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Prophage Map of Converting Corynebacteriophage Beta

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A prophage map for corynebacteriophage beta consisting of seven markers has been constructed and compared with the vegetative map. The mapping system utilizes heteroimmune double lysogens and capitalizes on the fact that these double lysogens are very unstable and throw off monolysogenic segregants. The prophage map, produced by characterizing the recombinant phage in these monolysogenic segregants, appears to be a cyclic permutation of the vegetative map with the gene for toxin at one end of the prophage map and the gene for phage immunity at the other. This permutation is in accord with the Campbell model for insertion of lambda phage if a site between the toxin and immunity genes in the vegetative map is designated as the phage attachment site. The position of the gene for toxin in the prophage map suggests that converting phages may have originated as specialized transducing phages for this gene.

The role of beta corynephage in the production of diphtheria toxin was first reported by Freeman in 1951 (6); it was confirmed and extended by Groman (7) and Barksdale and Pappenheimer (1). Subsequently, Groman (8) showed that the presence of the phage genome was essential for the maintenance of the toxigenic state and demonstrated by means of simple genetic crosses that the ability to convert to toxinogeny was not only phage specific but gene specific (10). Holmes and Barksdale (15) began the work of mapping the beta phage genome. Using vegetative crosses, they established the relationships between several host range markers (*h* and *h'*) and the genes for toxigenicity *(tox)* and phage immunity *(imm)*. Matsuda et al. (18) mapped some temperature-sensitive *(ts)* mutants of virulent beta phage, and Singer, after isolating an additional series of *ts* mutants, combined the *ts* markers with many of the previously characterized markers to construct an extensive linear vegetative map of beta phage (19).

The data obtained by Holmes and Barksdale (15) and Singer (19) indicate that the genes *tox* and *imm* are closely linked and without any apparent intervening markers. As a result, recombination between these genes should be rare. However, an observation made by one of us years ago (11) seemed to conflict with this prediction. It was observed then that nearly half of the free phage produced after spontaneous induction of the double lysogen C4 (γ^{tox-})(β^{tox+}) were recombinant for the *imm* and *tox* markers. Since this high rate of recombination was contrary to expectation, it suggested to us that the linkage relationships in the prophage might differ from those in the vegetative phage. As a result, we decided to investigate the nature of the prophage map of beta corynephage.

MATERIALS AND METHODS

Strains of bacteria and phage. *Corynebacterium diphtheriae* strains C7, C7(β), C7(γ), and C7/β were from our stock culture collection. R. Holmes generously sent us strains C7/β*vir* and C7(β*h9*) (14). All lysogenic strains to be described were derived by infecting stock strain C7 with the appropriate phage or phages. Phage strain β^{tox-45}, graciously sent to us by B. Iglewski, was isolated by Uchida et al. (20), and beta strains carrying *ts61h9h'3*, *ts45*, *ts45tox-30*, *ts31*, *ts30tox-30*, and *ts61h9h'2* were kindly provided by R. Singer (19). Lysogens of C7 carrying each of these phages were isolated.

Bacteriophage markers. All beta and gamma phages described in this paper plaque on strain C7. Phage carrying the *h'* marker also plaque on C7/β, whereas those with the *h* marker plaque on C7/β*vir*. The lysogens C7(β) and C7(γ) were used to test for phage immunity. Beta phage plates on C7(γ) but not on C7(β), whereas the reverse is true for gamma phage. We will designate phage immunity as *imm*-β and *imm*-γ. Phage carrying the *ts* markers were recognized by their ability to plaque on C7 at 30 C but not at 39 C.

The nomenclature proposed by Holmes for *tox* mutants (14) has been followed. The gene for toxin is identified as *tox*. The phenotypic designation CRM+ (cross-reacting material) is used when the product of the gene is identified as a protein serologically related to or identical with diphtheria toxin. A number following the CRM designation will indicate the molecular weight of the CRM. Thus a CRM of the same molecular weight as diphtheria toxin is pheno-

typically CRM62, one producing a product with a molecular weight of 45,000 is phenotypically CRM45, etc., while a strain producing no detectable cross-reacting material is designated CRM−. The *tox* allele with the CRM− phenotype that is used in this study was obtained from γ phage and is designated *tox*-118.

The in vitro gel immunodiffusion test using diphtheria antitoxin (13) was used to detect the CRMs. The procedure was modified by spotting inocula from the colonies to be tested around the antitoxin-containing filter paper strips at a distance of approximately 0.5 cm from the strip rather than streaking the colonies across the strips. By appropriate spacing of the inocula and by incorporating two strips into each standard agar plate, the number of tests that can be performed per plate is increased six- to sevenfold. We have found that it is possible to distinguish CRM62, CRM45, and CRM30 strains from one another by differences in the intensity of the lines of precipitation, the intensity decreasing significantly as the molecular weight decreases. A more specific check for these differences depending on spur formation is carried out by testing unknown strains next to each of the known strains.

Media. Bacteria were grown in tryptose-yeast extract (TYE) broth (21) or on TYE agar plates. Bacteria were occasionally grown in heart infusion broth (Difco). Tween-TYE broth or Tween-broth is made by adding Tween 80 (polyoxyethylene sorbitan monooleate) to TYE broth to a final concentration of 0.2%. It has been shown (9) that Tween 80 prevents adsorption of beta corynephage, and we have established a similar effect on gamma phage adsorption.

Antitoxin. Diphtheria antitoxin was purchased from the Connaught Laboratories Ltd., Ontario, Canada.

Phage assay. Phage were diluted in TYE broth. A 0.1-ml sample was mixed with 2 ml of TYE soft agar (0.5%) supplemented with 1.5% maltose and 10^{-3} M $CaCl_2$, after which 0.1 ml of indicator cells was added and the mixture was layered immediately onto TYE hard-agar (1.0%) plates. The indicator cells were prepared in TYE broth as a logarithmic-phase suspension grown to a concentration of 1×10^8 to 2×10^8 cells/ml.

Test for lysogeny. C7 indicator plates were prepared as in the phage assay, but the phage was omitted. When the soft-agar overlay had hardened, the colonies to be tested for lysogeny were first picked and then inoculated onto the indicator lawn with a sterile toothpick. The plate was irradiated with UV for 15 to 20 s and then incubated overnight. A Hanovia 83A1 25-W lamp set at 58 cm from the surface on which the plates were placed was used for UV irradiation. After incubation, lysogenic colonies were ringed by a halo of lysis several millimeters in diameter. The halo was slightly turbid due to growth of lysogenic cells.

Phage stocks. Stocks of temperate phage were obtained by inducing logarithmic-phase cells of a lysogenic strain with UV. The cells were grown to a density of 10^8/ml in TYE broth. Five milliliters of the broth suspension was pipetted into a petri dish (100 by 15 mm), after which the suspension was irradiated for 5 min. During irradiation the cells were agitated on a rotary shaker. The cells were grown for 3 h at 37 C after irradiation, and the lysate was filtered through a 0.45-μm membrane filter (Millipore Corp.). The filtrate commonly had a titer of 5×10^8 PFU/ml.

Incubation temperatures. In general, phage assays, tests for lysogeny, and the production of phage stocks were carried out at 30 C for phage carrying a *ts* marker and at 37 C for wild-type phage. Double lysogens carrying phage with a *ts* marker can be and were incubated at 37 C without affecting phage carriage.

Method of genetic analysis. None of the conventional techniques that might be used to determine gene order of integrated phages are available for *C. diphtheriae*. However, the method set forth by Campbell (4) for analyzing gene order of prophages in double lysogens is applicable. The method requires no advance knowledge of the order of genes in the prophage map, the order of the prophages, or the coupling of the markers in the phages. The order of the prophages and of the genes in the prophages is established by determining the frequency of the individual markers in monolysogenic segregants, all of which carry a recombinant prophage. The method requires only two assumptions: (i) that the principle of progressive rarity holds, and (ii) that the two phage genomes are inserted in tandem. The principle of progressive rarity states that single crossovers in a given region are more common than any higher-order crossovers (doubles, triples, etc.) that involve the same region. We have assumed the validity of the principle. Evidence for the tandem arrangement of the phages must of necessity be indirect for the present since no independent method for mapping the prophage genomes is available. However, the segregation of recombinant-carrying monolysogens at a high frequency in itself indicates that the prophages are close enough to engage in frequent pairing and, in addition, are so close that no essential bacterial genes lie between them. If the latter were not true, the excision that leads to the removal of one complete phage genome would also remove any bacterial genetic material lying between the prophages and thus preclude the production of viable monolysogens. Another observation to be discussed later also supports the tandem arrangement of the prophages. This is the observation that in a number of instances the insertion of the second prophage occurs as a result of recombination of the superinfecting phage with the resident prophage.

The process by which different monolysogenic segregants can be produced from double lysogens and by which phage is excised can be easily visualized by looking at Table 6. By examining any one of the double lysogens diagramed there, it can be seen that single-crossover events between phages in tandem, held in register by chromosomal homology, will produce various monolysogens in direct proportion to the distance between the various genes.

Formation and recognition of heteroimmune double lysogens. To produce heteroimmune double lysogens, indicator plates containing the appropriate monolysogenic strain were prepared as in the phage assay, minus any added phage. A drop containing approximately 10^5 heteroimmune phage was

spotted onto the indicator plate and allowed to dry. The plate was then incubated for 24 h at 30 C, and a turbid macroplaque formed. Material was then taken from the center of the plaque with a loop and was streaked onto three "thin" TYE plates, each containing 10 to 15 ml of TYE agar. Three plates were necessary to insure the isolation of the relatively rare doubly lysogenic clones, and thin plates made it easier to identify the various colony types. The plates were incubated at 30 C for 40 h.

The resulting plates have a very characteristic appearance. There is mass lysis in the primary streak area. Among the well-isolated colonies, some are smooth and intact and usually contain C7 cells cured of phage, C7 monolysogens that contain the original phage, or C7 cells in which the superinfecting phage has displaced the original phage. Some of the isolated colonies have large sectors or "bites" taken out of the edge of the colony, probably the result of phage infection of a cured or monolysogenic colony during development on the plate. Finally, there are a few colonies of double lysogens that can only be distinguished by observing the plate under a dissecting microscope. When light is passed through the bottom of the plate, colonies of the heteroimmune double lysogens exhibit a unique morphology. One or more lines of lysis can be seen extending radially through the colony, undoubtedly the result of infection of a sector of a monolysogen by a heteroimmune phage. This radially striated appearance is quite different from that of a colony in which a whole segment has been lysed from the edge of the colony. Occasionally a colony will display both radial striations and loss of a segment, but it is the former that is crucial to the identification of a potential double lysogen.

Each presumptive double lysogen is restreaked to thin TYE plates and grown again for 40 h at 30 C. If the colony picked is a true double lysogen, there is no macroscopic lysis in the primary streak area and 80% or more of the isolated colonies exhibit radial striations. On the other hand, extensive lysis in the primary streak area indicates that the colony selected contained a monolysogen that had become contaminated with a heteroimmune phage. Finally, presumed double lysogens are recloned several times.

The double lysogen is verified by checking it for the presence of all parental phage markers. A broth culture of the double is induced with UV as previously described, and a sample of the phage released is plaqued on strain C7. Phage from 50 plaques are then characterized for their markers. If the strain is to be useful, all markers of the parents should be found among these phages. It should be noted that early in this study, some of the clones that were scored as double lysogens solely on the basis of phage immunity proved to be homozygous for other markers such as *tox* and *ts*. Therefore, a complete check is necessary to establish the extent of heterozygosity.

Isolation of monolysogens. It is possible to perform a genetic analysis of a heteroimmune double lysogen by collecting and characterizing a representative sample of monolysogenic segregants derived from it. The fact that a double lysogen produces monolysogens rapidly both complicates and simplifies the collection of monolysogens. On the one hand, cultures of double lysogens are always "contaminated" with monolysogens and to a certain extent other double lysogens as a result of single- and double-crossover events, respectively. This means that to collect an unbiased sample of monolysogens for genetic analysis one must begin each experiment with a single cell of the double lysogen. In principle this can be accomplished by streaking out the double lysogen and then analyzing the monolysogenic segregants that are thrown off in one or in a series of colonies of the double lysogen. In practice, the secondary infection of monolysogens by phage in the developing colony can produce distortions in the sample. As a result, a streak plate method used in the early phases of this study was replaced by a broth culture method in which secondary infections can be controlled. Although this method has the advantage of simplifying the collection of a representative sample of monolysogens, care must be taken to limit and to assess the level of clonality that develops (see below).

The broth culture method of producing monolysogens involves two successive single-cell isolations of the double lysogen to insure purity of the culture, followed by extensive passage to allow monolysogenic segregants to accumulate. The proportion of monolysogens in a culture increases dramatically, and a large pool of monolysogens composed of representatives of different clones is created. The relative proportion of the various classes of monolysogens will reflect the degree of segregation between the various markers in the prophages. Hence, by examining a large number of monolysogens, one should be able to map the sequence of their traits in the original double lysogen.

The following are the details of the experimental procedure. A stock culture of a heteroimmune double lysogen is streaked to a thin TYE agar plate and incubated for 24 h at 37 C. An inoculum taken from the largest radially striated colony on the plate is seeded into Tween-broth and grown to a density of 2×10^8/ml. The culture is then diluted in Tween-broth to a concentration of 0.2 cells/ml and distributed in 0.5-ml amounts to 50 tubes. Tween is included in the medium to prevent super-infection by phage. The tubes are incubated without shaking for 19 h (approximately 21 generations). At this time the cell concentration in the positive cultures (approximately five according to the Poisson distribution) is 10^6 to 10^7/ml. An inoculum is taken from each positive tube, streaked onto a thin TYE plate, and incubated for 24 h. The remaining material is taken up in 4.5 ml of Tween-broth and refrigerated at 4 C.

After 24 h the colonies on each of the streak plates are examined with a dissecting microscope to determine whether the parent culture is monolysogenic or doubly lysogenic. Most of the colonies in a clone derived from a doubly lysogenic cell are marked by radial striations, whereas all of the colonies are intact in a clone derived from a single monolysogenic cell. One culture containing a clone of double lysogens is then chosen to continue the experiment.

The double lysogen is now carried through a second single-cell isolation. The culture containing the doubly lysogenic clone is taken from the refrigerator and incubated with shaking until the cell density has reached 2×10^8/ml; after dilution to 0.2 cells/ml, the culture is distributed in 0.5-ml amounts to 100 tubes, which are then incubated without shaking for 19 h. At this time the entire contents of the positive tubes, i.e., those in which a culture has arisen from a single cell, are taken up into 4.5 ml of Tween-broth, transferred into screw-capped tubes, and grown at 37 C with shaking. They are periodically diluted 1:50 with fresh Tween-broth before the cell concentration exceeds 2×10^8/ml, and thus are kept in the logarithmic phase. After 90 h (approximately 108 generations), during which time monolysogenic segregants have accumulated to a level of approximately 40%, the material from each culture is diluted into broth and a sample is spread onto thin TYE plates so as to yield 400 to 500 isolated colonies. After 24 h of incubation, the colonies are examined under a dissecting scope and the proportion of monolysogens is determined for each plate. The median value for the proportion of monolysogens in the various cultures is calculated, and those plates that contain a percentage of monolysogens greater than the median value are discarded. This step is used to limit the level of clonality, and its justification will be discussed in the next section. Finally, monolysogens are selected from each of the remaining plates and their phages are characterized.

Analysis of clonality. The experimental basis for mapping prophage is to determine the frequency of each class of monolysogens that accumulates during the growth of a double lysogen. A class is defined as sharing the same genotype. Maximum resolution of the map would be obtained if each monolysogen in a sample was derived from a different clone. Although this is not practicable, the high rate of segregation of monolysogens (see below) makes it possible to obtain a fairly representative sample by the procedure just described. The accuracy of the map then depends on the fidelity of the sample, i.e., the degree to which it accurately reflects the frequency of segregation of each class of monolysogens. The question of fidelity is related to the problem of clonality and to sampling error. The following is an analysis of clonality in the present experiments. Sampling error will be discussed later.

To estimate the extent of clonality, it is necessary to know the rate at which monolysogens segregate from a double lysogen. An experiment in which replicate samples from a single culture were used to determine the rate is given in Table 1. The data show that the average rate of segregation is high, 4.7×10^{-3}/cell per generation. Calculation of the individual rates for each clone shows that the rate is reproducible. In a second, more extensive experiment involving four independently isolated heteroimmune double lysogens (Table 2), the overall rate is again 5×10^{-3}/cell per generation, and this value will be used in the following discussion. In both experiments, the rates indicate about one segregation event in every 200 cell generations.

Given the rate of segregation of monolysogens, it is possible to estimate the probability that various levels of clonality will be established in a series of replicate cultures grown from small inocula. The level any given clone attains, i.e., the fraction of the final population it will constitute, is determined by the cell concentration at the time the first founding cell enters the population. For example, if a monolysogen is segregated at the 25-cell stage, then the clone it establishes will constitute 4% of the final population, and if a second monolysogen is segre-

TABLE 1. *Rate of segregation of monolysogens from a double lysogen*[a]

Tube no.	No. of monolysogens		No. colonies counted
	24th generation	35th generation	
1	20	35	269
2	25	52	365
3	32	45	307
4	35	67	395
5	33	48	381
6	30	39	312
7	27	41	336
Total	202	327	2,365

[a] The broth method for single-cell isolation described in Materials and Methods was used to produce a culture of C7(γ) (β) and to isolate seven clones derived from that culture. The proportion of monolysogens was determined at the 24th and 35th generations, growth being sustained by continuous passage. The same number of colonies were counted at both times. The rate of segregation of monolysogens $m = (P_2 - P_1/G_2 - G_1)$, where P is the proportion of monolysogens present and G, the number of generations, is 4.7×10^{-3}.

TABLE 2. *Rate of segregation of monolysogens from double lysogens*[a]

Sample characteristic	Double-lysogen strain no.			
	1	2	3	4
No. of replicate clones tested	21	26	28	25
Monolysogens (%)				
Range	8.5–25	5–20.5	9.5–30	5.5–21.5
Mean	15.0	12.5	18.5	13.0
Median	13.5	12.5	19.0	13.5
Variance	23.0	18.5	30.5	23.0
SD[b]	4.8	4.3	5.5	4.8
m ($\times 10^3$)	4.8	4.5	6.8	5.2

[a] The broth method for single-cell isolation described in Materials and Methods was used to produce four genetically distinct, independently isolated double lysogens. Replicate clones were prepared of each double lysogen in the same manner. The percentage of monolysogens present in each clone was determined at the 28th generation. The mean value for m (the rate of segregation of monolysogens per cell per generation) is 5×10^{-3}.

[b] SD, Standard deviation.

gated at the 50-cell stage, that clone will constitute 2% of the final population, etc. Thus, in every culture originating from a single, doubly lysogenic cell, each class of monolysogenic segregants will contain numerous clones, each fixed in the population at a given level.

The probability of a segregational event occurring within any specified number of cell generations can be calculated with the aid of the Poisson formula, $P(r) = N^r e^{-N}/r!$. In this formula $P(r)$ is the proportion of cultures that have sustained r segregation events, and N is the average number of such events per culture. N is given by the number of cells in the culture times the rate of segregation of monolysogens. Given this formulation, it is possible to calculate for any given cell concentration the proportion of cultures that will have experienced 0,1,2,3. . . segregation events. From this one can calculate the maximum level of clonality that any single clone can contribute to the final population of a culture and the probability that this will occur.

As will become apparent, the $P(O)$ term is the most useful statistic obtained from the Poisson formulation. It gives the proportion of cultures that will have experienced zero segregation events, i.e., the probability that any single culture has yet to experience such an event. By calculating the $P(O)$ term at various cell concentrations, the probability of various levels of clonality can be established. When a series of cultures has reached the 10-cell stage, $P(O) = e^{-N} = e^{-(10 \times 0.005)} = 0.95$, and by the time the culture has reached the 33-cell stage $P(O) = 0.85$. Thus, at the 33-cell stage, 85% of the cultures will not yet contain a monolysogenic segregant. It follows from this that the largest single clone this fraction of tubes will eventually contain can only constitute 3% or less of the final population. Stated another way, this means that in any single culture there is a probability of only 0.15 that a single clone will contribute 3% or more to the final population.

In the mapping experiment, the theoretical levels of clonality are reduced even further by a selective procedure. It will be recalled that all cultures in which the proportion of monolysogens exceeds the median value are discarded, leaving only those with the median value or less for sampling. It is obvious that this procedure tends to eliminate cultures in which a single monolysogenic clone has achieved a high level of clonality. However, it is possible to be more precise and to calculate the probable extent of clonality in those tubes that are saved. The data from the experiment described in Table 2 can be used as the basis for the calculations. These data, expressed in the form of a histogram in Fig. 1, show the number of cultures containing various proportions of monolysogens. It can be seen that the population of cultures is distributed in a normal curve except for the slight skew toward higher values. This skew most likely represents the contribution of clonality to what is otherwise a normal distribution.

Using the knowledge that the distribution pattern approaches that of a normal curve, it is now possible to reestimate the proportion of cultures that will contain clones above or below a given level of clonality after the discard step. We have previously

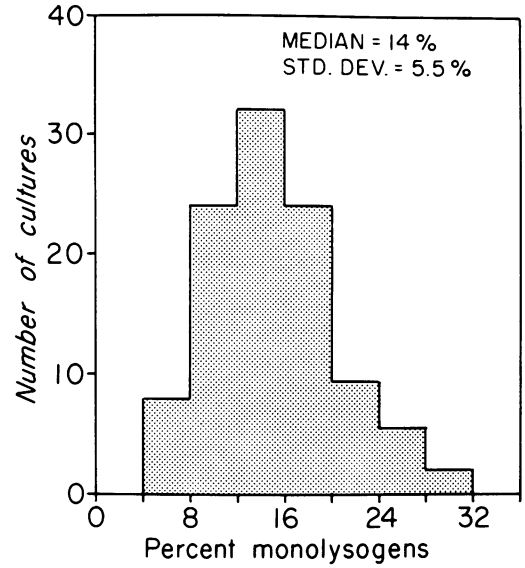

FIG. 1. *Accumulation of monolysogenic segregants in cultures of double lysogens. The data from the experiments summarized in Table 2 are pooled in this figure.*

calculated that 15% of the cultures will contain clones that individually constitute 3% of the final population. Given the median value and standard deviation of the distribution, it is possible to calculate the fraction of cultures that would contain various proportions of monolysogens exclusive of any clonality effects. For example, either by inspection of Fig. 1 or by using a statistical table of the area under a standard normal curve, it is posible to calculate that 50% of the cultures would normally contain a level of monolysogens of 14% or greater, 25% would contain 11 to 14%, whereas the remaining 25% would contain less than 11% monolysogens. It is apparent that if an additional 3% were added to any of 75% of the cultures, in this case those falling above the 11% level, they would contain more than 14% monolysogens and thus would be discarded with those above the median. The other 25% of the cultures, which is one-half of the group retained for sampling, could still hide a clone of 3%. Therefore, the probability that a 3% clone will occur in a culture and not be discarded is 0.15×0.50 (the probability of a 3% clone occurring times the probability that a 3% clone will not be discarded), or 0.075. Thus, the discard procedure decreases that fraction of cultures with clones of 3% or greater from 15 to 7.5% and conversely increases the fraction with clones of less than 3% from 85 to 92.5%. Similar calculations can be made for any level of clonality, but this illustration was chosen to demonstrate that there is less than a 10% chance that the level of clonality in a culture will be greater than 3%.

From this discussion it is clear that the contribution of any single clone to the final population of a culture will be less than 3%. However, its contribution to the population of monolysogens will depend

on the proportion of monolysogens finally attained in the experimental sample. In the mapping experiments, the cultures sampled contained 40% or more monolysogens. Thus, at the maximum, a clone that had achieved a 3% level in a culture would only contribute 7.5% (3/40%) to the sample of monolysogens. But in addition to the discard procedure, there is yet another aspect of the mapping experiment that reduces the impact of any individual clone on the final map. In the genetic crosses, monolysogenic segregants were collected from more than one sample, pooled, and then genetically characterized. As a result, the effect of a single clone appearing in one sample is reduced proportionately to its contribution to the total frequency of monolysogens in the pooled sample. It is obvious from this extended discussion that clonality is not a significant issue in the mapping experiments.

The analysis to this point has been concerned solely with the effect on clonality of the time at which a monolysogenic segregant appears. However, mutations affecting the growth rate of a monolysogenic segregant could also influence clonality. Since there is no practical way of estimating the frequency of such mutations, there is no way of estimating the probabilities of various levels of clonality as was done previously from the segregation frequency. However, the discard procedure does limit the level of clonality that could be introduced due to an increased growth rate in an early segregant. Early segregants with a decreased growth rate do not constitute a problem. It seems likely that growth rate mutations are rare and contribute little to clonality.

RESULTS

Analysis of double lysogens. A series of double lysogens was prepared as described in Materials and Methods. The original monolysogen, the heterologous superinfecting phage, and the derived double lysogen are listed in Table 3. The double lysogens in group A were used to determine the prophage map and will be discussed in this section. Those in group B will be discussed later. Monolysogenic segregants were isolated from the double lysogens in group A, and their prophages were characterized. The data are given in Tables 4 and 5.

The mode of analysis for each double lysogen was similar and will be illustrated with the data from double lysogen no. 1. First, the frequency of the markers in all the monolysogens was determined, and then the marker with the highest frequency was identified (Table 4). According to the Campbell analysis, this marker lies closest to one end of the double prophage. The high frequency shows that the largest

TABLE 3. *Heteroimmune double lysogens*[a]

Strain no.	Monolysogen	Superinfecting phage	Double lysogen isolated
Group A			
1	C7(*imm-βts30 tox-30*)	*imm-γ + tox-118*	C7(*imm-γ + tox-118*) (*imm-βts30 tox-30*)
2	C7(*imm-βts30 tox-30*)	*imm-γ + tox-118*	C7(*imm-β + tox-118*) (*imm-γts30 tox-30*)
3	C7(*imm-γ + tox-118*)	*imm-βts30 tox-30*	C7(*imm-γts30 tox-30*) (*imm-β + tox-118*)
4	C7(*imm-βts31 tox*$^+$)	*imm-γ + tox-118*	C7(*imm-β + tox-118*) (*imm-γts31 tox*$^+$)
5	C7(*imm-γ + tox-118*)	*imm-βts31 tox*$^+$	C7(*imm-βts31 tox*$^+$) (*imm-γ + tox-118*)
6	C7(*imm-βts45 tox-30*)	*imm-γ + tox-118*	C7(*imm-γ + tox-118*) (*imm-βts45 tox-30*)
7	C7(*imm-γ + tox-118*)	*imm-β h ts61 tox*$^+$	C7(*imm-β + h tox*$^+$) (*imm-γts61 + tox-118*)
Group B			
8	C7(*imm-β h tox-30*)	*imm-γ + tox-45*	C7(*imm-β + tox-45*) (*imm-γ h tox-30*)
9	C7(*imm-γ + tox-45*)	*imm-β h tox-30*	C7(*imm-γ h tox-30*) (*imm-β + tox-45*)
10	C7(*imm-γ + tox-118*)	*imm-βts30 tox*$^+$	C7(*imm-γts30 tox*$^+$) (*imm-β + tox-118*)
11	C7(*imm-γ + tox-118*)	*imm-βts31 tox*$^+$	C7(*imm-γts31 tox*$^+$) (*imm-β + tox-118*)
12	C7(*imm-γ + tox-118*)	*imm-βts45 tox*$^+$	C7(*imm-βts45 tox*$^+$) (*imm-γ + tox-118*)
13	C7(*imm-γ + tox-118*)	*imm-βts61 tox*$^+$	C7(*imm-βts61 tox*$^+$) (*imm-γ + tox-118*)

[a] The CRM phenotypes of the *tox* markers are: *tox-118*, CRM-; *tox-30*, CRM30; *tox-45*, CRM45; *tox*$^+$, CRM62.

TABLE 4. *Frequency of markers in monolysogenic segregants*

Double-lysogen strain no.	No. of monolysogens examined	Variable marker *V*	Marker frequency (%)						
			imm		*V*		Phenotype of *tox*		
			β	γ	*V*	+	CRM62	CRM30	CRM⁻
1	172	*ts30*	19	81	88	12		100	0
2	92	*ts30*	82	18	92	8		100	0
3	165	*ts30*	21	79	10	90		<1[a]	>99
4	170	*ts31*	21	79	76	24		>99	<1
5	169	*ts31*	78	22	27	73	2		98
6	254	*ts45*	24	76	50	50		100	0
7	340	*ts61*	79	21	54	46	2		98
7	340	*h*	79	21	6	94	2		98
8	432	*h*	82	18	77	23	<1	>99	
9	181	*h*	18	82	23	77	<1	>99[b]	

[a] In each cross in which the frequency of CRM is given in this manner, one segregant with the minority CRM designation was found.

[b] In cross 9, the CRM marker was actually CRM45 rather than CRM30.

TABLE 5. *Major classes of monolysogenic segregants*[a]

Double-lysogen strain no.	Variable markers		No. in classes			
	V	*tox*	*imm-γ V tox*	*imm-γ + tox*	*imm-β V tox*	*imm-β + tox*
1	*ts30*	*tox-30*	120	20	32	
2	*ts30*	*tox-30*	17		68	7
3	*ts30*	*tox-118*	17	112		34
4	*ts31*	*tox*⁺	93	40	36	
5	*ts31*	*tox-118*		38	45	86
6	*ts45*	*tox-30*	66	128	60	
7	*ts61*	*tox-118*	68		114	149
7	*h*	*tox-118*		71	20	243
8	*h*	*tox-30*	78		256	97
9	*h*	*tox-45*	41	107		32

[a] Monolysogens carrying phage of other minority classes were present in some of the samples and account for the differences in the totals from those given in Table 4.

amount of recombination occurs between this marker and all other markers or, conversely, that the smallest amount of recombination occurs between this marker and the end of the phage chromosome. The allele of this high-frequency marker is of course a terminal marker in the other prophage. Thus in double lysogen 1, *tox-30* is the marker at one end of the double prophage, and the derived gene sequence at this point is . . . *tox-118* . . .*tox-30*. The *tox* gene is terminal in all of the double lysogens that have been prepared, and the assignment of the *tox* alleles between the two prophages is determined directly from an inspection of their frequencies in the monolysogens. It should be noted that both *tox* alleles were present in each parent double lysogen. This was established when the phage from the double lysogen was checked to insure that all markers were present.

In the second step of the Campbell analysis, the coupling of the remaining markers is determined by examining those present in the class of segregants carrying the internal, terminal marker. In the case of double lysogen 1, this would have been the class carrying the *tox-118* marker. However, the *tox* marker is so close to the end of the prophage that this class of segregants is either missing, as in this cross, or is so small as to be a questionable basis for judgment. Thus, to determine the coupling of genes, a different method was used. As will be seen, this method generates two possible coupling arrangements for each double lysogen and ultimately two possible prophage maps.

The data in Table 5 show that three major classes of monolysogenic segregants were produced by each double lysogen. It is reasonable to assume that each of these major classes is the consequence of a single crossover event. With the position of each of the *tox* markers fixed in each cross, there are then eight possible ways in which the remaining genes could be arranged. Of these eight possible arrangements, only two from each cross will generate the major classes of segregants by means of single crossover events. The two possible coupling arrangements for each double lysogen are given in Table 6. One of the arrangements places the *imm-β* and *imm-γ* markers in a terminal position (orientation 1), and the other places them in an interior position (orientation 2). For each orientation, the relative distance of each gene from the *tox* markers, i.e., from the end of the prophage chromosome, can be determined from the frequency of the alleles linked on the same phage genome as the internal *tox* marker as given in Table 4. Thus with double lysogen 1 in orientation 1, the markers γ and + are coupled on the same genome with *tox-118*; therefore,

TABLE 6. *Possible prophage orientations in the double lysogens*[a]

Double-lysogen strain no.	Orientation 1	Orientation 2
1	---*imm-γ + tox-118* *imm-βts30 tox-30*---	---*ts30 imm-β tox-118* *+ imm-γ tox-30*---
2	---*imm-β + tox-118* *imm-γts30 tox-30*---	---*ts30 imm-γ tox-118* *+ imm-β tox-30*---
4	---*imm-γ + tox-118* *imm-βts31 tox*$^+$---	---*ts31 imm-β tox-118* *+ imm-γ tox*$^+$---
6	---*imm-γ + tox-118* *imm-βts45 tox-30*---	---*ts45 imm-β tox-118* *+ imm-γ tox-30*---
7	---*imm-β + tox*$^+$ *imm-γts61 tox-118*---	---*ts61 imm-γ tox*$^+$ *+ imm-β tox-118*---
7	---*imm-β h tox*$^+$ *imm-γ + tox-118*---	---*+ imm-γ tox*$^+$ *h imm-β tox-118*---
8	---*imm-β + tox-45* *imm-γ h tox-30*---	---*h imm-γ tox-45* *+ imm-β tox-30*---
9	---*imm-γ h tox-30* *imm-β + tox-45*---	---*+ imm-β tox-30* *h imm-γ tox-45*---

[a] In diagraming the double lysogens, the bacterial chromosome is represented by --- and connects directly with the prophage DNA. The first prophage is upper in the diagram, and the second is below it. The DNA of the two phages is continuous; i.e., the phages are in tandem.

the immunity gene is 81% and the *ts30* marker is 12% from the end of the phage chromosome. This last determination is identical with the third step of the Campbell analysis.

By applying this analysis to the double lysogens in group A, one can identify the position of all the genes relative to the *tox* marker and thus generate a prophage map for each possible orientation. In calculating map distances, the data for the *imm* and *tox* markers in all the experiments were combined, as were the data for the two experiments involving *ts30* and *ts31*. The maps are shown in Fig. 2 along with the vegetative map published by Singer (19). It is evident on inspection that both prophage maps represent a cyclic permutation of the vegetative map and could be generated by circularization of the vegetative phage chromosome followed by its insertion via an attachment site. The attachment site would lie between the *imm* and *tox* markers in orientation 1 and between *tox* and *h* in orientation 2. The mechanism of insertion follows that proposed by Campbell for lambda phage (3).

At the present time, an unequivocal choice cannot be made between the two prophage models. However, there is some evidence suggesting that orientation 1 is more likely than 2. Thus, among the minor classes of segregants isolated from double lysogen 7, two, *imm-β h tox+* and *imm-β + tox+*, contained *tox+* the internal *tox* marker. In each case they were present in large enough numbers, six each, so that the coupling relationships of the markers could have been determined in this experiment by identifying these alleles associated with *tox+*. If this is done, the coupling relationships coincide with those postulated in orientation 1. A similar correlation was also seen in one other cross in which the number of minority-class segregants was significant. A second piece of evidence supporting orientation 1 comes from an analysis of the mechanism by which double lysogens are produced. As will be seen, orientation 1 is most compatible with the findings.

Validity of the crosses. An inspection of the group A crosses in Table 3 shows that varying the position of markers in the double lysogen from cross to cross did not affect their relative map position. The frequencies varied no more than is expected from random sampling error. Thus, coupling relationships do not appear to influence the arrangement of the markers. The reproducibility of the data suggests that clonality did not produce any significant bias in the results. If there was any clonality, then it must have been hidden by the random sampling error, which in no experiment was greater than 3%. Therefore, the extent of clonality must have been less than 3%, just as predicted in the theoretical argument.

It is evident from an inspection of the data derived from double lysogens 8 and 9 (see Tables 4 and 5) that the *h* marker is considerably further from the *tox* marker in these crosses than it is in cross 7, on which the map is based. The difference between these crosses is that a recombinant phage, γ^{tox-45}, was used in crosses 8 and 9, whereas the wild-type γ phage was used in all group A crosses. Phage γ^{tox-45} was derived from a prophage cross between wild-type gamma, $\gamma^{tox-118}$, and β^{tox-45} and probably has a greater though unknown amount of homology with beta phage than with wild-type γ phage. Holmes and Barksdale (15) observed that crosses between beta and gamma phage yielded lower frequencies of recombination than did crosses between homologous beta phages. They postulated that this was due to regions of nonhomology between the heterologous phages and showed that the frequency of recombination in a given area increased as the degree of homology was increased. Thus, the higher frequency of recombination in the *h* region in crosses 8 and 9 compared with cross 7 is probably due to the greater homology of the phages in the former crosses. On the basis of this analysis, crosses 8 and 9 were not used in generating the prophage maps. The map was generated against a constant genetic background so that the relative distances are comparable from one cross to the other.

Sampling error has been computed for the

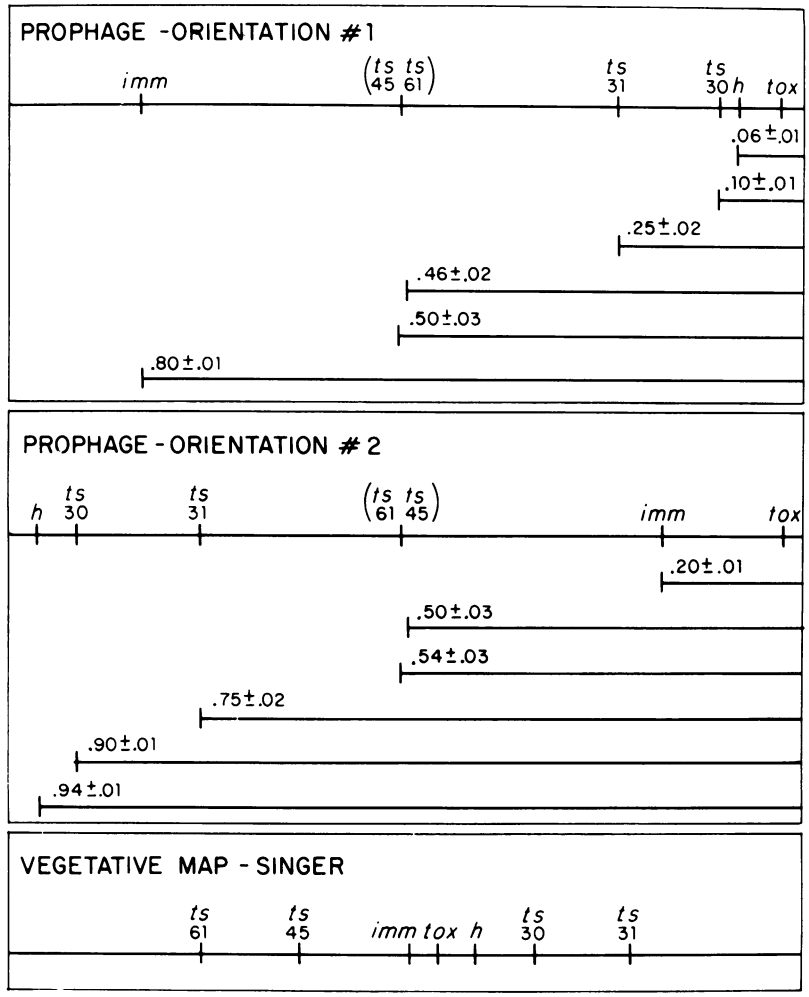

FIG. 2. *Two possible orientations of the prophage map of corynephage. The relative distances on the vegetative map are approximately those diagramed by Singer (19). The standard errors of the prophage map distances are indicated.*

map distances given in Fig. 2. The standard error is calculated from the formula $s = pq/n$, where p = the frequency of the characteristic counted, $q = 1 - p$, and n = the number of monolysogens characterized. As seen from the data, the only markers whose order is not clearly resolved are *ts45* and *ts61*.

Mechanism of integration of the second phage. The intergration of a second phage into a monolysogen in tandem with the first phage could occur by two basic mechanisms. The second phage could be inserted at either end of the resident phage through specific recombination at attachment sites, following the model developed by Campbell for insertion of lambda phage (3). Alternatively, it could be inserted through recombination with the resident prophage in any region of genetic homology (2). If insertion occurred through an attachment site or by means of recombination in the region that is outside the two markers terminal for the tandem prophages, then the parental coupling of the markers would be retained. However, the order of the prophages and hence the sequence of their markers would differ, depending upon at which end the insertion had occurred. If recombination occurred in regions between these markers, then the coupling relationships would differ from those present in the parental phages. Some of these possibilities can be distinguished from one another by the types of monolysogenic segregants produced by the double lysogen.

In the series of 13 double lysogens listed in Table 3, five, 1, 5, 6, 12, and 13, have retained the marker couplings of the parental phages,

the new phage having been inserted to the left of the terminal immunity marker. Seven of the double lysogens, 2, 3, 4, 8, 9, 10, and 11, appear to have been formed by a recombination event involving single crossovers in areas of genetic homology inside the terminal markers, and one, 7, seems to have been produced by multiple events that could have involved both types of mechanisms. These results suggest that recombination is frequently used in insertion. However, in those cases where parental phage gene couplings were retained, it is not possible to discriminate between the two mechanisms that might have been used. It should be noted that in over 30 double lysogens produced in this laboratory, there has been no case in which insertion occurred between the terminal *tox* gene and the beginning of the bacterial chromosome. This indicates that insertion is occurring preferentially at the other end of the prophage and suggests that the two terminal attachment sites are not coequal.

In the above analysis of insertion mechanisms, it was assumed that the gene sequence in the prophage map was given by orientation 1 (see Fig. 2). The logic to this selection is that orientation 1 permits each double lysogen to be derived by a single crossover event between resident and superinfecting phage. If orientation 2 were assumed, then minimally a second event involving a double crossover would have to follow the first single crossover. This is not to say that such combinations of events do not occur, as double lysogen 7 seems to attest, but rather that one would expect their frequency to be much lower. This predictive finding strengthens the argument that orientation 1 is correct for the prophage map.

DISCUSSION

The present study suggests that the corynephages beta and gamma are similar functionally to lambda phage of *Escherichia coli*. The production and behavior of the double lysogens, including their apparent mode of insertion and excision via the Campbell model, attests to their similarity. However, although the general patterns of activity appear to be broadly similar, it is well to point out that critical facts established for lambda are not yet known for the corynephages. Thus the linear insertion for lambda prophage into the host chromosome involving the interaction of phage and host attachment sites and mediated by a gene for integration (5) is without parallel in the corynephage system. Despite this, lambda seems a reasonable working model from which to speculate.

The major finding of this study is that the prophage map of corynephage beta appears to be a cyclic permutation of its vegetative map. In the prophage map, the gene for immunity is at one end of the map and the gene for toxin is at the other. The gene for toxin is immediately adjacent to the host chromosome. The data presented clearly established that *imm*, *ts30*, *ts31*, *h*, and *tox* genes and the gene pair *ts45* and *ts61* all fall into a sequence consonant with a cyclic permutation of the vegetative map. Given the data at hand, the position of the two genes *ts45* and *ts61* cannot be distinguished, though they appear to be properly placed on the basis of their known position on the vegetative map. Additional data agreeing with these general findings were obtained earlier in this study when a streak plate method was being used for collecting monolysogenic segregants. This method was abandoned when it was realized that uncontrollable interactions between monolysogenic segregants and free phage in the colony could obscure the characteristics of the primary monolysogenic segregants. Despite this, the data obtained in this way were in good agreement with those derived from the more controllable tube method.

The terminal position of the *tox* marker is established regardless of which orientation of the prophage is used. This fact is of some importance in speculating about the origin of the *tox* gene in phage. The position of the *tox* gene is consonant with the hypothesis that a bacterial *tox* gene or its precursor was incorporated into the phage through an abnormal excision. Though this possibility makes it less necessary to consider a phage gene as participating in whole or in part in the formation of *tox*, the report by Goff (12) that an ADP-ribosylating gene is produced during the infection of *E. coli* by phage T4 suggests that such a possibility cannot be ignored. A eukaryotic origin for *tox* has also been postulated (20), but Goff's finding and the recent discovery (16) that an extracellular toxin produced by *Pseudomonas aeruginosa* also has ADP-ribosylating activity makes this hypothesis less attractive.

Singer (19) observed three inconsistencies when data obtained from superinfection of an induced monolysogen were analyzed on the basis of the vegetative map derived from mixed infection. He noted that in certain crosses recombination frequencies between *h'* and *ts* markers to the right of *h'* were higher than expected, whereas those to the left were lower than expected. In other crosses, recombination frequencies between *ts* markers spanning the *imm. . . .h* region were asymmetric. Finally, in crosses in which the *h'* and *imm* markers were present on the same phage genome as *ts*

markers, he observed that the frequency of these nonselected markers in the non-*ts* recombinants was unexpectedly high. Two of these inconsistencies are better understood if one postulates, as did Singer, that the superinfecting phage and the resident prophage interact.

The first inconsistency can be rationalized by noting that the increase in the frequency of the right-hand *ts* markers is a logical consequence of the insertion of prophage into the host chromosome via a break that splits the *h'* marker from the right hand markers. The distance between these markers would be increased in the prophage and result in a higher rate of recombination. However, the corresponding decrease in the recombination of the left hand *ts* markers can only be explained on this formulation by invoking more complex interactions. The second inconsistency does not require invocation of a cyclically permuted prophage, but, as Singer has suggested, it could be explained by preferential insertion of superinfecting phage at one end of the resident prophage. Our data on integration appear to support just such a preferential insertion. Finally, there is no explanation for the last inconsistency, and the details given in Singer's paper do not provide an adequate basis for speculation.

This investigation began with the observation that phage produced by induced doubly lysogenic cells exhibited a high rate of recombination between the *imm* and *tox* markers. That observation was made on an extensively passaged doubly lysogen and probably reflected the fact that monolysogenic segregants had accumulated to a high level in the culture. Nevertheless, the results did indicate that a high rate of recombination had occurred overall. An examination of the data on which this general observation was made (11) established the high rate of recombination between these markers in an even more convincing manner. In a study of single bursts of a double lysogen, both recombinant classes involving *imm* and *tox* were produced in 11 out of 16 bursts, and the proportion of recombinants in the total population of these bursts achieved a minimum level of 34%. This high rate of recombination accompanying induction is a phenomenon that itself requires an explanation, but the fact of its occurrence between these two markers suggests that *imm* and *tox* were widely separated at the time recombination occurred. In contrast, both Holmes and Barksdale (15) and Singer (19) found that the rate of recombination between these markers in vegetative crosses approached 1% at best. Although these map distances are not directly comparable even when normalized, their differences suggest that the distance involved in prophage recombination is much larger. Finally, it should be pointed out that the high rate of recombination observed in prophage crosses also favors the first orientation of the prophage map, since the distance between the *imm* and *tox* markers is increased in this orientation and is unchanged in the second when compared with the distance between them in the vegetative map.

Another point of interest is our finding that in the prophage map the *imm* gene is located either 20% from the end of the prophage genome (orientation 1) or 20% from the *tox* gene (orientation 2). This implies that there is considerable distance between the *imm* and *tox* markers on the vegetative map, a finding somewhat at variance with Singer's results (19). One possible explanation is that the distance in the prophage map is more apparent than real. If one prophage is preferentially excised in the double lysogens, as, for example, at the same site at which phage is preferentially inserted, then this would elevate the population of monolysogenic segregants containing the other *imm* marker, giving the appearance of recombination in the region between the *imm* marker and the bacterial chromosome when in fact none had occurred. At the present time we have no firm evidence with which to support this speculation.

Finally, in the dilution method for single-cell isolations used in producing a representative population of monolysogens, the possibility that more than one cell was added to any one tube at the time of inoculation needs to be considered. To reduce or eliminate clumping, the samples were agitated vigorously by vortexing at the time of dilution. Nevertheless, it is possible and even likely that more than one cell was present in some tubes since cell separation is often incomplete after division of *C. diphtheriae*. The crucial question is whether any given tube received cells with different genetic compositions. This question is formally equivalent to asking what the probability is that a single, doubly lysogenic cell will segregate a monolysogen, or produce a new double lysogen either by mutation or internal recombination within the first few divisions. Since the probability of segregating a monolysogen is much greater than either of the other two events, the analysis of clonality undertaken in Materials and Methods sets the limits on the impact that these events may have on the final population. As indicated in that discussion, there is a probability of less than 0.075 that any single monolysogenic clone will contribute 3% or more to the final popula-

tion of monolysogens from which the positions of the genes on the prophage map are determined.

ADDENDUM

The recent work of Holmes (14) and Laird and Groman (17) establishing the orientation of the *tox* gene in vegetative phage and prophage, respectively, is compatible with the hypothesis that the beta phage attachment site lies between the *imm* and *tox* genes. This establishes orientation 1 of the prophage map even more firmly.

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LITERATURE CITED

1. **Barksdale, W. L., and A. M. Pappenheimer, Jr.** 1954. Phage-host relationships in nontoxigenic and toxigenic diphtheria bacilli. J. Bacteriol. 67:220-232.
2. **Calef, E.** 1967. Mapping of integration and excision crossover in superinfection double lysogens for phage lambda in *Escherichia coli*. Genetics 55:547-556.
3. **Campbell, A. M.** 1962. Episomes. Adv. Genet. 11:101-145.
4. **Campbell, A. M.** 1969. Episomes. Harper & Row Publishers, New York.
5. **Campbell, A. M.** 1971. Introduction to lambda, p. 3-44. *In* A.D. Hershey (ed.), The bacteriophage lambda. The Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. **Freeman, V. J.** 1951. Studies on the virulence of bacteriophage-infected strains of *Corynebacterium diphtheriae*. J. Bacteriol. 61:675-688.
7. **Groman, N. B.** 1953. Evidence for the induced nature of the change from non-toxigenicity to toxigenicity in *Corynebacterium diphtheriae* as a result of exposure to specific bacteriophage. J. Bacteriol. 66:184-191.
8. **Groman, N. B.** 1955. Evidence for the active role of bacteriophage in the conversion of nontoxigenic *Corynebacterium diphtheriae* to toxin production. J. Bacteriol. 69:9-15.
9. **Groman, N. B., and D. Bobb.** 1955. The inhibition of adsorption of *Corynebacterium diphtheriae* phage by Tween 80. Virology 1:313-323.
10. **Groman, N. B. and M. Eaton.** 1955. Genetic factors in *Corynebacterium diphtheriae* conversion. J. Bacteriol. 70:637-640.
11. **Groman, N. B., M. Eaton, Z. K. Booher.** 1958. Studies of mono- and polylysogenic *Corynebacterium diphtheriae*. J. Bacteriol. 75:320-325.
12. **Goff, C. G.** 1974. Chemical structure of a modification of the *Escherichia coli* ribonucleic acid polymerase and polypeptides induced by bacteriophage T4 infection. J. Biol. Chem. 249:6181-6190.
13. **Hermann, G. J., M. S. Moore, and E. I. Parsons.** 1958. A substitute for serum in the diphtheria *in vitro* toxigenic test. Am. J. Clin. Pathol. 29:709-712.
14. **Holmes, R. K.** 1976. Characterization and genetic mapping of nontoxinogenic *(tox)* mutants of corynebacteriophage beta. J. Virol. 19:195-207.
15. **Holmes, R. K., and L. Barksdale.** 1969. Genetic analysis of tox^+ and tox^- bacteriophages of *Corynebacterium diphtheriae*. J. Virol. 3:586-598.
16. **Iglewski, B. N., and D. Kabat.** 1975. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. Proc. Natl. Acad. Sci. U.S.A. 72:2284-2288.
17. **Laird, W., and N. Groman.** 1976. Orientation of the *tox* gene in the prophage of corynebacteriophage beta. J. Virol. 19:228-231.
18. **Matsuda, M., C. Kanei, and M. Yoneda.** 1971. Temperature sensitive mutants of nonlysogenizing corynebacteriophage: their isolation, characterization and relation to toxinogenesis. Biken J. 14:119-130.
19. **Singer, R.** 1973. Temperature sensitive mutants of toxinogenic corynebacteriophage. Virology 55:347-356.
20. **Uchida, T., D. M. Gill, and A. M. Pappenheimer. Jr.** 1971. Mutation in the structural gene for diphtheria toxin carried by temperate phage B. Nature (London) New Biol. 233:8-11.
21. **Uchida, T., A. M. Pappenheimer, Jr., and R. Greany.** 1973. Diphtheria toxin and related proteins 1. Isolation and properties of mutant proteins serologically related to diphtheria toxin. J. Biol. Chem. 248:3838-3844.