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Comparative Studies with *tox*$^+$ and *tox*$^-$ Corynebacteriophages[1]

RANDALL K. HOLMES[2] AND LANE BARKSDALE
Department of Microbiology, New York University School of Medicine, New York, New York 10016

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The characteristics of nine inducible temperate corynebacteriophages designated α^{tox+}, β^{tox+}, P^{tox+}, γ^{tox-}, π^{tox+}, K^{tox-}, ρ^{tox-}, L^{tox+}, and δ^{tox+} have been compared. Virion morphology and ability to recombine genetically with the well-studied phage β^{tox+} have been correlated with other properties of the phages, and the distribution of the genetic marker *tox*+ among related and relatively unrelated corynebacteriophages has been analyzed. The immunity specificity, host range, and plaque morphology of each phage were determined. The phages can be separated into five groups with different immunity specificities. Each type of host range previously recognized in mutants of phage β^{tox+} was present in one or more of the phages included in the present study, and the phages were found to produce plaques of several different morphological types. Representative phages with each of the five types of immunity specificity were further characterized with respect to virion morphology, ability to recombine with phage β^{tox+}, latent period, average burst size, and neutralization by homologous and heterologous antiphage sera. All of these phages have polyhedral heads and long slender tails, but two distinct morphological types were distinguished by the sizes and proportions of the components of the virions. Only phages of the same morphological type as β^{tox+} were capable of genetic recombination with β^{tox+}, but morphological similarity between phages was not sufficient to insure interfertility. The phages which recombined with β^{tox+} resembled one another in plaque morphology, latent period, and average burst size, whereas phages which failed to recombine with β^{tox+} differed in these characteristics. The phages capable of genetic recombination with β^{tox+} were found to differ from each other in immunity specificity, host range, neutralization by antiphage sera, and toxinogenicity. Thus, these latter characteristics are of limited value in establishing the extent of relatedness between corynebacteriophages. The genetic marker *tox*+ was not consistently correlated with any other property of the corynebacteriophages analyzed in this study. The most striking finding regarding the distribution of the *tox*+ marker is its presence both in β^{tox+} and δ^{tox+}, phages which fail to recombine genetically and which differ in virion morphology. The presence of the *tox*+ marker in genetically unrelated corynebacteriophages poses many questions concerning the origin(s) of *tox*+ and the evolution of the phage-host interactions which determine the ability of corynebacteria to synthesize diphtherial toxin.

The discovery by Freeman (14, 15) that certain strains of *Corynebacterium diphtheriae* can acquire the ability to produce diphtherial toxin by phage-mediated conversion has stimulated considerable study of corynebacteriophages. At the present time, the best studied corynebacteriophages are β^{tox+}, a lineal descendant of Freeman's toxinogenic phage B (7), and γ^{tox-}, a nontoxinogenic phage (19) which can recombine genetically with phage β^{tox+} (19, 23, 25). Previous studies have established that β^{tox+} is a temperate (7), inducible (7), deoxyribonucleic acid (DNA)-containing (31) phage which is inactivated rapidly by heat and slowly by prolonged storage in the cold (7, 20). The virion of phage β^{tox+} has a polyhedral head and a long, slender tail (14, 30, 31). Lytic infection of susceptible cells in rich media yields a burst of 30 to 60 plaque-forming units (PFU)/cell after a latent period of approximately

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[2] Present address: Department of Medicine, Beth Israel Hospital, Boston, Mass. 02215.

1 hr (7, 20). Divalent calcium ions are required for the adsorption of phage β^{tox+} to sensitive bacteria (7), but adsorption is inhibited by Tweens (8, 18). A phage inhibitor, possibly related to the phage receptor material, is released from susceptible cells of *C. diphtheriae* in the presence of oleic acid (17, 21). A system for the genetic analysis of β^{tox+} and related corynebacteriophages has been described recently, and the position of the *tox*+ determinant on the genetic map of phage β^{tox+} has been determined (25).

Lysogenicity has been shown to occur frequently among strains of *C. diphtheriae*, and many corynebacteriophages have been isolated (10, 13, 22, 27, 41–43). A practical phage-typing scheme for *C. diphtheriae* has been developed (37, 38). In most cases, however, the characterization of the typing phages has been restricted to determination of their host ranges. In this paper, several *tox*+ and *tox*− bacteriophages which infect *mitis* strains of *C. diphtheriae* are characterized, and their ability to undergo genetic recombination with phage β^{tox+} is assessed. Our aim has been to determine the relatedness of these phages and incidentally to provide data for their classification. The extent to which the genetic marker *tox*+ is regularly associated with other specific properties of corynebacteriophages has been emphasized in our studies.

MATERIALS AND METHODS

Corynebacteria. *C. diphtheriae* strain $C7_s$ $(-)^{tox-}$, hereafter designated C7, was described previously (25). *C. diphtheriae* strains A028 and P17886, from which we have isolated some of the bacteriophages described below, are toxinogenic *mitis* strains kindly supplied by Groman and Memmer (22). All other bacterial strains used are mutants, or lysogenic derivatives of C7, or both. All strains derived from C7 are nontoxinogenic unless they carry *tox*+ prophages. We described strains C7(β), C7(γ), C7(L), C7/β^c, C7/β^{vir} types 1 and 2, and C7/β^{vir}/β^{hc} in an earlier communication (25). Strains C7(α) and C7(P) were characterized by Barksdale (4), and C7(K) was characterized by Barksdale, Garmise, and Rivera (6). All other strains, including C7(π), C7(ρ), C7(δ), and the strains used for phage-mating experiments, were prepared by methods previously reported (25).

Corynebacteriophages. Phages β^{tox+}, γ^{tox-}, L^{tox+}, the mutant phages designated β^{vir}, β^{h}, $\beta^{h'}$, $\beta^{hh'}$, β^{hc}, $\beta^{ch'}$, and γ^{c}, and the alleles of gene *h* of phage β^{tox+}, designated h_1 and h_9, were described in an earlier publication (25). Phages α^{tox+}, P^{tox+}, and K^{tox-} were obtained from the respective lysogenic derivatives of C7 mentioned above. Phage ρ^{tox-} was isolated from supernatants of *C. diphtheriae* strain A028 plated on indicator lawns of strain C7. Phage δ^{tox+} was subsequently isolated from supernatants of *C. diphtheriae* strain A028 by plating on indicator lawns of C7(ρ), and δ^{tox+} was then cloned by successive single-plaque isolations on C7 lawns. Phage π^{tox+} was isolated from supernatants of *C. diphtheriae* P17886 by using strain C7 as indicator. For convenience, the superscripts *tox*+ and *tox*− will usually be omitted.

Media and general methods. The culture media and the conditions for cultivation of corynebacteria, the procedures for phage assay, preparation of phage stocks, and phage matings, as well as the tests for presence of the *tox*+ gene in corynebacteriophages and for the production of diphtherial toxin by corynebacteria, were those reported previously (25).

One-step growth experiments. The protocol described by Adams (1) was modified for use with corynebacteriophages because of their slow adsorption to C7 and an apparent retardation of vegetative phage growth in infected cultures of C7 after large dilutions in PGT medium. Exponentially growing cultures of C7 at an optical density (OD) of 0.30 were mixed with 10^6 plaque-forming units (PFU) of phage per ml [multiplicity of infection (MOI) less than 0.01] and were incubated for 5 min. Adsorption was then terminated by collecting the bacteria on membrane filters (0.45 μm average pore diameter; Millipore Corp., Bedford, Mass.) and removing the unadsorbed phage by two washings with warmed PGT medium. The filters were placed in the original volumes of warmed PGT medium, and the almost simultaneously singly infected bacteria were resuspended by agitation with a Vortex Junior mixer. During further incubation, samples of the infected cultures were withdrawn at appropriate intervals and were immediately plated in the same manner as adsorption mixtures for phage assays. Minimum latent periods, rise periods, and average burst sizes were calculated in the usual manner.

Electron microscopy. High-titered stocks of corynebacteriophages (25) were placed on carbon-coated Formvar films supported by copper mesh grids and were negatively stained (9), either with 2% aqueous potassium phosphotungstate at *p*H 6.2 or with saturated aqueous uranyl acetate diluted immediately before use with an equal volume of distilled water. The grids were examined and photographed in an Hitachi HS-7S electron microscope.

Antiphage sera. High-titered stocks of corynebacteriophages (25) were emulsified with equal volumes of incomplete Freund adjuvant (11), and 4.0-ml samples of the freshly prepared antigens were injected in divided doses, both intramuscularly and into the foot pads of rabbits. Each rabbit received five sets of injections at approximately 3-week intervals. Antisera were prepared against phages β, γ, and K and were stored frozen at −15 C.

Phage neutralization tests. To 2.40-ml samples of phage at 2×10^7 PFU/ml in PGT medium, 0.10 ml samples of appropriately diluted antisera were added, and the mixtures were incubated at 36 C with rotary shaking at 240 rev/min. Samples were withdrawn at 0, 5, 10, 15, 20, and 30 min and were diluted 1:100 into cultures of C7 at an OD of 0.30 to stop further inactivation of phage by antiserum. These samples were treated as adsorption mixtures for phage assays. Dilutions of antisera were chosen to give inactivation of 90% of the homologous phage in approximately 10 to 15 min. Rate constants for neutralization, *K*

values, were calculated from the initial rates of inactivation as described by Adams (1).

RESULTS

Bacteriophages α, β, P, γ, π, K, ρ, δ, and L were originally isolated from lysogenic strains of *C. diphtheriae* and *C. ulcerans*. Each of these corynebacteriophages is temperate, and each can infect and lysogenize *C. diphtheriae* strain C7. Ultraviolet irradiation of lysogenic C7 derivatives induces each of these prophages to develop as vegetative phage. These nine corynebacteriophages have been compared with respect to plaque morphology, specificity of lysogenic immunity, host range, and toxinogenicity. Phages β, γ, K, ρ, δ, and L were selected as representative phages and further characterized with respect to latent period and burst size, ability to undergo genetic recombination with the well-studied corynebacteriophage β, morphology of the virion, and neutralization by homologous and heterologous antiphage sera. The results of these studies are summarized below.

Plaque morphology. The plaques produced on lawns of *C. diphtheriae* C7 by phages P, γ, π, and L are similar to the plaques of wild-type phage β illustrated previously (25). These plaques are turbid, are uniform in size, attain a maximum diameter of approximately 2 mm, and acquire densely turbid peripheral halos up to 4 mm in diameter during prolonged incubation. Phages α, K, and δ give rise to tiny, punctate, turbid plaques of uniform size about 0.4 mm in diameter. In contrast, phage ρ produces turbid plaques which are small, indistinct, variable in size, and less than 1 mm in diameter.

Specificity of lysogenic immunity. Lysogenic derivatives of *C. diphtheriae* C7 were prepared, each of which carried one of the nine corynebacteriophages as prophage. The immunity specificity of each phage was determined by testing its ability to grow on each of the nine lysogenic bacterial strains. The results are presented in Table 1. As previously described (4), α, β, and P form one group of coimmune phages. Phage γ is coimmune with phage π. The immunity specificities of phages K and ρ are unique. Phages δ and L appear to be coimmune. Phage δ fails to form plaques on the lysogenic C7 derivatives carrying as prophage the heteroimmune phage α, β, P, γ, or π. The mechanism(s) by which these prophages restrict the vegetative growth of superinfecting δ phage has not yet been established.

Host range. Nonlysogenic mutants of *C. diphtheriae* C7 which are resistant to wild-type phage β and which serve as selective indicators for several different host-range mutants of phage β have been described previously (25). In Table 2, the host ranges of the phages presently under consideration are compared with those of wild-type phage β and the mutant strains $\beta^{h'}$, β^{h}, and $\beta^{hh'}$. Only phage P shares the restricted host range of wild-type phage β. Phages γ, π, K, and L have the h' type of host range. Phage α has the host range designated h. Phages ρ and δ have both the h and h' types of host range. No wild-type or mutant phages have yet been found which are capable of growing in the bacterial strain C7/β^{vir} type 2.

TABLE 1. *Immunity relationships among corynebacteriophages*

Phages	Bacterial indicator strains				
	C7(β) C7(α) C7(P)	C7(γ) C7(π)	C7(K)	C7(ρ)	C7(δ) C7(L)
β, α, P	—[a]	t	t	t	t
γ, π	t	—	t	t	t
K	t	t	—	t	t
ρ	t	t	t	—	t
δ	—	—	t	t	—
L	t	t	t	t	—

[a] Symbols: t, turbid plaques; —, no plaques.

Toxinogenicity. The genetic marker *tox*+, which determines the ability of corynebacteriophages to direct the synthesis of diphtherial toxin during infection of bacterial strains such as C7, has been detected in phages from three of the five groups of heteroimmune phages described above. Phages α, β, P, π, δ, and L are *tox*+, but phages γ, K, and ρ are *tox*−. Lysogenic strains of C7 carrying each of these six *tox*+ phages as prophage produce diphtherial toxins which are completely neutralized by standard diphtherial antitoxin during intracutaneous tests in rabbits or guinea pigs. Thus, the *tox*+ genes of these six phages direct the synthesis of diphtherial toxins which have either identical or closely related antigenic determinants.

Latent period and burst size. One-step growth experiments were performed as described above. The patterns of vegetative growth of phages β, γ, and L were indistinguishable. The mean values from duplicate experiments with these three phages were as follows: average burst size, 37 phages/cell; minimum latent period, 65 min; and rise period, 18 min. Phages K, ρ, and δ each behaved in unique fashion. The minimum latent period for phage K was also 65 min, but the rise period was prolonged to at least 65 min, and the average burst size at the end of that period did not exceed 15 phages per cell. In contrast, the minimum latent periods for phages δ and ρ were approximately 80 min. Phage δ had a rise period of

TABLE 2. *Host ranges of corynebacteriophages*

Phages	Bacterial indicator strains				
	C7	C7/β^c	C7/β^{vir} type 1	C7/β^{vir} type 2	C7/β^{vir}/β^{hc}
β (wild type)	t[a]	—	—	—	—
P	t	—	—	—	—
$\beta^{h'}$	t	t	—	—	—
γ	t	t	—	—	—
π	t	t	—	—	—
K	t	t	—	—	—
L	t	t	—	—	—
β^{h}	t	—	t	—	—
α	t	—	t	—	—
$\beta^{hh'}$	t	t	t	—	t
ρ	t	t	t	—	t
δ	t	t	t	—	t

[a] Symbols: t, turbid plaques; —, no plaques.

approximately 40 min and an average burst size of about eight phages per cell. The rise period and average burst size for phage ρ could not be calculated because the titer of infectious centers decreased progressively after approximately 80 min. This presumably reflected lysis of the infected cells, but under the conditions of our experiments the efficiency of plating appeared to be much higher for cells infected with ρ than for free phage ρ.

The prolonged latent periods and small average burst sizes for phages δ and ρ may explain an observation made during the preparation of phage stocks. Mass lysis and clearing never occurred in cultures of *C. diphtheriae* C7 inoculated in the usual manner with single plaques of phage δ or ρ, and it was, therefore, necessary to prepare stocks of phages δ and ρ by induction of lysogenic C7 strains with ultraviolet light. The average phage yield per irradiated cell was approximately 1 to 2 for phage δ and 5 to 10 for phage ρ.

Genetic recombination. A system for the genetic analysis of *tox*+ and *tox*− bacteriophages of *C. diphtheriae* has been described previously, and recombination between the genes which control immunity specificity and type *h* host range has been demonstrated in biparental matings between phage β and the heteroimmune phages γ and L (25). As shown above, wild-type phages K, ρ, and δ have host-range phenotypes designated, respectively, *h'*, *hh'*, and *hh'*, but it is not known whether the genetic determinants of these host ranges in K, ρ, and δ are homologous to genes *h* and *h'* of phage β. Biparental matings between appropriate mutants of phage β and the heteroimmune phages K, ρ, and δ were performed by superinfection of induced lysogenic derivatives of C7 (25), and the progeny from each cross was examined for the presence of recombinant phages with the *h* host range of one parent and the immunity specificity of the other parent (Table 3). No recombinants were detected, and in each of the matings the upper limit for the frequency of possible recombinants represented the frequency of host-range mutants of the selected genotype in the parental phage stocks. Recombination thus appears to be absent in matings between phage β and the heteroimmune phages K, ρ, and δ.

Phages β, γ, and L which recombine genetically resemble one another in plaque morphology, latent period, and burst size. In contrast, phages K, ρ, and δ, which failed to recombine with β

TABLE 3. *Absence of genetic recombination between phage β and phage K, ρ, or δ*

Expt	Cross		Analysis of phage progeny			
	Induced lysogen	Superinfecting phage[a]	Parental type phage (avg yield per infected cell)		Recombinant phages	
			Prophage type[a]	Infecting type[a]	Selected properties[b]	Recombination frequency
17	C7(K)	β^{hc}	14.9	5.7	*h imm*^{K}*h'*	$\leq 2 \times 10^{-6}$
24	C7(K)	β^{hc}	9.4	3.9	*h imm*^{K}*h'*	$\leq 10^{-7}$
27	C7(ρ)	$\beta^{ch'}$	7.7	40.0	*h imm*$^{\beta}$*h'*	$\leq 2 \times 10^{-7}$
28	C7(ρ)	$\beta^{ch'}$	2.7	24.3	*h imm*$^{\beta}$*h'*	$\leq 2 \times 10^{-7}$
27	C7(δ)	$\beta^{ch'}$	0.38	44.7	*h imm*$^{\beta}$*h'*	$\leq 10^{-7}$
28	C7(δ)	$\beta^{ch'}$	0.28	28.7	*h imm*$^{\beta}$*h'*	$\leq 10^{-7}$

[a] Parental β phages carried the unselected marker *c* so that they could be distinguished from phages K, ρ, and δ by the formation of clear plaques on lawns containing C7 as indicator. In the last four crosses, parental β phages which carried the marker *h'* were used for technical convenience based on the expected characteristics of single recombinants and the availability of appropriate indicator strains of bacteria.

[b] Assayed on C7(*h imm*$^{\beta}$*h'*)/β^{vir}/β^{hc} in the first two crosses, on C7(*h imm*$^{\rho}$*h'*)/β^{vir}/β^{hc} in the second two crosses, and on C7(*h imm*$^{\delta}$*h'*)/β^{vir}/β^{hc} in the final two crosses. To increase the sensitivity of the assay for the detection of recombinants, the dilution of the progeny phage was minimized by using the selective indicator cells both for preadsorption of the phage progeny and for the bacterial lawns.

in our experiments, also differed from β with respect to these other properties.

Morphology. High-titered stocks of phages β, γ, K, ρ, δ, and L were examined by electron microscopy after negative staining either with potassium phosphotungstate (PTA) or with uranyl acetate (UA). All of these corynebacteriophages were found to have polyhedral heads and long, noncontractile tails. The average dimensions of each phage strain included in this study are presented in Table 4. To obtain a parameter which is independent of magnification, the ratio of the contour length of the tail to the length of the head was measured for each virion. The average value of this parameter for each phage strain is also presented in Table 4.

On the basis of the morphology of their virions, the corynebacteriophages in this study could be separated into two distinct groups. Phages β, γ, K, and L have similar absolute sizes and proportions, with the average ratios of tail length to head length in the range from 4.3 to 4.9. In contrast, phages ρ and δ have smaller heads, longer tails, and ratios of tail length to head length in the range from 6.1 to 6.6. The differences in the average dimensions of phages β, γ, K, and L may not be significant, because the size distributions of virions of these phages showed considerable overlap. For the same reason, the average dimensions of phages ρ and δ may not differ significantly. However, among individual virions of phages ρ and δ, the smallest measured value for the ratio of tail length to head length was 5.53, which exceeded the largest value of 5.38 observed for any virion of phages β, γ, K, and L. In addition, the choice of PTA or of UA as the negative stain did not significantly influence the observed dimensions of the phages, as shown in Table 4 for phages β, γ, and K.

The photomicrographs in Fig. 1 were chosen primarily to illustrate the differences in proportions of the corynebacteriophages belonging to the two morphological groups described above. Additional details of corynebacteriophage structure are revealed in the photomicrographs in Fig. 2 and 3. Phages with full heads are shown in Fig. 1A, 1F, 3D, and 3E. Central longitudinal channels in the phage tails, approximately 2 nm in diameter, are observed only in virions which also have empty heads. In preparations stained with UA, the phage tails appear to be composed of subunits and to show a periodicity of approximately 4 nm along the length of the tail, as illustrated for phage δ in Fig. 1E. In our phage stocks, many virions with empty heads were seen attached by the tips of their tails to bits of amorphous debris, presumably of bacterial origin. The tips of the phage tails are frequently separated by about 10 nm from such debris. Occasionally, one or two short, fine fibers were seen to originate from the tips of the phage tails (Fig. 2A and 2C) and to extend from the tips of the phage tails to debris (Fig. 2D and 2E).

The types of symmetry observed in bacteriophage head structures have been reviewed recently by Bradley (9). Our corynebacteriophages have regular polyhedral heads which appear hexagonal in outline in negatively stained preparations. The full heads of L phages in Fig. 3D and 3E appear to have octahedral symmetry, and the configurations of many of the empty heads of phages β, γ, K, and L in Fig. 2 and 3 appear to be consistent with slightly deformed regular octahedrons.

TABLE 4. *Average dimensions of corynebacteriophages (nm)*

Phages	No. of virions measured	Negative stain	Head[a]		Tail		Tail length/ head length
			Width	Length	Length[b]	Width[c]	
β	10	UA	52	56	270	9.0	4.9
β^{vir}	11	PTA	58	59	270	10.5	4.6
γ	10	UA	57	62	265	8.5	4.3
γ^{c}	22	PTA	55	56	260	9.5	4.6
K	10	UA	59	63	270	9.0	4.4
K	10	PTA	60	64	275	9.0	4.3
L	15	PTA	51	55	235	9.5	4.3
ρ	10	UA	51	53	325	7.5	6.1
δ	10	UA	44	50	330	7.5	6.6

[a] Rounded off to the nearest 1 nm.
[b] Rounded off to the nearest 5 nm.
[c] Rounded off to the nearest 0.5 nm.

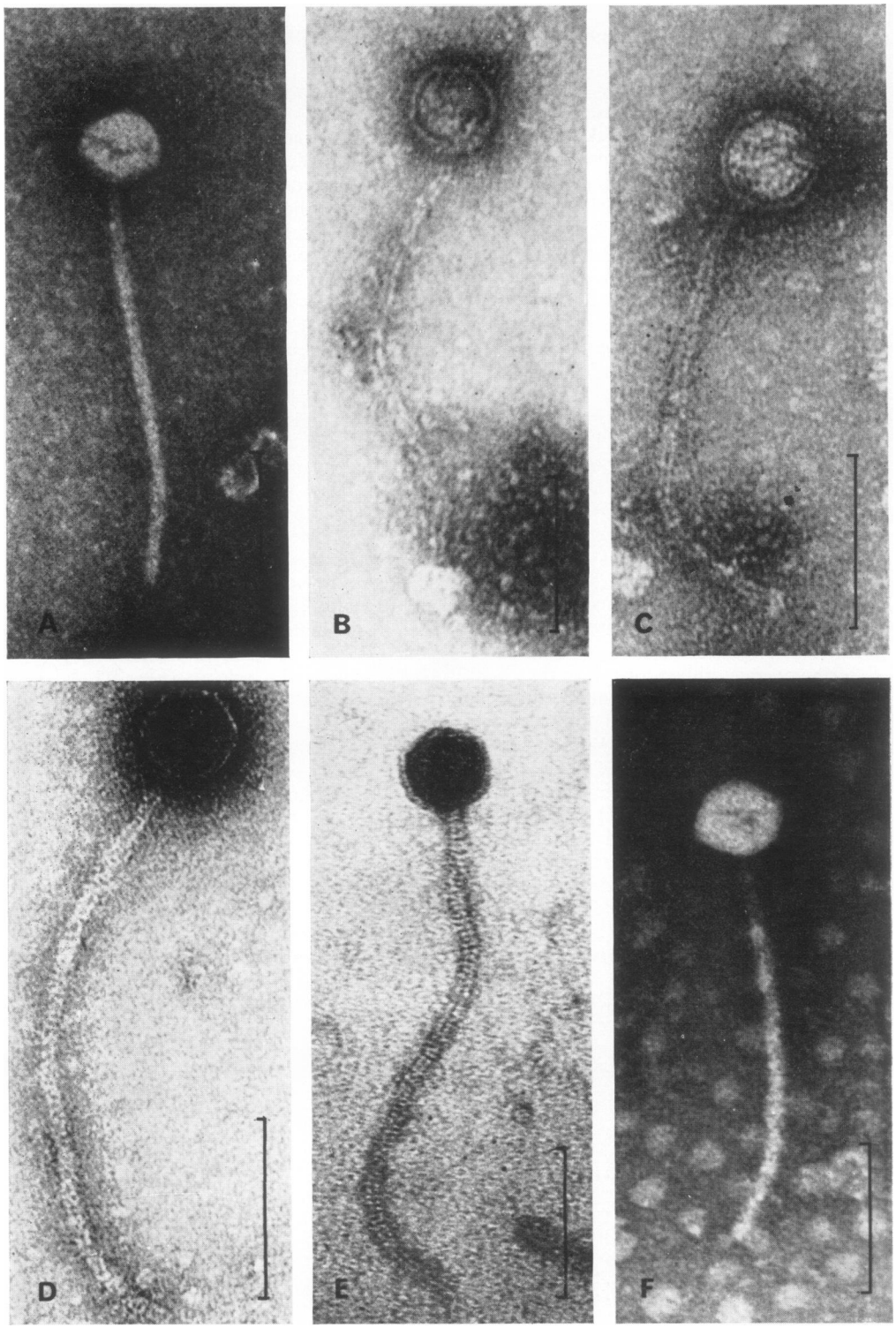

FIG. 1. *Morphology of corynebacteriophages in preparations negatively stained with potassium phosphotungstate (PTA) or uranyl acetate (UA). (A) β^{tox+}, 270,000 ×, PTA. (B) γ^{tox-}, 226,000 ×, UA. (C) K^{tox-}, 256,000 ×, UA. (D) ρ^{tox-}, 281,000 ×, UA. (E) δ^{tox+}, 226,000 ×, UA. (F) L^{tox+}, 228,000 ×, PTA. Phages in A and F have full heads. The periodicity in the tail of phage δ^{tox+} is illustrated in E. Scale markings indicate approximately 100 nm.*

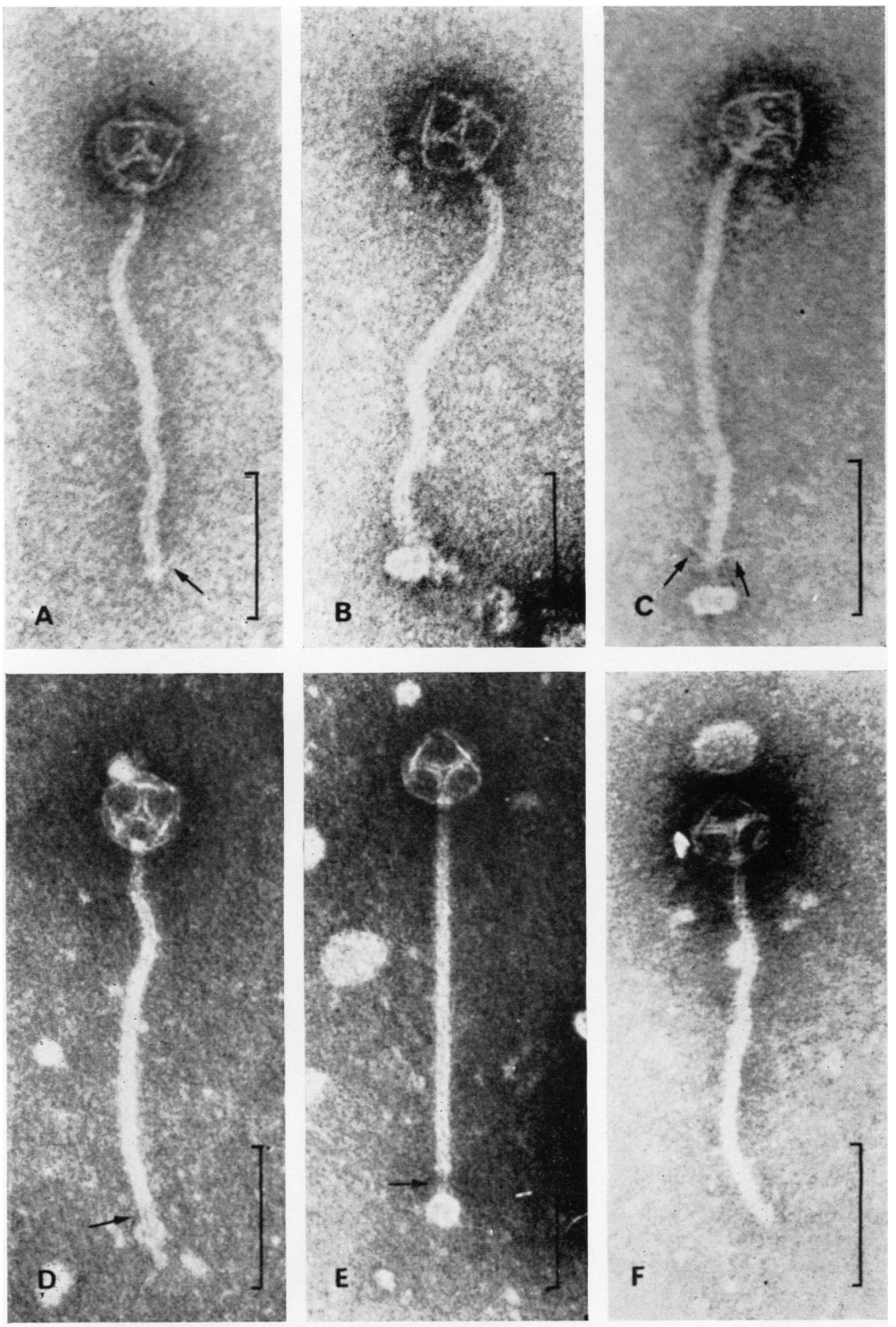

FIG. 2. *Morphology of empty virions of corynebacteriophages* β^{vir} *and* γ^{c} *negatively stained with potassium phosphotungstate. (A)* β^{vir}, *216,000* ×. *(B)* β^{vir}, *216,000* ×. *(C)* β^{vir}, *227,000* ×. *(D)* γ^{c}, *215,000* ×. *(E)* γ^{c}, *215,000* ×. *(F)* γ^{c}, *215,000* ×. *Scale markings indicate approximately 100 nm*

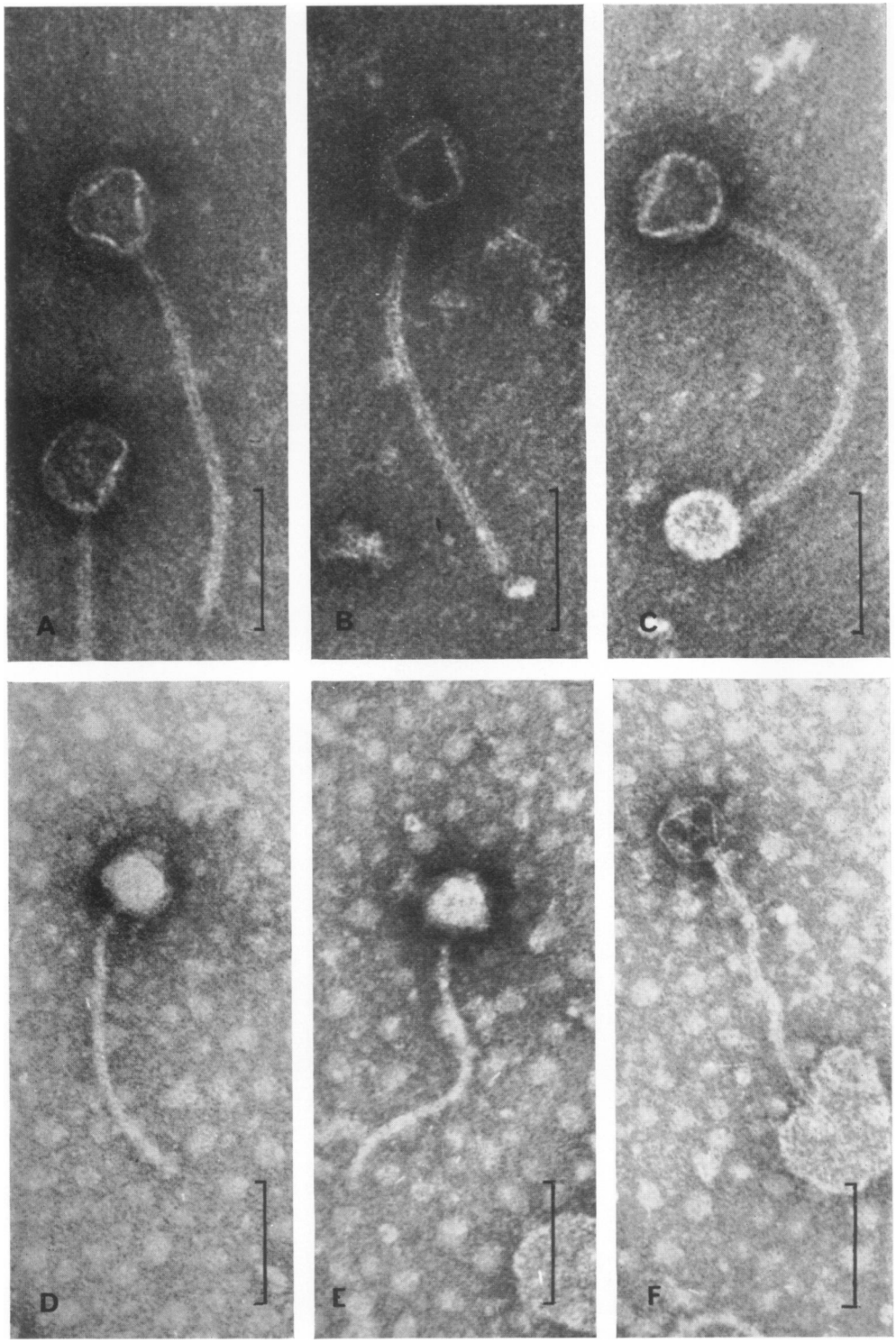

FIG. 3. *Morphology of phages* K^{tox-} *and* L^{tox+} *in preparations negatively stained with potassium phosphotungstate.* (*A, B, C*) K^{tox-}, 207,000 ×. (*D, E, F*) L^{tox+}, 180,000 ×. *Phages in D and E have full heads. Scale markings indicate approximately 100 nm.*

TABLE 5. *Rate constants for neutralization of corynebacteriophages by antiphage sera*

Antiserum	Serum dilution (*D*)	Phages					
		β	γ	K	L	ρ	δ
Anti-β	1:50	12	18	2.0	0.9	12	16
Anti-γ	1:50	2.5	10.4	1.1	1.4	1.7	1.7
Anti-K	1:100	0.2	2.7	26	0.8	0.4	0.2

Antigenic specificity. The abilities of rabbit antisera prepared against phages β, γ, and K to neutralize the infectivities of the six representative corynebacteriophages β, γ, K, ρ, δ, and L were determined. The results are presented in Table 5, and all *K* values are averages from two or more experiments. As controls, normal rabbit serum and an agglutinating antiserum directed against *C. diphtheriae* C7 were shown to cause no inactivation of any of these phages during the incubation period.

The four phages β, γ, K, and L have similar morphologies, and only phage K among these phages fails to recombine with phage β. Their responses to neutralizing antiphage sera can be summarized in the following manner. Phages β and γ are both neutralized by sera prepared against wild-type or mutant β phages, as previously observed (19, 31, 34). It is somewhat unexpected that the *K* values of our anti-β serum against phage γ are as high as those against the homologous phage β, but a similar behavior for an anti-β serum has been described previously (19). Our anti-γ serum neutralized phage γ at a rate approximately four times as fast as the heterologous phage β. Thus, considerable cross-reaction occurs between phages β and γ in neutralization by anti-β and anti-γ sera. The rate of neutralization of phage K by anti-β sera (31) and by anti-γ serum is slight. The infectivity of phage L is neutralized at barely measurable rates by these three antiphage sera. Thus, although phage L is genetically related to phage β, it appears to be antigenically distinct from β. Anti-K serum neutralized the infectivity of phage K at a rate at least 10 times greater than it neutralized any of the other five phages tested. Phage K thus appears to be both genetically and antigenically distinct from phage β.

Phages ρ and δ are morphologically distinct from phages β, γ, K, and L, and both ρ and δ appear to be genetically distinct from phage β. Phages ρ and δ fail to be neutralized as rapidly as homologous phages by anti-K serum or anti-γ serum. However, anti-β serum does react with phages ρ and δ, and the rates of neutralization of phages ρ and δ by anti-β serum are comparable to the rates for the homologous phage β.

DISCUSSION

The classification of viruses was recently reviewed by Lwoff and Tournier (28), who pointed out that the known evolutionary and phylogenetic relationships between viruses are too limited to provide a practical basis for virus classification. Lwoff, Horne, and Tournier (28, 29), therefore, proposed a system of classification based upon the chemical and morphological properties of the virion. In this paper, a group of corynebacteriophages was characterized with respect to several morphological, physiological, and antigenic properties. In addition, our previously described system for recombinational analysis of corynebacteriophages (25) was used to determine the extent of genetic relatedness between representative corynebacteriophages. We attempted, therefore, to ascertain the characteristics which are shared by several corynebacteriophages and to determine which of those properties are most consistently correlated with the ability to recombine genetically.

The well-studied corynebacteriophage β^{tox+} has been partially purified and is known to contain deoxyribonucleic acid (DNA; 31). The remaining corynebacteriophages have not been analyzed chemically. Attempts to demonstrate the type of nucleic acid in corynebacteriophages by fluorescence microscopy with acridine orange staining by the method of Anderson and collaborators (2) have been unsuccessful (Holmes and Barksdale, *unpublished data*). However, these corynebacteriophages possess two properties which have previously been found only in DNA phages and which suggest that their genetic material is DNA: (i) they are all temperate phages which can establish lysogeny and (ii) the representative phages studied by electron microscopy all have polyhedral heads attached to long slender tails. The virions of these phages are naked nucleocapsids lacking envelopes, and capsomeres of the phage heads were not visualized in our preparations. Thus, all of the corynebacteriophages included in our study are temperate, inducible phages whose virions possess polyhedral heads and noncontractile tails. Within this group of corynebacteriophages, however, two morphologically distinct subgroups could be recognized by differences in the absolute sizes and proportions of the virions.

Our observations on interfertility have revealed that the ability of corynebacteriophages to recombine genetically is correlated with morphological similarities between them. The virions of phages β, γ, L, and K are similar to each other but differ morphologically from the virions of ρ and δ. Phage β recombines genetically

with γ and L, although recombination frequencies between specific markers in matings between β and the heteroimmune phages γ and L are reduced in comparison to matings between parental β phages (25). Corynebacteriophages β, γ, and L are thus genetically related and have partial genetic homology. Their interrelationships are to a degree analogous to those of the lambdoid phages of *Escherichia coli* (26). In constrast, genetic recombination was not detected in matings of phage β, either with the morphologically similar phage K or the morphologically different phages ρ and δ. Among this sample of corynebacteriophages, then, morphological similarity between parental phages appears to be necessary for the occurrence of genetic recombination in phage matings but does not of itself guarantee fertility of the crosses. Genetic recombination was not observed between morphologically different phages in our experiments.

Several other characteristics of corynebacteriophages were observed to be correlated with the ability to recombine genetically. The interfertile phages β, γ, and L were similar in plaque morphology, latent period, and average burst size and differed in these properties from phages K, ρ, and δ which failed to recombine with β. Many examples of similarities in these physiological parameters among other closely related phages have been reported and have been summarized by Adams (1). Phages α, β, P, γ, π, and L, when present as prophages in lysogenic strains of *C. diphtheriae* C7, share the property of inhibiting the formation of plaques by phage δ (Table 1). It is likely that these six phages are all closely related, but α, P, and π have been characterized in less detail than phages β, γ, and L. The smaller size of the plaques formed by phage α on lawns of C7 may possibly be explained by the type *h* host range of α, since several mutants of phage β with type *h* host range have previously been shown to grow poorly and to produce smaller plaques than wild-type phage β (25; Holmes and Barksdale *unpublished data*). Phages δ and L, which differ morphologically and in their abilities to recombine with phage β, appear to be coimmune (Table 1). We expect that coimmune phages should be closely related and have, therefore, considered the possibility that the data in Table 1 do not necessarily reflect coimmunity of phages δ and L. Since phage L is closely related to β, it is possible that restriction of the development of superinfecting δ phage by prophages β and L occurs by the same undetermined mechanism. If, in addition, prophage δ possessed a mechanism which restricted the development of phage L but not of the other corynebacteriophages studied, then phages δ and L would appear to be coimmune. It is not known whether these restriction phenomena in corynebacteriophages are related to the phenomena of host-controlled modification (3) or of mutual exclusion between bacteriophages (1, 12).

Similarities in host range and antigenic cross-reactions with phage-neutralizing antisera did not correlate closely with interfertility among the corynebacteriophages studied. Each type of host range observed in the nine corynebacteriophages studied has previously been recognized in mutants of phage β (25). Although this can be ascribed to the choice of bacterial indicator strains used, it is evident that bacteriophage host range can vary readily by mutation and is of limited taxonomic value. The observation that antiserum prepared against phage β rapidly neutralized the infectivity of the morphologically and genetically different phages ρ and δ but failed to inactivate the genetically related phage L illustrates clearly the poor correlation between interfertility and antigenic cross-reactivity among these corynebacteriophages.

The most unusual attribute of corynebacteriophages is their role in directing the synthesis of diphtherial toxin by toxinogenic strains of *C. diphtheriae*. The production of diphtherial toxin appears to result from a complex interaction between a corynebacterial host and a corynebacteriophage which carries the marker *tox*+ within its genome. The extent to which the specific corynebacterial host, the *tox*+ corynebacteriophage, and the environmental conditions can determine or modify the expression of toxinogenicity has been discussed elsewhere (5, 25, 31, 36, 44). The new fact established by our data is the following: *tox*+ markers which direct the synthesis of antigenically similar diphtherial toxins are found in corynebacteriophages which do not appear to be closely related. The most striking example is the presence of *tox*+ markers both in β^{tox+} and δ^{tox+}, phages which differ in virion morphology and fail to recombine genetically. In addition, the presence of the *tox*+ marker was not correlated with virion morphology, interfertility with phage β, immunity specificity, plaque morphology, latent period, burst size, host range, or antigenic specificity in the corynebacteriophages studied.

The presence of the *tox*+ marker in corynebacteriophages which fail to recombine genetically raises many questions concerning the evolution of toxinogenicity in corynebacteria. The *tox*+ marker could be a structural gene which determines the primary structure of diphtherial toxin. If so, did it arise as a phage gene which has been retained during the evolution of diverse coryne-

bacterial species from a common ancestor, or was it once a bacterial gene which has become incorporated into the genomes of corynebacteriophages which may have evolved independently? These questions would also apply if *tox*+ were not a structural gene but instead provided some secondary function necessary for the synthesis of diphtherial toxin. In that case, however, the possibility would arise that *tox*+ genes could have evolved independently in different corynebacteriophages without necessarily leading to the production of diphtherial toxins which differ in any way. At present, no bacterial or phage mutations which alter the properties of diphtherial toxin have been described, and it is not yet possible to determine whether *tox*+ is a structural gene for diphtherial toxin. It is of interest that Sickles and O'Leary (40) recently described the isolation from *C. diphtheriae* C7 of a nontoxic protein whose properties are in some ways similar to those of diphtherial toxin from *C. diphtheriae* C7(β). However, the atoxic protein failed to cross-react immunologically with diphtherial toxin, and there was no direct evidence that the structure of the atoxic protein is homologous to diphtherial toxin. If the role of the phage gene *tox*+ is to release or modify a bacterial protein which becomes diphtherial toxin, then the precursor of diphtherial toxin must be widely distributed among corynebacterial species. The production of diphtherial toxin by corynebacteria lysogenized with appropriate *tox*+ corynebacteriophages has been reported in *C. diphtheriae* (14), *C. ulcerans* (32, 33), *C. belfanti* (16, 24, 35), and *C. ovis* (33).

All of the corynebacteriophages included in the present study share the common host C7, a *mitis* strain of *C. diphtheriae*. Many phages which attack *gravis* and *intermedius* strains of *C. diphtheriae* have been described previously (10, 37, 41, 42), and both toxinogenic and nontoxinogenic varieties have recently been found among clinical isolates of *C. diphtheriae* types *mitis*, *intermedius*, and *gravis* (39). The inclusion of corynebacteriophages active against *intermedius* and *gravis* strains in future comparative studies with corynebacteriophages may provide further information concerning the variability of corynebacteriophages and the natural history of the *tox*+ marker.

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