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TEMPERATURE-SENSITIVE MUTANTS OF *BACILLUS SUBTILIS* BACTERIOPHAGE SP3

I. ISOLATION AND CHARACTERIZATION

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ABSTRACT

NISHIHARA, MUTSUKO (University of California, Los Angeles), AND W. R. ROMIG. Temperature-sensitive mutants of *Bacillus subtilis* bacteriophage SP3. I. Isolation and characterization. J. Bacteriol. **88**:1220–1229. 1964.—Twelve temperature-sensitive mutants of *Bacillus subtilis* bacteriophage SP3 were isolated from a suspension of the wild type treated with nitrous acid. The mutants were detected because of an impaired ability to replicate in the host cell at 45 C, in contrast to the parental wild type which has essentially the same burst size at temperatures between 37 and 48 C. However, the latent period of the wild-type phages was decreased as growth temperatures were varied from 37 to 49 C. The reduction of burst size at temperatures above 48 C is probably the result of the effect of higher temperatures on the metabolism of the host, because the bacterial growth rate is decreased between 45 and 51 C. A temperature effect on the mutants was studied by infecting cells at 45 C, and then transferring portions of the mixture to 37 C at two time intervals. One transfer was made within the expected 45 C latent period, and the other was made about 10 min after the end of the latent period. In both cases, removal of the phage-bacterium complexes from the inhibitory condition of high temperature did not result in an immediate increase of plaque-forming units. A delay was observed which might correspond to the time necessary to complete the temperature-sensitive synthesis in the infected cells. In experiments with three of the mutants, cells infected at 37 C were transferred to 45 C at various times during the 37 C latent period. The extent of inhibition upon transfer to the high temperature was found to decrease progressively with increasing time of prior incubation at 37 C.

The isolation of temperature mutants of bacteriophages and genetic work with them has thus far been restricted to bacteriophages for *Escherichia coli*. These include Edgar's ts mutants of T_4 (Luria, 1962), some temperature-sensitive hs mutants of lambda (Campbell, 1961), two temperature-resistant mutants of lambda (Groman, 1962), a temperature-resistant mutant of T_5 (Adams and Lark, 1950), and some temperature-sensitive mutants of $\phi X174$ (Hutchison, *unpublished data*). In addition to these, a mutant of lambda which is noninducible at 37 C and also unable to lysogenize at 40 C was reported by Sussman and Jacob (1962).

Genetically, the most well studied are the ts mutants of T_4 and the hs mutants of lambda. The former were found to be randomly distributed over the entire genetic map of T_4, and the mutations apparently caused a variety of different defects in the synthesis of phage-related material when the infected bacteria were incubated at elevated temperatures (Luria, 1962; Edgar, *personal communication*). Some of the heat-sensitive lambda mutants were also suppressor mutants capable of propagating in one strain of *E. coli* but not another (Campbell, 1962). Nineteen of the mutants were reported to be temperature-sensitive but not suppressor-sensitive. The classification of temperature sensitivity was based on the inability of isolates to form plaques at 43 C.

Normal, wild-type lambda was found by Groman and Suzuki (1962) to have a burst at 44 C which was decreased to about 2 to 5% of the burst at 37 C. Their temperature-resistant lambda mutants gave better bursts than did the wild type at 44 C (Groman and Suzuki, 1961; Groman, 1962).

The temperature-resistant mutant of phage T_5 is inactivated to a lesser extent than is the wild type at 50 C in a low salt concentration (Adams and Lark, 1950). In broth or in high salt concentration, no difference was detected. This mutant is unlike the others, in that thermal

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stability is a property of the free phage rather than of the phage-bacterium complex. Some evidence was presented for an altered coat protein in this mutant (Lark, 1962).

The present work concerns some temperature-sensitive (t^s) mutants of bacteriophage SP3, whose host is *Bacillus subtilis* SB19. Some structural properties of the phage and host were described by Eiserling and Romig (1962); a physicochemical property of the phage nucleic acid, by Marmur and Cordes (1963); and some biological properties of the nucleic acid, by Romig (1962).

The mutation in these phages has resulted in a block at one of the numerous stages of intracellular development. As a possible way of determining the particular time of occurrence of the block during the latent period, some temperature shift experiments similar to those of Groman and Suzuki (1962) were performed with the *B. subtilis* phage SP3 mutants.

MATERIALS AND METHODS

Phages. The phages studied were all derived from *B. subtilis* bacteriophage SP3.

Bacterium. The bacterial indicator used throughout was *B. subtilis* SB19, a streptomycin-resistant prototrophic derivative, obtained by transformation, of the indole-requiring *B. subtilis* 168 (Nester and Lederberg, 1961).

Media. The medium used in all experiments was described by Romig and Brodetsky (1961). This is referred to as TY broth, and differs from it only in the omission of $CaCl_2$. The basal agar and soft agar for plating were made by adding 1.5 and 0.6% agar to TY broth.

Buffer. Phosphate buffer (pH 7.0) consisted of the following: Na_2HPO_4, 7 g; KH_2PO_4, 3 g; and deionized distilled water, 1,000 ml.

General phage and bacterial techniques. The soft-agar overlay technique with and without preadsorption was used throughout for phage assays (Adams, 1959). Indicator cells of *B. subtilis* SB19 were usually cultured in TY broth on an Eberbach reciprocal shaker at 37 C. For single-cycle growth experiments, the bacteria were grown in aeration tubes at 37 C for 3 to 5 hr to a concentration of approximately 10^8 to 5×10^8 cells per ml.

Phage dilutions were made in TY broth. Bacteria were diluted in phosphate buffer, and viable cells were assayed by the plate-spreading method.

Phage lysates were prepared from confluently lysed plates. For the more stable temperature mutants, a previous stock was diluted above the level of revertants present in the suspension, and approximately 10^4 to 10^6 phages were used for each plate. For the other mutants, plaques were picked into broth, and this suspension was used to prepare the confluent plates. All plates except one of each set were incubated at 37 C. The single control plate was incubated at 45 C; if a large number of revertants were found on this plate, the phages on the plates incubated at 37 C were not collected. Phages were harvested in TY broth, and then were chloroformed to kill residual bacteria and stored at 4 C.

Nitrous acid inactivation. The reaction mixture consisted of 0.23 ml of 0.1 M citric acid, 0.77 ml of 0.1 M sodium citrate, 0.5 ml of 1.0 M sodium nitrite, 0.3 ml of deionized distilled water, and 0.2 ml of a stock phage suspension. This gave a concentration of 0.25 M nitrous acid at pH 5.2.

The reaction was carried out in a 28 C water bath. A 0.1-ml portion of the above mixture was removed at 10-min intervals and was diluted in 0.9 ml of buffered TY broth. The buffered TY broth used as a diluent was made by adding 6.35 g of $NaH_2PO_4 \cdot H_2O$ and 22.4 g of Na_2HPO_4 to 836 ml of TY broth. This medium had a final pH of 7.0 when diluted with 0.1 volume of the nitrous acid reaction mixture. Unbuffered TY broth was found inadequate for raising the pH to 7.0 when it was desired to stop the reaction. Routine techniques were used for dilution and plating of samples.

Isolation of temperature mutants. Wild-type phage SP3 was exposed to nitrous acid for 30 to 35 min. The percentage inactivation was determined by dividing the titer of the phages immediately after mixing into the titer at the end of the reaction time. The treated phages were used to infect *B. subtilis* in TY broth, and were allowed to replicate for at least one cycle to segregate possible mutants from heterozygotes. This was done by adding 0.5 ml of the exposed population of phages to 4.5 ml of buffered TY broth, and 4.0 ml of this mixture to 4.0 ml of a culture of *B. subtilis*. The tube was then incubated at 28 C for 2 hr. Any bacterial cells remaining were killed with chloroform, and the lysate was diluted and plated to obtain discrete plaques. Approximately 30 to 40 plates were prepared, and

were incubated for 18 hr at 32 to 37 C. These plates were then refrigerated until it was convenient to transfer phages from the plaques to duplicate plates for the purpose of detecting the presence of temperature-sensitive mutants.

Plates to be used for the detection of mutants were poured 3 to 5 days prior to use, and were stored at room temperature. A 3- to 4-ml portion of soft agar seeded with the *B. subtilis* indicator culture was distributed on top of each plate with an aupette. The plaques formed by the nitrous acid-treated phages were transferred with toothpicks to marked areas on each of two identical indicator plates. Usually, 24 plaques were transferred to each pair of plates. One set of plates was incubated at 37 C; the duplicate plates were incubated at 45 C. After 7 to 10 hr of incubation, plates at 45 C were checked and refrigerated. Plates at the low temperature were incubated about 18 hr.

Areas which were clear on the plates incubated at 37 C, but which were turbid or altogether absent on the corresponding spot of the plate incubated at 45 C, were picked from the 37 C plate into broth. This suspension was used to prepare duplicate indicator plates: one to be incubated at 37 C, and the other, at 45 C. The two plates were compared and, if any difference indicative of less phage production at 45 C was found, it was assumed that a temperature-sensitive mutant had been isolated. In earlier experiments, the original spot had been transferred directly to duplicate plates with a toothpick rather than into broth. It was then realized that this procedure decreased the probability of detecting mutants which might be mixed with wild-type phages, because the mixture would form a clear area at 45 C, thereby masking possible mutants. Also, extremely leaky mutants, if spotted in large numbers, cause some clearing of the lawn at the higher temperature. In such cases, the presence of a mutant was suggested by the finding of a turbid, but not completely grown-over, area in the position spotted on the 45 C plate. Several subcultures were required for good isolation of most of the mutants.

Bacterial growth at 37, 45, and 51 C. Bacteria from a TY agar plate were used to inoculate 5 ml of TY broth. The broth culture was aerated for 1.5 hr at 37 C; equal portions were then added to two aeration tubes, each containing 40 ml of TY broth. One of these tubes was incubated at 37 C, and the other, at the higher temperature.

At 15- or 30-min intervals, samples were removed and the absorbance was determined on a Bausch & Lomb Spectronic-20 colorimeter at a wavelength of 600 mμ. Parallel samples were also diluted for viable-cell counts. The titers recorded are averages of the counts found for three to four plates.

One-cycle growth of SP3 at various temperatures. The procedure used for wild-type phage SP3 growth experiments was similar to that for the temperature shift experiments, except that host cells grown at the experimental temperature, rather than at 37 C, were used. Experiments were performed with a water bath (model 1079; Electric Hotpack Co., Philadelphia, Pa.) in which the water was mixed continually by bubbling air through a tube beneath the platform. Under these conditions, the temperature remained constant to within 0.5 C.

Temperature shift-down experiments. Cells to be infected were grown in TY broth with aeration at 37 C for 3 to 4 hr until a concentration of 10^8 to 2×10^8 cells per ml was attained. Bacteria and phage were mixed in a test tube (13 by 100 mm) with an aeration cap. The input phage-bacterium ratio was usually about 1; in a few cases, it was as high as 3. Duplicate tubes were prepared, and adsorption was permitted for 5 min by bubbling one tube at 45 C, and the other at 37 C. At 5 min postmixing, 0.1-ml samples were removed from each tube, and were blown into tubes containing 2 ml of TY broth and 1 ml of chloroform. The contents of the tubes were vigorously shaken by use of a Vortex Jr. mixer, and were then permitted to stand until the chloroform had settled. The broth portion was assayed for nonadsorbed phages.

At 5 min postinfection, antiserum was added to give 99.9% inactivation, and aeration continued for 10 min. At 10 min, samples were diluted in buffer for bacterial counts, and in broth plus chloroform to assay for residual phages not adsorbed and not inactivated by the antiserum.

The contents of the adsorption tubes were then diluted in prewarmed broth. Two dilutions were aerated at the high and at the low temperatures. At 35 and 50 min, samples were removed from the high-temperature tube containing the lower dilution, and were transferred to low-temperature tubes. A 1:5 dilution into broth prewarmed to

the low temperature was used. All tubes were vigorously aerated.

Plates for phage count were prepared by removing 0.1-ml samples at 2- to 10-min intervals from the appropriate growth tubes and adding them to tubes containing soft agar and indicator cells. All plates were incubated for about 16 hr at 37 C.

Temperature shift-up experiments. Shift-up experiments from low to high temperature were similar to those in the reverse direction. Only low-temperature adsorption was used. At appropriate intervals, samples were removed from the low-temperature "master" tube and were diluted 1:10 or 1:5 into tubes containing broth prewarmed to the high temperature. The growth tubes at the high temperature were then sampled at about 10-min intervals for as long as 130 min after mixing. A total of seven to eight counts were determined for each transfer tube. The maximal yield of phage was estimated from these points.

RESULTS

Nitrous acid inactivation and mutant isolation. The inactivation of phage SP3 by nitrous acid is similar to the type of inactivation observed with this agent for other viruses (Tessman, 1959; Vielmetter and Schuster, 1960; Bautz-Freese and Freese, 1961). The data for two experiments are shown in Fig. 1. On the basis of the inactivation curve, an exposure time of 30 to 35 min was selected for mutation induction. In three experiments, 12 temperature-sensitive mutants were isolated. These were numbered in the order in which it was possible to obtain lysates showing a definite difference in behavior at 45 C as compared with 37 C, rather than in the order of orignal isolation.

Only one mutant (t^{s-1}) was isolated from about 2,500 plaques in the first experiment. As skill in detection increased, a greater frequency of mutants was obtained. In the second experiment, with 99.6% inactivation, six mutants (t^{s-2}, t^{s-3}, t^{s-4}, t^{s-7}, t^{s-8}, and t^{s-9}) were isolated from approximately 4,000 plaques. The other five mutants (t^{s-5}, t^{s-6}, t^{s-10}, t^{s-11}, and t^{s-12}) were obtained from about 6,500 plaques in a third experiment with 99% inactivation. The frequency of nitrous acid-induced mutations of phage SP3 is about 1 per 1,000 survivors. Increasing the extent of inactivation may increase the mutation frequency; however, this was not investigated.

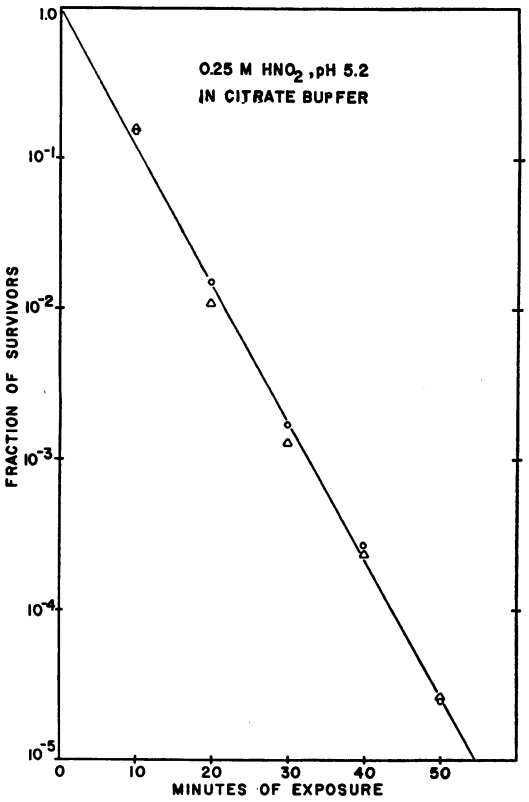

FIG. 1. *Nitrous acid inactivation of bacteriophage SP3. Points represent the results of two experiments.*

Effect of temperature on bacterial growth and phage reproduction. The temperature of 45 C was selected for mutant isolation because it was known that *B. subtilis* grows well at this temperature. The growth of the indicator strain at 37, 45, and 51 C is shown in Fig. 2 and 3. At 45 C, the bacteria have a slightly shorter generation time, and cell mass is equal to or greater than that of cells at 37 C. At 51 C, the generation time is longer than at 37 C, and the difference in growth rates (Fig. 2 and 3) suggests that the cells may be larger at the higher temperature. The rapid increase in viable titer at 51 C between 120 and 150 min (Fig. 2) could be explained as a sudden division of filamentous cells. This temperature was reported to be about maximum for growth of some strains of *B. subtilis* tested in a minimal medium (McDonald and Matney, 1963). Only two of their strains (168 and P1) were found to

grow at a higher temperature (57 C) in a complete medium.

The effect of temperature on the propagation of wild-type phage SP3 is shown in Fig. 4. The differences in burst size at 37, 45, and 48 C are probably not significant. At 49 C, there is a definite decrease. In three experiments, the burst was found to be between 30 and 50. At 50 C, phage replication is greatly reduced. As a control in one such experiment, a portion of the phage-bacterium suspension was transferred from 50 to 37 C at 15 min. At about 45 min, the transferred complexes began to lyse and gave a near-normal burst of approximately 70, indicating that the condition of continued high temperature was responsible for the reduced burst size and not an inability to adsorb or to inject nucleic acid.

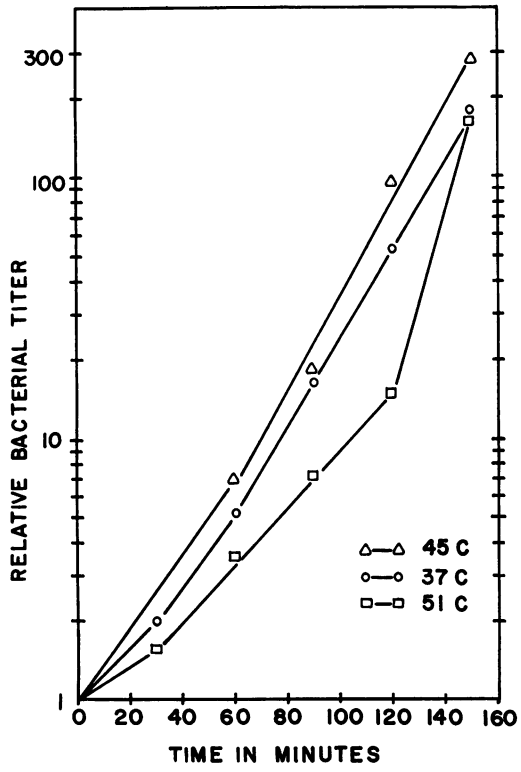

FIG. 2. *Effect of temperature on bacterial growth: viable cell count. Temperatures used were: 37 (average of two experiments), 45, and 51 C. Relative titers are plotted versus time in minutes. At 0 time, for the 37 C plot, 1.0 = 1.4 × 10⁶ bacteria per ml; for the 45 C, 1.0 = 1.7 × 10⁶ bacteria per ml; for the 51 C plot, 1.0 = 1.1 × 10⁶ bacteria per ml.*

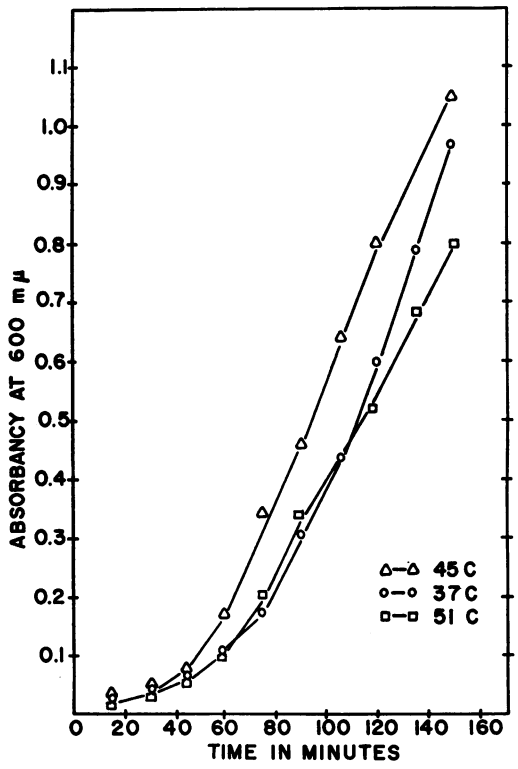

FIG. 3. *Effect of temperature on bacterial growth: absorbancy at 600 mμ. Temperatures used were 37 (average of two experiments), 45, and 51 C.*

There is also a temperature effect on the duration of the latent period. At 37 C, the latent period is 53 ± 2 min. At higher temperatures, this was found to be decreased to about 40 min. This decrease in latent period is in agreement with the type of results obtained with the coliphage lambda (Groman and Suzuki, 1963). However, the relationship between temperature and duration of the latent period is a complex one. The latent periods were almost identical at 45, 48, and 49 C. At 50 C, an increased latent period was found for the population of infected cells. This effect may be partly due to inactivation of some infected complexes which would tend to mask early bursts of others.

Temperature shift-down. The information obtained from the temperature shift-down experiments is summarized in Table 1. The values for the latent and lag periods are obtained from plots such as those shown in Fig. 5 and 6 of the one-cycle growth curves, and correspond to the time

interval between the last low plate count and the first increase in titer indicating the beginning of the burst. Samples were taken at 2-min intervals during the critical change period; there is a technical error involved in all values reported, corresponding to the time consumed in plating. The latent periods reported would tend to be shorter than the actual value by a maximum of 2 min. Where uncertainties exist, a range of values is indicated. If mutants leaked to a significant extent, the duration of the second lag period after transfer at 50 min was difficult to determine.

Estimation of the burst size was complicated by the apparent plateaus and steps in the rise portion of some of the one-cycle growth curves when experimental points were followed closely in drawing the plots (Fig. 5). In these and in other cases, exceptionally large bursts were observed. The usual burst size is about 100 to 150 for phage SP3 in *B. subtilis* SB19. The plateaus and large bursts may be due to a mixed population of cells lysing at different times.

Bacterial cell division after infection with phage SP3 was observed microscopically (Fred Eiserling, *personal communication*). This phenomenon was also reported for *E. coli* phage $\phi X174$

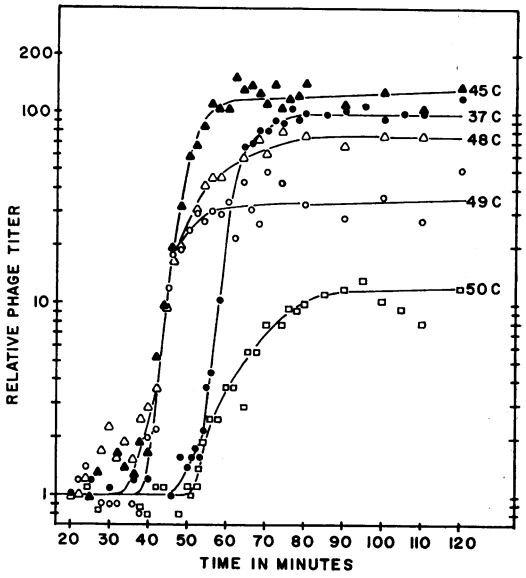

FIG. 4. *One-cycle growth of phage SP3 at various temperatures. Plots are based on relative titers, which were calculated from plate counts for the first samples taken. The temperatures used were: 37, ●; 45, ▲; 48, △; 49, ○; and 50 C, □.*

TABLE 1. *Temperature shift-down of t^s mutants*

Mutant	Latent period		Burst size		Burst after transfer		Lag after transfer		Approximate percentage of low temp during latent period
	Low temp	High temp	Low temp	High temp	35 min	50 min*	35 min	50 min	
t^{s-1}	53	—	110	0.16–0.40	100	75	10	7	13–19
	55	—	100	0.40	50	27	10–11	9	16–20
t^{s-2}	55	39	75	40	115	65 (50)	12	11	20–22
	51	47	100	20	88	80 (77)	14–16	11	21
t^{s-3}	51	41	100	15	95	50 (39)	10	7–9	14–20
t^{s-4}	53	55	45	4.5	100	30–77	19	17	32–36
t^{s-5}	51–53	43–46	>80	3.5	140	42	12	13	23–25
t^{s-6}	51	39	90	2.2	60	40	9	9–11	18
t^{s-7}	51	49	200	20	200	150	15–16	15	29
t^{s-8}	51–52	38–43	100	16	70	30 (18)	10	9	18–20
t^{s-9}	51	39	150	40	160	120 (117)	10	13	20–26
t^{s-10}	55–59	49	160	22	130	60 (49)	16	14	24–27
	55	41	100	25	55	34 (17)	12	11	19–21
t^{s-11}	55	41	130	15	54	16–32 (10–26)	14	10	18–26
t^{s-12}	60–67	45	250	20	150	100 (94)	16	13	24–27
	59	41	70	20	84	50 (45)	14	13–15	24
	53	39	180	20	135	56 (50)	12	11–15	23

* Numbers in parentheses indicate increase over the number of infective centers at the time of transfer. Other values are calculated on the basis of the plaque counts present during the latent period.

(Stárka, 1962) and for a temperate phage of *B. megaterium* both after infection of sensitive cells and after induction of lysogenic cells with ultraviolet light (Lwoff, Siminovitch, and Kjeldgaard, 1950). Experimentally, this was an additional complication which could not be eliminated, because it was necessary to start the phage SP3 growth experiments with exponentially growing cells to get reasonable phage yields.

Temperature shift-up. The reverse experiment, temperature shift-up from low to high, was performed for three of the more stable mutants (Table 2). For all of these mutants, any increase in time of incubation at 37 C appeared to increase the yield of phage after transfer to 45 C. No base line with a sharp increase in titer was detected for the intervals tested; hence, the time at which the sensitive step occurs could not be accurately determined. Mutant t^{s-4}, in agreement with the shift-down experiments, seemed to give a relatively greater yield after an early shift than did the other two. However, the burst expected at the high temperature, relative to that at 37 C, is greater for this mutant; also, the latent period in this particular experiment was shorter.

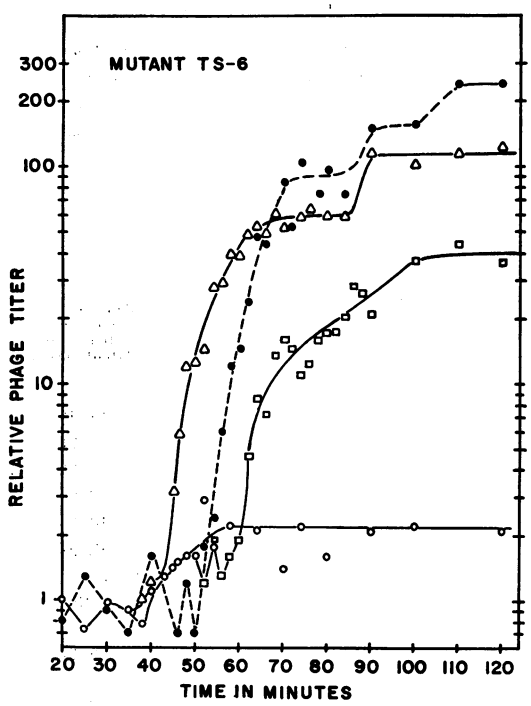

FIG. 5. *Temperature shift-down of mutant* t^{s-6}. *Relative titers are plotted as in Fig. 4. Growth curves are as follows: kept at low temperature,* ●; *kept at high temperature,* ○; *transferred high to low at 35 min,* △; *transferred high to low at 50 min,* □. *High temperature equaled about 45 C. Maximal range of variation was 44 to 47 C. Low temperature was about 37 C. The range in water-bath temperature was 35 to 37 C for shift-down experiments in general.*

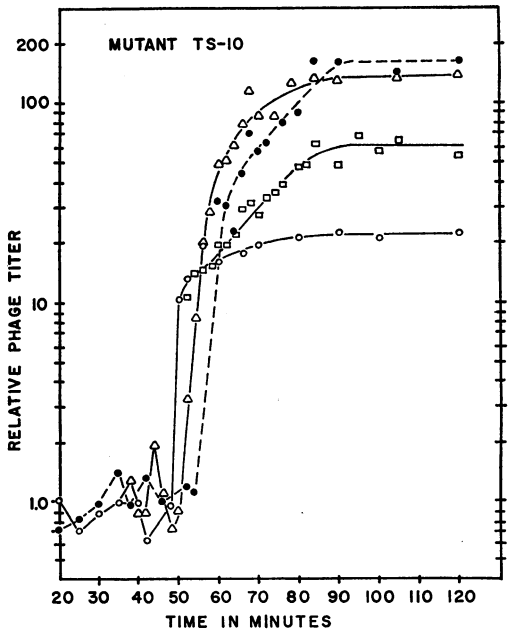

FIG. 6. *Temperature shift-down of mutant* t^{s-10}. *Relative titers are plotted as in Fig. 4. Growth curves are as follows: kept at low temperature,* ●; *kept at high temperature,* ○; *transferred high to low at 35 min,* △; *transferred high to low at 50 min,* □.

DISCUSSION

The study of the effects of temperature on the growth of *B. subtilis* SP19 and on the replication of phage SP3 indicated the complexity of these relationships. Bacterial growth is more rapid at 45 than at 37 C. The lawns on plates incubated at this temperature appear sooner and tend to be thicker than on duplicate plates incubated at 37 C. However, at 51 C in broth, a slight decrease in growth rate was observed, some of which might have been caused by use of cells grown at 37 C as an inoculum for this experiment. Some ideas on the effect of temperature on bacterial growth were reviewed by Ingraham (1962), and will not be considered here.

Propagation of wild-type phage SP3 approaches

TABLE 2. *Temperature shift-up of some t^s mutants*

Mutant	High temp burst*	Latent time at low temp	Maximal burst at low temp (100%)	Per cent maximal burst after transfer at time (min)									
				30	34	36	38	40	42	44	46	48	50
		min											
t^{s-1}	0.4	56	100			0.4		4.5	8.0	15.5	19.0	26.0	
t^{s-4}	10.0	51	27	1.7	6.1	11.1	17.8			28.8		40.7	
				−4.1									
t^{s-6}	2.4	54	90			0.8		5.6	12.2	16.7	17.8		50.0
						−1.1							

* Values calculated from data of temperature shift-down experiments, and expressed as percentage of maximum.

the upper temperature limit for growth of the bacterial host. Moreover, the duration of the latent period of the phage seems to have a minimal value at temperatures corresponding roughly to those at which the maximal rate of bacterial growth occurs. However, if these properties are determined by the genetic material of the phage, other *B. subtilis* phages may have different maxima and minima.

From Table 1, which summarizes the results obtained in temperature-shift experiments, the following generalizations may be made about the phage mutants. (i) The isolates are mutant, in the sense that the burst size is much lower at the high temperature than at the low temperature. However, the mutants leak to various degrees at 45 C, the replication of some being more temperature-sensitive than others. (ii) The burst size after the first transfer is usually equal to or slightly less than the burst at the lower temperature. The burst after the second transfer is definitely less than the burst after the first transfer, indicating an inactivation or loss of complexes with increasing time at the high temperature. However, because in almost every case there is a small burst after transfer to the lower temperature, some of the phage-bacterium complexes can survive in a blocked state at the high temperature for 50 min. (iii) For a particular mutant, there is a rough agreement in the duration of the lag before a burst after the first and the second transfer from high temperature to low. Differences are usually 2 min or less, which is within the expected experimental variation. (iv) For different mutants, the estimated lag periods are not greatly different, although the range between the longest and the shortest time interval observed is significant. (v) In cases where leakage of the mutants permits this to be determined, the latent period was found to be shorter at the high temperature, in agreement with results obtained with wild-type SP3 (Fig. 2). However, the time values were not the same for different mutants or for different experiments with the same mutant. In most cases, the latent period at the high temperature was about 40 min. Some variation can also be noted in the latent periods at the low temperature. With the exception of t^{s-10} and t^{s-12}, the range of values was 51 to 55 min. The inconsistencies may partly be due to fluctuations in water-bath temperature, the extent of aeration of the growth tubes, and errors in time of sampling.

The temperature shift-down experiments (Table 2) suggested that there are small differences among the mutants either in the time of the latent period at which the sensitive step occurs or in the time necessary to complete the synthesis of phage material beyond the block. If the lag periods observed were to represent the particular time of occurrence of the block during the latent period, it would be necessary to make the following assumptions. (i) The synthesis of any particular phage-mediated substance occurs at a characteristic time of the latent period. (ii) The replication of different phage-induced material is interrelated and sequential. The first assumption is supported by the work with T-even phages of *E. coli* (Lwoff, 1961). The second assumption may be valid for only certain steps involved in phage replication, such as the synthesis of phage nucleic acid which may depend on the prior synthesis or increased synthesis of early enzymes (Cohen, 1961). As yet, little is known on the molecular level about the mechanism of synthesis of other phage components, such as heads, tails, tail fibers, and cores. The possibility

exists, also, that different bacteriophages may differ in the pathways of macromolecular synthesis.

In spite of the uncertainties, it is evident that the duration of the lag would correspond to at least the time necessary to synthesize a sufficient quantity of the primary blocked component, which may or may not be necessary for the synthesis of other phage parts. If the synthesis of other phage components were independent of the temperature-sensitive process, the lag observed may not give any indication of the time point of the latent period at which the sensitive process is normally initiated, because processes which start at a later time would not be inhibited, and the products synthesized would accumulate. The lag would reflect only the time necessary to complete the temperature-sensitive synthesis.

The lag periods, corrected for differences in latent period (Table 1), indicate that t^{s-4} and t^{s-7} are either blocked early in the replication cycle or require more time to synthesize the sensitive material. Mutant t^{s-5} has a fairly long lag which cannot be clearly distinguished from those of t^{s-9}, t^{s-10}, and t^{s-11}. The duration of the lag for these mutants is somewhat uncertain. Mutant t^{s-12} appears to be similar to t^{s-5}. The lag for t^{s-2} is shorter than that of t^{s-5}, but may be longer than those of t^{s-3} and t^{s-8}. Mutants t^{s-1} and t^{s-6} appear to fall in the range of the latter, the short lags indicating a late blockage or rapid synthesis.

The shift-up experiments are not conclusive, but would seem to indicate, for at least t^{s-1} and t^{s-6}, that the observed lags represent only the time necessary to complete synthesis. On the basis of the shift-down experiments, the expected time of block for these mutants would be 42 to 44 min, with a 53-min latent period. A significant amount of material, exceeding the leakage expected at the high temperature, was synthesized by 38 to 40 min (Table 2). However, for t^{s-4}, the calculated time from the shift-down experiment of 34 to 36 min is in reasonably good agreement with the relative number of phages produced after transfer from low to high temperature at 36 min. Interpretation is complicated by the possibility that the metabolic reactions accelerated at the high temperature may continue at an increased rate after a shift-down to a lower temperature. This would tend to shorten the observed lag and, hence, to increase the apparent time of blockage. On the other hand, the similarity and short duration of the lag times among the mutants do not strongly support a time-of-blockage explanation.

One distinguishing characteristic of the mutants appears to be the extent of leakage at 45 C. Among the isolates, t^{s-1}, t^{s-4}, t^{s-5}, and t^{s-6} were found to leak to the least extent. The others gave reduced but significant bursts at 45 C. Mutant t^{s-4} tends to give a smaller burst than do the others at 37 C.

In addition to the slight differences in the lag periods and differences in the extent of leakage at the high temperature, there are some differences in plaque size among the mutants. There is a tendency for t^{s-1} and t^{s-4} to form smaller plaques on plates at 37 C than the others. Mutant t^{s-6} tends to form a mixture of small and large plaques. The large plaques are two to three times the diameter of the smaller ones. Mutant t^{s-10} forms intermediate size plaques at 37 C. Plaque-size tendencies are clearly discernible when pure populations of different mutants are compared, but it should be pointed out that it is not a stable characteristic for phage SP3. The wild type forms a mixture of sizes. Passage by single-plaque isolations will tend to result in a homogeneous plaque size, but the population of a mass lysate will usually give rise to a variety of sizes. Therefore, plaque morphology is not a good genetic marker for SP3.

Host range mutants for this phage are also difficult to obtain. Repeated attempts in this laboratory to isolate a *B. subtilis* SP19 resistant to phage SP3 have failed. Thus, at the current time, the only good genetic markers on this phage are the temperature-sensitive ones studied here.

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