# Chapter 8
# Phage Biopesticides and Soil Bacteria: Multilayered and Complex Interactions

**Antonet M. Svircev, Susan M. Lehman, Peter Sholberg, Dwayne Roach, and Alan J. Castle**

## 8.1 Phages as Biopesticides

Control of plant pathogens in agriculture faces many unique challenges. These include development of pathogen resistance to conventional pesticides, absence of resistant plant host material, the requirement for environmentally friendly alternatives to traditional pesticide, and the gradual regulatory removal of traditional chemicals used for plant pathogen control. The resurgence in research on the development of bacteriophage-based control programs is evident in the inspection of current literature. Debate continues on the suitability of phages as biological control agents (BCAs) (Stewart 2001). Recently, phages have been tested as BCAs or biopesticides for the control of bacterial spot of tomato (Flaherty et al. 2000; Balogh et al. 2003; Obradovic et al. 2004; Obradovic et al. 2005; Jones et al. 2005; Balogh et al. 2005; Iriarte et al. 2007), bacterial wilt of tomato (Kumar et al. 2006; Yamada et al. 2007); bacterial blight of geranium (Flaherty et al. 2001), citrus canker (Balogh et al. 2008), fire blight in pear and apple (Schnabel et al. 1999; Schnabel and Jones 2001; Svircev et al. 2002a; Gill et al. 2003; Svircev et al. 2006; Lehman 2007), soft-rot of calla lilies (Svircev et al. 2002b; Ravensdale et al. 2007), leaf blight of onion (Lang et al. 2007), seed treatments (Goyer 2005), and bacterial

A.M. Svircev (✉)
Agriculture and Agri-Food, Victoria Ave. North, Vineland Station, ON L0R 2G0, Canada
e-mail: Antonet.Svircev@agr.gc.ca
S.M. Lehman
Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mail Stop C-16, Atlanta, GA 30333, USA
P. Sholberg
Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, Summerland, BC V0H 1Z0, Canada
D. Roach and A.J. Castle
Department of Biological Science, Brock University, 500, Glenridge Avenue St. Catharines, ON L2S 3A1, Canada

G. Witzany (ed.), *Biocommunication in Soil Microorganisms*, Soil Biology 23,
DOI 10.1007/978-3-642-14512-4_8, © Springer-Verlag Berlin Heidelberg 2011

spot of peach (Zaccardelli et al. 1992; Saccardi et al. 1993). Jones et al. (2007) provide a thorough and up-to-date review on the use of bacteriophages as agricultural biopesticides. In addition, the authors discuss the advantages, challenges, and novel approaches that are utilized for the integration of phages into modern diseases control programs.

### 8.1.1 Aerial Application of Phage Biopesticides and Impact on Soil Ecology

The aerial surfaces of plants provide a unique habitat for bacterial microorganisms. The complex interactions between these organisms as a whole are only beginning to be examined and studied (Andrews and Harris 2000). In the apple and pear orchard, *Erwinia amylovora* is the pathogen responsible for a necrotic wilt disease commonly named fire blight (Vanneste 2000). Fire blight biological control programs have been developed and implemented with commercially available bacterial BCAs (Johnson and Stockwell 2000). While *E. amylovora* is commonly present in the aerial portions of the canopy, it has been isolated from the orchard soil and a soil-inhabiting microarthropod (Hildebrand et al. 2001). The authors postulate that the insect may be responsible for reducing the pathogen population in the soil. Little is understood on the impact of *Erwinia* spp. phages on the removal of *E. amylovora* from soil. However, majority of the phages isolated by Gill et al. (2003) originated from the soil in the immediate vicinity of active fire blight infections.

The aerial application of phage biopesticide for the control of the fire blight pathogen occurs in the spring when the blossoms are fully open (Lehman 2007). Biopesticides are commonly applied at 0–25%, 25–50%, and 75–100% bloom, to help establish the BCA populations ahead of the pathogen and prevent the ingression of the pathogen *E. amylovora* into the host via the flower cup or hypanthium. In the phage-based biopesticide program, the common orchard epiphyte *Pantoea agglomerans* performs as a phage “carrier” and incubator. The carrier and phage are simultaneously applied to the opened blossoms at multiplicity of infection (MOI=1) or 1:1 ratio of bacteria:phage at $10^8$ CFU and PFU/ml. Real-time PCR technology was used to monitor in situ the phage, carrier, and the artificially introduced pathogen populations during field trials (Lehman 2007). Disease control was obtained in in vivo bioassays and field trials when phages were able to decrease the population of the pathogen below $10^4$ CFU/ml (Lehman 2007).

The impact of the aerial application of phages on the orchard soil ecology is poorly understood. Biological control programs generally focus on understanding the interaction(s) between the biological agent and the pathogen ignoring the more complex interactions of pathogen–epiphyte–BCA–plant-associated microorganisms on aerial and soil surfaces. Studying the impact of the phage biopesticide on the soil ecosystem is hampered by the lack of specific technologies. The techniques available for the study of phages in soil ecosystems are further discussed later in this

chapter. We cannot distinguish between the applied phage-carrier and the indigenous phage populations. However, molecular techniques that follow overall populations of phage, *P. agglomerans* and *E. amylovora* have been developed (Lehman 2007) and serve an important role in following the population dynamics in the flower and, potentially, in the orchard soil. The yearly application of phage and/or bacterial biologicals may alter the ecology of the orchard soil but, to date, there are no reported studies that study directly the impact of phage biopesticides on the soil microbial communities.

## 8.2 Phage Biopesticides in Greenhouse Soils: Control of *Pectobacterium carotovorum*

The use of bacteriophages to control disease in greenhouse soil mixtures creates unique challenges since the plant pathogen and biopesticide interaction takes place on soil particles in an arid-aqueous environment. Soils and water solutes, including fertilizers, affect the survival of phage biopesticides and their interactions with the pathogen. In this section, we look at the use of bacteriophages that inhibit *Pectobacterium carotovorum* soft-rot as a model system for the biocontrol of greenhouse diseases and the problems that are encountered during the development of such a biocontrol system.

*Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*, Jones 1901) Hauben et al. 1999 (= *Erwinia carotovora* subsp. *carotovora*) and its relatives, most notably *P. carotovorum* subsp. *atroseptica*, are the causative agents of soft-rot disease of numerous plant species. These bacteria are common in surface waters (Harrison et al. 1987), are distributed worldwide, and in the case of *Pcc* show little host specificity (Pérombelon and Kelmon 1980). These characteristics contribute to significant crop damage in a broad range of host species.

Ravensdale et al. (2007) were the first group to assess the feasibility of using bacteriophages as BCAs for soft-rot caused by *Pcc*. Fourteen bacteriophage isolates, classified by morphology to the order *Caudovirales*, have host ranges specific to *Pcc* isolates from calla lilies. *Pcc* is a devastating pathogen of calla lilies (*Zantedeschia* spp.) and can cause anywhere from $<3\%$ to complete loss of greenhouse crops (Blom and Brown 1999).

Prior to greenhouse trials, Ravensdale et al. (2007) tested phage survival in preplant-treatment and fertilizer solutions. Phages were completely inactivated by 48 h in three preplant-treatment solutions. The mechanism of inactivation is unknown but may be attributed to deleterious effects of copper (Sagripanti 1992) or nonionic surfactants (Chattopadhyay et al. 2002). Fertilizer solutions did not inhibit the phages, while the choice of water source was critical for the phage-based biopesticide. Reverse osmosis removes an inhibitory agent in tap water, most likely chlorine (Berg et al. 1989) or a cation scrubbed from piping. Later work on bacteriophages that attack *Erwinia amylovora* showed that EDTA amendment

increased phage survival in sterile tap water thus supporting the "cation inhibitor" hypothesis (Svircev and Castle, unpublished data).

Three tests for the effects of the phages on *Pcc* populations were reported by Ravensdale et al. (2007). These tests included inhibition in liquid media, on tissue plugs from calla lily tubers and on intact calla lilies grown in the greenhouse. The tests in liquid media included phage and *Pcc* in nutrient broth (Ravensdale et al. 2007), in fertilizer solution containing Fe-EDTA or in fertilizer solution lacking Fe-EDTA. The phages reduced *Pcc* populations in nutrient broth and fertilizer lacking Fe-EDTA but were ineffective in the presence of Fe-EDTA. The authors proposed two plausible mechanisms for this inhibitory effect. First, bacteriophage replication is dependent upon the activity of bacteriophage-encoded ribonucleotide reductase, an iron-dependent enzyme. An iron chelator such as EDTA could reduce effective, unbound iron concentrations within the host cell thereby inhibiting bacteriophage propagation (Romeo et al. 2001). Alternatively, free EDTA could bind other essential cations such as magnesium or calcium that stabilize many phages.

Several bacteriophages, alone or in mixtures, reduce soft-rot of calla lilies by up to 70% in greenhouse trials. These tests indicated that bacteriophages show promise as biocontrol agents and warrant further study. Several routes for optimization of biocontrol efficacy could be followed. For example, MOI of 100 was used in these studies. Higher MOIs may give better control by allowing greater diffusion of phages within the soil. Bacteriophages can diffuse up to 10 cm in porous soils and can persist at relatively stable concentrations for several weeks (Assadian et al. 2005). Lower effective MOIs may be achieved with phages that are difficult to amplify but nevertheless give promising biocontrol results in laboratory conditions. Testing biocontrol efficacy for crops in different soils would determine the overall applicability of these agents. Different soils also generate a new set of problems to be addressed, including the effects of various microbial populations on bacteriophage propagation and survival and sorption of applied phages to particulate matter (Chattopadhyay et al. 2002).

The Ravensdale et al. (2007) study gives a glimpse of the broad spectrum of factors that should be considered when developing a new bacteriophage-based biocontrol agent for bacterial diseases of greenhouse and, by extension, field crops. One must test all potential factors such as preplant treatment, fertilizer, water source, or surfactants that may come in contact with the agent. Soil type, delivery vehicle, multiplicity of infection, and timing of application should also be considered for optimal results. Despite this complexity, however, useable biocontrol agents may be realized even at intermediate developmental stages.

## 8.3 Phages and Rhizobacteria

Soil and plant-associated phages have been reviewed before with different focuses each time. Brüssow and Kutter (2005a) stated that the work is in its infancy compared with phage research in marine environments. They see the plant

rhizosphere as an important area of study for bacteriophages in the soil environment especially regarding bacterial populations and gene transfer. The review by Kimura et al. (2008) is the most up-to-date on strictly soil-associated phages and provides a good ecological summary with references of all that is currently known about phages in the soil environment. The review by Weinbauer (2004) is an all encompassing ecological view of prokaryotic phages and presents a detailed picture of phage evolution, their interactions between one another and their bacterial hosts, and their potentially huge influence on food webs. Three other reviews that should be mentioned in relation to soil-associated bacteriophages are the reviews by Marsh and Wellington (1994) on phage–host interactions in soil, the review on plant interactions by Gill and Abedon (2003) and the recent review on plant disease control by Jones et al. (2007).

### 8.3.1 Effect of Soil on Phages

The soil has a profound effect on the microflora that lives in it and indirectly on the phages that parasitize these organisms. Some important factors that affect viral inactivation and infectivity are temperature, pH, clay type, organic matter, heavy metals, acid pollutants, aerobicity, ionic strength (Assadian et al. 2005), and moisture content (Kimura et al. 2008). In general, lower temperatures resulted in longer periods of survival, longer latent periods, and reduced burst size of bacteriophages. Phages were not found in soils with a pH value lower than 6.1 despite the presence of potentially susceptible acidophilic hosts in these soils (Sykes et al. 1981). This could be because they are hydrophilic and their adsorption depends on the electrostatic properties of the soil surface which largely depends on pH. A variety of factors influence the binding of phages to soil particles. Kimura et al. (2008) list them as (1) type of clay minerals; (2) cation exchange capacity of the soil; (3) soil-associated and dissolved organic matter; (4) soil pH; (5) ionic strength and its constituent; and (6) type of phage. These parameters interact in negatively charged phages with soils having both negatively and positively charged sites. The net result depends on soil pH and ionic strength and its constituents that determine the binding force between phage and soil. This is influenced by organic matter which weakens the electrostatic binding of phages to soils. The ionic environment of the phage affects life cycle and survival. Divalent cations such as magnesium and/or calcium appear to have a positive affect whereas sodium has a variable affect.

Bacteriophages can move horizontally and vertically in soil for several meters even though soils are efficient adsorbers of them. In soil, desiccation and adsorption to soil colloids could be major reasons for phage destruction. On the other hand, some phages adsorb to colloids together with their hosts allowing the phage to find its host (Weinbauer 2004). An experiment in which host cells and a particular phage were introduced into the soil showed that host cells were lysed in the presence of added nutrients, and that the phage was more abundant in fertilized soils (Smit et al. 1996). Encapsulation of the host cells in alginate beads inhibited lysis by the phage

in soil. The lysogenic lifestyle is a distinct advantage for bacteriophages in soil where long periods of host inactivity can be survived by residence within host populations (Marsh and Wellington 1994).

### 8.3.2 Phages in the Rhizosphere

The rhizosphere is that portion of the soil which is subject to the influence of the plant root system and supports greater microbial activity than soil more distant from roots (Katznelson et al. 1948). It is a unique zone exerting a powerful stimulation on soil microorganisms which varies with type, variety, age, and vigor of the plant and the type, treatment, and moisture content of the soil in which it grows. An important factor in the study of rhizosphere microorganisms is the rhizosphere:soil (R:S) ratio or the number of organisms in the rhizosphere soil divided by the number in the soil at a distance from the root. This ratio is of fundamental importance for evaluating the influence of soil type, treatment, and other factors on the root surface microflora (Katznelson et al. 1948). Phage can attack bacteria directly associated with the rhizosphere (Gracia-Garza et al. 2004). Phage ecology in the rhizosphere is thought to approximate solid-phase growth in laboratory media to some extent. It is speculated that if the soil is not disturbed, bacterial microcolonies within the rhizosphere display periods of boom or bust with regard to phage attack that is influenced by microcolony size which increases the likelihood of phage-microcolony encounter. Infection of one bacterium within a localized bacterial clone could result in the destruction of part or all of a genetically homogenous bacterial microcolony. However, bacteria have several ways to thwart such an attack such as variation in life cycle, motility, and sequestration away such as infection of a root nodule.

The challenge of using phages to control plant pathogens in the rhizosphere has been summarized by Jones et al. (2007) (Hagens and Offerhaus 2008). They listed several factors that can hinder success of disease control in the rhizosphere. The rate of diffusion through soil is low and changes as a function of available free water. Phages can become trapped in biofilms, adsorbed to clay particles, and inactivated by low soil pH. For these reasons, only a low number of viable phages are available to lyse target bacteria and a high population is needed to insure bacterial lysis. In soils, there are "hot spots" for microorganisms where they proliferate very actively (Kimura et al. 2008). These are the habitats around plant roots or the rhizosphere where the lytic life may be favorable to phages because the host turnover rate is short enough. The number of host cells required for bacteriophages to increase has been studied in natural ecosystems. Bacteriophages required a host cell population of at least 100–1,000 per g of soil in order to multiply (Germida 1986). Wiggins and Alexander (1985) found that the minimum density of a strain of *Bacillus subtilis* to increase the number of a specific bacteriophage was $3 \times 10^4$ CFU/ml. They concluded that phages do not affect the number of bacteria in environments where the density of the host species is below $10^4$ CFU/ml. Research on the soil rhizosphere in specific crops has been conducted in barley, wheat, and sugar beets. The earliest

research was on the rhizosphere soil of sugarbeets by Stephens et al. (1987). They found that a key factor in decline of a beneficial fluorescent pseudomonas strain were bacteriophages and phages antagonistic toward *Pseudomonas* strain B2/6 that was present in 43% of the soils from the major sugarbeet growing regions of Ireland. Five bacteriophages infecting *Pseudomonas fluorescens* and *P. putida* were isolated from barley rhizosphere soil (Campbell et al. 1995). Four of the phages belonged to the Myoviridae family with large isometrical heads on contractile tails and had complex protein and DNA profiles. The ecological importance of these phages could be their slow multiplication rates suggesting a possible mechanism of balanced phage–host coexistance in the rhizosphere. Slow multiplication might reflect complexity in formation of large bacteriophages. Free *Pseudomonas* spp. bacterial hosts in the rhizosphere of wheat were only slightly affected by the addition of bacteriophages, while cells escaping from alginate beads were effectively lysed (Blom and Brown 1999). It is hypothesized that the cells escaping from beads might be in a more active metabolic state allowing phage infection to occur. Phages could potentially infect cells from beads preventing them from colonizing wheat roots.

### *8.3.3 Effect of Phages on Root Nodulation*

Root-nodule bacteria of leguminous plants are classified into two genera: *Rhizobium* and *Bradyrhizobium*. Inoculation of soybean with *B. japonicum* is often unsuccessful because the inoculum strains do not nodulate soybeans in the presence of indigenous strains. Studies have shown that it is possible to reduce nodulation with indigenous strains by amending the soil with a bacteriophage specific for the indigenous strain. Nodulation was increased from 48 to 82% by coating the seed with a phage and *B. japonicum* (Basit et al. 1992). Therefore, the elimination of a single strain of rhizobia from soil enhanced nodule occupancy by the inoculum strain in this case. Unfortunately soils often contain numerous strains of rhizobia, so phage coating of seed only has limited value. Nitrogen fixation by bacteria is influenced by phage infection. KIeczkowska (1957) found that as long as the phage is present, phage-resistant mutants are also present that may be more effective at nitrogen fixation. Novikova et al. (1993) provided evidence using phages specific to *R. loti* that *Rhizobium* strains nodulating *Astragalus*, *Hedysarum*, *Glycyrrhiza*, and *Ononis* plant species are related to each other.

### *8.3.4 Effect of Phages on Yield and Disease Control*

*Pseudomonas fluorescens*, a plant growth promoting rhizobacterium (PGPR), is optimally infected by bacteriophages at 26°C (Sillankorva et al. 2006). The prevalence of bacteriophages in PGPR bacteria was investigated in four bacterial species belonging to the genus *Azospirillum* (Harrison et al. 1987). The study showed that

there were many phages present because 11 strains out of 24 released phage particles. Moreover, each type of bacteriophage seemed to be associated with a specific bacterial species because only "big" phages were found for *A. brasilense* and "small" phages for *A. lipoferum* strains. A successful use of PGPR bacteria resistant to phage was given by Suslow (1986) with his patented technique in which yield of root crops such as potatoes, sugar beets, and radishes are increased. In this process, bacteriophage-resistant strains are applied to seeds or root pieces at concentrations of $10^5$ to $10^9$ cells/ml in an acceptable carrier medium. The technique is not without problems because the soil presents several obstacles to the successful use of bacteriophages-resistant strains. The use of phages for disease control is an expanding area of plant protection but a major problem has been the development of bacterial strains resistant to the phage. A patented process was developed to prevent occurrence of phage-resistant mutants (Hagens and Offerhaus 2008). Mixtures of mutant phages are prepared that are able to lyse bacterial strains that are resistant to the parent phage, while still capable of lysing the wild-type bacterium. This gives them an extended host compared with the parent strain and has been shown to be an effective strategy for phage application and disease control.

## 8.4 Lysogeny and Soil–Phage Interaction

Lysogenic replication by temperate bacteriophages involves the suppression of lytic functions by down-regulating specific gene expression to establish a quiescent state inside the host (Campbell 2006). The bacteriophage genome (Canchaya et al. 2004) is stably maintained as an integrated part of the bacterial chromosome or as an autonomous extrachromosomal element. Prophage replication is coordinated with host-genome replication and is present in the progeny of the lysogenic parent bacterium. It is latent and so its presence does not promote cell death or the production of bacteriophage virions. Reversion to the lytic functions occurs at a certain frequency in growing lysogenic populations, although prophage induction is usually caused by environmental signals that cause physiological stress to the host cell. The stress causes loss of expression of the repressor protein(s), subsequent reinstatement of lytic functions, and release of bacteriophage progeny (Campbell 2006).

### *8.4.1 Lysogeny as a Bacteriophage Survival Mechanism*

While little is known about the factors leading to the establishment of lysogeny in nature, it is generally believed that lysogeny provides a refuge for temperate bacteriophages when conditions are unfavorable for robust replication or when host abundance is low (Marsh and Wellington 1994; Weinbauer 2004). A study of the interactions between *Bacillus subtilis* and its bacteriophages in soil ecosystems is consistent with this idea that lysogeny increases bacteriophage survival

(Pantastico-Caldas et al. 1992). At equilibrium, both temperate and virulent bacteriophages were much less abundant than the bacterial host. The temperate bacteriophage did not reduce the equilibrium host titre while the virulent bacteriophage reduced the titre tenfold as compared with soil lacking phage. The authors suggested that the dynamics of this system was the result of the acidic soil, which caused a rapid and permanent inactivation of free bacteriophages. This inactivation drives selection for temperate bacteriophages capable of forming a lysogenic association. Pantastico-Caldas et al. (1992) suggest that a temperate life cycle would be commonplace in harsh soil environments. Another study of soil ecosystems also has shown correlations between host-cell density, nutrient availability, and the frequency of temperate bacteriophages (Ashelford et al. 1999). The authors determined that in a sugar beet rhizosphere a high level of *Serratia* temperate bacteriophages occurred during periods of high host-cell density and elevated metabolic activity early in the growing season. Virion titres were much lower when cell densities were low due to nutrient depletion late in the growing season. These data suggest that lytic replication by temperate bacteriophages occurs during times of rich resources in the rhizosphere microbial community, and lysogeny is favored when host growth is limited.

When considering bacteriophage as BCAs, one question to be addressed concerns their artificial predominance in a particular soil microenvironment. In virulent bacteriophage populations, the only route for cell-to-cell transmission of genetic material is through horizontal gene transfer (HGT) by generalized or abortive transduction. Lysogenic populations, through lysogeny, are capable of cell-to-cell transmission by various forms of specialized transduction where host genes are excised along with the prophage and carried to another host. Furthermore, lysogenic infections can result in lysogenic conversion of the host where the expression of novel genes on the bacteriophage genome alters the host phenotype by the addition or loss of various characteristics (Herron 2004; Brüssow and Kutter 2005b). This phenomenon can provide the host with new phenotypic characteristics conferring pathogenicity or enhanced virulence addition of toxins.

### *8.4.2 Phage Gene Transfer in Soil*

There are two mechanisms by which bacteriophages can mediate bacterial gene transfer, phage conversion, and transduction. Phage conversion occurs when the phenotype of the host changes due to a gene within the genome of a temperate phage and has been the most studied aspect of phage-mediated gene transfer (Herron 2004). Transduction is the phage-mediated gene transfer between a donor and a recipient host cell followed by expression of the genetic traits in the progeny of the recipient (Weinbauer 2004). Only a small fraction of generalized transducing bacteriophages have been characterized presumably because most are not culturable. Sander and Schmieger (2001) developed a host-independent method

to detect these phages, and the method is being used to estimate the contribution of generalized transduction to HGT.

Generalized transduction was observed for a number of phages among strains of *R. meliloti* and it was thought that rhizobiophages capable of specialized transduction could be useful for genetic studies in *B. japonicum* (Abebe et al. 1992). Integration of phage V into the genome of a phage-resistant isolate was accompanied by the inability of that isolate to nodulate soybean plants. The fact that the phage V integrated into the genome of some strains offers hope for the development of a *B. japonicum*-specific transducing system that would greatly facilitate genetic studies. *R. leguminosarum* bacteriophages were isolated from a field where survival of a genetically modified host had been monitored for several years (Mendum et al. 2001). The authors found evidence of infrequent generalized transduction of a plasmid-located gene for neomycin resistance. It is thought that a small proportion of phage particles enclosed fragments of the host genome, although the maximum length of host DNA that can be packaged is not known. The presence of sequences from both virulent and temperate phage in indigenous bacteria indicates that phage–bacteria interactions occur in soil. The authors believe that where phage and susceptible bacteria coincide, such as in the rhizosphere, infection will occur to make gene transduction possible. The virulent phages could provide a reservoir of bacterial genes in conditions where the host might not survive. Polymerase-chain reaction (PCR) studies with phage-specific primers show promise for future studies on the ecology of phages in soil.

## 8.5 Detection of Phages in Soil Systems

Direct observation of phage populations is conducted for many different purposes. Metagenomic studies require the isolation of large amounts of genomic phage DNA, without regard to individual viral particles. Ecological studies often involve the recovery of infectious particles and can be either qualitative (presence/absence) or quantitative (O'Brien and Lindow 1989). A growing interest in phages as biopesticides has increased the need for tools that allow investigators to monitor the population dynamics of their therapeutically applied phages over the course of treatment and then to trace the environmental fate of those phages. Ecological and biopesticide studies are increasingly looking toward molecular genetic methods of phage enumeration, but these will not replace the direct recovery of viable virions in all situations.

The great diversity of soil types, and the wide range of physical and chemical properties observed in even one soil type, makes it difficult to establish one, or even a few, standard methods for detecting phages in soil systems. This is particularly true if quantitative detection is desired, since accurate enumeration is so easily inhibited by soil chemistry and by direct interactions of phages with soil particles and other components of the soil environment. The following discussion considers some of the challenges and uses of various approaches to detecting and enumerating

phage communities in soil. These topics are mostly considered in the context of phage biopesticides, but are equally applicable to studies of general soil ecology and of viral persistence and transport in wastewater- and biosolids-amended soils.

## *8.5.1 Isolation of Phage Particles*

### 8.5.1.1 Enrichment Methods

Whole phages can be isolated from soil using either enrichment techniques or direct recovery of existing virions. Enrichment methods rely on the basic principle that phages will replicate if they have access to a susceptible host in a suitable environment. If even one viable phage is present in a sample, it should be detectable in far greater numbers following enrichment on an appropriate bacterial host culture. In reality, the efficiency of enrichment can be greatly affected by the enrichment conditions. Infective phages may be temporarily unavailable to host bacteria as a result of interactions with charged substances; the nutritional status of the host cell can alter expression of the cell surface molecules that are required for phage adsorption; the energetic state of the host cell can affect the ability of the phage genome to replicate within an infected host; and factors such as pH, temperature, and the availability of cofactors such as $Ca^{2+}$ or $Mg^{2+}$ can also influence the efficiency of phage replication and thus which phages are detected (Guttman et al. 2005). The choice of host culture will also influence which phages are enriched. An environmental soil sample can be reasonably expected to contain multiple phage types. Obviously, the choice of a host species influences which phages are recovered, but within a host species, the number and type of strains used will favor the preferential enrichment and recovery of some phages over others. For example, the use of multiple host strains vs. a single host strain preferentially enriches phages having broader host ranges (Jensen et al. 1998; Gill et al. 2003; Lehman 2007).

*E. amylovora* phages are easily isolated from soil beneath rosaceous hosts exhibiting signs of an active fire blight infection (Erskine 1973; Schnabel and Jones 2001; Gill et al. 2003) but are not normally recovered from soil beneath healthy trees that have not been treated with phages, even when using enrichment techniques (Ritchie and Klos 1977; Ritchie 1978; Ritchie and Klos 1979; A. M. Svircev, unpublished data). Similarly, free *Bacillus* phages were recovered from environmental soil samples in very low numbers until the population of endogenous bacterial hosts was increased by adding rich media (Tan and Reanney 1976). In both cases, the presence of actively growing host cells and high concentrations of multiple phage strains were recovered. If the potential for future population growth of a therapeutically applied phages is the main consideration, then enrichment methods also have the benefit of detecting phages that are present in the soil environment within lysogenic, pseudolysogenic, or poorly replicating cells such as those in stationary phase or biofilms [for a comprehensive discussion of these particular phage–host relationships see Abedon (2008)].

Basic enrichment methods have been well described (Adams 1959; Carlson 2005; Van Twest and Kropinski 2009).Van Twest and Kropinski (2009) report substantially better recovery from soil samples that are allowed to dry before adding enrichment media. Traditionally, enrichment has been limited to phage–host systems for which, at a minimum, the host can be isolated and grown in pure culture. This is an important restriction, since it has been estimated that as few as 1–5% of endogenous soil bacteria are currently culturable (Torsvik et al. 1990).

#### 8.5.1.2 Elution Methods

Recovery of phages from soil is hindered by adsorption of phages to soil particles. Adsorption is mediated by pH-dependent electrostatic interactions (Burge and Enkiri 1978a, b; Taylor et al. 1981; Dowd et al. 1998) and seems to be greater for phages with longer tails (Williamson et al. 2003; Ashelford et al. 2003). For a detailed review of the principles of viral adsorption to soil, see Kimura et al. (2008).

Several detailed studies of elution techniques have been published, but tend to lack the type of truly systematic approach that is needed to ascertain which particular factors influenced the relative success of a particular method. In some studies, different sample handling procedures have been used with each elution medium (Hu 1998), or each medium has been used at a different pH (Williamson et al. 2003), making it difficult to determine which factor is responsible for differential recovery efficacies. It should also be noted that some studies report pH of the elution medium prior to use, while others report the pH of the soil–eluent mixture. Perhaps the most useful single study of phage elution was conducted by Lanning and Williams (1982). They tested recovery of actinophages from multiple soil types using media at a fixed pH, and then examined the effect of pH on recovery using the best-performing medium. Based largely on the results of Lanning and Williams (1982), we have compared the elution of *E. amylovora* phages from sandy orchard soil using five media, all at pH 8.0: nutrient broth, nutrient broth with 0.1% egg albumin, 250 mM glycine, 10 mM tetrasodium pyrophosphate, and 10 mM potassium phosphate buffer amended with sodium chloride and magnesium chloride (Lehman 2007). In this case, no significant difference in phage elution efficiency was observed. Proteinaceous substances and egg albumin in particular have been found to aid the release of phages from soil in some cases (Lanning and Williams 1982) but not all (Lehman 2007). Since adsorption is mediated by pH-dependent electrostatic processes (Taylor et al. 1981; Dowd et al. 1998), it is possible that there is no detectable difference between elution media at a near-optimal pH, but that at nonoptimal pH levels, the specific types of charged species in each medium are differentially successful in disrupting the interactions between phages and soil particles.

Apart from the chemical factors affecting phage recovery from soil, the physical treatment of the sample also appears to be important. Actinophage recovery by Lanning and Williams (1982) was generally more efficient when samples were agitated using a reciprocal flask shaker vs. an orbital shaker or magnetic stirrer.

Sonication is fairly common practice for viral recovery from marine and freshwater sediments (Danovaro et al. 2001; Leroy et al. 2008) and does not appear to reduce the viability of myoviruses (Fu et al. 2009). Guzmán et al. (2007) compared the effects of homogenization procedures, centrifugation, and filtration on recovery of coliphages and F-specific RNA phages. Centrifugation and filtration reduced bacterial contamination without reducing phage viable counts, but unlike in the Lanning and Williams (1982) study, no one method proved superior. Despite the variation in methodology among all of these studies, three common themes emerge: the efficacy of any given elution technique varies with soil type, is lower for phages with long tails, and tends to be better when using eluents at slightly basic pHs.

### *8.5.2 Direct Detection by Microscopy*

Transmission electron microscopy (TEM) is commonly used to directly count phages in soils (Borsheim et al. 1990; Weinbauer and Suttle 1997; Ashelford et al. 2003; Williamson et al. 2005; Yamada et al. 2007; Srinivasiah et al. 2008; Swanson et al. 2009). The reported populations of virus-like particles (VLPs) were at least 350-fold higher than those estimated from viable plaque counts (Ashelford et al. 2003). However, the ecological impact of these extra VLPs is questionable since some of these samples did not yield any viable phages even after enrichment on multiple hosts. Epifluorescence microscopy (EFM) may be employed to assess phage abundance in soils. Danovaro et al. (2001) successfully applied EFM to study viral abundance in marine sediments.

EFM is more sensitive than TEM when applied to marine water samples (Hennes and Suttle 1995; Weinbauer and Suttle 1997), but may be less specific since the lower magnification reveals approximate dimensions rather than detailed particle morphology (Borsheim et al. 1990; Brussaard et al. 1996). The use of high concentrations of SYBR Green may improve threshold-based differentiation of viruses from extremely small bacteria (Danovaro et al. 2001). The sensitivity of microscopic detection methods is also impacted by the same factors that limit phage elution from soil, since viruses attached to soil particles cannot be resolved, which necessitates a preparatory phage elution step. Using a combination of techniques, Swanson et al. (2009) estimated 0.87–1.1 $\times$ $10^9$ virions/g dry agricultural soil.

### *8.5.3 Direct Detection of Biopesticides by Molecular Methods*

In the case of phage-based biopesticides, there is a clear need to monitor the populations of at least one phage and one host over time, and often there will be multiple types or strains of each. The population dynamics of the phages, their target, and any alternative bacterial hosts can confirm that disease control is attributable to phage action or can suggest reasons for treatment failure (Lehman 2007). Even if the therapeutic outcome of interest occurs on aerial plant tissue, the

surrounding soil should be expected to act as both a sink for applied phages (due to inactivation and adsorption) and as a protected reservoir (since not all phages washed down into soil will be inactivated or otherwise lost). In following the fate of the phage biopesticide, there may sometimes only be a need to know whether a phage persists, in which case enrichment and subsequent identification of a particular phage type is sufficient. However, there is often interest in the more detailed ecology of surviving phages. Two such cases have been presented in this chapter: when phage biopesticides are being applied to subsurface plant tissues such as tubers, making biopesticide efficacy directly dependent on phage interactions with the rhizosphere; and when a nonpathogen that also supports replication of the therapeutic phages is present in abundance, as in the case of *P. agglomerans* and phages that target *E. amylovora*. Quantitative tracking of phage biopesticides may also become a regulatory issue that must be addressed to register a phage-based biopesticide.

This type of tracking is a challenge for culture-based detection methods, even if only two or three different phages are being used to target a single host species since true therapeutic cocktails are likely to contain 4–6 phages in the mixture. (Markoishvili et al. 2002; Guenther et al. 2009). Plaque morphologies often cannot be used to reliably distinguish among phage types, and there is no phage plating equivalent to selective bacterial culture media. Rather than attempting to separate phage types within a mixture, it is far easier to apply DNA detection methods such as PCR. Endpoint PCR can be used for detection, real-time PCR for quantification, and reverse transcriptase steps can be incorporated into either if the target is an RNA virus or a transcription product.

When a phage enrichment step is not desirable, PCR is performed after community DNA extraction or the elution of phage particles. However, successful extraction or elution does not necessarily equate to successful detection and quantification, since soil contains many substances that are known to inhibit PCR, and that tend to be extracted or washed from the soil along with the recovered phages. As little as 10 ng humic acid can inhibit a conventional, endpoint PCR reaction (Tsai and Olsen 1992). It has been postulated that phenolic moities in humic substances react with, and covalently bind to, DNA and protein, preventing the necessary interactions between the polymerase and the target DNA, or between primers and target DNA (Young et al. 1993). Humic acids may also interfere with the fluorescence processes upon which real-time PCR depends by quenching fluorescence of SYBR Green, Hoescht 33258, and PicoGreen complexed to DNA (Bachoon et al. 2001; Zipper et al. 2003).

A variety of metal ions can inhibit PCR, apparently by interfering with the binding and activity of the polymerase enzyme. Calcium ions in milk can interfere with PCR amplification (Bickley et al. 1996). Iron and other heavy metals are generally present in soil, whether in high levels as pollutants or levels appropriate for micronutrition of plants, and are known PCR inhibitors (Wilson 1997; Ogram 1998; Hao et al. 2002). Any substances that sequester $Mg^{2+}$ will also inhibit the DNA polymerase enzyme, which requires the ion as a cofactor and is sensitive to changes in its concentration (Satsangi et al. 1994; Wilson 1997). Polyamines

(Ahokas and Erkkila 1993), phenol (Katcher and Schwartz 1994), and plant polysaccharides (Demeke and Adams 1992) can inhibit amplification by directly affecting the DNA polymerase. Foulds et al. (2002) were able to remove PCR inhibitors by washing *E. coli* cells collected from environmental water samples with EDTA, a metal ion chelator, prior to DNA extraction. Extensive work has also been done to develop methods of removing these inhibitors in the course of extracting total community DNA from soil or soil eluates (Zhou et al. 1996; Sjöstedt et al. 1997; Miller et al. 1999; Desai and Madamwar 2006).

There are many studies describing DNA extraction techniques that can be used, with varying success, to remove soil-derived PCR inhibitors. The Tth and Tfl polymerases have been shown to be much more resistant than Taq polymerase to inhibitors that directly affect the DNA polymerase (Katcher and Schwartz 1994; Wiedbrauk et al. 1995) and may offer some improvement in DNA amplification from soil extracts. The SDS-based method of Zhou et al. (1996) was used successfully with several soil types. The Ultraclean Soil DNA Isolation kit from MO BIO Laboratories has been used to extract total bacterial DNA for real-time PCR detection of *E. coli* O157:H7 (Ibekwe et al. 2002), though Desai and Madamwar (2006) describe a protocol that removes metallic and organic inhibitors more efficiently.

Few studies bother to mention attempts to amplify microbial DNA directly from soil eluate, and those that do, report consistent failure (Sjöstedt et al. 1997; Lehman 2007). Some commonly used eluents that help dissociate phages from soil particles are not optimal for direct PCR, and any that may help chelate soil-derived PCR inhibitors will also tend to chelate cofactors required for PCR. For example, tetrasodium pyrophosphate has been used by soil scientists to dissolve organic matter and extract metals bound to humic substances (McKeague et al. 1971; Manninen et al. 1996), but it reduces the sensitivity of real-time PCR (Lehman 2007), presumably by sequestering $Mg^{2+}$ ions. The addition of small amounts of EDTA to soil eluate, followed by ultrafiltration with buffer replacement, may remove most soil-derived PCR inhibitors, but the overall detection efficiency is still dominated by the elution process (Lehman 2007).

## 8.6 Summary

Phages can have strong influences on the performance of microbial food webs, microbe diversity, and biogeochemical cycles in various environments, although many specific details on the mechanisms of these influences are lacking (Weinbauer 2004). We are at the verge of understanding the influence of phages on links between ecosystem stability, functioning, and diversity. With respect to phages effects in soils, circumstantial evidence suggests that these viruses play important roles in biogeochemical nutrient cycles and as genomic reservoirs similar to those in the sea (Kimura et al. 2008). Phages may regulate host populations by lysis, but this regulation is probably extremely limited in soil where populations have periods of inactivity (Marsh and Wellington 1994). Changes in environmental parameters

such as moisture content, temperature, pH, and aerobicity frequently fluctuate because of weather and field management. These changes have a direct impact on microbial activities and may induce lysogenic changes in soil bacteria. Lysogeny can influence host bacteria in two ways, by permitting survival and in rare circumstances in soils mediating HGT. Lysogeny represents a compromise between hosts and phage where both parties are granted advantages in terms of improved survival capabilities in return for reduced abundance.

In regard to applied biocontrol of soil-borne plant diseases, bacteriophages have great potential because they are widely present, are self-replicating, can be targeted against specific bacterial receptors, are nontoxic to eukaryotes, and are specific to bacteria (Hagens and Offerhaus 2008).

Our ability to study phage ecology in soil systems generally lags behind our ability to study phages in water systems. For phage-based biopesticides, the principle value of good phage detection methods is to permit a quantitative assessment of phage activity and to explain why a treatment is successful or not. This is especially important when one considers how many of the substances that we use to fertilize agricultural soils are known to impact phage survival and activity (Ravensdale et al. 2007; Kimura et al. 2008; Bouzari et al. 2008). DNA-based methods of phage detection and quantification are generally the most sensitive. These require some specific sample processing to remove soil-derived enzymatic and fluorescence inhibitors, but with elution techniques yielding only 40–60% recovery in most cases (Lanning and Williams 1982; Danovaro et al. 2001; Lehman 2007), only the most abundant phage types will be reliably detected by elution-dependent methods. This makes DNA extraction directly from soil, followed by PCR-based quantification, the most effective technique currently available for quantitative studies of soil phage communities.

To date, limited information is available on the large-scale impact of bacteriophages applied as biopesticides on the soil ecosystem. While many phages that are used as biopestides have originated from soil (Svircev et al. 2002b; Gill et al. 2003; Goyer 2005; Svircev et al. 2006; Jones et al. 2007; Ravensdale et al. 2007), determining the environmental fate of the phages has been restricted to laboratory and greenhouse. Future developments in molecular detection technologies may permit study of biopesticide-related phages in agricultural ecosystems and should lead to greater elucidation of the impact and importance of phages on bacterial activities in soils in general.

## References

Abebe HM, Sadowsky MJ, Kinkle BK, Schmidt EL (1992) Lysogeny in *Bradyrhizobium japonicum* and its effect on soybean nodulation. Appl Environ Microbiol 58:3360–3366

Abedon ST (2008) Phage, ecology, and evolution. In: Abedon ST (ed) Bacteriophage ecology. Advances in molecular and cellular microbiology, vol 15. Cambridge University Press, Cambridge UK, pp 1–28

Adams MH (1959) Bacteriophages. Interscience Publishers, New York

Ahokas H, Erkkila MJ (1993) Interference of PCR amplification by the polyamines, spermine and spermidine. PCR Methods Appl 3:65–68

Andrews JH, Harris RF (2000) The ecology and biogeography of microorganisms on plant surfaces. Ann Rev Phytopathol 38:145–180

Ashelford KE, Day MJ, Bailey MJ, Lilley AK, Fry JC (1999) In situ population dynamics of bacterial viruses in a terrestrial environment. Appl Environ Microbiol 65:169–174

Ashelford KE, Day MJ, Fry JC (2003) Elavated abundance of bacteriophage infecting bacteria in soil. Appl Environ Microbiol 69:285–289

Assadian NW, Giovanni GD, Enciso J, Iglesias J, Lindemann W (2005) The transport of water-borne solutes and bacteriophage in soil subirrigated with a wastewater blend. Agric Ecosyst Environ 111:279–291

Bachoon DS, Otero E, Hodson RE (2001) Effects of humic substances on fluorometric DNA quantification and DNA hybridization. J Microbiol Methods 47:73–82

Balogh B, Canteros BI, Stall RE, Jones JB (2008) Control of citrus canker and citrus bacterial spot with bacteriophages. Plant Dis 92:1048–1052

Balogh B, Jones JB, Momol MT, Olson SM (2005) Persistence of bacteriophages as biocontrol agents in the tomato canopy. Acta Hortic 695:299–302

Balogh B, Jones JB, Momol MT, Olson SM, Obradovic A, King P, Jackson LE (2003) Improved efficacy of newly formulated bacteriophages for management of bacterial spot on tomato. Plant Dis 87:949–954

Basit HA, Angle JS, Salem S, Gewaily EM (1992) Phage coating of soybean seed reduces nodulation by indigenous soil bradyrhizobia. Can J Microbiol 38:1264–1269

Berg G, Sanjaghsaz H, Wangwongwatana S (1989) Potentiation of the virucidal effectiveness of free chlorine by substances in drinking water. Appl Environ Microbiol 55:390–393

Bickley J, Short SK, McDowell DG, Parkes HC (1996) Polymerase chain reaction (PCR) detection of *Listeria monocytogenes* in diluted milk and reversal of PCR inhibition caused by calcium ions. Lett Appl Microbiol 22:153–158

Blom TJ, Brown W (1999) Preplant copper-based compounds reduce *Erwinia* soft rot on calla lilies. HortTechnol 9:56–59

Borsheim KY, Bratbak G, Heldal M (1990) Enumeration and biomass estimation of planktonic bacteria and viruses by transmission electron microscopy. Appl Environ Microbiol 56:352–356

Bouzari M, Emtiazi G, Mehdipour-Moghaddam MJ (2008) The effects of heavy metals and chelating agents on phage development and enumeration of *Rhizobium* by phage counting in different soils. Am Eurasian J Agric Environ Sci 3:420–424

Brussaard CPD, Kempers RS, Kop A, Riegman R, Heldal M (1996) Virus-like particles in a summer bloom of *Emiliana huleyi* in the North Sea. Aquat Microb Ecol 10:105–113

Brüssow H, Kutter E (2005a) Phage ecology. In: Kutter E, Sulakvelidze A (eds) Bacteriophages: biology and applications. CRC Press, Boca Raton, pp 129–163

Brüssow H, Kutter E (2005b) Genomics and evolution of tailed phages. In: Kutter E, Sulakvelidze A (eds) Bacteriophages: biology and applications. CRC Press, Boca Raton, pp 91–128

Burge WD, Enkiri NK (1978a) Virus adsorption by five soils. J Environ Qual 7:73–76

Burge WD, Enkiri NK (1978b) Adsorption kinetics of bacteriophage P29103000X-174 on soil. J Environ Qual 7:536–541

Campbell A (2006) General aspects of lysogeny. In: Calendar R (ed) The bacteriophages, 2nd edn. Oxford University Press, New York

Campbell JIA, Albrechtsen M, Sorensen J (1995) Large *Pseudomonas* phages isolated from barley rhizosphere. FEMS Microbiol Ecol 18:63–74

Canchaya C, Fournous G, Brüssow H (2004) The impact of prophages on bacterial chromosomes. Mol Microbiol 53:9–18

Carlson K (2005) Working with bacteriophages: common techniques and methodological approaches. In: Kutter E, Sulakvelidze A (eds) Bacteriophages: biology and applications. CRC Press, Boca Raton, pp 437–487

Chattopadhyay D, Chattopadhyay S, Lyon WG, Wilson JT (2002) Effect of surfactants on the survival and sorption of viruses. Environ Sci Technol 36:4017–4024

Danovaro R, Dell'Anno A, Trucco A, Serresi M, Vanucci S (2001) Determination of virus abundance in marine sediments. Appl Environ Microbiol 67:1384–1387

Demeke T, Adams R (1992) The effects of plant polysaccharides and buffer additives on PCR. BioTechniques 12:333–334

Desai C, Madamwar D (2006) Extraction of inhibitor-free metagenomic DNA from polluted sediments, compatible with molecular diversity analysis using adsorption and ion-exchange treatments. Bioresour Technol 98:761–768

Dowd SE, Pillai SD, SookYun W, Corapcioglu MY (1998) Delineating the specific influence of virus isoelectric point and size on virus adsorption and transport through sandy soils. Appl Environ Microbiol 64:405–410

Erskine JM (1973) Characteristics of *Erwinia amylovora* bacteriophage and its possible role in the epidemiology of fire blight. Can J Microbiol 19:837–845

Flaherty JE, Harbaugh BK, Jones JB, Somodi GC, Jackson LE (2001) H-mutant bacteriophages as a potential biocontrol of bacterial blight of geranium. HortSci 36:98–100

Flaherty JE, Jones JB, Harbaugh BK, Somodi GC, Jackson LE (2000) Control of bacterial spot on tomato in the greenhouse and field with h-mutant bacteriophages. HortSci 35:882–884

Foulds IV, Granacki A, Xiao C, Krull UJ, Castle AJ, Horton PJ (2002) Quantification of microcystin-producing cyanobacteria and *E. coli* in water by 5′-nuclease PCR. J Appl Microbiol 93:825–834

Fu W, Forster T, Meyer O, Curtin JJ, Lehman SM, Donlan RM (2009) Bacteriophage cocktail for the prevention of biofilm formation by *Pseudomonas aeruginosa* on catheters in an in vitro model system. Antimicrob Agents Chemother. doi:10.1128/AAC.00669-09

Germida JJ (1986) Population dynamics of *Azospirillum brasilense* and its bacteriophage in soil. Plant Soil 90:117–128

Gill J, Abedon ST (2003) Bacteriophage ecology and plants. APSnet http://www.apsnet.org/online/feature/phages/ Cited: July 2009

Gill JJ, Svircev AM, Smith R, Castle AJ (2003) Bacteriophages of *Erwinia amylovora*. Appl Environ Microbiol 69:2133–2138

Goyer C (2005) Isolation and characterization of phages Stsc1 and Stsc3 infecting *Streptomyces scabiei* and their potential as biocontrol agents. Can J Plant Pathol 27:210–216

Gracia-Garza JA, Blom TJ, Brown W, Roberts DP, Schneider K, Freisen M, Gombert D (2004) Increased incidence of *Erwinia* soft-rot on calla lilies in the presence of phosphorous. Eur J Plant Pathol 110:293–298

Guenther S, Huwyler D, Richard S, Loessner MJ (2009) Virulent bacteriophage for efficient biocontrol of *Listeria monocytogenes* in ready-to-eat foods. Appl Environ Microbiol 75:93–100

Guzmán C, Jofre J, Blanch AR, Luana F (2007) Development of a feasible method to extract somatic coliphages from sludge, soil and treated biowaste virol Meth 144:41–48

Guttman B, Raya R, Kutter E (2005) Basic phage biology. CRC Press, Boca Raton, FL

Hagens S, Offerhaus ML (2008) Bacteriophages – new weapons for food safety. Food Technol Chicago 62:46

Hao Y, Dick WA, Tuovinen OH (2002) PCR amplification of 16S rDNA sequences in Fe-rich sediment of coal refuse drainage. Biotechnol Lett 24:1049–1053

Harrison MD, Franc DG, Maddox DA, Michaud JE, McCater-Zorner NJ (1987) Presence of *Erwinia carotovora* in surface water in North America. J Appl Bacteriol 62:565–570

Hennes JE, Suttle CA (1995) Direct counts of viruses in natural waters and laboratory cultures by epifluorescence microscopy. Limnol Oceanogr 40:1050–1055

Herron, PR (2004) Phage ecology and genetic exchange in soil. In: Kowalchuk GA, Bruijn, FJD, Head IM, Akkermans ADL and Elsas JDV (eds) Vol. II, Molecular microbial ecology manual, pp 1173–1183

Hildebrand M, Tebbe CC, Geider K (2001) Survival studies with the fire blight pathogen *Erwinia amylovora* in soil and in a soil-inhabiting insect. J Phytopath 149:635–639

Hu TL (1998) A comparison of two methods to recover phages from soil samples. Bioresour Technol 65:167–169

Ibekwe AM, Watt PM, Grieve CM, Sharma VK, Lyons SR (2002) Multiplex fluorogenic real-time PCR for detection and quantification of *Escherichia coli* O157:H7 in dairy wastewater wetlands. Appl Environ Microbiol 68:4853–4862

Iriarte FB, Balogh B, Momol MT, Smith LM, Wilson M, Jones JB (2007) Factors affecting survival of bacteriophage on tomato leaf surfaces. Appl Environ Microbiol 73:1704–1711

Jensen EC, Schrader HS, Rieland B, Thompson TL, Lee KW, Nickerson KW, Kokjohn TA (1998) Prevalence of broad-host-range lytic bacteriophages of *Sphaerotilus natans*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Appl Environ Microbiol 64:575–580

Johnson KB, Stockwell VO (2000) Biological control of fire blight. In: Vanneste JL (ed) Fire blight: the disease and its causative agent, *Erwinia amylovora*. CaB International, Wallingford, Oxon, pp 319–337

Jones JB, Jackson LE, Balogh B, Obradovic A, Iriarte FB, Momol MT (2007) Bacteriophages for plant disease control. Ann Rev Phytopathol 45:245–262

Jones JB, Momol MT, Obradovic A, Balogh B, Olson SM (2005) Bacterial spot management on tomatoes. Vol. 695 Acta Hortic 119–124

Katcher HL, Schwartz I (1994) A distinctive property of *Tth* DNA polymerase: enzymatic amplification in the presence of phenol. BioTechniques 16:84–92

Katznelson H, Lochhead AG, Timonin MI (1948) Soil microorganisms and the rhizosphere. Bot Rev 14:543–587

KIeczkowska J (1957) A study of the distribution and the effects of bacteriophage of root nodule bacteria in soil. Can J Microbiol 3:171–180

Kimura M, Jia Z-J, Nakayama N, Asakawa S (2008) Ecology of viruses in soils: past, present and future perspectives. Soil Sci Plant Nut 54:1–32

Kumar MKP, Khan ANA, Reddy CNL, Basavarajappa MP, Venkataravanappa V, Basha Z (2006) Biological control of bacterial wilt of tomato caused by *Ralstonia solanacearum*, race-1, biovar-III. J Plant Dis Sci 1:176–181

Lang JM, Gent DH, Schwartz HF (2007) Management of *Xanthomonas* leaf blight of onion with bacteriophages and a plant activator. Plant Dis 91:871–878

Lanning S, Williams ST (1982) Methods for the direct isolation and enumeration of actinophages in soil. J Gen Microbiol 128:2063–2071

Lehman SM (2007) Development of a bacteriophage-based biopesticide for fire blight. PhD thesis, Brock University

Leroy M, Prigent MDM, Confalonieri F, Dubow M (2008) Bacteriophage morphotype and genome diversity in Seine River sediment. Freshw Biol 53:1176–1185

Manninen A, Hall GEM, Vaive JE, MacLaurin AI (1996) Analytical aspects of the application of sodium pyrophosphate reagent in the specific extraction of the labile organic component of humus and soils. J Geochem Explor 56:23–36

Markoishvili K, Tsitlanadze G, Katsarava R, Morris JGJ, Sulakvelidze A (2002) A novel sustained-release matrix based on biodegradable polyester amides impregnated with bacteriophages and an antibiotic shows promise in management of infected venous stasis ulcers and other poorly healing wounds. Int J Dermatol 41:453–458

Marsh P, Wellington EMH (1994) Phage–host interactions in soil. FEMS Microbiol Ecol 15:99–107

McKeague JA, Brydon JE, Miles NM (1971) Soil Sci. Soc Am Proc 35:33–38

Mendum TA, Clark IM, Hirsch PR (2001) Characterization of two novel *Rhizobium leguminosarum* bacteriophages from a field release site of genetically-modified rhizobia. Antonie Van Leeuwenhoek 79:189–197

Miller DN, Bryant JE, Madsen EL, Ghiorse WC (1999) Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Appl Environ Microbiol 65:4715–4724

Novikova NI, Pavlova EA, Limeshchenko EV (1993) Phage sensitivity and host range of *Rhizobium* strains isolated from root nodules of temperate legumes. Plant Soil 151:45–53

O'Brien RD, Lindow SE (1989) Effet of plant species and environmental conditions on epiphytic population sizes of *Pseudomonas syringae* and other bacteria. Phytopathology 79:619–627

Obradovic A, Jones JB, Momol MT, Balogh B, Olson SM (2004) Management of tomato bacterial spot in the field by foliar applications of bacteriophages and SAR inducers. Plant Dis 88:736–740

Obradovic A, Jones JB, Momol MT, Olson SM, Jackson LE, Balogh B, Guven K, Iriarte FB (2005) Integration of biological control agents and systemic acquired resistance inducers against bacterial spot on tomato. Plant Dis 89:712–716

Ogram A (1998) Isolation of nucleic acids from environmental samples. In: Burlage RS, Atlas R, Stahl D, Geesey G, Sayler G (eds) Techniques in microbial ecology. Oxford University Press, Oxford UK, pp 273–288

Pantastico-Caldas M, Duncan KE, Istock CA, Bell JA (1992) Population dynamics of bacteriophage and *Bacillus subtilis* in soil. Ecology 73:1888–1902

Pérombelon MCM, Kelmon A (1980) Ecology of the soft rot *Erwinias*. Ann Rev Phytopathol 18:361–387

Ravensdale M, Blom TJ, Gracia Garza JA, Svircev AM, Smith RJ (2007) Bacteriophages and the control of *Erwinia carotovora* subsp. *carotovora*. Can J Plant Pathol 29:121–130

Ritchie DF (1978) Bacteriophages of *Erwinia amyovora*: Their isolation, distribution, characterization, and possible involvement in the etiology and epidemiology of fire blight. PhD thesis, Michigan State University

Ritchie DF, Klos EJ (1977) Isolation of *Erwinia amyolovora* bacteriophage from aerial parts of apple trees. Phytopathology 67:101–104

Ritchie DF, Klos EJ (1979) Some properties of *Erwinia amylovora* bacteriophages. Phytopathology 69:1078–1083

Romeo AM, Christen L, Niles EG, Kosman DJ (2001) Intracellular chelation of iron by bipyridyl inhibits DNA virus replication. J Biol Chem 24:301–308

Saccardi A, Gambin E, Zaccardelli M, Barone G, Mazzucchi U (1993) *Xanthomonas campestris* pv. *pruni* control trials with phage treatments on peach in the orchard. Phytopathol Mediterr 32:206–210

Sagripanti J-L (1992) Metal-based formulations with high microbiocidal activity. Appl Environ Microbiol 58:3157–3162

Sander M, Schmieger H (2001) Method for host-independent detection of generalized transducing bacteriophages in natural habitats. Appl Environ Microbiol 67:1490–1493

Satsangi J, Jewell DP, Welsh K, Bunce M, Bell JI (1994) Effect of heparin on polymerase chain reaction. Lancet 343:1509–1510

Schnabel EL, Fernando WGD, Meyer MP, Jones AL, Jackson LE (1999) Bacteriophage of *Erwinia amylovora* and their potential for biocontrol. Acta Hortic 489:649–653

Schnabel EL, Jones AL (2001) Isolation and characterization of five *Erwinia amylovora* bacteriophages and assessment of phage resistance in strains of *Erwinia amylovora*. Appl Environ Microbiol 67:59–64

Sillankorva S, Oliveira R, Viera MJ, Sutherland I, Azeredo J (2006) *Pseudomonas fluorescens* infection by bacteriophage øS1: the influence of temperature, host growth phase and media. FEMS Microbiol Lett 241:13–20

Sjöstedt A, Eriksson U, Ramisse V, Garrigue H (1997) Detection of *Bacillus anthracis* spores in soil by PCR. FEMS Microbiol Ecol 23:159–168

Smit E, Wolters AC, Lee H, Trevors JT, van Elsas JD (1996) Interactions between a genetically marked *Pseudomonas fluorescens* strain and bacteriophage FR2f in soil: effects of nutrients, alginate encapsulation, and the wheat rhizosphere. Microb Ecol 31:125–140

Srinivasiah S, Bhavsar J, Thapar K, Liles M, Schoenfeld T, Wommack KE (2008) Phages across the biosphere: contrasts of viruses in soil and aquatic environments. Res Microbiol 159:349–357

Stephens PM, O'Sullivan M, Gara F (1987) Effect of bacteriophages on colonization of sugar beet roots by *Pseudomonas* spp. Appl Environ Microbiol 53:1164–1167

Stewart A (2001) Commercial biocontrol – reality or fantasy? Australas. Plant Pathol 30:127–131

Suslow TV (1986) Bacteriophage-resistant plant growth promoting rhizobactria. USA Patent 4584274

Svircev AM, Gill JJ, Sholberg P (2002a) *Erwinia amylovora* (Burrill) Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith, fire blight (*Enterobacteriaceae*). In: Mason PG, Huber JT (eds) Biological control programmes in Canada. CaBI Publishing, Wallingford UK, pp 448–451

Svircev AM, Lehman SM, Kim W-S, Barszcz E, Schneider KE, Castle AJ (2006) Control of the fire blight pathogen with bacteriophages. In: Proceedings of the 1st International Symposium on Biological control of Bacterial Plant Diseases. Zeller W and Ullrich C (eds) Arno Brynda, Berlin Germany, pp 259–261

Svircev AM, Smith R, Gracia-Garza JA, Gill JJ, Schneider K (2002b) Biocontrol of *Erwinia* with bacteriophages. Bull OILB/SROP 25:10, p. 139–142

Swanson MM, Fraser G, Daniell TJ, Torrance L, Gregory PJ, Taliansky M (2009) Viruses in soils: morphological diversity and abundance in the rhizosphere. Ann Appl Biol 155:51–60

Sykes IK, Lanning S, St W (1981) The effect of pH on soil actinophage. Microbiol 122:271–280

Tan JSH, Reanney DC (1976) Interactions between bacteriophages and bacteria in soil. Soil Biol Biochem 8:145–150

Taylor DH, Moore RS, Sturman LS (1981) Influence of pH and electrolyte composition on adsorption of poliovirus by soils and minerals. Appl Environ Microbiol 42:976–984

Torsvik V, Goksr̆yr J, Daae FL (1990) High diversity in DNA of soil bacteria. Appl Environ Microbiol 56:782–787

Tsai YL, Olsen B (1992) Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl Environ Microbiol 58:2292–2295

Vanneste JL (ed) (2000) Fire blight: the disease and its causative agent, *Erwinia amylovora*. CaB International, Wallingford, UK

Van Twest R, Kropinski AM (2009) Bacteriophage enrichment from water and soil. In: Clokie MRJ, Kropinski AM (eds) Bacteriophages, methods and protocols, Vol. 1: Isolation, characterization and interactions. Methods in molecular biology, Walker JM (series ed). Humana Press part of Springer Science + Business Media, Totowa, NJ

Weinbauer MG (2004) Ecology of prokaryotic viruses. FEMS Microbiol Rev 28:127–181

Weinbauer MG, Suttle CA (1997) Comparison of epifluorescence and transmission electron microscopy for counting viruses in natural marine waters. Aquat Microb Ecol 13:225–232

Wiedbrauk DL, Werner JC, Drevon AM (1995) Inhibition of PCR by aqueous and vitreous fluids. J Clin Microbiol 33:2643–2646

Wiggins BA, Alexander M (1985) Minimum bacterial density for bacteriophage replication: implications for significance of bacteriophages in natural ecosystems. Appl Environ Microbiol 49:19–23

Williamson KE, Radosevich M, Wommack KE (2005) Abundance and diversity of viruses in six Delaware soils. Appl Environ Microbiol 71:3119–3125

Williamson KE, Wommack KE, Radosevich M (2003) Sampling natural viral communities from soil for culture-independent analyses. Appl Environ Microbiol 69:6628–6633

Wilson IG (1997) Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 63:3741–3751

Yamada T, Kawasaki T, Nagata S, Fujiwara A, Usami S, Fujie M (2007) New bacteriophages that infect the phytopathogen Ralstonia solanacearum. Microbiology 153:2630–2639

Young CC, Burghoff RL, Keim LG, Minak-Bernero V, Lute JR, Hinton SM (1993) Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. Appl Environ Microbiol 59:1971–1974

Zaccardelli M, Saccardi A, Gambin E, Mazzucchi U (1992) *Xanthomonas campestris* pv. *pruni* bacteriophages on peach trees and their potential use for biological control. Phytopathol mediterr 31:133–140

Zhou J, Bruns MA, Tiedje JM (1996) DNA recovery from soils of diverse composition. Appl Environ Microbiol 62:316–322

Zipper H, Buta C, Lämmle K, Brunner H, Bernhagen J, Vitzthum F (2003) Mechanisms underlying the impact of humic acids on DNA quantification by SYBR Green I and consequences for the analysis of soils and aquatic sediments. Nucleic Acids Res 31:e39