

Chapter 2

Phage for Biocontrol

Abstract Bacteriophage (phage) therapy, or the therapeutic use of phage for the treatment of bacterial diseases, is a classical approach that was originally disregarded due to inconsistent results and with the advent of antibiotic drugs. However, with a greater understanding of phage biology and the pressing need for new and innovative antimicrobial strategies to challenge the ever-increasing prevalence of multidrug-resistant bacterial pathogens, phage therapy is seen to have great potential for reintroduction as antimicrobial strategy, although not without many limitations. In this chapter, by pointing out the limitations of native bacteriophage (phage) therapy, engineered phage-based bactericidal delivery vehicles will be introduced as a treatment approach for the biocontrol of a variety of important pathogens. Such an efficient approach would be suitable for concurrent treatment with standard antibiotics and possibly become a suitable replacement. The bacterial infections to be considered will include those due to: *Escherichia coli*, *Staphylococcus aureus*, *Chlamydia trachomatis*, *Pseudomonas aeruginosa*, and *Helicobacter pylori*. The pathogens will be described along with the efficiency of the phage-based methods to be investigated.

1 Introduction

One of the most concerning problems in therapeutic medicine today is the emergence of multi-drug resistant bacteria and fungi (Sulakvelidze et al. 2011). Bacterial infections are among the most prevalent causes of illness and mortality in clinical settings (Georgiev 2009). The increase of immunosuppressed patients in the present era results in more serious diseases and prolonged hospitalizations with bacterial pathogens (Sulakvelidze et al. 2011; Lu and Collins 2009). Moreover, new antibiotics are not being produced at a sufficient rate to replace the previous medicines which are less effective (Coates and Hu 2007; Kutateladze and Adamia 2010). The economic burden of antibiotic resistance is continuously increasing and

is currently exceeding an estimated 55 billion dollars annually in the United States alone (Smith and Coast 2013). Additionally, the potential cost of the future development of drug resistance is still unknown. Therefore, adequate attention and the devotion of resources devoted towards resolving the problem of antibiotic-resistant bacteria is one of the first priorities in modern medicine (Sulakvelidze et al. 2011).

Bacteriophages are among the most well studied and abundant organisms on the planet (Clokiet al. 2011). They are distinguished as viral entities that exclusively infect bacterial cells and are composed of a DNA or an RNA genome surrounded by a protein coat. There are two typical phage growth cycles: lytic and non-lytic (Petty et al. 2007), both of which use the host bacterium as a source for their own replication (for a full description about phage types and cycles, refer to Chap. 1: Phage Basics). Considering that phage have a natural capacity to target, exponentially replicate within, and kill their bacterial hosts, they have been deemed a considerable potential option in treatment of bacterial infections (Merril et al. 2003).

Phage therapy can be defined as the therapeutic use of bacteriophage to cure bacterial infections. The history of the usage of bacteriophage therapy for bacterial infections in humans is extensive and dates back to initial studies in this field, as early as 1919 when the co-discoverer of the bacteriophage Felix d'Herelle suggested the use of phage for the treatment of bacterial-induced diarrhoea (Brüssow 2005). Phage-based therapies were sold by American pharmaceutical companies in the 1930s and were used by soldiers in the Second World War to fight off dysentery (Brüssow 2005). The use of phage therapy in the West was thwarted by the invention and practical application of antibiotics for the treatment of bacterial infections (Matsuzaki et al. 2005). However, phage therapy continues to be a common treatment method in the Soviet Union where a number of companies, namely Microgen Inc., sell a long list of different phage cocktails due to a shortage of antibiotics (Hagens et al. 2004; Alisky et al. 1998; Hanlon 2007). Recently, the increasing rate of emergence of multi-drug-resistant bacteria has motivated medical scientists to reconsider phage therapy as a therapeutic option for bacterial infections that are not treatable by conventional antibiotic therapy (Matsuzaki et al. 2005). Even though it is unlikely that antibiotics will be replaced by phages in near future, they offer a great alternative for treatment of drug-resistant pathogens either as monotherapy or in combination with other antibiotics (Kutateladze and Adamia 2010; Smith and Huggins 1982; Kutter et al. 2010).

As a result of problems encountered in using the native phages for treatment of infectious disease, scientists have recently presented the idea of creating genetically modified phages with high killing efficiencies (Hagens et al. 2004). In this chapter we will describe using genetically modified bacteriophage, herein referred to as recombinant phage, for the treatment of bacterial infections. Furthermore, new applications using engineered phages for treatment of drug addictions such as that for cocaine will be briefly discussed.

2 The Importance of Using Recombinant Phage

The past use of native phages for the treatment of bacterial pathogens has not been without its difficulties and consequences, and the stigma arising from these difficulties has led to a false understanding about the potential of phage-based therapeutics (Brüssow 2012). With our current understanding of phage properties and genetics, the limitations of phage therapy using lytic phages can be circumvented with the use of recombinant phages, with their distinct set of properties, for the effective treatment of bacterial diseases. In this section, some of the limitations of native phages will be discussed as well as the alternatives that recombinant phage can offer.

Lysis of bacterial cells, normally associated with lytic phage, will result in the disintegration of cell wall components and consequently the release of endotoxin, typically resulting in inflammation and seen as circulatory shock or sepsis in treated subjects (Paul et al. 2011; Matsuda et al. 2005). To address this limitation, delivery agents for lethal cargoes have been designed using phage-based in vivo packaging systems to create a lysis-deficient phage and/or non-replicative phage that will have bactericidal activity without destroying the cell wall (Goodridge 2010). This system benefits from being able to selectively kill the target cell without releasing the cell contents, which could potentially cause sepsis or release the intracellular toxin that has been delivered. Methods for developing lethal delivery agents may be based on the elimination of the lysis genes from otherwise lytic phage or may use phages that are intrinsically lysis-deficient (Goodridge 2010).

Hagens and Blasi (2003) evaluated a recombinant M13 filamentous phage encoding lethal proteins for killing bacteria without host-cell lysis. Bacterial survival was determined after infection of a growing *Escherichia coli* culture with bacteriophage M13 that encoded either the restriction endonuclease BglII (phage M13R) gene or two modified phage λ S holin genes. Infection of bacteria with either of the recombinant phage led to a high killing efficiency, notably 99.9 % of the host cells were killed within 6 h after treatment with the phage expressing restriction endonuclease BglII (Hagens and Blasi 2003). Furthermore, all treatments succeeded in leaving the host cells intact. Bacterial growth did however resume between 2 and 3 h following infection due to the emergence of phage-resistant mutants (Hagens and Blasi 2003).

In another study by Hagens et al. (2004) engineered non-replicating, non-lytic phages were used to treat *Pseudomonas aeruginosa*. The modified phage killed the bacterial pathogen with minimal release of the host endotoxin (Hagens et al. 2004). It also has been found that modified lysis-deficient *Staphylococcal* phages are efficient in killing of methicillin-resistant *S. aureus* without inducing lysis (Paul et al. 2011). In another study, Matsuda et al. (2005) used a modified *E. coli* phage (*t* amber A3 T4) that was genetically altered to contain a mutation in the holin gene, which prevented lysis of the bacterial cells after infection. The phages were able to effectively infect and replicate within the host bacterial cells; however, their inability to lyse the cells prevented liberation of potentially dangerous endotoxins. The bacterial cells were

killed, but remained intact. This phage treatment was demonstrated to improve survival using a murine peritonitis model (Matsuda et al. 2005).

The limited bacteriophage host range is another limitation of phage therapy that should be considered. Each phage species will typically have a very limited and specific host range, in that they can only target one species and even in some cases one single strain of bacteria. Therefore, developing modified phages with an expanded host range through the means of synthetic biology is an important priority in the field. Evidence for the value of expanding the phage host range can be seen in the research from Timothy Lu's lab at MIT (Lu and Collins 2009), including an initial study that involved the grafting the gene 3 protein (g3p) of one filamentous bacteriophage (Ike) to another (Fd), thereby extending the filamentous phage host range. (Ike and Fd are two similar filamentous bacteriophages that target their host by attaching to the pili on host surface membranes. Fd infects bacteria bearing F pili, while Ike infects bacteria bearing N or I pili). In this study the recombinant phage was able to infect bacteria bearing either N or F pili (Lu and Collins 2009).

A further challenge is that phage therapy typically results in a bacterial resistance, often within hours *in vitro*, to the phages. There is an ever-continuing arms race between phage infection and bacterial resistance to phages, where bacteria have established immunity mechanisms as crucial survival phenotypes. These phenotypes include but not limited to: preventing phage absorption (Labrie et al. 2010; Samson et al. 2013), blocking phage DNA entry (Labrie et al. 2010; Samson et al. 2013), restriction-modification systems (Labrie et al. 2010; Samson et al. 2013), the CRISPR/Cas system (Hatoum-Aslan and Marraffini 2014; Deveau et al. 2010), and abortive infection systems (Labrie et al. 2010; Samson et al. 2013; Amati 1961). Therefore, new techniques are needed to reduce the rate of phage resistance. One of these techniques can be to combine the phage with antibiotics (Lu and Koeris 2011). The use of 'phage cocktails' and/or cycling between different phage treatments is another strategy, as well as specifically considering disrupting the phage-resistance mechanisms while designing the engineered phage (Goodridge 2010).

3 Recombinant Phage for the Treatment of Bacterial Infections

The exponential growth of antibiotic resistance has encouraged researchers to find alternative modalities for treatment of bacterial infections. Pathogens showed resistance to penicillin as early as the 1940s and this became clinically significant leading into the 1960s (Alisky et al. 1998). Currently, there are many pathogens that show resistance not only to penicillin but also to third-generation cephalosporin and even vancomycin (Alisky et al. 1998). Lytic phage therapy has been shown to be effective in treatment of drug-resistant pathogens, at least in uncontrolled clinical studies (Brüssow 2012; Goodridge 2010). In this Section, different studies that employ recombinant phages for the treatment of specific bacterial infections will be discussed.

3.1 *Escherichia coli* (*E. coli*)

E. coli, a gram-negative bacillus, is considered one of the important health concerns in the Western world. An example of this organism (*E. coli* O157:H7) which is the most common and most studied member of this group was identified as the causative agent of two outbreaks of bloody diarrheal syndrome in 1982 (Griffin and Tauxe 1991; Rangel et al. 2005). *E. coli* and its relatives can cause an impressive range of diseases. In general, the pathogens can be described as gram-negative bacilli, facultative aerobes and members of the Enterobacteriaceae family. They make up a substantial portion of the human colonic flora, and develop there as early as a few hours after birth (Nataro and Kaper 1998). *E. coli* is typically non-pathogenic when confined to the lumen of the gastrointestinal tract; however, certain strains of this species cause human disease when introduced to other areas of the body. There are many different strains, each with a different clinical outcome. Some strains are considered more pathogenic than others, although most are capable of causing disease, especially in immunocompromised hosts. *E. coli* is the predominant culprit in illnesses such as urinary tract infection (UTI) (Karlowsky et al. 2002). Management of these diseases is complicated by drug-resistant infections. Fluoroquinolone and trimethoprim-sulfamethoxazole resistance limit outpatient treatment while cephalosporin resistance limits inpatient treatment (Johnson et al. 2010).

It has been demonstrated that suppressing SOS network (a global response to DNA damage in which the cell cycle is arrested and DNA repair and mutagenesis are induced) in *E. coli* using engineered M13 bacteriophage heightened quinolone efficiency by several orders of magnitude in vitro. SOS network task is repairing the DNA damage (Echols 1981). To disrupt the SOS response, the *lexA* 3 SOS suppressing gene was inserted into the phage genome. Moreover, treatment of infected mice with modified phage plus the fluoroquinolone antibiotic Ofloxacin, significantly increased their survival compared to unmodified phage plus antibiotic or no phage plus antibiotic. The level of antibiotic-resistant cells was dramatically reduced with the engineered phage. According to this study, the use of phage in combination with antibiotics could decrease antibiotic-resistant mutants that come from the bacterial population exposed to bactericidal agents (Lu and Collins 2009).

Though this is a unique technique for manipulating bacteriophage targeting the SOS network, which is a beneficial pathway in *E. coli*, could weaken the bacteria harboring the phage (Lu and Collins 2009; Citorik et al. 2014). Following this study, Edgar et al. introduced a system using genetically-engineered phage in order to reverse the pathogen's antibiotic resistance (Edgar et al. 2012). *E. coli* mutants resistant to streptomycin due to mutations in the *rpsL* gene were isolated and transformed with plasmids containing wild type (WT) *rpsL*. The delivery of WT *rpsL* gene to the streptomycin-resistant *E. coli* made the mutants significantly more sensitive (approximately a 10-fold increase in bactericidal activity) to this antibiotic. Furthermore, the group was also able to produce an increase in the bactericidal activity of streptomycin on the *rpsL* mutants through lysogenization with an

engineered bacteriophage lambda (λ) strain modified to carry *rpsL* gene. To establish whether the system is expandable to other antibiotics, phage λ was engineered to contain wild-type copies of *gyrA*, then lysogenized with nalidixic acid-resistant bacteria. The recombinant phage was able to restore the *E. coli* strain sensitivity to the nalidixic acid antibiotic. According to this study, the proposed system may be practical for treatment of difficult drug resistant bacterial infections (Edgar et al. 2012).

In another study, Westwater et al. (2003) added Gef and ChpBk toxins to the M13 phagemid system to investigate the possibility of using phage as a lethal-agent delivery vehicle. The bacterial loads were reduced by several orders of magnitude both in vitro and in vivo in mice models infected by *E. coli* following the phage-mediated delivery of bactericidal agents. This technology may open new doors in treatment of multi-drug resistant bacterial pathogens (Westwater et al. 2003).

3.2 *Staphylococcus aureus* (*S. aureus*)

S. aureus is one of the main causes of hospital- and community-acquired disease (Hiramatsu et al. 2001). The organism has readily developed resistance against therapeutic agents used over the past 50 years. Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most notable example of this phenomenon and was discovered in 1961 (Hiramatsu et al. 2001). MRSA is now a frequent culprit of skin and soft tissue infections in the United States (Klevens et al. 2007). In hospitalized patients, MRSA infections are correlated with longer hospitalization, increased mortality and morbidity, and higher expenses (Klevens et al. 2007). The emergence of multi-drug resistant *S. aureus* has motivated the re-evaluation of phage therapy for this pathogen.

Recently, it has been shown that a recombinant lysis-deficient *S. aureus* phage P954 could rescue immunocompromised mice infected by MRSA without lysing bacterial cells and releasing endotoxin (Paul et al. 2011). Bacteriophage P954 is a temperate phage that was amplified in *S. aureus* strain RN4220. In order to construct the new plasmid, the native endolysin gene was replaced with an endolysin gene disrupted by the chloramphenicol acetyl transferase (CAT) gene. Induction of the parent plasmid with mitomycin resulted in cell lysis while the endolysin-deficient phage P954 did not lyse. The bactericidal activities of parent and recombinant plasmids were comparable and the host range was the same (Paul et al. 2011).

3.3 *Chlamydia trachomatis*

Chlamydia trachomatis (CT) is an obligate intracellular pathogen which is responsible for genital tract infections in young sexually active women (Somani et al. 2000; Béb  ar and de Barbeyrac 2009; Dean et al. 2000). Recently, it has been indicated that

chronic asymptomatic chlamydial infections can cause infertility in women (Somani et al. 2000). A high rate of recurrence of chlamydial infections is common in a sexually active population and has been associated with the development of antibiotic-resistant organisms (Bébéar and de Barbeyrac 2009; Somani et al. 2000). The treatment of CT by conventional phage is challenging, because of its intracellular nature. To overcome this problem, Bhattarai et al. (2012) engineered a M13 phage to express integrin-binding peptide Arg-Gly-Asp (RGD), which is a eukaryotic adhesion motif, to facilitate internalization of the phage into the cells. Moreover, CT peptide (polymorphic membrane protein D) was added to RGD-M13 to interfere with CT infection. In this study, the modified phage reduced the CT infection significantly in primary endocervical cells compared to CT infection alone. The engineered M13 phage enhanced cellular internalization and could be considered as a new modality for treatment of CT infection and other sexually transmitted disease (Bhattarai et al. 2012).

3.4 *Pseudomonas aeruginosa* (PA)

P. aeruginosa (PA) is a common, gram-negative, opportunistic pathogen that is found to be the culprit in many challenging infections in the airways, epithelium and blood systems. As PA is common in immunocompromised and hospitalized patients, it would be ideal to have a treatment strategy that comes with minimal negative health outcomes to the patient (Hilf et al. 1989; Dzuliashvili et al. 2007).

In one study for treatment of *P. aeruginosa* infection, genetically modified non-replicating, non-lytic phage were produced (Hagens et al. 2004). The PA filamentous phage (Pf3) was armed through recombinant DNA technology with the BglII restriction endonuclease gene. The recombinant pf3 phage (Pf3R) was able to significantly reduce PA infection in mice with minimum release of endotoxin, showing good potential for this recombinant phage technology (Hagens et al. 2004). Treatment of infected mice by *P. aeruginosa* with three times the minimal lethal dose (MLD) of either Pf3R or replicating lytic phage resulted in a cure of mice in both cases. In spite of that, when mice were challenged by 5 times the MLD, the survival rate of Pf3R treatment was significantly higher than that of mice treated by lytic phage therapy. This might be due to a reduced inflammatory response in Pf3R-treated mice compared to mice treated by lytic phage. This study demonstrates that treatment of experimental bacterial infection by non-replicative phage can be as effective as replicative phage. Moreover, the use of non-replicative phage would give us the opportunity to specify the therapeutic phage dose, which is not feasible by replicative phage as they increase exponentially (Hagens et al. 2004).

3.5 *Helicobacter pylori* (*H. pylori*)

Helicobacter pylori infection is one of the most common pathogens associated with gastritis and both gastric and duodenal ulcers. *H. pylori* has also been connected with mucosa-associated lymphoid tissue (MALT) lymphomas, which have been linked with gastric cancer. Antibiotics currently remain the antibacterial therapeutic choice for *H. pylori* infections; however, there is a need for new and improved strategies (Cao et al. 2000). Cao et al. (2000) have shown that infection by the recombinant ScFv-expressing phage reduced the concentration of all six strains of *H. pylori* in vitro. Moreover, phage treatment of mice infected with *H. pylori* results in a significant decrease in bacterial colonization in the gastric mucosa. To produce this phage, *H. pylori*-antigen-single-chain variable fragments were extracted from murine hybridomas secreting monoclonal antibodies and then expressed as a fusion protein on a filamentous M13 phage (Cao et al. 2000). According to this data, engineered bacteriophages have a good potential in treatment of *H. pylori* and other bacterial pathogens.

4 Phage as Drug Delivery Vehicles for the Treatment of Bacterial Infections

In nanobiotechnology, bacteriophages have been exploited as the gene-delivery cargo for the transfer of gene into mammalian cells since the original identification of internalized phages from libraries of phage-displayed peptide. Recent studies have demonstrated that phage can be a good potential carrier of cytotoxic drugs to apply against both cancer cells and bacterial infections (Yacoby and Benhar 2008; Bar et al. 2008). In one study, filamentous bacteriophages were genetically modified to display P8 coat protein molecule on their surface while chloramphenicol was attached to the bacteriophage through chemical conjugation. Then, the phages were targeted to attach to *S. aureus* bacteria. The results show a retardation of growth of *S. aureus* following treatment with the chloramphenicol-conjugated *S. aureus* targeted phages in comparison to *S. aureus* treated by phages without the cytotoxic drug. In this study the reduction in bacterial growth was not significant because of hydrophobicity of the chloramphenicol, which results in an irreversible precipitation with conjugation of more than 3000 chloramphenicol molecules. To address this limitation, Yacoby and Benhar (2008) applied aminoglycoside antibiotics as a solubility-enhancing linker to connect the hydrophobic drug (i.e. Chloramphenicol) to the phage. The ability of targeted drug-carrying phages to inhibit the growth of methicillin-resistant *Staphylococcus*, *Streptococcus pyogenes*, and pathogenic *E. coli* O15787 were tested and complete growth inhibition was obtained (Yacoby and Benhar 2008; Yacoby et al. 2006). To assess the effect of the drug-carrying phages on animals, mice were injected with the recombinant phage. Neomycin-chloramphenicol (Neo-CAM) phages have shown low toxicity in vivo. Moreover,

Neo-CAM carrying phages were less immunogenic in comparison to native unconjugated phage particles (Vaks and Benhar 2011). Targeted drug-delivery may open up new ways in treatment of resistant bacterial pathogens. Furthermore, some potent bactericidal agents are inefficient due to lack of selectivity and this can be solved by targeted therapy (Yacoby et al. 2006).

5 Summary

In this chapter the importance of finding new strategies for the treatment of antibiotic-resistant bacteria as a first priority in modern medicine was emphasized. Native phage therapy was introduced as one of well-known approaches and its limitations were discussed. To overcome the limitations of phage therapy and make it more efficient than other approaches, the genetically-modified phage was introduced and the results of research on different bacterial infections was presented; including but not limited to, *E. coli*, *S. aureus*, *C. trachomatis*, *P. aeruginosa* (PA), and *H. pylori*.

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