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Prospectus of Bacteriophages as Potent Antimicrobial Agents Against Phytopathogenic Bacteria

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Abstract: Bacteriophages are the viruses of prokaryotes, acts as natural bacterial killers, that infects bacteria and can either instantly kill a bacterial cell. Although phage typing was discovered during the second decade of the 20th century, a widespread appreciation of their viral character of specific bacteria were being used in as biocontrol agents in plant protection. Control of plant pathogenic bacteria is a serious problem in production of many agricultural crops. High multiplication rate, adaptability and life inside plant tissue make bacteria unsuitable and inaccessible for most of control measures. Consequently, the list of bactericides available for plant protection is very short. Lately, biological control measures have been intensively studied as a potential solution for the current problems in agriculture. Investigation of bacteriophages is a fast-expanding area of research in plant protection. Several experiments have shown that they can be used as a very efficient tool for control of plant pathogenic bacteria. The fact that they are natural omniLytics for bacteria, availability of simplified protocols for cultivation, host-specific, human and environment friendly, provide a great advantage for the application of phages over other bactericides.

Key words: Phytopathogenic bacteria · Bacteriophages · Biological control · Bactericides · Integrated disease management

INTRODUCTION

Challenges for Controlling Bacterial Diseases in Agricultural Plants: Plant diseases caused by bacteria are a major economic liability to agricultural production. Disease control has been a major challenge for many bacterial diseases Bacterial plant diseases affecting important agricultural crops can result in considerable damage and serious economic loss worldwide. They are becoming more difficult to control because bactericides in present-day use are not as effective as they have been in the past. Antimicrobials for prophylactic treatment of bacterial diseases of plants are limited in availability, use and efficacy and therapeutic use is largely ineffective. Most applications are by spray treatments in orchards. Monitoring and surveillance for drug resistance are not routinely done. In the United States, data on use of antimicrobials for treatment of bacterial diseases of plants are limited to streptomycin and oxytetracycline. Resistance to streptomycin has become widespread among bacterial phytopathogens; no resistance among these bacteria has yet been reported for oxytetracycline. No human health effects have been documented since inception of use of antimicrobials in plants in the 1950s. Transfer of antimicrobial resistance from marker genes in transgenic plants to bacteria has not been documented under natural conditions in field-grown plants. However, antimicrobial-resistance genes are being eliminated from use as marker genes because of concerns about possible transfer from plant genomes back to bacteria, with further horizontal transfer to the bacteria in the environment, or from plant genomes to animals by plant consumption. No new antimicrobials agents such as phages (Phage therapy) are expected to be used in plant agriculture because of high costs of development, regulatory constraints and environmental and human health

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concerns. Alternatives to antimicrobials, such as biocontrol agents, transgenic plants and novel chemicals, are being developed and marketed, although their efficacy remains to be determined.

Phage Biology: Phages are a kingdom of viruses that infect bacteria and are distinct from the animal and plant viruses. Phages can have either a "lytic" or a "lysogenic" life cycle. The lytic phages are the most suitable candidates for phage therapy, because they quickly reproduce within and lyse the bacteria in their host range, growing exponentially in number in the process. All phages are composed of nucleic acid encapsulated by a protein coat; the genome and the capsid, respectively. Phage genomes can be double-stranded DNA, single stranded DNA, or single-stranded RNA. Capsids come in many forms, ranging from small hexagonal structures, to filaments, to highly complex structures consisting of a head and a tail. These virion particles, like their eukaryotic counterparts, are metabolically inert. This all changes upon bacterial infection which begins, in a process called adsorption, with capsid binding to the cell surface and subsequent genome uptake into the cell cytoplasm.

Upon infection phages can exhibit one of two distinct life cycles: (i) active infection, in which phage virion particles are assembled within the cytoplasm of an infected bacterium, or (ii) lysogeny, in which the phage genome integrates (as a prophage) into the bacterial chromosome as a giant gene complex. To produce virion progeny the prophage, which together with the host bacterium is described as a lysogen, must be induced. Induction can occur as a stochastic process, or may be forced via the application of DNA damaging agents. Many phages, described as temperate, are capable of displaying either active infection or reduction to lysogeny upon bacterial infection. Other, non-temperate phages are obligated to actively produce phage progeny upon infection of actively metabolizing bacteria. Depending on the species and conditions, each "parent" phage can produce on average approximately 200 "daughters" per lytic cycle. If each daughter infects and kills a host bacterium there will be 40,000 progeny at the end of the 2nd cycle; 8 million at the end of the 3rd cycle; 1.6 billion at the end of the 4th cycle; and so on [1].

Active phage infections, whether immediately active upon phage adsorption or the product of lysogen induction, may be further distinguished by their means of progeny-phage release from the infected bacterium, with release occurring, depending on the phage, either by lysis or by extrusion. Lysis is by far the most common and involves the destruction of the bacterial cell envelope by so-called lytic phages to allow intracellularly matured phage progeny to leak into the extracellular environment [2]. Lysis caused by the phage kills the infected bacterium and terminates the phage infection. By contrast, another mode of infection leads to the movement of filamentous virions out of cells-allows phage-progeny release without causing bacterial death. Phages can also be differentiated based on various phage infection strategies in terms of their utility. Temperate phages (particularly the *E. coli* phages are used for cloning; filamentous phages (such as the coli phage M13) are employed as platforms for protein display in genetic engineering experiments; and obligatory lytic bacteriophages (described as virulent) are the phages of choice for bacteriophage mediated biocontrol and phage therapy.

Bacteriophages are biodegradable organisms chemically they are composed of protein and nucleic acid, eventually they will be broken down by proteases and nucleases secreted by various species of soil bacteria and fungi. Amino acids, nucleotides and other end products of microbial hydrolase activity will be absorbed as nutrients by soil inhabitants including plants. Unlike many agrochemicals such as copper and other heavy metals, phages do not persist in the environment. Bacteriophage compositions offer a bio-control alternative for bacterial phtyto pathogens with no biological risk or environmental pollution.

Early Studies of Phages in Plant Protection: Bacteriophages are ubiquitous in the environment and they are thought to be ecologically important by controlling bacterial numbers and activity, affecting composition and diversity of bacterial populations and facilitating gene transfer between bacteria via transduction [3-7]. In soil, phages are likely to be abundant in nutrient-rich habitats such as the rhizosphere (i.e. an ecological niche which can support high population densities of metabolically active bacteria). In a limited number of studies, populations' dynamics of phages and their host bacteria in the rhizosphere were monitored in microcosms [8].

Bacteriophages constitute a majority of biological organisms on the earth and have crucial influences on the evolution of bacteria [9-12]. The double-stranded DNA (dsDNA)-containing tailed phages (the Caudoviridales) represent the most numerous, most widespread and probably the oldest group o f bacteriophages [13]. Three families (i.e. Myoviridae, Podoviridae and Siphoviridae) belong to the order Caudovirales and

approximately 25% of the members of this order are myoviruses [14]. Myoviridae phages classically represented by T4-like phages have a contractile tail sheath and infect a broad range of bacterial hosts. From analyses of T4- like phage genomes, it has been suggested that myovirus genomes are mosaic of conserved core genes, which include structural genes encoding head and tail proteins and enzyme genes for DNA and nucleotide processing [15] and the remaining accessory non-core genes, which are not conserved across species and correspond to the vast majority of the huge gene pool of phages. The functions of the non-core genes are mostly unknown, although it is commonly assumed that they provide a selective benefit to phages [16].

As a result, there is a critical present-day need to identify other effective bactericides for bacterial plant pathogens. This has been exemplified by studies directing toward evaluating different chemical agents for control of bacterial speck of tomato [17]. Specific phage-host interactions have been investigated in marine environments [4] and also, though to a lesser extent, in soil environments [18-23].Until recently plant pathologists have generally rejected the use of phages because there was no way to circumvent the appearance of phage resistant mutants.

Antimicrobial Resistance in Plant Pathogens: Antimicrobial resistance in plant pathogenic target bacteria began to appear as early as the 1960s, a few years after introduction of use of streptomycin [24, 25]. Resistance has also been found to be linked with copper resistance [26, 27]. Genetically, resistance genes may be chromosomal or carried on plasmids or transposons; all genetic forms are found in environmental, human and plant pathogenic strains [28]. Tetracycline resistance has not been reported in target bacteria-that is, the pathogen-but it has been found in plant surface-associated (phylloplane) bacteria [29].

Although there is at present no evidence for a correlation between the agricultural use of azoles as fungicides and fungal resistance in humans, such concerns have been expressed [30] and research on this issue is merited. In principle, the same concerns that apply to development of resistance with the use of bacterial antimicrobials are applicable to anti fungal. The reverse concern may apply to antiviral agents, which have not yet been used in plants.

Antimicrobial-resistance genes have been used as selectable markers in producing transgenic plants. Under optimized laboratory conditions, the *nptII* gene (conferring resistance to kanamycin) could be transferred from transgenic sugar beets to the soil bacterium *Acinetobacter* sp. BD413 gene [31].This gene can also be transferred from transgenic potatoes to *Acinetobacter* BD413 and *Pseudomonas stutzeri* ATCC 17587, both of which harbor plasmids carrying the *nptII* gene with a small deletion [32]. In these experiments, detectable marker rescue was dependent on sequence homology in the recipient cells. Even if such transfer were to occur, findings of the research [31] pointed out that the promoter sequences used in the transgenic constructions are not active in most bacteria, so that the recipients would not express a kanamycin resistance phenotype. Also, most of the antimicrobial-resistance genes used as marker genes are widely disseminated in environmental bacteria. Nevertheless, such use is being phased out because of concerns about potential transfer of these bacterial antimicrobial resistance genes from plant chromosomes back to bacteria, with subsequent horizontal transfer among bacteria in the environment [33].

At the genetic level, little information exists on the extent of antimicrobial susceptibility and resistance occurring naturally in environmental bacteria. Consequently, implications for human health from resistance arising from these sources remain problematic. Alternatives to antimicrobials under investigation include biocontrol agents [34, 35] transgenic plants and novel chemicals. Some of these agents or compounds have been recently marketed, but efficacy and safety over time still remain to be determined.

The Phage-Therapy Advantage: Phage therapy has specific advantages compared with chemical antimicrobials. The first advantage is that phages are not small molecules capable of modifying and thereby degrading animal metabolisms but, instead, exert their effects on the animal host through bacteria. The second advantage is that phages are self-replicating. As a consequence, a given phage dosage can self-amplify over the course of treatment, greatly increasing efficacy. The third advantage is that this self amplification can allow phages to burrow into bacterial infections, one infected bacterium at a time, resulting in greater penetration than chemical antimicrobials, even though the small size of the latter would seem to bestow a diffusion advantage. The

fourth advantage comes from the ubiquity and diversity of phages. Typically these phages may be employed in cocktails-the phage equivalent of multi-drug therapy-so that phage resistance is countered right from the start of therapy. The fifth advantage of phage therapy stems [36], nevertheless, from the narrow spectrum of phage activity, particularly as compared to many chemical antimicrobials. Not only do many phages display monovalence, i.e. a propensity to infect only a single species or even only a small selection of strains within a given species of bacteria, but common phage-enrichment schemes likely select for phages that are specialized for propagation on a small range of bacterial types [37]. Even when they are multivalent and thereby able to infect numerous bacterial types, one would still classify phages as relatively narrow spectrum antibacterial. In fact, even given the employment of cocktails of numerous phage types, the spectrum of activity remains narrow due to the narrow and overlapping spectrums of activity of the individual phages making up a cocktail. Phages consequently can be very selective in the bacterial populations that they attack, reducing the likelihood of super infection and other complications of normal-flora reduction that can often result following treatment with chemical anti-bacterial.

Use of Bacteriophages as Antimicrobials in Agriculture: Bacteriophages (phages) specific to a particular species or subspecific group of bacteria have been used to help identify plant bacterial pathogens [38- 42]. The reason for its prevailing use is that the technique is more rapid, simple and effective than conventional time-consuming procedure. Phage lysis zones usually become visible within 18-24 h of incubation such as spot inoculation of phage solution. The occurrence and distribution of lysotypes of plant pathogenic bacteria [43-49]. Three phages highly specific to bacterial pathogens related to citrus plants namely, CP1, CP2 [49] and CP3 [50] have been described previously.

A new technology was introduced, which unlike the common past use of only one phage for prevention of disease utilizes a mixture of three to eight different phages including h- (host-range) phages [51,52]. H-phages are spontaneously derived from their wild-type parent phages and have been found to lyse not only their parent wild-type bacteria but also phage-resistant mutants originating from their parent bacteria. They were named host-range mutants because of their capacity for attacking this extended range or number of hosts. If a bacterial control mixture is composed of phages including h-phages, any phage-resistant mutants arising in a bacterial-pathogen population will be destroyed by heterologous phages in the multiphage composition. Antimicrobials originated from microorganisms isolated from the environment [53]. The extent of naturally occurring antimicrobial resistance is not well known because, except for monitoring the target pathogen treated with antimicrobials, even fewer studies have monitored the resistance of nontreated, wild-type pathogens [54, 55].

However there are some studies of phenotypic antimicrobial resistance and a few studies of genetic determinants associated with resistance in natural isolates of commensal and phytopathogenic bacteria, this study critically pointed out that there are no systematic studies of microbes in an ecosystem. This lack of data is the case even for environments in which antimicrobials are used for managing bacterial plant diseases of fruit trees, for which antimicrobial use in the United States has proven to be economical [56].

A study found that virulent citrusphages are highly specific to *X. axonopodis* pv. *citri* strains among other strains of xanthomonads tested [57]. An investigation in North America revealed that Several *Erwinia amylovora* phages have been partially characterized before by restriction digests and proposed for control of fire blight [58].

The genomic studies of two phages revealed the use direct repeats in their genomes for replication as concatemers, similar to *E. coli* phage T7 [59]. A 54-mer is repeated twice and is present in both genomes. A study on the comparison of two different phages, Ø Ea104 with Ø Ea21-4 revealed 98% identity for their genomes of 84,565 and 84,576 bp, respectively. Regions with high levels of mismatch but 97% protein identity were noted for genes encoding RIIA, a hypothetical protein and DNA polymerase. BLAST search revealed low similarities to *Salmonella* phage Felix O1 (AF320576) [60].

Experiments were designed to evaluate the feasibility of utilizing phage mixtures as a viable alternative for controlling bacterial plant diseases using bacterial wilt and bacterial spot of tomato as test systems. Unlike most agrochemicals, phage exhibit narrow specificity of action, killing only targeted, pathogenic bacteria. Phages will not attack other bacteria, many of which are beneficial to plants and soil ecosystems.

Case Studies and Recent Use of Bacteriophages in Plant Protection: Nevertheless, investigation on bacteriophages is a fast expanding area over recent decades, several experiments have shown the use of bacteriophage as biocontrol agent to control the growth of plant-based bacterial pathogens, which has been explored with increased interest. The fact that they are natural enemies of the bacteria, the host specific, acts as bactericides, mode of infection and simple for cultivation and management. A investigation on complete genome analysis of bacteriophage specific for *Erwinia amylovora* a causative agent Fire blight, a plant disease. Investigators of the study have been reported that *Erwinia amylovora* may be controlled by the application of bacteriophages. The outcome of this research finds new avenues in bacteriophage research at genomic level. The study provided the complete genome sequences and the annotation of three *E. amylovora*-specific phages were isolated in North America and genomic information of *E. amylovora* resembles two already-reported viral genomes in BLAST [61]. Recently one of the study anticipated the use of alternative bacteriophage mediated biocontrol method using a unique phage, such as ϕRSL1, This study anticipated coexistence of bacterial cells and the phage resulted in effective prevention of wilting caused by *Ralstonia solanacearum*. Infection with ϕRSA1 and ϕRSB1, either alone or in combination with the other phages, resulted in a rapid decrease in the host bacterial cell density. The three phages ϕRSA1, ϕRSB1 and ϕRSL1 appear to be useful in the eradication of the bacterial wilt pathogen [62].

A recent research on bacteriophages speci?c to *Salmonella* strains were isolated from sewage effluent and characterized. They could able to demonstrate efficacy of bacteriophage mixture applied to dairy manure compost inoculated with *Salmonella enterica* serotype *Typhimurium*. This study has been suggested that use of bacteriophage treatment resulted in a greater reduction of *Salmonella* and the findings of the research was anticipated that, naturally occurring bacteriophages can be isolated from the environment and applied to compost surfaces contaminated with *Salmonella* [63].

A genomic study on specific phages infecting *Lactococcus lactis* revealed a typical icosahedral capsid connected to one of the smallest noncontractile tails found in a *lactococcal* phage of the *Siphoviridae* family. This study has pointed out that a burst size of 72 virions released per infected host cell and a latent period of 90 min. The host range of phage specific bacteria was limited to 3 out of the 60 *lactococcal* strains tested. The genomic organization was similar to those of other *siphophages.* All genes were on the same coding strand and in the same orientation. The proteomic analyses showed that several phage structural proteins shared similarity with reported bacteriophages [64].

The prevalence of bacteriophages was investigated in 24 strains of four species of plant growth-promoting rhizobacteria belonging to the genus *Azospirillum*. Upon induction by mitomycin C, the release of phage particles was observed in 11 strains from three species. The studies on transmission electron microscopy revealed two distinct sizes of particles of the *Azospirillum* species, typical of the *Siphoviridae* family. DNA based study revealed that all phages isolated from *A. lipoferum* and *A. doebereinerae* strains had a size of about 10 kb whereas all phages isolated from *A. brasilense* strains displayed genome sizes ranging from 62 to 65 kb. The complete sequence of the *A. brasilense* Cd bacteriophage (Ø Ab-Cd) genome was determined as a double-stranded DNA circular molecule of 62,337 pb that encodes 95 predicted proteins [65].

One of the research on experimental contamination of various vegetables revealed that bacteriophage cocktail (designated ECP-100) containing three *Myoviridae* phages lytic for *Escherichia coli* O157:H7 was examined for its ability to reduce experimental contamination of hard surfaces (glass coverslips and gypsum boards), tomato, spinach, broccoli and ground beef by three virulent strains of the bacterium. The treatment with the least concentrated preparation that elicited significantly less contamination of the hard surfaces (i.e. 109 PFU/ml) also significantly reduced the number of viable *E. coli* O157:H7 organisms on the four food samples. This study has been suggest that naturally occurring bacteriophages may be useful for reducing contamination of various hard surfaces, fruits, vegetables and ground beef by *E. coli* O157:H7 [66]. Isolation and characterization of several different kinds of phage was studied that specifically infected *R. solanacearum* strains belonging to different races and/or biovars. Phage ϕRSA1 is a P2-like head-tail virus (*Myoviridae*) with a very wide host range; all 15 strains tested from race 1, 3, or 4 and biovar 1, N2, 3, or 4 were susceptible to this phage [67].

A study reported that total of 33 *Rhizobium meliloti* bacteriophages were analysed. Of those, 21 were isolated in northern France from field soil in which *Medicago*

sativa (L.) was grown. The other 12 phages were obtained by UV light and mitomycin C induction from 46 R. *meliloti* strains. *Rhizobiophages* were characterized by their morphology, host range, serological properties, restriction endonuclease patterns, DNA-DNA homologies and DNA molecular weights. This study revealed that different morphotypes were observed showing tailed phages with icosahedral heads. The categories of morphotypes included the *Myoviridae* (11 phages), *Siphoviridae* (3 morphotypes and 20 phages) and *Podoviridae* (2 phages) [68].

Bacteriophages were isolated against *Ralstonia solanacearum*, a soil-borne Gram-negative bacterium that is the causative agent of bacterial wilt in many important crops. These phages may be useful as a tool not only for molecular biological studies of *R. solanacearum* pathogenicity but also for specific and efficient detection and control of harmful *Ralstonia* pathogens in cropping ecosystems as well as growing crops [69]. One of the research demonstrated the different kinds of phage that specifically infected to *R. solanacearum* strains belonging to different biovars. Phage ϕRSA1 is a P2-like head-tail virus (*Myoviridae*) shown wide host range [70]. However the the practical use of phage against on *R. solanacearum* infecting ϕRSL1 is another *myovirus* containing 231 kb in its genome; this phage was able to lyse 10 of 15 tested strains and can be used as a biocontrol agent for wilt disease caused by *Ralstonia solanacearum* [71]. A research on Lytic bacteriophages were recovered from five different fermenting cucumber tanks that were inoculated with *Pediococcus* sp. a lytic phage, was characterized for efficient lytic activity against *Pediococcus* sp, the findings of the research was anticipating the efficient use of phages as biocontrol agent [72].

Studies on activity of the lysozyme from *E. amylovora* phage against *E. amylovora* strains and other plant-associated gram-negative bacteria was investigated for its ability to inhibit growth of bacteria and compared with the lysozyme from *Escherichia coli* phage T, this study strongly suggested efficacy of lysosomic activity of phage mediated bacterial control in agriculture [73]. One the research to illustrate the potential of phages to impair biocontrol performance of beneficial bacteria released into the natural soil environment. This research provides new insights into biotic factors that can influence survival, root colonization and biocontrol capacity of plant-beneficial *pseudomonas*. The exploitation of phage-resistant variants may be a way to improve persistence and beneficial activity of bacterial inoculants for use in natural soil environments. The findings of the research have been revealed the better understanding of the dynamics of phage-host interactions in habitats like the rhizosphere, which was considered to be “hot spots” for bacterial activity [74].

CONCLUSION

Bacteriophages are viruses that infect bacteria. Since their discovery in the early part of the twentieth century, they have been evaluated extensively for control of all kinds of bacterial infected diseases, including plant diseases. In the recent past, phages have been under evaluation for controlling fire blight on apple and pear, bacterial wilt on tobacco, citrus bacterial canker, citrus bacterial spot, bacterial blight on geranium, Bacteriophages have great potential because they are widely present in nature; are self-replicating; can be targeted against bacterial receptors that are essential for pathogenesis; are nontoxic to eukaryotes; and are specific to certain bacterial species or strains without damaging other, possibly beneficial members of the indigenous flora. Phages have been proposed as natural antimicrobial agents to fight bacterial infections in crops of agricultural importance. These proposals have a long history, but are currently going through a kind of renaissance as documented by a spate of recent reviews. Phages are fairly easy and inexpensive to isolate, produce and store. They may also have application for use in technologically less developed regions. In other instances, phages were coapplied with bacteria that served as phage hosts as well as biological control agents providing two levels of biological control simultaneously. However, we think that there is a arena of literature and sufficient data on bacteriophage studies may show promise for treating antibiotic resistant phytopathogenic bacteria. To facilitate further progress in phytopathology, directions for future research, we are proposing to find out new alternative treatment modalities in the field of phage therapy research.

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