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Genetic Analysis of *tox*$^+$ and *tox*$^-$ Bacteriophages of *Corynebacterium diphtheriae*[1]

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A series of mutants derived from the temperate corynebacteriophages β^{tox+}, γ^{tox-}, and L^{tox+} was isolated and characterized. In three-factor crosses between mutant β phages the relative map order of the genetic markers determining extended host ranges (*h* and *h'*) and loss of ability to lysogenize (*c*) was found to be *h--c--h'*. Recombination between markers was observed in matings between phage β and the heteroimmune corynebacteriophages γ and L. In such matings between heteroimmune phages the *c* markers of phages β and γ failed to segregate from the *imm* markers which determine the specificity of lysogenic immunity in these phages. The factor which directs the synthesis of diphtherial toxin during infection of appropriate corynebacterial hosts by toxinogenic corynebacteriophages is designated *tox*$^+$. It was possible to show that the *tox*$^+$ determinant of phage β behaves as a single genetic element which occupies a position between the loci *h* and *imm* on the genetic map of this phage. Genetic recombination between mutants of phage β occurred at very low frequencies in biparental matings performed by mixed infection of *Corynebacterium diphtheriae* $C7_s(-)^{tox-}$. Considerably higher recombination frequencies were observed when lysogenic bacterial strains carrying one parental phage as prophage were induced by ultraviolet irradiation and then superinfected by the second parental phage. Maximal stimulation of genetic recombination between mutant β phages was detected when superinfection followed ultraviolet irradiation of the lysogenic cells within a limited period of time. In matings between phages with incomplete genetic homology, the stimulation of recombination by ultraviolet radiation was much less effective.

Freeman (11) and Freeman and Morse (12) first observed that nontoxinogenic strains of *Corynebacterium diphtheriae* could acquire the ability to produce diphtherial toxin through phage-mediated conversion. Subsequent studies have clarified the essential features of phage conversion in *C. diphtheriae* (6, 36). Only corynebacteriophages which carry a specific genetic determinant designated *tox*$^+$ can direct the synthesis of toxin in phage-infected cells (5, 15, 19), and the *tox*$^+$ genotype of such phages appears to be expressed only in certain strains of *C. diphtheriae* (6, 16, 32). When an appropriate phage-sensitive bacterial host is exposed to a temperate *tox*$^+$ phage, conversion to toxinogenicity occurs at high frequency (13, 14). There is then an exact correlation between the acquisition of toxinogenicity and of lysogenicity for the *tox*$^+$ phage (9, 11), and the persistence of toxinogenicity requires the continued presence of the *tox*$^+$ prophage (15). In addition, diphtherial toxin can be produced during the lytic infection of appropriate hosts by nonlysogenizing mutants of *tox*$^+$ corynebacteriophages (8, 27, 28). Deoxyribonuclease fails to inhibit phage-mediated conversion of *C. diphtheriae* to toxinogenicity (14). Thus, conversion differs both from transformation of bacteria by deoxyribonucleic acid (3) and from generalized transduction of bacteria mediated by phage (38).

Information concerning the genetic behavior of corynebacteriophages is limited. Groman and collaborators (17, 18) observed that surviving cells from cultures of *C. diphtheriae* C4(γ) lysed by phage β sometimes liberated phage particles which differed in toxinogenicity or host range from the parental phages β^{tox+} and γ^{tox-}. These new phage types were also detected in spontaneously occurring "single" bursts in cultures of the doubly lysogenic strain C4(γ)(β). Groman and

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collaborators concluded that the new phage types had originated by recombination between phages β and γ. The studies communicated here were undertaken to investigate further the genetic basis for the control of toxinogenicity by corynebacteriophages. Toward this end, a series of mutants of the well-studied corynebacteriophage β was isolated and characterized. A system for the genetic analysis of β and related phages has been developed, and the position of the tox^+ determinant on the genetic map of phage β has been established.

MATERIALS AND METHODS

Corynebacteria. *C. diphtheriae* strains $C7_s(-)^{tox-}$, $C7_s(\beta)^{tox+}$, and $C7_s(\gamma)^{tox-}$ have been previously described (9, 28). *C. diphtheriae* strains C4/β and c4(γ)(β) were kindly provided by Neal Groman (17). Strain $C7_s(-)^{tox-}$ is nontoxinogenic (tox^-), has smooth colonial morphology (subscripts), and has not been shown to be lysogenic. For convenience, strain $C7_s(-)^{tox-}$ will hereafter be designated C7. For lysogenic strains derived from C7, the names of the prophages will be indicated in parentheses, for example, C7(β) and C7(γ). All strains derived from C7 by lysogenization or by mutation (or both) were found to be nontoxinogenic except when they were lysogenized by tox^+ phages.

New lysogenic strains were prepared by exposing lawns of phage-sensitive bacterial strains to drops of bacteria-free, isogenic phage stocks. Surviving bacteria were isolated and tested for lysogenic immunity, for phage release, and in most cases for inducibility with ultraviolet light. Strain C7(L) was prepared and kindly provided by Sheldon Arden.

Nonlysogenic, phage-resistant mutants were obtained by exposing lawns of phage-sensitive bacteria to nonlysogenizing mutant phages. Strains C7/β^c and c7/β^{vir} were selected for resistance to phages β^c and β^{vir}, respectively. Two colonial types of C7/β^{vir} designated type 1 and type 2 were subsequently distinguished. Type 1 colonies are flatter and firmer than type 2 colonies. Strain C7/β^{vir}/β^{hc} was isolated from a population of C7/β^{vir} type 1 by using as a selective agent the phage β^{hc}. All newly derived bacterial strains were cloned by at least three successive single-colony isolations.

Corynebacteriophages. The corynebacteriophages β^{tox+} and γ^{tox-} were obtained from the respective lysogenic derivatives of C7. They will be referred to hereafter as β and γ. Phage $\beta^{hv64(tox+)}$ is a virulent, multistep mutant of β (27, 28), and will be referred to hereafter as β^{vir}; β^{vir} is serologically closely related to the parental phage β. Phage L^{tox+}, hereafter referred to as L, was isolated by Edward Goldzimmer as a mutant of phage l^{tox+} which had acquired the ability to grow on C7. Phage l^{tox+} was isolated by Edward Goldzimmer and Larry Frank from *C. ulcerans* strain 976/51 (20) utilizing as indicator *C. ulcerans* strain 603/50 (20).

Nonlysogenizing phage mutants were recognized by the formation of clear plaques on lawns of C7. The host-range mutants β^h and $\beta^{h'}$ were selected for their ability to form plaques on the bacterial indicator strains C7/β^{vir} type 1 and C7/β^c, respectively. Phages with both *h* and *h'* types of host range were selected by plaque formation on the bacterial indicator strain C7/β^{vir}/β^{hc}. Each mutant phage strain was cloned by at least three successive single-plaque isolations.

Media. PGT medium. The casein hydrolysate medium of Mueller and Miller (31) was modified according to Barksdale and Pappenheimer (9) and adjusted to *p*H 6.8 with 50% KOH. The undeferrated medium was supplemented prior to use with 10% (v/v) of 20% maltose solution containing (per liter): 0.40 ml of 1% $FeSO_4$ in 0.1 N HCl, 6.0 ml of 0.1% aqueous calcium pantothenate, and 30 ml of 10% aqueous $CaCl_2 \cdot 2H_2O$. PGT medium contained about 0.2 μg of Fe^{++} per ml.

Tryptose-hard agar and tryptose-soft agar for phage assays were prepared as previously described (8), with the agar concentrations reduced to 1.0 and 0.5%, respectively. Plates for viable counts and for streaking of bacteria contained 1.5% agar and 1% of the sterile maltose supplement for PGT medium.

Cultivation of corynebacteria. All bacterial strains were maintained as frozen stocks prepared from overnight cultures of cloned cells. Experiments were carried out with cultures in the exponential phase of growth in PGT medium. Cultures were incubated at 36 C in a water bath (model G-76, New Brunswick Scientific Co.) with aeration provided by rotary shaking at 240 rev/min.

The ratio of viable cells to optical density (OD) was approximately 1.0×10^8 cells per ml per OD 0.3 (Bausch & Lomb colorimeter, type 33-29-40). Viable counts were determined by spreading samples of appropriately diluted cultures on tryptose-agar.

Phage assay. The procedure of Barksdale et al. (8) was used with minor modifications. Adsorption mixtures were diluted serially in unsupplemented PGT. Separate 0.10-ml samples of the diluted adsorption mixtures and of appropriate indicator cells were added to 2.0-ml samples of tryptose-soft agar and layered onto hard agar plates. Indicator cells were prepared as OD 0.3 suspensions in PGT, except for strains C7/β^{vir} type 1, C7/β^{vir}/β^{hc}, and their lysogenic derivatives, which were prepared at OD 0.5. Plates were incubated at 30 C for at least 1 day.

For several purposes described below, bacterial lawns were prepared by adding bacteria without phage to tryptose-soft agar overlays.

Preparation of phage stocks. A single phage plaque in a lawn of *C. diphtheriae* C7 was picked and inoculated into a 5.0-ml culture of C7 at OD 0.3. Two hours later 15.0 ml of PGT was added. Mass lysis of the infected culture occurred between 5 and 7 hr after infection. Supernatant fluids from such cultures usually contained 10^9 or more plaque-forming units (PFU)/ml.

High-titered phage stocks were prepared by infecting cultures of C7 with phage to give an OD of 1.5 and a phage titer of 5×10^8 PFU/ml. Supernatant fluids obtained at the onset of mass lysis (135 to 150 min, two cycles of viral growth) contained more than 10^{10} PFU/ml. Phage yields of approximately 10^{11}

PFU/ml were achieved by adding sodium citrate to a final concentration of 0.07 M just prior to the onset of mass lysis. Phage stocks were filtered to remove bacteria, tested for sterility, and stored at 4 C.

Phage stocks were also prepared by induction of phage development in lysogenic corynebacteria essentially as described by Barksdale and Pappenheimer (9). Log-phase bacterial cultures at OD 0.3 were induced by exposure for 135 sec to ultraviolet radiation at 1,750 (±5%) ergs per cm^2 per sec. Sodium citrate (0.07 M) was added at 50 min after irradiation, and the supernatant fluids were collected at 4 hr postirradiation. Titers of 10^9 PFU/ml were obtained.

Induction of mutations in corynebacteriophages. (i) The ultraviolet irradiation method was based on the observations of Weigle (37). In two experiments, several independently grown stocks of phage β in PGT medium were irradiated with ultraviolet light for 15 min (experiment I) and 20 min (experiment II), with phage survivals varying from 1.8 to 4.8%. The irradiated phage was adsorbed under assay conditions to cells of strain C7 at OD 0.3, previously irradiated with ultraviolet light for 120 sec (experiment I) or 90 sec (experiment II). The irradiated and infected cells were diluted and plated in the same manner as adsorption mixtures for phage assays.

(ii) The chemical mutagen *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (K & K Laboratories, Jamaica, N.Y.), hereafter designated NG, was used according to the two protocols described below. Stock solution containing 4 mg of NG per ml in 0.3 M acetate buffer (*p*H 4.9) was prepared just before each experiment and was sterilized by filtration.

Method I was the method of D. Krieg as described by Baldwin and collaborators (4) and modified slightly for use with *C. diphtheriae*. Single colonies of C7 (β) or C7 (γ) were picked into tryptone broth (Difco) in roller tubes, incubated overnight at 30 C in a roller drum (New Brunswick Scientific Co.) at 40 rev/min, diluted 1:10 into fresh tryptone broth, and subcultured for 4 hr. The actively growing cultures were centrifuged, washed once with 0.3 M acetate buffer, and mutagenized with approximately 500 μg of NG per ml for 2 hr at 30 C without shaking. The mutagenized, lysogenic cells were washed once with tryptone broth, resuspended in tryptone broth containing 0.07 M sodium citrate, and incubated overnight at 30 C in roller tubes. Supernatant fluids of overnight cultures were examined for mutant phages.

In method II, exponentially growing cultures of C7 with OD between 0.3 and 1.5 were infected with phage to yield at least 5 PFU/bacterium. NG was added at 20 min postinfection, and sodium citrate (0.07 M) was added at 50 min postinfection. With final NG concentrations of 18 and 90 μg/ml, mass lysis began at 75 and 110 min after infection, and the yields of treated phages were equal to normal yields. At 440 μg/ml of NG, mass lysis of the culture and phage release failed to occur within 3.5 hr. Both methods gave effective mutagenesis, but much higher titers of mutant phages were obtained with method II.

Complementation tests. The method of Kaiser (24) was used to determine whether individually isolated nonlysogenizing mutants of phage β could complement in establishing lysogeny. Drops from pairs of stocks of independently isolated β^c mutants were placed on overlapping regions of lawns of C7. The plates were examined after incubation for 1 day at 30 C for turbidity due to the outgrowth of lysogenic cells in the areas infected with pairs of β^c phages.

Recombination between corynebacteriophages. Each phage mating was performed by inducing a lysogenic derivative of C7 with ultraviolet light, as described previously, and infecting the irradiated bacteria with an isogenic stock of phage differing from the prophage. In each cross, samples were taken for measurement of the following: (i) viable count of lysogenic cells immediately before irradiation, (ii) titer of the superinfecting phage stock, (iii) titer of unadsorbed superinfecting phage, and (iv) titer of phage progeny. As controls, both the superinfecting phage stock and a lysate from the induced but uninfected lysogenic cells were assayed to exclude the possibility that mutant phages with recombinant genotypes might be present.

Duplicate 2.0-ml samples of induced lysogenic cells were transferred to tubes (15 by 100 mm) and incubated in the dark. At 10 min postirradiation, 0.50 ml of appropriately diluted superinfecting phage was added to one tube to give approximately 3 PFU per irradiated cell, and 0.50 ml of PGT was added to the control tube. At 50 min postirradiation, 0.20-ml samples were removed, diluted 1:100 in PGT, and assayed for unadsorbed phage after centrifugation. Sodium citrate was added at 50 min postirradiation to yield approximately 0.07 M. At 4.5 hr after irradiation, supernatant fluids were collected and assayed on C7 to detect all viable phage progeny and on appropriate bacterial indicator strains to detect selectable recombinant phages. Plates were incubated for 2 days at 30 C.

Yields of phage progeny were expressed as the average number of infectious particles of each genotype released per infected bacterial cell. Recombination frequencies were expressed as percentages of reciprocal recombinants among all progeny phages, and the assumption was made that reciprocal recombinants were equally frequent among the progeny.

Tests for toxinogenicity. Bacterial strains were tested for their *ability to produce diphtherial toxin* by injecting 0.2-ml samples of log-phase cultures in PGT medium at OD 0.30 intradermally into the depilated backs of rabbits or guinea pigs as described elsewhere (7, 28). Phages were tested for the *presence of the tox$^+$ character* in the following manner. Plaques were picked into 2.0-ml roller-tube cultures of C7 in PGT at OD 0.30 and incubated in a roller drum for 3 hr at 30 C. To each culture, 8.0 ml of PGT medium was then added and incubation was continued overnight. Supernatant fluids of overnight cultures were diluted 1:100 in unsupplemented PGT, and 0.20-ml samples were tested for the presence of toxin by intradermal injection into rabbits or guinea pigs as described above. When the tests with diluted culture supernatant fluids revealed no toxin, the supernatant fluids were retested without dilution to confirm the absence of diphtherial toxin. Phages were designated *tox*$^-$ only

if no toxin could be detected by skin tests with the undiluted phage lysates.

RESULTS

Phages β^{tox+}, γ^{tox-}, and L^{tox+} are temperate, heteroimmune bacteriophages which produce turbid plaques of similar morphology on lawns of *C. diphtheriae* strain C7. They are defined here as wild-type phages. In designating their genetic determinants, we have, where relevant, employed terminology used for the temperate coliphage λ (1, 24, 26). The genetic markers in these phages which we have analyzed to date are summarized in Table 1.

The symbol *imm* refers to the marker(s) which determines the specificity of lysogenic immunity. The immunity specificities of wild-type corynebacteriophages and their nonlysogenizing mutants are summarized in Table 2. Clear mutants (*c*) fail to lysogenize but respect homologous lysogenic immunity. Thus *c* mutants fail to produce plaques on lawns of bacteria which carry an homologous prophage. Spontaneously occurring clear mutants of phage β were not detected, but induced clear mutants were observed at frequencies of about 10^{-5} after mutagenesis with ultraviolet radiation and at frequencies up to 8×10^{-3} after mutagenesis with NG. In pairwise tests with 14 independently isolated clear mutants of phage β, no complementation in establishing lysogeny was detected, thus suggesting that the mutation in each of these strains had occurred within the same cistron. On lawns of C7, the *c* mutants of phage β produce clear plaques with a uniformly speckled appearance due to the growth within the plaques of many colonies of phage-resistant cells. The phenotype of these *c* mutants of β is altered by the presence of the *h'* marker described below, and double mutants with the genotype *ch'* produce modified plaques which have completely clear centers and which contain phage-resistant colonies located in narrow rings at the edges. The appearance of turbid (t), clear (c), and modified clear (c*) plaques produced on lawns of C7 by wild-type and mutant β phages is shown in Fig. 1. The genetic behavior of the *c* and *imm* markers will be discussed subsequently.

Virulent mutants (*vir*) can overcome homologous lysogenic immunity and thus are able to develop within cells carrying an homologous prophage. Virulent mutants of phage β are extremely rare and appear to arise as multistep mutants (28, 29). The mutant β^{vir} has the *h'* type of host range described below. In preliminary matings with phage β^{vir}, no segregation of virulence from *h'* host range was detected, but it is not known whether the *h'* locus (or the closely linked loci *c* and *imm*, *see below*) has any role in determining virulence in phage β. Spontaneously occurring virulent mutants of phage L are quite common and frequently give rise to lysed areas in old cultures of strains C7(L) growing on solid medium.

Among the phages investigated here, the host range of phage β is most limited and is defined as wild type. Two distinct classes of mutants with extended host ranges were isolated from phage β. The *h* mutants of β are defined by their ability to grow on the bacterial indicator strain $C7/\beta^{vir}$ Type 1 (Table 3); *h'* mutants are defined by growth on $C7/\beta^{c}$. Only mutant or recombinant phages with both *h* and *h'* host ranges can grow on the indicator strain $C7/\beta^{vir}/\beta^{hc}$. Thus, $C7/\beta^{vir}/\beta^{hc}$ can be used as a selective indicator to detect *hh'* recombinants in matings between phages with *h* and *h'* host ranges. The wild-type phages γ and L have host ranges of the *h'* type,

TABLE 1. *Genetic markers of corynebacteriophages*

Markers	Associated functions	Mutant phenotypes
imm	Determines immunity specificity	
c	Required for lysogenization	Clear plaques on C7
vir	Fails to respect lysogenic immunity	Grows on lysogenic hosts Carrying homologous prophage
h	Extended host range	Infects $C7/\beta^{vir}$
h'	Extended host range	Infects $C7/\beta^{c}$
tox+	Required for production of diphtherial toxin	

TABLE 2. *Immunity specificities of wild-type corynebacteriophages and nonlysogenizing mutants*

Ancestral phage	Phage genotype	Genotype of indicator cells			
		C7	C7(β)	C7(γ)	C7(L)
β	Wild type	t[a]	–	t	t
	c	c	–	c	c
	vir	c*	c*	c*	c*
γ	Wild type	t	t	–	t
	c	c*	c*	–	c*
L	Wild type	t	t	t	–
	c	c*	c*	c*	c*

[a] Symbols: (t) turbid plaques; (c) clear plaques formed by β^{c}; (c*) modified clear plaques formed by non-lysogenizing phages with *h'* type host range; (–) no plaques.

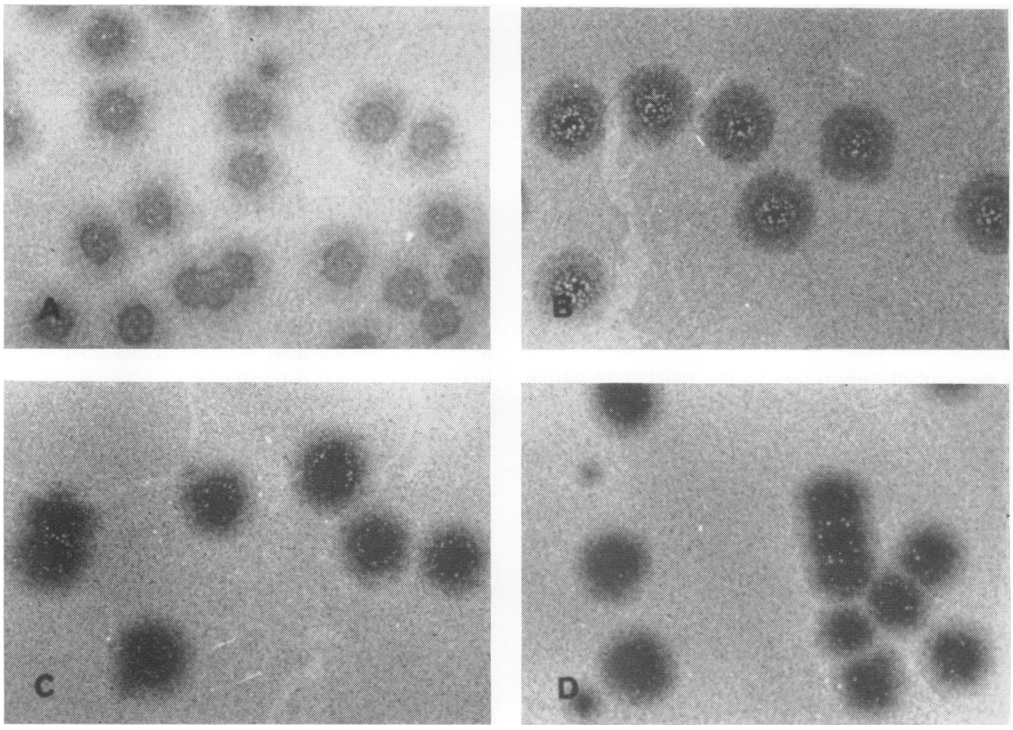

FIG. 1. *Morphology of plaques produced on lawns of C. diphtheriae C7 by phage β and by mutants derived from it. (A) Wild type β produces turbid plaques. (B) The nonlysogenizing mutant c produces clear (c) plaques speckled by colonies of mutant C7/β^{c} cells. The nonlysogenizing phage mutants (C) ch′ and (D) vir can lyse mutant cells like C7/β^{c} and thus produce modified clear (c*) plaques with completely clear centers. Note turbid halos surrounding plaques of all these phages. ×3.5.*

TABLE 3. *Host range phenotypes of corynebacteriophages*

Ancestral phage	Phage genotype	Genotypes of nontoxinogenic indicator cells				
		C7	C7/β^{vir} type 1	C7/β^{vir} type 2	C7/β^{c}	C7/β^{vir}/β^{hc}
β	+++ (Wild type)	t[a]	–	–	–	–
	+c+	c	–	–	–	–
	h++	t	t	–	–	–
	hc+	c	c	–	–	–
	++h′	t	–	–	t	–
	+ch′	c*	–	–	c*	–
	h+h′	t	t	–	t	t
	hch′	c*	c*	–	c*	c*
	vir	c*	–	–	c*	–
γ	Wild type	t	–	–	t	–
L	Wild type	t	–	–	t	–

[a] Symbols: (t) turbid plaques; (c) clear plaques; (c*) modified clear plaques formed by nonlysogenizing phages with *h′* type host range; (–) no plaques.

as does the multistep virulent mutant β^{vir}. Phage β^{vir} is not known to have been selected for extended host range of type *h′*. No phage mutants have been observed which can grow on strain C7/β^{vir} type 2.

Several experiments were performed in an attempt to determine the physiological basis for phage resistance in our mutant strains of *C. diphtheriae*. Under standard assay conditions, wild-type phage β is adsorbed at the same rate, approximately 70 to 80% in 20 min, to the bacterial strains C7 and C7/β^{c}, but only C7 is killed by phage β. The resistance of strain C7/β^{c} to phage β is not due to failure to adsorb β. In this respect, strain C7/β^{c} resembles strain C4/β previously described by Groman and Eaton (17). When log-phase C7/β^{c} is mixedly infected with at least 5 PFU per cell of phage β$^{h'+}$ and of β^{+c}, more than 99.9% of the phage progeny released by cell lysis has the genotype β$^{h'+}$. Thus the multiplication in strain C7/β^{c} of β phages which lack the *h′* marker appears to be inhibited at some early step after phage adsorption, and in mixed infections this inhibition cannot be reversed by complementation with a phage carrying the *h′* marker. In

addition, no phenotypic mixing of the h' host range could be detected either among the progeny from standard crosses of β phages possessing and lacking the h' marker or among the progeny liberated from C4(γ, β) after induction with ultraviolet light. However, the detailed physiological basis for phage resistance in C7/β^c remains unknown. Strain C7/β^{vir} type 1 adsorbs phage β at approximately 75% of the rate of adsorption to C7, but the interactions of C7/β^{vir} type 1 with phage β have not yet been analyzed in detail.

The tox^+ marker is present in wild-type phages β^{tox+} and L^{tox+} but absent in phage γ^{tox-}. Mutations affecting toxinogenicity have not yet been observed in any of these phages.

Preliminary mating experiments with h, c, and h' mutants of phage β, performed by mixed infection of C7 with approximately 5 PFU of each parental phage per cell, revealed recombinants at low frequencies not exceeding 10^{-3} (Holmes and Barksdale, Bacteriol. Proc., 1967, p. 155). Stimulation of genetic recombination (23) was obtained when phage matings were carried out by inducing lysogenic derivatives of C7 by ultraviolet irradiation and superinfecting the induced bacteria with phages differing from the prophage (Holmes and Barksdale, Bacteriol. Proc., 1968, p. 160). The recombination frequencies obtained by this latter method were found to depend markedly on the time at which the superinfecting parental phages were added to the irradiated lysogenic cells. The data in Table 4 illustrate that the recombination frequency between the h and h' markers in matings with mutant β phages decreased progessively from 2.3 to 0.14% as the time of superinfection was delayed from 12 to 150 min postirradiation. As superinfection was delayed from 12 to 60 min postirradiation, the ratio of the yields of the parental phage types among the progeny remained virtually unchanged, although the recombination frequency decreased approximately fivefold. In further crosses between mutants of phages, β superinfection was routinely carried out at 10 min postirradiation.

The relative order of the loci h, c, and h' on the genetic map of phage β was determined by three-factor crosses between mutant phages carrying these markers in several different arrangements. Table 5 describes the origins of the parental β phages used in these matings, and the experimental data are summarized in Table 6. Each type of cross was performed at least twice, and the data presented illustrate the maximum variability found. The independently isolated markers h_1 and h_9 were both analyzed because they produce slightly different phenotypes. In the preparation of isogenic phage stocks derived from single phage plaques, β phages carrying h_1 or h_9 require longer periods to produce mass lysis of C7 cultures and yield lower-titered stocks than does wild-type phage β. This retarded development produced by h_1 is more marked than that produced by h_9. All of the data in Table 6 are consistent with the assignment h--c--h' for the relative order of these three markers on the genetic map of phage β; the genetic behavior of the markers h_1 and h_9 is identical, suggesting that they are both alleles at the h locus. The frequencies of recombination between the loci h and c varied from 1.6 to 4.2%, and the recombination frequencies between c and h' varied from 0.01 to 0.05%. In the first four crosses in Table 6, double recombinants were 4.1 to 6.2 times more frequent than expected from the frequencies of single recombinants. The relative frequencies of double and single recombinants indicate that the loci h, c, and h' are all linked, but c is much more closely linked to h' than to h. Small but consistent differences in recombination frequencies were detected when the markers were present in different arrangements in the parental phages. In each of the crosses in Table 6, the progeny contained more phages with the genotype of the superinfecting parent than with the genotype of the prophage parent. The superinfecting phage appeared to have a slight growth advantage relative to the prophage even when the h allele was present in the superinfecting phage.

In matings between the heteroimmune phages β and γ the relationship between recombination frequency and time of superinfection was found to be very different from that previously observed in crosses between mutants of phage β (Table 4). The data in Table 7 illustrate that the frequency of recombinants receiving the h_1 marker of the β parent and the h' marker of the γ parent did not decrease but remained nearly constant as super-

TABLE 4. *Effect of time of superinfection on recombination between mutants of phage β; cross: $h_1+ \times +h'$*

Time of superinfection with $+h'$	Avg yield of progeny phage per infected cell			Recombination frequency between h and h'
	h_1+	$+h'$	h_1h'	
min				%
Uninfected control	3.0			
12	14.6	18.3	0.38	2.3
30	11.7	17.8	0.19	1.3
60	8.9	10.5	0.048	0.49
90	17.0	7.3	0.044	0.37
150	4.6	1.3	0.0040	0.14[a]

[a] Based on a count of five recombinant plaques of genotype h_1h. In each of the other crosses more than 50 h_1h recombinants were observed.

TABLE 5. *Origins of parental β phages for three-factor crosses*

Phage genotype	Origin	Ancestral phages
$+c+$	Mutagenesis with ultraviolet light	Wild type β = $+++$
$+ch'$	Spontaneous mutation	$+c+$
$++h'$	Recombination	$+ch' \times +++$ (expt 3)[a]
h_1++	Mutagenesis with NG, method I	$+++$
h_1+h'	Recombination	$h_1++ \times ++h'$ (expt 7)
h_9c+	Mutagenesis with NG, method II	$+c+$
h_9++	Recombination	$h_9c+ \times +++$ (expt 17)
h_9+h'	Recombination	$h_9c+ \times ++h'$ (expt 17)

[a] Cross performed by method of mixed infection of C7.

TABLE 6. *Three-factor crosses between mutants of phage β*

Expt	Cross (induced prophage × superinfecting phage)	Analysis of phage progeny					
		Parental type phages (avg yield per infected cell)		Recombination frequencies for selected genotype[a]			
		Prophage type	Superinfecting type	$hc+$	$+ch'$	hch'	$h+h'$
				%	%	%	%
9	$h_1+h' \times +c+$	4.6	30.0	1.6	0.037	0.0041[b]	—[c]
26	$h_1+h' \times +c+$	3.1	31.8	2.0	0.035	0.0043	—
19	$h_9+h' \times +c+$	2.7	16.7	1.7	0.052	0.0039	—
26	$h_9+h' \times +c+$	2.8	27.4	1.8	0.037	0.0046	—
9	$h_1++ \times +ch'$	5.7	27.8	—	—	2.5	0.011[b]
26	$h_1++ \times +ch'$	7.9	46.6	0.009	—	3.0	0.0092
19	$h_9++ \times +ch'$	4.3	17.9	0.01	—	2.4	0.014
25	$h_9++ \times +ch'$	5.8	34.7	0.01	—	3.3	0.014
17	$++h' \times h_9c+$	5.3	8.7	—	—	0.028	3.7
20	$++h' \times h_9c+$	7.0	11.1	—	—	0.025	4.2

[a] Recombinants $h+h'$ and hch' were selected as t and c^* plaques, respectively, on C7/β^{vir}/β^{hc}; $hc+$ recombinants were selected as c plaques on C7/β^{vir} type 1; $+ch'$ recombinants were selected as c^* plaques on C7/β^{c}, and the titer was corrected by subtracting the titer of hch' recombinants. Recombination frequencies were calculated for each pair of reciprocal recombinants.

[b] Less than 30 recombinant-type plaques scored.

[c] Either not measured or not a recombinant genotype.

TABLE 7. *Effects of time of superinfection and multiplicity of infection upon recombination between phages β and γ; cross: $h_1imm^{\beta}c^{+}+ \times +imm^{\gamma}ch'$*

Time of superinfection with $+imm^{\gamma}ch'$	Multiplicity of infection for superinfecting $+imm^{\gamma}ch'$	Avg yield of progeny phage per infected cell[a]			Recombination frequency between h and h'
		$\bullet\bullet imm^{\beta}\bullet$	$\bullet\bullet imm^{\gamma}\bullet$	$h_1\bullet\bullet h'$	
min					%
10	1.6	12.9	35.3	0.024 (34)	0.10
10	3.4	5.6	45.5	0.033 (60)	0.13
60	1.7	13.3	23.2	0.014 (20)	0.077
60	3.5	9.0	16.3	0.016 (28)	02.3
120	1.7	11.7	3.5	0.0088 (13)	0.12
120	3.4	10.9	12.0	0.013 (23)	0.11

[a] A dot (•) in the phage genotype designates that the marker at the locus specified was unselected. The $\bullet\bullet imm^{\beta}\bullet$ and $\bullet\bullet imm^{\gamma}\bullet$ progeny were selected on C7(γ) and C7(β), respectively. The $h_1\bullet\bullet h'$ recombinants were selected on C7/β^{vir}/β^{hc}. Figures in parentheses indicate the numbers of $h_1\bullet\bullet h'$ recombinants observed.

infection was delayed from 10 to 120 min post-irradiation. When the multiplicity of infection was changed by a factor of two at each time of superinfection, small differences were observed in the recombination frequencies. Recombination between the *h* and *h'* markers of these heteroimmune phages was approximately 20 times less frequent than the maximal value observed previously in matings between mutants of phage β (Table 4). The *hh'* recombinants from the matings in Table 7 also were analyzed to determine the distribution of the unselected markers *imm* and *c*. Among the 178 *hh'* recombinants observed, all produced clear plaques. From two independent crosses of this type, 91 *hch'* recombinants were analyzed for immunity specificity, and all carried the imm^{γ} marker. Thus, in these crosses between the heteroimmune phages β and γ, no segregation was observed between the *c*, imm^{γ}, and *h'* markers of the γ^{tox-} parent. The loci *c*, *imm*, and *h'* in phages β and γ thus appear to be closely linked.

Three types of matings (Fig. 2) were performed to analyze the genetic factor(s) controlling toxinogenicity in phages β^{tox+} and γ^{tox-}. The five genetic markers *h*, *tox*, *imm*, *c*, and *h'* were employed in these crosses. The arrangement of these five loci on the genetic map of phage is presented at the top of Fig. 2, in which the relative position of *c* and *imm* is not established. In each cross, the segments of the parental phage genomes derived from phage β are drawn as fine lines and those derived from phage γ are drawn as heavy lines. Only the segments of the genomes represented by fine lines in both parental phages are known to represent genetically homologous regions. In each cross, recombinants were selected for the host range or immunity markers (or both), and groups of the selected recombinants were then analyzed for the presence or absence of the tox^{+} marker (Table 8). The first three crosses in Table 8 are of type 1, the next two crosses are of type 2, and the final cross is of type 3.

The frequencies of recombination between the loci *h* and *imm* (or *c*) in crosses of types 1, 2, and 3 were about 0.1, 0.9, and 2.5%, respectively. These data, together with the segregation data presented below, suggest that the genomes of phages β and γ contain regions of limited homology within which the frequency of recombination is reduced in both segments A and B of the genetic map (Fig. 2). In the cross of type 3, the recombination frequency between *h* and *c* was 2.5% and was approximately equal to that previously obtained between the same loci in crosses between mutant β phages (1.6 to 2%; Table 6). Thus, either the regions of the genetic map carrying tox^{+} and tox^{-} are homologous, or the genetic length of

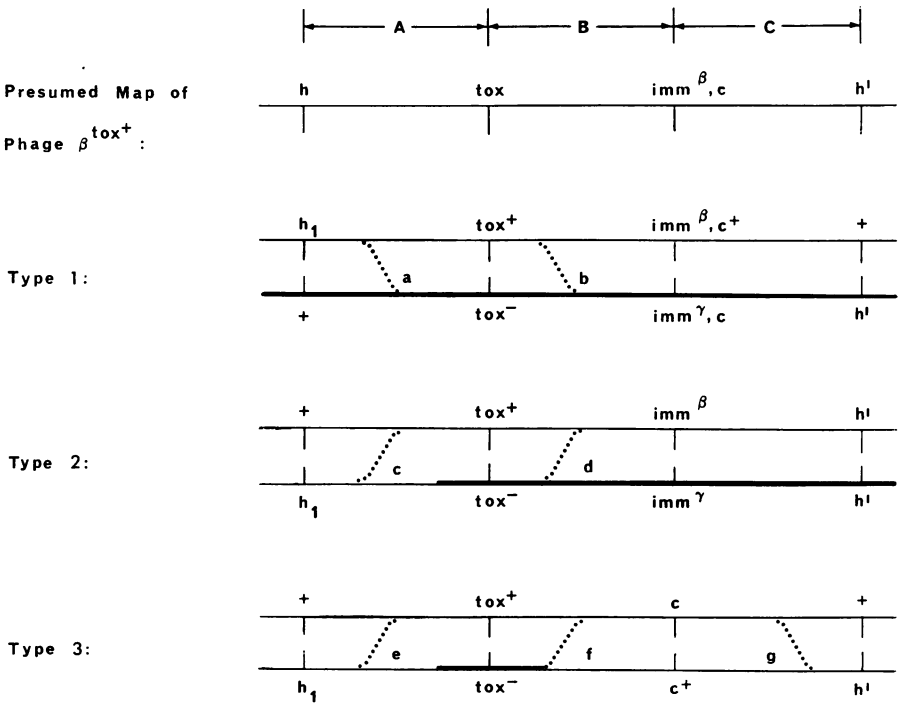

FIG. 2. *Genetic map of phage* β^{tox+}.

the *tox* region is short relative to the genetic length of the *h--imm* interval.

The analyses for *segregation* of the tox^+ marker revealed several facts (Table 8). Among 94 selected recombinants from type 1 crosses, 74 were tox^+ and had arisen from crossovers of the kind labeled *b* in Fig. 2, and 20 were tox^- and had originated from crossovers labeled *a*. Thus, in type 1 matings, the *tox* locus had segregated preferentially with the *h* locus. In contrast, among 115 selected recombinants from type 2 crosses, 112 were tox^+ and had arisen from crossovers labeled *c*, and only 3 were tox^- and had originated from crossovers labeled *d*. Thus, in crosses of type 2, tox^+ had segregated preferentially with *imm* rather than with *h*. This observation is consistent with the assignment of the *tox* locus to a position between *h* and *imm* on the genetic map of phage β. In type 2 crosses, the introduction of a region of complete homology between *h* and *tox* in the genomes of the parental phages may well explain both the increased frequency of recombination in *h--imm* interval relative to type 1 crosses and the preference within that interval for crossovers labeled *c* rather than *d*. The assignment of the *tox* locus to a position between *h* and *imm* on the genetic map of phage β was confirmed by the third type of cross, in which the segregation of the tox^+ marker was analyzed in three separate classes of nonreciprocal recombinants. No segregation of tox^+ occurred among 43 single recombinants formed by crossovers labeled *g* occurring in the *imm--h'* interval of the genetic map. Segregation of tox^+ did occur when recombination occurred between the loci *h* and *imm*. Of 46 single recombinants of genotype $h_1 \cdot c+$, where the dot (·) indicates the unselected tox marker, 34 were tox^+ and 12 were tox^-; of 5 $h_1 \cdot ch'$ double recombinants scored, 1 was tox^+. However, most $h_1 \cdot ch'$ double recombinants would be expected to be tox^+, like most of the $h_1 \cdot c^+$ single recombinants. This discrepancy could be due to sampling error, since only five double recombinants were scored. It could also be explained by negative interference if double crossovers occurred with increased probability per unit map length over short genetic distances, thus increasing the frequency with which type *f* and *g* crossovers would occur together in double recombinants. All of these data are consistent with the hypothesis that toxinogenicity in phage β is determined by a single genetic marker, tox^+, which is absent or nonfunctional in phage γ, and which is located on the genetic map of phage β between the linked loci *h* and *imm*.

The occurrence of genetic recombination between the heteroimmune phages β and L is demonstrated in Table 9. In each cross, recom-

TABLE 8. *Multifactor crosses between tox^+ and tox^- phages derived from β and γ*

Expt no.	Cross (induced prophage × superinfecting phage)	Analysis of phage progeny					
		Parental type phage (avg yield per infected cell)		Recombinant types			
		Prophage type	Superinfecting type	Selected genotype	Recombination frequency	No. of tox^+	No. of tox^-
					%		
8	$h_1 tox^+ imm^\beta c^+ + \times + tox^- imm^\gamma ch'$	5.6	45.4	$h_1 \cdot imm^\gamma ch'$[a]	0.13	33	12
10[b]	$h_1 tox^+ imm^\beta c^+ + \times + tox^- imm^\gamma ch'$	1.6	15.4	$h_1 \cdot imm^\gamma ch'$[a]	0.12	39	7
10[b]	$h_1 tox^+ imm^\beta c^+ + \times + tox^- imm^\gamma c^+ h'$	—[c]	—[c]	$h_1 \cdot imm^\gamma c^+ h'$[a]	0.12	2	1[e]
13	$h_1 tox^- imm^\gamma h' \times + tox^+ imm^\beta h'$	1.9	27.2	$h_1 \cdot imm^\beta h'$[d]	0.77	10	1
14	$h_1 tox^- imm^\gamma h' \times + tox^+ imm^\beta h'$	1.2	15.9	$h_1 \cdot imm^\beta h'$[d]	1.07	102	2
28	$h_1 tox^- c^+ h' \times + tox^+ c+$	4.8	25.5	$h_1 \cdot c+$[e]	2.48	34	12
				$+ \cdot ch'$[e]	0.044	43	0
				$h_1 \cdot ch'$[e]	0.0033	1	4

[a] Selected on $C7/\beta^{vir}/\beta^{hc}$. No segregation was observed between the imm^γ, *c*, and *h'* markers of the γ parental phage.

[b] Superinfecting phage added at 60 min postirradiation.

[c] Total of prophage and superinfecting types was 15.4.

[d] Selected on $C7(h_1 tox^- imm^\gamma c^+ h')/\beta^{vir}/\beta^{hc}$. Cloned on $C7/\beta^{vir}/\beta^{hc}$ and $h_1 \cdot imm^\beta h'$ genotype confirmed before testing for presence of tox^+ marker. Both parental phages are c^+.

[e] See Table 6 for selective indicators. Both parental phages are imm^β.

[f] This is the $h_1 tox^- imm^\gamma h'$ parental phage in experiments 13 and 14.

[g] This is the $h_1 tox^- c^+ h'$ parental phage in experiment 28.

TABLE 9. *Genetic recombination between phages β and L*

Expt	Cross (induced prophage × superinfecting phage)	Analysis of phage progeny			
		Parental type phage (avg yield per infected cell)		Recombinants	
		Prophage type[a]	Infecting type[b]	Selected genotype[c]	Recombination frequency
23	$h_9imm^{\beta}+ \times +imm^{L}h'$	0.43	53.6	$h_9imm^{L}h'$	0.10
24	$h_9imm^{\beta}+ \times +imm^{L}h'$	0.28	49.9	$h_9imm^{L}h'$	0.074
24	$+imm^{L}c^{+}h' \times h_9imm^{\beta}c+$	1.3	4.0	$h_9imm^{L}{\cdot}h'$	0.41
25	$+imm^{L}c^{+}h' \times h_9imm^{\beta}c+$	3.3	2.9	$h_9imm^{L}{\cdot}h'$	0.77

[a] Assayed on C7(L) in the first two crosses and on C7(β) in the last two crosses.
[b] Assayed on C7(β) in the first two crosses and C7(L) in the last two crosses.
[c] Assayed on $C7(h_1tox^{+}imm^{\beta}c^{+}h')/\beta^{vir}/\beta^{hc}$.

binants were selected which had received the *h* marker from the β parent and the imm^{L} and *h'* markers from the L parent. In the final two crosses, the clear (*c*) marker of the β parent was present as an unselected character. When the β parent originated as prophage, the frequencies of recombination did not exceed 0.10%, and phages of the prophage genotype accounted for less than 1% of the phage progeny. In contrast, when the L parent originated as prophage, the recombination frequencies were several times greater and the two parental genotypes occurred at comparable frequencies among the phage progeny. In both types of crosses, however, the recombination frequencies were significantly lower than in crosses between β phages carrying the markers *h* and *h'* (*see* Table 6). Thus, incomplete genetic homology exists between the *h--imm* segments of the genomes of phages β and L. Among 1,239 $h_9immL{\cdot}h'$ recombinants from the final two crosses in Table 9, all produced turbid plaques like the L parent and none produced clear plaques like the β parent. Thus, the *c* marker of the β parent failed to segregate from imm^{β} in these heteroimmune crosses and, like the *c* marker of phage γ^{tox-} discussed previously, appears to be clearly linked to the *imm* locus.

DISCUSSION

To obtain increased frequencies of genetic recombination between corynebacteriophages, the matings described here were performed by superinfection of lysogenic corynebacteria in which phage development had been induced by previous ultraviolet irradiation. Our procedure was based on the discovery of Jacob and Wollman (23) that ultraviolet irradiation of λ coliphages stimulated genetic recombination between them. Jacob and Wollman demonstrated that the observed frequency of recombination was directly proportional at low doses to the amount of ultraviolet radiation delivered to the parental phage genomes. Stimulation of recombination was obtained by ultraviolet irradiation either of extracellular phages or of lysogenic host cells prior to superinfection, but irradiation of nonlysogenic host bacteria prior to infection by nonirradiated phages did not stimulate phage recombination. From the proportions of recombinant phages in single-burst experiments, they concluded that recombination between irradiated phage genomes was an early event in phage development.

Our observations on matings between corynebacteriophages revealed an additional feature concerning the stimulation by ultraviolet radiation of recombination between phages. In matings between mutants of phage β, the observed frequency of recombination decreased progressively as the superinfecting phage was added to the ultraviolet-induced cells after increasingly long intervals (Table 4). However, this decrease in recombination frequency was out of proportion to any variation seen in the ratio of the parental type phages in the progeny from these same crosses. This seems to be direct evidence that the stimulation of recombination between phages in our system is an event which occurs within a limited range of time after ultraviolet irradiation of the lysogenic host cells. Our interpretation of these data is that the product of ultraviolet radiation which effects this stimulation of recombination may be unstable or may be subject to an as yet undetermined repair mechanism.

The results of matings between the heteroimmune phages β and γ differed from those just described in two significant respects (Table 7): the recombination frequencies were approximately equal at all times of superinfection investigated, and they were lower than the smallest value observed in crosses between mutants of

phage β. In matings between phages β and γ, no preferential stimulation of recombination was observed when superinfection occurred at short intervals after ultraviolet irradiation. This apparent absence of the stimulatory effect of ultraviolet radiation on recombination between phages β and γ probably reflects the incomplete genetic homology between these phages. When recombinant phages derived from crosses between phages β and γ were mated with mutants of phage β, the recombination frequencies increased dramatically as the extent of known homology between the parental phages increased (Table 8, Fig. 2). It seems possible that the incomplete genetic homology between phages β and γ may be associated with a sufficient delay in recombination during matings between them so that the ultraviolet-induced stimulation of recombination, which appears to be a transient phenomenon, does not occur.

Several of the genetic markers described here for corynebacteriophages resemble markers previously characterized in other bacteriophage systems. The *c* mutants of phages β and γ appear to be similar to the c_I group of mutants of coliphage λ (24, 25). In phages from each of these groups, the *c* mutation is closely linked to the *imm* locus (loci) and is associated with loss of the ability to lysogenize sensitive cells, formation of clear plaques on lawns of sensitive cells, and inability to grow on bacteria lysogenic for co-immune phages. These properties of the *c* mutants of corynebacteriophages can be interpreted by the model of Jacob and Monod (21) which postulates that the *c* marker is the structural gene for an immunity substance or repressor which inhibits vegetative phage development during the establishment and maintenance of the lysogenic state. It is postulated that *c* mutants are unable to produce active repressor. Direct evidence for the existence of immunity repressor has been obtained recently in the λ coliphage system (33, 34), but data of this type are not yet available for corynebacteriophages.

Jacob and Monod (21) postulated that virulence in temperate bacteriophages arises by mutations in operator loci which result in the loss of the ability of the phage genome to respond to the immunity repressor produced by coimmune phages. Virulent mutants of phage β are exceedingly rare and may arise as multistep mutants (28, 29), like the virulent mutants of coliphage λ (10, 22). In contrast, virulent mutants of phage L are common and probably arise from wild-type L as single-step mutants. It appears possible that the number of mutational steps required to establish virulence in a phage may reflect the number of operator genes controlled by the immunity substance to which that phage is sensitive.

Our phage-resistant mutants of *C. diphtheriae* C7 are capable of adsorbing the phages to which they are resistant, in contrast to the well-characterized mutants of *Escherichia coli* resistant to phage λ (1). Our current data reveal that the replication of phage β in *C. diphtheriae* C7/β^c is inhibited at an early postadsorption step prior to the destruction of the viability of the host bacterium. However, these data do not establish whether the phage germinal substance is unable to penetrate into the bacterial cytoplasm or is prevented from functioning normally during intracellular phage development. The mechanism of resistance to phage β in strain C7/β^c seems distinct from lysogenic immunity and from the usual forms of restriction associated with host-controlled modifications of bacteriophages (2). Strain C7/β^c is not known to be lysogenic and can be lysogenized normally by the extended host range mutants designated $\beta^{h'}$, and the $\beta^{h'}$ mutants maintain their extended host range as an heritable property during propagation on the phage-sensitive, ancestral host bacterium C7. A detailed elucidation of the interactions of phage β with the mutant strain *C. diphtheriae* C7/β^c may provide an additional model system for understanding the mechanisms by which bacteria acquire resistance to their viral parasites.

The fundamental observations supporting the hypothesis that conversion to toxinogenicity in *C. diphtheriae* requires the presence within an appropriate host bacterial strain of a tox^+ bacteriophage were summarized above. In the experiments of Groman and collaborators (17, 18), recombination was detected between genetic markers determining toxinogenicity and host range in corynebacteriophages β and γ. However, since their data were obtained from a single type of two-factor phage cross, the existence of linkage between the *tox* marker and other phage genetic markers could not be established.

In our current investigations, observations were made concerning the genetic control of toxinogenicity by corynebacteriophages. In phage β, the tox^+ determinant behaves as an heritable element which is present in all the progeny produced by lytic growth of phage β in the nontoxinogenic host C7. In matings between tox^+ and tox^- phages, the tox^+ determinant behaves like a genetic element of the corynebacteriophages. In phage β, all of the known genetic markers were found to comprise a single linkage group. The *tox* locus was found to be linked to the *h*, *c*, and *imm* loci and to be located between *h* and *c* on the genetic map of phage β. Our data are consistent

with the hypothesis that *tox*$^+$ is a single gene. Our observations thus support the hypothesis that the *tox*$^+$ marker is an integral part of the genomes of toxinogenic corynebacteriophages and that phage-mediated conversion to toxinogenicity results from the intracellular presence of a phage whose genome includes the *tox*$^+$ marker.

This and other evidence cited below is at variance with the proposal of Rajadhyaksha and Rao (35) that the *tox*$^+$ marker in toxinogenic strains of *C. diphtheriae* is carried by an episome which is distinct from corynebacteriophages but which can be introduced into nontoxinogenic cells by phage-mediated transduction. Their proposal was based primarily on the following three observations. (i) Treatment of toxinogenic, lysogenic strains of *C. diphtheriae* with small doses of the dye acriflavine could eliminate their prophage without eliminating the *tox*$^+$ factor; (ii) treatment of such cells with larger doses of acriflavine could eliminate both their prophage and their *tox*$^+$ factor; (iii) treatment of extracellular *tox*$^+$ phages with acriflavine could render the surviving phages nontoxinogenic. However, Miller and Pappenheimer (30) found that a sample of the reportedly toxinogenic Weissensee G substrain of *C. diphtheriae* PW8, obtained from S. S. Rao, contained both phage-resistant, toxinogenic cells and nonlysogenic, nontoxinogenic, phage-sensitive cells which closely resemble strain C7. They concluded that the results of the acriflavine curing experiments could be explained by a mixture of cell types in the cultures of the PW8 substrain which had been used.

A number of important questions concerning the role of the *tox*$^+$ marker in the production of diphtherial toxin remain unanswered. It is not yet known whether *tox*$^+$ is a structural gene for diphtherial toxin or whether diphtherial toxin is a secondary product whose synthesis requires some phage-directed function. It is not clear whether *tox*$^+$ and *tox*$^-$ are alleles at the *tox* locus or whether *tox*$^-$ represents a deletion of the genetic information of the *tox*$^+$ marker. In addition, although diphtherial toxin is a well-characterized protein (36) which appears intracellularly at early times during the latent period of phage development in cells lytically infected with *tox*$^+$ phages (28), there is at present no information concerning the physiological role which diphtherial toxin might play in the growth cycle of corynebacteriophages.

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