Bacteriophages; Major Applications and Phage Therapy

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Abstract

An introduction to bacteriophages along with the general areas of application is described with special and detailed discussion of therapeutic use of phages and phage enzymes. Phage therapy being safer and faster than conventional chemotherapy has been employed for a while but its incorporation into general disease combating methods has to make a few pit stops those being the proper understanding of phage behaviour in vivo among others. The experiments done on model organisms and humans are described. Industrial benefits of phages, their surface cleaning abilities as well as use of bacteriophages in livestock and poultry development are highlighted. Phages as therapeutics hold a promising future due to their novel nature and mechanism of action for an underdeveloped country like ours.

Key words: Bacteriophage, Phage therapy, Antibiotics, lysin, Inhibition.

Introduction

Bacteriophage or phage (short for from Greek "phagein" that means "to eat" or "to nibble") are highly specific viruses that invade bacterial cells. They are obligate intracellular parasites that multiply inside bacteria by making use of some or all of the host biosynthetic machinery (i.e., viruses that infect bacteria) ultimately resulting in death of bacteria.

First reported discovery of viruses infecting bacteria was by Twort in the year 1915. He studied excretory samples of some patients of diarrhea and was able to isolate a peculiar filterable infectious agent from the samples. This discovery was further validated when in 1917 d'Hérelle also reported a similar finding. This discovery occurred about 20 years before practical application of penicillin, the first antibiotic. At that time phage therapy was considered as possible treatment method against bacterial infections (Ho K 2001). Although phage therapy is being practiced in Soviet unions and Eastern Europe (Chanishvili *et al.* 2002) but it was abandoned by West in 1940s with the discovery of antibiotics. However antibiotic resistance emergences in bacteria i.e. multi drug resistant (MDR) and extremely drug resistant bacteria (XRD) are major developing threats that have recently motivated the western scientific community to reevaluate phage therapy for bacterial infections that are incurable by conventional chemotherapy (Shigenobu *et al.* 2005).

Phage as tools in molecular biology

Phage research has had a key impact on molecular biology. The T series of bacteriophages had a central role in the development of molecular biology. In their book *Phage and the Origins of Molecular Biology*, Cairns *et al.* show how phages have contributed not only to the understanding of essential cellular processes, but also to the development of a considerable number of important genetic and biochemical tools. For example, the realization that viable bacteriophage lambda particles could be constructed with a significant portion of their genome deleted led to the development of insertional and replacement vectors, as well as cosmids and integrative plasmids. Phage serine integrates, particularly those of *Streptomyces* phage C31 have been exploited by Michele P Calos (Stanford University, USA) to integrate foreign DNA into mammalian cells and Drosophila (Groth *et al.* 2004) with the goal of producing transgenic animals or curing biochemical defects (Ginsburg *et al.* 2005), (Held *et al.* 2005). In addition, phage packaging signals, promoters and terminators, together with a great variety of enzymes, are used in today's molecular biology laboratory including polynucleotide kinases, DNA ligases, DNA polymerases, RNA polymerases, recombinases, single stranded DNA binding proteins, endo and exonucleases, and even methylases and restriction endonucleases (Roberts RJ *et al.* 2003).

Medical applications and industrial applications

Phage therapy is an area of phage applications that is being explored extensively due to emergence of antibiotic resistant bacterial strains. Specific phages can be administered to infected individuals that can lyse the bacteria thus ridding the patient of infection. Proteins and antibodies are purified by phage display technology (Zilka *et al.* 2003). These can then be used as therapeutics that would act either as agonists or through the inhibition of receptor ligand interactions. Another important use of phage is in diagnosing bacterial infections and in epidemiological studies. Phage typing is a technique used to diagnose bacterial infection by using phages. Phage typing is also used in epidemiological studies i.e. to identify if two epidemics are caused by the same bacterial strain or not (Sharp *et al.* 2001). Phages are used in various diagnostic procedures for example, determining antibiotic sensitivity for slow growing Mycobacterium tuberculosis (M Pai *et al.* 2005). Phages can also be tagged with a flourescein dye like antibodies and used in diagnostic; the tag can be detected using microscopy techniques (Hennes

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et al. 1995).

Bacteriophages are used in food processing industries to avoid bacterial contamination of food as well as eradication of biofilms which in turn increases shelf life of products. Bacterial contamination in food industries is a hazard that causes the industry immense damage. The most damaging bacterium of food industry is *Listeria monocytogenes* and phages against this are used in production and processing of food. Sometimes foods need a threshold level of bacteria for the enzymes to work, anything more than that value can be poisonous for the enzymes used in the process. Phages are used to control these bacterial loads (Flaherty *et al.* 2001), (Leverentz *et al. 2001*). Bacteria exist as biofilms in nature and these biofilms act as a shield to antimicrobials and biocides. Surfaces that are in contact with food can be decontaminated of any bacterial colonization using phages (Bassett *et al.* 2007). Phages are used to take out these biofilms so that the antimicrobials and biocides can effectively remove the contaminants. Bacteriophages are also used in mining industries to assist bacteria in different steps of metal and coal processing. A bacteriophage called phi Ac1 has been reported to assist bacteria in mining operations (Ward *et al.* 1992) which help bacteria remove sulphur from coal prior to burning.

Phages in agriculture & live stock

Phages are used in agricultural settings to control plant pathogenic bacteria by introducing specific phages to the fields (J.B. Jones *et al.* 2007). Fire blight in apple trees as well as Tomato and Pepper spots are targets of phage therapy (Gill JJ *et al.* 2003). Also phage systems like Cre lox P can be used to generate transgenic plants. Phage can also play a very important role in the decontamination of meat by treating the animal with phages before its slaughter (Sklar *et al.* 2001)

Antibiotic resistance and future problems

S. aureus is not the only problem, the CDC estimates that in some areas, 30% of pneumonia caused by *Streptococcus pneumoniae* is resistant to penicillin, whereas virtually all cases were susceptible in the 1970s. Vancomycin started failing to keep some *Enterococcus* (*faecium and faecalis*) infections in check in late 1988, necessitating new aggressive combination regimens. By 1993, according to the NIH, more than 10% of hospital acquired enterococci infections reported to the CDC were attributed to vancomycin resistant *Enterococcus* (VRE) *faecium*.

Aventis's (Strasbourg, France) drug Synercid was introduced in 1999 as a new weapon against VRE, but some resistance was observed before it even reached the market (G.M. Eliopoulos *et al.* 1998). Figure 1.

Phage therapy

Phage therapy is in fact the use of specific bacteriophages to kill pathogenic bacterial strains. Classically it implies the use of whole phage that infects bacterial cells and causes its lysis, however recently phage Lytic enzymes are being used to cause lysis of bacterial cells. Whole phage

Antimicrobial	MRSA n = 82	MSSA n = 18
Vancomycin	0%	0%
Clindamycin	73.2%	66.7%
Erythromycin	90.2%	66.7%
TMP/SMX[3]	42.7%	22.2%
Ciprofloxacin	79.3%	72.2%
Tetracycline	87.8%	77.7%

Fig. 1. MRSA=Methicillin-resistant S. aureus, MSSA= Methicillin-susceptible S. aureus, P/SMX=Trimethoprim/ sulfamethoxazol. Antimicrobial resistances of MRSA and MSSA isolated from infections in ICU (Aysen et al. 2006).

infects bacterial cell, multiplies and causes lysis of the cell thus releasing large number of phage progeny. These newly released phages further infect bacterial cells and radically decrease the bacterial load in the infected organism. Phages can be administered orally, topically or directly into tissues via injections. Besides using phages to cause bacterial lysis, phages can be used to deliver non phage genes to infected cells coding for antimicrobials (West water *et al.* 2003). Figure 2.

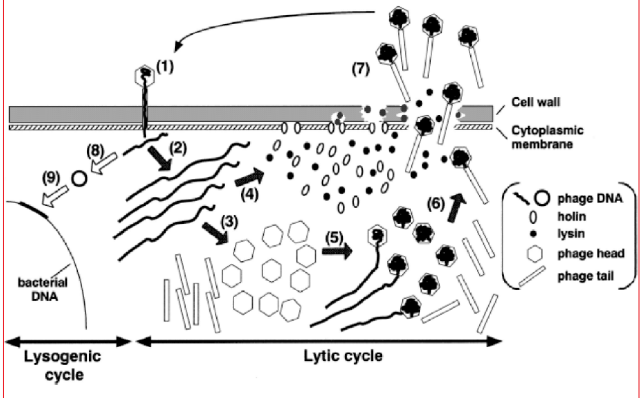

Fig.2. Schematic illustration of phage induced bacteriolysis. (1) Adsorption and DNA injection; (2) DNA replication; (3) production of head and tail; (4) synthesis of holin and lysin; (5) DNA packaging; (6) completion of phage particle; (7) disruption of the cell wall and release of the progeny; (8) circularization of phage DNA; (9) integration of the phage DNA into the host genome. (Shigenobu et al.2005).

Work on efficacy of phage therapy in living organisms was initiated in 1980's by Smith *et al.*via experimentation in veterinary animals. According to Smith's experiments, one dose of phage either intramuscular or intracerebral is far more effective against *E.coli* K1 infection in mice as compared to treatment with antibiotics like ampicillin, chloramphenicol or trimethoprim. This opened the way for further experiments on model organisms against a wide range of bacterial infections like *E. coli*, (Merril CR *et al.* 1996), (Chibani Chennoufi S *et al.* 2004) *Pseudomonas aeruginosa* (Ahmad SI *et al.* 2002), *Acinetobacter baumanii* (Soothill JS. *et al.* 1992), *Klebsiella pneumoniae*, *Enterococcus faecium* (vancomycin resistant strain, VRE),

(Biswas B *et al.* 2000) *Vibrio vulnificus*, (Cerveny KE *et al.* 2002) and *Salmonella* sp.

Staphylococcus aureus is a pathogen responsible for inflammatory diseases, toxic shock syndrome and food poisoning. Some of the antibiotic resistant strains of *S. aureus* include the methicillin resistant *staphylococcus aureus* (MRSA) (Hiramatsu K *et al.*2001), (Shimada K *et al.* 2004) and vancomycin resistant *S. aureus* (VRSA) (Chang S *et al.*2003), (Kacica M. *et al.* 2004). The drug resistant strains of this specie are rapidly on rise and efforts must be devoted to combat this bacterial dissemination through phage therapy. Also *S. aureus* strains that are resistant to a relatively new antibiotic linezolid are already being reported in USA and Europe (Pillai SK *et al.* 2002). According to the CDC, methicillin resistant *Staphylococcus aureus* (MRSA) accounted for nearly 60% of nosocomial *S. aureus* infections in 2001, a figure that had nearly doubled over the previous decade. Although most MRSA can still be treated with powerful antibiotics, some super bugs can shrug off even the strongest drugs. The first reported case of resistance to Pfizer's (Groton, CT, USA) Zyvox, the last line of defense against MRSA, was reported a little more than a year after the drug was approved. (S. Tslodras *et al.* 2001).

Pseudomonas aeroginosa is rapidly becoming resistant to antibiotics. Regarding this bacterium, Yun *et al's* recent work is worth mentioning. In their work, they identified two phages (MPK1 and MPK6) against *pseudomonas aeroginosa* strain PAO1. In experiments on mouse models, MPK1 administration; and to a lesser extent MPK6 administration decreased the mortality rate associated with PAO1 induced peritonitis sepsis. Also the mice treated with either one of these bacteriophages had lesser bacterial loads in livers, lungs and spleens. Another animal model used was Drosophila melanogester. Both of the phages were introduced via feeding and resulted in delayed PAO1 induced killing of D. melanogester. This experiment further substantiated the efficacy of MPK1 and MPK6 for *pseudomonas aeroginosa* infections.

Another important practical example of phage therapy usage was presented by Naka *et al.*who saved a large number of fish infected by *Lactococcus garvieae* and *Pseudomonas plecoglossicida* (Nakai *et al.* 1999), (Park *et al.* 2003). Phages have also been reported successful in fight against food poisoning in eliminating pathogens like *salmonella sp*. (Goode *et al.* 2003), *Campylobacter jejuni*, and *Listeria monocytogene*, ((Leverentz *et al.* 2004).

Research is also being done on the statistical and mathematical aspects of phage therapy to better elucidate phage host interaction mechanisms and if a threshold quantity of host cells are required for viable infection with phages or not (Payne *et al* .2000-2002). A hand wash solution enriched with phages has been reported to reduce staphylococcal contamination by 100 fold as compared to a hand wash solution that is phage free. In another experiment, application of a staphylococcal phage prevented abscess formation in rabbit model of wound infection (Wills *et al* .2005).

Using phage lytic enzymes

The mechanism of phage infection involves the progeny release step which is necessary for viability of infection. In order to release the newly formed phage particles, bacteriophages direct the synthesis of two proteins holin and lysin. Holin is responsible for making a hole in the cell membrane through which the lysin which is an amide, an endopeptidase or an N-acetylmuramidase can pass through and degrade the cell wall. Thus, application of lysin can lyse the cell wall of uninfected bacterial cells resulting in "lysis from without". This phenomenon is being used in treating and preventing bacterial infections (Loessner *et al.* 2002).

A lysin has a C terminal that has the binding activity and N terminal controls the catalytic activity. So until the C terminal binds with the target, no catalytic activity can take place. The enzyme works by targeting the peptidoglycan cell wall and by making holes in it. Due to high osmotic pressure inside, the holes cause the cell to burst. Another important phenomenon observed in lysis is targeted killing. The lytic enzyme only targets the bacteria for which the phage has specificity (Loessner *et al.* 2002). Research has also shown that bacteria do not become resistant to lytic enzymes even if they are applied repetitively.

Another interesting aspect of phage lytic enzyme is that they don't elicit damaging immune response. A number of experiments show increase in antibody titer against PlyG lysin but the antibodies have no affect on the enzyme and the enzyme works efficiently (Loeffler *et al.* 2002). Studies related to streptococci and pneumococcal lytic enzymes are worth mentioning. Lytic enzyme therapy is also being pursued as a probable anti biowarfare mechanism. Studies have been performed against B. anthracis responsible for anthrax, (Raymond *et al.* 2002). Studies in mouse models have shown promise for post exposure intravenous treatment of anthrax. The lytic enzyme used was PlyG. 90% of mice treated with lytic enzyme survived whereas only 10% of the mice not treated with PlyG survived.

Future of phage therapy in Pakistan

Keeping in mind, the ground realities while working for masses in Pakistan it should be seen that a large population is poverty stricken. We need to work on alternative treatments that are cost effective and economy friendly. Pakistan is basically an agricultural country and our exports include crops and livestock. To increase the yield and decrease the losses associated with crop and livestock, phage therapy can be effectively used. When quality and quantity of our crops and livestock will increase, automatically our foreign exchange reserves will benefit. Phage therapy is cost effective as number of doses needed as compared to antibiotics is far less and time for hospitalizations is also reduced due to speedy recovery. Tuberculosis is a widespread disease of our country and the treatment cost is just preposterous for the masses. Phage therapy against the bacterium can be both affordable and effective for the patients.

Conclusion

Phages being ubiquitous in nature can be easily sought, isolated, identified and put to use. Comparatively easy cell biology of prokaryotes and switching of lytic and lysogenic life cycle of bacteriophages gave many answers about gene regulations and developed a better understanding of molecular biology. Different enzymatic products such as holins and endolysin provide a simple solution for the killing of many Gram positive bacteria.

A lytic phage replicates in a limited time span, producing large population of new phages thus minimizing the number of pathogenic bacteria and controlling the infection. Bacteria although having short generation time still, are no match for the rapid phage replication and in few hours' phages outnumber the pathogenic bacteria. Unless they lurk in inaccessible locations, they are sought out and destroyed.

Unlike antibiotics, bacteriophages are 'living' organisms; they have been infecting bacteria since the beginning of life on this planet. Bacteria evolve to resist phage, but phage evolves too at an amazing rate. Chemists tinkering with new generations of antibiotics can never cope with ever mutating antibiotic resistant bacteria so it seems that phages provide the ultimate antibacterial therapy: lethal, adaptive, highly efficient, safe to humans.

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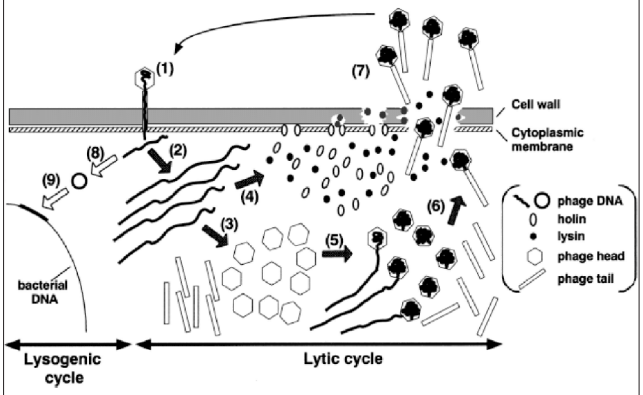

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