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Characterization of *Bacillus subtilis* Bacteriophages

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ABSTRACT

BRODETSKY, ANNA M. (University of California, Los Angeles), AND W. R. ROMIG. Characterization of *Bacillus subtilis* bacteriophages. J. Bacteriol. 90:1655–1663. 1965.—A group of six phages, SP5, SP6, SP7, SP8, SP9, and SP13, which use the Marburg strain of *Bacillus subtilis* as host was characterized. These phages, referred to as group 1, were examined for the following properties: host range, plaque morphology, stability, adsorption kinetics, one-step growth characteristics, calcium requirements, serum neutralization, thermal inactivation, and inactivation by ultraviolet irradiation. Five unrelated *B. subtilis* phages, SP3, SP10, PBS1, SP alpha, and SP beta, were included in the studies. When first isolated, none of the group 1 phages was able to replicate efficiently on *B. subtilis* SB19, a mutant of the "transforming" *B. subtilis* 168. Host range mutants capable of growth in SB19 were isolated for all of the group 1 phages except SP13, and are designated the "star" phages (SP5* through SP9*). For characterization, SB19 was used as host for the star phages, and another *B. subtilis* mutant, 168B, was host for SP13.

Discovery of the deoxyribonucleic acid (DNA)-mediated transformation of biochemical characteristics in *Bacillus subtilis* (Spizizen, 1958) made this organism more useful for studies in bacterial genetics. Since bacteriophages have been valuable tools for the investigation of genetic phenomena in other bacterial species, phages active against strains of *B. subtilis* were isolated. Of these phages, six were selected for further study. They were numbered in the order of isolation as SP(*subtilis* phage)5, SP6, SP7, SP8, SP9, and SP13. The method of isolation and some preliminary characterization studies have been reported (Romig and Brodetsky, 1961). On the basis of serological relatedness, as revealed by serum neutralization, they were designated the group 1 phages (Brodetsky and Romig, 1964).

The following properties of the group 1 phages have been investigated: (i) plaque morphology, (ii) stability, (iii) host range, (iv) adsorption kinetics, (v) one-step growth characteristics, (vi) calcium requirements, (vii) serum neutralization, (viii) thermal inactivation, and (ix) inactivation by ultraviolet (UV) irradiation.

Other *B. subtilis* phages included in these studies were: phage SP3 (Romig, 1962); the transducing phage SP10 (Thorne, 1961); the temperate phages SP alpha and SP beta (Eiserling and Romig, 1964); and the transducing phage PBS1 (Takahashi, 1961).

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Phage SP3 was isolated in the same manner as the group 1 phages, but at a later date. Isolated SP3 DNA is infectious for competent *B. subtilis* (Romig, 1962) as is the DNA from SP8 (Romig, *unpublished data*).

Although none of the group 1 phages transduces bacterial markers, several phages capable of mediating generalized transduction between strains of *B. subtilis* have been reported. Phages SP10 and PBS1 were the only transducing phages available when these characterization studies were initiated. Thorne (1961, 1962) isolated SP10 and SP15, temperate phages of *B. subtilis* and *B. licheniformis*, which transduce mutants of either strain (Taylor and Thorne, 1963). Phage PBS1 (Takahashi, 1961) can transduce *B. subtilis* to streptomycin resistance, nutritional independence, and sporogenesis. Other transducing *B. subtilis* phages have been reported by Ivanovics and Csiszar (1962) and Takagi and Ikeda (1962).

Results of the characterization studies on the group 1 phages are reported here. The properties which have been examined served to set these phages apart from other *B. subtilis* phages and to distinguish them from each other.

MATERIALS AND METHODS

Bacterial strains. The Marburg strain of *B. subtilis* used in these studies was obtained from C. Yanofsky. Strain SB19, furnished by E. W. Nester, is a streptomycin-resistant mutant of a prototrophic transformant of the indole-requiring

strain 168 (Nester and Lederberg, 1961). Strain 168B was derived from a surviving colony of UV-irradiated 168-2, an indole- and leucine-requiring diauxotroph isolated from strain 168 by UV irradiation of spores (Romig, 1962). Strain W23S^r is a prototrophic derivative of the threonine-requiring strain 23 (Spizizen, 1958) and is streptomycin-resistant. This organism and strain W23S^r-L9, lysogenic for phage SP10 (Taylor and Thorne, 1963), were received from C. B. Thorne.

Bacteriophages. The group 1 *B. subtilis* phages SP5, SP6, SP7, SP8, SP9, and SP13 were isolated from soil (Romig and Brodetsky, 1961), and were selected specifically for their ability to lyse the Marburg strain. The propagating host is designated as follows. The symbol of the phage is followed by the symbol of the host bacterium in which it was propagated, the two symbols being separated by a dash. Thus, SP8-M means SP8 propagated in the Marburg strain, SP8-W signifies that the phage was grown in strain W23S^r, and SP8-19 and SP8-168B represent the phages grown in strains SB19 and 168B, respectively. The mutant phages, capable of growth in SB19, are symbolized as SP5*, SP6*, SP7*, SP8*, and SP9*, and will be referred to as the star phages.

Phage SP3 has been described (Romig, 1962; Eiserling and Romig, 1962). Phage PSB1 was obtained from I. Takahashi. The source of SP10 was a suspension of spores of the lysogenic strain W23S^r-L9. Strain SB19 was used as host for SP3 and PBS1. Strain W23S^r was employed as host for SP10, which does not form plaques on SB19.

Media. The medium used for propagation and dilution of phages and bacteria was TY broth (Romig, 1962). Bottom layer and soft agars for plating contained 1.5 and 0.75% agar in TY broth. When $CaCl_2$ was added to TY broth for one-step growth experiments, it was present at a final concentration of 0.018 M. The minimal medium described by Spizizen (1958), modified by the omission of glucose, was used as diluent for the phages in the UV inactivation studies.

Preparation and assay of phage lysates. The phages were propagated by the plate lysis technique (Adams, 1959). Phage lysates were sterilized by filtration through Millipore membrane filters (0.45-mμ pore size). Lysates were assayed by the conventional agar-layer method (Adams, 1959), except that suspensions of bacterial spores were sometimes used as indicator cells instead of logarithmically growing bacterial cultures. The phages formed plaques with equal efficiency on spores or vegetative cells.

Host range determinations. Host range was examined by the spotting method previously described (Romig and Brodetsky, 1961), modified by use of serial 10-fold dilutions of phage lysates (10^{-1} to 10^{-7}), or by the agar-layer method.

Phage antisera. Antisera for the group 1 phages were prepared in rabbits by use of six intraperitoneal injections of phage lysates. Antiserum against phages SP alpha and SP beta was obtained from F. A. Eiserling. Source of the phages used in preparation of this antiserum was an UV-induced lysate of strain SB19 lysogenic for both SP alpha and SP beta. Antiserum against phage PBS1 was provided by W. Pritikin.

Neutralization procedures. The method outlined by Adams (1959) was used to determine the kinetics of neutralization by homologous antiserum. For determining relatedness of the group 1 phages, antiserum and phage were mixed as in the standard procedure, and the mixture was assayed after 5 min of incubation. Group 1 antisera were pooled for testing ability to neutralize other *B. subtilis* phages, and the incubation period was extended to 30 min.

Adsorption of phages to bacteria. A method modified from that described by Adams (1959) was used to determine adsorption rates. Logarithmically growing bacteria were mixed with phage in a ratio of less than one phage per bacterium. Samples were withdrawn from the aerated mixture at intervals, freed from bacterial cells by filtration through Millipore filters, and assayed for free phage. Successive filtration of phage suspensions did not result in a measurable loss of infectious titer.

One-step growth experiments. Growth characteristics were determined by one-step growth experiments (Ellis and Delbrück, 1939; Adams, 1959). The host bacteria were grown with aeration to a final concentration of about 5×10^8 colony-forming units per ml. The multiplicity of infection in the adsorption tube was always less than one. Adsorption was allowed to proceed for 10 min; a sample of the adsorption mixture was then exposed to antiserum action for 5 min, and dilutions were made into growth tubes. Samples were withdrawn and assayed for plaque count until 100 min had elapsed, at which time lysis of infected bacterial cells was essentially complete. The experiments were performed in a water bath at 37 C, and the tubes were aerated by gently bubbling air through them.

Thermal inactivation. For determination of the kinetics of heat inactivation, a tube containing 3.6 ml of TY broth was maintained at 60 $\pm$ 0.5 C and 0.4 ml of phage suspension was added to it and thoroughly mixed. At intervals, samples were delivered into ice-cold broth and assayed for infectious phage.

Inactivation by UV irradiation. The UV source used was a 15-w General Electric germicidal lamp having an energy emission of 10 ergs per sec per mm^2, as determined from a control inactivation curve of coliphage T2 (Latarjet, Morenne, and Berger, 1953). Phage suspensions were diluted in Spizizen's minimal medium and irradiated in petri dishes. Samples taken after various intervals of exposure to UV light were diluted in TY broth and assayed for viable phage.

The plate method of Dulbecco (1950) was used for investigation of photoreactivation. UV-irradiated samples were assayed in parallel. One series of plates was immediately incubated in the dark; the other series was exposed to the light of two

15-w fluorescent lamps at a distance of 7 cm for 30 or 60 min at room temperature, and then was incubated in the dark at 37 C.

Effect of pH. Autoclaved samples of TY broth adjusted to desired *p*H values with HCl and NaOH were used to determine the stability of the phages over a range of *p*H values. Phage suspensions diluted in each broth were incubated for 1 hr at 37 C, and assayed for surviving phage.

RESULTS

Plaque morphology. Morphology of the plaques formed by the group 1 phages on *B. subtilis* Marburg has been described (Romig and Brodetsky, 1961). Upon further propagation by the plate lysis method, the clear plaques formed by SP5, SP7, SP8, and SP9 were unchanged. The turbid plaques of SP6 and SP13 were replaced by clear plaques similar to those formed by the other four phages. When strains SB19 and 168B were used as hosts, plaque morphology was unchanged, but each plaque was surrounded by a large halo which appeared shiny in comparison with the bacterial lawn. The halos resembled those produced in T2-infected *Escherichia coli* by the action of phage lysozyme (Koch and Dryer, 1958; Anfinsen, 1961). Murphy (1957) also described halos surrounding plaques of phage-infected *B. megaterium.*

Stability. Of the *B. subtilis* phages originally isolated, several were entirely inactive after storage at 4 C for 3 weeks, and some were inactivated when treated with chloroform. The six phages of group 1 were more stable under these conditions, and their response to various conditions of storage was investigated. Phage suspensions were stored at 4 C, were quickly frozen with solid CO_2 in ethyl alcohol, or were lyophilized. After various periods of time, samples were assayed for viable phage. Addition of substances known to increase the stability of viruses, such as glycerol or sucrose, did not affect recovery of viable phage to a significant degree. None of the group 1 phages survived lyophilization, and titers were drastically reduced (10^{-5} to 10^{-6}) after quick freezing. The phages were best preserved when stored in TY broth at 4 C. Under these conditions, 8 to 9% of viable phages initially present were recovered after 1 year.

Examination of the effect of *p*H on phage stability yielded the results shown in Table 1. SP5 and SP8 were more stable at alkaline *p*H, although infectivity was lost between *p*H 9 and 10. Similar results were obtained with the other group 1 phages.

Host range. Since transformation and transduction have been demonstrated with *B. subtilis* 168 and its mutants, it seemed desirable to use SB19 as host for the characterization studies, rather than our Marburg strain which does not become competent. Initial attempts to propagate the phages in strain SB19 were unsuccessful. The efficiency of plating (EOP) of the Marburg-grown phages on SB19 was 10^{-4} to 10^{-5} that on the Marburg host. Plaques were pinpoint in size and indistinct, and single-plaque isolation did not yield high-titered phage suspensions. However, when agar plates were inoculated with mixtures containing 10^8 plaque-forming units (PFU) per ml of phage and 10^7 bacterial cells per milliliter, growth of the bacterial lawns was inhibited. The filtrates obtained from these plates contained phage which plated efficiently on SB19, and propagation of these phages in SB19 yielded high-titered lysates (10^9 to 10^{10} PFU/ml). All of the phages except SP13 were thus "adapted" to grow in SB19. They were designated SP5*, SP6*, SP7*, SP8*, and SP9*, and were used in the characterization studies, with SB19 as host.

The fraction of a Marburg-grown phage population which could form plaques on SB19 might represent either mutants with extended host range, or phages which had undergone host-induced modification (Luria and Human, 1952). Stocks of the star phages grown in SB19, when assayed on SB19, gave consistently high EOP values (about 10^2) relative to assay on W23S^r or the Marburg strain. The star phages retained their high relative EOP after one cycle of growth in either W23S^r or the Marburg strain if they were mutants and not host-modified. To distinguish between these two possibilities, SP8* was chosen to represent the star phages. One growth cycle of SP8*-19 in W23S^r did not alter the plating efficiency of the progeny phage on either SB19 or W23S^r, and the EOP on SB19 was still about 10^2 relative to that on W23S^r.

TABLE 1. *Effect of pH on SP5 and SP8**

*p*H	Recovery of viable phage†	
	SP5	SP8
4	0	0
5	62	60
6	70	71
7	100	100
8	89	88
9	81	82
10	0	0

* The phages were suspended in broths at various *p*H levels, and were incubated for 1 hr at 37 C.

† Per cent of plaque-forming units recovered from *p*H 7 broth.

Similar results were obtained with the Marburg host. It was concluded that ability of SP8* to grow in SB19 was due to mutation, and it was therefore assumed that the star phages are mutants with an extended host range.

Phage SP13-M was not adsorbed by SB19 (Table 2). However, SP13-M produced high-titered lysates when propagated in 168B, and this strain was employed as host for SP13 in the characterization studies.

Adsorption rates. The kinetics of adsorption of the star phages by SB19, and of SP13-168B by 168B, were determined. Rate constants for the reactions (Adams, 1959) are shown in Table 2. Adsorption rates of Marburg-grown phages by the Marburg strain were very similar. Both hosts adsorbed 85 to 95% of input phage in 5 min, but neither host adsorbed phage SP13 as efficiently as the other phages. Only 47% of input phage SP13 was adsorbed in 5 min by the Marburg strain, suggesting a difference in tail structure between SP13 and the other group 1 phages.

One-step growth characteristics. One-step growth experiments were done with each phage in calcium-supplemented broth, and latent periods and average burst sizes were determined (Table 3). All of the star phages showed very similar growth characteristics in host SB19, except for the amount of abortive infection observed. A typical growth curve, that of SP9* in calcium-supplemented broth, is shown in Fig. 1. Phage SP13-168B had a significantly larger burst in 168B, although its latent period was the same as that of the other phages, and abortive infection was also observed.

TABLE 2. *Adsorption rates of the group 1 phages*

Phage	Input ratio*	Host	Adsorption rate constant
			ml/min
SP5*	0.14	SB19	1.9×10^{-9}
SP6*	0.11	SB19	6.7×10^{-10}
SP7*	0.18	SB19	5.4×10^{-9}
SP8*	0.20	SB19	4.3×10^{-9}
SP9*	0.11	SB19	7.6×10^{-10}
SP13-M	10.00	SB19	0.0
SP13-M	0.18	Marburg	1.4×10^{-8}
SP13-168B	0.11	168B	2.0×10^{-9}

* Input ratio = ratio of phage to bacteria in the adsorption mixture.

TABLE 3. *One-step growth characteristics of the star phages in host SB19, and of SP13-168B in host 168B*

Phage	Latent period	Avg burst size	IBC recovered*
	min		%
SP5*	37–40	96	39
SP6*	35–40	110	72
SP7*	34–38	117	11
SP8*	35–40	120	96
SP9*	34–38	87	35
SP13-168B	35–40	195	85

* Infectious bacterial centers recovered, expressed as per cent of adsorbed phage.

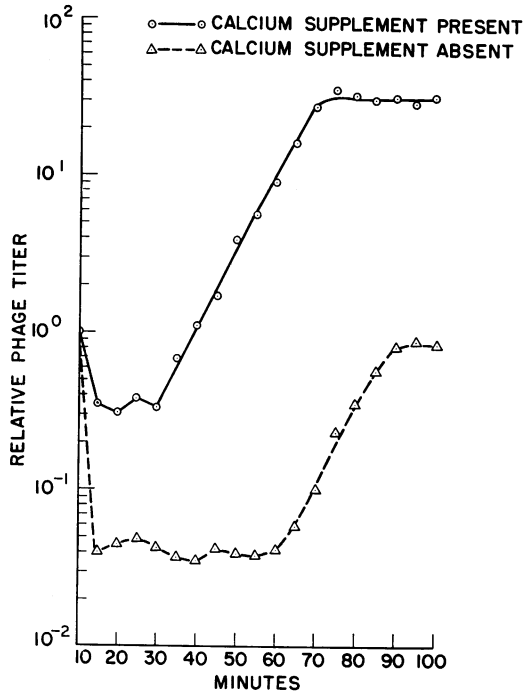

FIG. 1. *One-step growth curves of SP9* in the presence and absence of calcium supplementation.*

In these experiments, recovery of infectious bacterial centers was always less than the number of bacteria which adsorbed phage, varying from 4 to 89% (Table 3). Phage adsorption was efficient and the multiplicity of infection varied between 0.07 and 0.09. At such multiplicities less than 1% of the bacteria would have adsorbed more than one phage particle. This would not account for the loss of input phage observed. The "killing" titer of an SP8* lysate, calculated from viable counts made on infected and uninfected bacteria, agreed very closely with the titer of infectious phage determined by plaque assay. Thus, productive infection is initiated only in a certain fraction of the bacteria which adsorb phage and are killed.

Very little abortive infection was seen with SP8*, whereas almost 90% of input phage SP7*

TABLE 4. *Average burst size and recovery of infectious bacterial centers in absence of calcium supplementation*

Phage	Avg burst size	IBC*
		%
SP5*	30	6
SP6*	16	22
SP7*	38	65
SP8*	5	2
SP9*	20	4
SP13-168B	30	3

* IBC = infectious bacterial centers recovered, expressed as per cent of adsorbed phage.

TABLE 5. *Per cent neutralization of the group 1 phages after exposure to antiserum action for 5 min*

Phage	Antiserum					
	5	6	7	8	9	13
SP5*	81	80	77	63	79	84
SP6*	79	84	72	72	62	74
SP7*	94	91	92	79	87	89
SP8*	91	72	68	54	76	74
SP9*	84	82	74	63	71	86
SP13-168B	88	85	83	62	79	84

was lost in nonproductive infections. Phages SP5* and SP9* also showed a high proportion of abortive infection, but SP6* and SP13-168B did not.

Calcium requirements. All the group 1 phages required additional calcium (final concentration, 0.018 M) for maximal phage production in broth. In unsupplemented broth, abortive infection was greatly increased, latent periods were almost twice as long, and bursts were reduced by 70 to 90%. Figure 1 shows a set of typical one-step growth curves, those for SP9*, in broth with and without calcium supplementation. Adsorption was not increased by addition of the ion.

Recovery of infectious bacterial centers and average burst size were affected to varying degrees by calcium deprivation (Table 4). The effect on average burst size did not always correlate with the effect on recovery of infectious centers, since in the case of SP7* average burst size was materially reduced when recovery of infectious centers was not (see Table 3). Only with SP8* was the average burst reduced to the same extent as recovery of infectious centers. The other phages varied in their response to added calcium. Only SP5* and SP9* appeared to resemble each other in their calcium requirement.

Serum neutralization. Homologous neutralization rate constants (K values) were determined for the group 1 antisera. The reactions followed first-order kinetics to the point of 99% neutralization. The K values ranged from 174 for SP8* antiserum to 1,932 for SP13-168B antiserum. Cross-neutralization was tested by reacting each antiserum with all six of the group 1 phages. The results appear in Table 5, expressed as per cent neutralization after 5-min exposure of phage to antiserum action. Since all the antisera neutralized all the phages to about the same degree, it was concluded that the phages are closely related serologically.

Heterologous rate constants for a pooled group 1 antiserum were determined with phages SP3, SP10, and PBS1. SP3 and PBS1 antisera were also tested with all the group 1 phages. No antiserum for SP10 was available, so the reciprocal test could not be done with this phage. Since no bacterial strain has been found which is susceptible to SP alpha and SP beta, only the effect of the SP alpha-SP beta antiserum on the other phages could be determined. Rate constants calculated from the data obtained in these

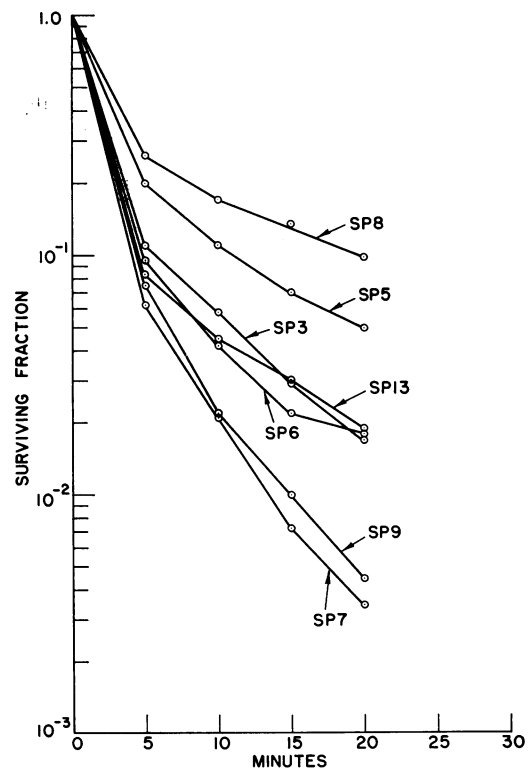

FIG. 2. *Thermal inactivation of the group 1 phages and SP3 at 60 C.*

experiments are shown in Table 6. The heterologous values were less than one in all cases. These fractional K values were within the limits of experimental error for the method, and were considered to indicate nonrelatedness of the phages.

From these results it was concluded that the group 1 phages are not serologically related to SP3, SP10, or PBS1, nor are the latter three phages related to each other. The data also indicate that neither SP alpha nor SP beta is related to the group 1 phages or to the other phages. This conclusion is supported by electron microscopy, which has revealed that SP alpha and SP beta differ morphologically not only from each other, but also from SP3, SP10, PBS1, and the group 1 phages (Eiserling, 1964).

Thermal inactivation. At 65 C the group 1 phages and SP3 were inactivated too rapidly to determine the rate of this process. Results of heating the phages at 60 C are shown in Fig. 2. Although the initial inactivation rates were similar, after 5 min the curves deviated in a manner suggesting different heat sensitivities in the phage populations. SP5* and SP8* showed similar sensitivity. SP6* and SP13-168B were nearly identical, as were SP7* and SP9*. Serologically unrelated phage SP3 was inactivated at the same rate as SP6* and SP13-168B.

Inactivation by UV irradiation. Inactivation of all the group 1 phages occurred exponentially after 30 to 45 sec of irradiation. During the exponential period, the surviving fraction decreased by four to five orders of magnitude. The group 1 phages were considerably more resistant than were SP3, SP10, PBS1, and coliphage T2, which was used to calibrate the UV lamp. Inactivation curves for these phages appear in Fig. 3, along with an abbreviated curve for SP8*. The other three *B. subtilis* phages differed markedly from the group 1 phages in their irradiation kinetics, exhibiting a gradual decrease in the inactivation rate as the surviving fraction decreased by about two orders of magnitude.

TABLE 6. *Neutralization rate constants (K min^{-1}) determined by exposure of phages to heterologous antisera for 30 min*

Phage	Antiserum			
	Pooled	SP3	SP alpha-SP beta	PBS1
SP5*		0.1		0.2
SP6*		0.2		0.1
SP7*		0.1		0.2
SP8*	174*	0.3	0.4	0.0
SP9*		0.1		0.2
SP13-168B		0.0	0.3	0.0
SP3	0.3	47*	0.0	0.0
SP10	0.2	0.0	0.0	0.0
PBS1	0.3	0.0	0.5	2,100*

* Homologous neutralization rate constant.

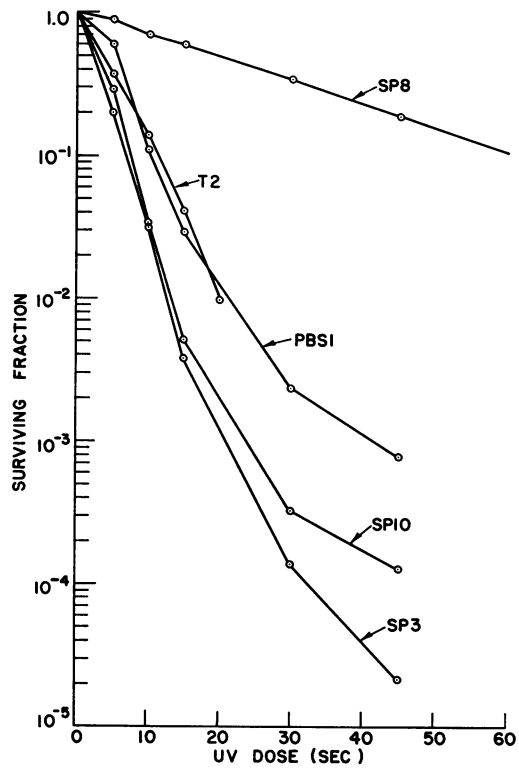

FIG. 3. *Inactivation of SP3, SP10, PBS1, SP8*, and coliphage T2 by ultraviolet irradiation.*

TABLE 7. *Energy required for 99% inactivation of subtilis phages*

Phage	Energy (ergs per mm²)
SP5*	1,270
SP6*	1,550
SP7*	1,100
SP8*	1,380
SP9*	1,450
SP13-168B	1,440
SP3	130
SP10	130
PBS1	210
T2	200

Photoreactivation did not occur to a significant degree with the *B. subtilis* phages. For example, a suspension of SP8* was irradiated with an UV dose which would inactivate all but 0.1% of the

phage, and was diluted to yield an average of 150 plaques when incubated in the dark. Samples from the same dilution tube produced an average of 160 plaques after exposure to fluorescent light for 1 hr. Dulbecco (1950) found that photoreactivation of the coliphages was 1,000-fold or greater under similar conditions.

Table 7 groups the phages in terms of their UV sensitivity. The values were determined from the inactivation curves and are expressed as the amount of energy required to inactivate 99% of a given phage population. Values for the group 1 phages were similar, those for SP10 and SP3 were identical, and the value for PBS1 was very close to that for coliphage T2. The group 1 phages were almost 10 times more resistant to irradiation than SP3 and SP10, and about six times more resistant than PBS1 and T2.

The characterization studies showed that each of the group 1 phages differs from the others in at least one property. Only thermal inactivation differentiates SP5 from SP9. Although they have a number of properties in common, none of the group 1 phages proved to be identical.

DISCUSSION

The criteria used for characterizing the *B. subtilis* phages were those advanced by Adams (1953, 1959) as most useful for phage classification. On the basis of serological relationship, SP5, SP6, SP7, SP8, SP9, and SP13 were tentatively assigned to a single group (Brodetsky and Romig, 1964). By this criterion, the other *B. subtilis* phages examined are not related to the group 1 phages, nor are they related to each other. This grouping is supported by the fact that the group 1 phages have other properties in common. They appear to be morphologically identical, since electron microscopy did not reveal any differences in the size and morphology of the phage particles (Eiserling, 1964). Of the other phages, only the transducing phage SP10 resembles the group 1 phages morphologically.

The DNA of SP8 was found to contain 5-hydroxymethyl uracil in place of thymine (Kallen, Simon, and Marmur, 1962). Data obtained from thermal denaturation and density-gradient centrifugation studies strongly suggest that the DNA of the other group 1 phages also contains this unusual base (Marmur and Cordes, 1963). The fact that the DNA preparations of all the group 1 phages have the same buoyant density and denaturation temperature provides two further criteria for classifying these phages within a single group.

The phages assigned to group 1 also had similar growth characteristics and were inactivated at nearly the same rate by UV irradiation. The presence of 5-hydroxymethyl uracil in the DNA of the group 1 phages could be responsible for their greater resistance to UV irradiation. Among the chemical modifications which occur when DNA absorbs UV radiation are the dimers formed between adjacent thymine bases in the polynucleotide chains of bacterial DNA (Wacker, 1963). It is thought that such dimerization creates the lesions primarily responsible for inactivation of irradiated phage particles (Stent, 1963). Wacker (1963) reported that irradiation of 5-hydroxymethyl uracil gave rise to a smaller amount of dimerization than irradiation of either thymine or uracil. The group 1 phages showed a greater resistance to irradiation than the other *B. subtilis* phages, which do not appear to contain this unusual base.

In addition, none of the group 1 phages could be photoreactivated to a significant extent. Available evidence indicates that photoreactivation in *E. coli* is mediated by a bacterial enzyme system which repairs UV-induced lesions in phage nucleic acid (Lennox, Luria, and Benzer, 1954; Goodgal, Rupert, and Herriott, 1957), and that the enzyme is normally present in the cells of a photoreactivating system (Metzger, 1964). Absence of photoreactivation with the *B. subtilis* phages might be understood in the light of Kelner's (1964*a*, *b*) observation that photoreactivation of *B. subtilis* 168 was of an unusual, oxygen-dependent type, occurring only when UV-irradiated cells were incubated under forced aeration. Under assay conditions where phage-infected bacteria were not aerated, photoreactivation would not be expected to occur.

Though these phages were placed in a group because of similarities in the properties just discussed, further characterization revealed that each of the phages within group 1 differed from the other members in one or more properties. Calcium requirements, thermal inactivation, and the production of abortive infections were important in differentiating the phages within the group.

It is known that many phages have a calcium requirement, and that related phages may differ in their calcium requirements. Rountree (1951) showed that the staphylococcal phages required divalent cations, and that each phage had a characteristic calcium requirement that could not be met with either magnesium or strontium (Rountree, 1955). Failure of the group 1 phages to initiate productive infections in broth without calcium supplementation could mean that there is insufficient calcium for DNA injection. The small bursts produced by the phages in unsupplemented broth are an indication that some step(s) in their replication is calcium-dependent. Shafia

and Thompson (1964), working with a *B. stearothermophilus* phage, found that both penetration of DNA into the host cell and synthesis of new phage particles required calcium. Aposhian (1965) also reported a requirement for calcium for propagation of SP5.

Even in the presence of calcium supplement a significant fraction of adsorbed phage was lost in abortive infection with some of the group 1 phages. A similar observation of nonproductive infections with phage PBS1 was reported by Takashashi (1961), who found that only 15% of the bacteria killed by this phage gave rise to plaques. He suggested that the phage lysates might contain defective particles which kill the bacteria but do not initiate plaque formation.

Characterization of the *B. subtilis* phages has shown that, among the criteria advanced by Adams (1959), serology, size and morphology, chemical composition, and latent period serve to place phages SP5, SP6, SP7, SP8, SP9, and SP13 in a group. The criteria of susceptibility to inactivation and of distinctive physiological properties serve to distinguish members of this group. Similarity in the basic properties of morphology, serology, and nucleic acid content make it logical to exclude from this group the other *B. subtilis* phages examined.

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