ANALYTICAL LETTERS
Vol. 36, No. 15, pp. 3241–3259, 2003

Diagnostic and Therapeutic Applications of Lytic Phages

Rosemonde Mandeville,[1,*] Mansel Griffiths,[2] Larry Goodridge,[3] Lynn McIntyre,[4] and T. Toney Ilenchuk[1]

[1]Biophage Pharma Inc., Montreal, Quebec, Canada
[2]Canadian Research Institute for Food Safety, University of Guelph, Guelph, Ontario, Canada
[3]Department of Animal Science, University of Wyoming, Laramie, Wyoming, USA
[4]Institute of Food, Nutrition and Human Health, Massey University, Private Bag, North Shore MSC, Auckland, New Zealand

ABSTRACT

The ability of lytic phages to rapidly kill and lyse infected bacteria, the specificity of phages for particular bacteria, and the ability of phages to increase in number during the infection process make phages excellent potential diagnostic and therapeutic agents for fighting bacterial disease. However, temperate phages are of little use in phage diagnostics and therapy.

*Correspondence: Rosemonde Mandeville, Biophage Pharma Inc., 6100 Royalmount, Montreal, Quebec H4P2R2, Canada; E-mail: rosemonde.mandeville@biophage.com.

DOI: 10.1081/AL-120026569
0003-2719 (Print); 1532-236X (Online)

Key Words: Lytic phages; Phages; Diagnosis; Therapy; Bacteria; Reporter genes; Luciferase.

INTRODUCTION

Bacterial phages, or simply phages, are viruses that infect a variety of Gram-negative bacteria using pili as receptors. Phages have been categorized into 12 families and a number of genera based on their morphology, the type of nucleic acids, nucleic acid homology, and serology.[1] Only a few phages represent cubic, filamentous, or pleomorphic morphology. The ability of a phage to infect is determined largely by surface receptors, which do not necessarily correspond to the phylogenic grouping of bacteria. Based on their life styles, phages can be divided into two groups: the virulent phages capable of only lytic propagation, and the temperate phages capable of either lytic or lysogenic propagation. The virulent life cycle of lytic phages consists of infection of the host, replication of the phage genome, production of phage structural components, phage assembly, and release of the progeny phages. This last step usually, but not always (e.g., filamentous phages f1 and M13 of *E. coli*), involves phage-induced lysis of the host cell and release of hundreds of progeny phage. This process can occur in as little as 15 min. The progeny phage can then continue the cycle by infecting other bacterial cells. In contrast to virulent phages, in temperate phages the genome exists as a prophage resulting in co-existence of phage and the host bacterial cell indefinitely. Usually this is achieved by integration of the phage genome into the host bacterium's chromosome by a site-specific integration mechanism.

DIAGNOSTIC APPLICATIONS

There has been much recent interest in the use of phages as a tool to aid in the detection of foodborne, and clinical bacterial pathogens. Indeed, this tool also has applications to monitor post-treatment utility of phage or conventional antibiotic treatment. In this regard, an abundance of approaches centered on phages are currently being investigated as indicated by the fullness of the literature. For the purposes of this review we will focus briefly overview the breadth of approaches

which cover phages as lytic agents, detection of inhibition of metabolism and growth, phages as staining agents. The remainder of the review will focus on the transducing activities of phages (e.g., reporter gene).

Detection of Intracellular Components Released During Phage Lysis

This approach has focused on phages capable of specifically lysing groups of microorganisms. Very simply, the lysis of bacterial cells by phage will result in release of intracellular materials that can be assayed using a variety of techniques. The method that has received the greatest attention is the use of the firefly luciferase/luciferin enzyme system to detect intracellular ATP released following lysis of bacterial cells by phage. The concentration of ATP in the assay is directly proportional to the quantity of light produced by the luciferase/luciferin reaction.[2,3] In order to improve sensitivity Squirrell and Murphy[4] suggested measuring the amount of released adenylate kinase (AK) as a means to monitor phage lysis. In the presence of excess amounts of ADP the equilibrium of the reaction could be shifted towards the anabolism of ATP, which may then be assayed using the ATP bioluminescence assay described above. Other intracellular constituents can also be used to monitor phage-mediated cell lysis: release of β-galactosidase[5]; the endolysins (enzymes produced as late gene products during phage replication in order to release progeny phage from the bacterial cell) have been used to specifically lyse bacterial cells; this is known as a lysis from within.[6] The continued characterization of phage lytic enzymes will not only greatly enhance our knowledge of the molecular action of such enzymes, but, as well, will increase their applicability in other areas of microbiology. In addition, they offer potential as anti-microbial agents to eliminate pathogens in the food industry and in medical applications.

Detection Based on Inhibition of Metabolism and Growth

As lytic agents, phage infection results in the death of cells. A number of methodologies are available to monitor metabolic activity and microbial growth of microbial populations. These include electrical *impedance*,[3,7,8] conductance,[9] turbidimetry, and colorimetry.[3,7] What these methods have in common, and what has primarily limited their application for pathogen detection in foods, is that they rely on differential

or selective media to identify pathogens. Generally they are not capable of differentiating to the species level, and can also be affected by the growth of contaminating microorganisms associated with the background microflora of foods, resulting in false-positive detections.

Detection of Phage Particles

Following cell lysis by phage, several tens or hundreds of phage particles are released. This natural amplification cycle has formed the basis of several methods of bacterial detection. The so-called 'phage amplification assay' has been applied for the rapid detection and identification of specific pathogenic bacteria.[10] In common with other phage-based detection methods, resistance to a particular phage is the most likely problem associated with this approach, but the use of cocktails of phages can circumvent this problem. In a modification of the phage amplification assay, Favrin et al.[11] used immuno-magnetic separation (IMS) to remove infected cells from the suspension containing exogenous phage. Numerous other methods have been described for the detection of amplified phage and these include: HPLC,[12,13] MALDI-TOF,[14] love wave sensors,[15] quartz crystal microbalance sensors,[16] and immunoassays.[17] To facilitate these assays it would be desirable to produce a reagent based on immobilized phage that could be used in much the same way as IMS. The Sapphire lytic phage from Amersham International has been evaluated in a novel immobilized capture method for the separation and concentration of *Salmonella* strains by Bennett et al.[18] In order to improve the efficiency of capture, Sun et al.[19] investigated covalent binding of phage to paramagnetic beads using the biotin-streptavidin reaction. Effective methods for immobilization of phage will lead to one-step assays incorporating cell concentration and detection.

Phage as Staining Agents

Fluorescent stains have been reported as a useful accessory to the use of phage probes, as a means of labeling phage nucleic acid within infected cells. Sanders et al.[20] reported the use of two fluorescent dyes, chromomycin A3 (CA3) and Hoechst 33258 (H33258), to bind and label G-C- and A-T-rich regions, respectively, of phage T4 DNA present inside infected *E. coli* cells. Fluorescent labeling of nucleic acid packaged within the virus head has also been used to directly count viruses in

samples from freshwater and marine environments using epifluorescence microscopy.[21,22] Labeling of phage DNA has been adapted to enable fluorescent detection of *E. coli* O157:H7.[23,24]

Phage as Transducing Agents: The Reporter Phage Approach

There has been much recent interest in the use of reporter phages as a tool to aid in the detection of foodborne, and clinical bacterial pathogens. Indeed, this tool also has applications to monitor post-treatment utility of phage or conventional antibiotic treatment. The reporter phage concept provides a sensitive method for bacterial detection and sensitivity to antimicrobial agents. The key elements of this approach include: bacterial and eukaryotic luciferases, the ice nucleation protein, and the *E.coli*-galactosidase reporter genes. In addition, examples that demonstrate the usefulness of reporter phage as tools to detect foodborne bacterial contamination will be discussed.

The reporter phage concept provides a sensitive method for bacterial detection and sensitivity to antimicrobial agents. The key elements of this approach include: bacterial and eukaryotic luciferases, the ice nucleation protein, and the *E. coli*-galactosidase reporter genes. In this technology, DNA carrying a reporter gene is introduced into a target bacterium via a phage. Once the reporter gene has been introduced to the bacterium, it is expressed, thereby allowing bacterial cells to be rapidly identified. Since phages need host cells to replicate, the phages will remain "dark" (i.e., the reporter gene will not be expressed until the phage DNA has been injected into the host). Therefore, expression of the reporter gene is indicative of the presence of the infected organism. To this juncture, the reporter genes employed in this system are the *prokaryotic*, and *eukaryotic luciferase* (*lux* and *luc*) *genes*, the *bacterial ice nucleation (inaW) gene*, and the *E. coli-galactosidase (lacZ) gene*.[25–27] To date, reporter phages have been developed that can detect *E. coli*, Mycobacteria, *Salmonella* spp., *Staphylococcus aureus*, and *Listeria monocytogenes*.[27–31] Several reviews have described some of the work accomplished with lux^+ phage mediated detection of bacteria.[32–34]

Luciferase Reporter Systems: Prokaryotic Luciferase

The majority of transducing phage detection assays have employed the bioluminescence (*lux* and *luc*) genes as the reporter system. The many species of luminescent bacteria that have been studied include the

marine bacteria *Vibrio harveyi, Vibrio fischeri, Photobacterium spp.* and the terrestrial bacterium *Photorhabdus luminescens.*[35] The detection system to be applicable requires several components, which combined, elicit the bioluminescent reaction to occur. These include an *oxidant* (usually O_2) a *protein catalyst* (luciferase), a *substrate* (luciferin), a *cofactor* (FMN or ATP), a *cation*, and an *ancillary protein.*[36] The light emitting reaction involves an intracellular luciferase-catalyzed oxidation of the reduced form of flavin mononucleotide ($FMNH_2$) and a long chain aliphatic aldehyde (RCHO), such as dodecanal, by molecular oxygen.[34,37]

Luciferase Reporter Systems: Eukaryotic Luciferase

The single polypeptide *luc* gene, encoding a 62 kDa luciferase from the North American firefly *Photinus pyralis*, has been cloned and expressed in *E. coli.*[38] The luciferase of *P. pyralis* emits in the yellow-green range, with peak emission at 560 nm, while other eukaryotic organisms, including different firefly species and click beetles, produce light ranging from green (547 nm) to red (604 nm). The production of light in the *luc*-based bioluminescence system occurs when adenosine triphosphate (ATP), reacts with the heterocyclic carboxylic acid, luciferin, in the presence of molecular dioxygen (O_2). The reaction is catalyzed by the firefly enzyme luciferase and produces a highly efficient flash of light, with an estimated quantum yield of 0.88, which is more efficient than the light generated by bacterial luciferase.[39] The fact that *luc* based bioluminescence systems produce a flash of light is a disadvantage in bacterial detection systems; however the use of Coenzyme A could prolong the half-life of light to 10 min. LucLite™, is a series of reagents developed to specifically improve the light output of *luc*-based reactions to over 5 h, with the production of a glow type light signal.[40] Additionally, it has been shown that a single serine to threonine substitution at position 198 of the *luc* polypeptide chain results in an enzyme with a 150-fold increase in half-time for light emission decay.[41]

Luciferase Applications

Ulitzur and Barak[27] first showed that phages carrying a reporter gene could be used to detect microorganisms. These researchers engineered phage λ Charon 30, with the *lux* genes from *Vibrio fischeri.* Using the lux^+ phage, it was possible to detect between 10 and 100 *E. coli*

cells/ml of milk or urine, within one hour.[4] Since then, reporter phage assays have been developed that are capable of detecting several pathogenic food-borne microorganisms (e.g., *E.coli* O157:H7[42]; *Salmonella* spp[28]; *Listeria monocytogenes*,[43] *Campylobacter*[44]). This technology provides sensitivity, specificity, and timeliness into the foodborne pathogen detection arena. For example, with a 6 h pre-incubation, 10 CFU/mL of *Salmonella* in original broth culture were detectable. The detection system also allowed *Salmonella* to be detected in whole eggs by the direct addition of the recombinant phages into the eggs. After 24 h of incubation, as few as 10 CFU/egg could be detected, thereby offering the ability to not only detect bacterial contamination in whole eggs, but also to determine its location.[29] Luminescence could be detected using a variety of instruments, including a photon-counting charge-coupled device (CCD) camera, a luminometer, or X-ray film.

Indirect Detection Applications

As an alternative to the assay of bacterial pathogens, microbial contamination can also be identified via the detection of indicator bacteria. By definition, these are microorganisms present in significant numbers within a food, which, while not pathogenic, can be related through increasing count to the increasing probability of pathogen contamination. Kodikara et al.[45] have endeavored to explore the use of *lux*$^+$ recombinant phage for detecting enteric indicator bacteria. The assay is simple and short; the evaluation of indicator strains in less than 1 h can be accomplished. However, while this is a simple and effective approach to detecting enteric indicator bacteria, at present, it constitutes only a prototype assay, since a different enteric flora might require additional phage constructs with different host ranges.[45] Several groups used luciferase-tagged bacteria to monitor environmental contamination.[46–49]

Applications in the Medical Setting

Reporter phages may help to reduce the time required for establishing antibiotic sensitivity of *M. tuberculosis* from weeks to days, which would accelerate screening for new anti-tuberculosis drugs.[31] In the presence of an antibiotic to which the host cell is sensitive, the infecting phage cannot complete its lytic cycle, and the amount of light emitted is therefore reduced, or abolished.[27] The majority of reporter phage testing

to determine antibiotic susceptibility has centered on the Mycobacteria. Jacobs et al.[31] proposed the use of a recombinant reporter mycobacteriophage carrying a firefly luciferase reporter gene (*lux*$^+$ phage L5:*FFlux*) as a tool for the rapid determination of *M. tuberculosis* drug susceptibilities. In this research, mutants of *M. bovis* BCG were selected that were resistant to rifampicin, streptomycin, or isoniazid. The results established that L5:*FFlux* is very efficient at revealing the patterns of drug susceptibility or resistance of *M. tuberculosis* strains,[31] and other researchers have shown that L5:*FFlux* can also determine the antibiotic susceptibility profile of other Mycobacteria.[50–54]

In brief, diagnostic technologies based on lytic phages offer rapid, reliable, and sensitive methods for the detection of specific food borne pathogens. Despite this, these technologies have not been widely accepted. One reason for this may be the fact that it is difficult to genetically modify phages, which has bacterial hosts that have not been genetically characterized. Hopefully, as the genetic sequences of more bacteria are determined, these technologies will become popular. Another potential problem is the fact that an individual phage may not possess the host range required to detect all isolates of a bacterial species. Therefore, it will be necessary to modify the host ranges of the phages used, or employ a cocktail of phages in order to develop a reporter assay that will be able to detect the desired target bacteria. The potential for a revolution in microbial testing can be discerned with the near on-line detection of food borne microorganisms. By defining the microorganisms present in significant numbers within a food, it will be possible, using lytic phages, to relate increasing bacterial counts to the increased probability of pathogen contamination.

THERAPEUTIC APPLICATIONS

Phage therapy was widely used around the world in the 1930s and 1940s, and it is still used in Eastern Europe and the former Soviet Union. Actually, the first report of a possible therapeutic effect of phages was published in 1917 by Felix d'Herelle.[55] Phage therapy was all but abandoned in the West after antibiotics became widely available. Moreover, early work on phage therapy was impeded by several factors based on: (a) the insufficient knowledge of the nature, diversity, and high specificity of phages; (b) the presence of toxins in crude phage lysate; (c) a lack of appreciation for the capacity of mammalian host defense systems, particularly the organs of the reticuloendothelial system, to remove phage particles from the circulation; and (d) the highly inadequate scientific methodologies used by practitioners at the time (e.g., their failure to

conduct placebo-controlled studies, to remove endotoxins from the preparations, and to re-confirm phage viability after adding sterilizing agents to the preparations).

In recent years, and in order to approach phage therapy more rigorously than is possible with human studies, well-controlled animal models were used. These studies have demonstrated that phages can rescue animals from a variety of fatal infections. In the 1980s, Smith et al.[56–58] performed phage treatment experiments on mice, calves, lambs, and piglets. In mice, phage treatment prevented death and caused a rapid reduction in the number of bacteria in all tissues examined. The only phage-resistant bacterial mutants that arose were K1- and thus were of low virulence. Interestingly, phage treatment was more effective than multiple doses of antibacterials.[59] The same group also demonstrated that spraying the bedding in barns with phage could prevent disease in calves. Similar results were also demonstrated in piglets.[60] Smith et al. suggested that by isolating phages that recognize different receptors on host bacteria and by selecting for mutant phages in the laboratory that have altered their receptor-binding activity, it should be possible to generate phages that will continue to be effective against spontaneously occurring phage-resistant bacteria.

In the 1990s, Barrow et al.[60,61] applied phage therapy to chickens and calves and Soothill[62] worked with guinea pigs and mice, while Ramesh et al. treated animals with ileocecitis caused by *C. difficile*.[63] Barrow et al.[60,61] reported success using phages to prevent salmonellosis in chickens in challenge studies where the animals were inoculated orally with *Salmonella typhimurium* and death was the end point. Oral treatment before (2 days before challenge) or at the same time as challenge provided protection from death, demonstrating the prophylactic and therapeutic use of phages. Findings such as these are encouraging to producers and processors who are searching for ways to reduce food borne bacteria, such as *Salmonella* and *Campylobacter*, in their products. While traditional treatment strategies are important in maintaining the health of livestock, it should also be recognized that the focus on food safety issues has become an important factor in developing novel and specific approaches in dealing with pathogens. In that regard a major target for this technology is to manage bacterial load in a manner as to reduce serendipitous "shedding" (e.g., *Salmonella* spp, *Campylobacter* spp., *E. coli* O157:H7) that occurs and associated with shipping and handling of livestock en route to slaughter.

At Biophage, we also worked with piglets[64] and confirmed earlier observations of the potential use of phages in treating *E. coli* infections in newborn piglets. In our studies treatment of piglets with phage BP64

against a challenge with the O64 *E. coli* strain resulted in a substantial decrease in the severity and duration of diarrhea (Fig. 1) and an increase in weight gain (Fig. 2) due to *E. coli* challenge, in comparison with piglets which had been challenged with the *E. coli* strain but not treated with the phage (Fig. 1). Furthermore, there was a reduced colonization of the ileum by O64 *E. coli* strain, a greatly reduced fecal excretion of the challenge strain, (Fig. 3). This reduced fecal excretion was observed immediately within the first day following treatment of piglets. The specificity of treatment was reflected in the observation that the total number of *E. coli* excreted in the feces of piglets up to 5 days following treatment remained constant (Fig. 4).

The potential for phages to control infection and disease without the use of antibiotics is important in terms of antibiotic resistance. Overuse of certain antibiotics may be responsible for their reduced effectiveness in treating disease, in both animals and humans. The powerful utility of phages in traditional treatment applications seems to be overlooked and wrongly viewed as a crude alternative to the use of antibiotics. In a comparative study with Gentamycin conducted at our facilities, a single

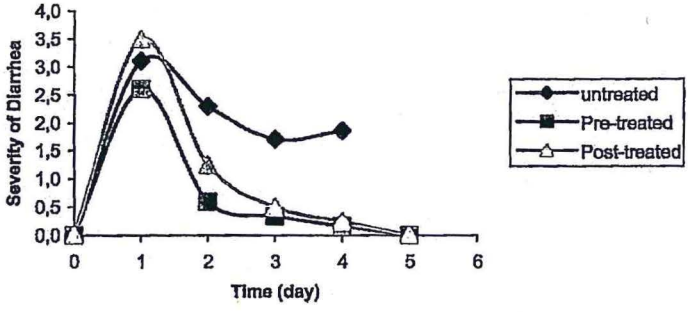

Figure 1. Effect of phage treatment on the severity of diarrhea.

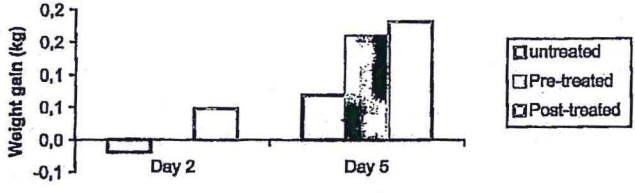

Figure 2. Effect of phage treatment on weight gain/day.

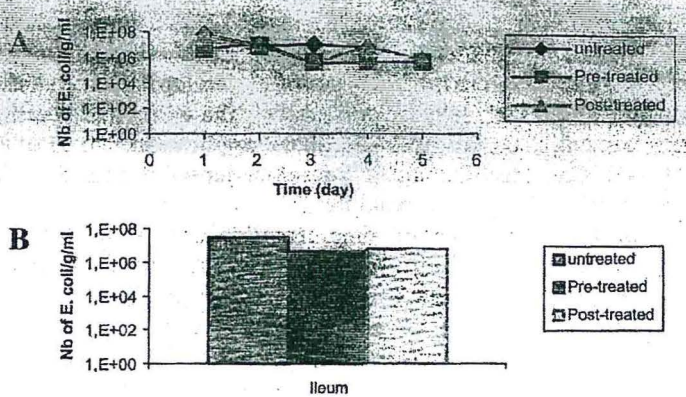

Figure 3. Effect of phage treatment on *E. coli* O64 count (A) in feces; (B) in ileum.

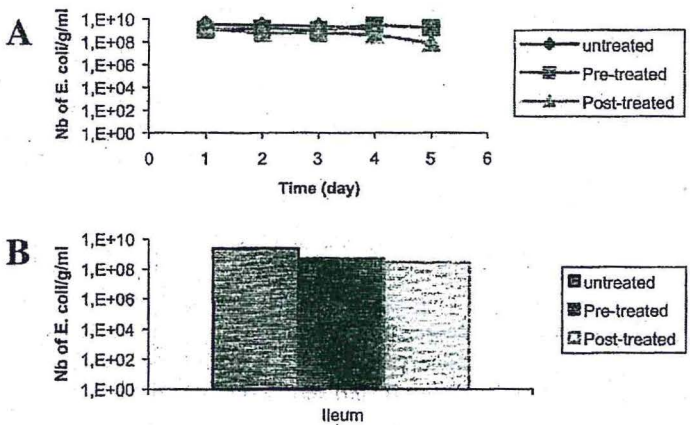

Figure 4. Effect of phage treatment on total *E. coli* counts (A) in feces; (B) in ileum.

treatment of an isolated and highly characterized naturally occurring phage was shown to substantially decrease the severity and duration of diarrhea as compared with a regimen of Gentamycin injections. Moreover, phage treatment resulted in lower fecal excretion of the challenge *E. coli* O64 strain while treatment with Gentamycin resulted in a slightly lower fecal excretion than that observed for treatment with the phage. Treatment with phage did not affect the total number

of *E. coli* excreted in the feces of piglets up to 7 days following treatment, whereas treatment with Gentamycin result in a slight decrease in the total number of *E. coli* excreted in the feces of piglets up to 5 days following treatment. Results such as these demonstrate the utility and specificity of phage treatment as compared to antibiotic application.

At NIH, Carl Merrill et al.[65,66] developed a serial-passage technique in mice to select for phage mutants able to remain in the circulatory system for longer periods of time. By this approach they isolated long-circulating mutants of *Escherichia coli* phage λ and of *Salmonella typhimurium* phage P22 and demonstrated that the long-circulating λ mutants also have greater capability as antibacterial agents than the corresponding parental strain in animals infected with lethal doses of bacteria.[65] More recently, the same group reported on the ability of bacterial viruses to rescue mice challenged with Vancomycin-Resistant Enterococci (VRE).[66] Matsuzaki et al.[67] demonstrated similar results using phages to treat animals infected with methicillin-resistant *Staphylococcus aureus*. Phages were also capable of treating antibiotic-resistant intracellular pathogens Broxmeyer et al.[68] recently described a system using *Mycobacterium smegmatis*, an avirulent mycobacterium, to deliver the lytic phage TM4 where both *M. avium* and *M. tuberculosis* reside within macrophages.

In aquaculture (reviewed by Nakai and Park[69]), recent studies have shown that phages can be used to treat bacterial diseases in fish[70–72] and oysters.[73] Reported results of field trials indicate the potential for phage control of gram-negative bacterial disease in fish and oysters. In aquiculture, Flaherty et al. reported on the potential application of phage therapy for the treatment of bacterial blight of geranium[74] and bacterial spots in tomatoes.[75] Leverentz and Conway[76] also investigated the potential of phage to reduce pathogens on fruits and vegetables- both whole and fresh-cut. They found that Salmonella-specific phages reduced Salmonella populations by approximately 3.5 logs on honeydew melon slices stored at 5–10°C and by approximately 2.5 logs on slices stored at 20°C, which is greater than the maximal amount achieved using chemical sanitizers.

In brief, these encouraging data have demonstrated that phages can be highly effective in treating many different types of bacterial infections. The lethality and specificity of phages for particular bacteria, the ability of phages to replicate within infected animal hosts, and the safety of phages make them efficacious antibacterial agents. When combined with the fact that drug-resistant bacteria have become a global crisis, these results have created a window of opportunity for phage therapy to be tested anew, this time using modern technologies

and placebo-controlled designs. The opportunities in both animal and human health are limitless not only from the perspective of treating infection but also from the perspective of food safety and the need to be prepared for threats of bio warfare. Although there are still several hurdles to be overcome, it appears likely that phage therapy will regain a role in both medical and veterinary treatment of infectious diseases, especially in the scenario of emerging antibacterial resistance. For the future, there is unique opportunity to combine the diagnostic and therapeutic applications into a dynamic approach in detecting and treating pathogens with high selectivity including those pathogens that are antibiotic resistant.

REFERENCES

1. Ackermann, H.W. Frequency of morphological phage descriptions. Arch Virol. **1992**, *124*, 201.
2. Griffiths, M.W. The role of ATP bioluminescence in the food industry: new light on old problems. Food Technology **1996**, *50*, 62–73.
3. McIntyre, L. Application and evaluation of bacterial viruses in rapid methodologies for the detection of foodborne pathogens. Ph.D thesis, University of Guelph, **1998**.
4. Squirrell, D.J.; Murphy, M.J. Adenylate kinase as a cell marker in bioluminescent assays. In *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspect*; 1995; 486 pp.
5. Stanek, J.E.; Falkingham, J.O. Raoid coliphage detection assay. J. Virol Meth. **2001**, *91*, 93.
6. Tarahovsky, Y.S.; Ivanitsky, G.R.; Khusainov, A.A. Lysis of *Escherichia coli* cells induced by bacteriophage T4. FEMS Microbiol. Lett. **2000**, *122*, 195.
7. McIntyre, L.; Griffiths, M.W. A bacteriophage-based impedimetric method for the detection of pathogens in dairy products. J. Dairy Sci. **1997**, *80*, 107.
8. Pugh, S.J.; Arnott, M.L. Automated conductimetric detection of *Salmonella* in cofectionary products. In *Rapid microbiological methods for foods, beverages & pharmaceuticals. Soc. Appl. Bacteriol. Tech. Series.* **1987**, *185*.
9. Chang, T.C.; Ding, H.C.; Chen, S. A conductance method for the identification of *Escherichia coli* O157:H7 using bacteriophage AR1. J. Food Protection **2002**, *65*, 12.
10. Stewart, G.S.A.B.; Jassim, S.A.A.; Denyer, S.P.; Newby, P.; Linley, K.; Dhir, V.K. The specific and sensitive detection of bacterial

pathogens within 4h using bacteriophage amplification. J. Appl. Microbiol. **1998**, *84*, 777.
11. Favrin, S.J.; Jassim, S.A.A.; Griffiths, M.W. Development and optimization of a novel immunomagnetic separation-bacteriophage assay for detection of *Salmonella enterica* serovar Enteritidis in broth. Appl. Environ. Microbiol. **2001**, *67*, 217.
12. Hirsh, D.C.; Martin, L.D. Rapid detection of *Salmonella* spp. using Felix O-1 bacteriophage and high-performance liquid chromatography. Appl. Environ. Microbiol. **1983a**, *45*, 260.
13. Hirsh, D.C.; Martin, L.D. Detection of *Salmonella* spp. in milk using Felix O-1 bacteriophage and high-performance liquid chromatography. Appl. Environ. Microbiol. **1983b**, *46*, 1243.
14. Madonna, A.J.; Van Cuyk, S.; Voorhees, K.J. Detection of *Escherichia coli* using immunomagnetic separation and bacteriophage amplification coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Comm. Mass Spec. **2003**, *17*, 257.
15. Tamarin, O.; Comeau, S.; Dejous, C.; Moynet, D.; Rebiere, D.; Bezian, J.; Pistre, J. Real time device for biosensing: design of a bacteriophage model using love acoustic waves. Biosens Bioelectron **2003**, *18*, 755.
16. Uttenthaler, E.; Schraeml, M.; Mandel, J.; Drost, S. Ultrasensitive quartz crystal microbalance sensors for detection of M13-Phages in liquids. Biosens Bioelectron **2001**, *16*, 735.
17. Block, T.; Miller, R.; Korngold, R.; Jungkind, D. A phage-linked immunosorbant system for the detection of pathological relevant antigens. Biotechniques **1989**, *7*, 756.
18. Bennett, A.R.; Davids, F.G.C.; Vlahodimou, S.; Banks, J.G.; Betts, R.P. The use of bacteriophage-based systems for the separation and concentration of *Salmonella*. J. Appl. Microbiol. **1997**, *83*, 259.
19. Sun, W.; Brovko, L.; Griffiths, M.W. Use of bioluminescent *Salmonella* for assessing the efficiency of constructed phage-based biosorbent. J. Indust. Microbiol. Biotechnol. **2001**, *27*, 126.
20. Sanders, C.A.; Yajko, D.M.; Nassos, P.S.; Hyun, W.C.; Fulwyler, M.J.; Hadley, W.K. Detection and analysis by dual-laser flow cytometry of bacteriophage T4 DNA inside *Escherichia coli*. Cytometry. **1991**, *12*, 167.
21. Hennes, K.P.; Suttle, C.A. Direct counts of viruses in natural waters and laboratory cultures by epifluorescence microscopy. Limnol. Oceanog. **1995**, *40*, 1050.
22. Hennes, K.P.; Suttle, C.A.; Chan, A.M. Fluorescently labeled virus probes show that natural virus populations can control the structure

of marine microbial communities. Appl. Environ. Microbiol. **1995**, *61*, 3623.

23. Goodridge, L.; Chen, J.; Griffiths, M.W. Development and characterization of a Fluorescent-Bacteriphage Assay for detection of *Escherichia coli* O157:H7 Appl. Environ. Microbiol. **1999a**, *65*, 1397.
24. Goodridge, L.; Chen, J.; Griffiths, M.W. The use of a fluorescent bacteriophage assay for detection of *Escherichia coli* O157:H7 in inoculated ground beef and raw milk. Intl. J. Food Microbiol. **1999b**, *47*, 4350.
25. Kelana, L.C.; Griffiths, M.W. Growth of autobioluminescent *Campylobacter jejuni* in response to various environmental stimuli. J. Food Prot. **2003**, *66*, 1190.
26. Wolber, P. Bacterial ice nucleation. Adv. Microb. Physiol. **1993**, *34*, 203.
27. Ulitzur, S.; Kuhn, J. Introduction of *lux* genes into bacteria, a new approach for specific determination of bacteria and their antibiotic susceptibility. In *Bioluminescence new perspectives*, Schölmerich, J.; Anreesen, R.; Japp, A.; Ernst, M.; Woods, W.G. Eds., John Wiley and Sons, Inc., New York, **1987**, 463 pp.
28. Loessner, M.J.; Rees, C.E.D.; Stewart, G.S.A.B.; Scherer, S. Construction of luciferase reporter bacteriophage A511:*luxAB* for rapid and sensitive detection of viable *Listeria* cells. Applied and Environmental Microbiology, **1996**, *62*, 1133.
29. Chen, J.; Griffiths, M.W. *Salmonella* detection in eggs using lux^+ phage. J. Food Protection **1996**, *59*, 908.
30. Pagotto, F.; Brovko, L.; Griffiths, M.W. Phage-mediated detection of *Staphylococcus aureus* and *Escherichia coli* O157:H7 using bioluminescence. Bacteriological quality of raw milk. IDF (special issue) **1996**, *9601*, 152.
31. Jacobs, W.R., Jr.; Barletta, R.G.; Udani, R.; Chan, J.; Kalkut, G.; Sosne, G.; Kieser; T.; Sarkis, G.J.; Hatfull; G.F.; Bloom, B.R. Rapid assessment of drug susceptibilities of Mycobacterium tuberculosis by means of luciferase reporter phages. Science **1993**, *260*, 829.
32. Billard, P.; DuBow, M.S. Bioluminescence-based assays for detection and characterization of bacteria and chemicals in clinical laboratories. Clin. Biochem. **1998**, *31*, 1.
33. Baker, J.M.; Griffiths, M.W.; Collins-Thompson, D.L. Bacterial bioluminescence: application in food microbiology. J. Food Protection **1992**, *55*, 62.

34. Stewart, G.S.A.B.; Williams, P. *Lux* genes and the applications of bacterial bioluminescence. J. General Microbiol. **1992**, *138*, 1289.
35. Campbell, A.K. Living light: biochemistry, function and biomedical applications. Essays Biochem. **1989**, *24*, 41.
36. Stewart, G.S.A.B.; Smith, T.; Denyer, S. Genetic engineering for bioluminescent bacteria. Food Science Technol. Today **1989**, *3*, 19.
37. Stewart, G.S.A.B.; Denyer, S.P.; Lewington, J. Microbiology illuminated: gene engineering and bioluminescence. Trends Food Science Technol. **1991**, *2*, 7.
38. De Wet, J.R.; Wood, K.V.; Helsinki, D.R.; Deluca, M. Cloning of firefly luciferase cDNA and the expression of active luciferase in *Escherichia coli*. PNAS **1985**, *82*, 7870.
39. Bronstein, I.; Fortin, J.; Stanley, P.E.; Stewart, G.S.; Kricka, L.J. Chemiluminescent and bioluminescent reporter gene assays. Anal. Biochem. **1994**, *219*, 169.
40. Roelant, C.H.; Burns, D.A.; Scheirer, W. Accelerating the pace of luciferase reporter gene assays. Biotechniques **1996**, *20*, 914.
41. Thompson, J.F.; Geoghegan, K.F.; Lloyd, D.B.; Lanzetti, A.J.; Magyar, R.A.; Anderson, S.M.; Branchini, B.R. Mutation of a protease-sensitive region in firefly luciferase alters light emission properties. J. Biol. Chemistry **1997**, *272*, 18766.
42. Waddell, T.E.; Poppe, C. Construction of mini-Tn10*luxABcam*/*Ptac*-ATS and its use for developing a bacteriophage that transduces bioluminescence to *Escherichia coli* O157:H7. FEMS Microbiol. Letters **2000**, *182*, 285.
43. Loessner, M.J.; Rudolf, M.; Scherer, S. Evaluation of luciferase reporter bacteriophage A511:*luxAB* for detection of *Listeria monocytogenes* in contaminated foods. Applied Environ. Microbiol. **1997**, *63*, 2961.
44. Allen, K.J.; Griffiths, M.W. Research Note: Use of luminescent *Campylobacter jejuni* ATCC 33291 to assess eggshell colonization and penetration in fresh and retail eggs. J. Food Prot. **2001**, *64*, 2058.
45. Kodikara, C.; Crew, H.H.; Stewart, G.S.A.B. Near on-line detection of enteric bacteria using *lux* recombinant bacteriophage. FEMS Microbiol. Letters **1991**, *83*, 261.
46. Belkin, S. Detection of oxidants using lux fusions to oxidative stress promoters. Methods Mol. Biol. **1998**, *102*, 247.
47. Gu, M.B.; Choi, S.H. Monitoring and classification of toxicity using recombinant bioluminescent bacteria. Water Sci. Technol. **2001**, *43*, 147.

48. Gu, M.B.; Gil, G.C. A multi-channel continuous toxicity monitoring system using recombinant bioluminescent bacteria for classification of toxicity. Biosens. Bioelectron **2001**, *16*, 661.
49. Moller, A.; Jansson, J.K. Detection of firefly luciferase-tagged bacteria in environmental samples. Methods Mol. Biol. **1998**, *102*, 269.
50. Sarkis, G.J.; Jacobs, W.R., Jr.; Hatfull, G.F. L5 luciferase reporter mycobacteriophages: a sensitive tool for the detection and assay of live mycobacteria. Molec. Microbiol. **1995**, *15*, 1055.
51. Riska, P.F.; Jacobs, W.R., Jr. The use of luciferase-reporter phage for antibiotic-susceptibility testing of mycobacteria. Methods Mol. Biol. **1998**, *101*, 431.
52. Carriere, C.; Riska, P.F.; Zimhony, O.; Kriakov, J.; Bardarov, S.; Burns, J.; Chan, J.; Jacobs, W.R. Jr. Conditionally replicating luciferase reporter phages: improved sensitivity for rapid detection and assessment of drug susceptibility of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **1997**, *35*, 3232.
53. Gordon, S.; Chung, G.; Andrew, S. The application of bacterial luciferase as a reporter gene in mycobacteria. Methods Mol. Biol. **1998**, *101*, 235.
54. Banaiee, N.; Bobadilla, M.; Riska, P.F.; Bardarov, S. Jr.; Small, P.M., Ponce-de-Leon, A.; Jacobs, W.R. Jr.; Hatfull, G.F.; Sifuentes-Osornio, J. Rapid identification and susceptibility testing of *Mycobacterium tuberculosis* from MGIT cultures with luciferase reporter mycobacteriophages. J. Med. Microbiol. **2003**, *52*, 557.
55. d'Herelle, F. Sur un microbe invisible antagoniste de bacikkes dysenteriques. CR. Acad. Sci. **1917**, *165*, 373.
56. Smith, H.W.; Huggins, M.B. Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. J. Gen. Microbiol. **1982**, *128*, 307.
57. Smith, H.W.; Huggins, M.B. Effectiveness of phages in treating experimental *Escherichia coli* diarrhoea in calves, piglets and lambs. J. Gen. Microbiol. **1983**, *129*, 2659.
58. Smith, H.W.; Huggins, M.B.; Shaw, K.M. The control of experimental *Escherichia coli* diarrhoea in calves by means of bacteriophages. J. Gen. Microbiol. **1987**, *133*, 1111.
59. Barrow, P.; Lovell, A.; Berchieri, A. Use of lytic bacteriophage for control of *Escherichia coli* septicemia and meningitis in chickens and calves. Clin. Diagn. Lab. Immunol. **1998**, *5*, 294.
60. Berchieri, A.; Lovell, A.; Barrow, P. The activity in chicken alimentary tract of bacteriophages lytic to *Salmonella thyphimurium*. Res. Microbiol. **1991**, *142*, 541.

61. Soothill, J.S. Treatment of experimental infections in mice with bacteriophages. J. Med. Microbiol. **1992**, *37*, 258.
62. Ramesh, V.; Fralick, J.A.; Rolfe, R.D. Prevention of Clostridium difficile-induced ileocecitis with bacteriophage. Anaerobe **1999**, *5*, 69.
63. Murthy, K.; Allain, B.; Roman, J.L.; Durand, S.; Fairbrother, J.; Quessy, S.; Mandeville, R. Phage therapy: an innovative approach to treat antibiotic-resistant bacterial infections. Am. Ass. Swine Vet. **1999**, 217.
64. Merril, C.R.; Biswas, B.; Carlton, R.; Jensen, N.C.; Creed, G.J.; Zullo, S.; Adhya, S. Long-circulating bacteriophage as antibacterial agents. PNAS **1996**, *93*, 3188.
65. Biswas, B.; Adhya, S.; Washart, P.; Paul, B.; Trostel, A.N.; Powell, B.; Carlton, R.; Merril, C.R. Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant *Enterococcus faecium*. Infect. Immun. **2002**, *70*, 204.
66. Matsuzaki, S.; Yasuda, M.; Nishikawa, H.; Kuroda, M.; Ujihara, T.; Shuin, T.; Shen, Y.; Jin, Z.; Fujimoto, S.; Nasimuzzaman, M.D.; Wakiguchi, H.; Sugihara, S.; Sugiura, T.; Koda, S.; Muraoka, A.; Imai, S. Experimental protection of mice against lethal *Staphylococcus aureus* infection by novel bacteriophage phi MR11. J. Infect. Dis. **2003**, *187*, 613.
67. Broxmeyer, L.; Sosnowska, D.; Miltner, E.; Chacon, O.; Wagner, D.; McGarvey, J.; Barletta, R.G.; Bermudez, L.E. Killing of Mycobacterium avium and Mycobacterium tuberculosis by a mycobacteriophage delivered by a nonvirulent mycobacterium: a model for phage therapy of intracellular bacterial pathogens. J. Infect. Dis. **2002**, *186*, 1155.
68. Nakai, T.; Park, S.C.: Bacteriophage therapy of infectious diseases in aquaculture. Res. Microbio. **2002**, *1153*, 13.
69. Fattal, B.; Dotan, A.; Tchorsh, Y.; Parpari, L.; Shuval, H.I. Penetration of *E. coli* and F2 bacteriophage into fish tissues. Schriftenr Ver Wasser Boden Lufthyg **1988**, *78*, 27.
70. Nakai, T.; Sugimoto, R.; Park, K.H.; Matsuoka, S.; Mori, K.; Nishioka, T.; Maruyama, K. Protective effects of bacteriophage on experimental *Lactococcus garvieae* infection in yellowtail. Dis. Aquat. Organ. **1999**, *37*, 33.
71. Park, S.C.; Shimamura, I.; Fukunaga, M. Isolation of bacteriophages specific to fish pathogen, *Pseudomonas plecoglossicida*, as a candidate for disease control. Applied Envir. Microbiol. **2000**, *66*, 1416.

72. Park, S.C.; Nakai, T. Bacteriophage control of *Pseudomonas plecoglossicida* infection in ayu Plecoglossus altivelis. Dis. Aquat. Organ. **2003**, *53*, 33.
73. Cerveny, K.E.; DePaola, A.; Duckworth, D.H.; Gulig, P.A. Phage therapy of local and systemic disease caused by *Vibrio vulnificus* in iron-dextran-treated mice. Infect. Immunol. **2002**, *70*, 6251.
74. Flaherty, J.E.; Harbaugh, B.K.; Jones, J.B.; Somodi, G.C.; Jackson, L.E. Control of bacterial spots on tomato in the greenhouse and field with H-Mutant bacteriophages. Hortscience **2001**, *36*, 90.
75. Flaherty, J.E.; Harbaugh, B.K.; Jones, J.B.; Somodi, G.C.; Jackson, L.E. H-mutant bacteriophages as a potential biocontrol of bacterial blight of geraniums. Hortscience **2000**, *35*, 882.
76. Leverentz, B.; Conway, W.S.; Alavidze, Z.; Janisiewicz, W.J.; Fuchs, Y.; Campbel, M.J.; Chighladze, A. Examinatión of bacteriophage as a biocontrol method for *salmonella* on fresh-cut fruit: a model study. J. Food Prot. **2001**, *64*, 1116.

Received August 12, 2003
Accepted August 18, 2003