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# DNA Relationships among Some *tox*-Bearing Corynebacteriophages

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**The DNA genomes of a number of *tox*-bearing, temperate corynebacteriophages isolated from strains of *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* were compared. With one exception, these phages displayed similarities in their restriction enzyme digest profiles and extensive homology with prototypic β converting phage. The exception, phage δ, had a unique restriction profile and exhibited homology with β over a limited portion of its genome. DNAs of phages from each host contained cohesive ends and integrated as prophage by a mechanism analogous to that employed by coliphage lambda. It is proposed that these *tox*-bearing phages belong to a common family, the β family. The role of the β family in the movement of the *tox* gene between strains of *C. diphtheriae* and *C. ulcerans* is discussed.**

A number of bacteriophages isolated from *Corynebacterium diphtheriae* strains carry the structural gene (*tox*$^+$) for diphtheria toxin (DT) or a mutated allele of that gene. These *tox*$^+$ phages convert nontoxinogenic *C. diphtheriae* to toxinogeny, and in some cases they convert *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* strains as well. Phages that can convert nontoxinogenic strains of the same three species have in a few instances also been isolated from natural DT-producing strains of *C. ulcerans* (8). A question of interest in the natural history of diphtheria is the relationship of the various *tox*-bearing phages to each other.

The first comparative study of corynephages was reported by Holmes and Barksdale over 15 years ago (10). Converting (*tox*$^+$) phages α, β, δ, L, P, and π and nonconverting phages γ, K, and ρ were compared morphologically, serologically, and genetically. With the exception of L phage, which originated in a strain of *C. ulcerans*, these phages were isolated from *C. diphtheriae* strains. All the phages had polyhedral heads and long slender tails, although they fell into two groups in terms of tail-to-head-length ratio. Some recombined genetically with β phage and were neutralized by anti-β serum. Five different immunity groups were distinguished. Holmes and Barksdale summarized their results as follows. "*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 in both $\beta^{tox+}$ and $\delta^{tox+}$, 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, immune specificity, plaque morphology, latent period, burst size, host range, or antigenic specificity in the corynebacteriophages studied."

With the advent of molecular genetic techniques, the DNAs of some of these phages have been compared, and it has been shown that nonconverting, *tox*-bearing γ phage (4) and converting ω phage isolated from the classical PW8 strain of *C. diphtheriae* (15) are closely related to the prototypic β-converting phage. In the present paper, we compared the DNAs of all of the phages described by Holmes and Barksdale as well as some phages recently isolated from DT-producing strains of *C. ulcerans*. We have shown by means of restriction endonuclease patterns and ability to hybridize with β DNA that with the exception of $\delta^{tox+}$, the DNAs of all the *tox*-bearing phages from both *C. diphtheriae* and *C. ulcerans* have similar restriction profiles and exhibit extensive homology with β phage DNA.

## MATERIALS AND METHODS

**Bacteria and phages.** *C. diphtheriae* strains C7, C7/β, C7(β), C7(β-*tsr*-3), and C7(γ-*tsr*-2) were previously described (1). C7 lysogens carrying phages α, P, π, L, δ, K, and ρ were generously provided by R. K. Holmes and W. L. Barksdale, who originally described these phages (9, 10). All of these phages carry the wild-type *tox* allele for DT, except γ which carries a mutated *tox* allele and K and ρ which are *tox*-less, i.e., *tox*$^0$ phages. Heat-inducible phage mutants α-*tsr*-1, P-*tsr*-1, π-*tsr*-1, L-*tsr*-1, and δ-*tsr*-1 were isolated from their respective wild-type phages as previously described (6). C7 lysogens of each were isolated, except the lysogen of δ-*tsr*-1, which was produced in C7/β.

Virtually all of the *C. ulcerans* strains and the strain of *C. pseudotuberculosis* which will be referred to have already been described (7). They, along with the following strains, were obtained from P. Maximescu, Institute Cantacuzino, Romania. The additional strains were *C. ulcerans* 15542, A238, and 987 (12) (our numbers 823, 872, and 876, respectively) and *C. diphtheriae* (*belfanti*) 1030. *C. ulcerans* phages 731, 761, and 876 are designated by the numbers of their strains of origin. *C. ulcerans* typing phages a and h originated in strains 823 and 762, respectively. It should be noted that strain 876 was the original source of phage L. Purified isolates of phage 731, 761, 876, and a were maintained in lysogens of *C. ulcerans* 712, and a purified isolate of phage h was maintained in a lysogen of *C. diphtheriae* (*belfanti*) 1030. These latter strains were used when high-titer stocks were required for the extraction of phage DNA.

The media, general methods for culturing bacteria, phage procedures, and in vitro test for phage-converting activity followed published procedures (11). Phage stocks were produced either by UV induction or by heat induction of *tsr* mutants in appropriate lysogens. When high-titer phage stocks were required for DNA extraction, the UV-induced phages were scaled up in titer by two cycles of lytic infection

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in which *C. diphtheriae* C7 was used for K and ρ, *C. ulcerans* 712 was used for phages 731, 761, 876, and a, and *C. diphtheriae* (*belfanti*) 1030 was used for phage h. The stocks of heat-inducible mutants did not require scaling up. All strains into which phages were introduced to form lysogens or which were used for scaling up phage titers were $tox^0$, i.e., did not carry the structural gene for DT as determined by lack of hybridization with the *tox* B-specific DNA probe (5).

**Analytical procedures.** Phage and bacterial DNAs were purified as described before (1, 5). Purified DNAs were treated with restriction endonucleases, electrophoresed in agarose gels, and visualized as previously described (1). Also described previously were nick translation with $^{32}$P-deoxyribonucleotides, Southern blots, and hybridization reactions (1). The latter were performed at a stringency permitting a 10 to 12% mismatch.

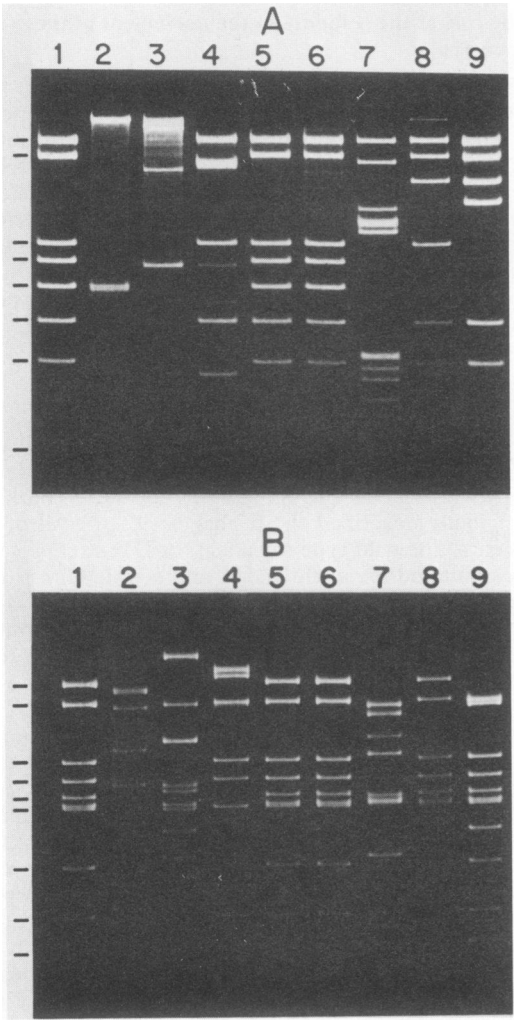


FIG. 1. Corynebacteriophage DNA restriction profiles. (A) *Bam*HI restriction digests. (B) *Eco*RI restriction digests. DNA from each phage was treated with the restriction endonuclease indicated and electrophoresed at 100 V for 2 h in a vertical 0.7% agarose gel. Lanes: 1, β; 2, ρ; 3, K; 4, L; 5, α; 6, P; 7, δ; 8, π; 9, γ. Fragments are referred to in the text as A, B, C, etc., starting from the largest. Hashmarks show positions of the β phage restriction fragments which are 11, 8.5, 3.9, 3.4, 3.0, 2.4, 1.9, and 1.0 kb for the *Bam*HI digest (panel A) and 6.8, 5.8, 3.8, 3.4, 3.1, 3.0, 2.2, 1.6, and 1.4 kb for the *Eco*RI digest (panel B). The very large faint fragment in the *Bam*HI digest of π is a partial digestion product that was not present in other *Bam*HI digests of this DNA.

TABLE 1. β restriction fragments that comigrate with L fragments

| *Bam*HI | *Hin*dIII | *Eco*RI | *Sal*I |
|---|---|---|---|
| A | B | B | $A_1$ |
| C[a] | D | C | $A_2$ |
| F | E | D | B[a] |
| H | G | $F_2$ | D |
| | I | H | I[b] |
| | | I | |

[a] Fragment containing *att*P and *tox*.
[b] Fragment not mapped.

## RESULTS

**Restriction analysis of corynebacteriophage DNAs.** The DNA genomes of $tox^+$ corynebacteriophages α, β, δ, L, P, and π, the *tox* phage mutant γ, and the $tox^0$ K and ρ were compared after treatment with restriction endonucleases and electrophoresis on agarose gels (Fig. 1). In the following description of results, the phages are grouped largely according to the immunity relationships described by Holmes and Barksdale (10).

**Phages β, α, and P.** The restriction profiles of the $tox^+$ homoimmune phages β, α, and P were consistently identical with restriction endonucleases *Bam*HI, *Eco*RI (Fig. 1A and B respectively; lanes 1, 5, and 6), *Hin*dIII, and *Sal*I (data not shown).

**Phages π and γ.** The restriction profiles of homoimmune phages $\pi^{tox^+}$ and $\gamma^{tox}$ (Fig. 1A and B, lanes 8 and 9) differed only slightly from each other and from the profiles of the phages with β immunity. As previously noted (1, 4), the differences between the profiles of γ and β are due to a small 1.2- to 1.4-kilobase (kb) insertion (DI-2) in the γ-*tox* gene and a 100-base-pair substitution in the proximity of genes responsible for tail synthesis. Thus, for the *Bam*HI digest, the *tox*-bearing 3.9-kb β fragment C was replaced by the 5.3 kb γ fragment D which carries a *tox* gene inactivated by the DI-2 insertion. In addition, differences in the DNA sequences of the phages within the substitution resulted in the replacement of the 3.0- and 3.4-kb β fragments D and E with the 6.4-kb γ fragment C.

The restriction profiles of π DNA were most like those of homoimmune γ DNA. The *Bam*HI profile of π was identical to that of γ, except that the 5.3-kb fragment which carries *tox* and DI-2 was replaced in π by a fragment which comigrated with the 3.9-kb *tox*-bearing fragment of β phage. These results suggest that π DNA is similar to γ DNA but does not contain the DI-2 insertion in *tox*. Comparison of the *Eco*RI, *Hin*dIII, and *Sal*I restriction profiles of these phages reinforced this interpretation.

**Phages L and δ.** L and δ are homoimmune phages with different restriction profiles. The restriction profiles of $L^{tox^+}$ contained many fragments comigrating with those of $\beta^{tox^+}$ and $\gamma^{tox}$ (Fig. 1, lanes 4; Table 1). Most of the comigrating fragments are located in the right arm of the β genome (1). The *Bam*HI and *Sal*I digests of L DNA contained fragments the same size as the *tox*-bearing 3.9-kb *Bam*HI C and the 5.7-kb *Sal*I B fragments of β, but the *tox*-bearing 15-kb *Hin*dIII A and 7.4-kb *Eco*RI A fragments of β DNA were not present in the corresponding digests of phage L.

Some of the L restriction fragments, e.g., the 3.4- and 2.7-kb *Bam*HI fragments E and F, were less intense than expected. (Note that fragments B and C comigrate in Fig. 1.)

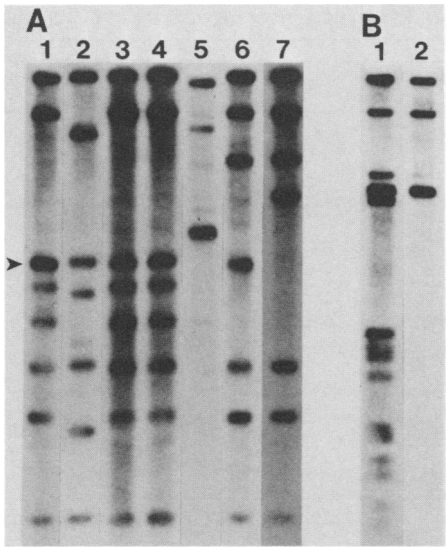


FIG. 2. Hybridization of corynebacteriophages with β phage DNA. (A) *Bam*HI restriction digests of the corynephage DNAs hybridized with $^{32}$P-labeled β DNA. The DNAs were digested with *Bam*HI, electrophoresed in a horizontal 0.7% agarose gel, blotted to nitrocellulose filter paper, and hybridized to β DNA labeled by nick translation. Lanes: 1, β; 2, L; 3, α; 4, P; 5, δ; 6, π; 7, γ. The arrowhead identifies the 3.9-kb β fragment that contains *tox* and *att*P. The sizes of the β fragments are given in the legend to Fig. 1. (B) *Bam*HI restriction profiles of δ phage DNA. DNA from δ was digested, electrophoresed, blotted, and hybridized as described above. (But note that gel A was electrophoresed for a longer time than gel B.) Lane 1, hybridized to $^{32}$P-labeled δ DNA; lane 2, hybridized to labeled β DNA.

We previously observed similar heterogeneity in the restriction profiles of a heat-inducible mutant of γ phage (2). This property was caused by abnormal excision of a significant fraction of heat-induced prophages which resulted in vegetative genomes carrying a small insertion of bacterial DNA in the phage attachment site *att*P. The DNA insert contained a *Bam*HI cleavage site, and as a result the normal *att*P-bearing fragment in *Bam*HI digests of these phages was split into two fragments. These two fragments were less intense than normal fragments because only a fraction of the heat-induced prophages acquired the insertion. Heterogeneous fragments similar in size to those found in the L phage preparations have been seen in *Bam*HI digests of some β phage recombinants (3). Because the *att*P-bearing *Bam*HI fragments of β and L are similar in size (unpublished observations), we conclude that the heterogeneity observed in digests of DNA from the heat-inducible L phage was caused by abnormal excision.

In contrast to the similarities observed among the restriction profiles of the other *tox* bearing phages, the profiles of $\delta^{tox^+}$ were unique (Fig. 1A and B, lanes 7). The one or two fragments of δ DNA in each digest that were similar in size to β fragments in the corresponding digest did not map to any particular region of the β genome. The relationship of the β and δ DNAs will be taken up later.

**Phages ρ and K.** The restriction profiles of $tox^0$ phages ρ and K were different from each other and from the profiles of all of the *tox*-bearing phages (Fig. 1A and B, lanes 2 and 3). Phages ρ and K belong to distinct immunity groups.

**Hybridization analysis of corynebacteriophage DNAs.** To further evaluate the relationships among these phages, we subjected their DNAs to Southern filter hybridization analysis with $^{32}$P-labeled β DNA as probe. The results of an experiment with *Bam*HI restriction digests of these phages is shown in Fig. 2A. Phages K and ρ did not hybridize with β DNA (data not shown) and thus are not included in the following description of these experiments. This lack of hybridization with β showed that K and ρ were $tox^0$ phages.

As expected from the analysis of the restriction profiles, all of the fragments of homoimmune phage $\alpha^{tox^+}$ and $P^{tox^+}$ hybridized strongly with the β probe (Fig. 2A, lanes 3 and 4). Similarly, despite at least some known regions of nonhomology, all the fragments of heteroimmune phage $\pi^{tox^+}$ and $\gamma^{tox}$ were also labeled (lanes 6 and 7).

With phage L (Fig. 2A, lane 2), two of the nine *Bam*HI fragments, B and F, did not hybridize strongly with β DNA probe. Fragment B, which is the upper band of a doublet formed by fragments B and C, probably contains L phage sequences that are not completely homologous to β DNA. As suggested above, the fragments E and F are probably formed when a segment of bacterial DNA is incorporated into *att*P in fragment D during abnormal excision of the heat-induced prophage. If fragment F contains most of the bacterial DNA, it would not be expected to hybridize as strongly to β DNA.

In contrast to the other converting phages, very few of the 15 *Bam*HI fragments of $\delta^{tox^+}$ DNA hybridized with the β probe (Fig. 2A, lane 5). A comparison of the total *Bam*HI digest of δ phage visualized by hybridization with $^{32}$P-labeled δ DNA and β DNA is given in Fig. 2B. Only 3 of the 15 δ fragments hybridized with β DNA (Fig. 2B, lane 2). Similar experiments at lower stringencies of hybridization did not change these hybridization profiles (data not shown). These results show that although some regions of the β and δ genomes are homologous, for the most part they are not.

**Regions of homology between $\beta^{tox^+}$ and $\delta^{tox^+}$ DNAs.** We previously showed that β phage integrates into the bacterial chromosome by a mechanism similar to that used by *Escherichia coli* phage λ (1). In this process, the vegetative DNA is circularized by ligation of the left and right cohesive ends *cos*L and *cos*R, and the phage DNA integrates into the chromosome via recombination between specific phage and bacterial attachment sites, *att*P and *att*B, respectively. These events lead to predictable differences in the restriction profiles of vegetative and prophage DNAs. The differences in the *Bam*HI profiles of β DNA are seen in Fig. 3 (lanes 1 and 2).

To localize the regions of homology between the genomes of β and δ, we hybridized $^{32}$P-labeled δ DNA to nitrocellulose filter blots of *Bam*HI-treated vegetative β DNA and genomic DNA from a C7(β) lysogen (Fig. 3, lanes 3 and 4). Vegetative β fragments E and H, which are not altered in prophage DNA, were similarly labeled in both digests. The δ probe also hybridized to vegetative fragments COS-L and ATT-P and to prophage fragments COS and ATT-L. Because δ DNA hybridized to ATT-L but not to ATT-R, it appears that the region of homology between the DNAs of δ and β includes the portion of vegetative fragment ATT-P that carries *tox* but does not extend into the region incorporated into ATT-R. It also appears that the region of homology on the prophage fragment COS does not extend across the entire fragment, because δ DNA hybridized to COS-L but not to COS-R.

**Bacteriophages from *C. ulcerans*.** Although bacteriophages have been isolated from *C. ulcerans* strains, only two converting phages have been identified. Phage L, one of the phages already described, converted the C7 strain *C. diphtheriae* (9), and phage h, a *C. ulcerans* typing phage,

converted *C. diphtheriae* (*belfanti*) 1030 as well as some *C. ulcerans* strains (12). A number of DT-producing *C. ulcerans* strains were recently identified (7), and UV-induced lysates of a representative sample were screened for the presence of converting or *tox* bearing phages. The sample was drawn from four groups that had been defined by their restriction enzyme profiles. Converting phages were isolated from all eight strains representative of the restriction enzme II group. However, none was found in any of the nine DT-producing strains identified in the other three groups, although other phages were detected in lysates of eight of these strains. In addition, *tox*-bearing phages were not detected in these nonconverting lysates when plaques formed by ca. $10^4$ phages in each lysate were tested by a blot assay with a $^{32}$P-labeled *tox* B probe.

Four converting phages, 731, 761, 876, and h, were selected for comparison with β phage based on their ability to produce high enough titers for DNA extraction and to cross species barriers (Table 2). The immune specificities of these phages were compared with those of the other *tox*-bearing phages (Table 3). In other tests not shown in Table 3, phage L failed to plaque on C7 lysogens of phages 731 and 761, whereas phages h and 782, the latter coimmune with β, each failed to plaque on 1030 lysogens of the other. From these results it was concluded that phages 731, 761, and 876 were coimmune with L phage and that phage h belonged to the β immunity group. Phage a was not coimmune with either the L or h groups, but its relation to β or γ immunity could not be determined.

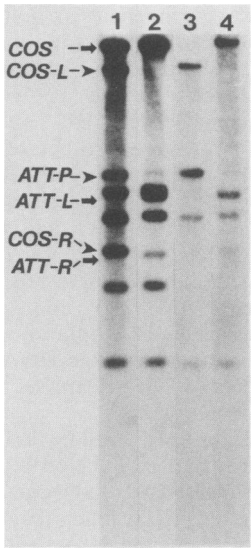


FIG. 3. Localization of β DNA sequences that hybridize to δ phage. DNAs from β (lanes 1 and 3) and C7(β) (lanes 2 and 4) were treated with *Bam*HI, electrophoresed, blotted, and hybridized as described in the legend to Fig. 2. Probes of β and δ were $^{32}$P-labeled. Lanes: 1, β DNA hybridized to β probe; 2, C7(β) DNA hybridized to β probe; 3, β DNA hybridized to δ probe; 4, C7(β) DNA hybridized to δ probe. Vegetative fragments (lanes 1 and 3) are identified with arrowheads, and prophage fragments (lanes 2 and 4) are identified with arrows. The sizes of the β fragments are given in the legend to Fig. 1. COS-L and COS-R, vegetative β phage fragments bearing *cos*L and *cos*R, respectively; COS, prophage fragment bearing ligated *cos* sites (note that COS migrates with the uppermost fragment in the prophage digests; i.e., the uppermost fragment in lane 2 is a doublet containing fragment A and COS); ATT-P, vegetative fragment bearing *att*P and *tox*; ATT-L and ATT-R, prophage fragments bearing hybrid phage-bacterial sites *att*L and *att*R, respectively.

TABLE 2. Lytic and converting activity of *C. ulcerans* phages

| Bacterial indicator[a] | Activity of phage[b] | | | |
|---|---|---|---|---|
| | 731 | 761 | h | 876 |
| *C. diphtheriae* | | | | |
| C4 | − | C | − | + |
| C7 | − | C | − | C |
| *C. diphtheriae* (*belfanti*) 1030 | C | C | C | C |
| *C. ulcerans* | | | | |
| RII 712 | C | C | − | C |
| 714 | − | − | ND | ND |
| 738 | + | + | ND | + |
| 754 | − | + | C | + |
| 755 | + | + | + | + |
| 872 | C | C | C | C |
| RIII 713 | − | + | ND | ND |
| Miscellaneous 719 | − | + | ND | ND |
| *C. pseudotuberculosis* 724 | − | C | − | C |

[a] All indicators are *tox*-less (*tox*$^0$) strains, i.e., do not carry DNA that hybridizes with the *tox* B probe for DT. R, restriction enzyme.

[b] UV-induced, filter-sterilized lysates of the originating strain of each phage were used. −, No lysis; +, lysis, not tested for conversion; C, lysis, conversion shown; ND, not done.

**Restriction endonuclease analysis and DNA homology of *C. ulcerans* phages.** The restriction enzyme profiles of vegetative DNAs of converting phages 731, 761, 876, h, and *tox*$^0$ typing phage a were compared with those of β and γ phages (Fig. 4A). Some degree of similarity in the *Eco*RI profiles was observed between all of these phages except for typing phage a. The profile of h phage was virtually identical with that of β. The profiles of phages 731, 761, and 876 (lanes 4, 5, and 6) were identical, and *Bam*HI and *Hin*dIII digestions yielded the same finding (data not shown). In addition, they

TABLE 3. Comparison of the immune specificity of corynephages

| Bacterial indicator | Activity of phage[a] | | | | |
|---|---|---|---|---|---|
| | β | γ | h | 731, 761, 876 | a |
| *C. diphtheriae* | | | | | |
| C7 | + | + | − | + | − |
| C7(β) | − | + | ND | + | ND |
| C7(γ) | + | − | ND | + | ND |
| C7(731)[b] | + | + | ND | − | ND |
| C7(L) | + | + | ND | − | ND |
| *C. diphtheriae* (*belfanti*) | | | | | |
| 1030 | + | + | + | + | − |
| 1030(γ) | + | − | + | + | ND |
| 1030(h) | − | + | − | + | ND |
| 1030(731)[b] | + | + | + | − | ND |
| *C. ulcerans* | | | | | |
| 872 | − | − | + | + | + |
| 872(h) | ND | ND | − | + | + |
| 872(731)[b] | ND | ND | + | − | + |
| 872(a) | ND | ND | + | + | − |

[a] In each set of tests, corynephages were UV induced from lysogens of the same strain used as indicator to avoid any inhibition by restriction systems. +, Lysis; −, no lysis; ND, not done because the phage did not infect the common indicator strain.

[b] Lysogens of 731, 761, and 876 gave identical results.

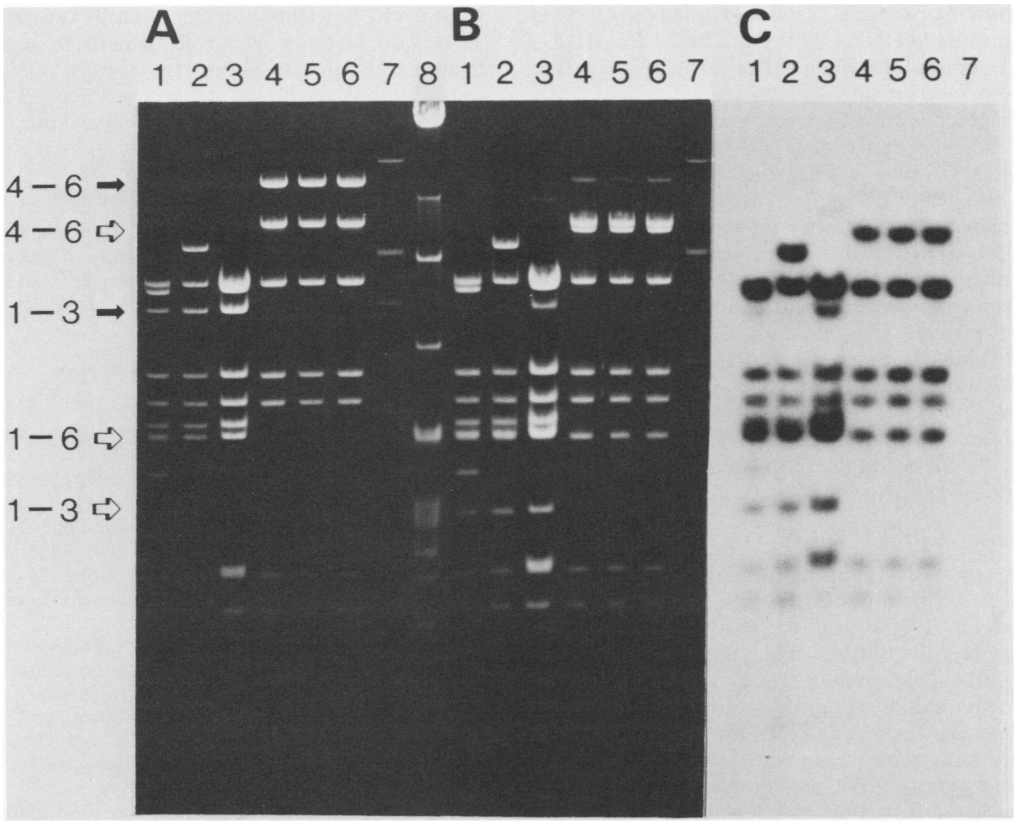


FIG. 4. Comparison of *Eco*RI cleavage fragments of corynephages. Restriction fragments were separated by vertical agarose gel electrophoresis. (A) DNA samples were not heated before electrophoresis. (B) DNA samples were heated at 75°C for 10 min and chilled on ice for 10 min to dissociate the terminal *cos* site-bearing fragments before electrophoresis. Solid arrows point to *cos* site-bearing fragments joined by the annealed *cos* sites. Open arrows point to *cos* site-bearing fragments dissociated by heat. The number next to the arrow designates the sample to which the arrows refers. Note that in samples 1 through 3, the larger of the two heat-dissociated *cos* site-bearing fragments comigrated with another fragment. The changes caused by heating sample 7 were not labeled. (C) DNA samples in panel B were Southern blotted and hybridized with $^{32}$P-labeled β-*tsr*-3 phage DNA under high stringency conditions. Lanes: 1, γ-*tsr*-2; 2, β-*tsr*-3; 3, h; 4, 731; 5, 761; 6, 876; 7, a; 8, *Hin*dIII digest of *E. coli* λ phage DNA as molecular weight markers.

were identical with the profile of phage L. Because phage 876 originated in the same strain as L and is probably identical with it, it is evident that the passage of L through a second *C. ulcerans* strain and into *C. diphtheriae* C7 (9) did not alter its genome. The genomic sizes of the phages estimated from the *Eco*RI restriction digests were 35.2 kb for h, 36.2 kb for phages 731, 761, and 876, 37.2 kb for a, and 35 and 36.2 kb, respectively, for β and γ. They were consistent with estimates based on *Bam*HI and *Hin*dIII digestions.

TABLE 4. Restriction fragments of corynephages hybridizing with the *att*P-bearing fragment of β

| Phage | Hybridizing DNA fragments (kb)[a] | | | | |
|---|---|---|---|---|---|
| | Vegetative phage | Prophage in strain C7 | | Prophage in strain 1030 | |
| | | Site 1 | Site 2 | Site 1 | Site 2 |
| β | 3.9 | — | 3.4, 2.2 | ND | ND |
| γ | 5.3 | 6.0, 2.7 | — | 6.0, 2.7 | 4.8, 2.2 |
| 731 | 3.9 | 4.6, 2.7 | — | 4.6, 2.7 | — |
| 761 | 3.9 | 4.6, 2.7 | 3.4, 2.2 | 4.6, 2.7 | 3.4, 2.2 |
| 876 | 3.9 | 4.6, 2.7 | — | — | 3.4, 2.2 |
| h | 7.1 | ND | ND | — | 6.6, 2.2 |

[a] *Bam*HI digests of vegetative and prophage DNA were hybridized with the $^{32}$P-labeled *Bam*HI C, *att*P-bearing fragment of vegetative β DNA. The hybridizing fragments are grouped at the two sites on the basis of one or two common-sized fragments. —, Site not occupied in this lysogen; ND, not done because the strain is insensitive to this phage.

DNA homology was examined by hybridizing these phages with labeled β phage (Fig. 4C). All the *Eco*RI fragments of the four *C. ulcerans* converting phages hybridized with the β probe, indicating extensive homology. The same homology relationships were observed when labeled h or 761 DNA was used as the probe. Phage a did not exhibit homology with any of these probes and failed to do so even when low stringency conditions allowing a ca. 30% base pair mismatch were used.

**Mechanism and sites of integration of *C. ulcerans* phages into bacterial chromosomes.** We previously showed that β phage DNA integrates into the *C. diphtheriae* C7 chromosome by first circularizing at its terminal cohesive (*cos*) sites and then integrating by a recombinational mechanism involving specific phage (*att*P) and bacterial (*att*B) attachment sites (1). It has been shown that γ and ω phages also integrate in the same manner and that these phages utilize two bacterial sites (*att*B1 and *att*B2) at which they form mono- or polylysogens (14). The presence of *cos* sites in all five *C. ulcerans* phages is shown in Fig. 4A and B. The presence of *att*P sites on the four *C. ulcerans* converting phages was

shown by hybridizing *Bam*HI restriction digests of their vegetative and prophage DNAs with the labeled *Bam*HI-C, *att*P-containing fragment of β phage. The results for a series of strains (gels not shown) are summarized in Table 4. In each case, as expected with phages integrating via *att*P sites (1), a single *Bam*HI vegetative DNA fragment hybridized and was replaced by two hybridizing *Bam*HI prophage fragments.

Rappuoli has shown (13) that when β prophage in strain C7 is probed with *Bam*HI-C, bands of 4.6 and 2.7 kb appear when integration occurs at the *att*B1 site, and bands of 3.4 and 2.2 kb appear when integration occurs at *att*B2. From the sizes of the β prophage fragments in C7 that hybridized with *Bam*HI-C (Table 4), it can be deduced that sites 1 and 2 represent *att*B1 and *att*B2, respectively. In lysogens of C7 and 1030 carrying phages 731, 761, and 876, similar sized fragments hybridized with *Bam*HI-C. This indicates that these phages use the same sites as β and suggests that strain 1030 has *att*B sites similar to those of C7.

## DISCUSSION

We compared the DNAs of a number of *tox*-bearing phages isolated from strains of *C. diphtheriae* and *C. ulcerans* and the DNA of prototypic converting phage β. With one exception, the DNAs had similar or identical restriction enzyme digest patterns, and all their *Bam*HI restriction fragments exhibited homology with β DNA. The exception, δ phage, had a unique restriction profile but did exhibit homology with some sequences located on the left arm of the β phage genome. We also showed that some *tox*-bearing phages isolated from both species use the same sites and mechanism of integration in lysogenizing common strains of *C. diphtheriae*. Although there are a number of phenotypic differences among this group of phages (10; this paper), including differences in immune specificity and host range, it was apparent that the *tox*-bearing phages are related, and we have proposed (8) that they all be considered members of one family of phage, the β family. The relatedness of the *tox*-bearing phages from natural isolates of *C. diphtheriae* and *C. ulcerans* suggests that phages of the β family have been responsible at least in part for distributing the *tox* gene among these organisms. Because there are *tox*-bearing phages capable of moving from each of these species to the other (8), the movement of *tox* is potentially bidirectional. The origins of δ phage must for the time being remain speculative. It is unlikely that a single recombinational event between a δ-like phage and β phage can explain the origin of δ, because the *Bam*HI fragments sharing homology with δ are not all contiguous on the β genetic map. In the β *Bam*HI restriction map sequence B, G, H, C, E, (1), δ failed to hybridize with fragment G and part of C.

We have shown that *tox*-bearing corynephages isolated from strains of *C. diphtheriae* and *C. ulcerans* are members of the β family of phage. Nevertheless, there are a number of DT-producing strains of *C. ulcerans* from which we have been unable to isolate a β-related phage. Some of these strains carry DNA homologous with β in excess of the *tox* gene itself, but some appear to carry only the *tox* gene (7). There are many ways to explain these observations. For example, the putative converting phage may be defective or noninducible, or the indicators used may have been insensitive. Finally, it is possible that phages completely unrelated to β carry the *tox* gene.

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## LITERATURE CITED

1. **Buck, G. A., and N. B. Groman.** 1981. Physical mapping of β-converting and γ-nonconverting corynebacteriophage genomes. J. Bacteriol. **148**:131–142.
2. **Buck, G. A., and N. B. Groman.** 1981. Genetic elements novel for *Corynebacterium diphtheriae*: specialized transducing elements and transposons. J. Bacteriol. **148**:143–152.
3. **Buck, G. A., and N. B. Groman.** 1981. Identification of deoxyribonucleic acid restriction fragments of β-converting corynebacteriophages that carry the gene for diphtheria toxin. J. Bacteriol. **148**:153–162.
4. **Buck, G. A., N. B. Groman, and S. Falkow.** 1978. Relationship between β-converting and γ-nonconverting corynebacteriophage DNA. Nature (London) **271**:683–685.
5. **Groman, N., N. Cianciotto, M. Bjorn, and M. Rabin.** 1983. Detection and expression of DNA homologous to the *tox* gene in nontoxinogenic isolates of *Corynebacterium diphtheriae*. Infect. Immun. **42**:48–56.
6. **Groman, N., and W. Laird.** 1977. Heat-inducible mutants of corynebacteriophage. J. Virol. **23**:587–591.
7. **Groman, N., J. Schiller, and J. Russell.** 1984. *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* responses to DNA probes derived from corynephage β and *Corynebacterium diphtheriae*. Infect. Immun. **45**:511–517.
8. **Groman, N. B.** 1984. Conversion by corynephages and its role in the natural history of diphtheria. J. Hyg. **93**:405–417.
9. **Holmes, R. K., and L. Barksdale.** 1969. Genetic analysis of $tox^+$ and $tox^-$ bacteriophages of *Corynebacterium diphtheriae*. J. Virol. **3**:586–598.
10. **Holmes, R. K., and L. Barksdale.** 1970. Comparative studies with $tox^+$ and $tox^-$ corynebacteriophages. J. Virol. **5**:783–794.
11. **Laird, W., and N. Groman.** 1976. Prophage map of converting corynebacteriophage beta. J. Virol. **19**:208–219.
12. **Maximescu, P., A. Oprisan, A. Pop, and E. Potorac.** 1974. Further studies on *Corynebacterium* species capable of producing diphtheria toxin (*C. diphtheriae*, *C. ulcerans*, *C. ovis*). J. Gen. Microbiol. **82**:49–56.
13. **Rappuoli, R.** 1983. Isolation and characterization of *Corynebacterium diphtheriae* nontandem double lysogens hyperproducing CRM197. Appl. Environ. Microbiol. **46**:560–564.
14. **Rappuoli, R., J. L. Michel, and J. R. Murphy.** 1983. Integration of corynebacteriophages $\beta^{tox^+}$, $\omega^{tox^+}$, and $\gamma^{tox^-}$ into two attachment sites on the *Corynebacterium diphtheriae* chromosome. J. Bacteriol. **153**:1202–1210.
15. **Rappuoli, R., J. L. Michel, and J. R. Murphy.** 1983. Restriction endonuclease map of corynebacteriophage $\omega_c^{tox^+}$ isolated from the Park-Williams no. 8 strain of *Corynebacterium diphtheriae*. J. Virol. **45**:524–530.