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Research Article

Biochemical and Molecular Characteristics of Pc1 Virulent Phage Isolate Infecting *Pectobacterium carotovorum*

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Abstract

Background and Objective: *Pectobacterium carotovorum* subsp. *carotovorum* is a plant-pathogenic bacterium. It is a post-harvest pathogen and causes soft rot diseases in infected plants. Different virulent bacteriophages have been isolated from different regions in the world. These bacteriophages were tolerant to high concentrations of calcium chloride and magnesium chloride. Whereas, the high concentrations of zinc chloride and aluminum chloride decreased the activity and stability of phages. Therefore, the present research aimed to study the biology of *P. carotovorum* phage (Pc1) by using a one-step growth experiment, its stability to different concentrations of some chemicals and molecular characteristics of this phage isolate. **Materials and Methods:** One step growth experiment, chemical stability, and molecular characteristics by using RAPD-PCR of *P. carotovorum* phage (Pc1) were studied. **Results:** The *P. carotovorum* phage (Pc1) isolate was found to have a latent period of 20 min and its burst size is about 92 pfu cell⁻¹. Calcium chloride, magnesium chloride, and copper sulphate (from 0.1-0.5 mM) increased the infectivity of Pc1 phage, while, zinc chloride in the same concentrations reduced its infectivity. RAPD-PCR amplification was indicated that the total amplified products were 32 bands with size ranged from 0.179-2.365 Kbp. **Conclusion:** Since, zinc chloride (at concentrations of 0.1-0.5 mM) reduced infectivity of Pc1 phage isolate, therefore, any chemical compounds containing zinc must be avoided in designing biocontrol strategy by using phages against soft rot bacterium (*P. carotovorum*) in potatoes.

Key words: *Pectobacterium carotovorum*, phage, one-step growth experiment, chemical stability, RAPD-PCR

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pectobacterium carotovorum subsp. *carotovorum* is a plant-pathogenic bacterium which produces different enzymes that analyze plant cell wall to cause soft rot diseases in infected plants¹⁻³. *Pectobacterium carotovorum* is a post-harvest pathogen that causes economic losses in potato wherever it is stored. Potato is an important food crop after wheat, rice, and corn. It is grown worldwide and propagates vegetatively⁴. *Pectobacterium* spp. are soft rot enterobacterial pathogens of wide host ranges and global distribution. These bacteria were known formerly as the soft rot *Erwinia* spp.⁵⁻⁷.

Several and different virulent phages have been isolated from different regions in the world. Lim *et al.*⁸ studied the host range to virulent phage (PP1) specific to *Pectobacterium carotovorum* subsp. *Carotovorum* and found that PP1 phage showed high specificity for *P. carotovorum* subsp. *Carotovorum* and several bacteria belonging to different species. These phages can be used as a novel biocontrol agent against plant-pathogenic bacteria⁹⁻¹².

The effect of different metal ions concentrations (from 0.1-0.5 mM) on the stability and infectivity of phages was studied by Paunika *et al.*¹³, Al-Khazindar *et al.*¹⁴. They found that the higher concentrations of calcium chloride and magnesium chloride increased the activity and stability of phages, whereas, the higher concentrations of zinc chloride and aluminum chloride decreased the activity and stability of phages.

RAPD-PCR technique was used to study the Enterobacter phages, quick typing, and detection of phage isolates. Molecular characterization of *P. carotovora* subsp. *Carotovora* virulent bacteriophages was studied by Toth *et al.*¹⁵, Gill *et al.*¹⁶, Lehman *et al.*¹⁷, Müller *et al.*¹⁸. They found that the virulent phage isolates containing ds-DNA.

The present research aimed to study the biology of *P. carotovorum* phage (Pc1) by using a one-step growth experiment. Moreover, the effect of some chemicals (i.e., CaCl₂.2H₂O, MgCl₂.6H₂O, CuSO₄.5H₂O and ZnCl₂) at different concentrations on Pc1 phage infectivity and molecular characteristics of this phage isolate (DNA restriction pattern and RAPD-PCR) were also studied.

MATERIALS AND METHODS

This study was carried out during the year 2018-2019 in the Department of Microbiology, Faculty of Agriculture, Ain Shams University.

Bacterial host and its specific phage: *Pectobacterium carotovorum* was kindly supplied by Department of Microbiology, Faculty of Agriculture, Minia University, Minia, Egypt.

Pc1 bacteriophage is specific to *P. carotovorum* which was previously isolated and characterized by Marei *et al.*¹² used in this study.

Propagation of Pc1 phage isolate: Liquid enrichment technique was carried out by using nutrient broth medium¹⁹ as described by Sambrook *et al.*²⁰ to prepare high titer suspension of Pc1 phage. A titer of the prepared suspension was determined according to Kiraly *et al.*²¹

One-step growth experiment of Pc1 phage: The method of Ellis and Delbruck²² modified by Czajkowski *et al.*²³ was used to carry out the one-step growth experiment. The burst size was estimated according to Sharaf *et al.*²⁴.

Effect of different chemicals on Pc1 phage: Different chemicals (i.e., CaCl₂.2H₂O, MgCl₂.6H₂O, CuSO₄.5H₂O, and ZnCl₂) each at a concentration of 100 mM was prepared and diluted into 0.1, 0.2, 0.3, 0.4 and 0.5 mM^{12,13}. Effect of these chemicals on Pc1 phage was tested as described by Czajkowski *et al.*²⁵, Hammad *et al.*²⁶.

Extraction of bacteriophage DNA: High titer phage suspension (15 mL) was purified and precipitated as described by Marei *et al.*¹². The pellet (Pc1 phage particles) was re-suspended in 500 µL of SM medium²⁶. Genomic DNA of Pc1 phage isolate was extracted as described by Maniatis *et al.*²⁷

Determination of DNA concentration: The concentration of Pc1 phage extracted DNA was estimated by using optical density at 260 nm by UV-1601-UV/VIS Japan, spectrophotometer. The quality of the extracted DNA was assayed by using the 260-280 nm ratio and gel electrophoresis.

Digestion of Pc1 phage DNA: The DNA restriction pattern of the Pc1 phage isolate was studied. DNA was digested with Hind III as described by Peacock and Dingman²⁸.

Table 1: Nucleotide sequence of the used primers

Primer name	Primer sequence (5'-3')
OPH-01	GGTCGGAGAA
OPH-02	TCGGACGTGA
SCoT-7	ACAATGGCTACCACTGAC
SCoT-8	ACAATGGCTACCACTGAG
SCoT-9	ACAATGGCTACCACTGCC

Bands were documented and analyzed in the Genetics Department, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh, Egypt as described by Marei²⁹.

RAPD-PCR reaction of Pc1 phage: PCR amplification was carried out by using 2 random 10-mer arbitrary primers as described by Marei²⁹. Sequences of the used RAPD primers were presented in Table 1.

The PCR amplification was carried out according to Williams *et al.*³⁰ as described by Marei²⁹.

Bands were documented and analyzed in Genetics Department, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh, Egypt as described by Marei²⁹.

RESULTS

One step growth experiment of Pc1 phage: The one-step growth curve analysis was used to determine the latent period and the burst sizes of Pc1 phage (Fig. 1). The latent period of Pc1 phage was estimated to be 20 min. The burst size was calculated to be 92 pfu cell⁻¹.

Chemical stability of Pc1 phage: Effects of four chemicals (i.e., CaCl₂.2H₂O, MgCl₂.6H₂O, CuSO₄.5H₂O and ZnCl₂) with different concentrations (0.1-0.5 mM) on infectivity of Pc1 bacteriophage were tested. As shown in Fig. 2 the tested chemicals varied in their effect on the infectivity of Pc1 phage. The highest numbers of phage particles were recorded in the presence of calcium chloride as compared to the other chemicals tested. The numbers of Pc1 phage particles increased gradually with increasing the concentration of calcium chloride. The highest number of Pc1 phage particles was achieved at 0.5 mM.

Magnesium chloride and copper sulphate exhibited a stimulative effect on the infectivity of Pc1 phage. Both chemicals increased the number of phage particles with increasing the concentration. The highest numbers were recorded at a concentration of 0.4 mM magnesium chloride and 0.5 mM copper sulphate.

On the other hand, lower numbers of Pc1 phage were recorded in the presence of zinc chloride at any concentration as compared to the other chemicals.

Molecular characteristics of Pc1 phage

Genomic DNA concentration and purity: DNA concentration of the Pc1 phage isolate was estimated to be 515.62 µg mL⁻¹. The DNA purity of the Pc1 phage isolate was calculated to be 1.83 as the ratio of A260/A280.

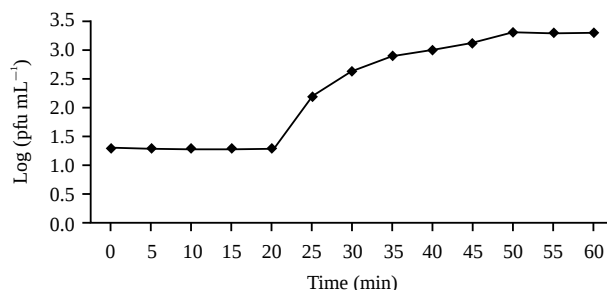


Fig. 1: One-step growth curve of Pc1 lytic phage specific to *P. carotovorum*

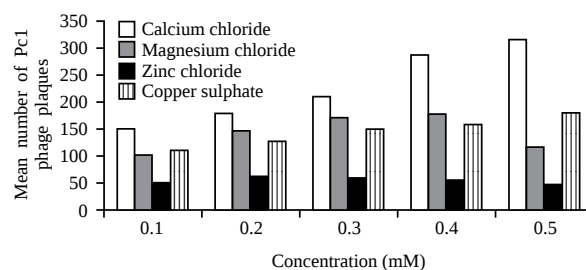


Fig. 2: Effect of different chemicals concentrations on the chemical stability and activity of Pc1 phage

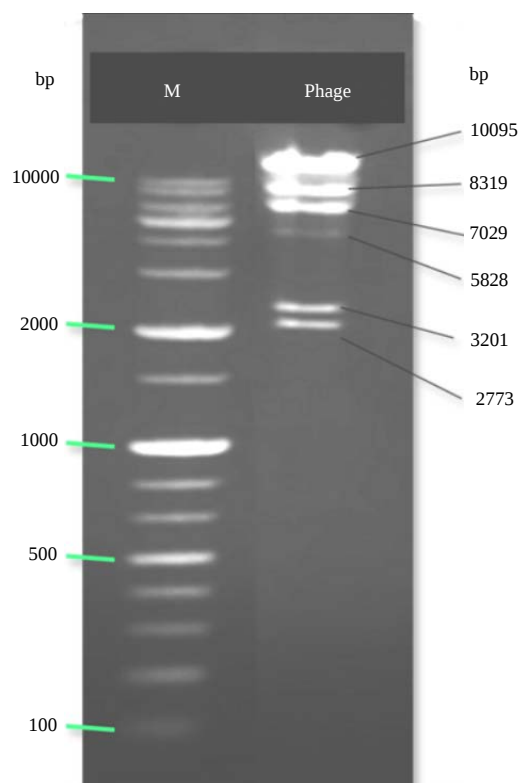


Fig. 3: DNA restriction pattern of Pc1 phage DNA. M = 1Kb (+) DNA ladder

DNA restriction pattern: The extracted DNA of the Pc1 phage isolate was digested with *Hind* III. Six fragments were produced due to digestion with *Hind* III (Fig. 3). The molecular sizes of the fragments ranged from 2773-10095 bp.

RAPD-PCR of Pc1 Phage: The polymorphic DNA products of Pc1 phage were determined by using RAPD-PCR. Reproducible polymorphic DNA products were generated by five primers. Thirty two bands were the total amplified products and their sizes ranged from 0.179 -2.365 Kbp.

Nine bands were generated by OPH-01 as RAPD-PCR amplified products. There bands were 6 monomorphic and 3 polymorphic when compared with other primers. OPH-02 had 3 bands (3 monomorphic when compared another primer), SCOT-7 had 5 bands (5 monomorphic when compared with other primers), SCOT-8 had 5 bands (5 monomorphic when compared with other primers) SCOT-9 had 10 bands (8 monomorphic and 2 polymorphic when compared with other primers). The results are analyzed by using the size of

amplified fragments ranged from 0.179-2.365 Kbp. Table 2 as illustrated in Fig. 4. Five bands were unique polymorphic with OPH-01 and SCOT-9 used primers when compared with other primers.

DISCUSSION

Soft rot bacteria *Erwinia carotovora* subsp. *carotovora* (Ecc) can infect potato and causes losses in potato yield which considered one of the most important vegetable crops³¹.

The virulent phage isolate of *P. carotovorum* (Pc1) was previously used against soft rot disease in potato tubers as a bio-control agent¹².

One-step growth experiment of *P. carotovorum* virulent phage (Pc1) isolates observed that the latent period was 20 min and the burst size was about 92 pfu cell⁻¹. Similar results were obtained by Czajkowski *et al.*²⁵ who found that the latent period of phage ϕ PD23.1 infecting *P. carotovorum* subsp. *carotovorum wasabiae* 3193 was found to be 20 min and the burst size was 95 $\pm$ 5 pfu cell⁻¹. Moreover, Czajkowski *et al.*³², found that the latent period of ϕ D7 phage specific to *Dickeya dadantii* and *D. solani* was 20-30 min and the burst size was 87 $\pm$ 8 pfu cell⁻¹.

The increase in concentrations of calcium chloride, magnesium chloride, and copper sulphate (0.1-0.5 mM) increased the infectivity of *P. carotovorum* virulent phage (Pc1) isolate. While the increase in the concentration of zinc chloride (from 0.1-0.5 mM) resulted in a reduction of infectivity and activity of Pc1 phage. This may be due to that metal ions are an integral part of some viral proteins and play an important role in their survival and pathogenesis. The commonest metal ion that binds with viral proteins is Zinc³³. These results are in agreement with those obtained by Paunika *et al.*¹³, AlKhazindar *et al.*¹⁴.

The DNA of the Pc1 phage isolate was digested with *Hind* III. The results showed that the viral genome of Pc1 phage was linear double-strand (ds) DNA. This result is in agreement with those of^{34,35}.

RAPD-PCR analysis of Pc1 virulent phage DNA showed that among the total amplified products 32 bands with all used different primers, five bands are unique polymorphic with OPH-01 and SCOT-9 used primers when compared to other primers. The size of the amplified fragments ranged from 0.179-2.365 Kbp. Similar results were obtained by Vesa and Kristina³⁶, Gutierrez *et al.*³⁷.

In this study, the different characteristics of this phage were determined to provide suitable conditions for

Table 2: Number of bands and molecular size by using RAPD-PCR

Primer name	Total number of amplified bands	Molecular size of bands (Kbp)
OPH-01	9	0.289-2.099
OPH-02	3	0.289-2.365
SCoT-7	5	0.351-2.365
SCoT-8	5	0.179-0.424
SCoT-9	10	0.179-1.389

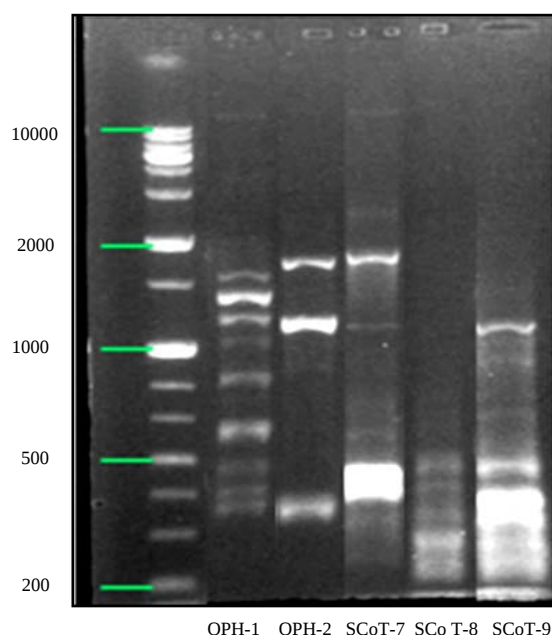


Fig. 4: RAPD-PCR products of Pc1 phage DNA generated by five primers
M = 1 Kb (+) DNA ladder

this phage in designing a biocontrol strategy against soft rot bacterium *Pectobacterium carotovorum* in potatoes. e.g., any chemical compounds containing zinc must be avoided.

CONCLUSION

Bacteriophage, Pc1 specific to *Pectobacterium carotovorum* was previously used as a biocontrol agent against soft rot bacterium (*P. carotovorum*) in potatoes. Therefore, it is of particular interest to study the characteristics of this phage to provide suitable conditions for the phage in designing a biocontrol strategy. This phage was found to have a latent period of 20 min and its burst size is about 92 pfu cell⁻¹. Calcium chloride, magnesium chloride, and copper sulphate increased the infectivity of Pc1 phage, while, zinc chloride reduced its infectivity. Therefore, any chemical compounds containing zinc must be avoided in designing biocontrol strategy.

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SIGNIFICANT STATEMENT

Bacteriophage Pc1 of *Pectobacterium carotovorum* was previously used as a biocontrol agent. This study confirmed that *P. carotovorum* phage (Pc1) isolate was found to be stable and active in different conditions of calcium chloride, magnesium chloride, and copper sulphate (at concentrations of 0.1-0.5 mM), while, zinc chloride in the same concentrations reduced its infectivity. In this study, the different characteristics of this phage were determined to provide suitable conditions for this phage in designing a biocontrol strategy against soft rot bacterium *Pectobacterium carotovorum* in potatoes. e.g. any chemical compounds containing zinc must be avoided.

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