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Research Article

Isolation and Characterization of a Myovirus Phage Infecting *Ralstonia Solanacearum*the Causative Agent of Bacterial Wilt in Northern Border Region in Saudi Arabia

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ABSTRACT

In this study, we isolated and characterized a myovirus phage isolated from the soil of Northern Border Region in Saudi Arabia. The phage was designated as ϕ**RSNB1** and characterized using Transmission Electron Microscopy (TEM), Restriction Digestion of its DNA and Host Range. Electron micrograph indicated that ϕ**RSNB1**phage has an icosahedral head with diameter of about 110±5 nm and contractile tail with length of about 170±10 nm and width of about 25±2 nm, respectively. On the basis of the **ϕRSNB1**phage morphology, we thus propose that ϕ**RSNB1**represents a member of *myoviridae*phages. The ϕ**RSNB1** phage was shown to be able to infect *Ralstonia solanacearum* and has no effect on other tested bacteria. ϕ**RSNB1** was stable over the pH range of 5-9,chloroformresistant and stable at 4°C.Furthermore, the result of one step growth curve of ϕ**RSNB1**showed that ϕ**RSNB1**phage has a latent period of approximately 60 minutes and a burst size of about 100 per infected. The size of the genome was estimated to be ~200 kbp. To our knowledge, this is the first report on the isolation of a *Ralstonia solanacearum* phage from Northern Border Region in Saudi Arabia.

Keywords: *Ralstonia solanacearum*, *myoviridae* phage, Bacterial wilt disease.

INTRODUCTION

It is noticed obviously that large number of economically important plant diseases are caused by pathogenic bacteria that infected plants in the world. These bacterial outbreaks are a problem of control due to the evolution of resistance in addition to the lack of effective bactericides [1]. One of these important economically plant diseases worldwide are the *Ralstonia solanacearum* bacterial wilts. In addition, this destructive bacterial pathogen causes bacterial wilt for more than 200 species of plants belonging to more than 28 families, especially in tropical and subtropical regions. The host range includes many economically important crop plants such as Tomato, Eggplant, Banana, Potato in addition to Tobacco. It is widely known as one of the most destructive plant pathogens in the world and it has been reported not only in temperate regions but

also in many other parts of the world [2]. Recently, it was found also that *R. solanacearum*expanded its host to the cut roses cultivation in the Netherlands [3].*R. solanacearum* is one of the most important plant pathogens in the world, because of its wide host range, aggressive, in addition to its great geographical distribution and its long persistence in soil and water environments. Earlier in the 20th century; exactly in 1896, *R. solanacearum* was described by Erwin F. Smith as *Pseudomonas solanacearum* that causes the bacterial wilt for plants belonging to the family *Solanaceae*[4, 5].Many research studies had been done on this bacterium, and also now many investigations of the molecular determinants are under processing in order to control this bacterial pathogen that infected some of the important crops all over the world [6, 7]. The increasing and enhancement of crop production requires an effort in controlling and overcoming these plant pathogens worldwide. Hence, development of effective disease management strategies and improvements in detection and monitoring tools are required. In the past there were several studies described the development of control methods against *R. solanacearum* bacterial wilt disease using cultural, chemical, physical, and suppression mechanisms in addition to biocontrol efficacy [8]. A relatively new technique called bacteriophage therapy was used to control the bacterial wilt disease. Various kinds of bacteriophages with characteristic features have been isolated during the past decade [9, 10], and paved the way for new methods of biological control of bacterial wilt disease. These bacteriophages may be useful tools for effectively detecting pathogens in cropping ecosystems and in growing crops [11, 12]. They also have potential uses to eliminate pathogens from contaminated soil or to prevent bacterial wilt in crops of economic importance. As with other methods of biological control, an important feature of the biological control of phage is to reduce the use of chemical compounds against pathogens. This method of phage therapy or phage biocontrol prevents the problems of multiple environmental pollution, ecosystem disruption, and residual chemicals on the crops. In recent decades, the use of phage biological control has been explored to restrict the growth of bacterial plant pathogens with increased zeal. For the practical use of phage as biological control agents against bacterial wilt, multiple phages are required with extensive ranges of hosts and strong lytic activity. To our knowledge; in the Northern Border Region, Saudi Arabia; up to date there is no study on the isolation and characterization of *R. solanacearum* bacteriophage. Therefore, we carried out this work in order to investigate the morphological, physical, chemical and genomic features of the *R. solanacearum* phage.

MATERIAL & METHODS

2.1. Bacterial strains and growth conditions:

Different bacterial strains of *Ralstoniasolanacearum* were isolated from soil samples that collected from different regions in the Northern Border Region, Saudi Arabia. The strains were identified and confirmed as *R. solanacearum*. *R. solanacearum* was used as indicator strain for bacteriophage isolation. The bacterial cells were cultured in CPG medium containing 0.1% casamino acids, 1.0% peptone and 0.5% glucose [13] at 28°C with shaking at 200-300 rpm. Stock cultures were stored in LB broth containing 20% glycerol at -20°C.

2.2. Bacteriophage isolation:

We collected five soil samples from different localities in the Northern Border Region, Saudi Arabia. The presence of bacteriophages in the collected samples was determined qualitatively by the spot test technique method [14]. Each collected soil sample was enriched with 50 ml of LB broth medium containing 1 ml of *R. solanacearum* as a host bacterium (10^8cfu/ml) and incubated on a rotary shaker for 48 h at 30°C, the culture supernatant was harvested by centrifugation for 10 min at 6000 rpm. We added a chloroform to the supernatant as 1/10 (v/v) rate with vigorously shaking and phages were obtained from the upper layer. In order to check the presence of bacteriophages by simple qualitative

method, the spot test technique was used for this purpose. Quantitative assaying of the phages was carried out as described by the soft-agar overlay (double layer) method as reported byYamada et al.,[9].

2.3. Bacteriophage propagation and concentration:

High titer phage stock of the *R. solanacearum* bacteriophage lysate was obtained using the culture method as following: 100 ml of LB culture medium were prepared and was inoculated with *R. solanacearum* (10^8cfu/ml), phage particles (10^7pfu/ml) were added in a ratio of (1:10, v/v) in an Erlenmeyer flask (250 ml) then incubated at 30°C for 48h. Cultures after incubation period were centrifuged for 15 minutes at 6000 rpm and then chloroform was added to the supernatant (1:10, v/v). The chloroform mixture was shaken vigorously for 3-5 minutes and left for 30 minutes to clarify. Then, the suspension containing bacteriophage was transferred into sterile bottles and stored at 4°C with traces of chloroform. The purification of *R.solanacearum* bacteriophage was performed by ammonium sulphate precipitation according to Johnson et al.[15]. The highly concentrated phage was assayed quantitatively afterconcentration, and the titer was 10^{10}pfu/ml. The phage was stored at 4°C for further investigations.

2.4.Electron microscopy for ϕRSNB1 phage:

We examined the isolated bacteriophage according to the methods of Bradley[16]. The bacteriophage drop suspension (10^8pfu/ml) was placed on 200 mesh copper grids with carbon-coat formvar films, and filter paper was used in drawn off the extra. After that a 1% uranyl acetate saturated solution was placed on the grids and the extra suspension was drawn off as before. The specimens were examined with Transmission Electron Microscope (Model JEM 1011) (Electron Microscopy Unit, Central Laboratory, King Saud University, Riyadh, Saudi Arabia.

2.5.Host specificity for ϕRSNB1 phage:

Table No. (1) is showing the isolates of *R. solanacearum* as well as different bacterial strains used in this study. All of these strains were checked for its sensitivity to the isolated bacteriophage by the spot test technique method. 10 µl of concentrated phage suspensions containing 10^9 pfu/ml was dropped onto a LB plate overlaid with different bacterial strains (10^8cfu/ml). The target plates were examined for lytic activity after incubation period for 24h at 30°C.

2.6.One-step growth curve of ϕRSNB1 phage:

The phage was added to the host bacterium *R. solanacearum* culture (10^8cfu/ml) at an MOI of 1.0 and the mixture was incubated at 28°C for 10min. Cells were collected by centrifugation at 9000 rpm for 10 min and were resuspended in 1 ml of fresh LB medium. This step was repeated twice in order to remove unadsorbed phage particles then the cell suspension added to the 100 ml LB broth and incubated with shaking at 28°C for 3 hours. Phage titer in the culture was measured by the double layer agar technique at a 15 min interval. The relative burst size at different times was plotted against time to determine the latent and rise period.

2.7.Effect of Temperature on the ϕRSNB1 phage stability:

Two ml of bacteriophage suspension (10^8pfu/ml) was incubated at different temperatures; 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90 and 95°C for 10 minutes. Phage infectivity was assayed qualitatively by the spot test.

2.8.Effect of pH on the ϕRSNB1 phage stability:

The stability of the isolated ϕRSNB1phage was carried out under different pH values according to the method of Jamalludeen et al. [17]. We carried out this experiment as the following; phage suspension was exposed to a specific pH value ranged from 2 to 10 using 0.1 M NaOH or HCl over 16 h of incubation at 30°C and then checked for survival by the spot test technique method.

2.9.Extraction of ϕRSNB1 phage genomic DNA and restriction analysis:

The phenol/chloroform method was used to extract the ϕRSNB1phage DNA according to Sambrook and Russell [18]. Some common restriction enzymes were used to digest the

genomic DNA according to the company instructions (Qiagen). Gel electrophoresis at 100 V in a 1.0% agarose gel was done for the digested DNA mixtures of ϕRSNB1 phageand were stained with ethidium bromide using a DNA ladder as marker.

RESULTS

3.1. Identification of *R. solanacearum* strains:

Seven strains of *Ralstoniasolanacearum* were originally isolated from different regions in the Northern Border Region, Saudi Arabia (Table 1). The strains were identified as *Ralstonia Solanacearum* on the basis of the morphology, and conventional biochemical tests. All the strains of *R. solanacearum* were tested for their ability to ϕRSNB1 phage infection by using them individually as an indicator.

Table 1: Susceptibility of different bacterial hosts to ϕRSNB1 phage.

Host	ϕRSNB1
***R. solanacearum* N1**	–
***R. solanacearum* N2**	+
***R. solanacearum* N3**	+
***R. solanacearum* N4**	+
***R. solanacearum* N5**	–
***R. solanacearum* N6**	+
***R. solanacearum* N7**	+
Bacillus cereus	-
***Escherichia coli* (ATCC 7839)**	-
***Pseudomonas aeruginosa* (ATCC 9027)**	-

(+) Indicates that the strain is susceptible to the phage and produces plaques, while (−) indicates that no plaques were observed.

3.2. Isolation of *R. solanacearum*ϕRSNB1phage:

Five soil samples were collected from different regions in the Northern Border Region, Saudi Arabia, were tested for the presence of bacteriophages specific for *R. solanacearum* using the spot test technique and the double over layer agar technique methods (Fig.1A, B). From these, one single plaque with 2 mm in diameter on lawns of *R. solanacearum*as host was isolated for further purification, and characterization.

3.3. Morphology and the host specificity of ϕRSNB1 phage:

The purified particles of ϕRSNB1 phagewas negatively stained and examined by transmission electron microscopyto determine the morphotype of the phage. The ϕRSNB1phage had an icosahedral capsid of 110±5 nm in diameter and long non-contractile tail with length of about 170±10 nm and width of about 25±2 nm. The ϕRSNB1 phageappeared to be a member of the *Myoviridae* family based on their structure in electron micrograph (Fig. 1C). The host range of ϕRSNB1phagewas determined against different strains of *R. solanacearum*.The results in Table (1) showed that ϕRSNB1 phagecould infect only fiveof sevendifferent*R. solanacearum* strains used as host in this study and could not infect other bacterial genera indicating a quite broad host range of this phage (Table 1).

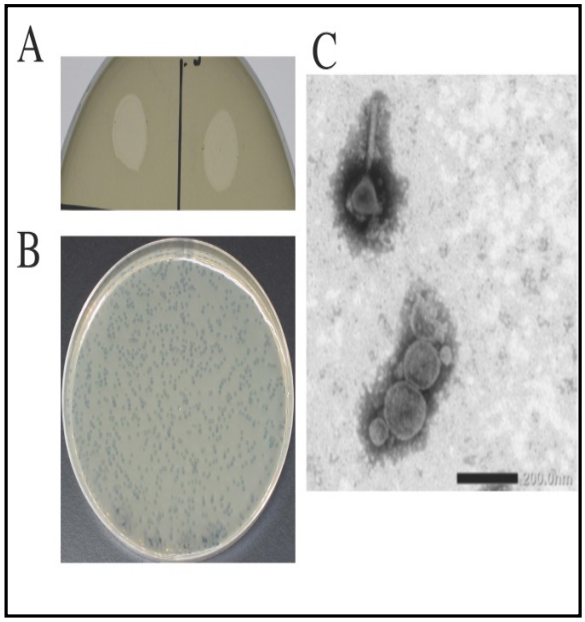

Fig. 1: **(A)** Spot test. 100 µl of ϕ**RSNB1**phage was spotted onto *R. solanacearum* as a host and incubation over night at 28°C. **(B)** Single plaques resulting from 10^{8} dilutions by overlayer agar technique. **(C)** Morphology of ϕ**RSNB1** phageparticles under TEM. Scale represents 200 nm.

3.4. One step growth curve of ϕRSNB1 phage:

The one step growth curve of ϕRSNB1 phage on *R. solanacearum*as a host was determined (Fig. 2). The growth characteristics of ϕRSNB1 phage revealed a latent period of 60 min, and the average burst size was 100 per infected cell (Fig.2).

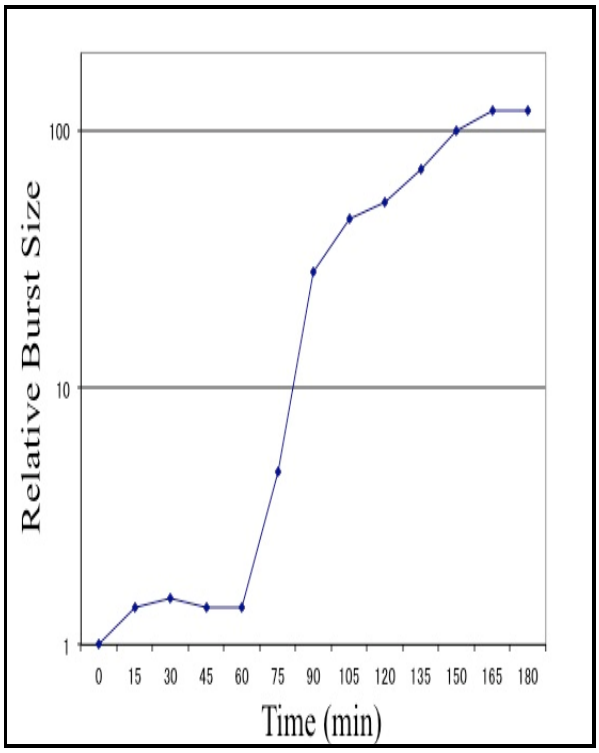

Fig. 2: One-step growth curve for ϕRSNB1 phage. The Relative burst size at different time post infections are shown. Samples ofϕRSNB1 phage were taken at intervals (every 15min up to 180 min).

3.5. Effect of Temperature and pHon the ϕRSNB1 phage stability:

The stability of ϕRSNB1 phage wasassayed by spot test and plaque assay methods. Data in Table (2) showed that ϕRSNB1 phage was extremely heat stable which is still remained active after 10 minutes exposure at 70°C; the phage loss its infectivity at 75°C Table (2). Different pH degrees on the ϕRSNB1 phage particles stability was examined at range of 2-10.

The results of the pH effect on the ϕRSNB1 phage is in Table 3 showed that ϕRSNB1 phage maintained its infectivity when incubated in a pH range between4 and 9.

However, the ϕRSNB1 phage could not be detected at less than pH 4 and more than 9.

Table 2: Effect of different Temperatures on the ϕRSNB1phage stability.

Temperature	Spot Test	PFU/ml
40°C	+	2×10^8
50°C	+	1.9×10^8
60°C	+	1.6×10^7
70°C	+	10^6
80°C	-	-

(+) Indicates that the ϕRSNB1phage produces plaques, while (−) indicates that no plaques were observed.

Table 3: Effect of different pH on the ϕRSNB1 phage stability.

pH	Spot Test	PFU/ml
2	-	-
3	-	-
4	+	8.5×10^7
5	+	10^8
6	+	1.9×10^8
7	+	2.1×10^8
8	+	3.5×10^8
9	-	-
10	-	-

(+) Indicates that the ϕRSNB1phage produces plaques, while (−) indicates that no plaques were observed.

3.6. Genomic characterization of *R. solanacearum* ϕRSNB1phage:

R. solanacearum ϕRSNB1phagegenomic DNA was extracted and treated with three different restriction enzymes (*Eco*RI, *Hinc*II and *Eco*RV). The genomic DNA of ϕRSNB1phagewas successfully cleavage indicating that ϕRSNB1 phagecontains dsDNA (Fig. 3).

The results in Fig. (3) showed separation patterns of ϕRSNB1 phage DNA fragments produced by *Eco*RI, *Hinc*II and *Eco*RV digestion. Generally, many bands appeared sizes summed up to 200 kbp in size.

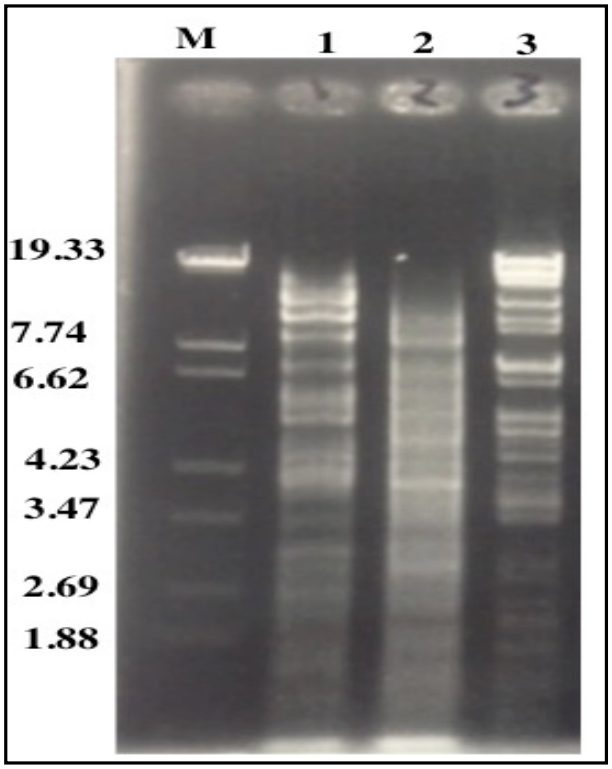

Fig. 3: Genomic DNA restriction enzyme digestion patterns of *R. solanacearum* ϕRSNB1phageand followed by resolution on 1 % agarose gel. (Lanes 1–3). **Lane 1**, ϕRSNB1 phagedigested with *Eco*RI; **Lane 2**, ϕRSNB1 phagedigested with *Hinc*II; **Lane 3**, ϕRSNB1phagedigested with *Eco*RV and (**M**) λ/StyI DNA Marker.

DISCUSSION

The wilt disease resulted from the infection by *R. solanacearum* bacterium is one the most problematic important plant diseases in the world. It affects many crops that haveeconomical importance in agricultural systems [19, 20]. It is difficult to control bacterial wilt disease in the soil [21]**.**Various control strategies and methods have been developed to control and suppress the disease, including host resistance and biological control[22]. Biocontrol of *R. solanacearum* has been described with either lytic or lysogenic bacteriophages and has been carried out in different ways [11, 12, 20, 23].

In this study, we isolated and characterized a ϕRSNB1phage infecting *R. solanacearum* from soil sample collected from the Northern Border Region, Saudi Arabia. The ϕRSNB1 phageshowed typical myovirus morphology with long, flexible and contractile tail of about 170±10 nm and width of about 25±2 nm and an isometric head of about 110±5 nm in diameter (Figure 1C). Interestingly, the morphology of ϕRSNB1phage showed a similarity to those previously reported for *R.solanacearum*specific phages [23, 24]**.**The ϕRSNB1phage was specific for *R.solanacearum,* anddid not infect any other bacteria tested (Table 1). Physical characteristics of ϕRSNB1phage including effect of heat and stability at different pH, and stability during storage were examined. Interestingly, the results in Table 2 showed that ϕRSNB1phage was thermostable, where is still remained active after 10 minutes exposure at 70°C.

Furthermore, the ϕRSNB1phage was able to grow in wide range of pH values (Table 2). Interestingly, the average size of the ϕRSNB1genome was estimated approximately 200 kbp from the sum of DNA bands generated using the restriction enzymes (*Hinc*II,*Eco*RI and *Eco*RV)digestion. A priority objective of our future research will be to identify the nucleotide sequence and protein of the isolated ϕRSNB1phage. In conclusion, we present ϕRSNB1; a myovirus phage isolated from the Northern Border Region, Saudi Arabia that is specific to different strains of *R.solanacearum.* We hope that the isolated ϕRSNB1 phage that is specific for *R. solanacearum* will be used as biocontrol against *R.solanacearum*pathogenand decipher specific rules regarding the host range of phage in our further study. Promising approaches to increase efficacy against bacterial wilt disease caused by *R.solanacearum* will be the integration of bacteriophages as biological control methods that can be combined with other biological control strategies into an integrated control program [20]**.**

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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