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Bacterial Wilt Causing *Ralstonia solanacearum* F_1C_1 Biocontrol Using Phage Treatment

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ABSTRACT

Bacteriophages that show lytic replication cycle resulting in bacterial cytolysis form excellent biocontrol agents for phytopathogens. *Ralstonia solanacearum* is the causative agent of bacterial wilt of economically important crop plants worldwide for which bacteriophage-based control therapy is currently being evaluated. In this study, we report the isolation and characterization of Myoviridae phage vB_RsoMSS4 (ϕSS4) capable of reducing *R. solanacearum* infection in potato tubers as well as tomato plantings. The select phage also showed host specificity with no lytic activity against beneficial plant growth promoting rhizobacteria.

***Key words*:** *Ralstonia solanacearum*, Bacteriophage, Potato tuber, Plaque, Phage, Biocontrol

Ralstonia solanacearum, formerly *Pseudomonas solanacearum* are Gram negative bacterial phytopathogens capable of causing wilt disease in over 200 different crop species of economic importance [1-2]. Upon infection, the pathogen colonizes in the vascular tissue, causing xylem destruction resulting in leaves and stem wilting with ultimately collapse and death of the plant. *R. solanacearum* are reported to be resistant to copper and antimicrobial based chemical treatments with the ability to persist in the soil environments for long time periods using alternative hosts causing devastating crop damage [3-4]. Yield losses caused by *R. solanacearum* varying according to their host plant; 0 to 91% in the tomato, 33 to 90% in the potato, 10 to 30% in tobacco, 80 to 100% in the banana, and up to 20% in the groundnut [5]. n India, total yield loss due to bacterial wilt is 25-75% in farmers' field [6].

Use of lytic and *R. solanacearum* specific bacteriophages for the effective biocontrol of the phytopathogen offers an effective, ecofriendly and economical alternative therapy [7-10]. Bacteriophages (phage) are obligate intracellular viral parasites whose lytic replication cycle can be used for biocontrol by cell death of targeted phytopathogen. Phages are naturally present in abundance in the environment and their population is regulated by the presence of host bacterium. Bacteriophages as biopesticides [11] are gaining popularity and support due to their several advantages which include narrow host specificity, safety to beneficial organisms, genetic plasticity, non-toxic nature to eukaryotic cells, abundance in nature and ease of application. Applications of lytic bacteriophages in the environment are also self- controlled by the presence of living host and this is also referred to as the auto dosing effect [12]. Bacteriophages for the control of phytopathogens have been studied for *Xanthomonas oryzae* (bacterial blight and spot disease) [13-14], *Pseudomonas syringae* (bacterial blight of leek, kiwifruits canker) [15-16], *Erwinia amylovora* (fire blight of apple, pear and raspberry) [17], soft rot Enterobacteriaceae [18-19], as well as *Ralstonia solanacearum* (bacterial wilt) etc., [20-22]. The effective use of phage application for the control of plant diseases has been used to develop commercial formulations such as Agriphages (Omnilytics), Erwiphages, biolyse for soft rot Enterobacteriaceae. Several patents have also been granted for phage biocontrol of food and agriculture industry.

Phages specific to infecting particular races of *R. solanacearum* can also be used for the development of highly specific phytopathogen diagnostic tools (phage typing) in soil or infected plant materials. Hence, isolation and characterization of bacteriophages can be very useful for the diagnosis as well as biocontrol of *R. solanacearum*. In this study, we report the isolation of *Ralstonia solanacearum* F_1C_1 specific phage vB_RsoMSS4 phage and study its effectiveness in controlling bacterial wilt disease in potato tubers and tomato plants.

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MATERIAL AND METHODS

Bacterial strain and culture condition

Ralstonia solanacearum F_1C_1 (obtained from Dr. Suvendra Roy, Department of Molecular & Biotechnology,

Tezpur University, Assam) was maintained on the Tryptone Soy Agar (TSA) or 2,4,6-Triphenyl tetrazolium chloride (TTC) medium (Hi Media, India) at 37°C. All chemicals and media were procured from HiMedia, Mumbai, India.

Bacteriophage spot assay and plaque assay

Phage SS4 was isolated using *Ralstonia solanacearum* F_1C_1 as host, supernatants were collected and filtered through PES membrane syringe (0.45µm pore size) (Himedia, Mumbai, India). To detect the presence of phages in supernatant, spot test was carried out as described by Chang et al**.** with some modifications**.** Briefly, 10 µl diluted phage were spot inoculated on molten agar (0**.**4% w/v) containing host cells of 10^7 CFU/ml**.** Clear zones of plaques were observed after incubating the plates overnight at 37°C. The phage titer was determined by plaque assay by employing double agar overlay technique.

Bacteriophage propagation and purification

All the isolated bacteriophages were purified by successive single-plaque isolation until homogenous plaques were obtained as per described by Sambrook *et. al*. [23]. Briefly, one well separated bacteriophage was picked with sterile inoculation loop with the surrounding cell mass and inoculated into 200 ml Tryptone Soy Broth (TSB), in which overnight culture of 10^9 host cells was added and incubated at 37°kC with agitation at 140 rpm for phages stock preparation. After complete lysis, the mixture was centrifuged (10,000 rpm, 10 min, 4°C), filter sterilized and treated with chloroform (1% v/v) to remove any bacterial contamination. Purified bacteriophages further concentrated in SM buffer (50 mmol l^{-1} Tris-HCl at pH 7.4, 100 mmol l^{-1} NaCl, 10 mmol l^{-1} MgSO4, and 0.01% gelatin) by using PEG-8000 method with some modifications [24]. Concentrated phages were stored in aliquots at -20ºC for long term storage. Short term stock preparations were maintained at 4°C for further use.

Host range determination

Host range was also determined by performing spot assay as described with different bacterial hosts**.** Log phage cultures of hosts such as *Pseudomonas aeruginosa* ATCC 15442, *E. coli*, fluorescent *Pseudomonas* GD2, *P. aeruginosa* R32 were used in spot assay as described previously.

Transmission electron microscopy

To observe bacteriophages morphology, transmission electron microscopy was performed as described by Goodridge et al, 2003 with some modifications. Drops of ultracentrifuge bacteriophages samples were dropped on copper coated grids (diameter, 3 mm; 300 meshes). After 5 min, the bacteriophages particles were stained with 2% (w/v) phosphotungstic acid (PTA) for 10s. The grids were allowed to dry for 20 min and examined under a transmission electron microscope (FEI Tecnai S Twin, at 200 kv) facility used at the All India Institute of Medical Sciences, New Delhi, India.

Plant bioassay

Potato tuber slices as well as tomato plant pot assays were used to determine the ability of the phage formulation to inhibit phytopathogen as described previously [25-26]. Infectivity of potato tuber was measured on days required for tuber browning and vascular collapse of lesion size. 10 days old tomato seedlings of variety New Uday were used for the plant bioassay study by soil drenching method. The experiment was designed in triplets for plastic pots containing 145 g soil per pot. The log culture was prepared in TSB and washed with phosphate buffer. Adjust the optical density 10^8 cfu/ml of log culture (10 ml for each pot). Fresh tomato plants were washed PBS and dipped plant roots in log culture of F1C1 for 10 min and then transfer to the pots and poured the culture around the root. For phage biocontrol, plantlings were treated with 1 ml phage suspension (1.0 MOI) mixed with 10 ml log culture (10^8 cfu/ml) dipped and transfer to the pots in the same way and for control tomato seedlings treated with phosphate buffer, no culture and no phages was used.

Subjective scale was used for disease symptoms in tomato plants**.** Grade1: 25% plant leaves wilted; Grade 2: 26-50% of plant leaves wilted; Grade 3: 51-75% plant leave wilted; Grade 4: 76% or more plant leaves wilted and stem collapse. All experiments were performed in triplicates.

Statistical analysis

Statistical analysis was done using student's t test. All experiments were repeated at least twice in performed in triplicates. $p \leq 0.05$ was considered as biologically significant.

RESULTS AND DISCUSSION

Bacteriophage isolation and characterization

Ralstonia solanacearum F1C1 was used as a host for spot assay to screen for phages that could show clear lytic activity**.** As shown in (Fig 1a), only phage ϕSS4 produced clearance in spot assay**.** When *R. solanacearum* F_1C_1 was infected with ϕSS4 phage on potato tubers and reisolated after 72 h, clear plaques of about 2-3 mm irregular boundary were again recovered (Fig 1b). Following single plaque isolation, the phage was propagated as described in materials and methods and its activity was assayed.

Transmission Electron Microscopy was performed for morphological study of the phage SS4 (Fig 2). TEM image shows the presence of tailed bacteriophage with an icosahedral head. The tail appears to be contractile and rigid. The size of the phage is approximately 200 ± 50 nm in length by 40 ± 10nm in width. Based on the TEM study, the phage was characterized to belong to Myoviridae and is designated as vB_RsoMSS4.

Negative staining is the simplest method can be performed by phosphotungstates (PT) and uranyl acetate (UA) salts for direct virus visualization under transmission electron microscope [27]. TEM is fast, easiest and advance technology contributes to a high level of phage classification to study virus's morphology, nature and genetic organization. Negative staining is much faster than genome analysis to perform and data deriving from virus visualization under TEM make available valuable information in short time. In addition to morphological analysis, it also helpful to understand about interaction study between phages and their host (adsorption of phages on bacterial cell surface) as well as correlation between phages and host present in the same environments. Bacteriophages are host dependent, occur wherever bacterial host present so helpful to identify these viruses as good indicators of bacterial contaminations (phage typing) hence bacteriophages to employ in prophylaxis (phage therapy) or in bio-decontamination [28].

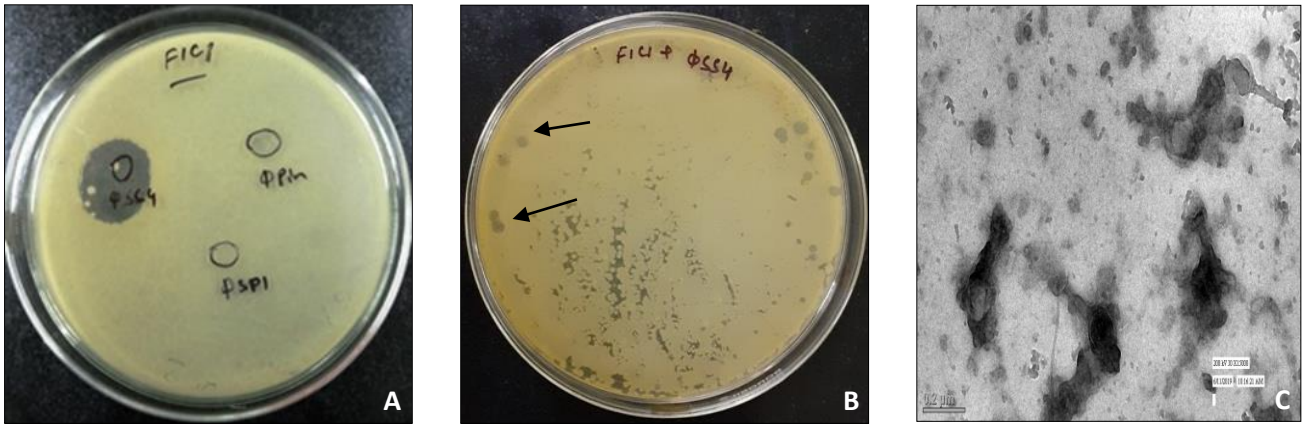
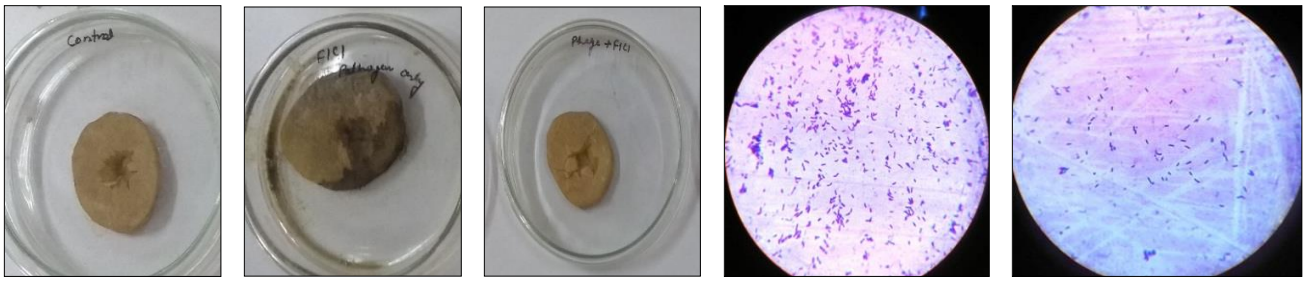


Fig 1 (A) Spot assay of phage ϕSS4 against *R. solanacearum* F_1C_1. (B) Plaques isolated using double overlay agar assay showing clear plaques. (C) TEM micrograph of phage ϕSS4 showing icosahedral head and rigid tail with base plate



A) Control uninfected potato tuber slice

B) *R. solanacearum* F_1C_1 infected potato tuber slice

C) Phage ϕSS4 + *R. solanacearum* F_1C_1 treated potato tuber

D) Methylene blue staining of material from wilted spot of F_1C_1 treated potato tuber slice

E) Methylene blue staining of material from wilted spot of ϕSS4 + F_1C_1 treated potato tuber slice

Fig 2 Phage ϕSS4 activity against *R. solanacearum* F_1C_1 infections in potato tubers

Fig 3 Tomato plantling experiment on treatment with phage vRsoMSS4

Diverse bacteriophages infecting different phytotypes of *Ralstonia solanacearum* have been isolated which include filamentous Inoviridae (ϕRSM-1 and ϕRSS-1 prophage), contractile tail containing Myoviridae (ϕRSA1) with wide host range, short non contractile tail Podoviridae (ϕRSB1) and non-contractile tail Siphoviridae (ϕRSC1) [29]. Amongst, only ϕRSL1 was found to 100% successful for bacterial wilt biocontrol depending on the condition, reported from Japan [30]. Other reports of lytic phage isolation against *Ralstonia solanacearum* include ϕRsS6 Podoviridae, found as effective biocontrol potential in tomato plants [31]. Phages PE204 of Podoviridae family, with a good potential of 30% to 100%, depending upon the multiplicity of infection of phages for bacterial wilt biocontrol was discovered in Korea [32]. The combination of two Podo-viruses (J2 and ϕRSB2) efficiently lysed *Ralstonia solanacearum* cells in contaminated soil but only J2 treatment enabled to prevent disease development in tomato plants [33]. Lytic phages ϕRP15 Myoviridae, infecting *Ralstonia solanacearum* was isolated in Thailand

[34]. Two bacteriophages ϕRSSKD1 & ϕRSSKD2 against *R. solanacearum* having wide host range have been reported from Indonesia [35]. Recently reported phage cocktail (P1) demonstrated different inhibition patterns upto 98% against growth of the bacterial wilt causing *Ralstonia solanacearum* [36]. The application of six lytic phages cocktail from Sri Lanka, applied to the rhizospheric soil of the tomato plants as soil drench resulted the reduction of bacterial wilt incidence by approximately 10–20% [37].

However, some lysogenic phages are also reported to reduce virulence of bacterial strain rather than complete loss of virulence. Filamentous phage ϕPE226, member of Inoviridae having the properties of both lytic and lysogeny showed high virulence against *Ralstonia solanacearum* strain [38]. Another filamentous phage ϕRs551 also demonstrated in reduced virulence strain of *R. solanacearum* race 3 biovar 2 strain by reducing EPS production reported by [39].

Table 1 Host range determination for phage SS4 on different phytopathogens and plant growth promoting bacteria

Host	Spot assay
Ralstonia solanacearum F_1C_1	+
Pseudomonas aeruginosa ATCC 15442	+
Pseudomonas aeruginosa R32	-
Escherichia coli	-
Staphylococcus aureus	-
Plant growth promoting Pseudomonad GD1	-
Raltonia solanacearum RS1	-

Host range determination

Host range experiments were set up using spot assay was carried with phage vB_RsoMSS4 on different bacteria such as plant growth promoting *Pseudomonads*, gram negative bacteria such as *E. coli*, Gram positive bacteria *Staphylococcus aureus* and *Ralstonia solanacearum* F_1C_1 as control (Table 2). The data indicates that the phage is host specific for *Ralstonia solanacearum* F_1C_1. Positive spot assay with *Pseudomonas aeruginosa* ATCC 15542 indicates some cross-reactive surface proteins between *R. solanacearum* F_1C_1 and P15. The phage is good candidate as biocontrol agents as it showed no lytic activity against plant growth promoting Pseudomonads and can be safely used without affecting beneficial organisms in the soil.

Table 2 Plant bioassay with phage treatment of *Ralstonia solanacearum* F_1C_1 infected potato tuber after 10 days

Experimental set-up	Lesion size	Colony count	Plaque isolated
Control	No lesions	O cfu/g	-
Pathogen control (F_1C_1)	3.2 cm × 1.8cm	2.7×10^9cfu/g	-
Phage treated (phageϕSS4 + F_1C_1)	No lesion	4.2×10^4 cfu/g	2.4×10^2 pfu/g

Biocontrol activity

In order to test the efficacy of phage treatment on *Ralstonia solanacearum* (F_1C_1) pathogenicity the potato tuber model as well tomato plant bioassay was used. Sterile potato tubers of approximately equal sizes were inoculated with 10^8 cfu in 100 µl of F_1C_1 isolate or F1C1+vB_RsoMSS4 phage in the form of a spot in potato tuber center. Uninoculated tubers served as negative control. The potato tubers were observed for any changes in the tissue architecture. (Fig 3a) shows that the infected tuber showed symptoms of vascular browning and bacterial slime droplets. By 72h, severe browning and vascular collapse was observed in the infected tuber but no such features were observed in the phage treated F_1C_1 + vB_RsoMSS4 samples. Further, samples were withdrawn from the infected tuber to test for the presence of the pathogen (0.1g/ml of DW). Accordingly, (Table 1), no Ralstonia/micro-organisms or plaques were isolated from uninfected tuber, 10^8cfu/g from infected tuber and 4.2×10^4 cfu/g from F_1C_1 + vB_RsoMSS4 samples. Further when double layer agar overlay plaque assay was performed from the tuber material, 240 plaques/g tissues were obtained. Microbial methylene blue staining of the sample for the presence of F_1C_1 showed reduced bacterial samples in the phage treated tubers (Fig 3b). Hence phage treatment was capable of ameliorating infection of F_1C_1 in potato tuber by 50%, even as no symptoms were observed.

Table 3 Plant bioassay with phage treatment of *Ralstonia solanacearum* infected tomato plantings

Experimental set-up	Shoot height at day 10 (cm)	Days of wilting
Control	7.2 cm	-
F_1C_1 control	1 cm (Collapsed)	4
PhageϕSS4 treated	5.6 cm	-

Further, 6-7 days old tomato seedlings were inoculated with 10^8 cfu/ml *Ralstonia solanacearum* F_1C_1 and treated with 10^8 pfu/mlvB_RsoMSS4. Within 6 days of inoculation, almost all the tomato plantlings were killed by *R. solanacearum* F_1C_1 strain. The uninfected plant showed no wilting and height of the plant at day 10 was recorded to be 7.6 cm with about 6 leaves. Phage vB_RsoMSS4 + *Ralstonia solanacearum* treated planting showed no wilting

and was standing with leaves intact at 5.6 cm even as effects of browning could be seen in comparison to uninfected plant and was showed Grade 1 disease symptoms. The pathogen infected tomato plant showed wilting of grade 4.0 as shown in (Fig 4) as one of three similar experiments. Phages for *Ralstonia solanacearum* include ϕRSL-1 (Myoviridae), ϕRSM-3 (Inoviridae, filamentous), ϕRSB-1 which have been reported to have efficacious biocontrol activity against *Ralstonia solanacearum*.

CONCLUSION

vB_RsoMSS4 phage belonging to Myoviridae family was found to have the potential to reduce the pathogenic wilting activity of *Ralstonia solanacearum* F_1C_1 in potato tuber as well tomato plant. The phage is also highly host specific and showed no activity against beneficial plant growth promoting bacteria. Hence, these phages can be good candidates for biocontrol and strain typing agents. The phages show great potential in developing it as a biocontrol formulation for the control of Solanaceae crop infections caused by *Ralstonia solanacearum*.

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