SOME BIOLOGICAL PROPERTIES OF BACTERIOPHAGES S13 AND ϕ X-174¹

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Since Burnet (1927) first isolated bacteriophage S13, this phage has been the subject of numerous investigations.² These have dealt mainly with its physical properties; S13 is believed to be the smallest known phage, with a diameter estimated to be about 16 millimicrons. There have been few recent papers dealing with the biological properties of S13; of these, the one by Mills (1955) is the most comprehensive. The present paper is intended to fill in certain gaps in the information available on this phage.

Bacteriophage ϕ X-174 was first isolated by Sertic and Boulgakov (1935). It has been studied less thoroughly than S13, but there is evidence that it is of a similar size (Bonét-Maury and Bulgakov, 1944; Sinsheimer, 1957). It will be shown here that there is probably a relationship between ϕ X-174 and S13.

MATERIALS AND METHODS

Shigella paradysenteriae strain Y6R and phage S13 were obtained from Dr. Robert Wahl. *Salmonella typhosa* strain 373 and phage ϕ X-174 were obtained from Dr. N. Boulgakov. *Escherichia coli* strain C and a second sample of ϕ X-174 were obtained from Dr. E. S. Lennox.

Except for overlay agar, all media contained 1 per cent tryptone (Difco), 0.5 per cent yeast extract and 0.1 per cent glucose. Indicator bacteria were grown in this basal broth overnight with shaking; Y6R and its mutants were incubated at 30 C, while all other strains were incubated at 37 C. One drop of overnight culture was added to 3 ml of overlay agar for plating. Logarithmic phase cultures were started by

inoculating appropriate media with $\frac{1}{50}$ volume of an overnight culture and shaking at 37 C.

The base agar consisted of about 35 ml/plate of basal broth with 1 per cent powdered agar and 1.2 per cent glucose. Except where otherwise noted the base agar also contained 1 per cent NaCl and 0.0025 M CaCl₂ (base agar-NaCa).³ Overlay agar consisted of 0.8 per cent nutrient broth powder (Difco), 0.65 per cent agar, and the same concentration of NaCl as the base agar. Plating techniques were those described by Adams (1950).

Lysates were made in basal broth-NaCa which was inoculated with cells from an overnight culture (of Y6R, unless otherwise stated) and shaken at 37 C until the cell concentration reached about 10⁸/ml. Phage was added to a final concentration of 10⁴ to 10⁵/ml, and shaking was resumed. As soon as visible lysis started the culture was centrifuged at about 1000 $\times$ G for 20 min at room temperature. The supernatant was poured over CHCl₃, shaken, and stored at 4 C. Titers of wild type S13 and ϕ X-174 lysates made on Y6R were between 10¹⁰ and 2 $\times$ 10¹¹/ml. Titers were considerably lower if the cultures were centrifuged after they had cleared completely. It was also possible to make lysates containing 5 $\times$ 10¹⁰ S13/ml by infecting cultures of *E. coli* growing in synthetic M-9 medium (Watson, 1950).

Phage dilutions were made in basal broth except when the phage was to be used to infect cells in basal broth-NaCa, in which case the dilutions were made in the latter medium. Infected cells were diluted in basal broth-NaCa or in basal broth-Na. Infections and incubation of infected cells (including cells on plates) were carried out at 37 C. If extra time was needed for handling infected cells they were chilled 7 min after infection; the infection proceeded normally when they were again warmed to 37 C.

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² A bibliography of papers dealing with S13 and ϕ X-174 is available from the author upon request.

³ "Na" and "Ca" will be used to designate the incorporation of these concentrations of NaCl and CaCl₂, respectively, into any medium.

Plaque-type mutants were isolated on colored agar plates whose base agar contained 0.0625 per cent Erio chrome yellow 2 G (Geigy Company, Inc., 89-91 Barclay St., New York, N. Y.) and 0.01 per cent Suco soluble blue (Standard Ultramarine Co., Huntington, W. Va.) (Bresch, 1953; Bertani, 1954).

Antisera were made by inoculating rabbits with 1 to 5×10^{11} S13 particles. Intravenous inoculation, even of phage preparations which had been partially purified by high-speed centrifugation ($140,000 \times G$ for 4 hr), often resulted in death of the rabbit with symptoms similar to those of endotoxin poisoning. This was also true of lysates prepared from cells of *E. coli* strain C. Best results were obtained by suspending a high-titer lysate in Freund's adjuvant and inoculating 0.5 ml aliquots of the mixture subcutaneously into each of the subaxillary and inguinal regions. All antisera were heated at 56 C for 30 min before using. Controls for antiserum inactivation experiments were run with pooled normal rabbit serum, which never caused detectable phage inactivation under the conditions used.

Irradiation experiments were carried out by exposing diluted lysates to ultraviolet light from a 15 watt General Electric germicidal lamp. Dosages were standardized by means of a dosimeter as described by Latarjet *et al.* (1953). Five ml of suspension were placed in a 9 cm petri dish on a rotary shaker about 43 cm below the lamp. With phage suspended in basal broth-NaCa, irradiation for about 220 sec (5000 ergs/mm²) killed 99 per cent of S13 phage. Killing was exponential to 10^{-5} survivors.

Photoreactivation was brought about by adsorbing UV-killed S13 to cells of Y6R (about 10^8 /ml) in 12 ml conical centrifuge tubes, using 1 ml of suspension per tube. The tubes were kept in a 37 C water bath, and a 150 watt Sylvania Birdseye Super spot light was placed so that its light was reflected through the water by means of an aluminum mirror, directly onto the portion of the tubes containing the suspensions. After the suspensions were plated, the petri dishes were placed for 1 hr in the light of two General Electric no. A21 100 watt bulbs in Castle operating lamps, supplemented by four 40 watt fluorescent lamps at a distance of 150 cm. Adsorption could be carried out in the dark with only a slight loss in photoreactivation.

RESULTS

S13 and ϕ X-174 are virulent phages. Although several scores of resistant colonies have been picked and tested from plates seeded heavily with Y6R, *E. coli* strain C or *S. typhosa* strain 373 in the presence of excess phage, no evidence has been obtained that any of these colonies is lysogenic.

One-step growth curves of S13 and certain of its mutants on Y6R (with unadsorbed phage inactivated by antiserum) show that in basal broth-NaCa at 37 C with aeration, the latent period is 14 to 15 min; the burst size is 200 to 250. The adsorption rate with young cells is greater than 2×10^{-9} min⁻¹, but decreases rapidly when the culture reaches about 4×10^8 Y6R/ml.

Isolation of S13 plaque-type mutants. (See figure 1.) Plaques of S13 on Y6R on colored agar-NaCa plates are 3 or 4 mm in diameter, and are surrounded by a pale green halo less than 1 mm wide. Background cells are dark green. After 18 hr incubation, a small fraction of such plaques contain halos of which a sector is dark blue. From such sectors a mutant, S13a, can be isolated. S13a forms plaques which are 2 or 3 mm in diameter surrounded by a dark blue halo more than 1 mm wide. S13a and other mutants which form wide, dark halos on this medium are more heat-sensitive (especially in the presence of CaCl₂) than wild-type S13. Lysates of these dark-halo mutants seldom contain more than 10^9 phage/ml.

Plaques of S13 and of S13a on colored agar without added NaCl (colored agar-Ca) are about 4 mm in diameter and do not possess halos. A small fraction of such plaques possess small dark

MEDIUM	MUTANT					
	S13e	S13a	S13	S13d	S13e'	S13c
CANaCa						
CACa						

Figure 1. Diagrammatic representation of plaque-type mutants of S13 on colored agar with (colored agar-NaCa) and without (colored agar-Ca) added NaCl. Arrows indicate origin of mutants from sectors appearing in occasional plaques. S13c is present in lysates of wild-type phage.

blue sectors at their periphery after 18 hr incubation. From these sectors can be isolated mutant phages which form plaques on colored agar-Ca with blue halos about 1 mm wide. The mutant isolated from plaques of S13 retains the wild-type plaque morphology on colored agar-NaCa, and is called S13d. The mutant isolated from S13a retains the "a" plaque morphology on colored agar-NaCa, and is called S13e.

When S13d is plated on colored agar-NaCa its plaques are like those of S13, and occasionally possess dark blue sectors. From these sectors a phage may be isolated which appears to be identical to S13e, giving dark halos on colored agar-Ca and on colored agar-NaCa. This mutant is called S13e'.

S13 lysates contain a small fraction (10^{-4}) of mutants, S13c, which form haloless plaques about 2 mm in diameter on colored agar-NaCa and on colored agar-Ca.

The derivation of these plaque-type mutants is outlined in figure 1. The isolation of S13e from plaques of S13a, and the isolation of the apparently identical S13e' from plaques of S13d, strongly suggests that the formation of dark halos on colored agar-NaCa and on colored agar-Ca are mediated by two genes which mutate independently. Unfortunately, in the absence of recombination data (e. g., the appearance of S13 and S13e among the progeny of mixed infections of cells with S13d and S13a), it is impossible to exclude completely the possibility that each of these phenotypes is the result of mutation at a single locus, and that they are all allelic to each other. This possibility is made quite unlikely, however, by the fact that no mutant with the characteristics of S13e has ever been isolated directly from plaques of S13.

Host ranges and host-range mutants. On base agar-NaCa plates, S13 and ϕ X-174 form plaques with good efficiency on Y6R, *E. coli*, and *S. typhosa*. The plating efficiencies of the two phages on these three hosts in the absence of NaCl, CaCl_2 , or both, are given in table 1. It will be noticed that NaCl inhibits plaque formation by S13, and that this inhibition is relieved by CaCl_2 . The inhibition is, to some extent, host-dependent; some plaques are formed on *E. coli* in the presence of NaCl without CaCl_2 , but none can be observed on Y6R or on *S. typhosa*. The plaques formed on *E. coli* under these conditions do not contain host-range mutants.

TABLE 1

Efficiencies of plating of S13 and ϕ X-174 on Shigella paradysenteriae strain Y6R, Escherichia coli strain C and Salmonella typhosa strain 373

Host	S13				ϕ X-174*			
	With NaCl†		No NaCl		With NaCl		No NaCl	
	With CaCl_2 ‡	No CaCl_2	With CaCl_2	No CaCl_2	With CaCl_2	No CaCl_2	With CaCl_2	No CaCl_2
Y6R	1.0	$<10^{-8}$	1.0	0.9	1.0	$<10^{-8}$	1.0	10^{-8} *
C	0.5	0.05	0.7	0.6	0.8	10^{-7} *	1.5	1.5
373	0.5	$<10^{-8}$	1.0	0.6	1.0	$<10^{-8}$	1.5	10^{-5} *

* Host-range mutants were isolated under conditions marked with an asterisk (*).

Efficiencies of plating were calculated by assuming that the efficiency on Y6R in the presence of NaCl and CaCl_2 (base agar-NaCa) was 1.0.

† Base agar and overlay agar contain 1 per cent NaCl.

‡ Base agar contains 0.0025 M CaCl_2 .

Strain ϕ X-174 differs greatly in plating efficiency from S13 under three conditions listed in table 1: on Y6R with no added salts; on C with NaCl; and on 373 with no added salts. Under each of these conditions host-range mutants of ϕ X-174 which form plaques with fair efficiencies (0.05 to 0.5) can be isolated. The mutants differ from each other, each having an increased efficiency of plating only for the conditions (i. e., host and ionic composition of the medium) under which it was isolated. It is possible that by serial selection one might isolate a "triple mutant" of ϕ X-174 with efficiencies of plating similar to those of S13 under all conditions listed; this has not been attempted.

Strains of Y6R, C and 373 resistant to S13 can easily be isolated by seeding base agar-NaCa plates with the sensitive host and an excess of S13. Most strains so isolated are also resistant to ϕ X-174. Phage mutants capable of forming plaques on these resistant strains can sometimes be isolated. The host-range mutants (S13h) usually have efficiencies of plating on these resistant bacteria between 0.01 and 0.1 when compared to assays on Y6R; the plaques are small, irregular and difficult to count. The few host-range mutants which have been tested on Y6R on colored agar-NaCa plates form plaques with dark halos, similar to those of S13a; they

are more heat-sensitive than S13. When host-range mutants of S13 are isolated on different hosts, they differ among themselves in their efficiencies of plating on the different hosts; at least 10 different host-range mutants have been identified by this criterion. No cases of host-induced modification have been observed. It may be noted that some, but not all, strains of Y6R/13 are sensitive to soluble blue, and fail to grow on colored plates.

Strains of Y6R/13 isolated on base agar-NaCa plates are usually sensitive to S13 when tested in the absence of NaCl, e. g., on base agar-Ca plates. *E. coli* strain B acts like these strains of Y6R/13, being resistant to S13 on base agar-NaCa and sensitive on base agar-Ca. It is possible to isolate host-range mutants of S13 capable of forming plaques on strain B on base agar-NaCa with an efficiency of plating of about 0.01.

Genetic recombination experiments. Numerous experiments have been carried out with the intention of demonstrating genetic recombination between different mutants of S13. Single-cell burst experiments were usually done, using multiplicities of infection of about five for each mutant. Both host-range and plaque-type mutants were used. Infected cells regularly released progeny of both parental types. In no case was a recombinant phage found. The sensitivity of the tests was such that in most cases one recombinant per thousand parental-type progeny would have been observed.

Photoreactivation. As suggested by Mills (1955), photoreactivation of ultraviolet light-killed S13 can occur. "Light" and "dark" phage inactivation curves give straight lines when the logarithm of the fraction of surviving phage is plotted against the UV dose. The slope of the "light" curve ("nonphotoreactivable sector," Dulbecco, 1950) is 0.85 that of the "dark" curve.

Multiplicity reactivation. Mills' observation (1955) that ultraviolet light-killed S13 fail to kill host cells has been confirmed. It was not possible to determine if the small photoreactivable fraction of irradiated S13 is able to kill host cells, however. No multiplicity reactivation (Luria and Dulbecco, 1949) was seen when cells of strain Y6R were mixed with sufficient irradiated S13 to give multiplicities of infection between 2.5 and 8.4 (assuming that irradiated

Figure 2. Inactivation of S13 and ϕ X-174 by anti-S13 serum. Serum diluted 1:40,000. Temperature, 27 C. Medium, basal broth. Phage concentrations, 2×10^5 /ml before inactivation.

phage adsorbs to host cells as efficiently as unirradiated phage does.) The phage in these experiments received between two and four lethal UV hits per particle.

Serology. S13 is an excellent antigen. Neutralizing antisera with k values (Adam, 1950) of several thousand are easily obtained. All of the mutants of S13 which have been tested (S13a, S13c, S13h) are inactivated at the same rate as wild-type phage. As in the case with other phages (Kalmanson and Bronfenbrenner, 1942; Tanami and Miyajima, 1956), serum inactivation rates vary according to the host upon which the assay is made. Inactivation velocity constants (k) for S13 are usually about twice as great when the phage is assayed on Y6R as when it is assayed on *E. coli* strain C. When the logarithm of the fraction of phage surviving is plotted against time for a given serum dilution, the curve is usually slightly concave upwards (figure 2).

Antiserum against S13 inactivates ϕ X-174, though not as rapidly as it does homologous phage (figure 2). Two different anti-S13 sera have been tested against ϕ X-174 from two

sources in various concentrations of NaCl and at temperatures from 25 to 37 C. In all cases, the initial inactivation velocity constant for S13 has been between two and four times the initial inactivation velocity constant for ϕ X-174, when both phages were assayed on Y6R.

Miscellaneous observations. Burnet and McKie (1930) reported that S13 failed to move to the anode in an electrophoretic apparatus at physiological pH's. This has been confirmed with starch-gel electrophoresis using the methods of Rotman and Spiegelman (1954). After 15 hr of electrophoresis at 350 v and 11 ma in 0.025 M sodium pyrophosphate buffer at pH 6.5, about 95 per cent of the phage could be recovered from the column between the origin and the cathode, with a peak containing two-thirds of the phage located 2 to 4 cm from the origin. Phage recovery was quantitative.

A few exploratory experiments suggest that S13, suspended in distilled water, is quantitatively adsorbed to the anion-exchange resin Duolite A-4 if the column of resin is charged with 10 per cent Na_2HPO_4 and washed with distilled water. The phage may be eluted with 5 per cent NaCl.

DISCUSSION

Phage S13 has a wide variety of mutants. Aside from the ones discussed in this paper, a number of plaque-type mutants have been isolated whose plaques differ only slightly from those of wild-type phage or of one of the reported mutants. Furthermore, when S13-resistant bacteria are isolated on media other than base agar-NaCa, it is possible to isolate a whole new spectrum of phage host-range mutants. At least 30 different mutants have been observed in the course of these investigations.

It is of some interest that no genetic interaction between S13 phage particles has been observed. In mixed infections, less than one recombinant per thousand parental-type progeny is formed. When host cells are mixed with ultraviolet light-killed S13, less than 3 per cent of the killed phage particles enter into the genetic processes necessary for multiplicity reactivation. In the latter case, however, there is no evidence that the killed phage particles adsorb to the cells.

The main evidence that S13 and ϕ X-174 are related is serological. The two phages differ in

their ionic requirements for growth on various hosts, and in certain adsorptive characteristics (Guelin and Lépine, 1957). However, the plaques of wild-type S13 and ϕ X-174 are indistinguishable under those conditions in which they both form plaques. Plaque-type mutants similar to the ones described for S13 can be isolated from ϕ X-174. The evidence suggests that they are related, but differ from each other in a number of genetic characteristics.

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SUMMARY

Methods for the production of high-titer stocks of phages S13 and ϕ X-174 are given. The two phages are related serologically, but differ in their ionic requirements for growth on certain hosts.

Attempts to demonstrate genetic recombination between mutants of S13 failed, although most cells infected with two different mutants released both parental types upon lysis. Less than one recombinant, if any, is produced for every thousand parental types. Multiplicity reactivation could not be demonstrated.

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