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POTENTIAL OF THE BACTERIOPHAGE-BASED THERAPY FOR A MORE ECO-SUSTAINABLE AGRICULTURE

Belén Álvarez
Department of Microbiology and Ecology, Universitat de València, Valencia
Area of Applied Research and Agricultural Extension, IMIDRA, Madrid

Elena G. Biosca
Department of Microbiology and Ecology, Universitat de València, Valencia

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Abstract: Viruses have traditionally been associated with transmission of diseases and harmful effects. However, viruses that infect bacteria, called bacteriophages (phages), can be very beneficial. In recent years, there has been an increase in the research on phages with the main objective of developing therapies for the control of bacterial infections as an alternative or complement to the use of antibiotics and/or agrochemicals, in accordance with the One Health approach and the Sustainable Development Goals (SDGs). In agriculture, phages and/or phage cocktails with proven biocontrol activity in plants against important phytopathogenic bacteria such as *Ralstonia solanacearum*, *Erwinia amylovora* and *Xanthomonas* spp. have been successfully isolated and characterized. Additionally, in relation to the SDGs, phages connect with a number of them. Innovative bacteriophage-based therapy has the potential to improve crop protection and reduce agrochemicals, contributing to a more eco-sustainable agriculture and greater global food security and health.
Keywords: virus; bacteriophage; biocontrol; crop protection; ecosystem; SDGs; One Health

Viruses are among the most abundant and ubiquitous microorganisms on the planet. The number of virus particles on Earth was initially reported to be on the order of 10^{31} (Hendrix *et al.*, 1999), and more recent assessments conclude that this number is unlikely to be either much less or much more than that (Mushegian, 2020). They have traditionally been associated with the transmission of diseases and, therefore, with harmful effects on humans, animals and plants. It is believed that there are approximately 200 viral species that can cause disease in humans. The number increases to more than 800 viral species that can cause disease in animals and increases again to more than 2,000 viral species that can cause disease in plants (Tatineni and Hein, 2023). However, viruses can also negatively affect bacteria, and when it comes to pathogenic bacteria, the overall effects can be beneficial to humans. Viruses that infect and lyse bacteria are called bacteriophages (phages) and can establish mainly two types of interaction or life cycle with the bacterial cells: lytic and lysogenic (Kirk *et al.*, 2024). In the lytic cycle, after an initial specific recognition, the phages inject their DNA into the host bacterial cells, where it is replicated and translated to produce new virions; when these virions are released, the bacterial cells are lysed. In the lysogenic cycle, the phages integrate their DNA into that of the bacterial cells becoming prophages, which are replicated along with the bacterial DNA without lysis. Prophages may remain dormant over many replication cycles and, occasionally, there may be activation signals triggered by some kind of environmental stress, so that prophages can switch to a lytic cycle and produce virions. For phage therapy only lytic bacteriophages are suitable.

In recent years, there has been an increase in the research on phages, especially the lytic ones, with the main objective of developing therapies for the control of bacterial infections in humans, animals and plant crops of agricultural interest, proposing them as an alternative or complement to the use of antibiotics or agrochemicals (García *et al.*, 2023). This is in line with the 2018 European Parliament resolution that allowed for the adoption of a European action plan named "One Health" to fight antimicrobial resistance (AMR) in humans and animals (2017/2254/INI), as well as with the Sustainable Development Goals (SDGs), established in 2015 by the United Nations as part of the UN 2030 Agenda for Sustainable Development. In general, in relation to ecosystems and global health, phages could be used for water treatment (Ji *et al.*, 2021), in case of water that could have been exposed to fecal contamination, surface runoff water, lakes, ponds, wells, the sea, etc.; for food sanitation, in case of products such as vegetables that could have been irrigated with contaminated water; also, and in the same way, for poultry and/or meat decontamination, and even the treatment of animals (Gutiérrez *et al.*, 2019; Khalid *et al.*, 2021; Ranveer *et al.*, 2024). The phage therapy for humans, applied for the first time by D'Hérelle (Dublanchet and Fruciano, 2008), is also being actively investigated in the last 15 years to combat AMR, with different administration routes depending on the type of bacterial infection, although at the moment, it has been applied mainly as a personalized therapy in specific cases in which the treatment of the patient with antibiotics had failed (Pirnay *et al.*, 2024). Therefore, bacteriophage utilities are mainly focused on the treatment of multidrug-resistant bacteria but, there is also some interesting use in biofilm disintegration, phage-based pathogen detection and synthesis of lysis-related phage proteins (Bertolini *et al.*, 2023; Ranveer *et al.*, 2024). Globally, one of the advantages of the bacteriophages is that many of them are highly specific and generally infect only a single pathogenic bacterial species, without affecting the beneficial natural microbiome of the host or the surrounding environmental microbiota, making them environmentally friendly. Other advantages are that a low dosage is usually required, due to their replication and then multiplication at the infection site; possibility of combinations with other prevention and/or control strategies either sustainable or not; low production costs; and different administration routes, as irrigation or spraying in agriculture (Durbas and Machnik, 2022). Limitations due to narrow host range can be solved by the use of phage cocktails, that can be used against mixed infections and to overcome the appearance of bacterial resistances (Durbas and Machnik, 2022; Ranveer *et al.*, 2024).

In agriculture, phage-based biological control is emerging as a promising option compared to chemical pesticides or the use of antibiotics in non-European Union countries, taking into account the complex phage-bacteria-plant interactions. In recent years, phages and/or phage cocktails with proven biocontrol activity in plants against important phytopathogenic bacteria such as *Ralstonia solanacearum*, *Erwinia amylovora* and *Xanthomonas* spp., which represent relevant threats to the yield of staple crops for human consumption, have been isolated and characterized. This is especially important for phytopathogenic bacteria for which chemical control in the field has been observed to be inefficient or to have variable results, in addition to environmental and health impact. Such is the case of *R. solanacearum*, causal agent of bacterial wilt, one of the most damaging plant diseases worldwide, affecting a range of economically important solanaceous crops and ornamentals (Álvarez *et al.*, 2010). The pathogen is considered a quarantine organism in many countries (Anonymous, 2019; EPPO, 2024), being subjected to strict rules and regulations. Within the course of surveys to find *R. solanacearum*, lytic phages were detected in environmental water and three phage isolates were obtained from three distant sampling areas (Álvarez *et al.*, 2019). These phages were characterized, and then selected according to their lytic activity and specificity against this bacterial species (Fig. 1).

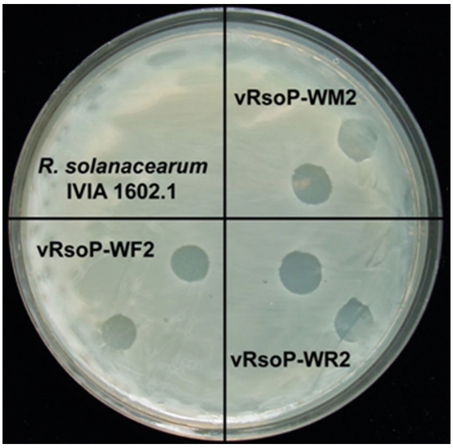

Figure 1. Lytic activity by specific bacteriophages against *Ralstonia solanacearum*. Three phage isolates (vRsoP-WF2, vRsoP-WM2 and vRsoP-WR2) were tested by pouring two drops of each of their suspensions onto a bacterial lawn (*R. solanacearum* strain IVIA 1602.1). Lytic activity was visualized by the appearance of areas of clearance by lysis of the *R. solanacearum* cells at the location where the drops of the phage suspensions were deposited.

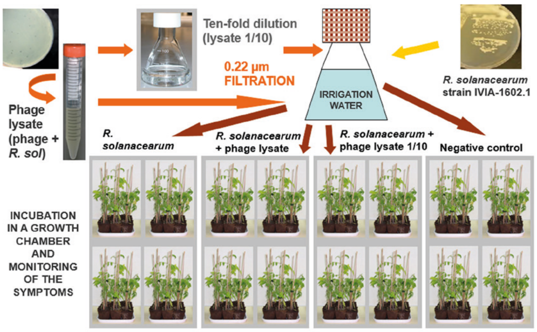

Figure 2. Bacteriophage-based therapy against *Ralstonia solanacearum* by watering application. Phage lysates were prepared and added to irrigation water along with a strain of *R. solanacearum* (IVIA 1602.1). Sets of tomato plants susceptible to *R. solanacearum* were co-inoculated by watering with the pathogen and the phage lysates at different proportions. Tomato plants treated with either the bacterial strain or water were included as controls. Plants were maintained in optimal conditions for disease development and periodically monitored to test phage efficiency for bacterial wilt biocontrol.

Ability of the selected phages to control *R. solanacearum* populations in irrigation water was proved in environmental water from different sources, and in sterile environmental and irrigation water for all the selected phages with one single phage, with bacterial populations decreasing from 10^9 CFU/ml to several thousands of cells in less than 24 h (Álvarez *et al.*, 2019). Biocontrol assays *in planta* were performed in susceptible tomato plants in conditions mimicking those of the field, with irrigation water previously contaminated with *R. solanacearum* and then treated or not with mixtures bacterium:phage with one single phage and their combinations (Fig. 2).

After watering the plants, they were incubated in optimal conditions for disease development, and reductions in a range from 45% to 100% in the incidence of the disease were achieved (Álvarez *et al.*, 2019). Significant decreases in bacterial wilt incidence were obtained, with the absence of symptoms in many of the plants. It was observed that mixing bacteriophages was more effective. The phages were classified by transmission electronic microscopy as belonging to the *Autographiviridae* family (former *Podoviridae*) and their genomes were characterized (Biosca *et al.*, 2021). The activity of the phages for the prevention and/or control of the bacterial wilt was patented (González Biosca *et al.*, 2017, 2019, 2020) and used for the development of an innovative biotechnological procedure for bacterial wilt management that can be easily applied in the field with less environmental impact than agrochemicals and less legal restrictions, opening new perspectives to the use of phages for a more ecological and sustainable agriculture.

Fire blight, caused by the phytopathogenic bacterium *E. amylovora*, is another highly contagious and difficult to control plant disease, which affects the *Rosaceae* family, including economically important fruit trees, as well as ornamental and wild plants (van der Zwet *et al.*, 2012). This bacterial pathogen is considered a protected zone quarantine pest in the European Union (Anonymous, 2019; EPPO, 2024). Recently, 124 phages isolated from material and natural sources were obtained, from which up to 28 proved to have lytic activity against *E. amylovora* (Fig. 3) (Biosca *et al.*, 2024).

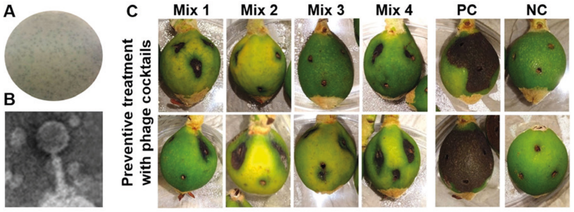

Figure 3. Bacteriophage-based therapy against *Erwinia amylovora*. A) Plaque morphology on *E. amylovora* double layer agar plate. B) Transmission electron micrograph of myovirus morphology of one representative Mediterranean *E. amylovora* phage. C) Biocontrol activity of a selection of four Mediterranean *E. amylovora* phage cocktails (Mix 1 to 4) against one Spanish (up) and one reference French (down) *E. amylovora* strains in detached inmature loquat fruits preventively treated with the phage mixes at 6 days postinoculation with the pathogen and maintained at 28°C, including positive and negative controls (PC and NC), respectively. [Adapted from Biosca *et al.* (2024)].

The phages were biologically characterized *in vitro* and *ex vivo* in fire blight-susceptible fruit, and up to 4 cocktails were designed and furtherly tested in immature loquat fruits to determine their biocontrol activity in preventive treatment and co-inoculation. Preventive application in the host was found to be better at both delaying the appearance of fire blight symptoms and reducing disease severity (Fig. 3), suggesting suitable biocontrol potential (Biosca *et al.*, 2024). These phages were molecularly characterized and morphologically identified as myoviruses (Fig. 3), from the present class of *Caudoviricetes* (Biosca *et al.*, 2024).

Phytopathogenic species of the genus *Xanthomonas* also pose a serious threat to global agricultural production because of the diseases they can cause in both herbaceous and woody plants. Therefore, there is a need to obtain new phages with activity against pathogenic *Xanthomonas* species, which may constitute new effective biological control agents (Stefani *et al.*, 2021). Studies have been initiated for the isolation and characterization of phages against some of these pathogenic species under certain environmental conditions.

Currently there are several phage cocktail-based products commercially available in the USA for the control of different important phytopathogenic bacteria (Omnilytics), including *Xylella fastidiosa* (A&P Inphatec) but, there are no commercial phage cocktails yet in the European Union (García *et al.*, 2023). Although some of these commercial phage products have demonstrated their efficacy against some strains of highly relevant phytopathogenic bacterial species, their efficacy seems to be highly dependent on environmental conditions, so the search for new bacteriophages needs to be continued.

The potential of phages in their integration with other sustainable agricultural practices is worth highlighting. In relation to the achievement of the[17] SDGs, phages connect with at least 8 of them: SDG 2 "Zero Hunger", because they can help increase crop productivity by reducing losses caused by bacterial diseases (Álvarez *et al.*, 2019; Holtappels *et al.*, 2021; Stefani *et al.*, 2021; Biosca *et al.*, 2024; Gdanetz *et al.*, 2024); SDG 3 "Good Health and Well-being", since their use can improve global health by reducing the need of agrochemicals and antibiotics in crops, which helps reduce the spread of resistant bacteria (Mohsin and Amin, 2023); SDG 6 "Clean Water and Sanitation", because phages can be used to eliminate or reduce populations of pathogenic bacteria that contaminate water in a more sustainable way (Álvarez *et al.*, 2019); SDG 12 "Responsible Consumption and Production", since phages can be applied for healthier and safer food production, by reducing the use of agrochemicals and antibiotics that generate resistance and accumulate in the environment and the living beings, and SDG 13 "Climate Action", as phages can also help reduce environmental pollution in agriculture, contributing to reduce dependence on agrochemicals and antibiotics, as well as to support the adaptation of microbial communities to environmental stress conditions for climate-smart agriculture (Huang *et al.*, 2024). Also SDG 14 "Life below Water" and SDG 15 "Life on Land", in relation to the benefits of using phages in aquaculture, livestock and agriculture (Sieiro *et al.*, 2020; Nachimuthu *et al.*, 2021; Garvey, 2022; Fiedler *et al.*, 2023).

Overall, the use of phages in agriculture constitutes an effective and natural alternative and/or complement for the control of bacterial plant diseases in line with the One Health approach, which can also contribute significantly to several SDGs by improving the production of healthier and safer food, protecting global health and promoting more sustainable and environmentally friendly agricultural practices. However, the acceptance of phage therapy and other phage applications by society can be challenging. The scarcity of societal knowledge about viruses, in general, and particularly about the viruses that infect bacteria, as new highly specific bactericidal biotools, is very little known, which may limit their social acceptance. Therefore, it is necessary to disseminate the benefits and safety of phages among society to favour their acceptance.

CONCLUSIONS

- Through research and innovation, bacteriophage-based therapy has the potential to improve crop protection and reduce the use of agrochemicals, allowing a more eco-sustainable agriculture and greater global food security.
- The beneficial activity of lytic bacteriophages against pathogenic bacteria contributes to achieving the SDGs by providing natural, safe and sustainable solutions to global health, agriculture and environmental problems.
- It is necessary to inform society about the beneficial effects of bacteriophages and their protective action against bacterial diseases, because not all viruses are harmful to humans, animals and plants, and have other applications.

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