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Physiological and Morphological Characteristics of PhagesInfecting *Bacillus thuringiensis* Isolated from Saudi Arabia Soil

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ABSTRACT

Bacillus sphaericus and *B. thuringiensis* are commonly used as a biological pesticide. To obtain phage-resistant *Bacillus*strains, the present study was carried out.Two phages were isolated fromsoil based on their ability to form plaques on four indicator hosts including*B.shpaericus* and three isolates of*Bacillus thuringiensis*. The purified phages were characterized by morphology and certain physiological characteristics including effect of storage period in refrigerator and freezer and effect of hightemperature on phage activity. The phages appeared to be of the *Myoviridae*(phages AZSBt1 and AZSBt2) family based on their structure in electron micrographs. The phage activity was not affected when stored in refrigerator or freezer up to 35 and 30 days, respectively. However, there was no activity when phages were stored in refrigerator or freezer for 90 and 150 days. On the other hand, phage activity was not affected by temperature up to 50°C; however, the activity was lost at 60°C. The phage AZSBt1 has broader host range than the phage AZSBt2.

INTRODUCTION

Bacillus spp. is Gram-positive, rod-shaped, spore-forming bacteria (Nissen *et al*. 2002; Lake *et al*. 2004). *Bacillus thuringiensis* is a commonly distributed bacterium that produces par asp oral inclusions during the stationary phase of its growth cycle. Many *B. thuringiensis* strains exhibit insecticidal activity against certain insect species among the orders Lepidoptera, Diptera, and Coleoptera (Hofte and Whiteley, 1989). However, some *B. thuringiensis* strains have also been shown to be active against other insect orders, such as Hymenoptera, Homoptera, Orthoptera, and Mallophaga (Schnepf *et al*., 1998), and against a much broader range of invertebrate pests including nematodes, and mites (Feitelson *et al*., 1992).

Viruses are considered obligate intracellular parasites requiring a specific host cell for its replication (Carlton, 1999; Mayer, 2005). Bacteriophages are commonly distributed in nature. About96% of phage investigated in the last 45 years, aretailed phage belonging to the Siphoviridae, Myoviridae,or Podoviridae families (Ackermann, 1996,1999). Siphoviridae is by far the most frequent phagegroup (61.7%), followed by the Myoviridae (24.5%)and Podoviridae (13.9%) (Ackermann, 1999).

The host specificity of viruses offer an enticing technology for fighting infections caused by bacteria or for the treatment of environments contaminated with pathogenic bacteria. Research into potential use of viral therapy is limited, but studies have shown success using this technology to treat infections in livestock, plants, aqua-cultured fish and humans (Sulakvelidze and Burrow, 2005; Sulakvelidze and Kutter, 2005). The use of phage in the treatment of bacterial infections is an attractive alternative to existing therapies (example, antibiotics), because unlike broad-spectrum antibiotics phage target a particular host and are unlikely to elicit resistance in untargeted bacterial strains (Sulakvelidze and Kutter, 2005). Also, unlike chemical therapeutic agents, phages are not susceptible to the onset of bacterial resistance because they have the ability to evolve with their host (Sulakvelidze and Kutter, 2005).The objective of this study was to isolate and characterize phages infecting *Bacillus thuringiensis* from Saudi Arabia soils to be used for isolation of phage-resistant strains of *B.shpaericus* and *B.thuringiensis*.

MATERIAL AND METHODS

Bacillus strains:

Bacillus thuringiensis CIP 105674, *Bacillus thuringiensis* var. *aizwi* CRBIP 3.2355, *Bacillus thuringiensis* var. *israelensis* CRBIP3.1047, *Bacillus thuringiensis var. israelensis* CRBIP3.495, and *Bacillussphearicus* CRBIP17.28 were obtained from Centre de Ressources Biologiques de l'Institut Pasteur, Pasteur Institute, France.

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Bacteriophage Isolation:

Ten gram of soil sample collected from sheep yard in Riyadh, KSA was added to 500 ml flasks containing 100 ml of sterile nutrient broth medium. The flasks were incubated in shaking water bath for 1 hr at 35°C. The broth was filtered using filter paper and the filtrate was centrifuged at 3000 r.p.m for 10 minutes. The supernatant was then subjected for ultrafiltration using Millipore filter (stericup, durapore 0.45 μm) and stored to be used as phage particles.

Plaque Assay Technique:

Nutrient agar plates were prepared and kept at 30°C for 24 hrto ensure sterility. Tubes of molten soft agar were kept at 45°C in a water bath. To a molten soft agar tube (3 ml), a 0.1 ml of the bacterial suspension (2 hr old culture) was added. The contents of the tube were swirled and poured on the surface of the basal agar medium.To another molten soft agar tube, a 0.1 ml of the bacterial suspension (2 hr old culture) and a 0.1 ml of the phage particles were added. The contents of the tube were then swirled and poured on the basal agar medium.Both inoculated plates were incubated at 30°C for 24-48 hr and then the plates were examined for the characteristic plaque appearance. The presence or absence of plaques indicates the presence or absence of bacteriophage.

Propagation of Bacteriophages:

Single plaque isolated and purified phages were propagated in 100 ml nutrient broth in 250 ml flasks as follows:

Bacterial growth on slants of 12-24 hr incubation period was transferred into test tubes containing 5 ml nutrient broth, each tube was rotated until a homogeneous suspension was obtained.A 0.5 ml the suspension was inoculated into a100 ml nutrient broth in a 250ml flask (2 flasks were inoculated; one was kept for check and the other was used for bacteriophage propagation).One flask was inoculated with 2ml of the upper phase of the bacteriophage inoculums, and incubated at 35°C with occasional shaking. The other flask was kept as check. Titration (plaque assay) was performed 24 hr post infection using different ten- fold dilutions.Plaque assay was carried out using culture of bacteria and the source of bacteriophage. If the titer of a bacteriophage was high, serial dilutions were made using ten-fold phosphate buffer or saline solution.

Factors affecting the activity of Bacillus phage:

Effect of storage in refrigerator:

The phage particle filtrate was distributed in sterile test tubes(5 ml) and stored in refrigerator at different time of period ranged from 3 up to 90 days. The phage activity was then determined by phage number in 1 ml using plaque forming unit (PFU) technique.

Effect of storage in freezer on phage ability of infection:

The phage particle filtrate was distributed in sterile test tubes (5 ml) and stored in freezer at different time of period ranged from 30 up to 150 days. The phage activity was then determined by phage number in 1 ml using plaque forming unit (PFU) technique

Effect of different temperature degree on the ability of the phage to infection:

The phage particle filtrate was distributed in sterile test tubes (5 ml) and each tube was separately exposed to different temperature ranged from 40 up to 60°C for 10 minutes. The phage activity was then determined by phage number in 1 ml using plaque forming unit (PFU) technique

Host range determination:

Overlays were inoculated with 0.1 ml of bacteria at10^8 CFU $ml^{-}1$, and poured on a base plate previouslylabelled with a grid-like format. Once the overlay wasdry, 10 μl of phage stock suspensions, serially diluted withSM buffer (0.05moll^{-}1 TRIS, 0.1 mol l^{-}1 NaCl, 0.008 mol l^{-}1 MgSO4,0.01% (w⁄v) gelatin pH 7.5) to corresponding final titres of 10^3, 10^5, 10^7PFU $ml^{-}1$, were transferred onto the gridded overlay. Theplates were left to dry and incubated at 37°C overnight.Inhibition of the bacteria shown as clear zones in thelawn indicated lysis of the host. Lysis from without was assumed to occur when clear zones were obtained at highconcentrations of phages but not at lower ones.

Electron Microscopy Examination:

The isolated phages were examined by Transmission Electron Microscope (TEM). The obtained page pellet was re-suspended in 10 μl of phage buffer. Phages preparations were negatively stained with phosphotangstic acid solution freshly prepared at pH=6.8. The stained phage was dropped directly to carbon-coated collodion grids of 200 meshes. After 1 to 2 min, the excess stain solution was removed with filter paper

(Hidaka, 1971). Air dried grids were examined in transmission electron microscope, Philips EM 400-end, Hoffman.

RESULTS AND DISCUSSION

Isolation of Bacillus thuringiensis phages:

The *Bacillus* phages investigated in this study were isolated from soil sample obtained from Jizan valley, KSA. The isolated phages were purified and their lytic effect to *Bacillus* strains was observed. Phages were examined using electron microscope. The electron micrographs revealed that two different phages formed plaques on *Bacillus thuringiensis*were observed and described (Figure 1). The two phages (AZSBt1 andAZSBt2) were characterized by presence of head and tail (Figure 1).

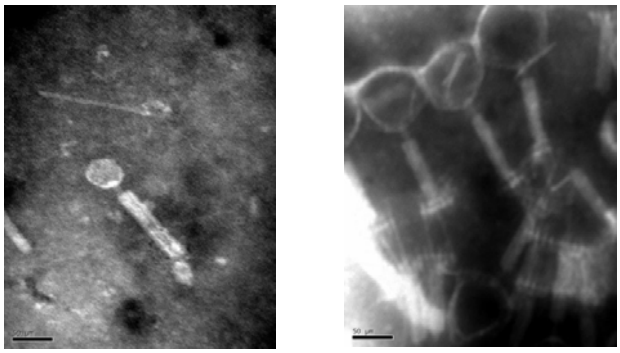
Morphological characters of Bacillus thuringiensis phages:

Phage AZSBt1:

The electron micrographs of the phage AZSBt1 revealed the presence of a head and a tail (Figure 1a). The phage has a head with icosahedral symmetry and a tail with helical symmetry. Thehead was 45-50 nm in diameter. The contractile, tubular tail was rigid, long and thick with cross-bands and has a length of 120-150 nm and a width of 18-22 nm. According to the International Committee on Taxonomy of Viruses (ICTV) the phage AZSBt1 belongs to the family Myoviridae (ICTV, 2006).

Phage AZSBt2:

The electron micrographs of the phage AZSBt2 revealed the presence of a head and a tail (Figure 1b). The phage has a head with icosahedral symmetry and a tail with helical symmetry. The head was 60-80 nm in diameter. The contractile, tubular tail was rigid and thick and has a length of 80-100 nm and a width of 14-18 nm. Tail has a collar, a base plate, spikes, and a sheath, which is separated from the head by a neck.According to the International Committee on Taxonomy of Viruses (ICTV) the phage AZSBt2 belongs to the family Myoviridae (ICTV, 2006).



(a) **(b)**

Fig. 1: Scanning Electron micrographs of *Bacillus* phage; AZSBt1 (a) and AZSBt2 (b) showing the head and tail.Bar lines = 50 nm.

The isolated phages (AZSBt1 and AZSBt2) were similar in morphology to SPO1 which infects *Bacillus subtilis* (Parker and Eiserling, 1983) and to FWLBc1 and FWLBc2 which infects *Bacillus cereus* (Lee *et al*., 2011). The tail length of AZSBt1 (120-150 nm) and AZSBt2 (80-100 nm) were shorter than that reported (around 210 nm) by Lee *et al*., 2011.

Physiological characters of Bacillus thuringiensis phages:

Effect of storage period:

Refrigerator storage:

The phage activity was affected by the period of storage in refrigerator (~ 4°C) (Table 1). The phage activity decreased by increasing of the storage period up to 90 days, when the phage showed no activity against all the tested strains. The phage activity was detected for all the investigated strains with storage period in refrigerator up to 35 days. However, after 42 days there was no phage activity against *B. sphaericus* CRBIP17.28 strain and after 70 days for *B. thuringiensis* var. *israelensis* CRBIP3.1047 and CRBIP3.495 strains. After 77 days the phage was inactive against the isolated *B. thuringiensis* CIP 105674strain.

Table 1: Effect of refrigerator storage on activity of *Bacillus*phage.

Refrigerator storage period (day)	Phage Number (PFU/mL)				
	Bacillus thuringiensis CIP 105674	*Bacillus thuringiensis* var.*aizwi* CRBIP3.2355	*Bacillusthuringiensis*var.*israelensis* CRBIP3.1047	*Bacillus thuringiensis* var.*israelensis* CRBIP3.495	*Bacillus sphaericus* CRBIP17.28
0(control)	112	094	164	170	062
7	060	310	162	125	033
14	050	190	084	070	020
21	032	075	050	050	011
28	020	066	025	028	005
35	020	060	026	020	001
42	020	058	020	020	000
49	020	061	017	020	000
56	016	058	010	013	000
63	010	040	003	008	000
70	005	034	000	000	000
77	000	012	000	000	000
85	000	006	000	000	000
90	000	000	000	000	000

Freezer storage:

The phage activity decreased by increasing of the period of storage in freezer(~ -10°C)up to 150 days, when the phage showed no activity against all the tested strains (Table 2).The phage activity was detected for all the investigated strains with storage period in freezer up to 30 days.However, after 30 days of freezer storage there was no phage activity against the isolated *B. thuringiensis*var.*israelensis*CRBIP3.1047strain and after 95 days for *B. thuringiensis* CIP 105674 strain. After 123 days the phage was inactive against *B. sphaericus*CRBIP17.28 strain. The activity of the phage against *B. thuringiensis*var.*israelensis*CRBIP3.495 strain was lost when stored in freezer for 140 days.

Effect of temperature:

The phage activity was affected by the temperature (Table 3). The phage filtrates were kept in different temperature degree for 10 minutes and then its activity was investigated. The phage activity was decreased by increasing of the temperature up to 60°C, when the phage showed no activity against all the investigated strains.However, at 55°C there was no phage activity against *B. thuringiensis var. israelensis*CRBIP3.1047, CRBIP3.495 and *B. sphaericus*CRBIP17.28 strains.

Table 2: Effect of freezer storage on activity of *Bacillus* phage.

Freezer storage period (day)	Phage Number (PFU/mL)				
	Bacillus thuringiensis CIP 105674	*Bacillus thuringiensis* var.*aizwi* CRBIP3.2355	*Bacillusthuringiensis*var.*israelensis* CRBIP3.1047	*Bacillus thuringiensis* var.*israelensis* CRBIP3.495	*Bacillus sphaericus* CRBIP17.28
30	048	065	021	156	300
45	030	054	000	132	225
60	027	103	000	093	190
75	011	074	000	059	114
95	000	045	000	046	095
123	000	024	000	013	000
140	000	013	000	000	000
150	000	000	000	000	000

Table 3: Effect of temperature on activity of *Bacillus* phage.

Temperature	Phage Number (PFU/mL)				
	Bacillus thuringiensis CIP 105674	*Bacillus thuringiensis* var.*aizwi* CRBIP3.2355	*Bacillusthuringiensis*var.*israelensis* CRBIP3.1047	*Bacillus thuringiensis* var.*israelensis* CRBIP3.495	*Bacillus sphaericus* CRBIP17.28
40°C	188	313	253	214	084
45°C	135	285	120	155	030
50°C	090	210	060	084	006
55°C	005	012	000	000	000
60°C	000	000	000	000	000

Iriarte *et al*. (2007) mentioned that the phages are clearly affected by thesurrounding environment, just as their target bacteria are, andtheir efficacy of control depends not only on the susceptibilityof the target bacterium but also on the environmental factorsthat affect their own survival. Those environmental factors

thathave been implicated in significantly affecting phage survivalon plant foliage are sunlight irradiation, especially in the UVrange, desiccation, temperature, precipitation, relative humidity,and presence of residual copper compounds. The current study revealed that the phage activity was affected by period of storage and temperature. When stored in refrigerator for 90 days the activity of phage against *Bacillus thuringiensis* strains was lost. On the other hand, the freezer activity for 150 days caused inactivation of the phage. The activity of phage was not affected by temperature up to 50°C, however, at 60°C there was no activity at all against all the investigated *Bacillus* strains.

Host range:

The host ranges of phages AZSBt1 and AZSBt2 wereassessed by assaying against several *Bacillus* spp. and *B. thuringiensis* strains. AZSBt2 lysed 8 of the 11 hosts, whileAZSBt1 lysed 10 of the 11 hosts, indicating abroader host range(Table 4).

Table 4: Host range of phages AZSBt1 and AZSBt2 at 10^3, 10^5, 10^7(PFU $ml^{-}1$).

Indicator hosts	AZSBt1 (PFU $ml^{-}1$)	AZSBt2 (PFU $ml^{-}1$)
Bacillus cereus ATCC 11778	10^5	10^5
Bacillus cereus DMS 14729	10^5	10^5
Bacillus cereus DMS 22652	-	-
*Bacillus sphaericus*CRBIP17.28	10^3	10^5
Bacillus subtilis DMS 2109	10^7	10^5
Bacillus subtilis DMS 30682	10^5	-
*Bacillus thuringiensis*CIP 105674	10^3	10^5
*Bacillus thuringiensis*var.*aizwi*CRBIP3.2355	10^3	10^5
*Bacillus thuringiensis*var.*israelensis*CRBIP3.1047	10^3	10^5
*Bacillus thuringiensis*var.*israelensis*CRBIP3.495	10^5	10^5
*Bacilluslaterosporus*ATCC 31932	10^5	-

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