Transducing Phages for *Bacillus subtilis*

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SUMMARY

Transducing phage PBS1 and a clear-plaque type mutant PBS2 derived from PBS1 show similar growth characteristics with a relatively small burst size (23 to 24), and are related serologically. They are morphologically indistinguishable and both have a dense head of about 100 m μ in diameter and a tail about 250 m μ in length. PBS1 is more efficient in transduction and its plaque-forming activity is more sensitive to ultraviolet (u.v.) irradiation than that of PBS2. The host range of the phages is limited to strains of *Bacillus subtilis*. All Marburg strains tested were sensitive to the lytic action of the phages. Prophages of PBS1 and PBS2 were not u.v.-inducible and the irradiation of lysogenic cultures with a relatively high dose of u.v. radiation resulted in considerable decrease of the free phage titre. However, transducing activities of lysates treated with a high dose of u.v. radiation were not affected significantly. Organisms lysogenic either for PBS1 or to PBS2 were not stable and reverted to the sensitive state.

INTRODUCTION

It has been shown that the temperate phage PBS1 is capable of transducing a number of genetic traits in *Bacillus subtilis* (Takahashi, 1961). A clear-plaque type mutant (PBS2) derived from PBS1 is also able to carry out general transduction, although the frequency of transduction is always lower than that of PBS1. Thorne (1961) also isolated a temperate phage from soil and demonstrated transduction in the same organism. However, the phages reported here differ in host range and other properties from the temperate phages for *B. subtilis* reported hitherto (Romig & Brodetsky, 1961; Thorne, 1962). This paper describes the general characteristics of transducing phages PBS1 and PBS2.

METHODS

The bacterial strains used in this work are listed in Table 1. Organisms grown overnight on Difco Tryptose Blood Agar Base (TBB agar) were used as inoculum. Soft TBB agar was made up to contain the same ingredients as the prepared product with the exception that the agar content was 1%. As the *Bacillus subtilis* strains used sporulated poorly on TBB agar, inocula were essentially all vegetative forms. Liquid cultures were obtained by growing the organisms in Penassay Broth (Difco) with shaking for 4 hr. The sporulation medium of Schaeffer (1961) was used to obtain spores. Medium Y (Cook & Lochhead, 1959) was used as diluent in the assay of plaque-forming particles. All incubations were at 37° unless otherwise stated.

Phages were isolated from soil samples collected at various places in Ottawa by a modification of the method described by Ivanovics & Lantos (1958). Samples of cultivated soil (about 50 g.) were suspended in 50 ml. tap water containing a small number (about 10^7) of streptomycin-resistant indicator bacteria *Bacillus subtilis* SB19. After 2 days at room temperature the soil suspensions were shaken vigorously and allowed to settle for 30 min. A sample of supernatant liquid was mixed with an equal volume of medium Y and shaken for 3 hr. To a 10 ml. portion of the mixture were added 5 ml. of indicator culture and streptomycin sulphate (Mann Research Laboratories, Inc.) to a final concentration of 500 $\mu\text{g./ml.}$, and incubation continued for a further 6 hr. with shaking. The number of plaque-forming particles in the culture was determined, after a brief centrifugation, on soft agar containing streptomycin sulphate 500 $\mu\text{g./ml.}$

Since the soft agar-layer method of Adams (1959) did not give satisfactory results in the assay of PBS1, the following modification of the pre-adsorption technique of Potter & Nelson (1952) was used. Three ml. samples of lysate diluted in medium Y were mixed in sterile test tubes (16×125 mm.) with 1.5 ml. medium Y containing about 5×10^7 indicator bacteria. After 5 min. at room temperature 4.5 ml. of soft TBB agar (previously melted and kept in a 55° water bath) were added to the tubes with a 10 ml. graduated pipette (large tip opening) and mixed thoroughly. Fresh TBB agar plates were overlaid with 3 ml. samples of this phage + bacteria mixture. By this modified technique the number of plaques that developed on each plate represented the number of plaque-forming particles/ml. lysate tested. The assay plates were made in duplicate. The soft agar plates were incubated overnight at 28° . The omission from medium Y of yeast extract and ferric chloride decreased the plaque counts by about 80 %. Under the same condition the adsorption of phage to host bacteria was also decreased to the same extent. Thus the low plaque counts observed were probably due to poor adsorption of phage to host.

Phage lysates were made by the following technique. Phage-sensitive cultures in Penassay Broth in the logarithmic growth phase were infected with phage at a multiplicity of infection of about 1.0 and shaken for one additional hour. The infected cultures were further incubated overnight without shaking. After a brief centrifugation at low speed, the lysates were filtered through Millipore filter membranes (0.45μ pore size). Lysates were also made by growing lysogenic cultures in Penassay Broth for 4 hr. with shaking, followed by overnight incubation without shaking. Plaque counts of lysates made by either methods were about $5 \times 10^9/\text{ml.}$ for PBS1 and 5×10^9 to 1×10^{10} for PBS2.

Ultraviolet irradiations of lysogenic cultures and phages were carried out at a distance of 20 cm. from a 'Mineralight' source (Ultraviolet Products Inc., South Pasadena, California) equipped with a 2537 Å filter.

Procedures for the transduction experiments were as previously described (Takahashi, 1961). In initial experiments, recipient organisms were grown in minimal media supplemented with growth factors. Higher frequencies of transduction, however, were obtained when the organisms were grown in a complex medium such as Penassay Broth.

RESULTS

Phage isolates. By using the method described it was possible to isolate, from 12 of 15 soil samples, phages active on *Bacillus subtilis* at a titre of 10^2 to 10^4 particles/ml. Among the phage isolates, temperate phage PBS1 was able to transduce prototrophy, resistance to antibiotics (streptomycin, erythromycin, neomycin) and sporogenesis in strains of *B. subtilis*.

When a large number of particles of phage PBS1 were plated on soft agar with *Bacillus subtilis* SB19 as indicator, the presence of clear plaques which were distinct from those of PBS1 was noted. This clear-plaque type mutant (PBS2) was also temperate and able to carry out general transduction in *B. subtilis*, although the frequency of transduction was lower than that with phage PBS1.

Plaque morphology. Plate 1, figs. 1, 2, show the plaque morphology of phages PBS1 and PBS2. PBS1 forms turbid plaques of about 1 mm. in diameter with peripheral rings. PBS2 forms clear plaques of about 1 mm. in diameter which become slightly turbid after prolonged incubation.

Morphology of the phage particles. Electron micrographs were prepared by the pseudo-replica technique described by Wyckoff (1951). Phages PBS1 and PBS2 were almost indistinguishable in their morphology (Pl. 1, figs. 3, 4). They were spermlike with a dense head (about 100 m μ diameter) and tail (about 250 m μ length). These dimensions are comparable to those of T4 phage which is one of the largest phages described (Luria, 1962).

Serology. Rabbit antisera were made and their velocity constants (K) of neutralization were measured according to the procedures of Adams (1959). An antiserum made against phage PBS1 having a K value of 1830 had a K value of 810 when measured against phage PBS2. It was found that the lysates of PBS1 and PBS2 contained, with different proportions, four density species determined in the CsCl density gradient; one was purely transducing and had no plaque-forming activities (unpublished results). This suggests that phage particles in the lysates of PBS1 and PBS2 are not homogeneous. Therefore, the difference in the rate of inactivation observed here may not be significant, since the K values were calculated only by the decrease in the number of plaque-forming particles. Nevertheless, the results obtained indicate that the two phages are closely related serologically.

Host range. The host range of the phages was determined by spotting a drop of cell-free lysate on a soft agar layer seeded with test organisms. Table 1 shows the results of host range determinations made with several aerobic spore-forming bacteria belonging to different groups. The Marburg strain of *Bacillus subtilis* and all its derivatives were susceptible to the lytic action of phages PBS1 and PBS2, and general transduction could be performed between these strains. Although other *B. subtilis* strains isolated from various sources were sensitive to the phages, attempts to transduce a streptomycin marker to these strains with *B. subtilis* SB19 as donor did not succeed.

Growth characteristics. Single-step growth experiments were made according to the procedures described by Adams (1959). The results obtained with *Bacillus subtilis* SB19 as host are summarized in Table 2. Phages PBS1 and PBS2 both have a relatively small burst size and their growth characteristics are very similar. In the same experiments, from viable counts made on infected and uninfected

bacteria, it was found that only 15 % of bacteria which were killed by phage PBS 1 gave rise to plaques. The proportion of plaque formers among bacteria infected by phage PBS 2 was higher (35 %). This observation suggests the presence in the lysates of defective particles which kill the host bacteria but do not initiate phage multiplication. These resemble 'killer particles' found in defective lysogenic transductants of *Shigella dysenteriae* (Luria, Adams & Ting, 1960).

Table 1. *Susceptibility of species of Bacillus to phages PBS 1 and PBS 2*

<i>Bacillus</i>	Source of culture	Lysis by phage*	
		PBS 1	PBS 2
<i>B. subtilis</i>	NCTC 3329	—	—
	NCTC 2586	+	+
	ATCC 6633	+	+
	Marburg ATCC 6051	+	+
	SB 19 Dr E. W. Nester (Stanford University)	+	+
	Wild type Dr J. Spizizen (University of Minnesota)	+	+
	168 Dr J. Spizizen (University of Minnesota)	+	+
<i>B. subtilis</i> var. <i>niger</i> †		+	+
<i>B. megaterium</i>	MRI‡ 151	—	—
<i>B. brevis</i>	MRI 515	—	—
<i>B. circulans</i>	MRI 602	—	—
<i>B. sphaericus</i>	MRI 566	—	—
<i>B. cereus</i>	MRI 247	—	—
<i>B. cereus</i>	MRI 818	—	—
<i>B. thuringiensis</i> var. <i>thuringiensis</i>	Dr D. Kushner (NRC)§	—	—
<i>B. thuringiensis</i> var. <i>alesti</i>	Dr D. Kushner (NRC)	—	—
<i>B. polymyxa</i>	ATCC 7070	—	—

* + = lysis; — = lack of lysis; † determined by Dr J. Marmur, Brandeis University; ‡ MRI: Microbiology Research Institute, Ottawa; NRC: § National Research Council of Canada, Ottawa.

Table 2. *Growth characteristics of phages PBS 1 and PBS 2**

Host: <i>Bacillus subtilis</i> SB 19			
Phage	Latent period (min.)	Rise period (min.)	Burst size
PBS 1	35	15	23
PBS 2	37	18	24

* Averages of 4 experiments for each phage.

Stability of the phages. The phages PBS 1 and PBS 2 appear to be fairly stable when stored at 5°. Plaque counts decreased only about 50 % when lysates of phages PBS 1 or PBS 2 were stored at 5° for 3 months. No measurable decrease in phage titre was noted when lysates were treated with chloroform. The transducing activity in lysates, on the other hand, declined at a much faster rate under the same conditions. In a typical experiment, the number of *indole*⁺ colonies developing from *Bacillus subtilis* 168 (*indole*[−]) when treated with 0.1 ml. of lysates made from *B. subtilis* SB 19 decreased from 3500 to 510 after 3 months at 5°, while the plaque count of phage PBS 1 decreased from 6.8×10^8 /ml. to 3.8×10^8 /ml.

Stability of lysogenic cultures. It was reported that stably lysogenic bacteria could be isolated from turbid plaques formed by phage PBS 1 (Takahashi, 1961). The

lysogenic cultures retained their prophage through repeated subcultures on sporulation agar and heating at 85° for 15 min. Control experiments showed that the same heat treatment decreased the phage titre of a lysate from 10^7 /ml. to < 1 /ml. It was found, however, that the individual lysogenic bacteria were not quite as stable as previously thought. When about 100 colonies derived from heated spores of *Bacillus subtilis* SB19 lysogenic for phage PBS1 were examined, 50 % of them were found to be in the typical lysogenic state while the remainder were immune to phage infection but had lost their ability to produce free phage. This at first suggested that the immune state was intermediate between sensitivity and lysogeny. However, in further experiments when a colony of lysogenic *B. subtilis* SB19 was grown in Penassay Broth containing phage antiserum at 1/500 (K value of the serum estimated at a dilution of 1/2000 being 1830) for 4 hr. and plated on TBB agar, 30 % of the colonies were fully lysogenic and the remainder were sensitive, there being no detectable colonies of the immune type. The necessity for postulating the intermediate state was thus eliminated since, during the initial growth of lysogenic *B. subtilis* SB19 in the absence of phage antiserum, the presence of a large number of free phages could have served to eliminate sensitive bacteria and to select resistant (immune) bacteria derived from them. Bacteria lysogenic for phage PBS2 were also unstable and eventually reverted to the sensitive state. Because of this instability of the lysogenic state, it was not possible to establish any exact relationship between transduction and lysogenization.

Induction by u.v. irradiation. Attempts to induce phage development by u.v. irradiation of lysogenic bacteria were unsuccessful. The bacteria were irradiated in physiological saline with a 30–95 % killing dose of u.v. radiation and transferred into Penassay Broth. At a relatively low dose of u.v. radiation in no case was there an appreciable increase in phage titre when compared with unirradiated cultures. Both irradiated and unirradiated lysogenic cultures gave a titre of about 10^9 /ml. after they were incubated overnight without shaking. Relatively high doses of u.v. radiation (95 % killing) decreased the free phage titre by about 90 %.

Table 3. *Effect of u.v. irradiation on plaque count and transducing activity of phage PBS1*

Recipient: *Bacillus subtilis* 168 (*ind*⁻). *B. subtilis* W-ery (prototrophic and resistant to 1 µg./ml. of erythromycin) was used as donor.

Dose of u.v. irradiation (sec.)	Plaque count/ml.	Fraction surviving	Prototrophs* (recipient:168)	Erythromycin resistant colonies* (recipient:168)
0	3.6×10^9	1.0	850	1400
20	5.4×10^8	0.15	630	1750
40	7.5×10^7	0.021	870	1570
80	9.5×10^6	0.0026	1020	1640
120	2.1×10^6	0.00058	1570	1470

* Number of transductants developed from 0.1 ml. of lysates and 0.9 ml. of recipient culture.

Effect of u.v. irradiation on the phages. Cell-free lysates were treated with various doses of u.v. radiation in very thin layers in Petri dishes which were agitated continuously. The plaque counts and transducing activities of the irradiated lysates are shown in Tables 3 and 4. Exponential decreases in the plaque count of phages

PBS1 and PBS2 were obtained up to 40 sec. exposure; thereafter, the inactivation proceeded at a somewhat lower rate. The lytic activity of phage PBS2 was more resistant to u.v. irradiation than that of phage PBS1. On the other hand, the transducing activity was apparently unaffected; it doubled for the indole marker even though the plaque count was decreased from 10^9 /ml. to 10^6 /ml.

Table 4. *Effect of u.v. irradiation on plaque count and transducing activity of phage PBS2*

Recipients: *Bacillus subtilis* 168 (ind⁻); *B. subtilis* S10. *B. subtilis* SB19 (prot, str-r) was used as donor. S10 was derived from the wild type strain of Spizizen (see Table 1) by u.v. irradiation and was glutamic acid⁻.

Dose of u.v. radiation (sec.)	Plaque count/ml.	Fraction surviving	Prototrophs* (recipient:168)	Prototrophs* (recipient:S10)
0	2.7×10^9	1.0	390	260
20	5.3×10^8	0.19	380	280
40	1.4×10^8	0.051	550	190
80	2.7×10^7	0.010	630	230
120	6.1×10^6	0.0022	840	260

* Number of transductants developed from 0.1 ml. of lysates and 0.9 ml. of recipient culture.

DISCUSSION

The host ranges of phages PBS1 and PBS2 seem to be restricted to certain strains of *Bacillus subtilis*, while the temperate phage SP 13 for *B. subtilis* isolated by Romig & Brodetsky (1961) is active on several *Bacillus* species, including *B. megaterium* and *B. brevis*. Thorne (1962) reported that his transducing phage SP 10 was active on *B. subtilis* W23 and its derivatives but had no lytic activity on *B. subtilis* 168, although this strain can be used as recipient in transduction. As shown in Table 1, phages PBS1 and PBS2 form plaques on *B. subtilis* 168 as well as on other derivatives of the Marburg strain. This difference in host range may be explained by the fact that phage SP 10 was isolated with *B. subtilis* W23 as indicator and, in the present report, *B. subtilis* SB19 was used for the isolation of phage PBS1. *B. subtilis* SB19 is a prototrophic derivative of *B. subtilis* 168 obtained by transformation with deoxyribonucleic acid (DNA) prepared from *B. subtilis* 23 (Nester & Lederberg, 1961) and presumably still possesses the genetic background of *B. subtilis* 168. Moreover, buoyant densities of the DNA prepared from phages PBS1 and SP 10 are quite different from each other (Dr J. Marmur, personal communication). It appears, therefore, that phage PBS1 belongs to a phage race which is distinct from phages SP 10 or SP 13, although they are all temperate.

Unexpectedly, during the single-step growth experiments, it was found that only a small portion of bacteria killed by the phage infection were able to form plaques. Lysates containing phage PBS1, therefore, seem to contain defective particles as well as plaque-forming particles. At present the nature of these killing particles and their relations to the transducing particles are not known. Investigations are being undertaken to clarify this situation.

Garen & Zinder (1955) reported that the transducing activity of phage P22 for *Salmonella typhimurium* was not significantly depressed by a high dose of u.v. radiation; similar observations have been made with phages PBS1 and PBS2.

Presumably the transducing activity of these phages also involves only a small portion of u.v.-sensitive material (DNA) of the phages.

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EXPLANATION OF PLATE 1

- Fig. 1. Plaques formed on *Bacillus subtilis* SB19 by phage PBS1. $\times 5$.
- Fig. 2. Plaques formed on *B. subtilis* SB19 by phage PBS2. $\times 5$.
- Fig. 3. Electron photomicrograph of phage PBS1. $\times 11,000$.
- Fig. 4. Electron photomicrograph of phage PBS2. $\times 11,000$.