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TEMPERATURE-SENSITIVE MUTANTS OF NONLYSOGENIZING CORYNEBACTERIOPHAGE β^{hv}: THEIR ISOLATION, CHARACTERIZATION AND RELATION TO TOXIONGENESIS[1]

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SUMMARY Temperature-sensitive (=*ts*) mutants of nonlysogenizing phage β^{hv}, which form plaques at 30 C but not at 38 C, were isolated after treatment with nitrous acid, 2-aminopurine or nitrosoguanidine and were characterized. By complementation tests 79 of the *ts* mutants thus isolated could be assigned to 10 cistrons and two-factor crosses between representative mutants from each cistron yielded a genetic map of the *ts* mutations of phage β^{hv} as one linkage group. Temperature-shift experiments on cells infected with the mutant phage showed that the temperature-sensitive steps for the mutations occurred at particular times for each cistron during the intracellular growth of the phage. The order of the times of these *ts* steps in the latent period was found to be roughly consistent with that of the locations of the corresponding cistrons on the genetic map. Analyses of phenotype were made on three mutants of different cistrons. The results indicate that two mutants have defects in synthesis of morphological components and the other a defect in DNA synthesis. With all of the *ts* mutants tested, synthesis of toxin was found to occur in nontoxinogenic diphtheria bacilli infected with mutant phages even under the nonpermissive condition (38 C) for the mutations. The relation of phage multiplication to toxin production is discussed.

INTRODUCTION

The role of specific phages (β group) in determining the ability of *Corynebacterium diphtheriae* to produce toxin has been thoroughly established (Freeman, 1951; Groman, 1953, 1955; Barksdale and Pappenheimer, 1954; Barksdale, 1955). However, the mechanism of control of toxin production by the phage is still unknwon. One approach to this problem would be genetic physiological analyses of the phage which carries the genetic factor controlling toxin production (" toxin gene "). So far, only a very few genetic studies have been made on corynebacteriophages (Groman and Eaton, 1955; Holmes and Barksdale, 1969, 1970) and

1 An outline of this work was reported at the 17th Symposium on Toxins at Hakone, on July 15, 1970.

they were all performed using a few classic markers, such as host range, plaque morphology and immunity in relation to the "toxin gene".

Recently, Matsuda and Barksdale (1966, 1967) demonstrated the production of toxin in nontoxinogenic diphtheria bacilli during multiplication of stable, nonlysogenizing phage β^{hv}. Such phage-directed synthesis of diphtheria toxin during one cycle of phage growth seemed a very good system for investigation of the genetic physiology of the phage in relation to toxin production, provided that suitable mutants of the phage were available. We thought that temperature-sensitive (=*ts*) mutants would be extremely useful for such a study, as *ts* mutations can occur in many genes controlling a variety of different functions of the genome of phage (e.g. T4D) and are conditional lethals that produce a lethal effect only under the conditions which the experimenter can control (Edgar and Lielausis, 1964; Edger et al., 1964). Attempts were therefore made to isolate *ts* mutants of β^{hv} phage.

This paper reports results on the isolation and characterization of *ts* mutants of β^{hv} phage and also some results of experiments on the effects of *ts* mutations on toxin production in nontoxinogenic diphtheria bacilli infected with β^{hv} phage.

MATERIALS AND METHODS

1. *Bacterial strain*

C7(−) is a nonlysogenic, nontoxinogenic *mitis* strain of *Corynebacterium diphtheriae* (Barksdale and Pappenheimer, 1954) and was used throughout this study as host bacteria for experiments and as plating indicator.

2. *Phage strain*

Corynebacteriophage β^{hv}, obtained as a multistep nonlysogenizing mutant of temperate phage β and similar to $\beta^{hv64tox^+}$ reported previously (Matsuda and Barksdale, 1967), was used as the wild-type for isolation of temperature-sensitive mutants.

3. *Media*

Deferrated PGT medium (Barksdale and Pappenheimer, 1954), to which known concentrations of iron were added if needed, was employed for growing bacteria and phage-infected bacteria in all except genetic cross experiments in which deferrated modified Pope's medium (Yoneda and Matsuda, 1961) was used as growth medium.

Prior to inoculation, a sterile 50% solution of maltose ("Difco" certified) deferrated by the calcium phosphate gel method (Mueller and Miller, 1941) was added to the medium at a final concentration of 4% and 3% for PGT medium and Pope's medium, respectively. Iron was also added to the medium before use as a sterile 0.01% solution of $FeSO_4 \cdot 7H_2O$ in 0.1% HCl.

Deferrated PG medium (PGT medium without tryptophane) was used for washing phage-infected bacteria without supplementation with maltose. Hard and soft agar for plating phages were prepared as previously described (Matsuda and Barksdale, 1967).

4. *Cultural methods*

Cultivation of bacteria in liquid medium was carried out in Erlenmeyer flasks (volume of medium equal to one-tenth of the volume of the flask) by incubation at 35 C in a rotary shaker in a warm-air incubator or in a water bath, at 160 or 130 rotations/min, respectively. Estimation of bacterial growth was made by reading the optical density (OD) at 590 mμ in a Bausch and Lomb spectrophotometer (one OD unit is equivalent to 1.3×10^9 colony forming bacteria/ml).

5. *Phage infection and one step growth*

Bacteria actively growing to a concentration of 2.0×10^9 bacteria/ml in medium containing an appropriate amount of iron (0.04 μg iron/ml for toxin production and for preparation of mutagen treated phage or 0.1 μg iron/ml for genetic cross experiments) were infected with phage or with phage mutants. The procedures used for synchronizing the infection and for one step growth experiments were as reported previously (Matsuda and Barksdale, 1967) with the following modifications. Deferrated TSNa buffer (Tris 0.05 M, Na succinate 0.01 M, NaCl 0.075 M, pH 7.4) prepared by omitting casamino acids, cystine and tryptophane from CST medium (Uchida and Yoneda, 1967) was used to resuspend the bacteria at a concentration of 4×10^9 bacteria/ml for adsorption of

phage. Depending on the experiment, aliquots of 4.5 ml or 5.0 ml, 10 ml and 15 ml of infected cultures were distributed in 100 ml, 300 ml and 500 ml Erlenmeyer flasks which had been cleaned with chromic acid. Lysis of infected cultures was determined by measuring the optical density of samples at intervals.

6. *Preparation of plating bacteria and phage assay*

An overnight culture of C7(−) bacteria in medium containing 0.1 μg iron/ml was diluted in medium containing 0.3 μg iron/ml to an OD of 0.03 and grown with shaking for about 3.5 hr. When the OD of the culture reached 0.3 (3.9×10^8 bacteria/ml), the bacteria were concentrated 4 times by centrifugation and resuspension in fresh medium. Aliquots of 0.1 ml of this preparation were used for plating. Samples for phage assay were first diluted appropriately with sterile phosphate buffered saline (NaCl 4.0 g, KH_2PO_4 3.0 g, Na_2HPO_4 7.0 g per liter) containing 0.001% gelatin (Difco) and 1 mM $MgSO_4$ and subjected to the procedure described by Adams (1959).

7. *Preparation of high titer phage stocks*

The procedures employed were the same in principle as those reported previously (Matsuda and Barksdale, 1967). In the study reported here, an actively growing culture (2.0×10^9 bacteria/ml) of C7(−) obtained by overnight growth in medium containing 0.04 μg iron/ml was infected with phage lysate obtained by extraction of confluent lysis plates. After infection all incubations were carried out at the low temperature (30 C) with gentle shaking (130 rotations/min) in a water bath. After adding $CaCl_2$ to a concentration of 4 mM, the phage-bacteria mixture was incubated for 40 min. Then the mixture was chilled and the phage-infected bacteria were concentrated to 4×10^9 bacteria/ml by centrifugation and gentle resuspension in fresh deferrated medium with no added iron and distributed in flasks as described in the section on *Phage infection and one step growth.* The infected culture was then incubated for about 6 hr to allow lysis. After centrifugation the supernatant of the lysed culture was filtered through a Millipore filter (HA 0.45 μ, Corp., Bedford, Mass.). These stocks had titers of about 10^{11} plaque forming particles/ml and were stored at 4 C.

8. *Isolation of mutants*

All the mutants described in this study were isolated from β^{hv} phage after treatment with one of the three mutagens as follows.

1) Preparation of nitrous acid treated phage:

Phage was treated for 40 min at 25 C with 0.2 M $NaNO_2$ at pH 4.2 in 0.2 M sodium acetate-acetic acid buffer. The initial phage titer was about 10^{10} plaque forming particles/ml of reaction mixture. The reaction was stopped by rapid 100 fold dilution in chilled phosphate buffered saline containing magnesium and gelatin as described in the section on *Phage assay.* The surviving fraction was approximately 10^{-4}. As a control, phage was incubated for the same time in the same buffer in the absence of $NaNO_2$. Very little loss of infectivity was observed.

2) Preparation of 2-aminopurine (2 AP) treated phage:

The phage-infected bacteria obtained as described in the section on *Phage infection and one step growth* were resuspended in fresh medium (1×10^8 infected bacteria/ml) containing 2-aminopurine nitrate (Sigma Chemical Co.) 1 mg/ml. The reaction mixture was incubated at 30 C with gentle shaking (130 rotations/min) for 170 min to allow lysis. This lysate was diluted and used as a source of mutants.

3) Preparation of nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine) treated phage:

The phage were treated in the same way as 2 AP treated phage. The concentrations of infected bacteria and of nitrosoguanidine were 2×10^8/ml and 10 μg/ml of medium, respectively.

Temperature-sensitive mutants of the *n* and *a* series were isolated at random from phage after nitrous acid treatment or 2 AP treatment as follows. The mutagen treated phage was plated after dilution to give 100 to 200 plaques per plate and incubated at 30 C overnight. Well separated plaques were each stabbed with a sterile pin and transferred to pairs of plates seeded with C7(−) bacteria. One of the duplicate plates was incubated overnight at 38 C, and the other at 30 C. Phage clones which formed plaques at 30 C but not at 38 C were picked up and retested three times by diluting and plating at 38 C and 30 C. After at least three successive single plaque isolations, the purified clones were propagated and the stocks were stored at 4 C.

The mutants of the *e* series were isolated by the special selection procedures described by Edgar and Lielausis(1964) as follows. Phage treated with nitrosoguanidine as described above was used to infect C7(−) bacteria with a multiplicity of about one phage per bacterium. The infected cells were incubated at 38 C in fresh medium in the presence of antiphage

serum (K=7 in the growth medium) with gentle shaking (130 rotations/min) for 200 min. After incubation the culture was diluted and plated at 30 C for surviving infective centers. From these surviving centers, mutants were isolated by the method used for the *n* and *a* series. Mutants with a reversion frequency of more than 10^{-4} were discarded.

Mutants were named as described by Edgar and Lielausis (1964).

9. *Complementation tests*

Warm tryptose agar plates were overlayed with soft agar plus indicator cells together with one of the known *ts* mutants (10^8 phage/plate). When the top agar became solid the plates were kept at 38 C for about 30 min, and then one droplet (about 0.01 ml) of each unknown mutant to be tested (10^9 phage/ml) was spotted on the plates and these were incubated at 38 C overnight. Complementation between pairs of mutants was always tested as follows: one member of the pair was spotted against the plate seeded with indicator cells together with the other member and *vice versa*. As a control, agar plates seeded with indicator cells only were used for spotting each test mutant. Only negative complementation was taken as significant.

10. *Genetic cross procedure*

A suspension of actively growing C7(−) cells (4×10^9 cells/ml) in TSNa buffer was prepared as described in the section on *Phage infection and one step growth*. Then 0.008 M KCN or chloramphenicol was added to the cell suspension at a final concentration of 200 μg/ml. The cells were mixed with an equal volume of phage mixture containing 4×10^{10} particles/ml of each parental type. 4 mM $CaCl_2$ was then added and the mixture was shaken gently at 30 C at 130 rotations/min for 22 min. Then anti β^{hv} phage serum (K=35, 0.15 ml/ml of reaction mixture) was added and incubation was continued for 7 min. The mixture was then diluted 100,000 fold with growth medium (deferrated modified Pope's medium containing 3% maltose and 0.1 μg iron/ml), and a 5.0 ml aliquot was put into a 100 ml flask and incubated at 30 C for 180 min with gentle shaking to allow lysis. After lysis samples were plated at 30 C to assay total progeny and at 38 C to measure wild-type (ts^+) recombinant progeny. As controls in each case the input phage was plated in parallel at comparable dilutions. Recombination values were calculated as twice the percentage of ts^+ progeny.

11. *Electron microscopy*

Samples were placed on grids with carbon-supported collodion films and excess liquid was removed by application of filter paper to the edge of the grids. The grids were dried in air, washed once with distilled water and then negatively stained with 1% uranyl acetate. Observations were made with a Hitachi electron microscope, type HU-11D-S. Fields of view were photographed at a magnification of $\times$41,000.

12. *DNA determination*

For measurement of DNA synthesis the method of Burton (1956) as modified by Epstein et al. (1963) was employed taking 1 ml samples containing 4×10^9 infected cells/ml from the experimental cultures.

13. *Assay of diphtheria toxin*

1) The MRD (minimal reacting dose) was determined with sterile culture filtrates (Millipore filter HA 0.45 μ) by a modification of the method of Jensen (1933) using standard diphtheria toxin, No. 108, obtained from the Vaccine Production Plant of our Institute (The Kanonji Institute of the Research Foundation for Microbial Diseases of Osaka University, Kagawa). Samples to be tested were diluted with M/15 phosphate buffer (pH 7.0) containing 0.02% gelatin. Aliquots of 0.1 ml of diluted samples were injected intracutaneously into the backs of depilated rabbits. The effect (erythema) was examined after 48 hr. A value of 10^5 MRD corresponds to 1 Lf unit (flocculating unit) which is equivalent to *ca* 1.9 μg of toxin protein.

2) Lf units (flocculating units) were determined semiquantitatively using Ouchterlony's agar double immunodiffusion technique (Ouchterlony, 1948) with pepsin-treated horse antitoxin No. 68 and a preparation of crystalline diphtheria toxin as a reference. The former contained 1300 limit flocculating units (Lf)/ml and was obtained from the Vaccine Production Plant of our Institute (The Kanonji Institute of the Research Foundation for Microbial Diseases of Osaka University, Kagawa). The latter was prepared as described by Hirai et al. (1965).

14. *Antiphage serum*

A purified β^{hv} phage preparation obtained after 4 cycles of differential centrifugation (35,000$\times g$, 100 min–10,000$\times g$, 10 min) was used to immunize rabbits. Rabbits were injected with 2×10^{11} plaque forming particles in incomplete Freund's adjuvant

(Freund, 1951). After 30 days the rabbits received a booster injection of the same dose and 8 days later they were bled to obtain sera. The K values of the antiphage sera obtained ranged from 15 to 35.

RESULTS

1. *Isolation of mutants and complementation tests*

Wild-type β^{hv} phage formed plaques with equal efficiency of plating (e.o.p.) at temperatures of 30 C to 38 C, but above about 39 C the growth of the plating bacteria was impaired and the e.o.p. of the phage decreased. Thus temperature-sensitive mutants which formed plaques at 30 C but not at 38 C were isolated as described in the MATERIALS AND METHODS.

Temperature-sensitive mutants of the *n* series were isolated at random from phage after treatment with nitrous acid. A small fraction of plaques obtained by plating this phage stock at 30 C failed to produce plaques on incubation at 38 C but did produce plaques at 30 C. More than one hundred mutants (*n* series) were isolated and stocks were prepared after successive single plaque isolations of each of them. Complementation tests were performed by modified spot tests. Analyses of fifty-six *n* mutants revealed some intragenic complementations in some of their combinations, but unfortunately all these mutations fell into one functional group, even though most of them were isolated independently.

Isolation of *ts* mutants of the *a* series at random from phage after 2-aminopurine treatment was found to be more difficult than in the case of *n* series mutants. The few mutants of the *a* series obtained were all assigned by complementation tests to one cistron which was different from that of the *n* series mutants.

When cells infected with *n* or *a* series mutants were kept at the elevated temperature (38 C), they lysed at the time characteristic of the wild-type phage but without production of viable progeny and thus these mutants appeared to be " late mutants ".

Since we were more interested in early mutants than in late mutants, as discussed later, in isolation of *e* series mutants, we attempted to " enrich " the population of mutagen-treated phage with respect to early mutants by taking advantage of differences between early and late mutants. In this manner twenty-one mutants were isolated.

To group mutants with a common functional defect and thus to reduce the number of crosses required to map the mutations, complementation tests were made between all possible combinations of pairs of mutants, before mapping. The results of these tests and the distribution of various mutations among the cistrons are summarized in Table 1.

TABLE 1. *Distribution of temperature-sensitive mutations of* β^{hv}

Cistron name	Representative mutant	Number of mutants
A	*e* 21	1
B	*e* 12	9
C	*e* 9	1
D	*e* 14	4
E	*a* 1	2
F	*e* 22	1
G	*e* 4	3
H	*e* 7	1
I	*n* 53	56
J	*e* 1	1
Number of cistrons 10		Total mutants 79

On the basis of the complementation tests, the seventy-nine *ts* mutants isolated were assigned to ten functional groups. As seen in the table, the mutations are not distributed uniformly over the genome; some cistron contain only a few mutations, while others have more than fifty.

2. *Mapping of mutations by two-factor crosses*

To construct the genetic map, two-factor crosses between *ts* mutants were performed with representatives from each cistron determined

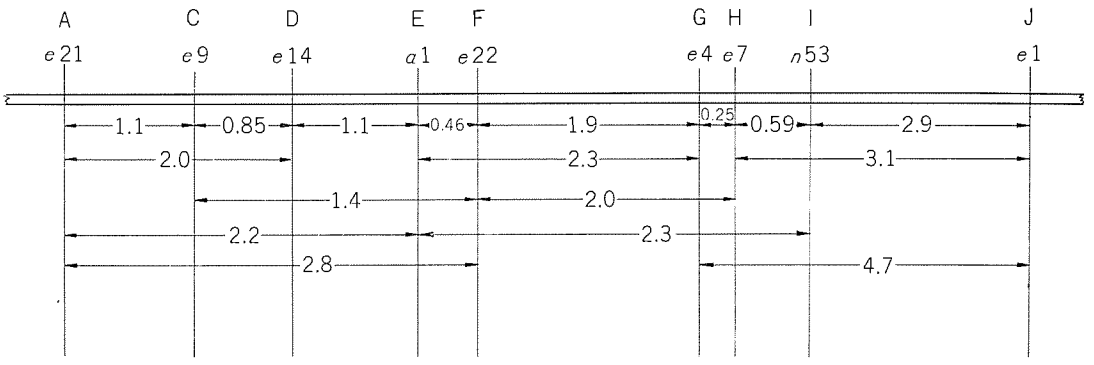


FIGURE 1. *Linkage map of ts mutations of corynebacteriophage* β^{hv}. *Recombination values (in %) are shown. Numbers prefixed by letters refer to temperature-sensitive (=ts) mutants.*

by complementation tests. Fig. 1 shows the results of these experiments as a tentative map of *ts* mutations.

As can be seen, all these mutations could be arranged in one linkage group. Recombination values in the β^{hv} phage did not show good additivity. In repeated experiments the recombination values varied somewhat, but results were in good agreement with respect to the order of mutations.

3. *Temperature-shift experiments on mutant phage-infected cells.*

To obtain information on the function of the genes oriented in this way by mapping, physiological studies were made on the mutations using a temperature-shift regime to reverse the phenotypes of the conditional lethals, temperature-sensitive mutants, in the middle of a single cycle of intracellular phage growth. These experiments were to determine the time of temperature-sensitive step for each mutation.

Actively growing C7(−) cells (4×10^9/ml) were infected synchronously with each *ts* mutant at a multiplicity of about five, as described in MATERIALS AND METHODS. Aliquots of 4.5 ml of suspensions of mutant phage-infected cells in fresh medium were distributed in 100 ml Erlenmeyer flasks and incubated with gentle shaking at 30 C. At various times after infection, flasks were transferred from the water bath at 30 C to the one at 38 C and incubated with shaking for 160 min. Then the cells were removed by centrifugation and the yield of plaque forming particles in the supernatant was assayed by plating at 30 C.

Fig. 2, 3, 4 and 5 show plotts of data from some of these temperature-shift experiments. As can be seen in these figures, when the culture was transferred to the high temperature before a particular time during the latent period, no plaque forming phage particles were produced, while on transfer of the infected cells after this time plaque forming particles were obtained.

TABLE 2. *Temperature-sensitive (=ts) steps of ts mutations in the latent period of phage growth*

Cistron	Mutant	*ts* step (min)
A	*e* 21	28
B	*e* 12	40
C	*e* 9	60
D	*e* 14	56
F	*e* 22	62
G	*e* 4	68
H	*e* 7	70
I	*n* 53	70
J	*e* 1	80

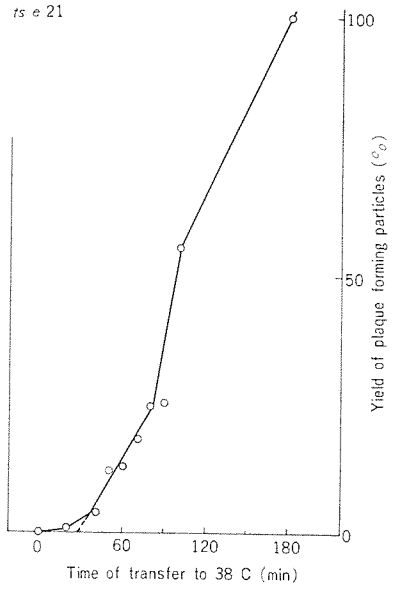


FIGURE 2. *Temperature-shift (30 C to 38 C) experiment on C7(−) cells infected with mutant phage ts e21.*

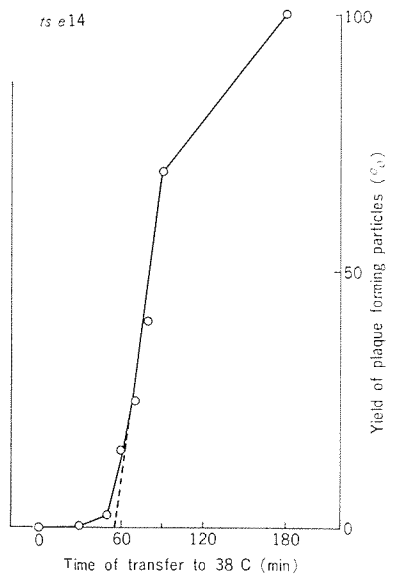


FIGURE 3. *Temperature-shift (30 C to 38 C) experiment on C7(−) cells infected with mutant phage ts e14.*

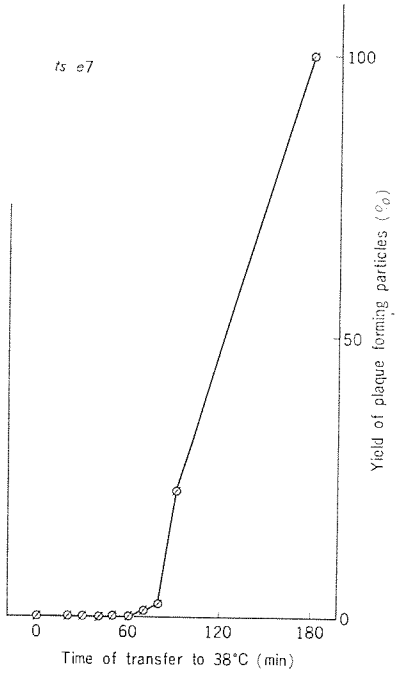


FIGURE 4. *Temperature-shift (30 C to 38 C) experiment on C7(−) cells infected with mutant phage ts e7.*

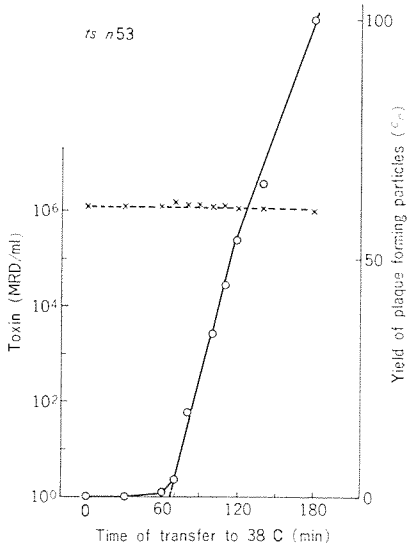


FIGURE 5. *Temperature-shift (30 C to 38 C) experiment on C7(−) cells infected with mutant phage ts n53.*
Yield of plaque forming particles (○- - - -○), *yield of toxin* (×- - - -×).

This clearly shows that the temperature-sensitive step occurred at a particular time. The results of these experiments are summarized in Table 2.

The table shows that the times of the temperature-sensitive steps during intracellular growth are characteristic of *ts* mutation from each cistron.

4. *Analyses of the phenotype of some mutants*

Fig. 6 shows data from a one step growth experiment on mutant *ts n*53 in C7(−) cells at low (30 C) and high temperature (38 C).

The mutant phage-infected cells lysed also 38 C at the time characteristic of the wild-type phage but they did not produce plaque forming progeny phage. This was also the case with mutant *ts a*1.

The lysates of both these mutants at high temperature were examined by electron microscopy for the synthesis of morphological components.

As shown in Fig. 7 (a) and 7 (b), in the lysate at 38 C by mutant *n*53 no complete phage particles were detectable and only head-like materials were observed.

The lysate at 38 C by mutant *a*1 showed no distinct morphological structures upon electron microscopic examination.

The total amount of DNA in the cells began to increase shortly after infection with the wild-type phage or *ts* mutants at low temperature. The DNA increased linearly with time and at the end of the latent period reached a value 2 to 3 times that at the time of infection. Even at high temperature (38 C) cells infected with mutant *n*53 or *a*1 showed DNA synthesis comparable to that at low temperature (30 C) or to that with the wild-type.

Another group of *ts* mutants did not lyse cells at the high temperature. The effect of one mutant in this group *e*21, which had the earliest *ts* step, on DNA synthesis was examined. It was found that there was no appreciable increase in DNA in cells infected with this mutant at the elevated temperature.

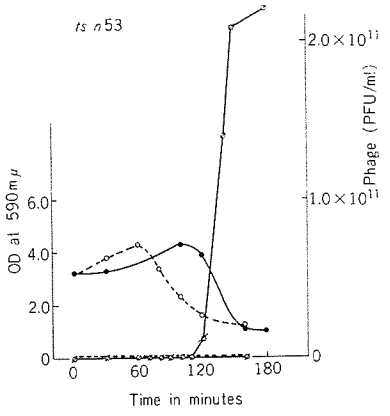


FIGURE 6. *One step growth of mutant phage ts n53 in C7(−) cells.*

∅——∅: plaque forming phage particles produced on incubation at 30 C.

∅- - -∅: plaque forming phage particles produced on incubation at 38 C.

●——●: optical density of culture at 30 C.

○- - -○: optical density of culture at 38 C.

▶

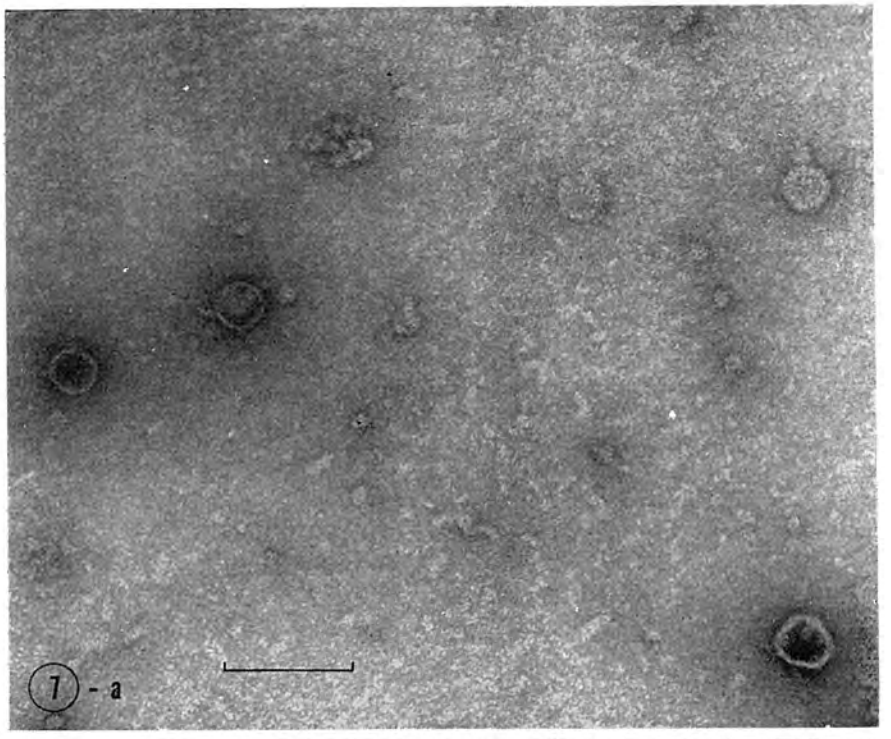
FIGURE 7 (a). *Electron micrograph of C7(−) cell lysate at 38 C by temperature-sensitive mutant, ts n53 of corynebacteriophage* β^{hv}.

Initial magnification ×41,000. Marker=1,000 A. Preparation negatively stained with uranyl acetate and examined under a Hitachi electron microscope, type HU-11D-S.

▶

FIGURE 7 (b). *Electron micrograph of C7(−) cell lysate at 30 C by temperature- sensitive mutant, ts n53 of corynebacteriophage* β^{hv}.

Initial magnification ×41,000. Marker=1,000 A. Preparation negatively stained with uranyl acetate and examined under a Hitachi electron microscope, type HU-11D-S.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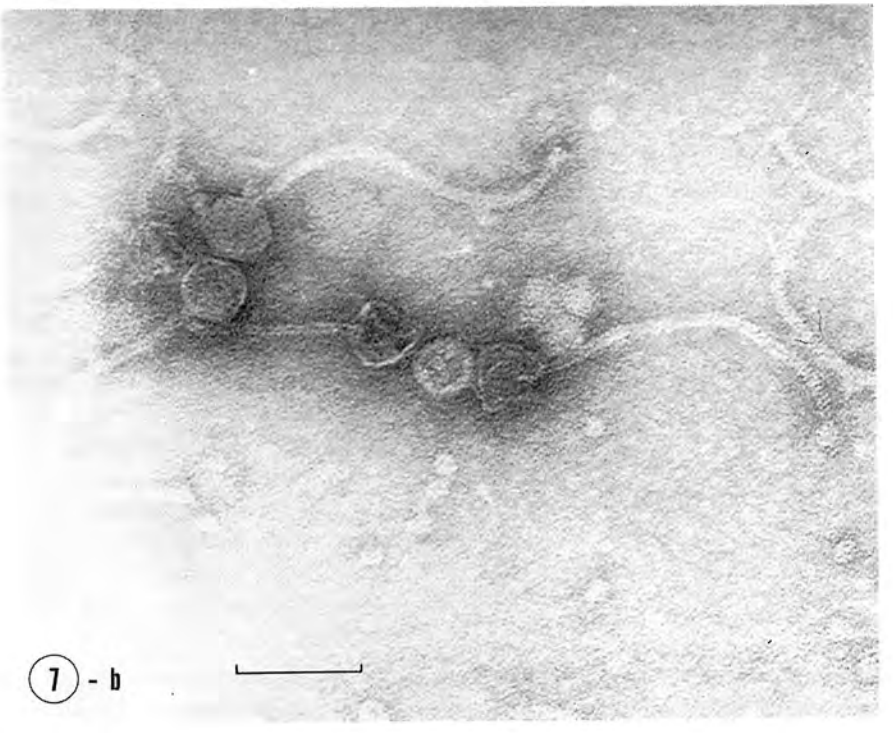
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5. *Effect of temperature-sensitive mutations of phage β^{hv} on toxin production in nontoxinogenic diphtheria bacilli*

The effect of the *ts* mutations on toxin production was studied under the condition (38 C) which was lethal to these mutants using the *ts* mutants which had been characterized.

Fig. 5 shows the yields of toxin and of plaque forming phage particles with cells infected with mutant *n*53 phage obtained in a temperature-shift expeiment. In sharp contrast to the yields of plaque forming phage particles, the yields of toxin were not affected by the temperature-shift and there was no difference in the yields of toxin at 38 C (transfer at 0 time) and at 30 C (transfer at 180 min).

With all the mutants, including the very early mutant *e*21 in which DNA synthesis was defective, even under the non-permissive condition (38 C) for these mutations, cells (4×10^9/ml) infected with the mutant phage produced as much toxin (about 6×10^5 MRD/ml or 5–10 Lf/ml) as under the permissive condition (30 C) for mutations or on infection with wild-type phage.

DISCUSSION

The results presented here show the successful isolation of the various temperature-sensitive (=*ts*) mutants of nonlysogenizing corynebacteriophage β^{hv}, which can be grouped into 10 cistrons by complementation tests, and that these cistrons can be mapped as one linkage group. Though the results of the complementation tests were not entirely unambiguous, that these were valid was supported by the results of temperature-shift experiments, which showed that the temperature-sensitive steps of the *ts* mutants each occurred at a time characteristic of the cistron to which the mutant belonged. The order of the times of the *ts* steps were almost consistent with the order of the location of the corresponding cistrons on the genetic map. This may be a reflection of the temporal sequential development of the phage during which the function of each cistron is expressed in order at a respective time. The gene order requires more precise determination by three-factor crosses. The questions of whether the linkage map of phage β^{hv} is circular and of the relative location of the "toxin gene" on the map also must await further study.

The present investigation showed that in all the *ts* mutants tested, phage-directed synthesis of diphtheria toxin in nontoxinogenic C7(−) cells could occur under the nonpermissive condition (38 C) for the mutant with which the cells were infected, just as under the permissive condition (30 C). This clearly indicates that the "toxin gene" can be expressed irrespective of the expressions of the various genes in the phage genome corresponding to the *ts* mutations tested and suggests that toxin synthesis can occur without processes in phage multiplication such as phage lysis, maturation or even DNA synthesis. Indeed, previous kinetic experiments with wild-type β^{hv} phage have already shown that the bulk of toxin begins to be produced at a time much earlier than the beginning of phage lysis (Matsuda and Barksdale, 1967; Matsuda, 1968). In the particular case of C7(−) cells infected with the mutant *ts e*21, toxin production was found to occur at 38 C, the nonpermissive temperature for the mutant, at which no DNA synthesis occurred and development of the phage was arrested at an early stage. Thus there appears to be no invariable association of phage multiplication with toxin production or if any relationship does exist, it may be in a very early stage of viral development. Isolation of "early *ts* mutants" related to very early functions are now in progress to elucidate this point.

Finally, it should be mentioned that the mutant *ts e*21 provides a much simpler system than that previously described (Matsuda and Barksdale, 1967) for investigating phage-directed synthesis of diphtheria toxin. Thus, in this new system, nontoxinogenic diphtheria bacilli such as C7(−) are infected with the *ts e*21 mutant instead of the wild-type phage and incubated under the nonpermissive condition

(38 C) for the mutant phage, so that the mechanism of toxin synthesis can be analyzed without the complications of the influence of most of the metabolism of the host and most of the process of viral development. Making use of this advantage, precise analyses of toxin synthesis in the C7(−)-*ts* *e*21 system are now in progress and results will be published elsewhere.

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