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*Research Article*

# Effect of RsPod1EGY Phage on *Ralstonia solanacearum In Vitro*

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**Keywords:**

Bacteriophage; longevity; bacterial wilt; *Ralstonia solanacearum*.

**Abstract:**

The bacterium *Ralstonia solanacearum* causes a serious wilt disease in potato plants, and it survives in soil, potato residues, and weeds for a long time. Bacteriophages offer an environmentally beneficial biocontrol method for these bacteria. In this investigation, the RsPod1EGY phage was initially isolated from Egyptian soil and propagated, and its host range was assessed against a wide range of pathogenic and antagonistic bacteria. The results revealed a unique specificity of the RsPod1EGY phage to the *R. solanacearum* strains used in this study. Furthermore, the RsPod1EGY phage showed *in vitro* high stability with various activities through a wide range of temperatures (2 up to 60 °C), pH (2 up to 10), and NaCl concentrations (2 up to 10%). In conclusion, the RsPod1EGY phage has an *in vitro* potential to suppress the growth of *R. solanacearum*, which may allow this phage to be applied in the field for the biocontrol strategy of this bacteria.

## 1. Introduction

*Ralstonia solanacearum*, a Gram-negative proteobacterium that causes bacterial wilt in potatoes, is a quarantine pathogen in Egypt with zero import or export tolerance. Its presence is impeding the smooth flow of Egyptian potatoes to various international markets, particularly to Russia and the European Union (Messiha et al., 2007). The disease known as bacterial wilt or brown rot caused significant losses in several important crops, such as tobacco, eggplant, tomato, and potato (Hayward, 2000). Given the significance of the potato crop to Egypt's economy, a unique approach to managing bacterial wilt in potatoes is desperately needed.

It has been shown that bacterial viruses, or bacteriophages, are successful natural nanomachines that taint their hosts with extraordinary accuracy and efficacy. Various phages have been previously used as biological controls against distinct plant diseases (Balogh et al., 2010). Several phage types with the ability to interact and infect different races of *R. solanacearum* were identified and characterized by several researchers (Yamada et al., 2007; Fujiwara et al., 2011; Askora et al., 2017; Elhalag et al., 2018). However, a few have been identified and assessed as biocontrol agents against bacterial wilt caused by *R. solanacearum* (Yamada et al., 2007; Fujiwara et al., 2011). The essential advantage of utilizing a phage as a biocontrol agent is the likely diminishing in the use of ineffective pesticides. By exclusively eliminating the intended pathogenic bacterial species, phages display a restricted specificity of action, preventing the accidental destruction of non-target microorganisms that might be pivotal components of the agroecosystem.

This study aims to evaluate the efficacy of previously isolated bacteriophage RsPod1EGY to suppress the growth of R. solanacearum *in vitro*. The stability of the phage was also studied *in vitro* through a

wide range of temperatures, pH, and NaCl concentrations.

## 2. Materials and Methods

### 2.1. Bacterial strains and growth conditions

The study employed two extremely virulent strains of *R. solanacearum* Phylotype IIa, sequevar 1 (race 3, biovar 2), namely K3, and K10. These strains were originally isolated from potato tubers exhibiting symptoms of brown rot, which is characterized by brownish discoloration of vascular bundles with slimy whitish bacterial ooze.

Cultural, and morphological properties were studied by growing on two types of media including Selective Medium South Africa (SMSA), and King's B media on Petri dishes at 28 °C ± 2 for 48 h (Elphinstone et al., 1996; King et al., 1954). Bacteria were routinely subcultured and grown at 28 °C on King's medium agar plates for 24–48 h in 9 cm Petri dishes.

The two *R. solanacearum* strains were identified by molecular tests using specific primers according to Pastrik et al. (2002). The strains were kept in 20% glycerol at –30 °C for long-term storage.

### 2.2. Phage used in this study

In a previous investigation, Elhalag et al. (2018) recovered the phage podovirus RsPod1EGY Ralstonia phage from the soil against R. solanacearum Phylotype IIa, sequevar 1. and its whole genome sequences were added to the gene bank under the accession number: MG711516. The phage was stored at 4oC for three years and continuously activated on its main host.

### 2.3. Propagation of the RsPod1EGY phage

The RsPod1EGY phage was multiplied as follows: a 500 ml flask containing a 100-fold dilution of a bacterial cell culture (cultivated for 24 hours in a liter of liquid King's medium and incubated at 28°C with a concentration of $3.2\times10^8$ colony forming units CFU/ml) was filled with 100 ml of fresh King's B medium. When the cultures reached 0.2 units at OD600 using a Spectrophotometer, the phage from the original concentration was introduced at a multiplicity of infection (MOI) of 0.1. The cells were extracted by centrifugation using a refrigerated centrifuge (SIGMA 3-18K, Sigma Laborzentrifugen GmbH, Germany) at 6000 rpm for 15 min at 4°C following a 20-hour incubation period. The phage particles were precipitated by centrifugation at 12500 rpm for two hours at 4°C after the supernatant was passed through a 0.2 µm membrane filter (Cobetter filtration, PES membrane, China). The particles were then suspended in SM buffer, which contains 50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 10 mM MgSO4, and 0.01% gelatin. Plaque assay was used to determine the final concentration, which was approximately $3.6\times10^{11}$ PFU/ml. Finally, the purified phages were kept at 4°C for further studies.

### 2.4. Assessment of the sensitivity of different bacteria to phage infection (phage specificity)

Using some antagonistic bacterial strains and some plant pathogenic bacteria that were kindly provided from the culture collection of the Department of Bacterial Diseases Research, Plant Pathology Research Center, Giza, Egypt. The pathogenic bacteria included *Pectobacterium atrosepticum* (MH3c, Fel2, MH1, MH2, MH8 and MH11), *Pectobacterium carotovorum* (GH1, GH2 and GH5) *Ralstonia solanacearum* (K3 and K10), *Pectobacterium brasiliense* (BR1), *Xanthomonas campestris pv. campestris* (Xcc), *Erwinia amylovora* (Ea), *Rhizobium radiobacter* (AGt), and *Robbsia andropogonis* (Ba). While antagonistic bacteria included *Serratia marcescens* (100B), *Bacillus subtilis* (500B), *Bacillus thuringiensis* (400B), and *Pseudomonas putida (*600B*).* Both the spot test (Armon et al., 1993) and the plaque assay (Maihara et al., 2016) were used to investigate the phage host ranges.

To conduct a spot test, three milliliters (0.75%) of soft agar containing 200 microliters of each bacteria cultured for 24 hours at 28°C (with a cell density of $10^8$ CFU/ml) were added individually as five replicates for each tested bacteria, gently mixed, and then poured onto the King's medium surface. After the King's plates were solidified, a solitary drop (5 µl) of

phage suspension ($2.9 \times 10^{11}$ PFU/ml) was spotted on the surface of the media, then incubated for an entire night at 28°C and observed for clear spots that indicate the lysis efficacy against each tested bacteria, lysed regions on the spot inoculations were monitored for 48 hours.

The assay for plaque was conducted by adding 100 µl of phage suspension (at the previously specified concentration) to 250 µl of each tested bacterial strain ($10^8$ CFU/ml) in a 1.5 ml tube and then incubated for 30 min at 28 °C to allow the phage to be adsorbed on the bacterial cell surface. The mixture was then added to a tube containing 3 ml of King's-soft agar containing 2% agar. After adding the mixture to the surface of King's agar plates, the mixture was left for 30 minutes to harden. Following a 24-hour incubation period at 28 °C, the plates were examined to ascertain the formation of plaques. As a control, non-phage-infected plates were employed. Results were averaged after the experiment was repeated twice with the same sets.

### 2.5. *In vitro* stability of phage at different temperatures, pH, and NaCl concentrations after the period of storage

The *in vitro* phage stability tests at various temperatures, pH levels, and salt concentrations were carried out after the serials of phage storage and reactivation for three years to ensure its activity using the techniques of Jamal et al. (2015). 1.5 ml Eppendorf tubes with a diluted solution of the bacteriophage were incubated for two hours at different temperatures (2, 8, 15, 28, 35, 40, 50, 60, 70, and 80°C) as described by Capra et al., 2006. After incubating the phage particles for three hours at 28°C, the phage stability was measured at various pH values (2, 4, 6, 8, 10, and 12) in King's broth. The phage particles that survived were promptly counted using the double-layer agar plate method, as previously mentioned. Additionally, the phage activity was evaluated under various NaCl concentrations (2, 4, 6, 8, 10, 15, and 20%). Three replicates of the above-described plaque assay were performed for each tested temperature, pH, and NaCl concentration.

### 2.6. Statistical analysis

All experiments were carried out using a completely randomized design. The analysis of variance (ANOVA) followed by post hoc pairwise comparisons using the Tukey honestly significant difference test (HSD) were used to compare variances between means of different treatments ($p \leq 0.05$). Moreover, a simple linear regression (SLR) analysis was carried out to better understand the relationship between the Log10 count of the phage (CFU/ml) and the studied factors (temperature, pH, or NaCl concentrations).

## 3. Results

### 3.1. Identification of *R. solanacearum* K3 and K10

Both strains of *R. solanacearum* (K3 and K10) showed the typical morphological growth on SMSA and King's media, after being incubated at 28 °C for 48 h (Fig. 1A and B). The colonies were irregularly round, slimy, and white with pink centers on the SMSA medium, while they were slimy, and creamy colonies with brownish discoloration on the king's medium.

Both bacterial strains were confirmed by conventional PCR assay using specific primers for *R. solanacearum* race 3 biovar 2, which showed an identical band at 718bp (Fig. 2).

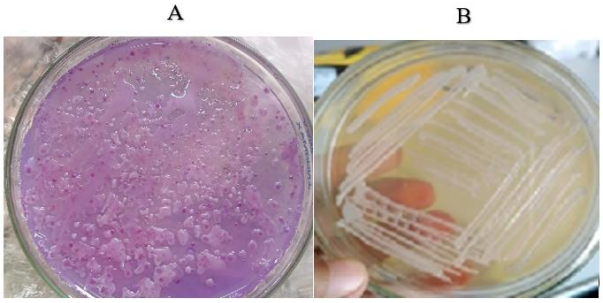


**Figure 1.** Morphology of R. solanacearum colonies race 3 biovar 2 on SMSA (A) and King's media (B).

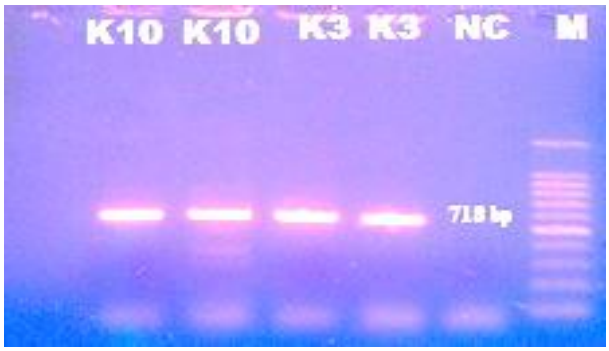


**Figure 2.** The PCR product's agarose gel electrophoresis pattern displays identical bands for each of the pathogenic bacterial strains employed in this investigation. M: ladder (100 bp), NC: Negative control (master mix only), and K3 and K10: *R. solanacearum* isolates with identical bands (718 bp), amplified by PCR using specific primers.

### 3.2. Sensitivity of various bacteria to phage infection (phage specificity)

A susceptibility test for RsPod1EGY infection was performed on twenty bacterial strains that belonged to the pathogenic and antagonistic bacteria indicated in Table (1). All strains except for *R. solanacearum* strains utilized in this experiment (K3, and K10) were found to be resistant to the phage based on the findings of the spot test and plaque assay (Fig. 3A and B).

**Table 1.** Sensitivity of different bacterial strains to RsPod1EGY phage infection

| Bacterial isolates | Strain | Spot test | Plaque assay |
|---|---|---|---|
| | **Pathogenic** | | |
| *Pectobacterium carotovorum* | GH1 | - | - |
| | GH2 | - | - |
| | GH5 | - | - |
| *P. atrosepticum* | MH3c | - | - |
| | Fel2 | - | - |
| | MH1 | - | - |
| | MH2 | - | - |
| | MH8 | - | - |
| | MH11 | - | - |
| *Ralstonia solanacearum* | K3 | + | + |
| | K10 | + | + |
| *Pectobacterium brasiliense* | BR1 | - | - |
| *Xanthomonas campestris pv. campestris* | Xcc | | |
| *Erwinia amylovora* | Ea | - | - |
| *Rhizobium radiobacter* | AGt | - | - |
| *Robbsia andropogonis* | Ba | - | - |
| | **Antagonistic** | | |
| *Serratia marcescens* | 100B | - | - |
| *Bacillus thuringiensis* | 400B | - | - |
| *Bacillus subtilis* | 500B | - | - |
| *Pseudomonas putida* | 600B | - | - |

(+) sensitive, (−) resistant.

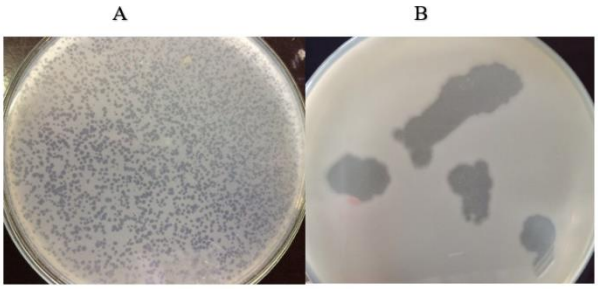


**Figure 3.** Plaque (A) and spot (B) assay showing the sensitivity of *R. solanacearum* (K10) to the infectious phage RsPod1EGY used in this study.

### 3.3. *In vitro* stability of phage at different temperatures, pH, and NaCl concentrations after the period of storage

Following three years of storage, the stability of RsPod1EGY demonstrated pronounced activity at various temperatures, pH levels, and salt concentrations. Up to 50 °C, the phage retained its stability; however, at temperatures above 60 °C, it was lost within two hours (Fig. 4A). The phage was most active at pH values between 6 and 8, and it was completely inactive at pH values greater than 10 (Fig. 4B). In addition, the phage continued to be active at NaCl concentrations up to 15% and stopped counting at 20% (Fig. 4C). The highest decline started after 8% NaCl concentration (Fig. 4C).

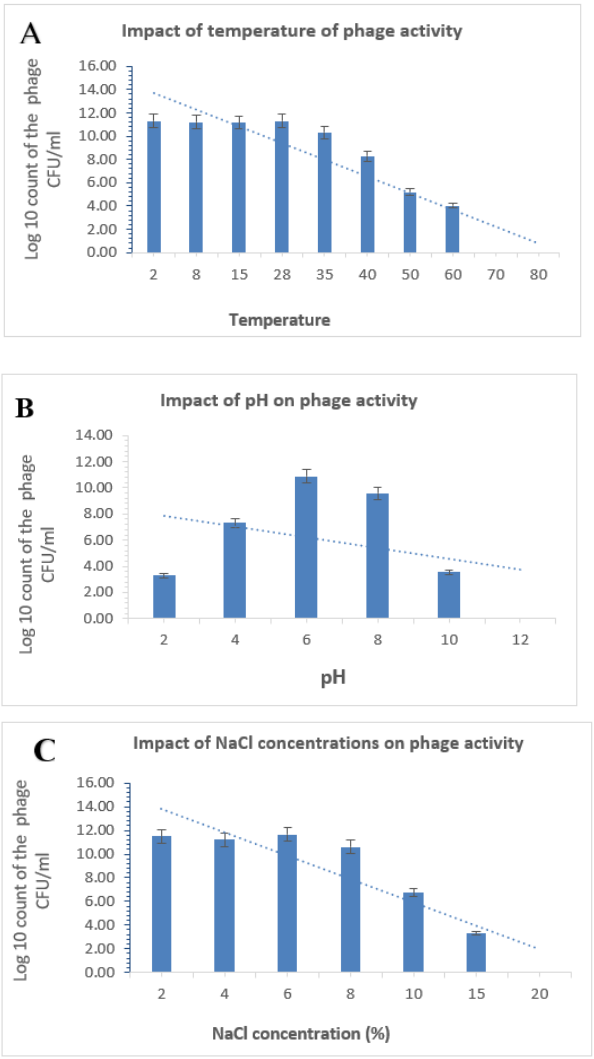


**Figure 4.** RsPod1EGY phage stability at different temperatures (A), pH (B), and NaCl concentration

All values represent means of three replicates ± SD.

## 4. Discussion

Bacterial wilt caused by *R. solanacearum* is considered an important disease that impacts and reduces the productivity of potatoes. It is a quarantine disease with zero tolerance (Farag et al., 1999). The modern approach in bacterial disease control aims to use environmentally friendly agents that have efficacy and safety in the field application. Bacteriophages represent good, efficient, and eco-friendly agents that have the opportunity to be an alternative control method for bacterial diseases in plants (Addy et al., 2012; Alomari et al., 2021). Bacteriophages are viruses that exclusively target bacteria, posing no threat to plants or animals.

The bacterial strains used in this study were identified by their cultural, and morphological properties and by molecular tests using PCR assay. On SMSA and King's medium (B), the morphology of all *R. solanacearum* strains (K3 and K10) was verified. This identification aligns with the previous work published by Engelbrecht (1994). Their molecular identity was confirmed using specific primers that showed specific bands at 718 bp, which agrees with Pastrik et al. (2002).

In our investigation, the antibacterial activity of the phage was assessed against a set of twenty bacterial strains that were classified as pathogenic and antagonistic. This revealed that the phage was specific to *R. solanacearum* strains that demonstrated distinct spots and plaques, as determined by the spot test and plaque assay. This finding is in agreement with a previous study on the same phage that was assayed by Abdelmonim et al. (2018) and Elhalag et al. (2018) against a different panel of bacterial strains used in this study.

The challenge in phage field application is the ability to sustain its survival under environmental conditions such as desiccation, ultraviolet (UV) radiation, pH, rain, and temperature that have an impact on the phage survival and persistence at the necessary site of action (Iriarte et al. 2007). In our study, the stability of the bacteriophage RsPod1EGY after three years of storage was assessed under various temperatures, pH, and sodium chloride concentrations. Our findings revealed that the phage was stable and exhibited noticeable activity at different pH values, temperatures, and salt concentrations. The phage was stable up to 50 °C, but it lost stability after two hours at temperatures over 60 °C. Furthermore, the phage was entirely inactive at pH levels higher than 10, while it was most active at pH values between 6 and 8. On the other hand, the phage remained active at NaCl concentrations up to 15% until ceasing to count at 20%. These findings support the conclusions stated by Elhalag et al. (2018).

According to a recent example of how pH affects phages, phage and other viral abundance enhanced when pH was raised from 3.5 to 5. The quantity of bacteriophages declined at increasing pH values (Narr et al., 2017). Phages vary in their pH requirements for survival, and the soil has an impact on the phages' development and activity. According to Oh et al. (2017) and Jin and Flury (2002), phages are bound to soil particles by pH, which prevents them from migrating and preserves the phage community. Soil pH plays a crucial role in dictating how well phages perform biocontrol in the soil.

## 5. Conclusion

Our study indicated that RsPod1EGY phage has the potential to suppress the growth of *R. solanacearum* the causal agent of the potato wilt disease under the laboratory bioassay. Moreover, our findings revealed that RsPod1EGY phage was stable under different temperatures, pH, and salt levels. Further studies are needed to ensure the use of RsPod1EGY phage as a biological component in the control strategy of potato wilt disease. These studies include the evaluation of the bacteriostatic potential of the phage under greenhouse and field conditions.

## References

Abdelmonim, A. A., Elhalag, K. M., Addy, H. S., Hussien, A. S., Nasr-Eldin, M. A. and Huang, Q. 2018. Sequencing, genome analysis and host range of a novel Ralstonia phage, RsoP1EGY, isolated in Egypt. Arch. Virol. 163:2271–2274.

Addy, H.S., Askora, A., Kawasaki, T., Fujie, M. and Yamada, T. 2012. Utilization of filamentous phage RSM3 to control Bacterial wilt caused by *Ralstonia solanacearum*. Plant Dis. 96(8): 1204-1208.

Alomari, M.M.M., Dec, M. and Urban-Chmiel, R. 2021. Bacteriophages as an alternative method for control of zoonotic and foodborne pathogens. Viruses, 13(12):2348.

Armon, R., Kott, Y. 1993. A simple, rapid and sensitive presence/absence detection test for bacteriophage in drinking water. J. Appl. Bacteriol. 74:490–6.

Askora, A., Kawasaki, T., Fujie, M. and Yamada, T. 2017. Lysogenic Conversion of the Phytopathogen Ralstonia solanacearum by the P2virus ϕRSY1. Front. Microbiol. 8:2212.

Balogh, B., Jones, J.B., Iriarte, F.B. and Momol, M.T. 2010. Phage therapy for plant disease control. Curr. Pharm. Biotechnol. 11:48-57.

Capra, M.L., Quiberoni, A., Reinheimer, J. 2006. Phages of *Lactobacillus casei*/*paracasei*: response to environmental factors and interaction with collection and commercial strains. J. Appl. Microbiol. 100:334–42.

Elhalag, K., Nasr-Eldin, M., Hussien, A. and Ahmad, A. 2018. Potential use of soilborne lytic Podoviridae phage as a biocontrol agent against *Ralstonia solanacearum*. J. Basic Microbiol. 58:658–669.

Elphinstone, J., Hennessey, J., Wilson, J., Stead, D. 1996. Sensitivity of different methods for the detection of *Pseudomonas solanacearum* in potato tuber extracts. Bull. OEPP/EPPO Bull. 26:663–78.

Engelbrecht, M.C. 1994. Modification of a semi-selective medium for the isolation and quantification of *Pseudomonas solanacearum*. Bact. Wilt Newsl. (10):3-5.

Farag, N.S., Stead, D.E. and Janse, J.D. 1999. *Ralstonia (Pseudomonas) solanacearum* race 3, biovar 2, detected in surface (irrigation) water in Egypt. J. Phytopathol. 147:485–487.

Fujiwara, A., Fujisawa, M., Hamasaki, R., Kawasaki, T., Fujie, M. and Yamada, T. 2011. Biocontrol of *Ralstonia solanacearum* by treatment with lytic bacteriophages. Appl. Environ. Microbiol. 77: 4155.

Hayward, A.C. 2000. *Ralstonia solanacearum*, In: Lederberg J (ed.). Encyclopedia of Microbiology; 4:p.32-42. Academic Press, San Diego.

Iriarte, F.B., Balogh, B., Momol, M.T., Smith, L.M.,

Wilson, M. and Jones, J.B. 2007. Factors affecting survival of bacteriophage on tomato leaf surfaces. Appl. Environ. Microbiol. 76(6):1704-1711.

Jamal, M., Hussain, T., Das, C. R., Andleeb, S. 2015. Isolation and characterization of a MyoviridaeMJ1 bacteriophage against multi-drug resistant *Escherichia coli* 3. Jundishapur J. Microbiol. 8:1–8.

Jin, Y. and Flury, M. 2002. Fate and transport of viruses in porous media. Adv. Agron. 77:39–102.

King, E.O. Word, M.K. and Rayney, D.E. 1954. Two simple media for demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44:301-307.

Mihara, T., Nasr-Eldin, M.A., Chatchawankanphanich, O. and Bhunchoth, A. 2016. A *Ralstonia solanacearum* phage ϕRP15 is closely related to Viunalike viruses and encodes 19 tRNA-related sequences. Virol. Rep. 6:61-73.

Messiha, N., Diepeningen, A., Farag, N. and Abdallah, S. 2007. *Stenotrophomonas maltophilia*: a new potential biocontrol agent of *Ralstonia solanacearum*, causal agent of potato brown rot. Eur. J. Plant Pathol. 118:211–225.

Narr, A., Nawaz, A., Wick, L.Y., Harms, H. and Chatzinotas, A. 2017. Soil viral communities vary temporally and along a land use transect as revealed by virus-like particle counting and a modified community fingerprinting approach (fRAPD). Front. Microbiol. 8:1975.

Oh, H., Seo, D.J., Jeon, S.B., Park, H., Jeong, S., Chun, H.S., Oh, M. and Choi, C. 2017. Isolation and characterization of *Bacillus cereus* bacteriophages from foods and soil. Food Environ. Virol. 9:260–269.

Pastrik, K.H., Elphinstone, J.G. and Pukall, R. 2002. Sequence analysis and detection of *Ralstonia solanacearum* by multiplex PCR amplification of 16S-23S ribosomal intergenic spacer region with internal positive control. Eur. J. Plant Pathol. 108:831-842.

Yamada, T., Kawasaki, T., Nagata, S. and Fujiwara, A. 2007. New bacteriophages that infect the phytopathogen *Ralstonia solanacearum*. Microbiol. 153:2630-2639.