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ISOLATION AND PRELIMINARY CHARACTERIZATION OF TWO BACTERIOPHAGES FOR *BACILLUS SUBTILIS*[1][2]

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Abstract

Two strains of *Bacillus subtilis* (designated as BS 1-1 and BS 4-4, respectively.) were identified from rice-fields. Physiologically they were quite similar to the standard strain *B. subtilis* PCI 219 except urease formation.

Two morphologically and serologically unrelated phages, phage BS 1-1 and phage BS 4-4, were also isolated from them. Phage BS 1-1 possesses a hexagonal head and a long tail with terminal appendage and tail fibers. The head diameter is about 700 Å. The dimension of tail is about 1700 Å in length and 170 Å in width. Phage 4-4 has an elliptical head and a long tail. The head diameter is about 465×580 Å. The tail is about 2090 Å in length and 100 Å in width. Phage BS 1-1 can attack both BS 1-1 and BS 4-4, but phage BS 4-4 can attack its own host only. Both phage BS 1-1 and phage BS 4-4 are DNA phages. Some physiological properties, host range, thermal inactivation, pH stability, UV inactivation and one-step growth, were also determined.

Introduction

Bacteria belonging to the genus *Bacillus* has been of academic and industrial importance. A survey of Bacillus species in Taiwan is under way by the auther. Two strains of *Bacillus subtilis* have been isolated. Two bacteriophages for them have also been isolated from rice-field water and soil samples. Some properties of these two phages are reported here.

Materials and Methods

Bacteria

Both bacteria and phages were isolated from rice-field water or rice-field soil samples collected from district of Taipei. The two strains of *B. subtilis*, designated as BS 1-1 and BS 4-4, respectively, were identified from 726 Gram positive bacilli isolated according to the Bergey's key and serological test.

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Physiologically they are quite similar to the standard strain *B. subtilis* PCI 219 except urease formation (Table 1). Serologically they are also related (Table 2). But they differed in their susceptibility to phage BS 1-1 and phage BS 4-4. *B. subtilis* PCI 219 were obtained from Department of Bacteriology, Medical School, Taiwan University.

Table 1. *Some physiological characters of* ***B. subtilis*** *PCI 219,* ***B. subtilis*** *1-1 and* ***B. subtilis*** *4-4*

Physiological tests \ Organisms	*B. subtilis* PIC 219	*B. subtilis* 1-1	*B. subtilis* 4-4
Growth at pH 6.0	+	+	+
M. R. test	−	−	−
V. P. tet	+	+	+
Gelatin hydrolization	+	+	+
Starch hydrolization (cm)	0.8	0.5	0.5
Nitrate reduction	++	+	++
Casein digestion (cm)	1.0	0.8	0.8
Urease formation	−	+	++
Motility test	+	+	+

−: No reaction
+: Positive reaction
++: Stronger than +

Table 2. *The agglutinaton reactions between* ***B. subtilis*** *PCI 219,* ***B. subtilis*** *1-1 and* ***B. subtilis*** *4-4*

Antisera \ Agglutinogens	*B. subtilis* 1-1	*B. subtilis* 4-4	*B. subtilis* PCI 219
B. subtilis 1-1	+	+	+
B. subtilis 4-4	+	+	+
B. subtilis PCI 219	+	+	+

Media

Both nutrient borth (Difco) and nutrient agar (Difco) were used for isolation and routine cultivation of *B. subtilis*. Those media were also satisfactory for the isolation and propagation of *B. subtilis* phages. Bottom layer and soft agars for plating contained 1.5% and 0.8% agar in nutrient broth.

Bateriophages

The two phages selected by their ability to lyse those two bacilli were designated as phage BS 1-1 and phage BS 4-4, respectively. Their isolation and propagation were carried out according to the method described by Adams

(1959).

Stock phages were prepared by centrifuging the plate lysate at 8,000 rpm for 15 minutes to remove the bacteria and debris. The supernatants were centrifuged at 20,000 rpm (Spinco L No. 30 rotor) for 120 minutes. The pelletes were resuspended in 0.01 M phosphate buffer (pH 7.0) or nutrient broth. The same procedure was repeated twice in order to remove the host cells completely. The 0.01 M phosphate buffer was added to the final pellet to make the phage suspension. Then the phage suspension was filtered throughout 0.45 μ pore size millipore filter and stored at 4°C. The titer of phage stock obtained was from 10^{12} to 10^{13} plaque-forming units (PFU) per ml.

Antisera

Antisera for host cells, phages and *B. subtilis* PCI 219 were prepared in rabbits by use of three-intramuscular injection of stock antigen suspension. Each rabbit was injected in sequence with 0.5 ml, 0.1 ml and 2.0 ml of antigen at 7 days interval. Ten days after the final injection, the injected rabbits were bled and the sera were separated. The neutralization titer (K value) of these antiphage sera ranged from 134 to 168. The agglutination titer of these antihost and antibacterial sera ranged from 2560 to 5120.

Electron microscopy

Purified phages were suspended in equal volume of 2% phosphotungstic acid, pH 6.8 and then dropped on copper grid and dried. Samples were observed in a Hitachi HU-11A type electron microscope.

Cross-neutralization reaction

The K value of the antisera and the cross-neutralization reaction were examined according to Adams' methods (1959).

Host range

Host range was determined by spot test; overnight culture of various *Bacillus* strains (such as BS 1-1, BS 4-4, *B. subtilis* PCI 219, *B. megatherium* and *B. natto*) grown in nutrient broth were overlaid on the basal agar plates, then one drop of phage suspension was spotted on the plates. The plates were incubated at 37°C over-night, and the resulting lysis area was examined.

Thermal inactivation

Tubes containing 3.6 ml of nutrient broth were maintained at 45°C, 50°C, 60°C, 70°C, 75°C etc and 0.4 ml of phage suspension was added. At various time intervals samples were delivered into ice-cold broth and assayed for surviving phages.

Effect of pH

0.1 ml of phage suspensions were added to 10 ml of nutrient broth. The pH of which has been adjusted to desired value (pH2, pH4, pH7, pH8, pH9, pH10, pH11 and pH12). The mixture were incubated at 37°C for 1 hr and

assayed for surviving phages.

Inactivation by UV irradiation

The source of ultraviolet was a mercury vapor quartz tube (Grid type) that emitted a high percentage of radiation at 2,537 Å with an intensity of about 155 microwatts per square centimeter at a distance of 44.5 cm. The distance of ultraviolet lamp to phage sample was 20 cm. Phage suspensions were diluted in nutrient broth and irradiated in petri dishes. Samples taken at various time intervals after exposure to UV light were diluted in nutrient broth and assayed for surviving phages.

Adsorption of phages to host cells

A method modified from that described by Adams (1959) was used to determine adsorption rates. Logarithmically growing host cells were mixed with phages in a ratio of less than one phage per bacterium. Samples were withdrawn from the mixture at intervals, freed from bacterial cells by centrifugation, and assayed for free phage.

One step growth experiment

Adams' (1959) method for determining latent period and burst size have been modified to some extent. Latent periods were determined by inoculating 10 ml of shake culture containing 2×10^7 indicator cells per ml at 37°C, with 0.1 ml of broth containing 1×10^7 phages particles. One-tenth ml of this mixture was plated out at various time intervals, and a sharp increase in phage titer could be observed as a time characteristics of each phage. Burst size determination was carried out by diluting a similar mixture. The multiplicity of infection in the adsorption tube was always less than one. Adsorption was allowed to proceed for 10 minutes, the adsorption mixture was then exposed to antiserum for 5 minutes. It was then diluted 10^6 times. Samples were withdrawn and assayed for plaque count at one minute interval until 200 minutes. The nutrient broth was used as a diluent. The experiments were carried out at 37°C in a water bath.

Nucleic acid determination

The purified phages were suspended in 0.01 M phosphate buffer and incubated with DNase (50 μg/ml) and RNase (50 μg/ml) in the presence 0.002 M of $MgCl_2$ for 3 hours. The suspensions were dialyzed against the phosphate buffer and phages were precipitated by centrifugation at 20,000 rpm for 1 hour. The phage pellets were suspended in 20 ml of buffered saline. To the phage suspension was added an equal volume of phenol saturated with water. The mixture in a glass-stoppered flask was shaken for 30 minutes at 4°C. The layers were separated by centrifugation, and the aqueous layer containing the nucleic acid was removed. Residual phenol was removed by dialysis against several changes of buffered saline. DNA and RNA were isolated by the pro-

cedure of Marmur (1961). Diphenylamine and orcinol reaction were used for qualitative determination of DNA and RNA.

Results

Plaque and phage morphology

The typical plaques of phage BS 1–1 and Phage BS 4–4 are shown in Fig. 1 and Fig. 2. The plaque size of phage BS 1–1 was about 1.5 to 2 mm in diameter and phage BS 4–4 was about 3 to 5 mm in diameter. Both phage BS 1–1 and phage BS 4–4 produced clear plaques but the later was surrounded by narrow halo with a width about 1.5 mm after 24 hours cultivation. The plaques formation of both phage BS 1–1 and phage BS 4–4 were about 7 to 8 hours. Electron micrographs of the two phages are shown in Fig. 3 and Fig. 4. The phage BS 1–1 has a hexagonal head and a long tail with its terminal appendage. The head diameter is about 700 Å. The dimensions of tail are about 1,700 Å in length and 170 Å in width. Sometimes tail fibers could be observed at the proximal end of the tail. The phage BS 4–4 has an elliptical head and a long tail. The head size is about 465×580 Å. The tail is about 2,090 Å in length and 100 Å in width. No tail fiber was observed.

Serological relationship

The K value of homologous neutralization rate constants was 134 for phage BS 1–1 antiserum and 168 for phage BS 4–4 antiserum. Cross-neutralization was tested by reacting each antiserum with two phages. The results are shown in Table 3, it can be seen that phage BS 1–1 and phage BS 4–4 are serologically unrelated to one another.

Table 3. *K value of antiphage sera against homologous and heterologous phage*

Antiphage sera \ Phages	*B. subtilis* 1–1	*B. subtilis* 4–4
B. subtilis 1–1	134	0
B. subtitis 4–4	0	168

Host range

The infectivities of phage BS 1–1 and phage BS 4–4 upon some *Bacilli* are shown in Table 4. The phage BS 1–1 attacks both BS 1–1 and BS 4–4, but not other *Bacillus* strains tested. The BS 4–4 phage infects its own host BS 4–4 only, but not other *Bacillus* strains tested.

Thermal inactivation

Phage BS 1–1 and phage BS 4–4 were inactivated at 60°C and 70°C respectively. They were drastically inactivated at temperature above 70°C and 75°C.

Table 4. *Infectivities of phage* ***B. subtilis*** *1-1 and phage* ***B. subtilis*** *4-4 upon some* ***Bacilli***

Hosts \ Phages	*B. subtilis* 1-1	*B. subtilis* 4-4
B. subtilis 1-1	+	−
B. subtilis 4-4	+	+
B. subtilis PCI 219	−	−
B. natto	−	−
B. megaterium	−	−

As shown in Fig. 5, phage BS 1-1 was more sensitive to temperature than phage BS 4-4.

Effect of pH.

Fig. 6 showed both phage BS 1-1 and phage BS 4-4 were stable at alkaline pH. The optimal pH value for the growth of phage BS 1-1 was 8, whereas that for phage BS 4-4 was from pH 7 to 9.

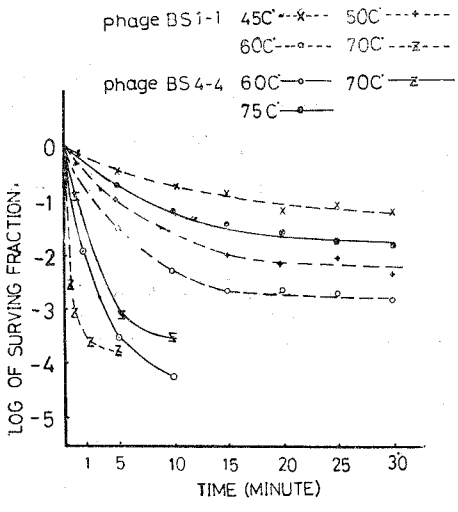

Fig. 5. Thermal inactivations of phage BS 1-1 and phage BS 4-4

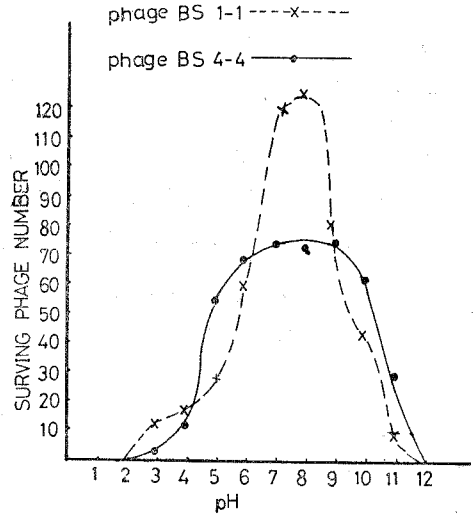

Fig. 6. PH stabilities of phage BS 1-1 and phage BS 4-4

UV inactivation

The inactivation of phage by UV irradiation was shown in Fig. 7. The phage BS 4-4 was more resistant to UV than phage BS 1-1.

Adsorption

95% of phage BS 1-1 was adsorbed on host bacteria in 14 minutes, while the same percentage of phage BS 4-4 was adsorbed in 12 minutes (Fig. 8).

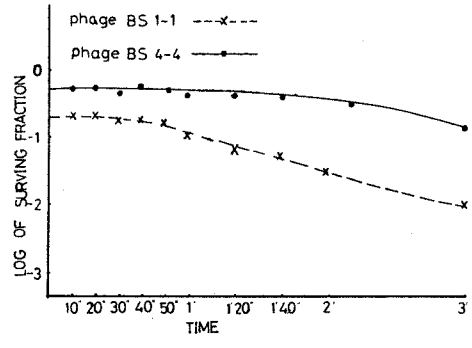

Fig. 7. Ultraviolet inactivations of phage BS 1-1 and phage BS 4-4

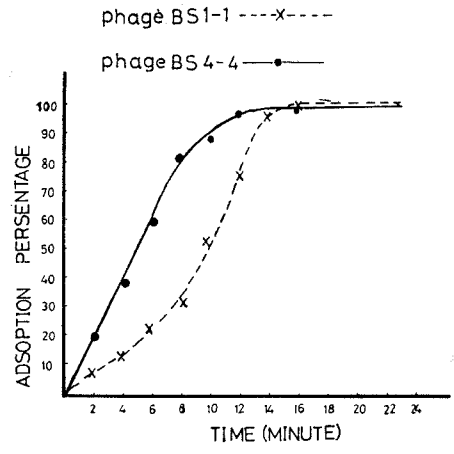

Fig. 8. Adsorption times of phage BS 1-1 and phage BS 4-4

One-step growth experiments

The latent periods for phage BS 1-1 and phage BS 4-4 were about 55 minutes (Fig. 9). The burst sizes of phage BS 1-1 and phage BS 4-4 were 86 and 160, respectively.

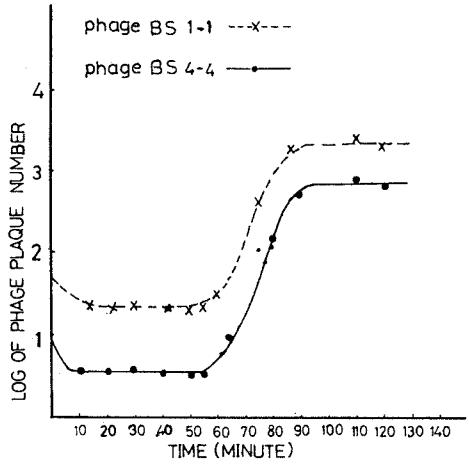

Fig. 9. One-step growth curves of phage BS 1-1 and phage BS 4-4

Nucleic acid determination

Positive diphenylamine reaction of the nucleic acids extracted from both phage BS 1-1 and phage BS 4-4 indicates that both phages contain DNA.

Discussion

The foregoing results describe two *B. subtilis* phages. They are morpho-

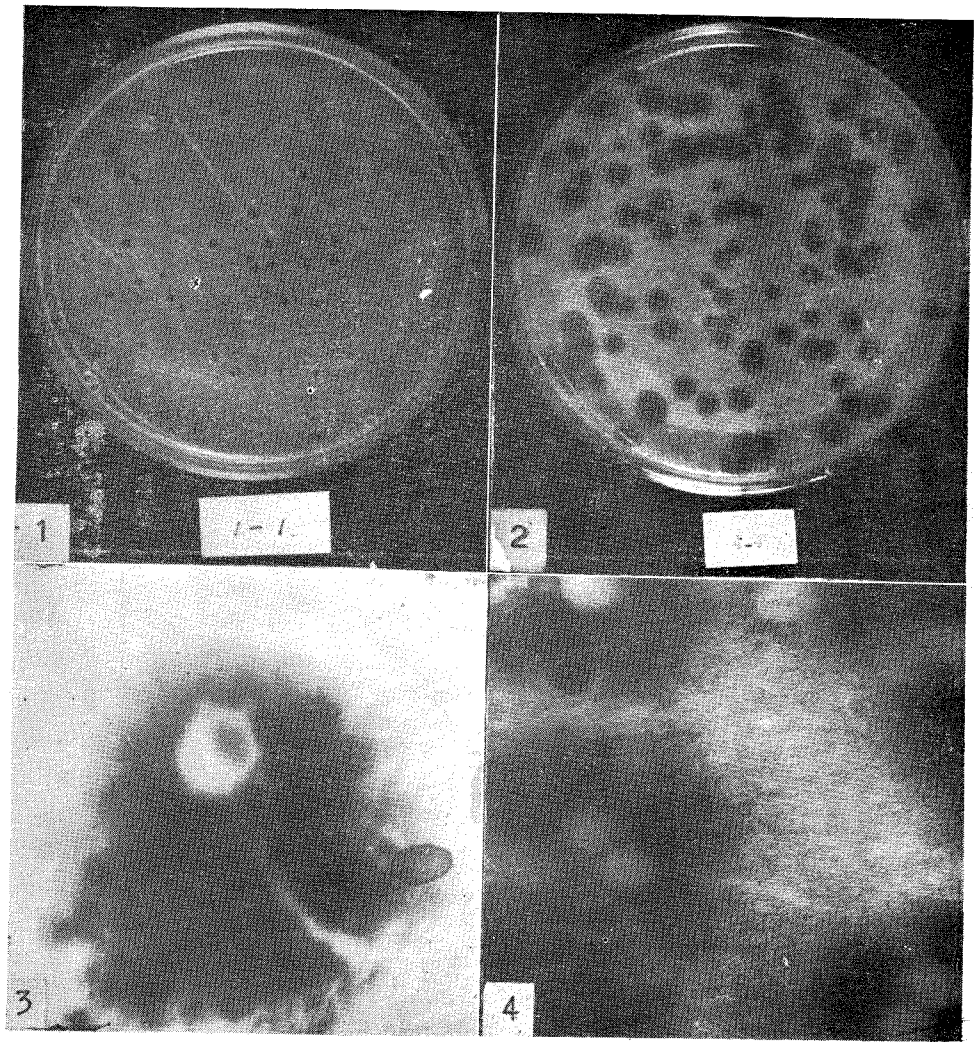

Fig. 1-4. Plagues and morphology of phage BS 1-1 and phage BS 4-4. Fig. 1. Plaques of phage BS 1-1. × $\frac{2}{3}$. Fig. 2. Plaques of phage BS 4-4. × $\frac{2}{3}$. Fig. 3. Electron micrography of phage BS 1-1. × 174,000. Fig. 4. Electron micrography of phage BS 4-4. × 129,000.

logically, serologically and physiologically different. The long tail and hexagonal head are also found in other *B. subtilis* phages: SP-8 (Davison, 1963), SP-50 (Eiserling *et al.*, 1965), AR-1, TSP-1 (LaMontagene *et al.*, 1972), and AR-1, AR-2 and AR-3 (TiKhonenko, 1970). Physiologically, although phage SP-8 and BS 1-1 are similar in response to pH variation, thermal inactivation and UV inactivation, they are different in adsorption times, latent periods and burst sizes (Brodetsky 1965).

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兩種枯草桿菌的噬菌體的分離和特性

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從169個水田泥土樣品中分離出 726株革蘭氏陽性桿菌，根據 Bergey 氏手冊和血清凝集反應，鑑定出兩株枯草桿菌（*Bacillus subtilis*），分別命名爲 BS 1-1 與 BS 4-4。它們和標準菌株 *B. subtilis* PIC 219 的不同處，是在能產生尿素酶。

從水田中又分離出以 BS 1-1 與 BS 4-4 爲宿主的噬菌體。BS 1-1 噬菌體的頭部是六角形，直徑是 700 Å，尾部之長度爲 1700 Å，寬度爲 170 Å。BS 4-4 噬菌體的頭部是楕圓形，大小爲 456×580 Å，尾部之長度是 2,090 Å，寬度是 100 Å。此二種噬菌體在血清反應上毫無相關。BS 1-1 噬菌體能在 BS 4-4 宿主上繁殖，但 BS 4-4 噬菌體只能在其本身宿主上繁殖。根據 diphenylamine 反應知道 BS 1-1 與 BS 4-4 噬菌體都是 DNA 噬菌體。它們的宿主範疇，熱不活化性，酸鹼穩定性，紫外線不活性，及一段增殖等特性，也都被測定。