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Conversion by corynephages and its role in the natural history of diphtheria

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SUMMARY

The conversion of non-toxinogenic *Corynebacterium diphtheriae* to toxinogeny has been reviewed. The biology of converting phage and the relationship of converting phages to nonconverting phages are summarized. The significance of these findings to the natural history and evolution of diphtheria is assessed.

INTRODUCTION

Freeman (1951) reported that following exposure of non-toxinogenic *Corynebacterium diphtheriae* to corynebacteriophage, diphtheria toxin (DT)-producing strains could be isolated. Most significantly he found that the toxinogenic strains were lysogenic, and went on to suggest that there was a relation between lysogeny and toxinogeny (Freeman & Morse, 1952). Subsequently it was shown that conversion to toxinogeny was phage-induced and not simply due to the selection of pre-existing toxinogenic mutants by the phage (Groman, 1953; Barksdale & Pappenheimer, 1954), that it was phage-specific (Groman, 1955), and that converting phage β carried a gene or genes responsible for toxin production (Groman & Eaton, 1955). Evidence was developed later showing that the structural gene for toxin (*tox*) was carried in the genome of the bacteriophage (Uchida, Gill & Pappenheimer, 1971; Murphy, Pappenheimer & deBorms, 1974). Thirty years after Freeman's original observation, a restriction enzyme map of β was established and a DNA fragment containing the structural gene for DT was identified in mutants of Freeman's original converting phage (Buck & Groman, 1981*a*, *b*; Costa *et al.* 1981). Within a few years the gene was cloned in *Escherichia coli* (Leong, Coleman & Murphy, 1983; Tweten & Collier, 1983) and the nucleotide sequence of the *tox* gene and associated elements was established for both β phage (Greenfield *et al.* 1983; Kaczorek *et al.* 1983) and ω, the β-related phage isolated from the PW8 strain of *C. diphtheriae* (Ratti, Rappuoli & Giannini, 1983).

THE BIOLOGY OF CONVERTING PHAGE

Although there has been much interest in conversion, only the barest outline of the life cycle of converting phage can be given. Most of our information has come from the study of β phage, and to the extent it has been described, β can be characterized as a counterpart to the intensely studied λ phage of *E. coli*. The sum

of our knowledge of β relative to that for λ is minuscule, but within the limits of the data the analogy is appropriate.

Beta phage has a regular polyhedral head roughly 52 nm wide and 56 nm long, and a long 270 nm tail (Holmes & Barksdale, 1970). Each particle contains a molecule of linear double-stranded DNA of approximately 23 Mdal (34·7 kb), which can be circularized by means of cohesive ends (Buck, Groman & Falkow, 1978). There is enough DNA to code for the synthesis of 30–40 proteins. The *tox* gene was mapped first and its location between *h* and *h'* established (Holmes & Barksdale, 1969). The most detailed map of β vegetative DNA was compiled by Singer (1976), based on his work and studies from other laboratories. A total of 15 cistrons and a number of specific markers were identified. An abbreviated map combining several other features of the β genome is given in Fig. 1. The cohesive ends (*cos*) are shown, and functions related to the synthesis and assembly of phage heads and tails are clustered at opposite ends of the map. The *h* and *h'* genes are host range markers. The regulatory gene *imm* (immunity) is concerned with homoimmunity and the more recently described *xcl* (exclusion) and *bin* (β-inhibitory) genes with heteroimmunity (Groman & Rabin, 1980, 1982). The regulatory genes are clustered to one side of the phage attachment site (*att*P), and the *tox* gene for DT is on the other side of this site. The *tox* gene is oriented so that transcription proceeds from *h* to *h'* with the carboxy terminus coding sequence proximal to *att*P (Holmes, 1976; Laird & Groman, 1976*c*; Buck & Groman, 1981*c*) and located within approximately 50 base pairs of the *att*P site (Michel *et al.* 1982; Kaczorek *et al.* 1983).

Beta phage is a temperate phage, and typically may enter a productive lytic cycle or lysogenize its host. The lytic cycle appears to be quite conventional. Adsorption of phage, probably via the tail tip, is followed by a latent period of *ca.* 65 min, a rise period of *ca.* 18 min, and a burst size averaging 30 (Holmes & Barksdale, 1970). It is reasonable to assume that following adsorption, linear DNA is injected into the cell and circularizes. However, virtually nothing is known about the processes of vegetative DNA replication and virion maturation which precede the release of mature particles by lysis. It can be concluded from a combination of genetic and molecular evidence (Laird & Groman, 1976*a*; Buck & Groman, 1981*a*; Michel *et al.* 1982) that, in lysogenization, the circularized DNA genome integrates into the host chromosome as prophage in a manner that formally conforms to the Campbell mechanism for the integration of λ phage into *E. coli* (Fig. 1). The prophage map is a cyclic permutation of the vegetative map, and the *imm* marker now lies at one end of the map and the *tox* marker at the other end. The number of sites at which β converting phage can integrate into the host chromosome appears to be limited, probably to two (Rappuoli, Michel & Murphy, 1983*a*). This suggests that a high degree of specificity is required for integration. Integration of β presumably occurs by site-specific recombination between a phage attachment site (*att*P) and a bacterial attachment site (*att*B), and recent findings indicate that these sites may contain common sequences of a 14 base pairs (bp) inverted repeat with a nine bp interruption (Kaczorek *et al.* 1983). When prophage is formed the *att* sites are split and form the *att*L and *att*R junctions with the host chromosome (Buck & Groman, 1981*a*). In the lysogenic bacterium most phage functions are repressed and the lysogen is immune to superinfection by homoimmune

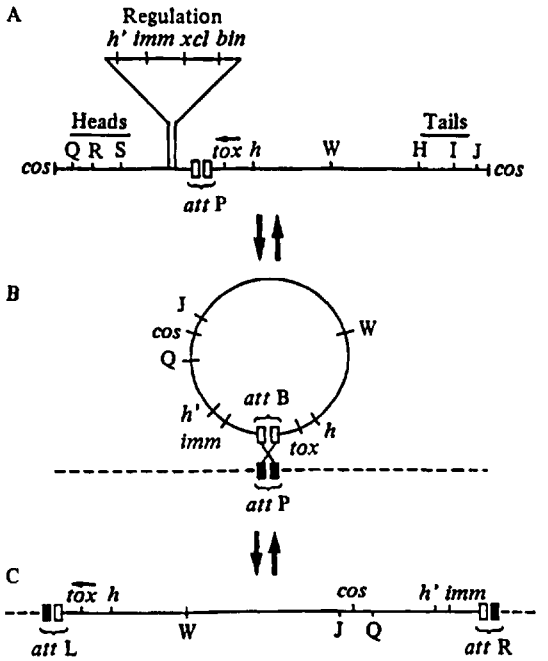

Fig. 1. Genetic map of β-converting phage. (A) Vegetative, linear phase; (B) circularized phage; (C) prophage. Genetic markers are italicized (see text for identification), and cistrons are capitalized. The horizontal arrow over the *tox* gene indicates the direction of transcription. The solid line is phage DNA and the interrupted line bacterial DNA.

phage. On induction, e.g. by u.v., repression is lifted and the induced phage enters and completes the lytic cycle (Barksdale & Pappenheimer, 1954).

Polylysogeny in *C. diphtheriae* was first described for strains carrying β phage and related but heteroimmune γ phage, and later for homoimmune β mutants (Groman, Eaton & Booher, 1958; Groman & Rabin, 1982). A recent report provides new information on double and triple homoimmune lysogens of β, γ and ω phage (Rappuoli, Michel & Murphy, 1983*a*). Two classes of double lysogens have been recognized. Members of the first class have a prophage integrated at each of two *att* sites and are very stable. Members of the second class are unstable and their prophages are aligned in tandem at one or the other *att* sites. The frequency of polylysogen formation seems to be a function of both the phage and the host strain. Tandem polylysogens are formed either through site-specific recombination between the *att*P of the superinfecting phage and the hybrid *att* sites of the resident prophage, or through recombination in a region of genomic homology (Laird & Groman, 1976*a*). Analysis of a limited number of double lysogens containing β and γ indicated that both mechanisms are used with approximately equal frequency. In general, double lysogens exhibit a higher frequency of induction than monolysogens (Groman, Eaton & Booher, 1958). It has been estimated that heteroimmune tandem double lysogens of β and γ undergo spontaneous induction and lysis at a rate approximately 100 times greater than monolysogens of either of the constituent phages (Laird & Groman, 1976*a*). They also recombine at a high rate,

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ca. 1/200 cell divisions, and over 50 % of the phage produced in both spontaneously and u.v.-induced cultures are recombinants (Groman & Laird, 1977). The model proposed by Campbell for λ polylysogens (1969) was invoked to explain the high frequency with which recombinants are recovered from tandem lysogens. In this model, recombination is followed by excision of one of the recombinants from the host chromosome while the other remains integrated. The excised recombinant is in a favourable position to reproduce and subsequently be released, since induction and lysis appear to be correlated with recombination in corynephage tandem lysogens (Groman & Laird, 1977) as they are in λ lysogens of *E. coli* (Roberts & Devoret, 1983). Apparently not all cells in which recombination occurs are induced or induce immediately, for monolysogens carrying the non-excised recombinant can be isolated and may constitute from 5 to 30 % of a fully grown culture (Laird & Groman, 1976*a*).

It is a matter of practical utility and theoretical importance that the frequency of recombinants in cultures of tandem polylysogens is higher by orders of magnitude than in lysates of cultures that have been multiply infected from the outside (Holmes & Barksdale, 1969). Furthermore, since the *imm* and *tox* markers are at opposite ends of the prophage map, all the single crossover events in a cross between two tandem prophages differing in these markers will be represented in recombinants between them. These characteristics of tandem polylysogens have been exploited in studying the genetics of β and of toxinogeny.

THE RELATIONSHIP OF CONVERTING AND NON-CONVERTING CORYNEBACTERIOPHAGES

A question of evolutionary and epidemiologic interest is whether converting phages belong to a closely related family of phages or whether they are a diverse group. The comparative study of both converting and non-converting corynephages by Holmes & Barksdale (1970) has provided the only significant published data on this question. Nine phages were examined; six were converting phages (α, β, δ, L, P, π) and a seventh, γ phage, though non-converting was subsequently shown to carry the *tox* gene cryptically (Laird & Groman, 1976*c*). Two non-converting phages K and ρ were also included in their study. Holmes & 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 β^{tox+} and δ^{tox+} phages which differ in virion morphology and fail to recombine genetically. In addition the presence of *tox*+ was not correlated with virion morphology, interfertility with phage β, immune specificity, plaque morphology, latent period, host range, or antigenic specificity in the corynebacteriophages studied

Recently we have examined the *Bam*H1 and *Hind*III restriction digest patterns of these phages (Buck *et al.*, unpublished observations), and find that α, β, γ, P, π and L have virtually identical patterns. All the fragments in their *Bam*H1 digest hybridize with β phage probe. Though there are phenotypic differences between these phages it seems clear they are closely related. In contrast, phages K and ρ are completely unrelated to β. Their restriction digest patterns are uniquely different from each other or from those of the β-related phages, and none of their

*Bam*H1 fragments hybridizes with β probe. They are non-converting, non-*tox*-carrying phages. The δ phage is in a class by itself. It too has a unique restriction digest pattern. Only three of its 15 *Bam*H1 fragments hybridize with β phage probe, while in the reverse hybridization with δ probe, four of β's eight fragments exhibit some homology. Mention should be made of ω phage, the tox^+ converting phage carried by the various PW8 substrains of *C. diphtheriae* (Maximescu, 1968), and originally described and designated P phage by Barksdale (1955). Morphologically ω phage is very similar to β phage. Its restriction endonuclease map and other characteristics of its DNA were recently described (Rappuoli, Michel & Murphy, 1983*b*). Its DNA, which also has cohesive ends, is slightly larger than β DNA (37·1 and 35·1 kb respectively). Except for three insertion-deletions the ω restriction map and the location of its *att* site and *tox* gene are all similar to those of β. In summary, most of the *tox*-bearing phages characterized thus far appear to be closely related to β. Though δ phage has only limited homology with β, it can by virtue of this homology be included in the family of β-related phages. The characteristics that define a phage family will be discussed in a later section.

There are two groups of β or β-related temperate phages that do not convert sensitive non-toxinogenic strains to toxinogeny. Some carry the *tox* gene, but are unable to convert because of a mutation in that gene, and some do not carry the *tox* gene. Beginning with the work of Uchida *et al.* (1971) a number of β non-converting *tox* mutants were produced in the laboratory by treating phage-producing cells with nitrosoguanidine (Holmes, 1976; Laird & Groman, 1976*b*). The *tox* mutations fall into two broad classes. One class contains mutations in the structural gene and codes for either a biologically inactive but full-length toxin molecule (62000 d) or for a fragment of the toxin molecule (20000–55000 d). The former are undoubtedly the result of mis-sense and the latter of nonsense mutations. The second class contains mutations in sites proximal to the region coding for the amino terminus of *tox*. This could include regulatory site mutations which prevent the formation of the toxin molecule, and mutations in the region coding for the signal peptide (Greenfield *et al.* 1983; Kaczorek *et al.* 1983). Mutations yielding small fragments of the toxin which cannot be detected by present procedures may masquerade in this class. Given the present capability to clone and sequence the *tox* gene, all ambiguities as to the classification of mutants can be resolved.

In theory, both classes of mutations should occur naturally, and undoubtedly they do, but until recently the only natural mutation that had been identified was the *tox* mutation in γ phage. Lysogens of γ phage do not produce any biologically, serologically or biochemically active toxin-related material as tested by animal inoculations, Elek tests and assays for ADP-ribosylating activity respectively (Groman *et al.* 1983). The absence of activity is due to the insertion of a fragment of bacterial DNA into either a regulatory site or the region coding for the amino terminal portion of the toxin molecule (Buck & Groman, 1981*b*). This paucity of natural *tox* mutations was due in part to the lack of a convenient technique for identifying non-toxinogenic strains carrying the *tox* gene. Now that probes for the *tox* gene are available this limitation has been removed (see next section).

The second group of non-converting β-related phages, those that do not carry the *tox* gene, has recently been identified (Pappenheimer, 1982; Groman *et al.* 1983).

The DNAs of these phages exhibit considerable homology with β phage. These phages are of interest for two reasons. First, they could be the precursor of the original converting phages, and second, from unpublished observations cited by Pappenheimer (1982) and our own unpublished observations (Cianciotto & Groman) it appears they may acquire the *tox* gene by recombining with related converting phages. In summary the β family of phages contains both converting and non-converting phages. Some of the non-converting phages are *tox* gene mutants, but some do not carry any *tox*-related DNA.

THE EPIDEMIOLOGICAL SIGNIFICANCE OF NON-TOXINOGENIC *C. DIPHTHERIAE*

The discovery of conversion led to a reconsideration of the role non-toxinogenic *C. diphtheriae* might play in the natural history of diphtheria. By definition, non-toxinogenic strains fail to produce detectable amounts of biologically active toxin. In principle, new toxinogenic strains could emerge by conversion if an individual carrying a toxinogenic strain lysogenic for a converting phage also carried a non-toxinogenic strain sensitive to that phage. Recently, Pappenheimer & Murphy (1983) provided the most convincing documentation for such an event. Both toxinogenic and non-toxinogenic strains had been isolated from the throats of four different children. When the restriction enzyme digest patterns of these strains were compared they were indistinguishable, thus strongly supporting their identity. However, when hybridized with a β-phage probe the toxinogenic strains each seemed to contain a β-related prophage, i.e. gave DNA fragment patterns identical to β's prophage, while the non-toxinogenic strains contained no β-related fragment. It is reasonable to assume that the β-related prophages had converted some of the non-toxinogenic organisms to toxinogeny, though the converting ability of these resident phages was not demonstrated. On the basis of the epidemiological evidence, the authors postulated that the converting phage had originated in an unrelated toxinogenic strain brought into the area by a healthy immune carrier, and that conversion of a resident sensitive strain was under way.

Theoretically, non-toxinogenic strains carrying a *tox* allele may also serve as a reservoir for toxinogeny. There are two classes of *tox*-bearing non-toxinogenic strains. Members of the first class carry a phage with a mutated *tox* gene. The strain from which γ phage was isolated (Groman, 1955) was the first example of this class. Recently, using a DNA *tox* probe, we identified two strains which also carry *tox* genes cryptically (Groman *et al.* 1983). In neither case was a toxin-related product detected in broth cultures of these organisms grown under low iron conditions and tested by any of the three methods previously described. A group of related strains which produce a fragment of toxin exhibiting ADP-ribosylating activity was also identified. All of these *tox*-bearing strains carry a β-related phage, and in two cases we have isolated a phage that cannot convert but carries a *tox* allele (Cianciotto & Groman, unpublished results). The second, and for the present purely hypothetical class of *tox*-bearing non-toxinogenic strains, would carry the wild type tox^+ gene. However, the organisms would be non-toxinogenic due to mutation of a host gene(s) affecting toxinogeny, for example a gene involved in regulating the synthesis or secretion of toxin. Transfer of this tox^+ gene to a 'normal' non-

toxinogenic strain by phage (or some other mechanism of genetic exchange) would of course produce a new toxinogenic strain.

Non-toxinogenic strains carrying a mutated *tox* gene would only be significant as a reservoir of toxinogeny if the gene reverted to wild type or if some of the gene was utilized in the reconstitution of a wild-type gene. A mutation could restore a tox^- gene to its tox^+ state, but the likelihood of such an event is extremely rare. As has been demonstrated (Laird & Groman, 1976; Buck & Groman, 1981*c*), a more promising possibility is reconstitution of a tox^+ gene through recombination, particularly between two tandem prophages carrying *tox* genes with different mutations. As we have seen, the fact that the β-related *tox*-bearing phages are heteroimmune makes this possible. Thus from the viewpoint of virulence, heteroimmunity among the converting phages is not only crucial to the spread of the *tox* gene to new strains of *C. diphtheriae*, but may also contribute to the reconstitution of wild-type *tox* genes from a reservoir of mutated genes. Some time ago we used recombination to explain the observation that *tox*+ converting phages were recovered following passage of a phage from one non-toxinogenic strain through a second non-toxinogenic strain (Parsons, 1955; Groman, 1956). Recently we tested some of the strains still available from those experiments with a *tox* probe and did not find any homology (Groman & Cianciotto, unpublished observations). Either the strains lost their *tox*-bearing phages since the original experiments, or the cultures became contaminated with converting phage during those experiments. The possibility of contamination was considered at the time but deemed remote.

TOXINOGENY AND PHAGE CONVERSION IN OTHER SPECIES OF *CORYNEBACTERIUM*

In addition to *C. diphtheriae* two other species of *Corynebacterium* produce DT. They are *C. ulcerans* and *C. pseudotuberculosis* (*C. ovis*). *C. ulcerans* is commonly found in humans and horses and in cows as well. It can produce an acute tonsillitis in humans and ulcerative lesions in domestic animals, but healthy carriers predominate. *C. pseudotuberculosis* causes ulcerative lymphangitis, abscesses, and other purulent infections in sheep, goats and horses, and occasional infections in man. In one sample of *C. ulcerans* strains (Maximescu *et al.* 1974) 35% (43/122) of the human and 17% (6/29) of the equine isolates produced DT, whereas only 3% (2/62) of the *C. pseudotuberculosis* strains produced it. Virtually all, and possibly all members of both species produce a second toxin, the so-called 'ovis' toxin which is phospholipase D (Barksdale *et al.* 1981).

Phages isolated from *C. diphtheriae* are active on some strains of *C. ulcerans* and *C. pseudotuberculosis* (Carne, 1968; Maximescu, 1968). These include some typing phages, the ω phage, and phage 76c. What is of great interest is that some of these phages can convert non-toxinogenic strains of *C. ulcerans* and *C. pseudotuberculosis* to toxinogeny (Maximescu *et al.* 1974; Maximescu, 1978). Conversely, though less commonly, phages isolated from various corynebacterial animal pathogens are also active on some *C. diphtheriae* strains (Saragea, Meitert & Bica-Popii, 1966; Maximescu *et al.* 1974) and in a few cases convert them to toxinogeny. The h phage, one of a set used in typing *C. ulcerans* and *C. pseudotuberculosis*, converts a non-toxinogenic strain of *C. diphtheriae* (*belfanti*) as well as strains of *C. ulcerans*,

whereas the b phage of this set does not. Conversion of *C. diphtheriae* by L phage, which originated in a *C. ulcerans* strain, has also been reported (Holmes & Barksdale, 1970). We recently examined 25 strains of DT-producing *C. ulcerans* for the presence of β-related phages. Seventeen of these strains had restriction enzyme fragments in addition to the *tox*-bearing fragment that hybridized with a β probe (Groman *et al.*, unpublished results). The relation of these phages to other converting phages is not known, and it remains to be established that the β-related phage in these strains are converting phages.

One is left with the impression that it is more common for phages originating in *C. diphtheriae* to infect and in some cases convert strains of *C. ulcerans* and *C. pseudotuberculosis* than the reverse. This may simply reflect the degree of effort expended on such studies. However, it may reflect an underlying biological factor such as the effect of restriction enzymes on the movement of phages.

Given that phages are exchanged between *C. diphtheriae* and *C. ulcerans* and *C. pseudotuberculosis*, and that toxinogenic strains and conversion to toxinogeny is common to all three species, it was of interest to know the relationship between these organisms. We have recently examined their DNA–DNA homologies employing the S1-exonuclease technique (Rabin & Groman, unpublished observations). There is very little homology (*ca.* 10%) between *C. diphtheriae* and the other two species, and a moderate amount of homology (*ca.* 40%) between *C. ulcerans* and *C. pseudotuberculosis*. In one respect these are surprising results, since the common property of DT production has sustained the idea that these organisms were closely related. It is clear that phage and *tox* can move across significant taxonomic distances.

EVOLUTIONARY CONSIDERATIONS

The discovery of conversion to toxinogeny by corynephages introduced a new perspective into the natural history of diphtheria, namely the realization that toxinogeny was a mobile trait by virtue of its association with phage. It is now apparent that the *tox* gene can be distributed not only over the host range of an individual phage but also over the range of a family of phages, the β family.

The concept of a family of phages was recently defined for the lambdoid phages by Campbell & Botstein (1983). According to their definition the family includes those with the ability to exchange genetic information by homologous recombination with λ itself. All members of the family have a similar genetic map in the sense that the order of genes relative to their specified functions is always the same. This makes it possible for phages to exchange segments or modules of DNA and still generate new, viable combinations. There is considerable diversity among members of the λ family including differences in DNA structure, morphology, host range and immune specificity. The diversity has probably arisen by a combination of mutational divergence in the various functions in different lines of λ descendants, and a shuffling of mutated segments between phages through recombination. For a family of phages the unit of selection is not the individual phage but rather the modules that can be exchanged, and each phage is a combination of modules favourable to its niche. As long as the common genetic organization and some areas of DNA homology for recombination are retained, a phage has the potential to function as a member of the family.

As in other areas, the comparative data for β-related phages are too meagre to define them as a family in the terms outlined above. However, the extensive DNA homology with β that most of them exhibit, the similarity in their restriction enzyme digest patterns, and the ability of some to recombine even in the limited tests performed thus far suggest that they are members of a family. Even δ phage with its more limited homology to β can be regarded as a member of the β family. The diversity in morphology, host range and immune specificity indicates that these phages have had a significant evolutionary history.

One of the characteristics of a phage family is the ability of some of its members to cross diverse genetic boundaries. Reanney (1976) in a study of bacillus phages observed that when phages from one ecological niche were tested against bacilli from another their activity was limited. In contrast, when phages and bacilli were isolated from the same niche there were extensive interactions and some of the phages crossed species barriers. Reanney proposed that the key to this interaction lay in ecological contact between the species, thus providing the opportunity for phages to evolve an extension of their host range. An interesting feature of this study was the network of interactions that could be traced between phages and bacteria. Although individual phages had their limits of host range, most of the bacterial strains were infected by two or more phages, so that if one assumed genetic exchanges between phages were possible, any piece of phage and potentially host information could be passed on to any other phage or strain in the network. Setting aside the general consideration of barriers to DNA exchanges, it is clear by extrapolating from this example that a gene could move across large physical and taxonomic distances provided there was a system of overlapping ecological niches and selection for the gene.

The concept of ecological opportunity has relevance to phage–host relationships among the pathogenic corynebacteria. Infection of some *C. diphtheriae* and *C. ulcerans* strains by diphtheria-derived phages, and of *C. ulcerans* and *C. pseudotuberculosis* by ulcerans-derived phages can be interpreted as a reflexion of the ecological niches they share; humans and horses in one case and domestic animals in the other. Just how these pairs of organisms entered or evolved into common niches is not known, but the domestication of animals may have contributed to their eventual ecological contact. One explanation for the ability of phages to evolve across species barriers is that species of *Corynebacterium* share some chemotaxonomic traits, among them cell wall composition (Keddie & Bousfield, 1980) and at least a limited amount of DNA homology. We have shown (Schiller *et al.* 1983), that a few of the restriction enzyme digest fragments of all the DT-producing organisms exhibit homology with the DNA of the others.

The converting phages belong to a group of accessory DNA elements which includes viruses, plasmids, transposons and insertion sequences. Campbell (1981) has examined the significance of these elements in evolution. Although genes in accessory elements may be 'selfish' DNA, i.e. function only for the survival of themselves, it is argued that the larger, complex phages because of their long evolutionary history must have survived because of some value to their hosts. Certainly prophages endow their host with immunity to superinfection, may provide some advantage by lysing competitors following their release as mature phage, or in a broader populational sense may facilitate exchange of genetic information. We assume that the non-converting β-related phages have a basis for

survival aside from carriage of the *tox* gene. However, the integration of the *tox* gene into such a phage can be viewed as enhancing the growth and survival of both the phage and its bacterial host under certain conditions, i.e. wherever some non-immune individuals or a community of non-immune individuals is available. The viewpoint that immunity is a selective factor is supported by an observation that the frequency of human carriers with toxinogenic strains declined when mass immunization was carried out (Pappenheimer, 1982). *A priori* one might reason that there should be an inverse relationship between population immunity and the carrier rate for toxinogenic strains. Certainly in physiological terms, synthesis of toxin in an immune individual would be a waste of energy and resources to a toxinogenic organism and place it at a competitive disadvantage with related as well as unrelated organisms. It is also conceivable, though there is no evidence, that some toxin-specific immune response eliminates toxinogenic organisms. Though antibacterial immunity (Barksdale, 1982) might contribute to the elimination of a given toxinogenic strain, other toxinogenic strains might be expected to compete for the niche unless there was a disadvantage to synthesizing toxin.

It is not known what happens to the converting phage or *tox* gene in the aftermath of a diphtheria outbreak or in the wake of an immunization programme. If we assume toxinogeny is a disadvantage in an immune population, then loss of converting phage might be advantageous, though some disadvantages might accrue through the loss of phage *per se*. A given toxinogenic strain might also have its immediate competitive position restored in mutants which lost the *tox* gene or in which a mutation made the gene cryptic. As noted, non-toxinogenic strains of the latter type were recently identified (Groman *et al.* 1983). So far as we know, the *tox* gene confers no other advantage to the phage or host bacterium other than toxin synthesis. In a cryptic state the *tox* gene might be carried for considerable periods of time without seriously handicapping its host bacterium, though eventually one would expect it to be eliminated in a consistently immune human host population. Like other genes on accessory DNAs, *tox* is only 'needed' occasionally, i.e. in situations where non-immunes are present, and one would expect that the frequency with which the wild-type *tox* gene is present in strains of *C. diphtheriae* is affected by the immune status of host populations, as well as the period over which immunity has been sustained in those populations. However, as long as there are pockets of susceptible individuals it can probably persist.

A question continuously posed is whether all toxinogenic strains are toxinogenic because they carry a converting phage. A strong correlation between these two elements was seen in a survey of *mitis* strains (Groman & Memmer, 1958). Furthermore, it might be argued, as Campbell (1981) has done, that a gene carried on an accessory element has probably adapted to that location as opposed to a euchromosomal location because it is not needed continuously. If true, then *tox* would almost always be phage-associated. Nevertheless, it is impossible to prove the proposition that *tox* is not associated with a phage. By employing β phage as a probe we can determine whether a toxinogenic strain carries related DNA. But reasonable proof of an association between a β-related phage and a *tox* gene requires isolating the phage and demonstrating that it carries the gene, while proof that the gene is *tox*$^+$ requires conversion by that phage. If a β-related prophage

were defective then at best one might be able to rescue β-related *tox*-associated components by genetic recombination. However, if the *tox* gene were on a phage unrelated to β, the phage might be missed for lack of an appropriate indicator or because it was defective. In either case it would appear as if *tox* were present independently of phage. Clearly the original question, whether toxinogeny is always associated with the presence of a converting phage, cannot be answered. At best we can determine the frequency of association between *tox* carriage and β phages or any other phage which is isolated and can be used as a probe. Certainly it is possible that some strains do carry the *tox* gene independently of any phage or phage genes. While this could represent the penultimate case of a *tox* gene that was never on a phage, it could be a case where the *tox* gene was left behind without a trace of the phage that carried it into this strain.

CONCLUSION

The discovery of conversion by Freeman in 1951 was immediately recognized as an important milestone in the history of diphtheria, but no one could have foreseen that it would ultimately lead to the isolation of the gene for DT. The developments in molecular biology and genetics promise more in the study of diphtheria, more detail concerning the regulation and synthesis of DT, and growing insight into the natural history of the disease. A central mystery in the study of diphtheria, the origin of the *tox* gene, remains unsolved. Arguments have been offered for both a eukaryotic (Pappenheimer, 1982) and a prokaryotic origin (Laird & Groman, 1976*a*), but the answer to this question must await new data and insight.

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