Bacteriophage therapy: a novel method of lytic phage delivery

Ratree Platt
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd
Part of the Microbiology Commons, and the Molecular Biology Commons

Recommended Citation
https://lib.dr.iastate.edu/rtd/13922

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI  48106-1346 USA

UMI®
800-521-0600
Bacteriophage therapy: A novel method of lytic phage delivery

by

Ratree Platt

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology

Major Professors: Donald L. Reynolds and Gregory J. Phillips

Iowa State University

Ames, Iowa

2000

Copyright © Ratree Platt, 2000. All rights reserved.
This is to certify that the Doctoral dissertation of

Ratree Platt

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Co-major Professor

Signature was redacted for privacy.

Co-major Professor

Signature was redacted for privacy.

For the Major Program

Signature was redacted for privacy.

For the Graduate College
# TABLE OF CONTENTS

## LIST OF FIGURES

v

## LIST OF TABLES

vi

## ABSTRACT

vii

## CHAPTER 1. GENERAL INTRODUCTION

1

- Introduction 1
- Dissertation Organization 4
- Literature Review 5
  - Discovery of bacteriophages 5
  - General characteristics of bacteriophages 6
  - Lytic versus lysogenic cycle 7
  - History of bacteriophage research 8
  - Application of bacteriophages 10
  - Pre-antibiotic bacteriophage therapy research 11
  - Post-antibiotic bacteriophage therapy research 13
  - \( \lambda \) and P22 phages 15
  - Repressor systems of \( \lambda \) and P22 phages 16
  - Site-specific recombination systems of \( \lambda \) and P22 phages 18
- References 21

## CHAPTER 2. GENETIC SYSTEM FOR REVERSIBLE INTEGRATION OF DNA CONSTRUCTS AND lacZ GENE FUSIONS INTO THE *ESCHERICHIA COLI* CHROMOSOME.

33

- Abstract 33
- Introduction 33
- Materials and Methods 37
- Results and Discussion 42
- Acknowledgements 46
- References 47

## CHAPTER 3. REVERSIBLE INTEGRATION OF DNA CONSTRUCTS INTO THE CHROMOSOMES OF *ESCHERICHIA COLI* AND *SALMONELLA TYPHIMURIUM* BY A BACTERIOPHAGE P22-MEDIATED SITE-SPECIFIC RECOMBINATION SYSTEM.

58

- Abstract 58
- Introduction 59
- Materials and Methods 61
- Results 69
- Discussion 72
- Acknowledgements 76
- References 77
### CHAPTER 4. BACTERIOPHAGE THERAPY: A NOVEL METHOD OF LYtic BACTERIOPHAGE DELIVERY

- Abstract 86
- Introduction 87
- Materials and Methods 90
- Results 93
- Discussion 95
- Acknowledgements 98
- References 98

### CHAPTER 5. GENERAL CONCLUSIONS

- General Summary 106
- General Discussion 108
- Recommendations for Future Research 110
- References 112

### ACKNOWLEDGEMENTS

113
LIST OF FIGURES

Figure 1.1 Right operator region of bacteriophage λ. 17

Figure 2.1 Plasmids for integration of DNA constructs into the E. coli chromosome at attB. 54

Figure 2.2 Vectors for construction and integration of lacZ gene fusions into the E. coli chromosome vectors. 55

Figure 2.3 Helper plasmids for integration and excision of integration vector. 56

Figure 2.4 Integration of suicide vectors by site-specific recombination and confirmation of integration by PCR. 57

Figure 3.1 Plasmid vectors for integration. 82

Figure 3.2 Helper plasmids for integration and excision. 83

Figure 3.3 PCR confirmation of plasmid integration in E. coli and S. typhimurium. 84

Figure 3.4 DNA sequences of attL and attR regions of E. coli chromosome. 85

Figure 4.1 Formation of a cointegrant by the homologous recombination. 103

Figure 4.2 PCR confirmation of the presence of the W30 KmR λ lysogen. 104

Figure 4.3 The in vitro test result. 105
LIST OF TABLES

Table 2.1  Bacterial strains.  
Table 2.2  Plasmids constructed in this study for site-specific recombination into the *E. coli* chromosome.  
Table 2.3  Retrieval of pCD11PlacZ by pXINT129-mediated site-specific recombination.  
Table 3.1  Bacterial strains and bacteriophages used in this study.  
Table 3.2  Plasmids used in this study and their relevant genotypes.  
Table 4.1  Bacteria, bacteriophages and plasmids involved in this study.
ABSTRACT

The widespread emergence of multi-antibiotic resistant bacteria has increased the need for alternatives to conventional antibiotic therapy. Accordingly, a significant amount of effort has been made to investigate the potential use of bacteriophages as prophylactic and therapeutic agents for bacterial infections. In this study, molecular biological techniques were applied to construct a lysogen of lytic bacteriophage \( \lambda \) in an attempt to combat with multi-antibiotic resistant bacteria by a novel method of lytic phage delivery.

To accomplish this goal, two plasmid-based site-specific recombination (SSR) systems for integration and recovery of DNA constructs from \textit{Escherichia coli} and \textit{Salmonella typhimurium} chromosomes were developed. The two systems are mediated by SSR machineries of bacteriophages \( \lambda \) of \textit{E. coli} and P22 of \textit{S. typhimurium}. These systems utilize plasmid vectors with conditional replicating origin of replication and provide stable chromosomal integration of genes at specific bacteriophage attachment sites without disruption of any host gene or a need for antibiotic selection. \textit{E. coli} contains attachment sites for both bacteriophages. When the two systems are applied consecutively, two different genes can be integrated at two specific locations. The integrated plasmids of both systems can also be completely excised and recovered from the host chromosomes to observe any genetic changes, \textit{e.g.} by DNA sequencing. Both systems are also very applicable in construction of bacterial strains as well as live \textit{E. coli} and \textit{S. typhimurium} recombinant vaccines expressing foreign genes of interest.

To construct a lysogen of lytic bacteriophage \( \lambda \), both SSR systems were applied. A lytic mutant (\( cI \)) of bacteriophage \( \lambda \) was marked with an antibiotic resistant gene cassette to
facilitate a lysogen selection. The P22 SSR system helped integrate functional λ repressor gene (cI) into a non-pathogenic E. coli strain and the marked lytic λ phage lysogenized in the presence of the helper plasmid of the λ SSR system. The lysogen demonstrated its efficacy in decreasing number of λ sensitive E. coli. This lytic phage lysogen construction strategy can be applied for other bacteriophages. A pool of different lysogens infects a wider range of bacteria and could be utilized as alternatives to the use of antibiotics to control bacterial infections.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

The widespread emergence of multiple antibiotic resistant pathogenic bacterial strains such as in *Escherichia coli*, *Staphylococcus sp.*, *Streptococcus sp.*, *Pseudomonas sp.*, etc., has become a significant problem in treating bacterial infections of humans and animals. Clearly, the need to identify alternatives to the use of antibiotics to control bacterial pathogens is becoming necessary. One suggested solution is the use of virulent bacteriophages (Barrow *et al.*, 1998). Bacteriophages are bacterial viruses that are able to infect specific bacterial species. Virulent phages lyse their host cells while temperate phages have the ability to either lyse the bacterial cells they infect or integrate into the host chromosome and form a lysogen. A population of lysogens will, however, continue to release progeny phages into the environment. These phage progeny will then repeat the infection cycle in their specific host species.

Since the bacteriophages were discovered in 1915 (Twort, 1915) and in 1917 (D’Hérelle, 1917), a significant amount of effort has been made to investigate the potential use of bacteriophages as prophylactic and therapeutic agents against bacterial infections. In the first 30 years after the discovery of bacteriophages, the experimental designs of most research contained inadequate controls and the results did not provide confident conclusions. The lack of understanding of the nature of bacteriophages also contributed to the variable success in bacteriophage therapy research. When antibiotics and sulfonamides were discovered in the 1940s, the interest in bacteriophage therapy dropped dramatically. It was not until multi-drug resistant bacteria and nosocomial infections increased significantly after
antibiotic usage that researchers turned their interests to the potential use of bacteriophages as an alternative to the use of antibiotics.

More recent attempts to use bacteriophages as therapeutic agents in both humans and animals have been applied with some success to treat a wide variety of antibiotic resistant pathogens (for a review see Alisky et al., 1998). For example, Smith and Huggins (1982) treated *E. coli*-infected mice using phages. They reported the superiority of phage therapy over antibiotics by demonstrating that a single intramuscular dose of the phages was more effective than multiple intramuscular doses of various antibiotics in curing mice of a potentially lethal induced infection of *E. coli*. This report inspired a new era of bacteriophage therapy researches both in human and veterinary medicine.

In medical applications, Slopek et al. (1981-1987) reported a series of results from both topical and oral administration of phage therapy in humans with 92.4% clinical improvement but also with certain side effects such as gastrointestinal intolerance after oral administration and allergic symptoms upon local application. Cislo et al. (1987) studied the effect of concomitant topical and oral phage therapy on infectious skin ulcers, with a high rate of outstanding results along with similar side effects seen with oral treatment. Soothill (1994) successfully applied bacteriophage BS24 to control *Pseudomonas aeruginosa* contamination of burn wounds that caused failure in skin isografting. In veterinary applications, Smith and Huggins (1982) performed several animal experiments to demonstrate the efficacy of bacteriophage therapy using mixed phages. The effectiveness of phages was reported in treating *E. coli*-induced diarrhea in piglets and lambs (Smith and Huggins, 1983), and in calves (Smith et al., 1987).
Merril et al. (1996) enhanced the therapeutic efficacy of selected bacteriophages by isolating bacteriophage mutants that were able to remain in the circulatory system for prolonged periods of time during serial passage in mice. These long-circulating bacteriophages were shown to have a greater capability as antibacterial agents than the corresponding parent strains in treating animals infected with lethal doses of bacteria. One of the most recent bacteriophage applications utilized lytic R phage, previously isolated from sewage. These phages attached to the K1 capsular antigen and prevented septicemia and a meningitis-like infection in chickens and calves caused by a K1+ bacteremic strain of E. coli (Barrow et al. 1998). These researchers reported promising results even when phages were inoculated intramuscularly after the animals showed signs of disease.

Bacteriophage therapy, however, has certain limitations. These limitations include the narrow specific host-range of bacteriophages, the ability of temperate phages to form lysogens, the sensitivity of bacteriophages to gastric acid when used in vivo and the lack of a continuous phage supply. When a temperate phage forms a lysogen in target bacteria, the lysogen then becomes immune to further phage infection and continues to proliferate in the presence of the phage. The sensitivity of bacteriophages to acid lysis requires gastric acid neutralization prior to oral treatment. The lack of a continuous supply of bacteriophages over the period of progressive bacterial infection may also contribute to the variable success of bacteriophage therapy.

The objective of this study is to develop an effective lytic bacteriophage delivery system, as an alternative to antibiotics, that overcomes some major bacteriophage therapy limitations. Molecular biological techniques were applied to construct a lysogen of lytic bacteriophages λ in a non-pathogenic E. coli strain. The lysogen will continuously release
lytic bacteriophage λ directly in the intestinal tract. In order to construct the lytic λ lysogen, two plasmid-based site-specific recombination (SSR) systems for the integration and recovery of DNA constructs from *E. coli* and *Salmonella typhimurium* chromosomes were developed. The two systems were mediated by site-specific recombination machineries of bacteriophages λ of *E. coli* and P22 of *S. typhimurium* and demonstrated to be efficient in integrating DNA constructs into their respective bacteriophage attachment sites on the bacterial chromosomes. Both SSR systems were applied to lysogenize a lytic bacteriophage λ mutant marked with an antibiotic resistant gene cassette into a non-pathogenic *E. coli* strain. The enterobacterial origin of this lytic bacteriophage lysogen encodes acid-regulated genes which provide tolerance in low pH environment (Hickey and Hirshfield, 1990) and can be administered orally with no requirement for gastric acidity neutralization. The lysogen also provides a continuous supply of lytic bacteriophage that is incapable of lysogen formation in the target bacteria. The efficiency of the lytic λ lysogen in decreasing number of susceptible bacteria *in vitro* was demonstrated.

**Dissertation Organization**

This dissertation is organized into five chapters. The first chapter contains the general introduction and the literature review. Three papers to be submitted to scholarly journals follow. The first paper (Chapter 2), "Genetic system for reversible integration of DNA constructs and *lacZ* gene fusions into the *Escherichia coli* chromosome" was published in *Plasmids* 2000 43:12-23. The second paper (Chapters 3), "Reversible integration of DNA constructs into the chromosomes of *E. coli* and *S. typhimurium* by a bacteriophage P22 mediated site-specific recombination system," is to be submitted to the *Journal of*
Bacteriology. These first two papers describe the development of genetic tools to integrate a gene of interest into bacterial chromosomes. These genetic tools were applied to construct lytic bacteriophage lysogens in the third paper (Chapter 4), "Bacteriophage therapy: A novel method of lytic bacteriophage delivery". The contents of the second paper were presented in a poster session (Session 44/H19) of the 99th General Meeting of the American Society of Microbiology in Chicago, on May 31, 1999. Chapter 5 presents the general conclusions of the research.

Literature Review


Discovery of bacteriophages

Bacteriophages are bacterial viruses that were first discovered in 1915 from Staphylococcus sp. by a British bacteriologist, Frederick William Twort (Twort, 1915), and independently in 1917 from Shigella dysenteriae by a Canadian medical bacteriologist, Félix Hubert d'Hérelle (D'Hérelle, 1917). These discoveries opened a new area in microbiology research (cited in Duckworth, 1976). D'Hérelle named the invisible microbe that was an obligate parasite of living bacteria he found "a bacteriophage" or by the shortened term "phage" and devoted the remainder of his scientific life conducting bacteriophage research.
The therapeutic potential of phages was also realized early on by d'Hérelle who laid the foundation for experimental phage work. After the first discovery, d'Hérelle found several additional phages from various bacterial species, including *Salmonella typhi*, *Bacillus subtilis*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Pasteurella multocida*, *Vibrio cholerae*, and *Yersinia pestis*. Since then, several researchers have reported discoveries of other new phages from a wide range of bacterial hosts and in the soil and water of the environment. By 1935, phages of many major human bacterial pathogens, mostly the enterobacteria and Gram-positive cocci, were discovered (Ackermann and Dubow, 1987).

**General characteristics of bacteriophages**

Bacteriophages are bacterial viruses that are able to infect specific bacterial species. Similar to other viruses, they are obligate parasites that are able to replicate only in living cells. A phage particle usually consists of a single nucleic acid molecule which may be single-stranded or double-stranded, linear or circular DNA, or single-stranded, linear RNA. The three major morphological classes of the phages are icosahedral, icosahedral tailed, and filamentous. Bacteriophages have a specific host range because they require specific receptors on the host cell surface to bind and initiate an infection.

Fundamental research of bacteriophages provided basic understanding of their physical and biological properties. Elford and Andrews (1932) used filters of graded pore size to determine the sizes of some phages. Burnett (1933) used particle size and host range of phages as well as resistance testing to subdivide enterobacterial phages into subgroups. Schlesinger (1933) purified phages by centrifugation. Ellis and Delbrück (1939) performed a one-step-growth experiment and introduced precise quantitative methods for bacteriophages.
Bacteriophages have two distinct life cycles, lytic and lysogenic. In the lytic cycle, bacteriophages utilize the host cells’ replication machinery and precursors to produce many phage particles, then end the cycle by lysing the host cells. In the lysogenic cycle, no progeny particles are produced and the phage DNA usually becomes part of the bacterial chromosome by site-specific recombination. Examples of lysogenic phage include λ of E. coli and P22 of S. typhimurium. Bacteriophage P1 of E. coli, alternatively exists in its lysogenic state as an episomal plasmid. The decision whether to utilize lytic or lysogenic growth depends on several factors, including the expression of the phage repressor and the nutritional status of the host. A phage capable only of lytic growth is called a virulent phage while a phage capable of both lytic and lysogenic growth is called a temperate phage. The bacterial host that contains a complete set of phage DNA in the chromosome is called a lysogen. Some prophages in a population of lysogens may switch to the lytic growth cycle and continue to release small amount of progeny phages into the environment. The progeny phages will then repeat the infection cycle in their susceptible host.

Lytic versus lysogenic cycle

When temperate phages such as λ and P22 infect their hosts, phage DNA is injected into the bacteria and phage replication begins using the host replication machinery. A temperate phage must enter one of two cycles after the infection, the lytic or the lysogenic cycle. If the phage enters the lytic cycle, various sets of phage genes turn on and off under a precisely regulated program to facilitate the phage DNA replication. New structural proteins are synthesized and new phage particles are formed within the bacterium. In the case of bacteriophage λ, within 45 minutes after infection the phage lyses the bacterium and releases
approximately 100 progeny phage particles. If the phage enters the lysogenic cycle, only one phage gene, the repressor gene, is turned on and a single phage DNA molecule integrates into the host chromosome by the phage-coded site-specific recombination system. When a lysogen grows and divides, the prophage is passively replicated and distributed to the progeny bacteria as a part of the chromosome and rarely produces progeny phages (Ptashne, 1986). A lysogen is generally quite stable and can replicate nearly indefinitely without release of phage. However, the prophage is able to enter the lytic cycle, lyse the host cell, and produce progeny phages when induced by agents that damage the host DNA such as ultraviolet light. The activation of the RecA protein of the SOS bacterial DNA repair system inactivates the phage repressor and causes the switch from lysogenic to lytic growth (Roberts et al. 1978).

**History of bacteriophage research**

Initially, the therapeutic potential of phages against bacterial infections in humans and animals received great attention as a research target. By 1940, before the first antibiotic was discovered, as many as 560 papers on phage therapy researches were published. Research in the basic biology of bacteriophage, including phage typing, lysogeny, genetics and biochemistry also attracted great interest during the period of 1940 to 1965. In 1950, for example, Lwoff and Gutmann described the nature of lysogens that carry "prophages" or phages in a non-infectious stage and pass them on to daughter cells for several passages (Lwoff and Gutmann, 1990). Lwoff et al. (1950) reported prophage induction by UV light and, in 1953, published the entire concept of phage lysogeny.
Bacteriophages research also resulted in two significant discoveries into the nature of genetic information. In 1952, bacteriophage T2 served as a model for Hershey and Chase to study the role of DNA and confirmed that DNA, and not protein, was the genetic material (Hershey and Chase, 1952). Watson and Crick, in 1953, published their famous discovery of the double helix structure of DNA by the x-ray crystallography experiment using T-even phage DNA. They demonstrated that the DNA configuration consisted of two antiparallel strands of polynucleotide wound around each other and the backbone of each strand consisted of alternating deoxyribose and phosphate groups (Watson and Crick, 1953). In 1959, Brenner and Horne introduced the thin sectioning and negative staining technique for electron microscopy which led to significant advances in phage ultrastructure and genetic research thus enabling researchers to gain a much better understanding of phage biology, identification and classification (Brenner and Horne, 1959). Electron microscopy also led to the extensive research of tailed phages and new phage groups, e.g., filamentous (M series) and pleomorphic (PM2) phages (Hofschneider, 1963, Marvin and Hoffmann-Berling, 1963, Zinder et al., 1963, Espejo and Canero, 1968). During this era, the search for new phages was constant. The phages of enterobacteria became the most known group and the majority of phage research was on coliphages. However, Gardner and Weiser discovered a Mycobacterium phage in 1947 (Gardner and Weiser, 1947), and in 1970 Gourlay isolated the Mycoplasma phage (Gourlay 1970). Currently, phages are known to exist in virtually all prokaryotes (Ackermann and Dubow, 1987).
Application of bacteriophages

Bacteriophages have been utilized extensively in molecular genetics ever since they were first discovered in 1915 and 1917. They are used as tools for biotechnology, e.g., cloning vectors for foreign DNA (Blattner et al., 1977, Benton and Davis, 1977, Enquist, 1980), or as specialized transducing phages (Miller, 1972, Glass, 1982). Bacteriophages were instrumental in the discovery and characterization of bacterial restriction and modification systems (Arber, 1974). DNA sequencing was made possible by using bacteriophages (Sanger, 1977, Messing et al., 1981, Messing and Vieira, 1982). Bacteriophages have yielded useful enzymes for molecular cloning (Dugaiczyk et al., 1975, Lobban and Kaiser, 1973, Maxam and Gilbert, 1977). Promoters from various phages have been used to study gene expression and regulation (Maniatis et al., 1982, Studier, 1973), and for biotechnological applications (Denarie et al., 1977, Faelen et al., 1977, Casadaban et al., 1977).

Phages have served as test objects for virucidal products (Drulak et al., 1979, Lepage and Romond, 1984, Menzel, 1984), and as indicators of bacterial pollution (Kenard and Valentine, 1974, Scarpino, 1971, Scarpino, 1975). They have been useful in the study of droplet infection (Colvin, 1932), the assay of air filters (Kewitsch, 1964), and in aerosol samplers (Morris et al., 1961, Harstad, 1965). Phages have been useful as tracers of water currents (Drury and Wheeler, 1982), ground and main water (Niemi, 1976), and waste water movement (Salkinoja-Salonen et al., 1981).

In microbiological applications, bacteriophages have been utilized as diagnostic reagents (Sonnenschein, 1925, Cherry et al., 1954, Seeliger and Holl, 1961, Welkos et al., 1974). Bacteria can be identified by phage typing (Schmidt and Jeffries, 1975, Engel, 1978;
Phages have also helped to detect specific bacteria (Katznelson and Sutton, 1951, Cook and Katznelson, 1960, Sutton, 1966), and in bacterial taxonomy (Keogh et al., 1938, Fisk, 1942, Conn et al., 1945). Phages have also been used for decontamination of bacteria from vaccines (Janzen and Wolff, 1922), tissue culture (Riche et al., 1978), or meat (Greer, 1986). The identification of potential carcinogens and antitumor antibiotics by prophage induction (Heinemann and Howard, 1964, Moreau et al., 1976, Moreau and Devoret, 1977) has provided simple, less expensive, and more rapid tests than the use of laboratory animals and cell cultures. Bacteriophage therapy and prophylaxis of infectious bacterial diseases in both man and animals have been widely practiced with optimistic results (Smith and Huggins, 1982-1983, Slopek et al., 1983-1985, Barrow et al., 1998).

Despite the advantages offered by bacteriophages, they can be harmful as well. For example, in the fermentation industry, harmful phages are those which contaminate the process. These phages also became a major problem in the dairy industry, causing considerable economic losses by destroying starter cultures and disrupting fermentation (Whitehead and Cox, 1935, Babel, 1962; Klaenhammer, 1984, Lawrence et al., 1976, Lawrence and Thomas, 1979). Other industries using bacteria for fermentation have experienced losses from phage contamination as well (Hongo, 1971, Ogata and Hongo, 1979, Ogata, 1980).

Pre-antibiotic bacteriophage therapy research

Before antibiotics, bacteriophages were perceived as a possible panacea to prevent and control bacterial infectious diseases in both humans and animals. Since the first
bacteriophage was discovered until 1965, some 900 papers on phage therapy research were published, with the highest peak of activity from 1930 to 1940. In 1921, the treatment of staphylococcal skin disease with phages initiated immediate widespread interest (Bruynoghe and Maisin, 1921). D’Hérelle, the medical bacteriologist who also independently discovered bacteriophage, used phages to treat plague bubos in Egypt in 1925 (D’Hérelle, 1925), and cholera in India in 1928 (D’Hérelle, 1928). Human diseases for which bacteriophage therapy was applied included pyogenic and urinary infections, cholera, dysentery, plague, typhoid and paratyphoid fevers, as well as phage prophylaxis of intestinal infections. Phage administration covered almost every route possible, including the intra-carotid artery and direct injection into lung tissue (Raettig, 1958, Raettig, 1967).

Phage therapy during that period resulted in a wide range of results, from spectacular successes to failures which led to a greater understanding of the nature and limitation of phages. The causes of the failures included the narrow host range of a particular phage and a specific phage was needed to treat a specific bacterial infection. If an infection was caused by mixed bacterial infection one phage could not stop the infection, rather a mixture of different phages specific to each bacterial pathogen was required. The emergence of phage resistant bacteria was also possible through the selection of mutants. Furthermore, lysogen of a temperate phage became immune to the superinfection with the same phage and was able to perpetuate the infection. Bacteriophages themselves were sensitive to acid lysis if administered orally, without prior neutralization of gastric pH.

The inactivation of phages by body defense mechanisms and antiseptics from skin lesion treatment also contributed to the failure of phage therapy. One significant, serious adverse effect from bacteriophage-mediated bacterial cell lysis was the liberation of
endotoxin that could cause a septic shock. The major weakness of bacteriophage therapy during the pre-antibiotic period was mainly due to the many unknown factors involved. Sometimes, unknown phages at unspecified titers were used to treat patients without prior bacteriological diagnosis, proper controls or placebos. Interpretations of treatments did not inspire confidence and large clinical studies were rarely reported. These circumstances, together with the discoveries of antibiotics and sulfonamides in the 1940s, resulted in a dramatic decline in phage therapy research. In 1959, the World Health Organization (WHO) concluded that there was no reason to continue investigations on the use of bacteriophage to control *Vibrio cholerae* infection because tetracycline therapy was successful (Pollitzer, 1959).

**Post-antibiotic bacteriophage therapy research**

The widespread emergence of antibiotic resistant bacteria and the discovery of antibiotic residues in food animals revived the interest in bacteriophage therapy to control long-term bacterial infection. Several research studies were conducted in both medical and veterinary settings. During this era, more careful selection of the specific phages, controls, and treatment protocols were applied. Most reports now have showed promising results that encourage further experimentation into the application of bacteriophage therapy.

**Medical applications**

Slopek *et al.* (1981-1987) reported a series of results from both topical and oral administration of phage therapy in humans in Poland, mainly against postoperative or spontaneous infections by antibiotic resistant *Staphylococcus, Salmonella, Klebsiella, Escherichia, Proteus, and Pseudomonas*. Oral administration was applied in cases of
gastrointestinal and generalized infections, with the repeated high doses of phages after gastric acidity neutralization. Positive results indicated clinical improvement without a proper control or an untreated group. However, side effects of oral phage treatment such as allergic symptoms and vomiting were also reported. Cislo et al. (1987) studied the effect of concomitant topical and oral phage therapy on infectious skin ulcers, with a high rate of positive results along with side effects similar to those seen with oral treatment. Weber-Dabrowska et al. (1987) reported the penetration of orally administered phages into the circulatory system and in urine. Kucharewicz-Krukowska and Slopek (1987) analyzed the antibody response of patients receiving oral phage therapy and found that most patients (77%) did not develop antiphage antibodies whereas some (14%) did at low levels, and only 4% generated high antibody titers. They concluded that this degree of immunogenicity did not interfere with phage treatment. More recently, Soothill (1994) successfully applied bacteriophage BS24 to control *Pseudomonas aeruginosa* contamination of burn wounds that caused failure in skin isografting.

**Veterinary applications**

Smith and Huggins (1982) performed several animal experiments to demonstrate the efficacy of bacteriophage therapy. They treated experimentally *E. coli*-infected mice using mixed phages. They reported the superiority of the phage therapy over antibiotics by showing that a single intramuscular dose of the phages was more effective than multiple intramuscular doses of various antibiotics in curing mice of a potentially lethal, induced infection with *E. coli*. Notably, the few phage-resistant mutants of *E. coli* found in the mice were shown to be of greatly reduced virulence.
The effectiveness of phages was reported in treating *E. coli*-induced diarrhea in piglets and lambs (Smith and Huggins, 1983), and in calves (Smith et al., 1987). It was observed that the phages multiplied rapidly after gaining entry to the *E. coli*-infected small intestine and quickly reduced the numbers of *E. coli* to levels that were virtually harmless.

Merril et al. (1996) enhanced the therapeutic efficacy of selected bacteriophages by serial passage in mice and isolating bacteriophage mutants that were able to remain in the circulatory system for prolonged periods of time. These ‘long-circulating’ bacteriophages were shown to have a greater capability as antibacterial agents than the corresponding parent strains in animals infected by lethal doses of bacteria.

One of the most recent bacteriophage applications utilized lytic R phage, previously isolated from sewage. These phages attached to the K1 capsular antigen and prevented septicemia and a meningitis-like infection in chickens and calves caused by a K1+ bacteremic strain of *E. coli*. Barrow et al. (1998) reported promising results even when phages were intramuscularly inoculated after the animals showed signs of disease.

**λ and P22 phages**

The two best-studied temperate phages are bacteriophage λ of *E. coli* and P22 of *S. typhimurium*. Bacteriophage λ is classified in the family Siphoviridae while P22 is in the Podoviridae. Both phages are well characterized genetically and although they belong to different families, they are closely related. The regions concerned with regulation, recombination, host integration, DNA replication, and lysis functions of both phages are arranged in the same order on their respective genetic maps. The phages also share major
DNA sequence homology and can even form viable hybrid phage when parts of P22 phage are replaced by analogous λ genes (Botstein and Herskowitz, 1974).

Although the two phages share these similarities they are clearly distinct from each other. For example, P22 DNA is packed in the phage head with direct sequence repeats at each end (Casjens et al., 1987) which recombine by homologous recombination to form a circular DNA molecule which is needed for rolling-circle replication (Rhoades et al., 1968). The λ DNA contains single-stranded cohesive end to circularize in vivo (Campbell, 1971). Morphologically, P22 has only a short baseplate structure and adsorbs only to S. typhimurium whereas λ has an elongated tail and adsorbs only to E. coli (Kellenberger and Edgar, 1971).

The control of the lysogeny of P22 is based on two regions essential for immunity and repression. P22 lysogens exclude superinfecting phages in ways extending beyond the immunity and repression system (Chan and Botstein, 1972). The λ phage has only one region for lysogeny control and the lysogens display solely the immunity provided by the single repressor protein to protect themselves from superinfection (Rao, 1968, Campbell, 1971, Susskind et al., 1971).

Repressor systems of λ and P22 phages

In either a λ or P22 lysogen, the only phage gene that is expressed is the repressor gene (cI of λ and c2 of P22). The repressor protein regulates phage gene expression by interaction with specific operator sites that overlap with the promoters for the repressor and cro genes. Repressor protein facilitates lysogenic growth while Cro protein does the lytic cycle.
As shown in Figure 1.1, the cl repressor and Cro proteins (transcribed from $P_R$) have their own promoters adjacent to each other with the transcripts diverging to opposite directions. There are three adjacent operator sites that overlap both promoters. The repressor and Cro proteins bind these sites with different affinities to regulate the activities of the two promoters. The repressor protein binds to its high affinity operator site $O_{R1}$ that overlaps the $P_R$ promoter. The binding of cl at $O_{R1}$ interferes with RNA polymerase binding and represses the transcription of Cro. The cooperative binding of cl repressors at $O_{R1}$ and $O_{R2}$ also facilitates RNA polymerase binding at cl promoter. When the repressor protein cl accumulates, it also binds the low affinity operator site $O_{R3}$ that overlaps its own promoter and turns off the transcription by autoregulation (Ptashne, 1986).

By binding to prophage operators, the repressor protein turns off all the phage genes required for lytic growth. As many as 100 copies of repressor protein are expressed in a lysogen (Ptashne, 1986). This number of repressor protein molecules provides immunity to the lysogen against superinfection by the same phage by binding to the operators of the newly introduced phage DNA and turning off all genes for phage replication. Factors that inactivate the repressor protein, such as activated RecA protein, will derepress the prophage genes and initiates the lytic growth cycle of the prophage (Ptashne, 1986).
The importance of the repressor gene in lysogeny was determined by isolation of lytic mutants of λ and P22 phages. The so-called clear plaque mutants encode non-functional repressor protein and therefore cannot establish lysogens and are always lytic to the host cells. These phage mutants grow lytically in any susceptible host but not in the lysogens of the same phage, since functional repressor protein encoded by prophages can repress the lytic growth of these lytic phages. Another type of clear plaque mutant resulted from mutations in the operator sequences recognized by the repressor protein. These mutants are unable to be repressed by functional repressor proteins and, consequently, can overcome superinfection immunity.

**Site-specific recombination systems of λ and P22 phages**

Site-specific recombination is a strategy used by a variety of temperate bacteriophages to lysogenize their hosts by reversibly integrating their DNA into the hosts' chromosomes. The characteristics of site-specific recombination are that it is site-specific, reciprocal, conservative, and does not involve DNA replication at the recombination site (Neidhardt et al., 1987), plus it does not require homologous recombination. In general, bacteriophages use the Int protein together with the integration host factor (IHF) of the host to recognize attachment sites of the phage DNA and the bacterial chromosome, to promote integration of the phage DNA. (Lindsey et al., 1992). Excision of a phage from a lysogen requires both the Int and Xis proteins to initiate the recombination event (Leong et al., 1985).

To form a lysogen in their host cells, temperate phages integrate their DNA into the host chromosomes. The specific attachment sites of each phage are different but share some common features including a high A+T composition. The attachment sites on both the
bacterial chromosome and the phages, as well as the resulting attachment sites, \textit{attL} and \textit{attR},
share common core sequences where recombination takes place. Bacteriophage \( \lambda \) has a 15-base pair while P22 has a 46-base pair common core. The core sequences of the two phages show no significant homology but there are dispersed regions of homology in the arm sequences that indicate that both \( \textit{att} \) sites are related (Leong \textit{et al.}, 1985). The integration host factor (IHF) binding sites of \textit{E. coli} in both phages are different, but the location and orientation of the binding sites in relation to the respective core regions are well conserved. In the \( \lambda \) system, the IHF interacts with three regions on the \( \textit{attP} \) and none on \( \textit{attB} \) (Craig and Nash, 1984). The IHF binds to a site to the left of the common core in the P22 phage \( \textit{att} \) site \( (\textit{attP}) \) and to a site to the right of the core in the P22 \( \textit{attP} \) and bacterial \( \textit{att} \) site \( (\textit{attP22}) \). The IHF has a significant role in \( \lambda \) SSR but is not required in the P22 system. Cho \textit{et al.} (1999) demonstrated that although the \( \textit{attP} \) region of P22 contained strong IHF binding sites, P22 phage performed site-specific recombination to its maximum efficiency in the absence of IHF.

In addition to the use of bacteriophage for a number of applications, specific phage genes have also been exploited as useful genetic tools. Specifically, the efficiency of bacteriophage site-specific recombination has been utilized in a number of plasmid-based systems to integrate various genes into host chromosomes at specific attachment sites. For example, systems have been developed that use phage Mx8 in \textit{Myxococcus xanthus} (Li and Shimkets, 1988), phage HP1 in \textit{Haemophilus influenzae} (Goodman and Scocca, 1989, Hauser and Scocca, 1992), pSAM2 in \textit{Streptomyces lividans} (Smokvina \textit{et al.}, 1990), in \textit{Mycobacterium smegmatis} (Martin \textit{et al.}, 1991), and in \textit{Mycobacterium leprae} (Eiglmeier \textit{et al.}, 1991), mycobacteriophage L5 in \textit{Mycobacterium smegmatis}, \textit{Mycobacterium}
tuberculosis, and bacille Calmette-Guerin (BCG) (Lee et al., 1991), phage phi adh in Lactobacillus gasseri (Raya et al., 1992), phage 16-3 in Rhizobium meliloti 41 (Hermesz et al., 1992), phage Tuc2009 (van de Gucht et al., 1994), phage mv4 in Lactobacillus plantaum (Dupont et al., 1995), actinophage RP3 in Streptomyces rimosus (Gabriel et al., 1995), phage phi CTX in Pseudomonas aeruginosa (Wang et al., 1995), phage phi AAU2 in Arthrobacter aureus (Le Marrec et al., 1996), phage T12 in Streptococcus pyogenes (McShan et al., 1997), phage phiLC3 (Lillehaug et al., 1997) and phage TP901-1 (Brondsted and Hammer, 1999) in Lactococcus lactis, phage A2 in Lactobacillus species, Lactococcus lactis, and E. coli (Alvarez et al., 1998), phage SfX in Shigella flexneri (Guan and Verma, 1998), phage P4 in Pseudomonas sp. (Ravattn et al., 1998), corynephage Phi16 in Corynebacterium glutamicum (Moreau et al., 1999), and λ phage in E. coli (Atlung et al., 1991, Diederich et al., 1992, Hasan et al., 1994, Platt et al., 1999). The advantages of these systems include the ability to study gene functions from a single copy of gene. For example, when employing a reporter gene such as lacZ to monitor gene expression, the result can be misinterpreted as the plasmid copy number can change depending upon the specific growth conditions or the genetic background of the bacterial strain. The experimental result can also be influenced by an unnaturally high level of a regulatory protein or cis-acting regulatory sequences present on the high copy number plasmids. Stable integration also obviates the need for continual antibiotic selection for the maintenance of the DNA construct. Moreover, integration at the specific λ phage attachment site assures that no functional host gene is inactivated. The system reported by Platt et al. (1999) utilizes conditionally replicating R6K ori plasmids as vectors for integration and also makes it feasible to easily retrieve the entire integrated plasmid. The plasmid-based systems of λ SSR have been widely applied to
construct bacterial strains and characterize specific genes (Atlung et al., 1991, Diederich et al., 1992, Hasan et al., 1994, Platt et al., 2000, Lee et al., 2000). This is also a promising model for future recombinant E. coli vector vaccines expressing foreign antigens.

References


CHAPTER 2. GENETIC SYSTEM FOR REVERSIBLE INTEGRATION OF DNA CONSTRUCTS AND \textit{lacZ} GENE FUSIONS INTO \textit{ESCHERICHIA COLI} CHROMOSOME

A paper published in Plasmid\textsuperscript{1}

Ratree Platt, Christopher Drescher, Sei-Kyoung Park and Gregory J. Phillips

ABSTRACT

A plasmid system for site-specific integration into, and excision and recovery of gene constructs and \textit{lacZ} gene fusions from the \textit{E. coli} chromosome was developed. Plasmid suicide vectors utilizing the origin of replication of R6K plasmids and containing the \textit{attP} sequence of bacteriophage \textit{\lambda}, multiple cloning site, and antibiotic resistance markers facilitate reversible integration into the \textit{E. coli} chromosome by site-specific recombination. Additional vectors permit construction of \textit{lacZ} gene fusions in 3 possible reading frames for recombination with the bacterial chromosome. These suicide vectors can be propagated in newly constructed \textit{E. coli} strains that harbor different \textit{pir} alleles. Two helper plasmids that encode the necessary gene products for integration (\textit{Int}) and excision (\textit{Int} and \textit{Xis}) were also constructed. This plasmid system was shown to be a reliable and efficient means to integrate and subsequently recover plasmids from the \textit{E. coli} \textit{attB} site.

INTRODUCTION

The characterization of specific genes \textit{in vivo} often requires creation of recombinant DNA constructs that places a target gene under control of a regulatable promoter, or that fuses a gene to a reporter sequence to monitor gene expression. Generally, such DNA

\textsuperscript{1} Reprinted by permission of the publisher. (Copyright © 2000 by Academic Press)
constructs are introduced and maintained in *E. coli* on multicopy plasmids. However, there can be disadvantages associated with the study and manipulation of gene function by the use of recombinant plasmids. For example, plasmid loss or rearrangement can occur if synthesis of a gene product is detrimental to cell growth. Recombinant plasmids may also be unstable and subsequently lost in the absence of continual selection. Likewise, results of experiments that employ a reporter gene, such as *lacZ*, to monitor gene expression can be misinterpreted as plasmid copy number can change depending upon the specific growth conditions, or genetic background of the bacterial strain. Moreover, experimental results can be influenced by unnaturally high level of a regulatory protein or cis-acting regulatory sequences present on high copy number plasmids.

It is with these problems in mind that various genetic systems have been constructed for integration of DNA constructs into the *E. coli* chromosome. For example, a number of cloning vectors derived from bacteriophage λ have been developed to integrate genes and gene fusions into the *E. coli* chromosome by specialized transduction (Howard and Gottesman, 1983; Bertrand *et al.*, 1984; Windle, 1986; Balakrishnan and Backman, 1988; Koob and Szybalski, 1990; Linn and St. Pierre, 1990; St. Pierre and Linn, 1996; see also Murray, 1991; Slauch and Silhavy, 1991). However, direct cloning of genetic constructs into bacteriophage λ vectors requires the manipulation of large DNA molecules, often with a limited number of available restriction sites. The recombinant molecules must subsequently be packaged *in vitro* prior to introduction to an *E. coli* host. Furthermore, the use of helper phage or plasmids are often required to generate a lysogen of the recombinant λ vector (Murray, 1991).
The assembly of specialized transducing phage can be aided by using specific λ vectors that have significant regions of homology to plasmid vectors that carry a gene construct or fusion. Upon recombination with the λ DNA, the phage carrying the gene construct can be introduced to the bacterial chromosome by lysogeny (Ostrow et al., 1986; Simon et al., 1987). These systems require construction of a specific recombinant plasmid prior to recombination into the λ vector. The large size of bacteriophage λ vectors also limits the ease at which the DNA can be further manipulated or studied after it is recovered from the host chromosome.

Additional systems have been described that make use of transposable elements to integrate DNA constructs into the bacterial chromosome (DeLorenzo et al., 1990; Bao et al., 1991; Alexeyev and Shokolenko, 1995; Peredelchuk and Bennett, 1997). A disadvantage of these approaches, however, is that the target for chromosomal insertion is not predictable as the transposable element can insert in a variety of locations. Further characterization of the insertion mutants is required to ensure that the transposable element has not altered the phenotype of the recipient strain.

A number of plasmid-based systems have also been described for introduction of DNA constructs into the E. coli chromosome, many of which utilize the efficient site-specific recombination machinery of bacteriophage λ to integrate DNA sequences into the bacterial attachment site for this phage (attB) (Atlung et al., 1991; Diederich et al., 1992; Hasan et al., 1994). In general, these plasmid-based systems first require creation of a circular DNA construct to be introduced to the bacterial chromosome. The construct must either be assembled in vitro (Atlung et al., 1991) or the origin of replication (ori) must be removed from a recombinant plasmid, either by restriction enzyme digestion (Diederich et al., 1992)
or by Cre-lox-mediated recombination (Hasan et al., 1994) prior to chromosomal integration. These systems utilize a helper plasmid that provides the int gene product in trans. While retrieval of the DNA constructs from the chromosome is not possible with the former systems (Atlung et al., 1991; Diederich et al., 1992), the latter system (Hasan et al., 1994) requires the use of two helper plasmids to retrieve the integrated DNA.

We report here a facile plasmid system for integration and recovery of DNA constructs from the E. coli chromosome that requires a minimal amount of manipulation for either integration or retrieval of the DNA constructs. The vectors described in this report utilize the R6Kyor to achieve conditional replication of the recombinant plasmids. The R6Kyor forms the foundation for a number of suicide vectors that cannot replicate in the absence of π, the protein product of the pir gene (Kolter et al., 1978). We have constructed a series of R6Kyor-based plasmids that carry attP, the attachment site from bacteriophage λ, a chloramphenicol resistance or spectinomycin resistance marker, and a multiple cloning site. Vectors have also been constructed to permit introduction of translational lacZ gene fusions to the chromosome. We show that these vectors faithfully integrated into the E. coli attB site when provided the int gene product via a conditionally replicating helper plasmid. The entire vector can subsequently then be retrieved from the bacterial chromosome by use of a second helper plasmid that provided the Integrase (Int) and Excisionase (Xis) proteins. Together, these vectors comprise a genetic system that should be useful for a number of applications where it is advantageous to stably maintain genetic constructs, including lacZ gene fusions, in single copy and retain the ability to retrieve the construct if desired. They should also foster novel genetic approaches to study bacterial gene function (Haldimann et al., 1996).
MATERIALS AND METHODS

Strains and plasmids

Bacterial strains are listed in Table 1 and Table 2 lists the plasmids constructed in this study.

Media and chemicals

Luria-Bertani (LB) medium (Sambrook et al., 1989) was used throughout the study. The concentrations of antibiotics were: ampicillin (Amp) 100 µg/ml; chloramphenicol (Cam), 20 µg/ml; kanamycin (Kan), 30 µg/ml; spectinomycin (Spc), 50 µg/ml, and tetracycline (Tet), 20 µg/ml. Bacterial cultures were grown at 37°C unless otherwise indicated. 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) and isopropyl β-D-thiogalactopyranoside (IPTG) were used at concentrations of 1% and 1 mM, respectively.

Techniques of recombinant DNA and bacterial genetics

Standard techniques of recombinant DNA were performed (Sambrook et al., 1989) for construction of plasmids. Enzymes and reagents were obtained commercially and used according to the manufacturers’ recommendations. Transformations were performed by either electroporation (Sheng et al., 1995) or chemical transformation (Inoue et al., 1990). Isolation of plasmid DNA was performed by a modification of the technique described by Carter and Milton (1993). Transductions with bacteriophage P1 were performed as described by Miller (1992). DH5αpir and DH5αpir116 were constructed by transducing zdg-232::Tn10 into BW19610 and BW19612. A P1vir lysate was prepared from the resulting strain and used to transduce DH5α that had been transformed with pRecATS (a plasmid with
temperature sensitive replication carrying recA\(^+\), (Park, S.-K. and G.J. Phillips, unpublished results). Tet\(^R\) transductants were cultured at 42°C to eliminate the complementing plasmid. The resulting transductants were screened for the ability to be transformed with pCD11PKS by selection for Cam\(^R\).

**Plasmid constructions**

Vectors for integration of DNA constructs into the *E. coli* chromosome include the Cam\(^R\) plasmids pCD11PKS and pCD11PSK and the Spc\(^R\) plasmids pCD13PKS and pCD13PSK. The former pair was constructed by introducing a 0.5 kb BamHI-BglII attP-containing fragment from pLDR11 (Diederich *et al.*, 1992) into the R6K\(\gamma\)ori suicide vector, pCD20 (Park, S.-K. and G.J. Phillips, manuscript in preparation). pLDR11 was first digested with EcoRI and Smal, treated with the Klenow fragment of DNA polymerase I, and religated to eliminate these restriction sites prior to insertion into pCD20. pCD13PKS and pCD13PSK were constructed by replacing an SpI Cam\(^R\) fragment from pCD11PKS and pCD11PSK with a 1.4 kb DraI fragment encoding Spc\(^R\) from pKRP13 (Reece and Phillips, 1995).

Vectors for construction and integration of lacZ gene fusions in three open reading frames (ORF) were made by first constructing a derivative of pCD11PSK where the lacZ gene from pMLB1034 (Silhavy *et al.*, 1984) was altered by site-specific mutagenesis to eliminate a SacI (Ecl136II) site from within lacZ. A 3.2 kb FspI fragment containing this modified lacZ was inserted into FspI-digested pCD11PSK. The unique Ecl136II site on this vector was converted to an AarII site by addition of linkers. A 1.5 kb AarII fragment from the vectors pLACZ1, pLACZ1a, pLACZ2, and pLACZ3 (Jain, 1993) carrying the multiple cloning site and four tandem copies of the transcriptional terminator from the *rrnB* operon...
was exchanged for the corresponding region of DNA in this vector creating pCD11PZ1, pCD11PZ1a, pCD11PZ2, and pCD11PZ3, respectively.

The helper plasmids for integration, pPICK, was constructed by modifying a 1 kb BstUI fragment encoding $\pi$, originating from pcos2EMBL (Ehrich et al., 1987) to contain EcoRI sites at each end by the addition of linkers and inserting it into a unique EcoRI site of pLDR8 (Diederich et al., 1992). pXINT129, the helper plasmid for production of Int and Xis proteins, was constructed by first cloning a 1 kb EcoRI-SacI fragment carrying xis from pPS2-3ARS (Numrych et al., 1992) into pSPORT2 (Gibco, BRL, Gaithersburg, MD). A second pSPORT2 derivative was also assembled by inserting a 2.3 kb EcoRI-PstI int fragment from pLDR8 (Diederich et al., 1992). A 2.4 kb BspHI-Bsal fragment from this latter vector was cloned into the former xis$^+$ plasmid generating a construct where xis and int were under control of the repressor encoded by lacI on pSPORT2. A 1.4 kb HindIII Kan$^R$ cassette from pKRP11 (Reece and Phillips, 1995) was then introduced. A Sapl DNA fragment carrying lacI, xis, int, and encoding Kan$^R$ was then excised, treated with the Klenow fragment of DNA polymerase I and inserted into the unique HincII site of pNoTA (Five Prime, Three Prime, Inc., Boulder, CO). A 4.4 kb Pmel cassette was then excised from this plasmid and joined to FspI-digested pWSK29 (Wang and Kushner, 1991) creating pXINT129.

**Chromosomal integration of plasmid DNA**

The *E. coli* strains MC4100 or DH5$\alpha$ served as hosts for plasmid integration. These strains were first transformed with the helper plasmid pPICK by selection for Kan$^R$ at 30°C. The temperature sensitive phenotype of pPICK was confirmed by showing that the
transformants were unable to form single colonies at 42°C in the presence of Kan. The pPICK transformants were then transformed with various pCD11P- or pCD13P-derivative plasmids by selection for Cam^R or Spc^R, respectively at 30°C. Transformants were subsequently restreaked for Cam^R or Spc^R at 42°C. Colonies that arose after overnight incubation were twice again restreaked under the same growth conditions. Finally, antibiotic resistant colonies were screened for Kan^S to confirm loss of pPICK.

**PCR assay for plasmid integration**

An approach similar to that reported by Powell *et al.* (1994) was used to confirm plasmid integration by site-specific recombination. A PCR primer pair was designed to amplify a 0.5 kb region flanking the *attB* site. The sequences of primer A and primer B were 5'-CGCCGCCTGAATCGTGCGGTAGTATG-3' (Tm = 57.3°C) and 5'-CAGTGTGTGGAGGGGAGTC3' (Tm = 58.3°C), respectively. Two additional PCR primers were designed to anneal to sequences within the pCD11P- and pCD13P-derivative plasmids. The sequence of primer C was 5'-GTAATAATTGAGGATATGATGTGGTAGGAGG-3' (Tm = 56.1°C) and primer D was 5'-GGTGTTGGGGTGTTTTGGTTG-3' (Tm = 57.9°C). When primer B was paired with primer D in a PCR reaction a 1.1 kb product was obtained only if the pCD11P- or pCD13P-derivative vector had correctly integrated into the bacterial chromosome at *attB*. Similarly, amplification with primers A and C yielded a 0.6 kb PCR product. If the vectors had integrated by site-specific recombination in tandem, i.e., two copies of the plasmid integrated at *attB*, a 1.2 kb product from the C-D primer pair was expected. PCR was performed on genomic DNA prepared by a commercial kit (Gentra, Inc., Minneapolis, MN) with 25 cycles of 94°C, 55°C, and 72°C for 30 seconds each using the GeneAmp PCR System 2400 (Perkin-Elmer, Foster City, CA).
Characterization of plasmid integration by P1 transduction

Fifty independently-isolated colonies of RP110, created by integrating pCD11PlacZ into the chromosome of MC4100, were chosen as recipients for bacteriophage P1vir grown on the gal-76::Tn10 strain N3030. The transductants were selected on LB+Tet plates and subsequently patched onto LB+Tet+X-gal plates. The patches were scored for blue or white color and then subsequently replica plated onto LB+Cam to score for loss of Cam^R.

Retrieval of integrated plasmids

The helper plasmid for excision, pXINT129, was transformed into RP110 by selection on LB+Kan+Cam plates. Six individual colonies were separately inoculated into six LB+Kan broth cultures. Each culture was then split into two tubes and IPTG was added to one of the tubes. After incubation at 37°C for 4 hours, plasmid DNA was prepared from one pair of cultures. The same procedure was repeated after growth for 5, 6, 7, and 8 hours and following overnight incubation. The plasmid DNA was then used to transform DH5αpir by selection for Cam^R in the presence of X-gal. The number of blue colonies arising from each transformation was recorded. RP110 served as a negative control for this experiment.

RESULTS AND DISCUSSIONS

Integration of plasmid DNA into the E. coli chromosome

The development of a plasmid system for reversible integration of DNA constructs into the E. coli chromosome required the use of vectors whose replication functions were conditionally. We have exploited the R6Kyori to construct plasmid vectors whose replication is conditional on the presence of π, a protein required to initiate replication of
plasmid R6K (Kolter et al., 1978). In the absence of Tc, stable transformants of a plasmid utilizing R6K\textit{yori} can be selected only if the plasmid integrates into the bacterial chromosome by recombination. As shown in Fig. 1, we have designed a series of R6K\textit{yori} vectors to facilitate introduction of DNA constructs into the \textit{E. coli} chromosome by site-specific recombination. These vectors carry, in addition to R6K\textit{yori}, the \textit{attP} site and impart resistance to Cam (pCD11PKS and pCD11PSK) or Spc (pCD13PKS and pCD13PSK). These plasmids, hereafter referred to as the “integration vectors,” also carry the multiple cloning site from pBlueScript (Stratagene, La Jolla, CA) to facilitate cloning of insert DNA, and screening by blue/white colony color in the presence of X-gal.

A series of integration vectors was also constructed to facilitate construction and introduction of \textit{lacZ} gene fusions to the \textit{E. coli} chromosome. As shown in Fig. 2, these vectors contain the multiple cloning site described by Jain (1993) where fusions between a target gene and \textit{lacZ} can be constructed in all possible ORFs. These vectors can also be modified to carry specific transcriptional fusions between a target gene and \textit{lacZ}.

The helper plasmid pPICK (Fig. 3) facilitates site-specific recombination of the integration vectors. This Kan\textsuperscript{R} plasmid is a derivative of pLDR8 (Diederich, \textit{et al.}, 1992) which expressed \textit{int} under control of the \textit{cl}_{857} repressor and whose replication is also thermosensitive. This plasmid was modified to encode \textit{pir}\textsuperscript{+}, creating pPICK, to enable cotransformation with the R6K\textit{yori} integration vectors. Subsequent culturing of the doubly transformed cells at 42\textdegree C results in loss of the \textit{pir}\textsuperscript{+} helper plasmid, concomitant with synthesis of the \textit{\lambda} Int protein. Cam\textsuperscript{R} colonies are selected and represent cells where the integration vectors have integrated by site-specific recombination.
To characterize the plasmid integration system we constructed a derivative of pCD11PKS that encoded lacZ*. This plasmid, pCD11PlacZ, was used to convert various lacZ mutants, including MC4100 and DH5α, to lacZ* and, therefore, was useful for a number of tests of the site-specific recombination system, as described below.

**Characterization of integrated DNA**

Fig. 4A shows the configuration of an integration plasmid that has been introduced to the *E. coli* chromosome by site-specific recombination. Also shown are the locations of PCR primers used to probe the results of an integration event. Fig. 4B shows the results of a PCR assay used to confirm integration of a pCD11P- vector into attB. PCR amplification of a prospective pCD11P- integrant in MC4100 yielded a 1.1 kb product from primers A-D, and a 0.6 kb product from primers B-C, while no product from the A-B primer pair was obtained. The MC4100 control yielded, as expected, a 0.5 kb band representing the intact attB site with primers A-B, but no product from either A-D or B-C primer pairs. Tandem plasmid integration was not detected using the C-B primer pair from any of the ten integrants tested. These results were consistent with the integration of a single copy of pCD11PlacZ into attB of MC4100.

To determine if pCD11PlacZ had integrated at multiple sites on the *E. coli* chromosome, i.e., at attB and at one of a number of secondary att sites (Shimada and Weisber, 1972; Shimada et al., 1973), a genetic test using bacteriophage P1 transduction was performed. We reasoned that transducing an attB::pCD11PlacZ host with a P1 lysate prepared from a gal::Tn10 donor should result in loss of CamR and lacZ* at a frequency representing the tight linkage between gal::Tn10 and attB. We anticipated that greater than 90% of the TetR transductants should have lost the integrated plasmid, as revealed by their
Cam\textsuperscript{R} phenotype and white colony color on X-gal, if pCD11PlacZ resided only at attB.

Conversely, transductants where pCD11PlacZ had integrated at another chromosomal location, unlinked to gal::Tn10, should remain Cam\textsuperscript{R} and blue on X-gal. The results of the transduction experiments revealed that out of 50 independent transductants of RP110, 100\% showed linkage to gal::Tn10, i.e. all of the Tet\textsuperscript{R} transductants became Cam\textsuperscript{R} and LacZ\textsuperscript{-} at a frequency that reflected the tight linkage between gal and attB. This observation led us to conclude that out of 50 independently derived integrants of pCD11PlacZ, all originated from an integration event exclusively at attB.

Construction of pir\textsuperscript{+} hosts

A number of E. coli strains have been constructed that incorporate pir, the structural gene encoding \( \pi \), into their chromosome permitting propagation of R6K\textit{yori} vectors (Simon \textit{et al.}, 1983; Metcalf \textit{et al.}, 1994). To improve the efficiency of selecting and analyzing recombinants of the integration plasmids we modified DH5\textit{a} by incorporating two pir alleles into its chromosome. These pir derivative strains were constructed using DH5\textit{a} as this strain is efficiently transformed with plasmid DNA, supports \( \alpha \)-complementation for blue/white screening of recombinants, and yields ample quantities of high quality plasmid. DH5\textit{a}pir is pir\textsuperscript{+}, and DH5\textit{a}pir116 carries pir116, a mutant version of pir that leads to increased copy number of R6K\textit{yori} plasmids (Greener \textit{et al.}, 1990). This latter strain is useful for preparing large quantities of the integration vectors, while the pir\textsuperscript{+} derivative should be useful for cloning genes that may be toxic to the host when overexpressed.
Retrieval of integrated plasmids

Genetic strategies such as isolation of regulatory mutants can provide a powerful approach to understanding gene expression and function. The plasmid integration system described here can facilitate isolation and characterization of mutants by providing a means to introduce genes or gene fusions in diploid, or to introduce heterologous sequences into the *E. coli* chromosome, and then to recover the sequences following mutagenesis (Haldimann et al., 1996). To retrieve the integration plasmids from the chromosome the Kan$^R$ helper plasmid pXINT129 was constructed. Retrieval of pCD11PlacZ was accomplished by transforming RP110 with pXINT129, isolating plasmid DNA after culturing the transformants for varying times and retransforming into DH5αpir. Table 3 shows that pCD11PlacZ was retrieved from the pXINT129 transformants at all growth times tested, while no plasmid was retrieved from cells that were not transformed with pXINT129. We routinely recover integrated plasmid DNA by culturing pXINT129 transformants in the presence of Kan and IPTG for 4 hrs. prior to isolation of plasmid DNA and subsequent transformation into DH5αpir or DH5αpir116.

Plasmids retrieved by excision from the *E. coli* chromosome were shown to be identical to the plasmids prior to integration. Essentially all of the DH5αpir transformants were LacZ$^+$, and no evidence of rearrangements or other alterations to pCD11PlacZ was observed following digestion with a number of restriction enzymes (data not shown). Plasmids that undergo a complete cycle of integration and retrieval apparently do so unaltered.
Homologous recombination vs. site-specific recombination

We have used the plasmid integration to construct partial diploid strains by integrating a second copy of an *E. coli* gene at *attB*. In a *recA* background, such as MC4100, we predicted that antibiotic resistant recombinants would also arise due to homologous recombination, in addition to site-specific recombination. Indeed, when integrating a pCD11PKS-derivative plasmid carrying a 2.0 kb insert with homology to the *E. coli* chromosome, we observed that up to 50% of the Cam\(^R\) transformants arose by homologous recombination (data not shown). We found this class of transformant to be relatively easy to identify and eliminate, however, as they frequently reverted to Cam\(^S\) when grown in the absence of selection. Moreover, they did not yield plasmid DNA following transformation with pXINT129. Integration by homologous recombination could also be avoided by using a *recA* mutant, such as DH5\(\alpha\), as a host strain.

The DNA sequences of the plasmids described have been assigned the following accession numbers: for pCD11PKS/SK, AF178449 and AF178450; for pCD11PZl, AF178451; and for pCD13PKS/SK, AF178452 and AF178453.

ACKNOWLEDGEMENTS

The authors thank Mary Berlin, Jeff Gardner, Tom Hill, Michele Igo, Sidney Kushner, Donald Reynolds, Jim Slauch, and Barry Wanner for bacterial strains, plasmids and helpful advice. This is journal paper number J-18080 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project number 3220, and supported by the National Institutes of Health (GM50836-04) and Hatch Act and State of Iowa funds.
REFERENCES


Table 2.1: Bacterial strains.

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Relevant genotype</th>
<th>Comments</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW19610</td>
<td>uidA::pir116</td>
<td>Host strain for R6Kyor plasmids</td>
<td>Metcalf et al. (1994)</td>
</tr>
<tr>
<td>BW19612</td>
<td>uidA::pir*</td>
<td>Host strain for R6Kyor plasmids</td>
<td>Metcalf et al. (1994)</td>
</tr>
<tr>
<td>DH5α</td>
<td>lac, recA</td>
<td>Host strain for helper plasmids and host for plasmid integration at attB</td>
<td>Gibco, BRL</td>
</tr>
<tr>
<td>DH5pir</td>
<td>pir*</td>
<td>Host strain for R6Kyor plasmids</td>
<td>This study</td>
</tr>
<tr>
<td>DH5pir116</td>
<td>pir116</td>
<td>Host strain for R6Kyor plasmids</td>
<td>This study</td>
</tr>
<tr>
<td>MC4100</td>
<td>λ, attB+, Δlac</td>
<td>Host strain for plasmid integration at attB</td>
<td>CGSC*, Silhavy et al. (1984)</td>
</tr>
<tr>
<td>N3030</td>
<td>gal-76::Tn10</td>
<td>Tn10 linked to attB</td>
<td>CGSC*</td>
</tr>
<tr>
<td>RP110</td>
<td>attB::pCD11PlacZ</td>
<td>MC4100 with pCD11PlacZ integrated at attB</td>
<td>This study</td>
</tr>
<tr>
<td>TH369</td>
<td>zdg-232::Tn10</td>
<td>Tn10 linked to uidA::pir</td>
<td>Thomas Hill</td>
</tr>
</tbody>
</table>

* E. coli Genetics Stock Center
Table 2.2: Plasmids constructed in this study for site-specific recombination into the *E. coli* chromosome.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant markers</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCD11PKS/Cam*</td>
<td>R6Kyor, attP,</td>
<td>Plasmid for integration of DNA constructs</td>
</tr>
<tr>
<td>pCD11PSK</td>
<td>MCS</td>
<td></td>
</tr>
<tr>
<td>pCD13PKS/Spc*</td>
<td>R6Kyor, attP,</td>
<td>Plasmid for integration of DNA constructs</td>
</tr>
<tr>
<td>pCD13PSK</td>
<td>MCS</td>
<td></td>
</tr>
<tr>
<td>pCD11PterZ</td>
<td>Cam*, R6Kyor, attP, lacZ</td>
<td>Control plasmid for integration of <em>lacZ</em></td>
</tr>
<tr>
<td>pCD11PZ1</td>
<td>Cam*, R6Kyor, attP, lacZ</td>
<td>Plasmid for integration of <em>lacZ</em> gene fusions (ORF 1)</td>
</tr>
<tr>
<td>pCD11PZ1a</td>
<td>Cam*, R6Kyor, attP, lacZ</td>
<td>Plasmid for integration of <em>lacZ</em> gene fusions (ORF 1)</td>
</tr>
<tr>
<td>pCD11PZ2</td>
<td>Cam*, R6Kyor, attP, lacZ</td>
<td>Plasmid for integration of <em>lacZ</em> gene fusions (ORF 2)</td>
</tr>
<tr>
<td>pCD11PZ3</td>
<td>Cam*, R6Kyor, attP, lacZ</td>
<td>Plasmid for integration of <em>lacZ</em> gene fusions (ORF 3)</td>
</tr>
<tr>
<td>pPICK</td>
<td>Kan*, oriTS*, cl857, int, pir*</td>
<td>Helper plasmid for integration of plasmids</td>
</tr>
<tr>
<td>pXINT129</td>
<td>Kan*, int, xis</td>
<td>Helper plasmid for excision of plasmids</td>
</tr>
</tbody>
</table>

*Temperature sensitive origin of replication
Table 2.3 Retrieval of pCD11PlacZ by pXINT129-mediated site-specific recombination.

<table>
<thead>
<tr>
<th>Plasmid DNA isolated</th>
<th>pXINT129</th>
<th>No plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+IPTG</td>
<td>-IPTG</td>
</tr>
<tr>
<td>After growth for:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 hr.</td>
<td>&gt;500</td>
<td>187</td>
</tr>
<tr>
<td>5 hr.</td>
<td>&gt;500</td>
<td>140</td>
</tr>
<tr>
<td>6 hr.</td>
<td>&gt;500</td>
<td>144</td>
</tr>
<tr>
<td>7 hr.</td>
<td>&gt;500</td>
<td>95</td>
</tr>
<tr>
<td>8 hr.</td>
<td>&gt;500</td>
<td>45</td>
</tr>
<tr>
<td>Overnight</td>
<td>388</td>
<td>6</td>
</tr>
</tbody>
</table>

Number of lacZ⁺, Cam⁺ colonies retrieved from RP110 transformed with:
Figure 2.1: Plasmids for integration of DNA constructs into the E. coli chromosome at attB. Top, pCD11PKS and pCD11PSK. Bottom, pCD13PKS and pCD13PSK. Shown are the location and orientation of the unique restriction sites within the multiple cloning sites as flanked by BssHII sites.
Figure 2.2 Vectors for construction and integration of lacZ gene fusions into the E. coli chromosome. The restriction sites and reading frame of lacZ is shown at the top for pCD11PZ1, pCD11PZ1a, pCD11PZ2, and pCD11PZ3. T1(4) designates four tandem copies of the transcriptional terminator from the rmpB operon.
Figure 2.3: Helper plasmids for integration and excision of integration vectors. Top, pPICK, the plasmid for production of λ Int protein. rep designates the gene encoding Rep protein from pSC101. Bottom, pXINT129, the helper plasmid for production of Int and Xis proteins.
Figure 2.4: Integration of suicide vectors by site-specific recombination and confirmation of integration by PCR.

A. Site-specific recombination between attP of an integration vector and attB. The integration of the suicide vectors is confirmed by PCR using primers A, B, C, and D that hybridize to the locations shown.

B. PCR confirmation of plasmid integration. Lanes 1-3 show the results of PCR of chromosomal DNA from MC4100 where a pCD1IP-derivative plasmid had been integrated at attB. Lanes 4-6 are from MC4100. The primer pairs are: lanes 1 and 5, A+B (0.5 kb product); lanes 2 and 6, A+C (0.6 kb product); lanes 3 and 7, B+D (1.1 kb product). Lane 4 shows a 1 kb ladder as a molecular weight standard.
CHAPTER 3. REVERSIBLE INTEGRATION
OF DNA CONSTRUCTS INTO THE CHROMOSOMES OF
ESCHERICHIA COLI AND SALMONELLA TYPHIMURIUM
BY A BACTERIOPHAGE P22-MEDIATED
SITE-SPECIFIC RECOMBINATION SYSTEM

A paper to be submitted to the Journal of Bacteriology

Ratree Platt and Gregory Phillips

ABSTRACT

A genetic system has been developed that utilizes the site-specific recombination (SSR) components of bacteriophage P22 to integrate DNA constructs into the *Salmonella typhimurium* and *Escherichia coli* chromosomes. Chromosomal integration by SSR overcomes problems associated with the overexpression of genes on a multicopy plasmid that may be detrimental to cell growth and can provide a more accurate measurement of gene expression as monitored by a reporter gene. Integration at *attA* or *attP22* sites also insures that no functional host gene is inactivated upon insertion. The system includes a conditionally replicating plasmid that carries the R6K*γ* origin of replication, bacteriophage P22 attachment site, multiple cloning sites, and imparts resistance to kanamycin, chloramphenicol, or spectinomycin. SSR is accomplished using a helper plasmid that expresses the P22 int gene and replicates using a temperature sensitive origin of replication. The recombinant formed as a result of P22 SSR was characterized by sequencing. Recovery of the integrated constructs can also be achieved using a second helper plasmid that carries both the P22 xis and int genes. This system was demonstrated to be efficient in both integration and excision of plasmid vector DNA in both *E. coli* and *S. typhimurium*. The successful integration and expression of *lacZ* in *S. typhimurium* by P22 SSR was also

**INTRODUCTION**

Site-specific recombination (SSR) is a strategy used by a variety of bacteriophages, including \( \lambda \) and P22 to lysogenize their host by reversibly integrating their genomic DNA into their host’s chromosome. SSR characteristically occurs between specific DNA sequences, so called \( \text{att} \) sites. Integration of phage DNA does not require homologous recombination and occurs by a reciprocal, conservative recombination event (Nash, 1996). The SSR machinery of bacteriophage \( \lambda \) has been exploited for the development of a number of systems to integrate genes into the chromosome of \textit{Escherichia coli} (Simons et al., 1987; Atlung et al., 1991; Diederich et al., 1992; Hasan et al, 1994; Yu and Court, 1998; Lee et al., 1999, Platt et al., 2000). These systems are convenient to characterize the function and regulation of a gene in single copy. This feature is especially important when employing a reporter gene such as \( \text{lacZ} \) to monitor gene expression since results can be misinterpreted as plasmid copy number can change with different growth conditions or genetic background of the bacterial strain. Experimental results can also be influenced by unnaturally high levels of a regulatory protein or \textit{cis}-acting regulatory sequences present on high copy number plasmids. Stable integration of DNA constructs also obviates the need for continual antibiotic selection for their maintenance. Moreover, since integration occurs at the specific
\(\lambda\) phage attachment site (\(attB\)) no functional host gene is inactivated. A system previously reported by Platt \textit{et al.} (2000) utilizes conditionally replicating plasmids as vectors for integration and making it feasible to easily retrieve the entire integrated plasmid.

To extend the utility of SSR for construction of new strains of \textit{Salmonella typhimurium} and \textit{E. coli}, an integration system based on P22, a lysogenic phage of \textit{S. typhimurium} (Zinder and Lederburg, 1952) was developed. By analogy with bacteriophage \(\lambda\), a specific attachment site for P22 (\(ataA\)) has been mapped on the \textit{S. typhimurium} chromosome at minute 7 near proBA (Sanderson and Roth, 1983). Interestingly, although \textit{E. coli} is not susceptible to P22 infection, it also contains an attachment site for P22 on its chromosome (Lindsey \textit{et al.}, 1992). The \(attP22\) site also maps near proBA, overlapping with \( thrW\), at minute 6. Conveniently, this makes the SSR system amenable for \textit{E.coli} as well as \textit{Salmonella}. Also, in a manner analogous with bacteriophage \(\lambda\), P22 uses the Int protein to promote integration of the P22 DNA (Lindsey \textit{et al.}, 1992) while excision of the phage requires both Int and Xis proteins (Leong \textit{et al.}, 1985).

In this study, we report the construction of a P22-mediated SSR system that utilizes the SSR machinery of P22 in combination with conditionally replicating plasmid vectors using the R6K\(\gamma\) origin of replication (\(ori\)). The R6K\(\gamma\) \(ori\) plasmid replicates only in the presence of the \(\Pi\) protein encoded by the \(pir\) gene (Kolter \textit{et al.}, 1978). In the absence of \(\Pi\), the R6K\(\gamma\) \(ori\) plasmid functions as a suicide vector and must integrate into its host's chromosome in order to obtain stable, antibiotic resistant recombinants. The plasmid vectors developed in this study contain the attachment site from P22 phage (P22\(attP\)), multiple cloning site (MCS) to facilitate insertion of DNA constructs to be integrated into the bacterial
chromosome, and either a kanamycin resistant (Km\textsuperscript{R}), chloramphenicol resistant (Cm\textsuperscript{R}), or spectinomycin resistant (Sp\textsuperscript{R}) marker. Two “helper” plasmids provide the Int and Int/Xis functions of P22 phage to promote integration and excision of the suicide vectors. These vectors are transiently maintained in the cells by use of a temperature sensitive (TS) origin of replication from pSC101. This system was effectively used for strain construction in both \textit{E. coli} and \textit{S. typhimurium}. The integration and expression of \textit{lacZ} gene in \textit{S. typhimurium} by P22 SSR was demonstrated. In addition, multiple gene insertions in \textit{E. coli} at both the \textit{\lambda} and P22 attachment sites is possible when the \textit{\lambda} and P22-mediated SSR systems are used in combination. These vectors should have broad application for the construction of \textit{E. coli} and \textit{S. typhimurium} to analyze gene function. Application of this system for the production of live vaccine strains expressing various antigens of interest is also discussed.

**MATERIALS AND METHODS**

**Strains and plasmids.** The bacterial strains, bacteriophages, and plasmids used in this study are listed in Tables 1 and 2. The maps of relevant plasmids are shown in Fig. 1.

**Media and chemicals.** Luria-Bertani (LB) medium (Sambrook \textit{et al.}, 1989) was used to grow all cultures. The concentrations of antibiotics were as follows: Ampicillin (Ap) 100 \mu g/ml, Cm 20 \mu g/ml, Km 30 \mu g/ml, Sp 50 \mu g/ml, and tetracycline (Tc) 20 \mu g/ml. Bacterial cultures were grown at 37\degree C unless otherwise indicated. The Lac phenotypes of the bacteria were demonstrated on agar plates spread with 100\mu l of 1% X-gal (5-bromo-4-chloro-3-indoyl-\beta-D-galactopyranoside).
Techniques of recombinant DNA and bacterial genetics. Standard techniques of recombinant DNA (Sambrook et al., 1989) were performed. Enzymes, reagents, bacteriophage DNA and bacterial genomic DNA preparation kits were obtained from commercial sources and used according to the manufacturers’ recommendations. Transformations were performed either by electroporation (Sheng et al., 1995) or chemical transformation (Inoue et al., 1990). Isolation of plasmid DNA was by a modified protocol reported by Carter and Milton (1993). Transductions with bacteriophages PI and P22 were performed as described by Miller (1992) and Maloy (1990), respectively. The DNA sequencing was performed by the DNA sequencing and synthesis facility, Iowa State University.

Polymerase chain reaction (PCR). All PCRs were performed in a total volume of 50 μl in a Perkin Elmer Gene Amp PCR System 2400 (Perkin-Elmer, Foster City, CA) thermocycler using 25 cycle of 94°C, 50°C, and 72°C, at 30 seconds each with a 2 minute hold at the end of the last cycle, unless otherwise indicated. The PCR products were visualized after gel electrophoresis and ethidium bromide staining.

Plasmid constructions.

Integrated plasmid vectors. Plasmid vectors for integration include the KmR plasmids pCD21PKS/SK, CmR plasmids pCD22PKS/SK, and SpR plasmids pCD23PKS/SK. pCD22PKS was derived from pCD11PKS by replacing λ attP with attP from bacteriophage P22. Two PCR primers (5' CCCATACACACAAAAAGCAAATACG 3' and 5' TTCAATCTCCGCTGACATAGTCCAGG3') were designed to amplify the P22 int gene
and the adjacent attP site from purified P22 DNA. The PCR product was digested with XmnI and inserted into pNoTA. The resulting plasmid was digested with BamH I and inserted adjacent to cat in a derivative of pKRP10 at a Bcl I site. A cat-attP22 cassette was liberated with BspH I digestion and was ligated with BspH I-digested pCD11PKS. The ligated product was transformed into DH5αPir116 and selected for CmR.

To construct pCD22PSK, pCD22PKS and pCD11PSK were both digested with Nhe I and Bgl I. The 2.5 kb fragment of pCD22PKS was ligated with the 1.0 kb fragment of pCD11PSK to obtain pCD22PSK. The 1.3 kb Stu I fragment containing Km resistant cassette from pBGS8 was ligated to the 2.6 kb Ssp I fragments of pCD22PKS/SK to generate pCD21PKS/SK. Similarly, the 2.0 kb Dra I fragment containing spectinomycin resistant gene cassette from pKRP13 was ligated with the 2.6 kb Ssp I fragments of pCD22PKS/SK to generate pCD23PKS/SK. pCD22PlacZ was constructed by digesting pCClacZS carrying lacZ with EcoRI and the 3.5 kb lacZ fragment was ligated with pCD22PKS digested with the same enzyme. All R6Kγ ori plasmids were transformed into DH5αPir116 and selected by appropriate antibiotic.

**Helper plasmids.** To construct the plasmid pXINT-A22 which serves as the helper plasmid for excision. Two PCR primers (5’ATGGATCCAGCGGAGTAAAACATGGAATCAC3’ and 5’AAGGATCTTTACGTATTATTCCGCCTTCC3’) were used to amplify a 1.6 kb fragment contains xis and int from purified P22 DNA. These primers were also synthesized with BamH I restriction site at the 5’ ends. The PCR product was digested with BamH I and was ligated to dephosphorylated BamH I digested pUC18 creating pXINT-A22. pXINT-K22
was constructed by ligating a *BamH* I fragment carrying P22 *xis* and *int* from pXINT-A22 with *BamH* I digested pWSK129.

The pUC18-derivative *ori* helper plasmid used for plasmid integration, pINT-P22, was derived by self-ligation of pXINT-A22 digested with *EcoR* V and *EclI36 II*. The TS *ori* helper plasmid, pINT-A22, was constructed by the ligation of an *EcoR* I and *Sca I* fragment encoding the P22 *int* gene from pINT-P22 and a 4 kb fragment of pTSA30 digested with the same enzymes. pINT-K22 was constructed by the ligation of an *EcoRV* and *Hinc II* fragment of pXINT-K22 and *EcoRV* digested pTSK29 with TS *ori*.

An additional helper plasmid for integration, pAPRIL, was also constructed to facilitate the integration of plasmids using both the λ and P22 SSR systems. pAPRIL was obtained by ligation of an *Apa I/Pvu I* fragment from pYWH101 carrying λ *int* gene and pTSA30.

**pPir1030.** This plasmid contained a *pir* gene that expressed a ΦI protein to support the replication of R6Kγ *ori* plasmid in the host without the *pir* gene. The plasmid was constructed by inserting a 1.0 kb *EcoR* I fragment from pTA-*pir* (Phillips, G. J. unpublished) into the low copy number cloning vector derived from pRSF1030 (Phillips, G. J. unpublished) at *EcoR* I.

**Isolation of a plasmid cointegrant.** Two PCR primers were designed to amplify the *attP22* sequence from the *E. coli* chromosome (primer A: 5'CGGCACACAACACTCCGAT3' and primer B: 5'GCCAAGGATGTATAGTGAGCGGA3'). The 561 bp PCR product was ligated with the plasmid pT-Adv, transformed into competent Top 10 cells and KmR, and ApR transformants
were selected on an X-gal containing plate. pT-attP22 was confirmed by restriction enzyme analysis and cotransformed into DH5α carrying pINT-P22 with selection for Km\textsuperscript{R} and Ap\textsuperscript{R}. Finally, pCD22PKS was transformed into DH5α carrying 2 plasmids, pT-attP22 and pINT-P22, Cam\textsuperscript{R} transformants were selected and subsequently screened for Km\textsuperscript{R} and Ap\textsuperscript{R}. The 7.7 kb cointegrant plasmid between pCD22PSK and pT-attP22 was digested with Ssp I and Pme I and self-ligated to eliminate a copy of lacZ\textalpha from pCD22PKS which would interfere with the DNA sequencing using primers internal to lacZ\textalpha. The resulting 5.4 kb plasmid, pTAP22 (Km\textsuperscript{R} and Ap\textsuperscript{R}), was used to determine the sequences of the attL and attR regions.

**Chromosomal integration of pCD2-P- derivative plasmids into E. coli.** pINT-A22 was transformed into MC4100. Ap\textsuperscript{R} transformants were selected at 30°C. pCD22PKS was then introduced into the transformant by selection for Cm\textsuperscript{R} at 30°C. The colonies were restreaked twice on LB + Cm at 42°C to eliminate the helper plasmid. Ampicillin sensitive (Ap\textsuperscript{S}) colonies were identified and the presence of the integrated plasmid was confirmed by PCR.

**Chromosomal integration of pCD2-P- derivative plasmids in S. typhimurium.** Due to the difference in the restriction-modification (R-M) system between E. coli and Salmonella, all plasmids originated from E. coli that was used for P22 SSR in Salmonella were modified to survive the Salmonella restriction system. The S. typhimurium mutant, LB5010 (r\textsuperscript{m+}) (Bullas and Ryu, 1983) was used for this plasmid modification purpose. LB5010 with pPir1030 was used to modify the R6Ky ori-derivative plasmids. The S. typhimurium modified plasmids were isolated from these strains and used for chromosomal integration in S. typhimurium. pCD22PlacZ was integrated and the Lac phenotype was
demonstrated by streaking on a LB + Cm + X-gal plate. The integrant was cultured in LB broth for 10 passages with no selection before plating on the X-gal containing plate to observe integration stability.

Characterization of plasmid integration by transduction. To determine if the plasmid vectors pCD2-P- had integrated at multiple sites on *E. coli* and *S. typhimurium* chromosomes, genetic tests using bacteriophages P1 and P22 transductions were performed. We reasoned that transducing an *attP22::pCD22PKS E. coli* host with a P1 lysate prepared from a *pro-81::Tn10* donor should result in loss of Cm<sup>R</sup> at a frequency representing the tight linkage between *pro-81::Tn10* and *attP22*. Similarly, transducing an *attA::pCD22PKS S. typhimurium* host with a high transduction (HT) P22 lysate prepared from a *proB1657::Tn10* donor should result in loss of Cm<sup>R</sup> at a frequency representing the tight linkage between *proB1657::Tn10* and *attA*.

In *E. coli*, 50 independently-isolated integrants of pCD22PKS in MC4100 were used as recipients for bacteriophage P1 transduction (Miller, 1992) using a P1<sup>vir</sup> lysate grown on CAG18447. In *S. typhimurium*, 50 independently-isolated integrants of pCD22PKS in *S. typhimurium* were used as recipients for bacteriophage P22 transduction (Maloy, 1990) using a P22 high transducing (HT) phage grown on JL2520. The transductants were selected and subsequently patched on LB + Tc plates. The patches were scored for Cm<sup>5</sup> by replica plating.

**PCR assay for plasmid integration in *E. coli***. The integration of a plasmid vector at *attP22* was also confirmed by PCR by a similar approach as the *attB* integration reported by Platt *et al.* (2000). In *E. coli*, the same PCR primer pair (primer A and B) employed to
amplify the intact \textit{attP22} site was used. Two additional primers were designed to anneal to sequences within the pCD2-P- derivatives (primer C: 5'GGTTCGCCGATATGCTCATC3' and primer D: 5'CAGCGGATTCGGAAAGGTCTG3'). If the pCD2-P- derivatives had correctly integrated into the \textit{E. coli} chromosome at \textit{attP22} site, primers A and C produced a 551 bp \textit{attL} product and primers B and D produced a 479 bp \textit{attR} product.

**PCR assay for plasmid integration in \textit{S. typhimurium}**. A primer pair, primer E: 5'ACTCATGGGCGCATGGAACAAGAA3' and primer F: 5'ATGAGGTTGTACATAAGTGA3' (Cho \textit{et al.} 1999), was designed from chromosomal sequence to amplify a 348 bp fragment represent the intact \textit{ataA} site of \textit{S. typhimurium}. In the integrant, primer E pairs with primer D were used to amplify a 470 bp \textit{attR} product while primers F and primer C were used to amplify 346 bp \textit{attL} product.

**Retrieval of integrated plasmids**. The helper plasmid for excision, either pXINT-A22 or pXINT-K22, was transformed into an \textit{E. coli} integrant of pCD22PKS and plated on LB + Ap + Cm or LB + Km + Cm plates, respectively. For the \textit{S. typhimurium} integrant, the helper plasmids for excision were initially transformed into LB5010 before being introduced into \textit{E. coli}. Six individual transformants were separately inoculated into 6 LB + Ap or LB + Km broth cultures. Each tube was collected for plasmid extraction at 4 hours after incubation and repeated at 5, 6, 7, 8 hours and following overnight incubation. The plasmid DNA preparations from both \textit{E. coli} and \textit{S. typhimurium} were used to transform DH5\textit{apir}116 and selected for Cm\textsuperscript{R}. The retrieved pCD22PKS was compared to the original plasmids by restriction enzyme analysis.
Integration of plasmids at two chromosomal locations in *E. coli*. The helper plasmid for integration by λ SSR, pAPRIL, was transformed into an *E. coli* strain when pCD22PKS had been integrated into *attB*. Ap^R^ colonies were selected at 30°C. pCD13PKS was transformed into the TS transformants, selected for Sp^R^ at 30°C and restreaked twice at 42°C. The integration of pCD13PKS at *attB* was confirmed by PCR (Platt *et al.* 2000).

Integration of plasmids at two chromosomal locations in *S. typhimurium*. The helper plasmid for integration by λ SSR, pAPRIL, was transformed into *S. typhimurium* strain LB5010 and selected for Ap^R^ at 30°C. pCD11PKS was transformed into the TS transformants, selected for Cm^R^ at 30°C and restreaked twice at 42°C to eliminate pAPRIL. The transformant was then transformed with pINT-A22 and selected for Ap^R^ at 30°C. The pINT-A22 transformant was transformed with pCD21PSK with selection for Km^R^. The pCD21PSK integration at *ataA* was confirmed by PCR.

Retrieval of integrated plasmids from an *E. coli* integrant of both SSR systems. pXINT-A22 was transformed into an *E. coli* integrant of both pCD13PKS and pCD22PKS (described previously) and plated on LB + Ap + Cm plates. The transformants were cultured in LB + Ap broth for 4 hours and the plasmids were extracted and transformed into DH5*spir116*. Likewise, pXINT129 was also transformed into the same *E. coli* integrant and plated on LB + Km + Sp plate. The transformants were cultured in LB + Km broth for 4 hours and the plasmids were extracted and transformed into DH5*spir116*. The transformants were plated on LB + Cm plate to detect the presence of pCD22PKS and LB + Sp plate to detect the excision of pCD13PSK.
RESULTS

Plasmid integration in *E. coli* and *S. typhimurium*. We constructed a series of plasmid vectors, including pCD21PKS/SK, pCD22PKS/SK, and pCD23PKS/SK, designed to facilitate the integration of plasmid DNA into either the *E. coli* or *S. typhimurium* chromosome. To test these vectors we co-transformed both *E. coli* and *S. typhimurium* with one of the pCD- plasmids, along with the helper plasmid expressing P22 Int and integrated the suicide vector into the bacterial chromosome, as described in Materials and Methods. The Cm$^R$ / Km$^S$ recombinants were screened for the presence of the pCD2-P- vector by generalized transduction and PCR.

Characterization of integrated DNA by transductions. If the pCD2-P- vectors had integrated into *ataA* of *S. typhimurium* or *attP22* of *E. coli*, we predicted that they should be tightly linked to genes adjacent to these sites on the chromosomes. As described in Materials and Methods, we transduced *E. coli* with P1 phage lysate prepared from a *pro-81::Tn10* donor and *S. typhimurium* integrants with P22 phage lysate prepared from a *proB1657::Tn10* donor respectively. All 50 P1 transductants of *E. coli* and P22 transductants of *S. typhimurium* integrants, when screened for chloramphenicol resistance, showed either all Cm$^S$ or mixed Cm$^S$ and Cm$^R$ phenotypes. These results confirmed the tight linkages between *pro-81::Tn10* and *attP22* in *E. coli* and between *proB1657::Tn10* and *ataA* in *S. typhimurium*. This experiment also confirmed that all of the integrants represented inserted pCD2-P- at a single locus since the acquired Tc$^R$ from Tn10 was almost parallel to the lost of Cm$^R$ which indicated the replacement of the Cm$^R$ gene by the Tc$^R$ gene at the same location.
If the plasmid integrated into the host chromosome at multiple loci, the Cm\(^R\) phenotype should remain in all Tc\(^R\) transductants.

**Confirmation of plasmid integration by PCR.** A PCR assay was used as a second approach to confirm that the pCD2-P- plasmid had recombined into *attA* or *attP22* as shown in Fig. 3.3. PCR primers A and B were used to amplify a 561 bp product representing the intact *attP22* site from *E. coli* in combination with other two primers (C and D) specific to the plasmid vector. In the *E. coli* pCD2-PKS/SK derivative integrants, primers A and C amplified a 551 bp *attL* product, and primers B and D amplified a 479 bp *attR* product. A similar result was observed with the *S. typhimurium* integrants by the generation of a 470 bp *attR* product by the PCR primers D and E and a 346 bp *attL* product by primers C and F (Fig. 3.3).

**Study of the integration pattern of P22 SSR in E. coli by DNA sequencing.** We also took advantage of this plasmid-based system to characterize the products of the P22 SSR reaction. To accomplish this, a cointegrant plasmid, pTAP22, was constructed as described in Materials and Methods. This plasmid was generated by integrating pCD22PKS into a pBR322-derivative plasmid carrying *E. coli* *attP22*. Its configuration was confirmed by restriction digestion as well as by DNA sequence analysis. Although the P22 *attL* and *attR* sites contain an identical 46 bp common core sequence, they can be differentiated by the DNA sequence of the surrounding DNA. The sequencing results showed that the *attL* and *attR* sites, contained one arm from the *E. coli* and the other from the P22 phage DNA. The same 46 bp common core of *attL* and *attR* of *E. coli* integrant was identical to the common core of the *attP22* of the *E. coli* chromosome and P22 *attP* of phage DNA (Fig. 3.4). The
orientation of plasmid integration was identical in all integrants, consistent with the results of
the PCR reactions.

**Expression of heterologous protein in *S. typhimurium***. To test the SSR system for
expression of genes in *S. typhimurium*, a derivative of pCD22PKS was constructed that
carried the *E. coli lacZ* gene. The resulting plasmid, pCD22PlacZ, was integrated into *S.
typhimurium* chromosome. The integrant stably expressed β-galactosidase when plated on
X-gal containing LB plates without antibiotic selection. This confirmed that *S. typhimurium*
can express heterologous protein integrated by site-specific recombination.

**Plasmid excision and recovery**. The vector system described here also permits
recovery of the integrated plasmids. The excision process mimics the SSR reaction that P22
utilizes to excise its DNA out of the host chromosome. The process requires both the Int and
Xis proteins to recognize the *attL* and *attR* sites for recombination. To recover the integrated
plasmids we introduced either pXINT-A22 or pXINT-K22 into *E. coli* and *S. typhimurium*
integrated with pCD22PKS. The helper plasmids, pXINT-A22 or pXINT-K22, expressed
both Int and Xis and promoted excision of pCD22PKS out of both the *E. coli* and *S.
typhimurium* chromosomes. pCD22PKS was retrieved after growth in DH5αpir116
encoding ΦII protein required for R6Kγ ori plasmid replication (Platt *et al.*, 2000), in the
presence of chloramphenicol. Characterization of retrieved plasmid by restriction enzyme
analysis showed the same fragment sizes from several restriction enzymes as the original
plasmid (data not shown). This confirmed the complete cycle of the whole plasmid vector
integration and excision by P22 SSR.
Integration of plasmids at two chromosomal locations in *E. coli*. A number of bacterial strain constructions may require introduction of DNA constructs to two different locations of the bacterial chromosome. We tested if the P22 SSR system could be used in combination with a previously described system using λ SSR (Platt *et al.*, 2000). When the *E. coli* integrant of pCD22PKS, integrated at *attP22* site, was transformed with pCD13PSK carrying different antibiotic resistant gene, pCD13PSK was successfully integrated at the *attB* site in the presence of λ Int protein provided by a helper plasmid for integration.

Integration of plasmids at two chromosomal locations in *S. typhimurium*. Even though *S. typhimurium* does not contain primary attachment site for bacteriophage λ, pCD11PKS plasmid integrated into *S. typhimurium* chromosome. The integration of the second R6K γ ori plasmid (pCD21PKS) with 3 kb homologous sequence at *ataA* site is possible in the presence of P22 Int provided by helper plasmid for integration. The plasmid integration at *ataA* site was confirmed by PCR.

**DISCUSSION**

The successful development of a plasmid-based integration system utilizing the λ SSR machinery by our laboratory (Platt *et al.*, 2000) prompted us to construct a similar bacteriophage P22-mediated integration system to expand the tools available for bacterial strain construction. The opportunity to stably express foreign antigens in *S. typhimurium* was also a motivation for developing a plasmid-based P22 SSR system. This system is an effective tool to integrate a gene of interest into the P22 attachment sites on the chromosomes of *E. coli* (*attP22*) and *S. typhimurium* (*ataA*). The recombinants were shown to represent
integration of the pCD2-P- vectors at the specific attachment site of bacteriophage P22 and were stably maintained without continual antibiotic selection. The application of the P22 SSR system described here is similar to the λ SSR system in *E. coli* (Platt *et al.*, 2000). This system also permits integration of different genes into both *E. coli* and *S. typhimurium* chromosomes at 2 specific locations when applied consecutively with the λ SSR system. Although there was DNA homology between the two R6Kγ ori plasmids the integration of the second plasmid was at *attP22* and *ataA* sites respectively as confirmed by PCR of each host. Depending on the amount of homology, the homologous recombination is 10³- to 10⁴-fold less efficient than the λ SSR system (Silhavy *et al.*, 1984).

Moreover, plasmid integration at the known attachment sites ensures that no essential host gene function is interrupted. Lindsey *et al.* (1992) reported the overlapping sequences of the 46 base common core sequence of *attP22* and the 3' end of *thrW* gene. The integration of P22 phage into *E. coli* chromosome replaced the host sequence at the 3' end with the exactly same sequence from the P22 phage DNA (Lindsey *et al.*, 1992). A similar integration pattern was also demonstrated in bacteriophage P4 (Pierson III and Kahn 1987). In this system, the replacement of the *attP* sequence of the P4 phage at the 3' end of tRNA *leu* gene in *E. coli* did not interrupt the expression and function of the tRNA despite the presence of the cryptic integrase sequences at the 3' end of the transcript.

Our previous report (Platt *et al.*, 2000) confirmed that the plasmid vector of the λ SSR system was integrated at the primary *attB* site with no evidence of multiple site integration. However, integration of the plasmid at a secondary *attB* site was detected in the host with primary *attB* site deletion (unpublished data). In this study, transduction experiments of both *E. coli* and *S. typhimurium* integrants confirmed that all integrants had
single site pCD2-P- integration. Although not tested, it is likely that second attP22 sites also exist on the *E. coli* and *S. typhimurium* chromosomes. The wide variety of antibiotic resistance in the plasmid vectors and helper plasmids expands the application of the P22 SSR system in a host that may contain other existing antibiotic resistance.

The plasmid pTAP22, derived from a cointegrant of pCD22PKS and pT-attP22 (a plasmid carrying *E. coli* attP22), was constructed to use as a sequencing template for P22 attL and attR. The DNA sequences showed the expected recombination product of P22 SSR at both attL and attR sites on *E. coli*. The 46 bp common core is identical in both the *E. coli* and *S. typhimurium* P22 attachment sites. Our results showed that the left arm of the attL and the right arm of the attR sequences were derived from the *E. coli* chromosome while the left arm of the attR and the right arm of the attL sequences were from the P22 phage DNA carried by pCD22PKS (Figure 3.4). The data also corresponded to the results of the PCR assay used to characterize the pattern of integration of pCD22P- on the *E. coli* and *S. typhimurium*. These results are consistent with the P22 phage lysogenization in *Salmonella* reported by Leong *et al.* (1985) identified by restriction enzyme analysis. This group, however, did not report the DNA sequencing data.

The \( \lambda \) Xis protein inhibits integrative recombination and is required for excisive recombination (Abremski and Gottesman, 1982, Bushman *et al.*, 1984, Moitoso and Landy, 1991). The role of Xis is to provide a distinct reactive structure at attR and allow an efficient paring between attL and attR (Better *et al.*, 1983). The regions of Xis binding present in both the attP and attR (Yin *et al.*, 1985). These reports confirmed the high specificity of Xis protein to initiate \( \lambda \) excisive recombination.
One specific application of the P22 SSR system for stable gene integration in bacterial chromosome is a promising model for future recombinant *E. coli* and *Salmonella* vector vaccines expressing foreign antigens. For example, normal flora *E. coli* that naturally resides in the digestive tract may serve as a host for expression of heterologous antigen. The establishment of a normal flora *E. coli* host in the gut will continually provide a supply of the foreign antigen. If it is needed, conditional expression of the gene product can be achieved using a different regulatory system rather than the constitutive Lac expression system used in pCD2-P- derivative plasmids. The conditional expression of the foreign gene may be advantageous to prevent the destruction of the live bacterial vaccine by specific host defense mechanisms and will avoid host tolerance due to continuous exposure to the foreign antigen (Kuby, 1994). The ability to stably integrate DNA constructs at two chromosomal locations in *E. coli* expands further applicability of the system to express more than one foreign gene at a time.

Even though the primary attachment site of bacteriophage λ was not mapped on the *S. typhimurium* chromosome, Lee, *et al.* (2000) successfully utilized the λ SSR system to integrate enterotoxigenic *E. coli* K88 gene cluster into the chromosome of *S. choleraesuis* strain 54 (SC54) to use as a vaccine candidate in animals. The expression of the K88 antigen was also demonstrated in this study. Although the integration site was not identified, maintenance of the integrated plasmid did not require antibiotic selection.

Attenuated *S. typhimurium* has been successfully used to express heterologous antigens or fusion proteins of either bacterial, viral, or protozoan origin or other novel antigens, e.g., cytokines and contraceptive antigens (Doggett and Brown, 1996). *Salmonella* tends to withstand macrophage killing and confers the advantage of being an expressing
vector with respect to survival in the host (Ramsey et al., 1994). Expression of the
heterologous antigens in Salmonella has been shown to induce both humoral, especially
mucosal immune responses, and cell-mediated immune responses to purified recombinant
antigens (Doggett and Brown, 1996). One problem encountered with the expression of
antigens by recombinant plasmids is the instability of the plasmids in the absence of
continual selection. To stabilize the antigen expression, integration of the gene into a
Salmonella chromosome has been practiced mainly by homologous recombination (Chatfield
et al., 1992; Cardenas and Clements, 1993) or a balanced-lethal host-vector system
(Nakayama et al., 1988). The recombinant plasmid, pCD22PlacZ, constructed in this study
demonstrated that stable expression of a heterologous gene integrated in S. typhimurium
chromosome by P22 SSR could be achieved.

In addition to facilitating strain construction, the described vector system can also be
used for analysis of gene function. For example, a gene can be cloned into the R6Kγ ori
plasmid vector and integrated into a host chromosome. A mutagenesis of the inserted gene
can be performed and screened for a specific mutant. The mutated gene can be easily
excised out of the host chromosome as described and recovered by transformation into the
host strain DH5αpir116 (Platt et al., 2000). The recovered plasmid can then be used directly
for DNA sequencing or further genetic manipulation.

ACKNOWLEDGEMENTS

The authors thank the E. coli Genetic Stock Center (CGSC), Salmonella Genetic
Stock Center (SGSC), and the National Animal Disease Center (NADC) for providing the
reference bacterial strains used in this study. This study is partly supported by the National Institutes of Health and ISU Carver Charitable Trust.

REFERENCES


Table 3.1  Bacterial strains and bacteriophages used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Comment</th>
<th>Source/ Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>recA, r-m+</td>
<td>Host for plasmid replication</td>
<td>Gibco-BRL, NY</td>
</tr>
<tr>
<td>DH5α pir116</td>
<td>recA, r-m+, pir116</td>
<td>Host for R6K γ ori plasmid replication</td>
<td>Platt et al., 2000</td>
</tr>
<tr>
<td>MC4100</td>
<td>r+m+</td>
<td>Host for homologous recombination</td>
<td>CGSC</td>
</tr>
<tr>
<td>MG1655</td>
<td>recA</td>
<td>Host for plasmid integration</td>
<td>Miller, 1992</td>
</tr>
<tr>
<td>CAG18447</td>
<td>pro-81::Tn10</td>
<td>Donor host for P1 transduction</td>
<td>SGSC, Singer et al., 1989</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JL2520</td>
<td>proB1657::Tn10</td>
<td>Host for plasmid integration</td>
<td>NADC, SGSC, Sanderson and Roth, 1983</td>
</tr>
<tr>
<td>LB5010</td>
<td>r-m+</td>
<td>Host for plasmid modification from E. coli system to S. typhimurium.</td>
<td>Bullas and Ryu, 1983</td>
</tr>
<tr>
<td>P22</td>
<td>wild type</td>
<td>Source of attP22 site, xis, and int genes</td>
<td>Miller, 1992</td>
</tr>
<tr>
<td>HT</td>
<td>vir</td>
<td>Transducing phage</td>
<td>Maloy, 1990</td>
</tr>
<tr>
<td>P1</td>
<td></td>
<td>Transducing phage</td>
<td>Miller, 1992</td>
</tr>
</tbody>
</table>

CGSC: E. coli Genetic Stock Center
SGSC: Salmonella Genetic Stock Center
NADC: National Animal Disease Center
Table 3.2  Plasmids used in this study and their relevant genotypes

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>ori</th>
<th>Relevant genotype</th>
<th>Ab*</th>
<th>Comment</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC18</td>
<td>CoIE1</td>
<td>lacZA, MCS</td>
<td>Ap</td>
<td>cloning vector</td>
<td>Pharmacia.</td>
</tr>
<tr>
<td>pNoTA</td>
<td>CoIE1</td>
<td>lacZA, MCS</td>
<td>Ap</td>
<td>cloning vector</td>
<td>5'→ 3' Inc.</td>
</tr>
<tr>
<td>pBG88</td>
<td>Co/El</td>
<td>lacZA, MCS</td>
<td>Km</td>
<td>cloning vector</td>
<td>Spratt et al., 1986</td>
</tr>
<tr>
<td>pCD11PKS/SK</td>
<td>R6Kγ</td>
<td>lacZA, MCS, λattP</td>
<td>Cm</td>
<td>plasmid vector for integration at attB</td>
<td>Platt et al., 2000</td>
</tr>
<tr>
<td>pCD13PKS/SK</td>
<td>R6Kγ</td>
<td>lacZA, MCS, λattP</td>
<td>Cm</td>
<td>plasmid vector for integration at attB</td>
<td>Platt et al., 2000</td>
</tr>
<tr>
<td>pCD21PKS/SK</td>
<td>R6Kγ</td>
<td>lacZA, MCS, P22</td>
<td>Sp</td>
<td>plasmid vector for integration at attB</td>
<td>This study</td>
</tr>
<tr>
<td>pCD22PKS/SK</td>
<td>R6Kγ</td>
<td>lacZA, MCS, P22</td>
<td>Sp</td>
<td>plasmid vector for integration at attB</td>
<td>This study</td>
</tr>
<tr>
<td>pCD23PKS/SK</td>
<td>R6Kγ</td>
<td>lacZA, MCS, P22</td>
<td>Sp</td>
<td>plasmid vector for integration at attB</td>
<td>This study</td>
</tr>
<tr>
<td>pCD22PlacZ</td>
<td>R6Kγ</td>
<td>lacZ, P22 attP</td>
<td>Sp</td>
<td>plasmid vector for integration of lacZ gene at attB or attA</td>
<td>This study</td>
</tr>
<tr>
<td>pPir1030</td>
<td>CoIE1</td>
<td>pir</td>
<td>Ap</td>
<td>provide π protein for R6Kγ ori</td>
<td>This study</td>
</tr>
<tr>
<td>pPIC3</td>
<td>pSC101TS</td>
<td>λint, pir</td>
<td>Km</td>
<td>helper plasmid for integration at attB with pir gene</td>
<td>Platt et al., 2000</td>
</tr>
<tr>
<td>pAPRIL</td>
<td>pSC101TS</td>
<td>λint</td>
<td>Ap</td>
<td>helper plasmid for integration at attB without pir gene</td>
<td>This study</td>
</tr>
<tr>
<td>pINT-P22</td>
<td>CoIE1</td>
<td>intP22</td>
<td>Ap</td>
<td>helper plasmid for integration at attB</td>
<td>This study</td>
</tr>
<tr>
<td>pINT-A22</td>
<td>pSC101TS</td>
<td>intP22</td>
<td>Ap</td>
<td>helper plasmid for integration at attB</td>
<td>This study</td>
</tr>
<tr>
<td>pINT-K22</td>
<td>pSC101TS</td>
<td>intP22</td>
<td>Ap</td>
<td>helper plasmid for integration at attB</td>
<td>This study</td>
</tr>
<tr>
<td>pXINT-A22</td>
<td>CoIE1</td>
<td>sstP22, intP22</td>
<td>Ap</td>
<td>helper plasmid for excision from attP22 or attA</td>
<td>This study</td>
</tr>
<tr>
<td>pXINT-K22</td>
<td>CoIE1</td>
<td>sstP22, intP22</td>
<td>Km</td>
<td>helper plasmid for excision from attP22 or attA</td>
<td>This study</td>
</tr>
<tr>
<td>pT-attP22</td>
<td>CoIE1</td>
<td>attP22</td>
<td>Ap</td>
<td>intermediate cointegrate plasmid for sequencing of P22 attL and attR.</td>
<td>This study</td>
</tr>
<tr>
<td>pTAP22</td>
<td>CoIE1</td>
<td>attP22::</td>
<td>Ap</td>
<td>cointegrate plasmid for sequencing of P22 attL and attR.</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Plasmids used in this study and their relevant genotypes.*
Figure 3.1 Plasmid vectors for integration.

The pCD2-PKS/SK contain the R6Kγ ori, attP from bacteriophage P22, antibiotic resistant cassette and the multiple cloning site within the lacZα coding region. The antibiotic resistant cassette in pCD21PKS/SK, pCD22PKS/SK, and pCD23PKS/SK are kanamycin, chloramphenicol, and spectinomycin respectively.
Figure 3.2 Helper plasmids for integration and excision.

The helper plasmid for integration, pINT-A22 / pINT-K22, carries int gene, ampicillin/kanamycin resistant cassette and pSC101<sup>TS</sup> ori.

The helper plasmid for excision, pXINT-A22 / pXINT-K22, carries both the xis and int genes, ampicillin/kanamycin resistant cassette and ColE1 ori.
Figure 3.3  PCR confirmation of plasmid integration in *E.coli* and *S.typhimurium*. Lane 1 is a 561 bp product of intact *attP22* of *E.coli* using primer pair AB. Lane 2 is a 551 bp product of *attL* of *E.coli* integrant using primer pair AC. Lane 3 is a 479 bp product of *attR* of *E.coli* integrant using primer pair BD. Lane 4 is a 100 bp DNA marker. Lane 5 is a 348 bp product of intact *ataA* of *S.typhimurium* using primer pair EF. Lane 6 is a 346 bp product of *attL* of *S.typhimurium* integrant using primer pair CF. Lane & is a 470 bp product of *attR* of *S.typhimurium* integrant using primer pair DE. The sequences of primers used are as follow, A: CGGCACACAACACTCCGAT, B: GCCAAGGATGTATAGTGAGCGAA, C: GGTTCGCCGATATGCTCATC, D: CAGCGGATTCGGAAAGGTCTG, E: ACTCATGGCGCATGGTAAACAAGA, F: ATGAGGTTGTACATAAGTG.
ATCGATCG represents primers A and B on the *E. coli* chromosome.
ATCGATCG represents primers C and D from pCD22PKS.
ATCGATCG represents the 46 bp common core sequence of *attL* and *attR*.
ATCGATCG represents nucleotide sequence originating from the *E. coli* chromosome.
ATCGATCG represents nucleotide sequence of pCD22PKS.

Figure 3.4 DNA sequences of the P22 *attL* and *attR* regions of *E. coli* chromosome. The left arm of the *attL* and the right arm of the *attR* sequences were derived from the *E. coli* chromosome while the left arm of the *attR* and the right arm of the *attL* sequences were from the P22 phage DNA carried by pCD22PKS.
CHAPTER 4. BACTERIOPHAGE THERAPY: A NOVEL METHOD OF LYTIC BACTERIOPHAGE DELIVERY

Ratree Platt, Donald L. Reynolds, and Gregory J. Phillips

ABSTRACT

Bacteriophage therapy is a potential alternative to the use of antibiotics that has been applied to control bacterial infections in both humans and animals. Despite its potential, multiple doses are required to provide a continuous supply of phage. A gastric acid neutralization step is also needed when phage particles were applied orally because of the susceptibility of the phages to acid lysis. To solve these limitations, a lysogen of a lytic mutant of bacteriophage λ in a non-pathogenic Escherichia coli strain was constructed for oral phage therapy. The lysogen of enteric origin bacteria is less susceptible to gastric acid than phage particles and acid neutralization should not be needed prior to treatment. The lysogen should also colonize in the intestinal tract and continuously release lytic λ phage that lyses the susceptible bacteria, thereby controlling the bacterial infection.

Two plasmid-based site-specific recombination (SSR) systems mediated by bacteriophages λ of E. coli and P22 of Salmonella typhimurium for integration of genes into the bacterial chromosome were used to construct a lysogen of a lytic bacteriophage λ. The P22 system was used to integrate the repressor gene cl from wild type λ into the chromosome of a non-pathogenic E. coli strain at the attP22. A lytic λ phage mutant, W30, encoding non-functional cl repressor protein is incapable of lysogeny and, consequently, only lyses λ-sensitive E. coli. W30 phage was marked with an antibiotic resistant gene cassette to
facilitate lysogen selection. The lysogen of W30 phage demonstrated the efficiency in decreasing the number of λ sensitive *E. coli*.

**INTRODUCTION**

The emergence of multiple antibiotic resistant pathogenic bacteria such as *Escherichia coli, Staphylococcus sp., Streptococcus sp., Pseudomonas sp.*, etc., has become a significant problem in the attempt to control both human and animal pathogens. Clearly, there is a need to develop alternatives to the use of antibiotics for elimination of pathogenic bacteria. One suggested solution is the use of lytic bacteriophages (Barrow *et al.*, 1998). Bacteriophages are bacterial viruses that are able to infect specific microbial species. Virulent phages lyse their hosts while temperate phages have the ability to either lyse the bacterial cells they infect or to integrate into the host chromosome and form a lysogen. A population of lysogens will, however, continue to release progeny phage into the environment. The progeny of phage will then repeat the infection cycle in their specific host.

Therapeutic uses of various bacteriophages in both humans and animals have been applied locally, orally, and systemically to treat a wide variety of antibiotic resistant pathogens with great success (reviewed in Alisky *et al.*, 1998). In more recent medical applications, Lang *et al.* (1979) applied bacteriophage therapy to treat long-term septic complications of orthopedic surgery involving antibiotic resistant organisms. The results were favorable in most of the reported cases. For example, topical applications of bacteriophages to control antibiotic-resistant staphylococcal suppurative skin infection in humans had been reported with "clinical improvement results" with no untreated control (Slopek *et al.*, 1983a, 1983b, 1984, 1985a, 1985b, 1985c, 1987, Cislo *et al.*, 1987).
Kochetkova et al. (1989) studied the efficacy of phage therapy of suppurative and inflammatory complications in oncological patients using medicinal phages manufactured by the Tbilisi Research Institute for Vaccine and Sera, Russia. They reported higher positive results from bacteriophage therapy than antibiotic treatment. The results depended on several factors, such as type of pyoinflammatory complications, the microflora pattern of the purulent foci, and characteristics of the therapeutic phages. Soothill (1994) successfully applied bacteriophage BS24 to control *Pseudomonas aeruginosa* contamination of burn wounds that caused failure in skin isografting.

In veterinary application, Smith and Huggins (1982) treated experimentally *E. coli* infected mice using phages. They also reported the superiority of phage therapy over antibiotics by showing that a single intramuscular dose of phage was more effective than multiple intramuscular doses of various antibiotics in curing mice of a potentially lethal induced infection of *E. coli*. Importantly, the few phage-resistant mutants of *E. coli* found in the mice were shown to be of greatly reduced virulence. Smith and Huggins (1983) also reported the effectiveness of phages in treating *E. coli*-induced diarrhea in piglets and lambs, and in calves (Smith et al., 1987a). They reported that the phages multiplied rapidly and profusely after gaining entry to the *E. coli*-infected small intestine and quickly reduced the *E. coli* to numbers that were virtually harmless. Smith et al. (1987a) reported the control of severe *E. coli* diarrhea in calves by a single oral dose of 10,000 phage organisms that were admixed with an oral inoculum of enteropathogenic *E. coli*. The "phage-infected" cows were also protected against diarrhea. Additional studies have also been performed to avoid loss of phage by the mammalian host defense systems that rapidly eliminate phages from the circulation. To avoid this limitation, Merril et al. (1996) isolated long-circulating
bacteriophage λ and P22 mutants that were maintained in the blood for a longer period of time. These phage mutants proved to have greater capability as antibacterial agents than the wild type phage in animals infected with lethal doses of bacteria. Barrow et al. (1998) applied a lytic bacteriophage to prevent septicemia and a meningitis-like infection in chickens caused by a K1+ bacteremic strain of E. coli. They reported a prolonged protection even when the phage administration was delayed until signs of disease appeared.

Bacteriophage therapy, on the other hand, has certain limitations. These include the necessity to neutralize gastric acidity prior to multiple dose of oral phage administration because the phage particles are more susceptible to acid lysis than the target bacteria (Smith et al., 1987b). Unpredictable results may also be contributed to by a narrow phage host-range, the formation of lysogens in the infected bacteria, the development of phage resistant bacteria, and the lack of a continuous supply of bacteriophages over the period of progressive bacterial infection. The goal of this study was to develop a novel and effective lytic phage delivery system that overcomes some of these limitations. The construction of bacterial strain lysogenic for a lytic phage for oral phage therapy from an enterobacteria origin should be able to overcome the requirements for multiple doses of the phage as well as the gastric acidity neutralization due to the acid resistance in enteric bacteria (Gorden and Small, 1993).

Bacteriophage λ is the best genetically characterized E. coli phage. Naturally, λ is a temperate phage that is able to lysogenize its DNA into the host chromosome by activity of the cl repressor protein. A phage mutant encoding a non-functional cl gene is essentially a lytic phage that is unable to form a lysogen in its susceptible host. In this study, a lysogen of a lytic λ phage mutant was constructed in a non-pathogenic E. coli strain. The lytic λ lysogen should be administered orally without a need for gastric acidity neutralization while ensuring
a continuous supply of the lytic phage. To construct this lysogen, lytic mutant of \(\lambda\) phage, W30, which contains a non-functional repressor gene \(cI\), was marked with an antibiotic resistant gene cassette to facilitate selection of a lysogen. Two plasmid-based site-specific recombination systems mediated by bacteriophages \(\lambda\) and P22 developed in our laboratory (Platt et al. 2000; Platt and Phillips, 2000) were applied as genetic tools to construct the lysogen. In vitro tests were performed to demonstrate the efficiency of the lysogens in decreasing the number of their susceptible hosts.

**MATERIALS AND METHODS**

**Strains and plasmids.** The bacterial strains, bacteriophages, and plasmids used in this study are listed in Table 1.

**Media and chemicals.** Luria-Bertani (LB) medium (Sambrook et al., 1989) was used throughout the study. The concentrations of antibiotics were: ampicillin (Ap), 100 \(\mu g/ml\); chloramphenicol (Cm), 20 \(\mu g/ml\); kanamycin (Km), 30 \(\mu g/ml\); and nalidixic acid (Nd), 30 \(\mu g/ml\). Bacterial cultures were grown at 37°C unless otherwise indicated. The Lac phenotype was demonstrated on an agar plate spread with 100 \(\mu l\) of 1% X-gal (5-bromo-4-chloro-3-indolyl \(\beta\)-D-galactopyranoside).

**Techniques of recombinant DNA and bacterial genetics.** Standard techniques of recombinant DNA were performed (Sambrook et al., 1989) for the construction of plasmid. Enzymes and reagents were obtained commercially and used according to the manufacturers' recommendations. Transformation was performed by either electroporation (Sheng et al.,
1995) or chemical transformation (Inoue et al., 1990). A modification of the technique described by Carter and Milton (1993) was used to isolate plasmid DNA. The DNA of λ and P22 phages were purified by using a commercial kit (Bio101, Vista, CA).

**Polymerase chain reaction (PCR).** All PCRs were performed in a total volume of 50 μl in a thermocycler (Perkin Elmer Gene Amp PCR System 2400, Perkin-Elmer, Foster City, CA). The reactions were performed using 25 cycles of 94°C, 50°C, and 72°C, at 1 minute each, with a 2-minute hold at 72°C at the end of the last cycle (unless otherwise indicated). The PCR products were visualized under ultraviolet illumination after agarose gel electrophoresis and ethidium bromide staining.

**Cloning of the cl repressor gene.** A 1.08 kb DNA fragment encoding λ cl gene was amplified from wild type λ DNA by PCR (5'GCTGGAATGTGTAAGAGCGGG3' and 5'TTGCTCAATTGTTATCAGCTATGCG3'). The amplified product was ligated to a pT-Adv plasmid vector and transformed into Top10 cells, and selected on LB + Km + X-gal plate. Transformants of this plasmid (pT-Adv-cl) showed immunity to wild type λ phage by a spot test (Maloy, 1990). pT-Adv-cl was digested with EcoRI and a fragment containing cl was isolated. The eluted fragment was ligated with EcoRI digested pCD22PKS, then transformed into DH5αpir116 with selection for CmR on LB + Cm + X-gal plates and yielded the recombinant plasmid pCD22Pcl. Immunity of the transformants of pCD22Pcl to wild type λ phage was again demonstrated by a spot test.

**Chromosomal integration of recombinant plasmid.** A recA E. coli strain GP353 served as host for plasmid integration. The strain was first transformed with the temperature sensitive helper plasmid for integration, pINT-A22, by selection for ampicillin resistance
(Ap<sup>R</sup>) at 30°C. The pINT-A22 transformant was then transformed with pCD22P<sub>cI</sub> by selection for Cm<sup>R</sup> at 30°C. GP353-c<sub>I</sub> transformants were subsequently restreaked twice for Cm<sup>R</sup> at 42°C to eliminate the helper plasmid. Finally, antibiotic resistant colonies were screened for ampicillin sensitivity (Ap<sup>S</sup>) to confirm loss of pINT-A22 and tested for immunity to λ phage by spot test. The integration of pCD22P<sub>cI</sub> was confirmed by PCR as previously described by Platt and Phillips (manuscript in preparation).

Construction of a λ W30 derivative. The E.coli strain MFH208 carrying pMFH49 (Henry and Cronan, Jr. 1991) was cultured in LB with Km and Cm and infected with lytic mutant of λ phage, W30. The progeny phages were harvested and used to infect a polA E. coli strain MFH210 to avoid transduction of pMFH49. The phages released from MFH210 were used for lytic λ phage lysogenization.

Lytic λ phage lysogenization. pAPRIL, the temperature sensitive (TS) plasmid encoding λ Int (Platt and Phillips, manuscript in preparation) was transformed into GP353-c<sub>I</sub> with selection for Ap<sup>R</sup>. The transformants were infected with a W30 phage lysate prepared from MFH210 and plated on LB with Ap and Km at 30°C. Single colonies were restreaked twice on Km plates at 42°C to eliminate the helper plasmid. The Km<sup>R</sup> colonies were screened for the release of lytic λ phage by the presence of clear plaques on a lawn of BNN102 by spot test. The Km<sup>R</sup> W30 lysogen (RP2000) was PCR confirmed for the Km<sup>R</sup> λ phage lysogenization at attB site. Two PCR primers designed from the right arm of the E. coli attB site, 5′CGCCGCTGTAATCGTGTTGATG3′ (Platt et al. 2000), and a wild type λ int gene, 5′ACTCGTCGCGAACCCTTTC3′ (Powell et al. 1994), were paired to amplify the 681 bp attR region of the lysogen.
**In vitro characterization.** The nalidixic acid resistant (Nd\(^R\)) *E. coli* strain ND1655 was isolated by culturing MG1655 overnight in LB + Nd broth followed by plating onto LB + Nd plates to isolate single colonies. ND1655 was used to test the efficacy of RP2000 *in vitro*. ND1655 and RP2000 were cultured overnight in LB + Nd and LB + Km broth respectively, then 50 µl of each culture were mixed in a tube of 5 ml LB broth. Fifty microlitres of the ND1655 culture was added into a second 5 ml LB broth tube and served as a control. The experimental and control cultures were harvested at 0, 1, 2, 3, 4, 5, 24 and 48 hours after inoculation. Dilutions of the samples were made and 100 µl each were plated onto LB + Nd plates. The resulting colonies were enumerated. One hundred microliter of diluted test samples were mixed with 3 ml of BNN102 containing 0.8% molten LB agar, and poured over LB plates. After overnight incubation at 37°C, phage plaques were enumerated. Three replications of the same experiment were performed and the results were averaged.

Ten colonies of ND1655 that arose on the LB + Nd plates collected at 24 hour post inoculation were tested for W30 phage resistance by a spot test (Maloy, 1990).

**RESULTS**

In order to construct a lysogen of the lytic λ mutant W30, a wild-type copy of the λ *cl* gene was integrated into the chromosome of GP353 at *attP22* site. GP353 was chosen as the host for W30 phage lysogenization as this *recA* *E. coli* strain is defective in homologous recombination. *λ cl* was integrated into GP353 by using pCD22Pcl in combination with the SSR system previously described (Platt and Phillips, manuscript in preparation). The integration of pCD22Pcl was confirmed by PCR (Fig. 4.2). The resulting strain, GP353-<i>cl</i>, expressed the λ repressor protein and, as predicted, imparted immunity to W30. Immunity
was demonstrated by a production of cloudy plaques when W30 was spotted on a GP353-cI lawn.

The lytic λ phage W30 was marked with a kanamycin resistant gene cassette by homologous recombination with pMFH49 which contained non-essential λ genes between the lysis genes and the right cohesive (cos) end (Henry and Cronan, Jr., 1991). pMFH49 also carried the λ cos site, along with chloramphenicol, and kanamycin resistant genes. Recombination between pMFH49 and W30 generated a cointegrant with 2 cos sites. These 2 cos sites, flanking W30 DNA and the kanamycin resistant gene, were the substrates for the λ packaging system resulting in the generation of KmR W30 phage particles (Fig. 4.1). The KmR W30 phage was used to infect GP353-cI that had been transformed with pAPRIL, a plasmid encoding λ Int (Platt and Phillips, manuscript in preparation). After growth at 42°C, KmR lysogens were selected generating the W30 lysogen RP2000. Lysogenization was confirmed by the generation of a 681 bp PCR fragment of the attR region of the lysogen (Fig. 4.2).

In order to demonstrate the effect of RP2000 on a susceptible E. coli strain, an in vitro test was performed. To distinguish between the phage sensitive strain (MG1655) and RP2000, the nalidixic acid resistant mutant ND1655 was isolated. The results showed that initially the phages released from RP2000 were effective at reducing the number of ND1655 when compared to the control culture (Fig. 4.3). The cell numbers dropped sharply after 2 hours post inoculation but increased after 24 hours. The number of W30 phage increased by 6 logs within 5 hours but slightly decreased after 24 and 48 hours. To better understand why the numbers of ND1655 did not continue to decline, 10 colonies that arose on the LB + Nd plates from samples collected at 24 hour post inoculation were tested for W30 sensitivity by
a spot test. All tested cultures yielded no plaques of W30 phage, demonstrating the cells had become phage resistant.

**DISCUSSION**


A temperate phage, such as λ, normally lysogenizes its host and forms a stable lysogen when the expression of its lytic genes is repressed by the specific repressor protein cl. On the contrary, a lytic λ mutant with a non-functional cl repressor gene is unable to lysogenize *E. coli* and, consequently, behaves essentially as a lytic phage. The lysogen of a temperate λ phage releases wild type phage within a culture of bacteria. This phage is capable of infecting another λ-sensitive host. The released phage may subsequently either lyse or lysogenize the new host. A mutation in the repressor gene of a temperate phage, e.g., cl of λ, renders the repressor protein unable to repress the phage replication, resulting in generation of a lytic mutant. Consequently, we reasoned that a lysogen of a lytic λ phage (cl') could be constructed by providing a functional copy of cl gene in the host. As cl gene is autoregulated (Ptashne, 1986), a single copy of the gene is needed to avoid overexpression. To accomplish this we developed two SSR systems to stably integrate DNA constructs into
the *E. coli* chromosome (Platt *et al.*, 2000, Platt and Phillips, manuscript in preparation). The stability afforded by this chromosomal integration system is also very practical if the system is to be applied in animal systems when antibiotic selection for plasmid maintenance cannot be used. Ideally, a culture of the lytic λ lysogen should release lytic λ phage while also maintaining the lysogen population.

To obtain a lytic λ lysogen, a non-pathogenic *E. coli* strain expressing the wild-type *cl* gene was constructed. A derivative of the plasmid vector pCD22P (Platt and Phillips, manuscript in preparation) system carrying the *cl* gene was constructed and integrated into the *E. coli* chromosome at the *attP22* site. The resulting *attP22::*pCD22P*cl* *E. coli* strain provided the functional repressor protein which, in turn, provided the opportunity for the lytic λ phage to lysogenize at its regular *attB* site. In order to facilitate selection of the rare W30 lysogens from the background of non-lysogens, the lytic λ mutant was marked with an antibiotic resistant cassette. The protocol developed by Henry and Cronan Jr. (1991) was used to recombine a Km\(^R\) cassette onto the W30 chromosome. To avoid transduction of kanamycin resistance by a plasmid-mediated event, a DNA polymerase I deficient (*polA*) *E. coli* strain, MFH210, was used to isolate the Km\(^R\) W30. Since this strain does not support *ColE1 ori* plasmid replication, it eliminated the chance of obtaining Km\(^R\) colonies from plasmid transduction. The host strain BNN102, an *hfl* (high frequency of lysogenization) mutant also proved useful in these studies. This strain is deficient in protease activity that permits easy differentiation between λ *cl* which forms clear plaque and wild type λ which forms cloudy plaques (Belfort and Wulff, 1973). In *cl* expressing cells, all the genes of the infecting phage including *int* are repressed. The helper plasmid for integration, pAPRIL, was
used as it provided the $\lambda$ Int protein necessary to initiate SSR. This plasmid contains a temperature-sensitive ori which made it possible to eliminate the vector at the non-permissive temperature of 42°C after phage lysogenization. The use of a recA host also helped avoid the chance of phage integration by homologous recombination with the chromosomally-integrated cl gene. It also assured that the released lytic phage would not revert to wild type due to cl homologous recombination.

The strain RP2000 was shown to effectively decrease the number of $\lambda$-sensitive E. coli in an in vitro assay shortly after inoculation. However, phage resistant mutants arose in the presence of a high concentration of W30 phage and multiplied back to a high number after 24 hours post inoculation. Smith and Huggins (1982) also reported the emergence of phage-resistant mutants of a pathogenic E. coli strain after lytic phage therapy in mice. The mutants, however, were shown to be of greatly reduced virulence. Since the bacterial strain ND1655 used in these experiments is a derivative of the strain K-12, we were unable to demonstrate an alteration of bacterial pathogenicity. The observation that the numbers of lytic phage rapidly increased in a relatively short period of time in the in vitro assay also confirmed that the phages were replicating in the RP2000 host, as well as demonstrated the phage’s lytic effect on the target cells. The propagation of the phage during exposure to the target host increases the likelihood of rapid and complete elimination of the target bacteria before the emergence of phage-resistant mutants.

Although the in vitro results did not show long-term protection, in vivo experiments in various experimental animals will also be necessary to evaluate the applicability of oral therapeutic and prophylactic administration of this lytic phage lysogen in animals. The new model of lytic phage delivery described here could potentially be applied to other temperate
bacteriophages that utilize similar repressor system such as P22 phage of *Salmonella typhimurium* to cover a wider range of bacterial hosts. The use of different combinations of lytic phage lysogens would also cover wider host ranges. To potentially overcome the observed limitation of a single phage application due to the emergence of phage-resistant mutants, several phage types that use different receptors on the same target bacteria may be applied simultaneously.

The construction of a lytic λ lysogen reported here offers another approach in the attempt to apply bacteriophage therapy to control multidrug-resistant bacteria. Although the phage-resistant bacteria will eventually develop, the current antibiotic resistant crisis suggests that bacteriophages may help combating deadly bacteria when no other means are available.

ACKNOWLEDGEMENTS

The authors thank the curators of the *Escherichia coli* Genetic Stock Center (CGSC) and *Salmonella* Genetic Stock Center (SGSC) for providing the reference bacterial strains used in this study. This study was partly supported by the National Institutes of Health and ISU Carver fund.

REFERENCES


<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>Comments</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC4100 $\lambda^c$, attB', Δlac</td>
<td>Host for homologous recombination.</td>
<td>CGSC 1</td>
</tr>
<tr>
<td>DH5α lac, recA</td>
<td>Host for plasmid replication.</td>
<td>Gibco, BRL, Gaithersburg, MD</td>
</tr>
<tr>
<td>DH5α pir116 piri16</td>
<td>Host for R6Kγori plasmid replication.</td>
<td>Platt et al., 2000</td>
</tr>
<tr>
<td>MFH208 hsdR</td>
<td>Host of pMFH49 plasmid.</td>
<td>Henry and Cronan, Jr. 1991</td>
</tr>
<tr>
<td>GP353 recA</td>
<td>Host for lytic λ lysogen construction.</td>
<td>Phillips, G.J. (lab collection)</td>
</tr>
<tr>
<td>BNN102 hflA::Tn10</td>
<td>Host for detection of cl- phase.</td>
<td>ATCC</td>
</tr>
<tr>
<td>ND1655 Nd6</td>
<td>Host for in vitro test.</td>
<td>This study</td>
</tr>
<tr>
<td><strong>λ phage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type Wild type</td>
<td>Phage for lytic phage construction and source of functional repressor gene.</td>
<td>Miller, 1992</td>
</tr>
<tr>
<td>W30 cl</td>
<td>Phage for testing of the repressor protein expression.</td>
<td>Silhavy and Beckwith, 1985</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT-Adv-cl Ap^R, λ cl</td>
<td>Plasmid vector carrying λ cl gene</td>
<td>This study</td>
</tr>
<tr>
<td>pCD22PKS Cm^R, R6Kγori, attP22, MCS</td>
<td>Plasmid vector for integration at attP22 site.</td>
<td>Platt and Phillips, manuscript in preparation</td>
</tr>
<tr>
<td>pCD22Pcl Cm^R, R6Kγori, attP22, λ cl</td>
<td>Plasmid for integration of λ cl gene at attP22 site.</td>
<td>This study</td>
</tr>
<tr>
<td>pINT-K22 Km^R, or1TE3, P22 int</td>
<td>Helper plasmid for integration of plasmids at attP22 site.</td>
<td>Platt and Phillips, manuscript in preparation</td>
</tr>
</tbody>
</table>

1 *E. coli* Genetic Stock Center.
2 American Type Culture Collection.
3 Temperature sensitive origin of replication
Figure 4.1 Formation of a cointegrant by the homologous recombination between W30 and pMFH49 carrying λ non-essential genes (Rz, R, and S) and cos site. The cointegrant carries 2 cos sites which are substrates for λ packaging machinery. The bars indicate the part of DNA that is packaged in Km^R W30 λ phage.
Figure 4.2 PCR confirmation of the presence of the W30 Km\(^R\) \(\lambda\) lysogen. Lane 1 and 8 are 100 bp molecular weight markers. Lane 2 and 3 represent the intact \(\text{attB}\) site amplification of the MC4100 and W30 Km\(^R\) \(\lambda\) lysogen DNA, respectively. In lane 3, as W30 Km\(^R\) \(\lambda\) forms a lysogen at \(\text{attB}\) site, the 494 bp \(\text{attB}\) fragment is absent. Lane 4 and 5 show the 681 bp fragments of the \(\text{attR}\) of \(\lambda\) lysogen. Lane 6 and 7 show the 479 bp fragments of the \(\text{attR}\) of pCD22\(cl\) integrant. MC4100 -\(\lambda\) lysogen and MC4100-pCD22\(Pcl\) integrant DNA were used as positive control templates in lane 4 and 6, respectively. W30 Km\(^R\) \(\lambda\) lysogen DNA was used as template in lane 5 and 7. The primers used in lane 2 and 3 were 5'CGCCGCTCTGATCGTGGGTATG3' and 5'CACTCTCTGACAGCGCGCTC3', lane 4 and 5 were 5'CGCCGCTCTGATCGTGGGTATG3' and 5'ACTCGTCTGATCGTGGGTATG3', lane 6 and 7 were 5'GCCAAGGATGTATAGTGAGCGAA3' and 5'CAGCGGATTTCGGAAAGTCTG3'.
Figure 4.2 *In vitro* test results: The effect of RP2000 in decreasing number of λ-sensitive cells. The control (black bars) and test (stippled bar) samples were collected at 0, 1, 2, 3, 4, 5, 24 and 48 hours after inoculation and enumerated for ND1655 on LB + nalidixic acid plates. Clear phage plaques from test samples were counted on a lawn of BNN102 on LB plates. The numbers of cell and phage counts are represented as log10 value of colony forming unit/ml (left) and plaque forming unit/ml (right) respectively.
CHAPTER 5. GENERAL CONCLUSIONS

General Summary

Three papers are presented in this dissertation. The construction of a new genetic tool for *E. coli* strain construction is described in the first paper in Chapter 2. A plasmid system was developed for site-specific integration into, and excision and recovery of gene constructs and *lacZ* gene fusions from an *E. coli* chromosome. Plasmid suicide vectors utilizing the origin of replication of R6K*γ* plasmids and containing the *attP* sequence of bacteriophage λ facilitate reversible integration into the *E. coli* chromosome by site-specific recombination (SSR). Additional vectors permit construction of *lacZ* gene fusions in three possible reading frames for recombination with the bacterial chromosome. These suicide vectors can be propagated in the newly constructed *E. coli* strains that harbor different *pir* alleles. Two helper plasmids that encode the necessary gene products for integration (Int) and excision (Int and Xis) were constructed. This plasmid system was shown to be a reliable and efficient means to integrate and subsequently recover plasmids from the *E. coli* *attB* site. Chromosomal integration by SSR overcomes problems associated with gene overexpression on a multi-copy plasmid that may be detrimental to cell growth. It also provides a more accurate measurement of gene expression as monitored by a reporter gene. Integration at *attB* insures that no functional host gene is inactivated upon insertion.

Chapter 3 presented the construction of an additional plasmid-based genetic system that uses the SSR machinery of bacteriophage P22 to reversibly integrate DNA constructs into the chromosomal P22 attachment site of *E. coli* (*attP*22) or *S. typhimurium* (*attA*). This system expands the use of SSR in the construction of bacterial strains in addition to the
previously constructed λ system. The P22 system also utilizes a conditionally replicating plasmid that carries the R6Kγ origin of replication and bacteriophage P22 attachment site. The P22 SSR reaction was initiated using a helper plasmid that carries the P22 int gene and replicates using a temperature sensitive origin of replication. Recovery of the integrated constructs was achieved using a second helper plasmid that carries both the P22 xis and int genes. This system was demonstrated to be efficient in both integration and excision of plasmid vector DNA in *E. coli* and *S. typhimurium*. In combination with a previously constructed λ SSR system, the P22 system permits integration of gene constructs at two bacterial chromosomal locations.

Chapter 4 described the application of the systems reported in Chapters 2 and 3. Both λ and P22 SSR systems were applied to construct a lysogen of lytic bacteriophage λ in *E. coli*. A lytic λ phage mutant was marked with an antibiotic resistant gene to facilitate selection of a lysogen. To construct a lysogen of the lytic λ phage mutant, the P22 SSR system was applied to integrate a functional λ repressor gene, *cl*, into the host chromosome. The λ SSR system then applied to facilitate the lysogenization of the lytic λ phage. *In vitro* tests were performed which demonstrated the high efficiency of the lytic λ phage lysogen in decreasing the number of susceptible bacteria. This lytic phage lysogen construction strategy can be applied as an alternative to the use of antibiotics to control pathogenic bacterial infections *in vivo*. 
General Discussion

Bacteriophage therapy can be applied as an alternative to the use of antibiotics when the prevalence of chronic antibiotic resistant bacterial infection increases. The appearance of antibiotic residue in food animals also favors the biological control of bacterial infection. Certain problems involved with bacteriophage therapy have limited its application in the past. Several attempts have been made to improve the efficiency of bacteriophage therapy, including the selection of specific lytic phages (Barrow et al., 1998) as well as the phages that can survive in the blood circulation for a longer period of time (Merril et al., 1996). These approaches were demonstrated to improve the success of bacteriophage therapy in vivo as an antimicrobial agent.

In this study, molecular biology techniques were applied to construct a lytic $\lambda$ phage lysogen in a non-pathogenic E. coli strain. Two plasmid-based bacteriophage-mediated site-specific recombination systems of bacteriophages $\lambda$ and P22 were developed. The plasmid vectors of the $\lambda$ SSR system integrated into the E. coli chromosome at the attB site while those of the P22 system integrated at the attP22 site of E. coli and the ataA site of S. typhimurium chromosomes. The integration into the host chromosome initiated by these systems is very stable with no need for continual antibiotic selection. Both systems are very useful for bacterial strain construction. The reversible nature of both systems also enables the retrieval of the integrated genes to facilitate the study of any genetic change, e.g., mutation when required. The consecutive use of both systems in E. coli makes it possible to integrate two different genes of interest into two chromosomal locations. Both systems were also proven to work in other Salmonella serovars, e.g., S. choleraesuis, S. pullorum (unpublished data), even though the primary attachment sites were not identified.
The application of the two SSR systems provides a promising model for construction of new *E. coli* and *Salmonella* live vaccines that express foreign antigens. The normal flora that colonizes naturally in the digestive tract may possibly be used as an expression host. The establishment of recombinant bacteria in the digestive tract will provide a continuous supply of the integrated foreign antigen. The stable gene integration in *E. coli* at two chromosomal locations expands the applicability of the system to express more than one foreign gene at a time. Furthermore, attenuated *S. typhimurium* has been successfully used to express heterologous antigens or fusion proteins from either bacterial, viral, or protozoan origin or other novel antigens, e.g., cytokines and contraceptive antigens (Doggett and Brown, 1996). *Salmonella* tends to withstand macrophage killing and confers the advantage of being an expressing vector with respect to the survival in the host (Ramsey et al., 1994). Expression of heterologous antigens in *Salmonella* has shown to induce both humoral, especially mucosal immune responses, and cell-mediated immune responses to purified recombinant antigens (Doggett and Brown, 1996). If necessary, conditional expression of the gene product can be achieved using a different genetic regulatory system. Conditional expression of the inserted gene will reduce the frequency of destruction of the live bacterial vaccine itself by specific host defense mechanisms and also reduces the chance of host tolerance due to continuous exposure to the antigen (Kuby, 1994).

In this research, both integration systems were applied to construct a lysogen of a lytic mutant of bacteriophage λ. The λ repressor gene was integrated into a non-pathogenic *E. coli* chromosome by P22 SSR. To facilitate selection of the lysogen, the lytic λ phage mutant was marked with a kanamycin resistant gene cassette. The marked lytic λ phage then lysogenized into the chromosome of the repressor expressing *E. coli* by λ SSR. The *in vitro*
test demonstrated the ability of the lytic λ lysogen in decreasing number of a λ-sensitive bacteria. This novel method of oral phage therapy may be able to solve the problem of requirements for multiple doses of the phage application as well as for gastric acidity neutralization prior to oral phage treatment.

Bacteriophage therapy is one of several approaches employed to combat multi-drug resistant bacteria. This strategy of lytic bacteriophage lysogen construction can be applied as a model to construct lysogens of other lytic bacteriophages to cover a wider range of bacterial hosts. *In vivo* experiments will be needed in various experimental animals to evaluate the applicability of this lytic phage lysogen for oral prophylactic and therapeutic purposes.

**Recommendations for Future Research**

This study consisted of two parts, the construction of molecular genetic tools and their application to construct a lytic bacteriophage lysogen. Future research will help strengthen their potential use. The following recommendations are made for future research:

1. *Construction of lytic λ lysogen by a different approach.* Lytic λ phage can be constructed by a complete replacement of repressor gene, *cl*, by an antibiotic resistant cassette. The constructed lytic phage will contain no homologous sequence to the integrated *cl* gene. This will eliminate the chance that mutated *cl* of lytic phage recombine with the integrated *cl* gene by homologous recombination and return to wild type phage. The similar approach can be applied to construct the lytic phage lysogen. The construction of a lytic λ phage has already begun in our laboratory.
2. **Construction of lysogens of other lytic bacteriophages.** The construction strategy of the lytic phage lysogen can be applied to other bacteriophages to expand the target pathogenic bacteria host range. The bacteriophage P22 of *S. typhimurium* has a very similar site-specific integration system as in the λ phage. The construction of a lytic P22 lysogen has already begun in our laboratory.

3. **Evaluate the performance of the lytic bacteriophage lysogen in various experimental animals.** Prior to prophylactic or therapeutic applications, the efficiency of lysogen in the digestive tract should be studied as well as any possible side effects. The locations of intestinal colonization by lysogen and the lytic phage release pattern should be identified. Various experimental animals may be used as different model for further in vivo applications.

4. **Construction of live bacterial vaccines expressing multiple foreign genes.** The *S. choleraesuis* strain 54 carrying the K88 antigen of *E. coli* was successfully constructed using the integration system of bacteriophage λ (Lee et al., unpublished data). Although the integration site for the λ phage in *S. choleraesuis* was not identified, *S. typhimurium* naturally contains a specific attachment site for the bacteriophage P22, and the λ integration system has been shown to be able to integrate into the *S. typhimurium* chromosome (unpublished data). A vaccine of *Salmonella* origin is also known to stimulate both humoral and cellular host defense systems.

5. **Application of the integration systems to control enteropathogenic *E. coli* infections.** The proteins " intimins" expressed by enteropathogenic *E. coli* are known to facilitate the adherence of the bacteria to intestinal mucosa and confer pathogenicity. The intimins' encoded genes have been identified (McGraw et al., 1999; Adu-bobie et al., 1998;
Beebakhee et al., 1992) and can be stably integrated into a non-pathogenic *E. coli* or *Salmonella* by site-specific recombination. The protein expression can be regulated by genetic regulatory system to prevent constitutive expression that may lead to the immune tolerance. At least two different intimin genes can be integrated into the same bacteria by the consecutive application of both SSR systems. The intimins expressing cells will then stimulate specific IgA production and can be used for prophylactic purpose in animal.

References


ACKNOWLEDGEMENTS

I would like to thank my co-major professors,
Dr. Donald L. Reynolds who gave me initial ideas, guidance and made it possible for me to participate in this interesting project.
Dr. Gregory J. Phillips who unselfishly shared valuable knowledge and his excellent laboratory facility. He put in a tremendous amount of time and effort to help me learn and improve myself. This dissertation is his as much as it is mine.

I also thank my POS committee members,
Dr. Jeffrey K. Beetham
Dr. James S. Dickson
Dr. Paul N. Hinz
Dr. Merlin L. Kaeberle
Dr. John P. Kluge
Dr. Theodore T. Kramer
Dr. John E. Mayfield.

I am in deep appreciation to
my home country, THAILAND
my parents, Mr. Charoen and Mrs. Ampa Yuadyong
my teachers in the past, present, and future, too many to mention
my sisters, Nittaya and Rung-a-run
my daughters, Oun and A
my friends, also too many to mention
and most of all to my husband, Dr. Kenneth B. Platt, who means the world to me.

Without them, their love, and support, I am not here today.

Thanks Thanks Thanks Thanks Thanks Thanks Thanks Thanks Thanks Thanks Thanks