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TEMPERATURE-SENSITIVE MUTANTS OF *BACILLUS SUBTILIS* BACTERIOPHAGE SP3

II. In Vivo Complementation Studies

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Abstract

Nishihara, Mutsuko (University of California, Los Angeles), and W. R. Romig. Temperature-sensitive mutants of *Bacillus subtilis* bacteriophage SP3. II. In vivo complementation studies. J. Bacteriol. **88**:1230–1239. 1964.—A plate-spotting procedure was used in initial attempts to group the temperature-sensitive *Bacillus subtilis* phage SP3 mutants by complementation. The results obtained did not show any clear patterns of reactions among the mutants. Crosses were, therefore, repeated in broth at a temperature of 49 C, which greatly reduced the extent of replication of each mutant type alone. The data on mixed infections indicated that there was a minimum of six complementation groups. Of the 12 isolates, 7 did not seem to complement with each other; the rest complemented with each other and with the seven noncomplementing mutants. There was a positive correlation between the complementation reaction of a pair and the recovery of wild-phenotype phages from a 49 C broth lysate. The relative proportion of phages capable of forming wild-phenotype plaques on plates incubated at 46 C to the total number of plaque-forming units was higher in a lysate of a mixed infection with two mutants than in lysates of each mutant alone. Moreover, this frequency was higher for a mixed lysate made at 49 C than for a lysate of the same two mutants made at 37 C. These observations suggested that genetic recombination might occur at 49 C, and that the increased recovery of wild-phenotype phages in lysates made at this temperature might be due to a selective advantage for these phages. Recombination experiments at 37 C with some complementing pairs gave frequencies of 2.0 to 4.8%. The ratio of wild-phenotype revertants to total phages in the stock lysates used for these crosses at 37 C was less than 10^{-6}. The noncomplementing mutants were not conclusively shown to be nonidentical.

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Mutations in microorganisms which result in the formation of enzymes with an increased temperature sensitivity include those affecting the production of: tyrosinase in *Neurospora crassa* (Horowitz and Fling, 1953; Horowitz et al., 1960), an enzyme for pantothenate synthesis in *Escherichia coli* (Maas and Davis, 1952), tryptophan synthetase of *E. coli* (Helinski and Yanofsky, 1962; Yanofsky, Helinski, and Maling, 1961), the lysozyme induced by T_4 bacteriophage in *E. coli* (Streisinger et al., 1961), and the endolysin induced by the phage lambda in *E. coli* (Campbell and del Campillo-Campbell, 1963). There is also some evidence that the deoxycytidylate hydroxymethylases induced by certain amber mutants of phage T_4 in permissive strains of *E. coli* are more temperature-sensitive than is the enzyme formed upon infection with wild-type T_4 (Dirksen, Hutson, and Buchanan, 1963).

It is not known whether the block in replication at high temperatures in temperature-sensitive (t^s) mutants of *Bacillus subtilis* phage SP3 is due to the production of an altered enzyme or some other phage-induced material. Because a number of different defects are possible, some grouping of the mutants isolated is desirable. The method frequently used for this purpose is complementation testing. Positive complementation for these bacteriophages would mean that temperature-sensitive progeny were produced in a mixed infection at a high temperature which prevents or reduces the production of mutant phages by cells infected with a single type. With two independently isolated t^s mutants present in the cell, the defective function of one may be supplied by the other. If the two phages were able to compensate for each others' defect, a burst yielding phages of both infecting types should occur at the high temperature. This report concerns work on the grouping of the t^s mutants of phage SP3 by in vivo complementation tests.

MATERIALS AND METHODS

Phages and bacterium. The bacteriophages used were all derived from SP3, and were described in a previous paper (Nishihara and Romig, 1964). The bacterial indicator was, as before, *B. subtilis* SB19.

Media. The media used were TY broth; TY agar; and soft agar, which was made by adding 0.6% agar to TY broth. TY broth differs from that described by Romig and Brodetsky (1961) only in the omission of $CaCl_2$.

Plate test for complementation. Preliminary crosses were performed by plating bacteria infected with one t^s mutant and spotting the other mutants on top of this layer. The concentration of phages plated was adjusted, when possible, so that control plates incubated at 37 C were confluently lysed, and those incubated at 45 C showed few, if any, plaques. With mutants which leaked to a significant extent, this concentration was about 10^3 to 10^4 phages per plate.

Spotting was initially performed with the use of 0.1-ml pipettes. Because this proved to be unsatisfactory, in later experiments either tuberculin syringes with 30-gauge needles or wooden applicator sticks were used. Four dilutions of the phage were spotted in each test. The dilutions were calculated to give 100 to 500 phages per 0.1 ml of the highest dilution. The other three concentrations were tenfold multiples of this. The spotting procedure was completed within the latent period of the plated phages.

The spotted plates were incubated right side up for about 16 hr at 45 C. The glass lids of all plates incubated at the high temperature were replaced with Brewer aluminum lids (BBL) lined with thick absorbent paper pads to collect the moisture accumulated at this temperature.

After incubation, plates were examined for clearing which would be indicative of phage production in the area spotted. If phages were produced, their phenotype was checked by transferring material to broth from the region of the highest dilution giving a reaction in the test. Duplicate indicator plates were prepared and were inoculated with the broth suspensions. One plate was incubated at 45 C, and the other, at 37 C. A comparison of the two plates permitted a decision to be made as to whether clearing on the original plate was due to t^s phages or to phages with a wild phenotype. In some cases, the plaques on the two plates were counted to determine whether differences existed which might be due to a mixed population of sensitive and resistant phages.

Complementation in broth. Cells to be used for infection were grown with aeration to a concentration of about 2×10^8 per ml at 49 C. Phage was added at a multiplicity of 3 to 6 in most experiments. Adsorption was conducted with aeration for 10 min at 49 C. At this time, samples were removed to assay for nonadsorbed phages, and antiserum was added. After further aeration for 10 min, the contents of the adsorption tubes were diluted and assayed for the number of infected centers. This procedure was completed by 35 min postmixing, so that the counts obtained would represent infected cells prior to the onset of lysis.

One dilution of each adsorption tube was aerated at 49 C. At 75 min, the growth tubes were assayed for total plaque-forming units. Further dilutions of the growth tube contents were made for plating, if necessary. Plates were incubated at 37 and 46 C. At 90 min, 2 ml from each growth tube were added to 1 ml of chloroform, and the mixture was vigorously shaken with a Vortex Jr. mixer. When the chloroform had settled, the broth portion was assayed for free phages. Plates were incubated at 37 and at 46 C to determine the total number of free phages and the number of recombinants or revertants present. All values for phage titers were based on averages of two or three plates.

Three different combinations and three controls were usually done at one time. The controls consisted of cells infected with a single type of temperature mutant at approximately the same input multiplicity of phage as in the mixed infections. The tests were combinations of the mutant types used in the controls. Scoring of the complementation reaction was based on the difference between the increase in phage titer of the mixture and the sum of the increases in titer of the two appropriate controls for that test.

Recombination of mutants. The procedure was similar to that used for complementation in broth, except that recombination experiments were performed at 37 C. Adsorption tubes containing a mixture of two mutants and each mutant separately were prepared at 37 C. Inactivation of nonadsorbed phages was performed as described in the previous section. Samples were removed from the growth tubes at 5-min intervals between

TABLE 1. *Plate tests of mutants**

Plated with	Spotted with temperature mutant											
	t^{s-1}	t^{s-4}	t^{s-5}	t^{s-6}	t^{s-10}	t^{s-2}	t^{s-3}	t^{s-7}	t^{s-8}	t^{s-9}	t^{s-11}	t^{s-12}
t^{s-1}	0	+(R) +(R) ++(R)	+(R2) ±(Rs) 0	++(M) +(MR)	++(R) ++(R)	++(M) ++(MR) ++(R2)	++(R)	++(R) ++(MR)	++(M) ++(M)	++(M) ++(M)	++(R) ++(R2) ++(M)	+(R4) ±
t^{s-4}	+(R) ++(R)	0	+(R2) ++(R) ±(MR)	+(R) ++(M)	+(R) +(R)	++(Rs)	++(M)	+(R) +(R) ++(R) ++(R)	++(R) ++(R2)	++(R) ++(R) ++(R2)	++(R) +(Rs)	+(R)
t^{s-5}	+(M)	+(R) ++(R) ++(M)	0	+(Rs) +(M) ±(MR)	++(R) ++(MR) +(M)	+(R)	++(R) ++(MR) ±(Rs)	+(R) +(R) ++(R)	++(R) ++(R)	++(R) ++(M)	++(R) ++(R4) ++(MR)	+(R) ++(R) ++(R)
t^{s-6}	++(R) ++(R) ++(R2)	++(R) ++(R)	+(R) ±	0	++(R)	+(R) ++(R)	++(R) ++(R2)	+(R3) ++(Rs3)	+(Rs) ±	++(R) ++(Rs)	+(R) ++(R)	+(R) ++(R) ++(M)
t^{s-10}	±(R)	++(R) ±	+(R) ++(R)	++(R2) ++(M)	++(M)	+(M) ±(Rs)	+(R) ±	++(Rs)	++(R) ++(R2) ++(M)	++(R) ++(R)	++(R) ++(M) ++(M)	+(M) +(M)
t^{s-2}	+(R) ±(R)	++(M) +(R)	++(M)	++(R2)	++(MRs) ++(R2)	±(M)	+(M) +(M)	++(Rs)	+(M)	+(M) ++(M)	++(M) ±(M)	++(M)
t^{s-3}	++(R) ±(R)	++(R) ++(R)	+(R3) ++(R)	+(R) ++(R)	+(MR)	++(M) ±(M) 0	+(M) +(R)	+(M) ++(M) ++(M)	++(M)	++(M)	++(M)	++(M)
t^{s-7}	+(Rs) ±	++(R)	++(M)	++(R)	++(R2)	+(MR)	+(MR)	+	++(M)	++(R)	++(R2)	++(M) ++(MR) 0
t^{s-8}	+(M)	++(Rs)	++(R)	++(R)	++(Rs2)	++(M)	+(R2)	++(M)	++(M)	+(M)	0	0

t^{s-9}	++(M) ±(R)	+(R) +(Rs) ++(Rs)	+(R) ++(R)	++(R) ++(Rs3)	++(R) ++(R2)	++(M) ++(Rs)	++(R) ++(M)	+(M) ++(M)	++(R) ++(M)	±	±	++(M) ±
t^{s-11}	±(R) ±(R)	+(R) ++(R)	+(R) +(M) ±(M)	+(R) +(Rs) ++(R)	+(R) ++(R)	+(R) ±(R) ±(M)	±(M) ±(M) ±(M)	+(M) ++(M)	++(M) ++(M)	++(M) ++(M)	± 0	+(M) 0
t^{s-12}	+(R) ±(R)	++(R) ±(R3)	±(R) ±(R)	++(R) ±(R2)	++(R) ++(R) ++(R)	+(M) ±(R2) 0	+(Rs) ±(M)	+(M) ++(M) ++(M)	++(M) ++(M) ++(M)	++(M) ++(M) ++(M)	+(M) ±	± 0

* Symbols: ++, clearing at highest or next to highest dilution; +, partial clearing at high dilution or complete clearing at low dilution; ±, clearing at lowest dilution only or partial clearing only at other dilutions; 0, no reaction; R, recombinant or revertant to wild phenotype; equal size and number of plaques at high and low temperature; Rs, equal number of plaques at both temperatures but plaques at high temperature are smaller than those at low temperature; some at high temperature may also be turbid; R2, R3, R4, more plaques at the low temperature; the approximate ratio is indicated by the number; thus, R2 indicates that there were twice as many; M, mutant phenotype; may be due to either leakage of the phage spotted or to complementation.

25 and 45 min after mixing to determine the number of infected centers prior to the onset of lysis. At 90 min, an assay was made for plaque-forming units at 37 and at 46 C; 2 ml of each growth tube were then chloroformed, and the broth portion was assayed, as before, for plaque-forming units at 37 and 46 C.

RESULTS

Interaction of mutants on plates. The results of crossing by spotting one phage mutant on cells infected with another are shown in Table 1. It will be noted that there are some differences between the reciprocal crosses. If one or both of the mutants leak extensively, a mutant type may be recovered in one cross but not in the reverse cross. The phage type spotted is probably in excess over the one plated in the area of interaction. Thus, if the spotted phage leaks to a significant extent, it may produce a clearing with a mutant which does not leak very much, whereas, in the reciprocal case, there would be no clearing. The control spottings give some indication of this. For example, t^{s-10} and t^{s-8} spotted in a homologous cross gave good responses with production of mutant phage types. Areas spotted with t^{s-1}, a mutant that leaks to a small extent, generally showed poor responses. Phages spotted on t^{s-1}-infected bacteria tended to give greater clearing of the bacterial lawn.

The results of repeated tests were fairly consistent in spite of difficulties in interpretation of the spotting patterns. The extensive leakage of some mutants again posed the problem of deciding whether the phages recovered from the spots were of a mutant or wild phenotype. In some cases, an equal number of plaques was observed on duplicate plates incubated at the two temperatures, but those on plates at the higher temperature were definitely smaller. The plaques of the parental wild type at 45 C would be the same size or larger than those at 37 C. In other cases, there seemed to be fewer plaques on the 45 C plates than on the 37 C plates. Actual counts revealed that, for a number of combinations, this difference amounted to there being one-half as many plaques at the higher temperature. It was thought that this difference might be caused by leakage or by complementation of a mutant with a revertant or recombinant to wild phenotype.

In two crosses (t^{s-4} spotted on t^{s-5} and t^{s-3} spotted on t^{s-10}), a distinctly new type of plaque was observed on the 45 C plates. The total number of plaques was about equal at both temperatures, and all plaques looked similar at 37 C. On the plates incubated at the higher temperature, about one-half of the plaques had hazy, ragged edges and distinct halos, in contrast to the sharp, clear edges and lack of halos around plaques of the usual wild-phenotype phages. It is not yet known whether this phage type arose by reversion of a single mutant or by recombination between the plated mutant and the one spotted in that area.

Complementation in broth. Since the results of the plate spotting were somewhat inconclusive, the crosses were repeated in broth. These results are shown in Table 2. In both Tables 1 and 2, the mutants were rearranged in accordance with certain similarities in their reaction patterns.

The temperature of 49 C was selected for phage production in broth, because it was known that the wild-type phage SP3 gave a reduced but significant burst at this temperature (Nishihara and Romig, 1964). Plates were incubated at 46 rather than 45 C to increase the distinction between pinpoints due to leakage and clear areas due to actual wild-phenotype plaques.

Positive complementation is indicated by a plus sign, no complementation by zero, and doubtful values are designated with a plus-minus sign. The extent of leakage of the mutants at 49 C (Table 3) is low. The bursts of cells infected by two mutant types was not very much greater. The largest burst observed was about 10. If the burst for the mixture was greater by a factor of 1 than the additive value of the bursts for cells infected with each mutant type singly, the pair was considered to complement. If the difference between the mixed infection and the sum of the two single infections was less than $+1$ but greater than -1, a $\pm$ was indicated. A lack of complementation was assumed if this difference was greater than -1. A positive result should be considered more significant than a negative one, because a number of factors could cause a negative result.

In the experiments in Table 2, the calculated input multiplicities of phage to bacterium ranged from 1.3 to 28. The input ratio of the two mutant types was between 1:1 and 1:2 for 70% of the crosses, and between 1:2 and 1:4 for 24% of the experiments. The greatest disproportion of input

TABLE 2. *Complementation at 49 C**

Mutant	t^{s-1}	t^{s-4}	t^{s-5}	t^{s-6}	t^{s-10}	t^{s-2}	t^{s-3}	t^{s-7}	t^{s-8}	t^{s-9}	t^{s-11}	t^{s-12}
t^{s-1}		±	±	+	+	±	±	0	+	0	+	±
t^{s-4}	.156		+	+	+	+	+	±	+	±	+	+
t^{s-5}	.017	.007		+	+	±	±	±	+	±	+	±
t^{s-6}	.048	.066	.094		+	+	±	+	+	±	+	+
t^{s-10}	.084	.085	.142	.111		+	±	±	±	+	+	+
t^{s-2}	.034	.070	.017	.054	.096		0	0	0	±	0	0
t^{s-3}	.015	.035	.065	.040	.153	<.001		0	±	0	0	0
t^{s-7}	.020	.056	.029	.019	.043	<.003	<.003		±	0	0	0
t^{s-8}	.124	.038	.075	.017	.063	<.002	<.003	<.001		0	0	0
t^{s-9}	.013	.015	.052	.033	.068	<.003	<.004	<.005	<.004		0	0
t^{s-11}	.051	.055	.041	.049	.042	<.005	<.002	<.002	<.002	<.001		±
t^{s-12}	.031	.020	.024	.013	.033	<.004	<.041	<.006	<.001	<.003	<.002	

* The complementation response is indicated by the signs above the diagonal. See text for the method of scoring. The ratio of plaques at 46 C to plaques at 37 C for the chloroformed lysate is shown below the diagonal line.

types was 1:7.5. If a question arose as to the effect of the input multiplicity or ratio on a particular cross, the experiment was repeated. However, experiments were not repeated if a positive result had been obtained.

The ratio of wild-phenotype phages to total number of phages in the bursts at 49 C is indicated in the lower portion of Table 2. The high values are believed to be due to recombination rather than reversion, because the frequency was found to be much lower (<0.005) for cells infected with a single type of mutant at 49 and also at 37 C.

Recombination experiments. The results of the recombination experiments are presented in Table 4. The titers recorded were based on averages of two to four plate counts. The frequency of recombinants can be determined from the ratio of plaques on plates incubated at 46 C to those on plates incubated at 37 C. With the controls infected with a single phage type, the dilution plated at 46 C proved to be too high to detect any revertant plaques. Therefore, these ratios were expressed in terms of the maximal values possible. The input phage stocks used for the first five experiments had ratios of revertants to mutants of less than 1.3×10^{-7} for t^{s-1}, about 1.2×10^{-6} for t^{s-4}, and about 4.4×10^{-7} for t^{s-6}.

Some crosses were repeated in an attempt to adjust the input multiplicities to an equal number of both mutants. But this did not appear to affect the results significantly. There is a rough agreement in the frequencies based on total plaque-forming units and those based on free phages obtained after chloroform treatment. In some cases, the use of chloroform caused a decrease in phage titer; in others, there was essentially no change. In experiment 4 (Table 4), the

TABLE 3. *Burst sizes of mutants at 49 C**

Mutant	No. of expt	Avg burst of cells infected with single type of mutant		Avg burst for mixed infection	
		Total PFU	Free phage	Total PFU	Free phage
t^{s-1}	7	0.51	0.25 ± 0.24	2.38	1.75
t^{s-2}	7	1.77	1.43 ± 0.19	2.47	2.24
t^{s-3}	6	2.18	1.95 ± 0.82	2.78	2.23
t^{s-4}	6	0.30	0.23 ± 0.33	3.77	3.13
t^{s-5}	6	0.46	0.42 ± 0.32	3.11	2.72
t^{s-6}	8	0.67	0.40 ± 0.29	4.05	3.42
t^{s-7}	7	2.58	1.99 ± 0.46	2.37	2.00
t^{s-8}	7	1.88	1.53 ± 0.47	2.46	2.56
t^{s-9}	6	2.70	2.11 ± 0.55	2.98	2.45
t^{s-10}	7	1.47	1.07 ± 0.30	5.24	3.96
t^{s-11}	6	1.56	1.33 ± 0.61	3.37	2.40
t^{s-12}	5	1.66	1.58 ± 0.36	2.36	1.90

* PFU, plaque-forming units. Average bursts for mixed infections are calculated from values for 11 crosses. Standard deviations are calculated for free phages liberated in bursts of cells infected with a single type of mutant.

loss of titer of t^{s-1} after chloroform treatment suggests that readsorption of phages liberated in the burst had occurred.

The burst sizes tend to be lower than expected. On the possibility that the maximal yield of phages had not been attained by 90 min, titers were determined at 120 min for the first cross with t^{s-1} and t^{s-6}. Because no change or a slight decrease was noted in the number of free phages, this procedure was discontinued. Mutant t^{s-4} consistently gives a lower burst than does the other member of the pair, and the value for the mixture is between those for the controls. The low bursts, in general, might be related to the age of the bacterial cells and to the high input multiplicities used in these experiments.

Two noncomplementing mutants were also tested for recombination. Mutants t^{s-3} and t^{s-7} were selected because they were indistinguishable on the basis of their burst sizes at high temperatures, and their patterns of complementation were similar. At the dilutions used, there did not appear to be any significant recombination. On one of two duplicate plates incubated at the high temperature, suggestions of plaques were noted for both the chloroformed and nonchloroformed suspensions of phages from the mixed burst. But even if these were counted, the maximal recombinant frequency would only be 0.006 to 0.008%.

DISCUSSION

The use of the plate-spotting technique to distinguish possible groupings of mutants did not give clear results. The lack of other genetic markers prevented easy identification of each input type in the high-temperature lysate. The recovery of wild-phenotype phages from the spotted areas suggested that recombination might occur at the high temperature. The crosses performed in broth with controls for comparison indicated that this was the case and that, in the burst at 49 C, the relative proportion of wild-phenotype phages to total number of plaque-forming units was higher than was the corresponding ratio for a burst at 37 C. This might be due to a selective advantage for the recombinant over the t^s parental type at the higher temperature.

The extent of leakage of cells infected with various mutants at 49 C (Table 3) agrees with that found at 45 C. Again, t^{s-1}, t^{s-4}, t^{s-5}, and t^{s-6} were found to be more temperature-sensitive than the rest. Among the others, t^{s-10} seems to leak to a lower extent, and t^{s-3}, t^{s-7}, and t^{s-9} are similar enough to be identical. Mutant t^{s-8} and t^{s-12} are also almost identical, whereas t^{s-2} and t^{s-11} were found to have slightly lower average burst sizes at 49 C than mutant t^{s-8} and t^{s-12}. Because there was a considerable range of values for most mutants, small differences between mutants in average burst size may not be indicative of actual nonidentity of the mutants. Conversely, two mutants known to be different may give similar average values for the burst at 49 C, such as those for t^{s-1} and t^{s-4}, and for t^{s-5} and t^{s-6}.

The values for total plaque-forming units at the time of assay for free phages tend to be higher than the corresponding titers for the chloroformed preparations both for the controls infected with a single mutant type and for the mixtures (Table 3). The difference was not great in most cases. Some loss of phages may have occurred in the chloroform layer. However, the distribution of phages among the two layers does not appear to be equal, because the difference between the total plaque-forming units and the free phages is not a constant fraction for all paired values. It had been assumed that the number of phages retained

TABLE 4. *Recombination at 37 C*

Expt	Mutant(s)	Input multiplicity[a]	Per cent adsorption[b]	Input no. of bacteria per ml (× 10^{-8})	Infected centers per ml[c] (× 10^{-8})	Burst			
						Total PFU[d]		Free phage[e]	
						Amt at 37 C	Per cent wild	Amt at 37 C	Per cent wild[f]
1	t^{s-1}	4	99.9+	3.0	1.1	75	—	58	<0.02
	t^{s-6}	10	99.9+		2.2	32	—	36	<0.01
	t^{s-1} t^{s-6}+	7	99.9+		3.2	50	0.8	47	1.2
2	t^{s-1}	18	99.9+	0.73	0.63	206	<0.008	121	<0.01
	t^{s-6}	25	99.8		0.58	103	<0.02	64	<0.03
	t^{s-1} t^{s-6}+	20	99.9+		0.53	185	1.0	109	1.0
3	t^{s-1}	6	99.9+	2.2	1.2	62	<0.01	74	<0.03
	t^{s-4}	28	94.4		1.9	19	<0.03	13	<0.04
	t^{s-1} t^{s-4}+	17	93.6		1.4	56	2.3	49	2.4
4	t^{s-1}	6	99.7	1.4	1.1	65	<0.01	27	<0.03
	t^{s-4}	10	94.1		0.91	19	<0.06	26	<0.04
	t^{s-1} t^{s-4}+	8	97.5		0.98	44	2.7	44	2.0
5	t^{s-6}	6	99.9+	1.9	1.8	31	<0.02	24	<0.02
	t^{s-4}	12	93.6		1.0	20	<0.05	18	<0.05
	t^{s-6} t^{s-4}+	9	94.8		1.4	29	0.8	22	1.3
6	t^{s-3}	5	61.1	1.1	1.0	130	<0.0008	130	<0.0008
	t^{s-7}	7	53.9		1.8	100	<0.0006	72	<0.0008
	t^{s-3} t^{s-7}+	6	63.1		1.3	123	(0.003)	108	(0.004)

[a] Input multiplicity of phage to bacterium. For mixed infections, the input ratio of one mutant to the other is the same as the ratio of the input multiplicities for the infections with each mutant alone.

[b] As determined from the nonadsorbed phages assayed by the chloroform method.

[c] Infected centers prior to the end of the latent period.

[d] Total PFU = increase in plaque-forming units over the number of infected centers present during the latent period.

[e] Free phage = burst size as determined from a chloroformed sample.

[f] Per cent phages forming plaques at 46 C as compared with the numbers at 37 C. If one assumes reciprocal exchanges, the recombinant frequencies would be twice these values.

in the chloroform was negligible compared with the amount in the broth portion.

There is a definite correlation between the frequency of wild-phenotype plaques recovered from mixed infections and the complementation results. In crosses where complementation was scored as + or ±, the proportion of wild-phenotype phages was found to be as high at 15%. Where complementation was not observed, the frequency of plaques on the high-temperature plates was below the level of detection at the dilutions used in the experiments. On the basis of these values, all pairs designated as ± should complement except, possibly, t^{s-3} with t^{s-8}, t^{s-2} with t^{s-9}, t^{s-7} with t^{s-8}, and t^{s-11} with t^{s-12}. Two negative results, t^{s-1} with t^{s-7} and t^{s-1} with t^{s-9}, might be expected to complement because the frequency of plaques at 46 C is relatively high.

As noted previously, t^{s-1} does not complement well with any of the other mutants.

The data indicate that t^{s-4}, t^{s-5}, t^{s-6}, and t^{s-10} complement with each other and with all other mutants. Mutant t^{s-1} also tends to complement with all of the other mutants. The remaining seven isolates do not complement well with each other. It would appear, therefore, that there is a minimum of six complementation groups at the sensitivity level of this test.

The correlation of high recombination frequency with complementation raises the question of whether recombination is necessary for detection of complementation at this level of sensitivity.The presence of genetic material for a wild phenotype, arising by recombination early in the infection process, may increase the extent of replication of mutant phages within the cells.

The finding that the mutants which did not seem to complement with each other were also those which leaked to the greatest extent at 49 C (Table 3) suggests the possibility that the greater leakage prevented detection of increases due to complementation. But, it is not likely that the complementation patterns simply reflect differences in extent of leakage. Mutant t^{s-10} gave the largest average burst for a mixed infection with another mutant (Table 3). This particular mutant, which seems to complement with all other mutants, leaks to almost the same extent as do some of the noncomplementing mutants. The next largest average bursts in mixed infections were also given by complementing mutants (t^{s-6}, t^{s-4}, and t^{s-5}).

In complementation tests where recombination was detectable, the frequency of occurrence appeared to vary for different combinations of mutants. However, the values observed are not usable for genetic mapping because of the variables involved. The limited number of recombination experiments at 37 C gave frequencies which were in the same range of magnitude as those for the extreme markers of a cistron in the rII region of *E. coli* phage T_4 (Chase and Doermann, 1958; Edgar et al., 1962). If reciprocal recombination is assumed, the recombinant frequency for t^{s-1} with t^{s-6} is 2.0 to 2.4%; for t^{s-4} with t^{s-6}, about 2.6%; and for t^{s-1} with t^{s-4}, between 4.0 and 4.8%. If these values were to be used for genetic mapping with the assumption of a linear arrangement, it would appear that the site for t^{s-6} was located between those for t^{s-1} and t^{s-4}.

As mentioned previously, the input ratio of the two phages did not seem to affect the recombination frequency in the expected way. Some data in Table 4 suggest that there are differences among the mutants in the efficiency of adsorption. Mutant t^{s-4} seemed to adsorb slightly less efficiently than did t^{s-1} and t^{s-6} when the chloroform method was used to assay for nonadsorbed phages. Because there is some uncertainty as to the actual values for nonadsorbed phages, and because the input multiplicities varied, no conclusion could be drawn from these experiments alone. The adsorption data for the complementation experiments and for other experiments at 37 and 45 C confirmed the slight differences among the mutants. Mutants t^{s-1} and t^{s-6} tend to adsorb at a higher efficiency than do all of the other mutants at 37, 45, and 49 C. The values observed were rarely below 60% and ranged above 99%. The figures obtained for t^{s-4} in these experiments were inaccurate, but indicated that adsorption was less than 50%. With regard to the recombination experiments, the greater input of t^{s-4} relative to that of the other mutant in the cross may have compensated slightly for adsorption differences, and this, together with the overall high-input multiplicities used, might explain some of the data. If the efficiency of adsorption proves to be significantly different for the various mutants, corrections would be necessary for accurate determination of recombination frequencies.

The recombination frequencies between noncomplementing mutants would be expected to be lower than those for complementing pairs. Only one such cross was attempted, and the results of that experiment confirmed the close identity of the two mutants. This would not be compatible with the longer lag period reported for t^{s-7} in a previous paper (Nishihara and Romig, 1964), unless one assumes that the lag observed was inaccurate because of the exceptionally large burst size at 37 C and the long latent period at 45 C. The other noncomplementing mutants were not clearly distinguished from each other. Although it is not likely that they are all genetically identical, this possibility has not yet been eliminated.

If some of these noncomplementing mutants were identical, the frequency of mutation induction with nitrous acid would be even lower than estimated (Nishihara and Romig, 1964). Those

which were isolated in the same experiment could arise from a single mutational event, because the treated phages were grown in indicator cells prior to selection for t^s mutants. However, not all of the noncomplementing mutants were obtained in one experiment and, therefore, could not be the progeny of a single mutant.

It is difficult to explain why there were no other duplications or groupings among the mutants except for this one group of seven noncomplementing isolates. One possibility is that this mutant or group of mutants is more stable through the isolation procedure, and the chances of detection are greater. Another possibility is that the region of the phage genome affected in these isolates mutates at a higher frequency than do other areas of the phage chromosome; this possibility is with the assumption, based on the frequency of recovery of wild-phenotype plaques in the crosses, that the mutants are closely located on the genetic map. The noncomplementing isolates may not necessarily be affected in the same function, because the level of sensitivity of the complementation test used is not very great. The test is, nevertheless, useful for preliminary grouping of the t^s mutants.

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