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Characterization of a newly isolated *Bacillus subtilis* virus vB_BsuS-Bot1 from soil

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The discovery of *Bacillus subtilis* phages is very important about their application in the agroindustry. We present the recently isolated soil-borne virus infecting *Bacillus subtilis*. The phage was designated as vB_BsuS-Bot1. It was propagated by the method of liquid enrichment and purified by polyethylene glycol and dextran sulfate two-phase system followed by centrifugation at high speed. Morphological investigation via transmission electron microscopy (TEM) revealed characteristics of the Siphoviridae family, but with a thin tail of virion particle of approximately 7 nm width and 180 nm length. Phage host range and sensitivity analyses exhibiting a narrow host range. The complete infection cycle of vB_BsuS-Bot1 was 120 min with a burst size of approximately 66 PFU/cell and a latent period of 50 min. Its optimal growth temperature was 30 °C, the optimum pH value was 7.0 and it was inactivated after 30 min from exposure to Ultraviolet (UV) irradiation. The virus lost its activity after storing it at -20 °C for 7 weeks. The genome of isolated vB_BsuS-Bot1 had double-stranded DNA. Electrophoresis of the SDS polyacrylamide gel of purified phage proteins was investigated. Protein pattern of the vB_BsuS-Bot1 revealed 9 structural proteins ranging from 34.7 to 88.1 kDa compared with the standard or markers proteins. The antiserum specific for vB_BsuS-Bot1 was prepared and serological identity analysis of vB_BsuS-Bot1 revealed the formation of precipitation bands against specific rabbit antiserum (As). This study has been performed to expand the knowledge of this virus isolated from wheat cultivated soil and infecting *Bacillus subtilis.*

Keywords: *Bacillus subtilis*, Bacteriophage, Characterization, Phage stability, Serology

INTRODUCTION

Soils are critical to global biogeochemical cycles that support all terrestrial ecosystems for biological diversity and the soil is a complex habitat for many different microorganisms such as the Gram-positive organism *Bacillus subtilis*, a member of the *Firmicutes* phylum. *Bacillus subtilis* (*B. subtilis*) is a facultative anaerobic, rod-shaped and endospore-forming bacterium (Logan and De Vos, 2009), which produces a wide array of extracellular enzymes and metabolites; therefore it plays an important role in soil and promote plant growth. The microbe was used previously as seed inoculant for wheat and pea (Arkhipova et al .2019; Khan et al. 2018). Streptomycin resistant strain of *B. subtilis* is an antagonistic to many plant pathogenic fungi was used in seed bacterization with some economically crop plants to inhibit root pathogens and improve wheat growth and yield (Milus and Rothrock, 1993; Wang et al. 2018, Khan et al. 2018). A phage is a virus that infects bacteria as a host. Because of the recent problems of antibiotic resistance, the control of harmful bacteria using a phage has been given considerable attention (Shin et al. 2011). The phage that infects beneficial bacteria

is therefore as important as those that kill pathogenic bacteria. Beneficial bacteria is susceptible for infection by many bacterial viruses (phages) causing a reduction of the total numbers in the soil (Furrer et al. 2020). Different authors isolated the phages specific for *Bacillus* from the free and rhizospheric soils cultivated with different plants and as well as from raw sewage (Okubo 1966; Walter and Baker, 2003; Furrer et al. 2020; Willms et al. 2017). We used *B. subtilis* as a bacterial host in this study to isolate the phage. Investigation of *B. subtilis* phages is as old as the establishment of the model organism itself (Hemphill and Whiteley, 1975); however, even for such an established and well-studied organism (Hedges, 2002), there are not many publicly available phage isolates. Phage-related *B. subtilis* investigations will help better assess the risks to agricultural application and better understand the diversity of viral predators in the environment. Here we characterized a new *B. subtilis* infecting phage isolated from wheat cultivated soil in Egypt.

MATERIALS AND METHODS

Assaying of total counts of soil bacteria

Soil extract agar medium was used for assaying of the soil bacteria and the spore-forming bacteria quantitatively by plate counts technique according to Ameh and Kawo (2017).

Detection of *Bacillus subtilis* phages

Twenty soil samples were collected between May and June 2019 from the soil cultivated with wheat at the farm of Agricultural Microbiology Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. We used the *Bacillus subtilis* laboratory strain (Othman, 1997), as the host strain for phage isolation. *B. subtilis* was grown in a test tube with 3 mL Luria–Bertani (LB) medium at 30 °C under shaking. Erlenmeyer flask (250 ml) containing 50 ml of LB was inoculated with 5 g of sieved soil; suspension of *B. subtilis* cells (10^8 CFU/ml) was added. The inoculated flask was incubated at 30 °C for 72 h, after incubation, the flask contents were clarified and centrifuged at 6000 rpm for 15 min, and then the supernatant was filtered through Millipore syringe filter (0.45um) to remove any contaminated bacteria from supernatant. Occurrence of phages specific for *B. subtilis* was detected in the prepared filtered suspension by spot test according to Othman (1997). Decimal dilutions of prepared virus suspension were done and the phages titres were obtained by the overlayer agar (plaque assay technique) according to Adams (1959).

Single plaque isolation

From the plates resulted from the plaque assay technique, single plaques were picked up depending on their morphology as diameter sizes, shape, presence or absence of halo and plaque clearness, and then single plaques were transferred to test tubes containing 2-3 ml suspension of *B. subtitles* cells (10^8 CFU/ml) followed by incubation at 30 °C for 24 – 48 h to obtain pure isolates of phages, the process repeated twice to confirm the purity.

Propagation of *Bacillus subtilis* phage

The propagation of phage was carried out using the liquid culture method (Sambrook et al., 1989). In brief, 1 ml of the *Bacillus subtilis* culture of the mid-exponential process (OD_{600} = 0.45-0.55) was inoculated to 100 ml of fresh Luria–Bertani (LB) medium containing 10 mM $CaCl_2$. Added to this 1 ml of phage lysate with at least 10^8 PFU/ml and aerobically incubated at 30 °C, shaking at 160 rpm for 12 h, or until lysis occurred. At 4°C the mixture was centrifuged for 10 min at 6000 rpm. The supernatant was filtered with (0.2 µm) syringe filter. The phage titre present in the lysate was estimated using plaque assay technique (Adams, 1959). High titre phage lysates were held at 4 °C until concentrated using polyethylene glycol (PEG).

Concentration and purification of the obtained phage stock lysates

The concentration of phage was carried out using a method described by Ackermann (2009). Dextran sulfate polyethylene glycol system was used for purification of the phage particles (Othman et al., 2008). The phage suspension was kept at 4 °C for further studies.

Determination of size and shape of viral particles

Drop of the high titer purified viral suspension was placed on a coated carbon copper grid for 2 min, then the prepared virus was negatively stained with 2 % (w/v) phosphotungstic acid (PTA) pH 6.8 and the preparation was examined under the electron microscope (Japan Electron Optics Laboratory Co., Ltd) at Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

Characterization of *B. subtilis* phage

Determination of host range and virus sensitivity

The virus host range was determined through plaque assays defined for virus isolation and spot test. For the latter, concentrated virion solution was spotted on nine different *Bacillus* strains on the overlay plates (see Table 1) and incubated for 24h at 30 °C. Bacterial strains that exhibit clear area at the phage suspension drop location were considered sensitive to the virus.

Determination of multiplicity of infection (MOI) and one-step growth curve

The optimal multiplicity of infection (MOI) of the phage was determined according to Li and Zhang (2014), *B. subtilis* used as host strain at 3 different MOI (0.01, 0.1 and 1) for 12 hours at 30 °C. For the one-step growth curve experiment, 1 mL of an overnight culture of *B. subtilis* (10^8 CFU mL^{-1}) was mixed with 100 µl of phage suspension, at the previously determined optimal MOI (0.1). After 15 minutes of incubation at 30 °C, the suspension was centrifuged at 6000 rpm for 3 minutes. Pellet was resuspended on LB broth in 2 mL. 100 µl of this broth was transferred to 50 mL of LB broth and incubated at 30 °C. Samples (in duplicate) were taken for 150 minutes every 10 minutes, and tested by the technique of double agar overlay (Adams, 1959).

Determination of virus stability

Test tubes containing of two ml of the crude lysate of the phage were exposed to different degree of temperature at 10, 20, 30, 40, 50, 60, 70, 80, 90 and100 °C for 10 min. followed by direct cooled water, then, assayed quantitatively to determine the thermal stability of the virus (Czajkowski et al., 2014).

In order to assess the stability of the phage against different pH values, pH ranging from 2 to 12 was tested, as described by Czajkowski et al. (2014).

To determine the phage stability under UV irradiation exposure, the UV lamp used was with a wavelength of 254.0 nm (general electric germicidal lamp). Phage suspensions were diluted and irradiated in petri dishes 30 cm from the light source. Samples taken at various intervals of UV light exposure were diluted in the LB broth and checked for a viable phage (Czajkowski et al. 2014).

Phage stock lysate samples were used to determine the stability of the phage after storing at 37°C, 4°C, -20°C over 6 months. Phage suspensions diluted in each sample, and assayed for surviving phage. The results of the virus stability were represented as a percentage of survival phage particles. All the experiments were performed in triplicates.

Extraction and detection of phage nucleic acid

Phage nucleic acid was extracted by using the phenol/chloroform method according to Sambrook and Russell (2001). Phage nucleic acid was treated with DNase I and RNase A, according to the supplier instructions (Thermo Scientific). The nucleic acid treated mixtures were analyzed by electrophoresis at 100 V in a 1.0% agarose gel stained with ethidium bromide using a DNA ladder as marker.

Determination of SDS–PAGE profile of virus particle

High titre phage was pelleted by cooling centrifugation and the pellets were resuspended in the phosphate buffer pH 7.2. Phage samples (50 µl) were added to 50 µl of Laemmli buffer containing 2- Mercapoethanol and boiled for 5-10 min, and then the viral particles were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) and stained by Coomassie brilliant blue R-250 (Bio-Rad, USA).

Serological characters of Bacillus subtilis phage

Two rabbits (white Newzeland) were immunized to produce the specific phage antiserum. Before immunization, the normal serum was taken as control. Purified phage (10^8 PFU/ml) was used for injection as antigen for injection intravenously in the marginal ear vein. Each rabbit was received 4 intravenous injections for production of antiserum, which the rabbit was immunized by injection with 0.2 ml of purified phage intravenously one a week for four consecutive weeks (Van Elsas and Penido 1981, Othman et al. 2008).

Ouchterlony double diffusion test

To determine the serological relationship between the antigen and specific rabbit antiserum, immunodiffusion test was carried out according to Ouchterlony (1968).

Electro-immunoassay (Rocket test)

The method was done as described by Laurell and Mckay (1981). In the technique, the antigen

was drown into a medium contain antiserum, where the two reactants meet in optimum proportions precipitation occurs in the form of cone or rocket. The area of precipitation was proportional to the quantity of the antigen.

RESULTS

Count of bacteria from cultivated soil

Soil sampled from the farm of Agricultural Microbiology Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt yielded $1.6x10^3$ and $4.3x10^2$ CFU/g for total counts of soil bacteria and total count of the spore forming bacteria respectively.

Isolation of *B. subtilis* phage

Viruses specific for *Bacillus subtilis* in the samples were assayed qualitatively by the spot test and the result showed positive reaction then quantitatively by the double over layer agar method and it was found that, titer or concentration of viruses was 9 $x10^5$ PFU/ml in the free soil and 4 $x10^5$ PFU/ml in the rhizosepheric soil.

From the plaques resulting from the plaque assay technique a single plaque with central clear area without halo with diameter of about 2 mm was picked up to prepare phage stock lysate. Two ml of 24 h incubation *B. subtilis* liquid culture was inoculated with obtained single plaque and incubated 24 h at 30 °C. The infected liquid culture was then centrifuged at 6000 rpm for 15 min. the supernatant was filtered through 0.22um Millipore filter to obtain purified phage lysate. This process was repeated twice to be sure that the isolated virus is purified (the particle have the same size and shape).

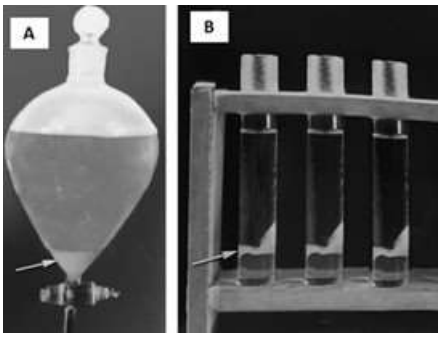

Figure 1: Purification and concentration of VB_BsuS-Bot1 by dextran sulphate-polyethylene glycol, two phase system. (A): Turbid precipitates in separating funnel containing the virus particles. (B): Intermediate phase (cake) containing the phage particles.

The biologically purified *Bacillus* phage was propagated by liquid enrichment method to obtain a large amount, and then the propagated phage was purified by dextran sulfate-polyethylene glycol two phases system (Figure 1) before characterization, and phage lysates were assayed by double agar plate method and the final titer was $9.5x10^8$ PFU/ml. The naming of phage isolate was based on Kropinski's proposed systematic scheme 2009 as vB_BsuS-Bot1 (Kropinski et al. 2009).

Morphology of the phage

The particle morphology of the isolated phage was determined by the transmission electron microscopy after negatively staining by 2 % PTA. As shown in Figure 2, a virion (vB_BsuS-Bot1) with head and tail morphology, typical of the Caudovirales order. The viral particle has long non contractile tail revealed that is a member Siphoviridae family. Tail dimensions of about 180 nm in length and about 7 nm in width in addition to isometric head with diameter of about 58.6 nm.

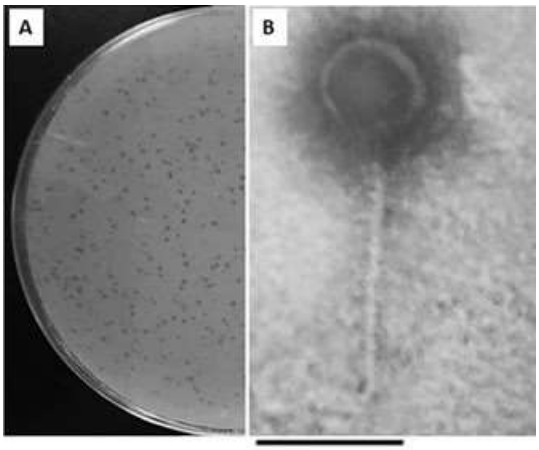

Figure 2: Characterization of bacteriophage VB_BsuS-Bot1 (A): Plaque morphology of VB_BsuS-Bot1 (B): Transmission electron micrograph of purified VB_BsuS-Bot1 (The scale bar represents 100nm).

Characters of vB_BsuS-Bot1

Phage host range

Phage vB_BsuS-Bot1 exhibited a narrow host range against different *Bacillus* strains. VB_BsuS-Bot1 showed only lytic activity on different strains of *B. subtilis*, and it did not infect other *B. subtilis* strains (Table 1). Plaque assays were carried out with the sensitive strains and observed on all strains, demonstrating their suitability for multiplication of phage.

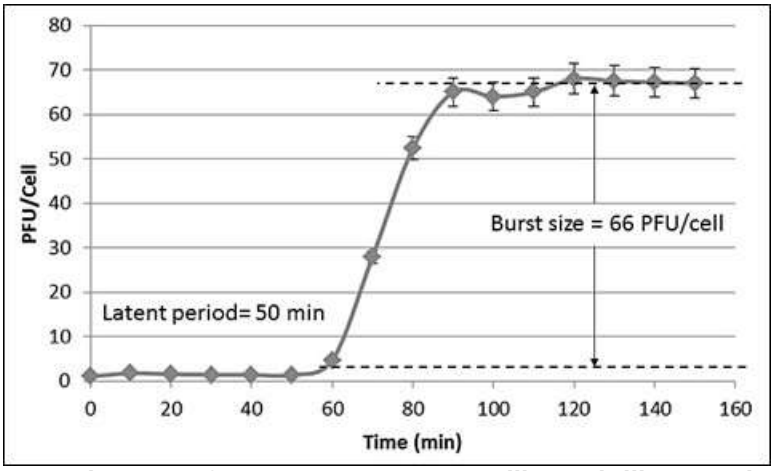

Figure 3: One step growth curve of vB_BsuS-Bot1 on *Bacillus subtilis*. Error bars represent ± the standard deviation.

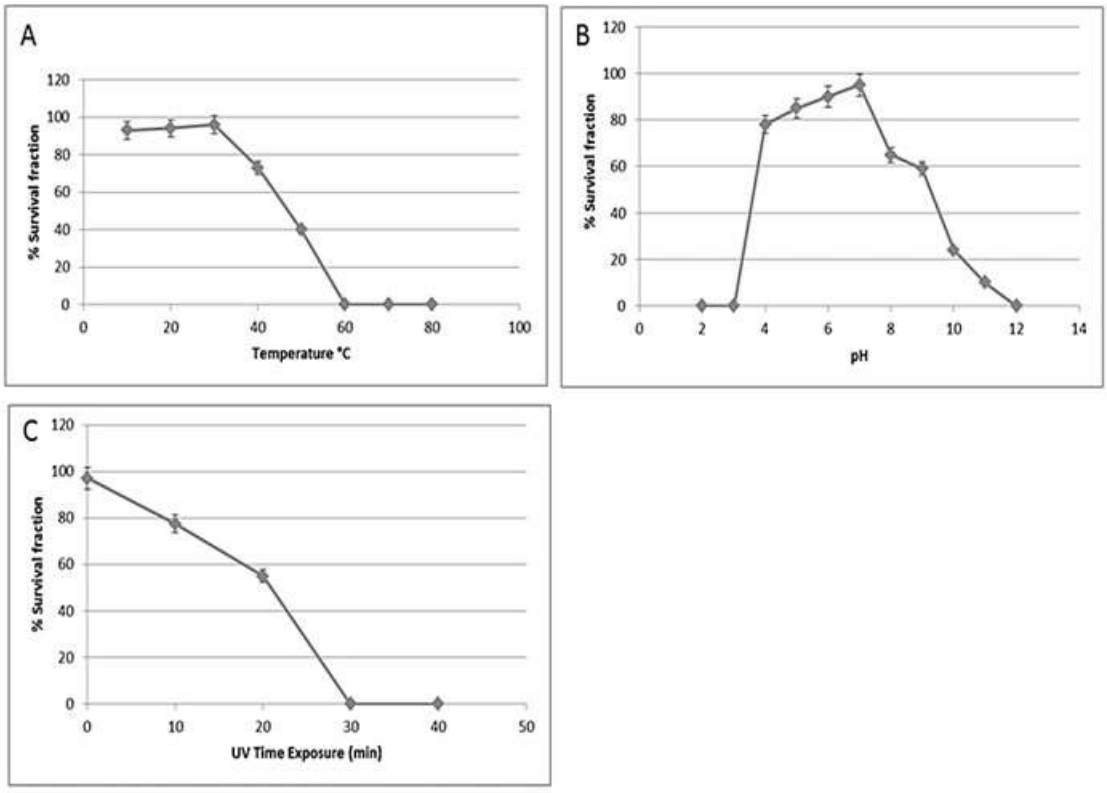

Figure 4: (A): Effect of temperature on VB_BsuS-Bot1 stability checked at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 °C after 10 min. (B): Effect of pH on phage stability, VB_BsuS-Bot1 lysate was treated at different pH values (2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) for one hour at 37 °C. (C): Effect of UV irradiation on the phage stability at distance 30 cm for 10, 20, 30, 40 and 50 min. Error bars represent ± the standard deviation.

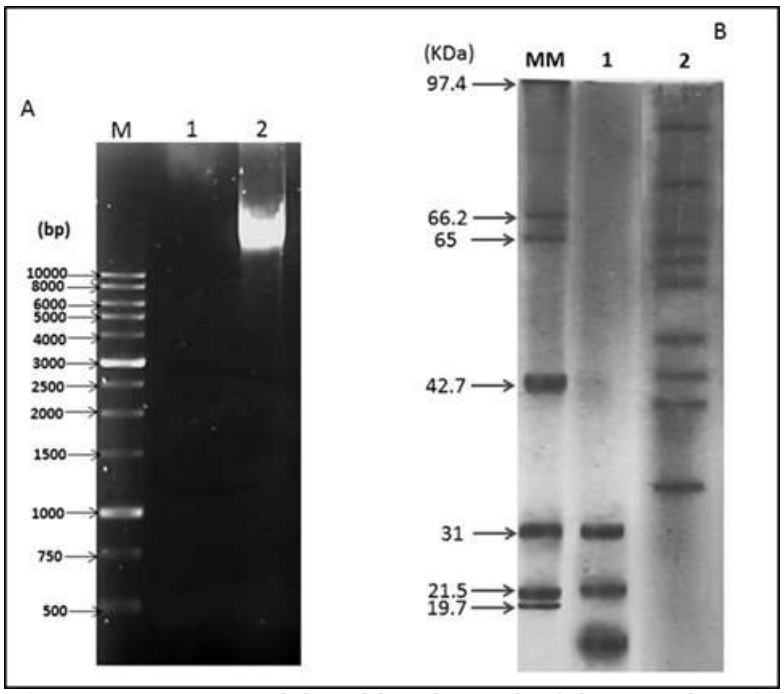

Figure 5: Analysis of vB_BsuS-Bot1 nucleic acid and protein. (A): Detection of phage nucleic acid. M: DNA marker; lane1: vB_BsuS-Bot1 DNA treated with DNase I; lane 2: vB_BsuS-Bot1 DNA treated with RNase A; (B) SDS-polyacrylamide gel electrophoresis analysis of vB_BsuS-Bot1 structural proteins. Protein bands were visualized by staining the gel with Coomassie brilliant blue R-250. MM: Medium molecular weight protein marker, lane 1: (LM) Low molecular weight protein marker, lane 2: The purified vB_BsuS-Bot1.

Table 1: Host range specificity of the isolated vB_BsuS-Bot1

Bacterial species	Source	Reaction	
		Spot test	Plaque assay
B. subtilis	Lab isolate, Ain Shams Univ.	+	+
B. subtilis 110	Lab isolate, Ain Shams Univ.	+	+
B. subtilis 136	Lab isolate, Ain Shams Univ.	+	+
B. cereus	Lab isolate, Ain Shams Univ.	-	-
B. circulans	Lab isolate, Ain Shams Univ.	-	-
B. thuringiensis	Lab isolate, Ain Shams Univ.	-	-
B. polymyxa 201	Lab isolate, Ain Shams Univ.	-	-
B. polymyxa 203	Lab isolate, Ain Shams Univ.	-	-
B. megaterium	Lab isolate, Ain Shams Univ.	-	-

A positive indication (+) means that the strain is sensitive to the phage, while a negative indication (-) means that no lysis or and no plaques were observed.

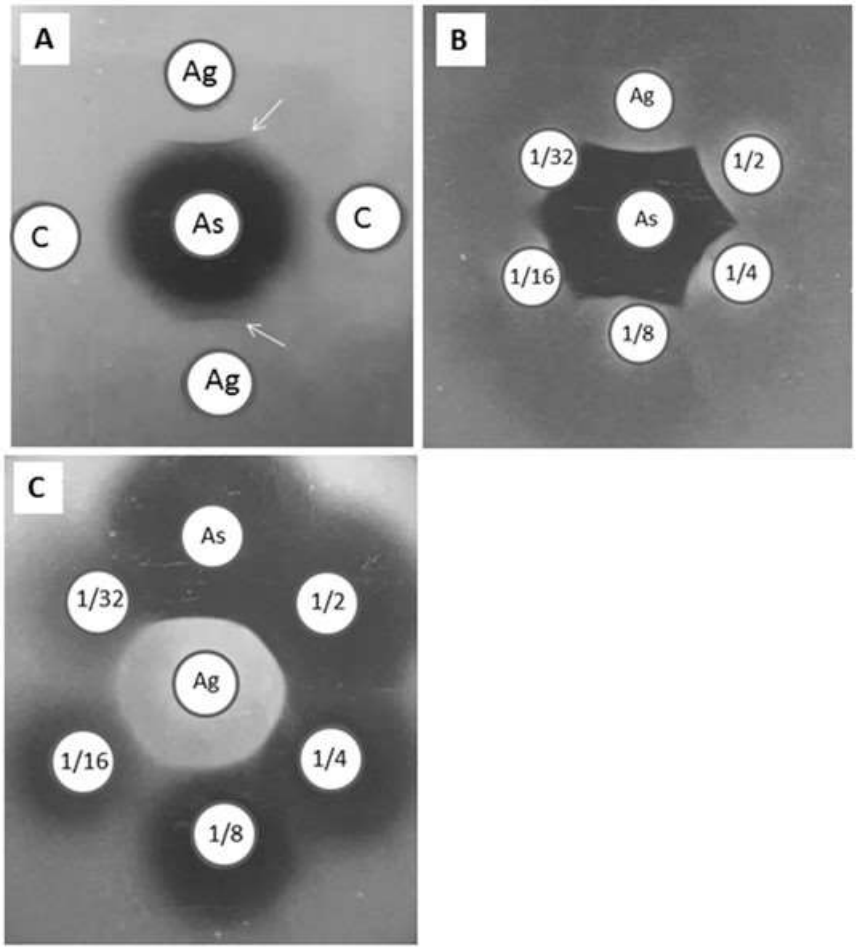

Figure 6: Serological analysis of vB_BsuS-Bot1 (A): Double diffusion test, central well contains antiserum (As) and two peripheral wells contain vB_BsuS-Bot1 (Ag) and other two wells contain phosphate buffer (c) as control. (B): Immuno diffusion test for determination of virus concentration, central well contains antiserum (As) to vB_BsuS-Bot1 and peripheral wells contain vB_BsuS-Bot1 (Ag), ½, ¼, 1/8, 1/16, 1/32 dilutions of antigen. (C): Immuno diffusion test for antiserum concentration, central well contains vB_BsuS-Bot1 (Ag) and peripheral wells contain antiserum (As), ½, ¼, 1/8, 1/16, 1/32 dilutions of antiserum.

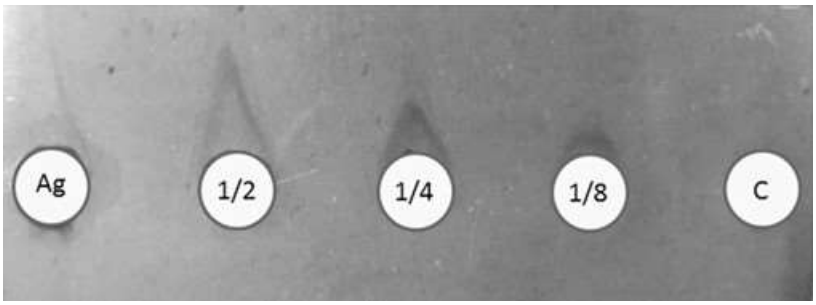

Figure 7: Immunoelectrophoresis technique, the wells contains 20 µl of purified vB_BsuS-Bot1 (Ag) and ½, ¼, 1/8 dilutions of antigen, (c) contain phosphate buffer as a control. The agarose contains specific antiserum (As) showed rocket shape of precipitation related to the concentrations of Ag.

Multiplicity of infection (MOI) and one-step growth curve

Phage vB_BsuS-Bot1 showed an optimal MOI of 0.1, a value used to carry out the assay of the one-step growth curve. A burst size of ~ 66 PFU/cell was observed with a latent period of ~ 50 min and 120 min for complete infection cycle (Figure 3).

Stability of the phage

As shown in Figure (4A), vB_BsuS-Bot1 had stable infection after incubation at 10, 20, and 30°C, more than 70% of vB_BsuS-Bot1 was still active after incubation at 40 °C for 10 min and lost its activity at 60 °C.

The optimum pH value for investigated vB_BsuS-Bot1 was 7.0 but its numbers was reduced in basic pHs (8-11) after incubation (Figure 4 B).Phage was inactivated in pH 3 and pH 12 after 1 h incubation.

Phage vB_BsuS-Bot1 didn`t lose their infectivity after exposure to the UV irradiation at distances of 30 cm from irradiation source for 20 min but it was readily inactivated with a 30 min UV exposure (Figure 4C).

The virus lost its activity after storing at -20 °C , 4°C and 37°C for 7, 15 and 11 weeks respectively, as no plaques were observed on the double-layer agar plates.

Nucleic acid and protein analysis of the isolated vB_BsuS-Bot1

Phage vB_BsuS-Bot1nucleic acid was sensitive to DNase I, and did not digest by treatment of RNase A. Therefore, the genome of isolated vB_BsuS-Bot1 had double-stranded DNA. The genome size was more than 10 kb (Figure 5A). Protein profile of the isolated *Bacillus* phage (vB_BsuS-Bot1) was determined by 12 % SDS-PAGE. Electrophoretic motilities of *bacillus* phage proteins were compared with the standard or markers proteins (medium molecular weight and low molecular weight marker proteins). As shown in Fig. 5B the phage had 9 structural proteins with molecular weight of 88.1, 74.3, 65, 61, 58.5, 49.6, 44.2, 41.5, and 34.7 kDa

Serological properties of the isolated *B. subtilis* phage

Polyclonal antiserum of *Bacillus subtilis* phage (vB_BsuS-Bot1) was obtained after immunization and the polyclonal antibodies were investigated for their specificity to the vB_BsuS-Bot1as antigen by Ouchterlony and Rocket methods. As shown in Figure 6A, the antiserum produced after immunization by vB_BsuS-Bot1 reacted with its antigen (*B. subtilis* phage) to form a single precipitin line comparing with control (phosphate buffer 0.01 M, pH 7.2). Concentration of vB_BsuS-Bot1 was determined serologically by immune-diffusion test. As shown in Figure 6B, the highest dilution at which precipitation occurred was 1/8 to the phage. The specific rabbit antiserum of vB_BsuS-Bot1 was determined by using immune-diffusion test which two fold dilutions of the antiserum was prepared till 1/32 and placed in the peripheral wells 0.8 cm distance from the central well containing the specific antigen. Data in Figure 6C showed that the titer was ½ for the antiserum specific for the vB_BsuS-Bot1. Purified vB_BsuS-Bot1 was placed in wells cut side by side at equal distance in agarose containing antiserum specific for it. As shown in Figure 7, formation of rocket shaped precipitation bands was appeared in case of relationship of vB_BsuS-Bot1 and its dilutions. The obtained results mean that, vB_BsuS-Bot1 serologically related to its antiserum. Immuno-electrophoresis method (Rocket test) is a quantitative method to determine the phage concentration in purified

suspension. The height of rocket shaped precipitation band is linear proportion to the concentration of the antigen.

DISCUSSION

Biological characterization of *Bacillus subtilis* phage was conducted to understand the phage interaction with its host. Results showed that phage specific for *Bacillus subtilis* was present in the clay soil cultivated with wheat plants. Virulent phages specific for *B. subtilis* were also isolated by Hammad (1999) from Egypt and by Krasowska et al. (2015) from Poland.

Handoko et al. (2019) isolated two novel *Bacillus* phages (vB_BspS_SplendidRed and vB_BspM_MarvelLand) from soil collected from a red chili plantation in Getasan Village, Indonesia. While in Korea, *Bacillus subtilis*-infecting phage BSP38 was isolated from a wastewater sample (Ghosh and Kim, 2019). Also the viruses Goe2 and Goe3 were isolated from the same sample of raw sewage from Germany (Willms et al. 2017). Culture lysis test was used just to indicate the presence or absence of *Bacillus subtilis* phage in the Egyptian soil by Hegazi (1980). In this investigation one of *Bacillus subtilis* phages was isolated by single plaque isolation method depending on plaque characters, i.e shape, size and plaque structure as well as many authors (Willms and Hertel, 2016; Ghosh et al. 2018). The isolated vB_BsuS-Bot1 produces circular small central clear area plaques with diameter of 0.2 cm (Figure 2). Ghosh et al. (2018) reported that, after 12 hours of incubation at 37 °C, the phage BSP10 showed a clear plaque in TA soft agar. Previously, Van Elsas and Penido (1981) used plaque morphology in characterization of sixteen *Bacillus pumilus* phage isolates. Willms et al. (2017) characterized 2 *B. subtilis* phage signed as Goe2 and Goe3 depending on virus plaque analysis which Goe2 forms wider plaques of about 1.1 mm in diameter with peripheral rings than Goe3 which forms clear plaques of about 0.6 mm in diameter. The isolated vB_BsuS-Bot1 was propagated to obtain a large amount of viral particles by the liquid propagation technique; it was purified by dextran sulfate polyethylene glycol two phases system in order to obtain purified concentrated particles. The method has been widely used to purify and concentrate the phages (Yamamoto et al., 1970; Othman et al., 1996; Othman et al. 2008; Iqbal et al., 2016). Morphology of the isolated *B. subtilis* phage and its dimensions was studied by the transmission electron microscope after negatively staining. The micro-electrograph observed that, the vB_BsuS-Bot1 has isometric head (58.6 nm in diameter) and long non contractile tail (180 nm in length and 7 nm in width). Similarly, many authors reported that, the phages specific for *B. subtilis* have an isometric polyhedron (icosahedron), i.e. Pecenkova and Paces (1999); Krasowska et al. 2015; Willms et al., 2017; Handoko et al. 2020).

The obtained results showed that vB_BsuS-Bot1 had a limited host range whereas, the phage infected *Bacillus subtilis* strains only. The results of host range analysis obtained in present study were similar from those reported by Willms and Hertel (2016) and Ghosh et al. (2018). Phage vB_BsuP-Goe1 showed only lytic activity on various strains of *B. subtilis*, except for *B. subtilis* natto (Willms and Hertel, 2016). Phage BSP10 displayed a narrow host range against *B. subtilis* (17 out of 52 strains) (Ghosh et al. 2018).

Phage vB_BsuS-Bot1 latency time (50 min) and burst size (approximately 66 PFU/cell) (Figure 3) were found to be significantly different compared to *B. subtilis*-infecting Myoviridae family phage, BSP10 (latency, 40 min; burst size approximately 185) (Ghosh et al. 2018), phiNIT1 (latency, 30 min; burst size approximately 50) (Ozaki et al. 2017), SPO1 (latency, 80 min; burst size, 70) (Wei and Stewart, 1993), and vB_BsuM-Goe3 (latency, 55 min; burst size approximately 114) (Willms et al. 2017). As suggested by other researchers (Bandara et al. 2012; Willms, et al. 2017; Ghosh et al. 2018), the distinct experimental conditions could be the reasons for the large variation in the latency period and burst sizes.

Temperature plays a key role in phage survival, attachment capacity and latent time length (Olson et al. 2004). Thermal inactivation point of the isolated vB_BsuS-Bot1 was 60 °C after 10 min of incubation. While, *Bacillus subtilis* infecting Siphoviridae phage (ARπ) lost its activity after 10 min at 70 °C and 3 h at 60 °C (Krasowska et al. 2015). Typically, members of Siphoviridae family are considered resistant to significant variations in temperature (Jonczyk et al. 2011).

Many essential factors affecting phage stability are the acidity and alkalinity of the environment. Phage vB_BsuS-Bot1 was stable between pH 4 and 7 but the phage titre was decreased in a pH of 8-11. On the contrary, phage ARπ was active only at a narrow pH range (6.0–8.0) (Krasowska et al. 2015).

Inactivation of vB_BsuS-Bot1 by ultraviolet (UV) occurred after 30 min of irradiation. On the contrary, El-Sayed et al. (2016) reported that

Bacillus cereus phage (BCP1) retained 58.7% of its activity after 75 min exposure to UV light. Moreover, Hazem (2002) stated that both *Bacillus* phages (46 and 80) were extremely resistant to UV exposure for 13 min and 20 min, respectively. Sunlight-mediated damage to phage particles is characterized by the formation of pyrimidine dimers (mostly thymine) in DNA caused by direct UV-light absorption (Ravanat et al. 2001).

Typically, a tailed phage possesses double-stranded (ds) DNA as its genomic nucleic acid. All of the *Bacillus* phages possess dsDNA (Ji et al. 2015). Genome analysis indicating that vB_BsuS-Bot1 consisted of dsDNA as previously reported by different authors (El-Arabi et al. 2013; Ji et al. 2015). The genome size of *B. subtilis* phages was known from 18 to 160 kb with different ranges of ORFs (15–250), which can be determined between 20–60 % of their protein functions (Handoko et al., 2020).

Polyacrylamide gel electrophoresis (SDS-PAGE) can determine the molecular weights of phage proteins, allowing individual protein molecules to be detected when viruses are isolated and identified from various environments (Zimmer et al. 2002).

Nine structural proteins with molecular weights ranged from 34.7 to 88.1 kDa were found in the chemical composition of isolated phage. The data are within the same range obtained by some investigators (Stewart et al. 2009; Krasowska et al. 2015, Ozaki et al. 2017). The proteins from purified *B. subtilis* virion (SPO1) were separated by electrophoresis of the SDS polyacrylamide gel to identify some virion protein genes and major head protein was calculated as 50 KDa (Stewart et al. 2009). *Bacillus subtilis* infecting phage (ARπ) produced three major protein bands at molecular masses around 42, 37, and 31 kDa and contained at least 14 structural proteins (Krasowska et al. 2015). Structural proteins of purified *B. subtilis* virion (ϕNIT1) were separated by SDS-PAGE and the molecular mass of resulting mature capsid protein was calculated as 48.8 KDa (Ozaki et al. 2017).

Ouchterlony double diffusion test is one of the most widely used technique for virus assaying, detection and diagnosis because its simplicity, low amount of antigen used and providing information about homogenicity and purity of antigens. Polyclonal antiserum specific for *Bacillus subtilis* phage in this investigation was prepared after immunization of rabbits and its specification to the phage as antigen was investigated by Ouchterlony double diffusion and Rocket tests. The both serological tests gave positive reactions and similar results were obtained by Van-Elsas and Penida (1981). Generally, the serological methods have an advantages reported by Matthews (1992), among these advantages, antisera can be stored and tests made over periods of years, the specificity of the reaction allows detection of antigen even in the presence of the host materials, results can be obtained with a short time and small volume of antiserum and antigen are sufficient.

CONCLUSION

A siphophage of *B. subtilis* (vB_BsuS-Bot1) was isolated from clay soil cultivated with wheat plants in Egypt and found to be a new phage with a thin tail and a narrow host range. Its lytic cycle was 120 min; and host strain specificity was strong. Its optimal growth temperature was 30 °C, the most suitable pH was 7.0, and was sensitive to ultraviolet radiation. *Bacillus subtilis* siphovirus had nine virion structural proteins ranging from 34.7 to 88.1 kDa. For future research, this study will provide biological characteristics of a virulent *B. subtilis*-infecting bacteriophage. Further studies will perform to assess genomic and proteomic properties of *B. subtilis*-infecting bacteriophage and also the phage typing.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Othman B.A. and Nasr-Eldin M.A. designed and performed the experiments. Othman B.A. and Nasr-Eldin M.A wrote the manuscript. All authors read and approved the final version.

with these terms.

REFERENCES

Ackermann HW, 2009. Basic Phage Electron Microscopy. In MRJ Clokie, AM Kropinski, eds, Bacteriophages: Methods and Protocols, Vol. 1, Springer, New York, pp113-26.

Adams MH, 1959. Bacteriophages. Wiley-Interscience, New York, N.Y.

Ameh AA, Kawo AH, 2017. Enumeration, Isolation and Identification of Bacteria and Fungi from Soil Contaminated with Petroleum Products Using Layer Chicken Droppings as an Amendment. BAJOPAS 10(1): 219 –225.

Arkhipova TN, Galimsyanova NF, Kuzmina LY, Vysotskaya LB, Sidorova LV, Gabbasova IM, Melentiev AI, Kudoyarova GR, 2019. Effect of seed bacterization with plant growth-promoting bacteria on wheat productivity and phosphorus mobility in the rhizosphere. Plant, Soil Environ. 65: 313–319.

Bandara N, Jo J, Ryu S, Kim K-P, 2012. Bacteriophages BCP1-1 and BCP8-2 require divalent cations for efficient control of *Bacillus cereus* in fermented foods. Food Microbiol.31: 9–16.

Czajkowski R, Ozymko Z, Lojkowska E, 2014. Isolation and characterization of novel soilborne lytic bacteriophages infecting *Dickeya* spp. biovar 3 (‘*D. Solani*’). Plant Pathol. 63:758–772.

El-Arabi TF, Griffiths MW, She YM, Villegas A, Lingohr EJ, Kropinski AM, 2013. Genome sequence and analysis of a broad host range lytic bacteriophage that infects the *Bacillus cereus* group. Virol. J.10: 48.

El-Sayed DG, Goda HA, Emam NF, 2016. Isolation and characterization of virulent bacteriophages infecting *Bacillus cereus*. Arab J. Biotech. 19 (2): 91-106.

Furrer AD, Bömeke M, Hoppert M et al., 2020. Phage vB_BveM-Goe7 represents a new genus in the subfamily Bastillevirinae. Arch Virol. 16: 959–962.

Ghosh K, Kang HS, Hyun WB, Kim KP, 2018. High prevalence of *Bacillus subtilis*-infecting bacteriophages in soybean-based fermented foods and its detrimental effects on the process and quality of Cheonggukjang. Food Microbiol. 76:196-203.

Ghosh K, Kim K-P, 2019. Complete nucleotide sequence analysis of a novel *Bacillus subtilis*-infecting phage, BSP38, possibly belonging to a new genus in the subfamily Spounavirinae. Arch Virol. 164:875–878.

Hammad AMM, 1999. Induction of bacteriophage-resistant mutants of nitrogen fixing and phosphate dissolving bacteria. Annals Agric. Sci. 44: 479-493

Handoko YA, Wardani A K, Sutrisno A, Widjanarko SB, 2020. Current status: the morphology diversity of *Bacillus subtilis* phages and their genome size IOP Conf. Series: Earth Environ. Sci. 475 (2020) 012088. doi:10.1088/1755-1315/475/1/012088

Handoko YA, Wardani AK, Sutrisno A, Widjanarko SB, Thurgood TL, Thompson DW, Sharma R, Grose JH, 2019. Genome sequences of two *Bacillus* phages isolated from Indonesia. Microbiol Resour Announc 8:e01058-19.

Hazem A, 2002. Effects of temperatures, pH-values, ultra-violet light, ethanol and chloroform on the growth of isolated thermophilic *Bacillus* phages. New Microbiol 25(4):469-476.

Hedges SB, 2002.The origin and evolution of model organisms. Nat Rev Genet 3:838–849.

Hegazi NA, Abou-El-Nasr MA, Othman BA, Allam EK, 1980. Ecological studies on bacteriophages specific for a number of soil bacteria with particular reference to Azotobacter. Proceedings of the 4th Conference of Microbiology, December 24-28, 1980, Cairo, Egypt, pp. 283-302.

Hemphill HE, Whiteley HR, 1975. Bacteriophages of *Bacillus subtilis.* Bacteriol Rev.39:257–315.

Iqbal M, Tao Y, Xie S et al., 2016. Aqueous two-phase system (ATPS): an overview and advances in its applications. Biol Proced Online 18, 18 https://doi.org/10.1186/s12575-016-0048-8

Ji X, Zhang C, Fang Y et al., 2015. Isolation and characterization of glacier VMY22, a novel lytic cold-active bacteriophage of *Bacillus cereus*. Virol. Sin. 30:52–58.

Jonczyk E, Klak M, Miedzybrodzki R, Gorski A, 2011. The influence of external factors on bacteriophages–review. Folia Microbiol. (Praha) 56:191–200.

Khan N, Martinez-Hidalgo P, Ice TA, Maymon M, Humm EA, Nejat N, Sanders ER, Kaplan D, Hirsch AM, 2018. Antifungal activity of *Bacillus* species against *Fusarium* and analysis of the potential mechanisms used in biocontrol. Front Microbiol. 9: 2363.

Krasowska A, Biegalska A, Augustyniak D, Łos´

M, Richert M, Łukaszewicz M, 2015. Isolation and characterization of phages infecting *Bacillus subtilis*. BioMed Res Int:179597.

Kropinski AM, Prangishvili D, Lavigne R, 2009.Position paper: the creation of a rational scheme for the nomenclature of viruses of Bacteria and Archaea. Environ Microbiol. 11 2775 7.

Laemmli UK, 1970. Cleavage of structural proteins during the assembly of bacteriophage T4. Nature227:680.

Laurel C, McKay EJ, 1981. Electroimmunoassy. Method Enzymol 73:339-369.

Li L, Zhang Z, 2014. Isolation and characterization of a virulent bacteriophage SPW 374 specific for *Staphylococcus aureus* isolated from bovine mastitis of lactating dairy 375 cattle. Mol Biol Rep 41:5829–5838.

LoganN A, De VosP, 2009. Genus I. Bacillus. In P Vos, GM Garrity, D Jones, NR Krieg, W Ludwig, FA Rainey, K-H Schleifer, WB Whitman, eds, Bergey's Manual of Systematic Bacteriology, The Firmicutes, Vol. 3, Springer, New York, pp 21–128.

Matthews REF, 1992. Fundamental of Plant Virology. Academic Press, New York.

Milus EA, Rothrock CS, 1993. Rhizosphere colonization of wheat by selected soil bacteria over diverse environments. Can. J. Microbiol. 39:335-341.

Okubo S, 1966. Bacteriophage of *Bacillus subtilis*. Tanpakushitsu Kakusan Koso 11:572–578.

Olson MR, Axler RP, Hicks R. E, 2004. Effects of freezing and storage temperature onMS2 viability. J. Virol. Methods, 122: 147–152.

Othman BA, El Nasr MAA, Shamloul AM, 1996. Biophysical and serological properties of two virulent phage isolates specific for *Erwinia carotovora*. Ann. Agric. Sci. 41(1): 141-153.

Othman BA, 1997. Isolation of lambdoiod bacteriophage B4EC from sewage polluted drinking water. Proceeding of the 9th Conference of Microbiology. March 25-27, Cairo, Egypt, pp. 78-88.

Othman BA, Askora AA, Awny NM, Abo-Senna ASM, 2008. Characterization of virulent bacteriophages for *Streptomyces griseoflavus* isolated from soil. Pak J Biotechnol, 5(1-2): 109-119.

Ouchterlony O, 1968. Handbook of Immunodiffusion and Immunoelectrophoresis. Ann Arbor Publ., Ann Arbor, Michigan. pp 215.

Ozaki T, Abe N, Kimura K, Suzuki A, Kaneko J, 2017. Genomic analysis of *Bacillus subtilis* lytic bacteriophage 'NIT1 capable of obstructing natto fermentation carrying genes for the capsule-lytic soluble enzymes poly- -glutamate hydrolase and levanase. Biosci. Biotechnol. Biochem. 81:135–146.

Pecenkova T, Paces V, 1999. Molecular phylogeny of phi29-like phages and their evolutionary relatedness to other protein-primed replicating phages and other phages hosted by gram-positive bacteria. J. Mol. Evol.48:197–208.

Ravanat JL, Douki T,Cadet J, 2001. Direct and indirect effects of UV radiation on DNA and its components. J Photochem Photobiol B Biol. 63:88-102.

Sambrook J, Fritsch EF, Maniatis T, 1989. Molecular cloning: a laboratory manual. Cold spring harbor laboratory press: NY, USA, 0879693096.

Sambrook J, Russell DW, 2001. Molecular cloning: A laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press.

Shin H, Bandara N, Shin E, Ryu S, Kim KP, 2011. Prevalence of *Bacillus cereus* bacteriophages in fermented foods and characterization of phage JBP901. Res. Microbiol.162:791–797.

Stewart CR, Casjens SR, Cresawn SG, et al., 2009. The genome of *Bacillus subtilis* bacteriophage SPO1. J. Mol. Biol. 388:48–70.

Van Elsas JD, Penido EGC, 1981. Isolation and characterization of *Bacillus pumilus* phages from Brazilian soil. Abl. Bakt. II. Abt., 136: 581-589.

Walter MH, Baker DD, 2003.Three *Bacillus anthracis* bacteriophages from top soil. Curr Microbiol. 47:0055–0058.

Wang XQ, Zhao DL, Shen LL, Jing CL, Zhang CS, 2018. Application and Mechanisms of *Bacillus subtilis* in Biological Control of Plant Disease. In: V Meena, eds, Role of Rhizospheric Microbes in Soil. Springer, Singapore.

Wei P, Stewart CR, 1993. A cytotoxic early gene of *Bacillus subtilis* bacteriophage SPO1. J. Bacteriol., 175: 7887–7900.

Willms IM, Hertel R, 2016. Phage vB_BsuP-Goe1: The smallest identified lytic phage of *Bacillus subtilis*. FEMS Microbiol. Lett., 363, fnw208.

Willms IM, Hoppert M, Hertel R, 2017. Characterization of *Bacillus subtilis* viruses vB_BsuM-Goe2 and vB_BsuM-Goe3. Viruses, 9, 146.

Yamamoto KR, Alberts BM, Benziger R,

Lawhorne L, Trejber G, 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large scale virus purification. Virology 40: 734-744.

Zimmer M, Scherer S, Loessner MJ, 2002. Genomic Analysis of *Clostridium perfringens* Bacteriophage Φ3626, which integrates into guaA and possibly affects sporulation. J. Bacteriol., 184:4359–4368.