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Orientation of the *tox* Gene in the Prophage of Corynebacteriophage Beta

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The orientation of the gene for diphtheria toxin, *tox*, in the prophage of converting corynebacteriophage beta has been determined. The orientation of *tox* in prophage and that reported simultaneously by Holmes (1976) for vegetative phage are compatible with the hypothesis that beta phage is inserted into the chromosome of its bacterial host by means of a mechanism similar to that described for lambda phage, and that the phage attachment site lies between the *tox* and *imm* genes. The position of three *tox* mutations that are phenotypically CRM- has also been determined. Relative to the *tox-45* mutation, they are located more proximally to the end of the *tox* structural gene that corresponds to the amino terminal of diphtheria toxin.

The genetic map of vegetative corynephage beta has been described by Singer (9). On this map, the structural gene for diphtheria toxin, *tox*, and the phage immunity gene, *imm*, are centrally located, flanked on one side by the genes for phage head synthesis and on the other by genes for tail synthesis. Recently we have mapped the prophage of beta (7). The prophage map appears to be a cyclic permutation of the vegetative map in which the *tox* gene is now positioned at one end of the prophage genome and the *imm* gene is positioned at the other. This permutation agrees with the Campbell model for the insertion of lambda phage (1), assuming that the phage attachment site lies between *tox* and *imm* on the vegetative map.

The position of the *tox* gene in beta prophage has suggested a mechanism for the origin of converting corynephage. Converting phage could have been formed as a result of an illegitimate recombination between an ancestral nonconverting phage and a strain of *Corynebacterium diphtheriae* carrying the *tox* gene. Further study of this possibility would be facilitated if the orientation of the *tox* gene in the phage genome were known. To determine this orientation, recombination experiments between selected *tox* mutants were performed.

Uchida et al. (10) isolated two *tox* mutants, β^{tox-30} and β^{tox-45}, that produce fragments of toxin with molecular weights of 30,000 and 45,000, respectively. Both fragments retain enzymatic activity associated with fragment A of diphtheria toxin, i.e., the ability to transfer adenosine diphosphate ribose from nicotinamide adenine dinucleotide to elongation factor 2. In addition, Michel et al. (8) and DeLange et al. (3) have established that the amino terminal of the toxin protein is in the A fragment. Given this information, the orientation of the *tox* gene can be determined by experiments in which phages carrying the *tox-30* and *tox-45* mutations are tested for their ability to produce tox^+ recombinants.

MATERIALS AND METHODS

Strains of phage and bacteria. *C. diphtheriae* strain C7 was taken from our stock collection. Phages β^{30} and β^{45} were kindly sent to us by A. M. Pappenheimer. Following the nomenclature proposed by Holmes for *tox* mutants (5), these strains will be referred to as β^{tox-30} and β^{tox-45}. Phages $\beta^{tox-116}$, $\beta^{tox-117}$, and $\gamma^{tox-118}$ were isolated and characterized in this laboratory (6), and phage γ^{tox-45} was produced by crossing β^{tox-45} and $\gamma^{tox-118}$. Phages β^{tox-30} and β^{tox-45} produce proteins that cross-react serologically with diphtheria toxin. The phenotypes of these strains are designated CRM30 and CRM45, respectively, and CRM62 will be used to designate strains that produce material serologically identical with diphtheria toxin. Phages containing the *tox-116*, *tox-117*, and *tox-118* alleles do not produce any detectable cross-reacting material and are designated CRM-.

Methods. The various methods used in producing and characterizing doubly lysogenic strains and isolating monolysogenic segregants, the phage methods, the in vitro method for determining the CRM phenotypes, and the media used were described previously. (6, 7). In the present study, 2 ml of calf serum was used as the supplement in preparing the in vitro plates. It is worth reiterating that the CRM30, CRM45, and CRM62 products of the *tox* gene can be distinguished from one another by the in vitro test.

Design of the experiment. The experiments to be described draw on the fact that many doubly lysogenic strains produce monolysogenic segregants that carry a recombinant prophage. By analyzing the phages from a large number of monolysogenic segregants, one can determine the order of the prophages in the parent double lysogen, the markers carried by each, and the extent of recombination between any of their genes (2, 7). In the present experiment, strain C7 of *C. diphtheriae* was lysogenized with two phages, β^{tox-30} and γ^{tox-45}, each carrying a different mutation in the *tox* gene. Monolysogenic segregants were isolated, and the *tox* genotype of each segregant was established from its CRM phenotype. The orientation of the *tox* gene was deduced from the specific mating conditions under which the greatest number of CRM62-producing recombinants was formed. To establish the location of the *tox* mutations in the CRM-strains, similar experiments were performed with double lysogens in which each of the CRM-mutants was coupled with a second phage carrying the *tox-45* allele.

The rationale for determining *tox* gene orientation is illustrated in Fig. 1. It can be seen that the order of insertion of the two prophages and the orientation of the *tox* gene both have an important bearing on the extent to which one expects to recover tox^+ (CRM62-producing) recombinants in the monolysogenic segregants. For each order of prophage insertion, only one hypothetical orientation of the *tox* gene is expected to yield significant numbers of tox^+ recombinants. One order requires only a single crossover, whereas the other demands a triple crossover. Since the order and genotypes of the prophages in double lysogens can be established experimentally, it follows that the orientation of the *tox* gene can be deduced by establishing which order yields the greatest number of tox^+ recombinants. It should be emphasized that this can be done because it is known that both *tox-30* and *tox-45* produce CRMs containing an enzymatically active A fragment, and it is this fragment that contains the NH_2 terminal of the toxin molecule.

RESULTS

Heteroimmune double lysogens C7(β^{tox-30}) (γ^{tox-45}) and C7(γ^{tox-45}) (β^{tox-30}) were isolated. Cultures derived from single cells of each strain were passaged in Tween-broth in order to allow monolysogenic segregants carrying phage recombinants to accumulate. After approximately 100 generations, the cells were diluted and plated to yield well-isolated colonies. Monolysogenic colonies, identified visually, were picked to master plates to tryptose-yeast extract agar with sterile toothpicks and after growth at 37 C were tested by the in vitro method for their CRM product. Monolysogeny was verified by checking each clone for the immunity pattern of its phage and by observing the clone both on the master and in vitro plates. Doubly lysogenic clones and carrier cultures containing heteroimmune phages exhibit lysis on these plates, whereas monolysogenic clones do not. Calf serum was used as the supplement on the in vitro plates since Tween 80 present in the alternate supplement inhibits the adsorption of corynephages β and γ (4) and hence would mask lysis.

The result of these first two experiments are given in Table 1. The data for the CRM phenotypes exhibited by the monolysogens establish the order of the prophage in the double lysogens and give the number of CRM62-producing recombinants formed. It can be seen that CRM62-producing strains were only recovered in experiment 2, in which the order of the two prophages was γ^{tox-45}, β^{tox-30}. From the argument detailed in Fig. 1, these data establish that orientation 2, in which the carboxyl terminal of the *tox* gene is immediately adjacent to the bacterial chromosome, is correct.

Additional crosses were performed to establish the position of the mutations in the CRM-strains relative to the mutation in the *tox-45* mutant (Experiments 3-8 in Table 1). In each case, CRM62-producing recombinants were isolated only when the order of the *tox* genes in the prophages was CRM45, CRM-. Given the orientation of *tox* as established in the first two crosses, this indicates that the mutations in the CRM- strains are located more proximally to the amino terminal in the *tox* structural gene than the mutation in the *tox-45* strain, assum-

TABLE 1. *Recombinant phages produced by doubly lysogenic C. diphtheriae*[a]

Expt no.	*tox* alleles		Monolysogens	
	Prophage 1	Prophage 2	No. examined	Phenotypes
1	*tox-30*	*tox-45*	1,649	1,649 CRM45
2	*tox-45*	*tox-30*	2,202	2,184 CRM30 6 CRM45 12 CRM62
3	*tox-45*	*tox-116*	1,220	1,200 CRM- 2 CRM45 18 CRM62
4	*tox-116*	*tox-45*	625	5 CRM- 620 CRM45
5	*tox-45*	*tox-117*	646	638 CRM- 2 CRM45 6 CRM62
6	*tox-117*	*tox-45*	889	16 CRM- 873 CRM45
7	*tox-45*	*tox-118*	1,066	1,054 CRM- 3 CRM45 9 CRM62
8	*tox-118*	*tox-45*	896	3 CRM- 893 CRM45

[a] The CRM phenotypes of the *tox* markers are: *tox-30*, CRM30; *tox-45*, CRM45; *tox-116*, *tox-117*, and *tox-118*, CRM-. The tox^+ recombinants are given the CRM62 designation.

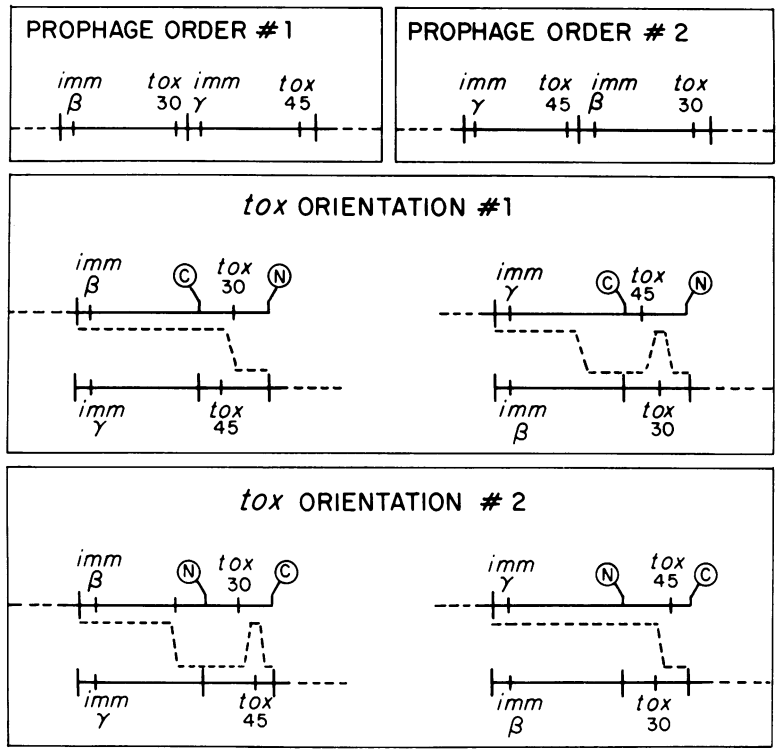

FIG. 1. *The effect of tox gene orientation on the production of tox⁺, CRM62-producing recombinants. In the upper portion of the diagram the two prophages are shown in tandem. Only the imm and tox markers are given. In the remainder of the diagram the two prophage genomes have been offset to facilitate visualization of the crosses. In addition, the tox gene has been greatly magnified and its boundaries defined by* (N) *and* (C)*, the locations of the codons for the NH_2-terminal and the COOH-terminal amino acids, respectively. Symbols: Phage chromosome (——), bacterial chromosome (-----).*

ing that they are indeed mutations in the *tox* gene.

DISCUSSION

The results of these experiments establish the orientation of the *tox* gene in the prophage of corynebacteriophage beta. They place the end of the gene corresponding to the carboxyl terminal of diphtheria toxin immediately adjacent to the bacterial chromosome. Holmes (5) has concurrently established the orientation of the *tox* gene in vegetative phage. If, as postulated (7), beta vegetative phage is inserted into the bacterial chromosome by the Campbell mechanism at an attachment site between the *tox* and *imm* genes, then the two orientations should be compatible. That they are indeed compatible is illustrated in Fig. 2. These results provide additional support for the argument that the phage attachment site lies between *tox* and *imm* and establishes the suggested orientation of the prophage map (7) more firmly.

The experiments with the three CRM- strains reaffirm the similarity of these strains to one another. From the results of these crosses, one can conclude that the mutations producing the CRM- phenotype are proximal to the *tox-45* mutation in the sense that they are closer to the end of the *tox* structural gene that corresponds to the amino terminal of diphtheria toxin. The frequency with which CRM62-producing segregants are formed in crosses between CRM45 and CRM- strains is roughly double that obtained in the CRM45 and CRM30 crosses. The greater distance between a proximal mutation in the *tox* gene and the *tox-45* mutation would be expected to account for this increased frequency, while the frequency of recombination is small enough to have been produced by an intragenic crossover. It is likely that the mutations yielding CRM- strains are in the *tox* gene or in an associated regulatory site. The possibility that they might be located in a gene immediately to the left of the *tox* gene seems less likely since CRM-- and CRM45-producing strains do not complement each other (6).

We have previously speculated that the *tox*

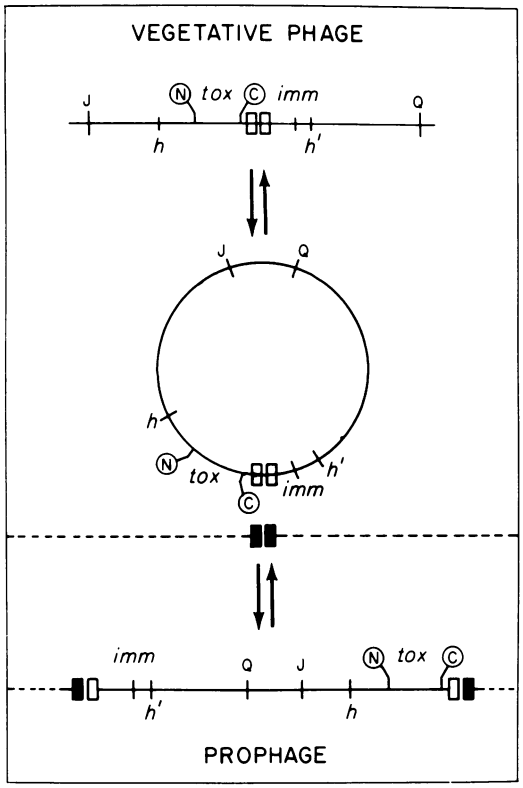

FIG. 2. *Orientation of the tox gene in beta vegetative phage and prophage. The figure shows the interconversion of the prophage and vegetative phage states via the postulated Campbell mechanism. The orientation of the tox gene in the vegetative map is as given by Holmes (5). The J and Q cistrons and the imm, tox, h, and h' markers are given. The tox gene has been enlarged and its boundaries are defined by* Ⓝ *and* Ⓒ*, the locations of the codons for the NH_2-terminal and COOH-terminal amino acids, respectively. Symbols: Phage chromosome (——); bacterial chromosome (-----). The open and closed rectangles represent phage and bacterial attachment sites, respectively.*

gene could have been incorporated into corynephage through an abnormal excision event in some ancestral strain of *C. diphtheriae*. Given the orientation of the *tox* gene, some simple models of how this event might have occurred can now be formulated. Such models can be expected to generate predictions regarding the relationships between the prophage genome of beta phage and the location of host genes that regulate toxin synthesis.

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