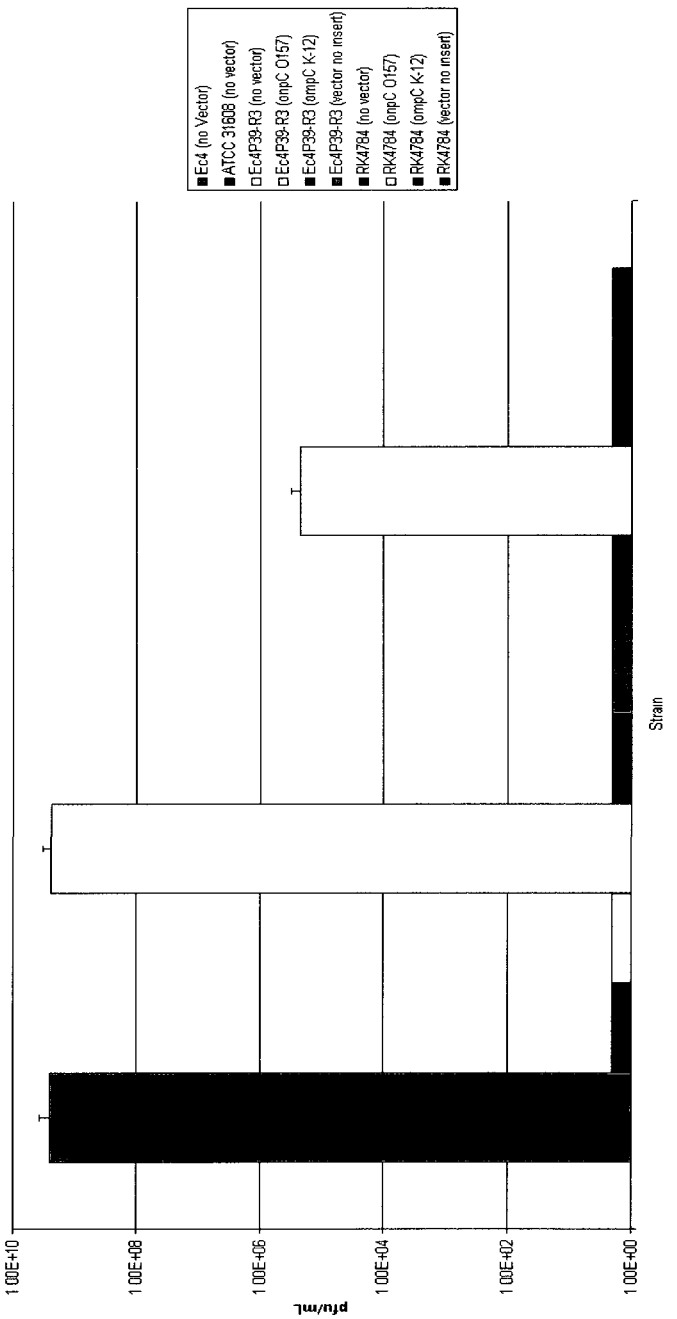
Error bars represent standard deviation (n=6). Complemented strain RK4784 (ompC K-12) was not significantly different than the control strain (ATCC 31608) (P >0.3 based on student t-test). All other resistant strains were significantly different from the control (P < 0.0011).

This may suggest that a secondary receptor is required for T4 to infect Ec4-derived strains. It has been shown previously that LPS and OmpC are both required for T4 infection (Henning and Jann, 1979; Yu and Mizushima, 1982). The plating efficiency of T4 with RK4784 transformed with OmpC from *E. coli* O157:H7 and with the vector with no insert was similar to control values of $3x10^6$ pfu/mL. However, when RK4784 was transformed with OmpC from *E. coli* K-12, the plating efficiency increased to approximately $1x10^9$ pfu/mL which is similar to that seen with the control strain ATCC31608. This confirms the observation that T4 requires OmpC for binding and infection of RK4784 (Henning and Jann, 1979; Yu and Mizushima, 1982).

The results of the plating efficiency studies using phage P39 are presented in **Fig. 28**. The control with Ec4 shows a plating efficiency of approximately $5x10^9$ pfu/mL while the second control strain, ATCC31608, showed no plaques. This was expected since P39 does not infect *E. coli* K-12. P39 also did not infect Ec4P39-R3, a P39-resistant mutant. The difference in plating efficiency was significant as determined by a student t-test ($P < 0.0011$). When Ec4P39-R3 was transformed with OmpC from *E. coli* O157:H7, the plating efficiency returned to positive control levels of $\sim 5x10^9$ pfu/mL. However, when Ec4P39-R3 was transformed with OmpC from *E. coli* K-12 and with the vector with no insert, no plaques were observed ($P < 0.0011$). This demonstrates clearly that the addition of OmpC from *E. coli* O157:H7 is all that is required to restore the plating efficiency of Ec4P39-R3 back to that observed for the parent strain. This result also confirms the specificity of P39 to *E. coli* O157:H7, since the addition of OmpC from *E. coli* K-12 did not alter the plating efficiency. P39 did not plate on RK4784, and RK4784 transformed with OmpC from *E. coli* K-12 and with the vector with no insert.

Figure 28: Complementation studies: P39.

Error bars represent standard deviation (n=6). Complemented strain Ec4P39-R3 (ompC O157) was not significantly different than the control strain (Ec4) ($P > 0.3$ based on student t-test). All other resistant strains, including RK4784 (ompC O157), were significantly different from the control ($P < 0.0011$).



However, when RK4784 was transformed with OmpC from *E. coli* O157:H7, the plating efficiency increased to approximately $5x10^5$ pfu/mL. This is significantly lower than the plating efficiency on Ec4 ($P < 0.0011$) but showed that the introduction of OmpC from *E. coli* O157:H7 was sufficient to allow P39 to bind to, and infect, *E. coli* K-12. The difference in plating efficiency as compared to the host strain Ec4, could be due to a missing secondary receptor such as LPS from *E. coli* O157:H7. This clearly highlights that OmpC is essential for phage P39 to bind to its host pathogen. This data also suggests that a secondary receptor may be involved as seen in the decreased plating efficiency for the K-12 strain. This would be similar to the known receptors identified in literature for bacteriophage T4.

4. Discussion

4.1 Isolating Phage Resistant Mutants

Initial efforts were focused on isolating mutants that were recovered from an overnight mixture of phage and bacteria. These isolates were tested both qualitatively and quantitatively to determine if the susceptibility of the isolate had changed in relation to the parent strain used for the initial inoculation. The Ec4 strain was sensitive to both bacteriophage P39 (T4-like) and R26 (T1-like) while the Ec2 strain was only sensitive to P39. Therefore, two combinations were prepared from the Ec4 parental strain: P39-resistant (Ec4P39) and R26-resistant (Ec4R26) mutants. Ten of the Ec4P39 mutants isolated were found to be resistant to P39 and sensitive to R26. These isolates were of interest for further characterization because deducing the mutation(s) leading to resistance to P39 could help identify a receptor site for P39. For Ec4R26, 12 isolates were characterized. Ten isolates were resistant to R26 and sensitive to P39 while two isolates (Ec4R26-R11 and -R12) were resistant to both P39 and R26. These latter two mutants were different from Ec4 in that they had a deep rough-like phenotype. All of these isolates where deemed to be of use in deducing the receptor site of R26. Three of the isolates that were selected that were resistant to R26 by the qualitative method, Ec4R26-R7, -R9 and -R10, had a reduction in plating efficiency of seven logs by the quantitative method. The remaining isolates Ec4R26-R1 to -R6, and -R8 were resistant to R26 based on the detection limit of the assay. For a subset of both the P39 and R26-resistant isolates, the possibility that loss of sensitivity was due to a change in the phage receptor site was explored. Mutations in the genes that regulate or code for receptor sites are expected to allow bacteria to escape from the selective pressure of infecting phage. By characterizing

these resistant mutants, it was expected that information could be obtained on these receptor sites (Forde and Fitzgerald, 1999; Tanji, *et al.*, 2004). In order to test whether the resistant mutants were in fact *E. coli* O157:H7-derived strains, a latex serology test for the *E. coli* O157 antigen was performed as well as PCR-based assays for six virulence factors characteristic of *E. coli* O157:H7 (Paton and Paton, 1998; Gannon *et al.,* 1997). With the exception of the two isolates, Ec4R26-R11 and -R12, all samples were serology positive. All of the isolates gave PCR products that matched the expected fragment sizes for all six virulence genes used for screening: $rfbE_{O157}$, stx_1, stx_2, *HlyA, EaeA,* and $fliC_{H7}$, including the two serology negative mutants. Based on these results, it was concluded that all 22 isolates were derived from *E. coli* O157:H7 and were further characterized for bacteriophage P39 and R26 receptor sites. The serology negative strains were of special interest since they were also resistant to bacteriophage P39 and had the most obvious phenotype difference from the parental strain. These two mutants appear to have significantly altered cell surface features to account for these observations.

4.2 Bacteriophage Adsorption

In order to determine the cause for resistance of the mutant strains, an adsorption assay was performed to determine if phage were able to bind to the bacteria, in spite of their inability to lyse the cells (Morita *et al.*, 2002). An adsorption assay that monitored the depletion of phage pfu from the supernatant over time when mixed with different isolates of bacteria was used. In order to ensure that loss of phage in handling and non-specific binding did not affect the results, controls were set-up with *E. coli* K-12 that does not bind these bacteriophage as well as a no bacteria control with broth substituted for bacteria. All values were compared to time '0' levels. In this assay, a reduction in

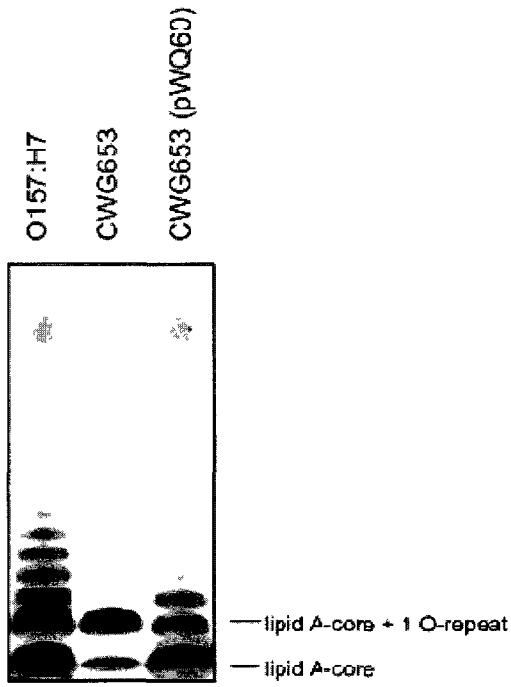
bacteriophage levels in the supernatant over time would indicate that the bacteriophage is adsorbing to the mutant bacterial strain while no change in pfu values in the filtrate would indicate that the bacteriophage is unable to bind, possibly due to a missing or incompatible receptor site on the bacterial surface (Morita *et al.*, 2002). This is an important distinction to make because it has been shown previously that some bacteria can digest phage DNA in the cytoplasm with restriction endonucleases as a defense mechanism against invading phage (Forde and Fitzgerald, 1999). Therefore, the resistance to the bacteriophage can be caused by different mechanisms. For bacteriophage P39, the sensitivity of the bacteria determined by the quantitative method matched the results observed by the adsorption assay; all strains that were resistant to P39 did not adsorb P39. This suggested that, as a group, the Ec4P39 mutants had an altered receptor site for this phage. A more complicated situation was observed for the R26-resistant mutants. R26 was able to bind to, but unable to plaque on, a number of Ec4R26 in addition to Ec2. R26 did not appear to have any modifications to its genomic DNA (as determined by GLSI) and is therefore susceptible to digestion by many different restriction enzymes. Therefore it is likely that restriction endonucleases present in the cytoplasm of Ec2 are able to digest R26's genome as it is introduced thus making the bacterium resistant to the phage (Forde and Fitzgerald, 1999). These assays provided some insight into strains with possible changes in receptor sites that are the most interesting to investigate for the project. Some of the mutants may also be of great interest for future research into other potential mechanisms for bacterial defense against bacteriophage, such as changes in restriction enzyme factors.

4.3 R26 Receptor Identification

It is well known that bacteriophage adhere to a surface component of the host cell, usually either a surface protein or LPS (Kutter and Sulakvelidxe, 2005; Mizoguchi *et al.*, 2003). Therefore, the LPS patterns of all the strains resistant to R26 were investigated to determine if a pattern emerged. Most of the Ec4R26 isolates had altered LPS patterns when compared to Ec4. Four isolates Ec4R26-R1, -R7, -R9, and -R10 had similar patterns to Ec4, and all except Ec4R26-R1 displayed some reactivity with phage R26. It is possible that these isolates had a minor change in the LPS at the receptor site that could not be resolved with this assay. Unfortunately the sensitivity of the LPS analysis allows detection of only major changes in the LPS profile and is not sufficient to deduce minor changes. The isolates with major changes in the LPS pattern fell into two groups. The first group consisted of Ec4R26-R2, -R3, -R4, -R5, -R6, and -R8 which showed truncated LPS but still contain at least one O-repeat as seen in **Fig. 29** (Kaniuk *et al.*, 2004). R26 is unable to bind to these mutants. This suggests that the portion of the LPS that is missing in these mutants is essential for R26 binding and that the difference between the LPS patterns of these isolates and those of Ec4R26-R7, -R9, and -R10 might explain why there is a difference in R26 sensitivity in these organisms. The second group consisted of Ec4R26-R11 and -R12 that portrayed a deep rough-like phenotype (Kaniuk *et al.*, 2004), and for which not even a single O-repeat was present (**Fig. 29**). This would suggest that the LPS is truncated in these mutants between the lipid A which is embedded in the membrane of the organism and the outer core region. Neither P39 not R26 phage bound to these isolates.

Figure 29: Typical LPS pattern for *E. coli* O157:H7 LPS mutant.

Illustrates the LPS profiles of *E. coli* O157:H7, a mutant strain of *E. coli* O157:H7 (CWG653) with truncated LPS, and the same mutant strain complemented with pWQ60 (to restore O antigen).



(Modified from Kaniuk *et al.*, 2004)

The observation that Ec4R26-R11 and -R12 were deep rough-like mutants could also explain the negative result for the serology test using the latex agglutination kit from Oxoid (Oxoid, Catalog # DR0620). If the O antigen is severely truncated or completely missing, then it is not likely to be recognized by the antibody directed against the O-antigen. This would explain why these isolates were negative by serology and positive by PCR. There are two possible explanations why the isolates did not react with the two bacteriophage. First, it is possible that a receptor site or some component of the receptor site is missing for both bacteriophage. In the case of R26, the primary receptor site may be missing in the truncated LPS, while a secondary receptor site in the core LPS may be missing for P39. This has been suggested by previous data for other T4-like phage. The second possibility is that because the LPS is severely modified in these isolates, the membrane is not stable and the target receptor sites are not available in the proper conformation for bacteriophage binding. Earlier studies have shown that LPS is required for proper membrane structure (Laird *et al.*, 1994). It should also be noted that the morphology of the deep rough mutants is very distinct; they are slimy and do not form discrete colonies like typical *E. coli*. LPS is closely associated with outer membrane proteins and it has been shown that deep rough mutants, that lack part of the LPS inner core, possess significantly reduced amounts of porin proteins (Laird *et al.*, 1994). It has also been shown that LPS mutants lacking several *rfa* genes that are responsible for the synthesis of LPS outer core and for phosphorylating the heptose residues of the inner core also produce reduced levels of porins (Laird *et al.*, 1994). Lack of phosphorylation prevents neighboring LPS molecules in the outer membrane from being cross-linked by divalent cations. This results in the destabilization of the outer membrane and leads to the

phenotype known as deep rough. Due to the improper expression of proteins on the cell surface in these mutants, the viability of these strains in a natural environment is compromised. Many of these cell surface proteins serve a function as ion channels or pumps for the organisms, and the cells may not survive if they are not expressed properly. Earlier studies have shown that deep rough mutants are unable to colonize in the large intestine (Laird *et al.*, 1994; Moller *et al.*, 2003). This would severely compromise the bacterium's viability and would bring in to question the relevance of these isolates in a natural setting. Due to the complexity of the LPS in *E. coli* species these mutants were not investigated in great depth. The deep rough-like mutants were pursued by examining the genes involved in attaching the LPS to the lipid A that is embedded in the membrane of the bacteria. The LPS is controlled by two gene clusters. The O antigen is controlled by a 13 gene cluster, while the inner/outer core is controlled by a 11 gene cluster (Samuel *et al.* 2004; Wang and Reeves 1998; Kaniuk *et al.*, 2004). For the deep rough-like mutants, selected genes involved in coding for LPS pathway components were investigated to determine the cause for the deep rough-like phenotype. Primers were designed for *kdtA*, *waaP, waaY, rfaC, and rfaF*. All five primer sets gave PCR products of the expected size. However, the PCR product for *rfaF* digested with *Hae*III showed a difference in the pattern. Comparing the sequences of PCR products from Ec4, Ec4R26-R11 and -R12 to the published sequence, a point mutation was identified in Ec4R26-R12. This point mutation eliminated the *Hae*III site (GGCC) and introduced a premature stop codon in the gene sequence that truncated the rfaF product to 127 amino acids from 348 amino acids. The *rfaF* gene generates a LPS heptosyltransferase II that is required to attach the second heptose to lipid A in the inner

core region of the LPS (Makino *et al.*, 1999; Kaniuk *et al.*, 2004). Based on the sequence and the LPS patterns for this isolate, it is likely that this protein is not functional and could lead to the observed deep rough-like phenotype of Ec4R26-R12. The mutation that caused the deep rough mutant Ec4R26-R11 was not identified in this study. The *rfaF* gene for Ec4R26-R11 was also sequenced and had 100% identity to that of Ec4 and the published sequence (Makino *et al.,* 1999). It is possible that a point mutation was present in one of the other genes involved in the LPS pathway. It is important to note that these two deep rough mutants are in fact distinct isolates suggesting that this is not necessarily a rare occurrence. As stated previously, the ability of these mutants to survive in a natural setting may be limited due to their unstable membrane and inability to colonize (Laird *et al.*, 1994 and Moller *et al.*, 2003). However the fact that these mutants are able to evade both bacteriophage is important. This could allow these deep rough mutants to have a competitive advantage in the presence of bacteriophage. However the low viability of these mutants would limit their impact. The possibility of these pathogens regaining viability through another mutation while maintaining their resistance to the present cocktail of phages cannot be ruled out. In such a scenario, these mutants could pose a concern in the future. However to address this concern, new bacteriophage that act on these mutants can be identified and used in the cocktail to eliminate them.

It is important to note that the phage cocktail needs to be dynamic because resistance to the cocktail might develop over a period of time. New phages that have already been characterized in detail and are available as part of a phage library can be screened and those shown to be effective against the newly resistant pathogens, can be added to eliminate new threats. To get a head start against the bacterium's efforts to

evade bacteriophage therapy, phage resistant mutants can be generated as described in this study and screened against a library of phages to identify potential candidates for future phage cocktails.

4.4 P39 Receptor Identification

It is well known that bacteriophage adhere to a cell surface component of the host bacterium, either a surface protein or LPS (Kutter and Sulakvelidxe, 2005; Mizoguchi *et al.*, 2003). At this point the LPS patterns of all the strains resistant to P39 were investigated to determine if a similar pattern emerged that was observed for the R26-resistant mutants. All of the Ec4P39 mutants displayed an LPS pattern that was comparable to the parent strain Ec4. This would suggest that the mutations either did not effect LPS , or any mutations that did occur in these strains were undetectable due to the lack of resolution capable with this assay. P39 is a T4-like phage as determined by GLSI (data not published). Literature searches revealed that OmpC and LPS are both involved in T4 binding (Yu and Mizushima, 1982). Since there were no obvious alterations in the LPS profiles in the P39-resistant mutants, possible mutations in OmpC were explored. Protein profiles were investigated but the resolution required to identify a single missing protein could not be achieved with 1D gels. Western blots for the OmpC protein would have been the ideal approach, but the required primary OmpC antibody was not commercially available and hence alternative approaches were evaluated. Another proposed approach was to probe non-denatured protein gels with P39 followed by detection of the phage using an anti-phage antibody. However the required antibodies against P39 were not available at GLSI at the time of this research. For all the above reasons, genomic analysis of *ompC* was carried out using Southern blot- and PCR-based

approaches. PCR primers that amplify the *ompC* gene were designed along with primers that were internal and external to the gene. None of the primers produced amplicons for Ec4P39-R1 and -R5, but did amplify the expected fragments for Ec4 (the parent strain). In order to determine if the *ompC* gene was present in these mutants, Southern blots were performed with the full length *ompC* PCR product from Ec4 as the probe that indicated a deletion had occurred between the two *Hin*dIII sites flanking the *ompC* gene in Ec4P39-R1 and -R5 that could explain the observed results. Based on the results of Southern blot analyses combined with the inability to amplify an *omp*C gene product by PCR using published internal and external primers, it was hypothesized that a portion of the *ompC* gene was deleted. An illustration of the inferred deletion is presented in **Fig. 30**.

In order to map this deletion further, new primers flanking the 5' and 3' ends of the *ompC* gene were designed. PCR studies using these new primer sets showed that only primers at the 3' end of the gene were able to amplify PCR products in the two mutants of interest further suggesting a deletion at the 5' end of the *ompC* gene. Primers designed within the *Eco*RI site internal to the *ompC* gene were not able to amplify PCR products from these mutants further confirming the Southern blot results. Based on these results, primers were designed using sequences at 500 bp intervals from the 5' end of the *ompC* gene. This was done to map the size of the deletion and also to obtain a PCR product that could be sequenced to confirm the exact site of the deletion. At this point, all Ec4P39 mutants were screened with the new primers and deletions of varying sizes were observed for all but one of these mutants. The isolates appeared to fall into four distinct groups as summarized in **Table 8**.

Figure 30: Proposed deletion in Ec4P39-R1 and -R5 *ompC* genes.

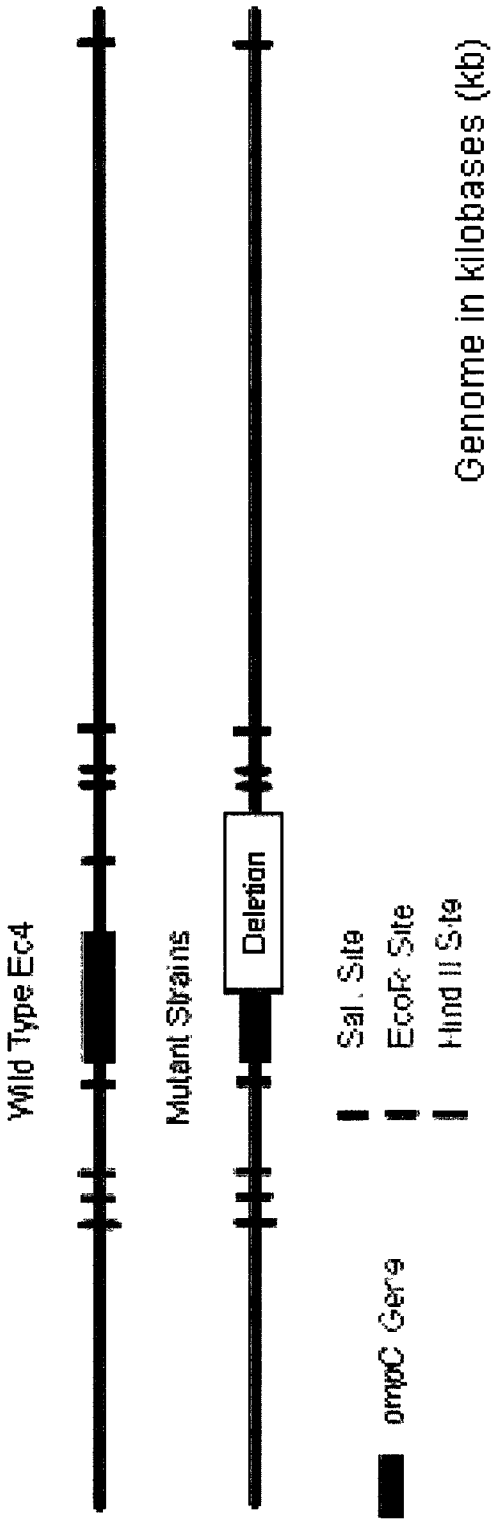


Table 8: Summary of deletion sizes for Ec4P39 mutants.

Isolate	Primers used to generate an amplicon	Size of PCR Product	Proposed Deletion Size
Ec4P39-R1, -R4, -R5, -R8, -R9 and -R10	OMPCintF / OMPCext3R	~600 bp	~1.8 kb
Ec4P39-R2 and -R6	No Amplicon Generated	N/A	>2.9 kb
Ec4P39-R3	OMPCintF / OMPCext4R	~350 bp	~2.55 kb
Ec4P39-R7	Oc5end / Oc3end	~1100 bp	N/A

PCR products from two deletion mutants, Ec4P39-R7 and -R3, were sequenced along with Ec4, the parent strain. Sequence obtained from Ec4 was identical to the published sequence of *E. coli* O157:H7 (Makino *et al.*, 1999). The Ec4P39-R7 sequence revealed a 46 base insertion near the 5' end of the *ompC* gene that would result in a premature stop codon and a truncated OmpC protein in this mutant. The insertion is a simple direct repeat slippage event (**Fig. 24**). Replication slippage is often invoked to explain small deletions and additions in DNA, which often lead to frame shift mutations as seen in this isolate (Tran *et al.*, 1995; Tippin *et al.*, 2004). The resulting severely truncated protein would not function as the full length protein and likely explains why there is a loss of reactivity with P39, further pointing towards OmpC being required for phage binding.

The sequence obtained from *ompC* from Ec4P39-R3 revealed a deletion, in comparison to the published sequence and Ec4, that includes 779 bp at the 5' end of the gene and 1790 bp 5' to the start of the gene including the *ompC* promoter for a total deletion of 2569 bp. Included in the large deletion is the *micF* regulatory sequence. This is normally located 334 bp from the 5' end of the *ompC* gene (Makino *et al.*, 1999). It is interesting to note that *E. coli* B has a truncated version of the *ompC* gene that is not functional similar to this isolate. The *IS1-13* element in *E. coli* B is associated with a deletion of the promoter and first 114 bp of *ompC*. *E. coli* B does not express OmpC, which is likely due to this deletion associated with *IS1-13* which also includes *micF* (Schneider *et al.*, 2002). Based on these observations with *E. coli* B, and data from the present study that indicated that a number of the P39-resistant mutants had deletions within the *ompC* region, it is possible that a similar *IS1-13* mechanism gave rise to the deletions observed in *ompC* of all but one of the Ec4P39 mutants in this study.

To confirm that the observed *ompC* mutations were responsible for the loss of P39 sensitivity, complementation studies were done using an expression vector carrying a copy of wild-type OmpC gene from *E. coli* O157:H7. The results of this assay showed that the plating efficiency of P39 on Ec4P39-R3 returned to wild-type levels when complemented with OmpC from *E. coli* O157:H7. Also, when a non-host strain, E. coli K-12, was complemented with the wild-type OmpC gene from *E. coli* O157:H7, P39 was able to titer on this strain. These observations strongly suggest that the OmpC from *E. coli* O157:H7 is a potential receptor essential for P39 binding.

Even though the results of this research clearly support the hypothesis that OmpC is a potential receptor for phage P39, similar to what has been observed with other T4-like phages, since displacement studies using the OmpC protein were not carried out, other hypotheses also have to be presented as potential mechanisms. These include the possiblity that OmpC could play the role of a secondary receptor or as a helper protein involved in stabilizing the actual receptor.

In this study only mutations in the OmpC gene were identified for the P39-resistant mutants. However, if more mutants were screened, it is possible that mutations in other regulatory genes that alter OmpC expression such as the OmpB operon involved in regulating OmpC expression may have been identified (Comeau *et al.*, 1985). Even though such a scenario of mutations in the regulatory region of the o*mpC* gene would point to a different mechanism of resistance development, it would still confirm the importance of the OmpC protein in phage P39 binding to *E. coli* O157:H7 since complementing the resistant clones with the OmpC protein was sufficient to revive P39 phage binding. The possibility of mutations in other surface proteins cannot be ruled out,

though it is unlikely since molecules other than LPS and OmpC have not been observed to be receptor molecules in the case of T4-like phages, the family to which phage P39 belongs.

In all the data presented in this thesis, it has been demonstrated that OmpC is clearly essential for P39 binding of *E. coli* O157:H7 and mutations to this gene allow the pathogen to escape infection from P39. In the case of the deep rough mutants that do not bind phage P39 the involvement of LPS as a secondary binding site is strongly suggested.

5. Conclusion

LPS was identified as a potential receptor site for R26. Eight out of twelve Ec4 mutants, resistant to R26, were shown to have truncated LPS patterns. Genomic DNA sequence analysis revealed a mutation in the *rfaF* gene of one of two deep rough-like mutants isolated. This gene is in the LPS biosynthetic pathway, and its loss of function would cause an altered LPS profile. It is reasonable to assume that other mutations within the LPS pathway could be the cause of the altered LPS profiles observed in the other R26 mutants. This hypothesis however requires further experimental verification.

All 10 mutants that were resistant to P39 were characterized and shown to contain mutations in the *ompC* gene by PCR, two of these were subsequently confirmed by DNA sequencing. By complementation studies, it was demonstrated that the plating efficiency of P39 on one of these confirmed *ompC* mutants (Ec4P39-R3) could be restored by complementation with *E. coli* O157:H7 *ompC*. This clearly showed that OmpC is an essential requirement for P39 binding to *E. coli* O157:H7. It was also demonstrated that P39 will plate with low efficiency on *E. coli* K-12 when complemented with OmpC from *E. coli* O157:H7, further reinforcing the role of this protein in P39 binding.
Through the use of the resistant mutants against bacteriophage P39 and R26 it was clearly demonstrated that P39 and R26 interact with different receptor sites on the bacterial surface of *E. coli* O157:H7. Such bacteriophage, which interact with unique receptor sites on the bacterial surface, would be suitable candidates for use in a phage cocktail to reduce *E. coli* O157:H7 levels in cattle.

6. Literature Cited

Agriculture and Agri-Food Canada, 2008, 2007 ANNUAL LIVESTOCK AND MEAT REPORT, Red Meat Market Information, http://www.agr.gc.ca/redmeat/almr2007.htm

Armstrong, G. L., Hollinsworth, J., and Morris, G. J. 1996, Emerging Foodborne Pathogens: *Escherichia coli* O157:H7 as a Model of entry of a New Pathogen into the Food Supply of the Developed World, Epidemiological reviews, **18**, 29-51.

Babalova, E. G., Katsitadze, K. T., Sakvarelidze, L. A., Imnaishvili, N. S., Sharashidze, T. G., Badashvili, V. A., Kiknadze, G. P., Meipariani, A. N., Gendzekhadze, N. D., Machavariani, E. V., Gogoberidze, K. L., Gozalov, E. I., and Dekanosidze, N. G., 1968, Prevenative value of dried dysentery bacteriophage, Zh. Mikrobiol. Epidemiol. Immunobiol, **2**, 143-145.

Barkocy-Gallagher, G. A., Arthur, T. M., Rivera-Betancourt, M., Xiangwu, N., Shackelford, S. D., Wheeler, T. L., and Koohmaraie, M., 2003 Seasonal prevalence of shiga toxin-producing Escherichia coli, including O157 :H7 and non-O157 serotypes, and Salmonella in commercial beef processing plants Journal of food protection , 66, 1978-1986.

Becker, G. S., 2006, Farm and Agriculture Issues, Kovinka, Chapter 5, 74.

Belson, K., and Fahim, K., 2007, After Extensive Beef Recall, Topps Goes Out of Business, New York Times.

Bio-Rad, InstaGene™ Matrix, Catalog #732-6030, LIT544 Rev G.

Bio-Rad, MicroPulser ™ Electroporation Apparatus Operating Instructions and Applications Guide, Catalog Number 165-2100.

Buchanan, R. L., Edelson, S.G., Snipes, K., and Boyd, G., 1998, Inactivation of *Escherichia coli* O157:H7 in Apple Juice by Irradiation, Applied and Environmental Microbiology, **64**, 4533–4535.

Calender, R., 1988, The Bacteriophage Volume 1, Plenum Press, New York, USA, 235-258.

Centers for Disease Control and Prevention, 2007, Preliminary FoodNet Data on the Incidence of Infection with Pathogens Transmitted Commonly Through Food --- 10 States, MMWR, **56**, 336-339.

Chart, H., Scotland, S. M., and Rowe, E. 1989, Serum Antibodies to *Escherichia coli* Serotype O157:H7 in Patients with Hemolytic Uremic Syndrome, Journal of Clinical Microbiology, **27**, 285-290.

Chart, H. 2000, Clinical Significance of Verocytotoxin-producing *Escherichia coli* O157, World Journal of Microbiology and Biotechnology, **16**, 719-724.

Comeau, D. E., Ikenaka, K., Tsung, K., and Yorinouye, M., 1985, Primary Characterization of the Protein Products of the *Escherichia coli ompB* Locus: Structure and Regulation of Synthesis of the OmpR and EnvZ Proteins, Journal of Bacteriology, **164**, 578-584.

Delbruck M. and Luria S. E., 1942, Interference between bacterial viruses. I. Interference between two bacterial viruses acting upon the same host and the mechanism of virus growth, Archs. Biochem. **1**, 111-141.

d'Herelle, F., 1917, Sur un microbe invisible antagoniste des bacilles dysenteriques, Compt Rend Acad, Sci, **165**, 373-375.

Forde, A., and Fitzgerald, G.F., 1999, Bacteriophage defence systems in lactic acid bacteria, Antonie van Leeuwenhoek, **76,** 89–113.

Gannon, V. P. J., D'Souza, S., Graham, T., King, R. K., Rahn, K., and Read, S., 1997, Use of the Flagellar H7 Gene as a Target in Multiplex PCR Assays and Improved Sensitivity in Identification of Enterohemorrhagic *Escherichia coli* Strains, Journal of Clinical Microbiology, **35**, 656-662.

Hankin, E. H., 1896, L'action bactericide des eaux de la Jumna et du Gange sur le vibrion du cholera. Ann. Inst. Pasteur, **10,** 511.

Hendrix, R. W., Smith, M. C. M., Burns, R. N., Ford, M. E., and Hatfull, G. F., 1999, Evolutionary relationships among diverse bacteriophages and prophages: All the world's a phage Proc. Natl. Acad. Sci., **96**, 2192–2197.

Henning, U., and Jann, K., Two-Component Nature of Bacteriophage T4 Receptor Activity in Escherichia coli K-12, 1979, Journal of Bacteriology, **137,** 664-666.

Hermos, J. A., Garcý, J. L., and Garcý, P., 2007, Taking aim on bacterial pathogens: from phage therapy to enzybiotics, Current Opinion in Microbiology **10**, 461–472.

Hussein, H. S., and Bollinger, L. M. 2005, Prevalence of Shiga Toxin-Producing *Escherichia coli* in Beef Cattle, Journal of Food Protection, **68**, 2224-2241.

Kaniuk, N. A., Vinogradov, E., Li, J., Monteiro, M. A., and Whitfield, C., 2004, Chromosomal and Plasmid-encoded Enzymes Are Required for Assembly of the R3-type Core Oligosaccharide in the Lipopolysaccharide of *Escherichia coli* O157:H7 The Journal of Biological Chemistry, **279**, 31237-31250.

Karch, H., Tarr, P. I., and Bielaszewska, M. 2005, Enterohaemorrhagic *Escherichia coli* in himan medicine, International Journal of Medical Microbiology, **295**, 405-418.

Killmann, H., Braun, M., Herrmann, C., and Braun, V., 2001, FhuA Barrel-Cork Hybrids Are Active Transporters and Receptors, Journal of Bacteriology. **183**, 3476-3487.

Kutter, E., and Sulakvelidxe, A., 2005, Bacteriophage Biology and Applications, CRC Press, Florida, USA 5-6, 30 and 42.

Laird, M. W., Kloser, A. W., and Misra, R., 1994, Assembly of LamB and OmpF in Deep Rough Lipopolysaccharide Mutants of *Escherichia coli* K-12, Journal of Bacteriology, **176**, 2259-2264.

Langenscheid, J., Killmann , H., Braun, V., 2004, A FhuA mutant of Escherichia coli is infected by phage T1-independent of TonB, FEMS Microbiology Letters, **234**, 133-137.

Law, D. 2000, The history and evolution of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*, World Journal of Microbiology and Biotechnology, **16**, 701-709.

Leiman, P. G., Kanamaru, S., V.V.Mesyanzhinov, V. V., Arisaka, F. and Rossmann M. G. 2003, Structure and morphogenesis of bacteriophage T4, Cellular and Molecular Life Sciences. **60,** 2356–2370.

Lorch, A., 1999, Bacteriophages: An alternative to antibiotics?, Biotechnology and Development Monitor, **39**, 14-17.

Makino, K., Yokoyama, K., Kubota, Y., Yutsudo, C.H., Kimura, S., Kurokawa, K., Ishii, K., Hattori, M., Tatsuno, I., Abe, H., Iida, T., Yamamoto, K., Onishi, M., Hayahi, T., Yasunaga, T., Honda, T., Sasakawa, C., and Shinagawa, H., 1999, Complete nucleotide sequence of the prophage VT2-Sakai carrying the verotoxin 2 genes of the enterohemorrhagic Escherichia coli O157:H7 derived from the Sakai outbreak, Genes and Genetic Systems, **74**, 227-39.

Mandeville, R., Griffiths, M., Goodridge, L., McIntyre, L., and Ilenchuk, T.T., 2003, Diagnostic and Therapeutic Applications of Lytic Bacteriophage, Analytical Letters, **36**, 3241 - 3259

Millipore, 2005, Immobilon-Ny+ Transfer Membrane Guide, P36007 Rev. D.

Mizoguchi, K., Morita, M., Fischer, C. R., Yoichi, M., Tanji, Y., and Unno, H., 2003, Coevolution of Bacteriophage PP01 and *Escherichia coli* O157:H7 in Continuous Culture, Applied and Environmental Microbiology, **69**, 170-176.

Møller, A. K., Leatham,M. P., Conway, T., Nuijten, P. J. M., de Haan, L. A. M., Krogfelt, K. A., and Cohen, P. S., 2003, An *Escherichia coli* MG1655 Lipopolysaccharide Deep-Rough Core Mutant Grows and Survives in Mouse Cecal Mucus but Fails To Colonize the Mouse Large Intestine, Infection and Immunity, **71**, 2142–2152.

Morita, M., Tanji, Y., Mizoguchi, K., Akitsu, T., Kijima, N., and Unno, H., 2002, Characterization of a virulent bacteriophage specific for *Escherichia coli* O157:H7 and analysis of its cellular receptor and two tail fiber genes, FEMS Microbiology Letters, **211**, 77-83.

National Cattlemen's Beef Association, 2006, *E. coli* O157 Solutions: The Pre-harvest Commitment. www.bifsco.org/uDocs/e_colisolutions_preharvest.pdf.

Oxoid, *E.coli* O157 Latex Test Kit, Catalog # DR0620.

Paton, C. J., and Paton, A. W., 1998, Pathogenesis and Diagnosis of Shiga Toxin-Producing *Escherichia coli* Infections, Clin Microbiol Rev., **11**, 450-79.

Public Health Agency of Canada. 2003, Investigation of an *E. coli* O157:H7 Outbreak in Brooks, Alberta, June-July 2002: The Role of Occult Cases in the Spread of Infection within a Daycare Setting, Canada Communicable Disease Report, **29**, 21-28

Public Health Agency of Canada. 2005, http://dsol-smed.phac-aspc.gc.ca/dsol-smed/ndis/index_e.html.

Public Health Agency of Canada, 2006, Notifiable Diseases by Province for Decmber 2006 (Preliminary), Notifiable Diseases Monthly Report, December, 1-3.

Qiagen, 2002, QIAquick® Spin Handbook.

Qiagen, 2005, QIAGEN® Plasmid Purification Handbook, 3rd Edition.

Riley, L. 1987, The epidemiologic, clinical and microbiologic features of hemorrhagic colitis, Annual Review of Microbiology, **41**, 383-407.

Roche, 2004, DIG DNA Labeling and Detection Kit, **October 2004**.

Rossmann, M. G., Mesyanzhinov, V. V., Arisaka, F., and Leiman, P. G., 2004, The bacteriophage T4 DNA injection machine, Current Opinion in Structural Biology, **14,** 171–180.

Samuel, G., Hogbin, J. P., Wang, L., and Reeves. P.R., 2004, Relationships of the *Escherichia coli* O157, O111, and O55 O-Antigen Gene Clusters with Those of *Salmonella enterica* and *Citrobacter freundii*, Which Express Identical O Antigens, Journal of Bacteriology, **186**, 6536-6543.

Schneider, D. Duperchy, E., Depeyrot, J., Coursange, E., Lenski, R. E., and Blot, M., 2002, Genomic comparisons among *Escherichia coli* strains B,K-12,and O157:H7 using IS elements as molecular markers, BMC Microbiology, **2,** 1-8.

Statistics Canada, 2007, Population and Dwelling Counts, 2006 Census, 97-550-XIE, 1-47.

Tanji Y., Shimada, T., Yoichi, M., Miyanaga, K., Hori, K., and Unno, H. 2004, Toward rational control of *Escherichia coli* O157:H7 by a phage cocktail, Applied Microbiology and Biotechnology, **64**, 270-274.

Thiel, K., 2004, Old dogma, new tricks – 21st Century phage therapy, Nature Biotechnology, **22**,

Tippin, B., Kobayashi, S., Bertram, J.G., and Goodman, M. F., 2004, To Slip or Skip, Visualizing Frameshift Mutation Dynamics for Error-prone DNA Polymerases' Journal of Biological Chemistry, **279**, 45360-45368.

Tran, H. T., Degtyareva, N. P., Koloteva, N.N., Sugino, A., Masumoto, H., Gordenin, D. A., and Resnick, M. A., 1995, Replication Slippage between Distant Short Repeats in *Saccharomyces cerevisiae* Depends on the Direction of Replication and the *RAD50* and *RAD52* Genes, Molecular and Cellular Biology, **15**, 5607-5617.

Twort, F. W., 1915, An investigation on the nature of ultra-microscopic viruses, Lancet, **186**, 1241–1243.

USDA NASS, 2008, Livestock Slaughter 2007 Summary, Issue 04-2008, http://www.nass.usda.gov/Statistics_by_State/Nebraska/Publications/Cattle_Feeders_News/2008/cfn0804.pdf.

Wang, L., and Reeves, P.R., 1998, Organization of *Escherichia coli* O157 O Antigen Gene Cluster and Identification of Its Specific Genes, Infect Immun, 663, 545-3551.

Yu, F., and Mizushima, S., 1982, Roles of Lipopolysaccharide and Outer Membrane Protein OmpC of Escherichia coli K-12 in the Receptor Function for Bacteriophage T4, Journal of Bacteriology, **151,** 718-722.

Yu, S. L., Ding, H. C., Seah, J. N., Wu, K. M., Chang, K. S. S., Tam, M. F., Syu, W. J., 1998, Characterization of a Phage Specfic to Hemorrhagic *Escherichia coli* O157:H7 and Disclosure of Variation in Host Outer Membrane Protein OmpC, J Biomed Sci., 5, 370-382.