GENETIC FACTORS IN CORYNEBACTERIUM DIPHTHERIAE CONVERSION

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The conversion to toxigenicity of nontoxigenic Corynebacterium diphtheriae strain C4 requires the active and continued participation of a specific bacteriophage (Groman, 1953, 1955). The facts supporting this statement are: (1) lysogenization with $\beta$ phage results in a simultaneous change to toxigenicity, and (2) reversion of toxigenic cells to the nonlysogenic state results in a return to the nontoxigenic state. The phage specificity in the conversion phenomenon is demonstrated by the isolation of a phage designated $\gamma$, capable of lysogenizing C4 without converting it to toxigenicity. In the present work the serological relationship of $\beta$ and $\gamma$ phage is described, as well as their interactions with host strain C4. Apparent recombination between $\beta$ and $\gamma$ phage is reported in which the ability of $\beta$ phage to convert to toxigenicity acts as a discrete genetic attribute of the phage.

MATERIALS AND METHODS

Phage and bacterial strains. The nomenclature for phage and bacterial strains was discussed in a previous paper (Groman, 1955) at which time phages $\beta$, $B$, and Bh were described. Phage $\gamma$ was isolated from C. diphtheriae strain 124 originally described by Freeman and Morse (1952). They recorded this strain as sensitive to $\beta$ phage but using current stocks and $\beta$ phage recovered from their original C4 ($\beta$) strain we could not confirm this. Temperate phage $\gamma$ is capable of lysogenizing strain C4 without converting it to toxigenicity. While there is good evidence for the close relationship of $\beta$ and $\gamma$ phage, the present nomenclature is being retained as a convenient means of associating names and characteristics.

Bacterial strains C4 and C7 and some of their lysogenic and resistant derivatives were previously described (Groman, 1955).

Techniques. Difco heart infusion medium was used in all phases of the work except for the in vitro toxigenicity tests. The plaque count, phage sensitivity, and toxigenicity testing procedures remain unchanged (Groman, 1953, 1955). The test for toxigeny has been modified. The indicator is now incorporated in the soft agar layer as for plaque counting, and a drop of the culture or culture supernate being tested is spotted on the overlay agar. This method is more efficient than the previously described “well” method (Groman, 1953) and is equally sensitive.

Temperate phage stocks were prepared by ultraviolet irradiation of the appropriate lysogenic strain (Booher, 1955). Virulent phage stocks were produced by passage on strain C4.

Attempts to produce $\beta$ and $\gamma$ phage antiserum yielded sera with a low neutralizing capacity. The immunization procedure described by Adams (1950) was followed. Prior to each injection, the rabbit received 800 to 1000 units of antitoxin to protect against toxin in the inoculum. Each inoculum contained only $5 \times 10^5$ to $5 \times 10^6$ phage particles indicating a possible reason for the lack of serum potency. A single booster shot administered after the initial series did not influence the antiphage titer.

Neutralization tests were performed at 37°C with 1:50 dilutions of the sera. Normal rabbit serum was tested at the same dilution.

RESULTS

Serological relationship of phages $\beta$ and $\gamma$. Homologous and heterologous $k$ values ($k$ = fractional rate of inactivation; Adams, 1950) were determined for phages $\beta$ and $\gamma$ with the specific antiserum of each phage. Although a number of tests were performed, the low $k$ values, and to some degree their lack of consistency, prevent detailed conclusions from these data. Nevertheless the data in table 1 strongly suggest a serological relationship between the two phages. An attempt to detect nonspecific phage neutralizing activity, which might have been produced as a result of the immunization process, yielded no evi-
The neutralization periods were 20 and 10 minutes, respectively, for \( \beta \) and \( \gamma \) antiserum during which time 60-90 per cent of the phage was inactivated. The \( k \) values, given above, were corrected for normal serum inactivation which did not exceed 15 per cent during the neutralization periods. The figures in parentheses indicate the number of determinations and represent an average in the case of anti-\( \beta \) and a range in the case of anti-\( \gamma \).

dence for such activity. Both \( \beta \) and \( \gamma \) phage antiserum were tested against a \textit{Bacillus megaterium} phage and in both cases neutralization fell slightly below the activity of normal rabbit serum. A test of a \textit{B. megaterium} phage antiserum against both \( \beta \) and \( \gamma \) phages gave similar results.

Characteristics of the phage-host system. The characteristics of the \textit{Corynebacterium diphtheriae} phages and their interactions with strain C4 are given in table 2.

The relationships of \( \gamma \) and \( \beta \) phage are important. As will be shown later, these phages are apparently capable of recombination, indicating a close relationship between them. The serological relatedness of these phages has already been described. In contrast, lysogenization with one does not exclude infection and lysis by the other, although double lysogenization with these two phages can occur. A significant distinction between the two phages is the inability of \( \gamma \) to convert C4 to toxigenicity. Thus each phage is labeled with a host range marker and a marker for the presence or absence of converting ability.

An interesting observation is noted in the immunity patterns of C4/\( \beta \) and C4 (\( \beta \)). Whereas the latter strain is protected against both \( \beta \) and B by the \( \beta \) prophage, strain C4/\( \beta \) is only immune to \( \beta \). Phage \( \beta \) is readily adsorbed by C4/\( \beta \), a characteristic not generally exhibited by phage resistant mutants. The origin of C4/\( \beta \) is currently under investigation. Attempts to isolate C4/\( \gamma \) strains have failed.

A set of C7 cultures behaved in an identical fashion to those of strain C4 given in table 2 with respect to lysogenicity, phage sensitivity pattern, and toxigenicity tests. Tests were also made in an attempt to discriminate between the A phage of Freeman (1951) and the currently designated \( \beta \) phage. With respect to host range, immunizing ability, and converting ability tested with both C4 and C7, this phage appears to be identical with \( \beta \) phage. Serological data, while supporting the relationship of these phages, fail to give a conclusive answer with respect to their identity.

The final observation of interest in table 2 is the complete resistance of C4/B to stock phages. This lack of sensitivity is reminiscent of strain C411, a strain isolated from the same case of diphtheria as C4 (Freeman, 1951). However, slide agglutination tests performed with antisera to C4 by the method of Minzel and Freeman (1950) conclusively demonstrated a serological relationship between C4/B and C4 but none between C4 and C411. Thus the simultaneous presence of C4 and C411 in a diphtheria patient can only be considered fortuitous at this time.

Superinfection of C4 (\( \gamma \)) by \( \beta \) phage. The infection of C4 (\( \gamma \)) by \( \beta \) was undertaken to determine whether double lysogenization was possible and what effect this might have on the conversion phenomenon. Strain C4 (\( \gamma \)) at a concentration of 10\(^6\) to 10\(^7\) cells per ml was exposed to \( \beta \) phage at a concentration of 2 \times 10\(^9\) particles per ml in heart infusion broth. The mixture was placed on a shaker and incubated at 34 \( \text{C} \). During the incubation period visible lysis occurred, and was followed by development of resistant organisms to a concentration of approximately 2 to 3 \times 10\(^7\) cells ml. Aliquots of the resistant growth were

### TABLE 1

Cross neutralization of phages \( \beta \) and \( \gamma \) by their respective antisera

<table>
<thead>
<tr>
<th>Phage</th>
<th>Serum Anti ( \beta )</th>
<th>Anti ( \gamma )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \beta )</td>
<td>2.0 (2)</td>
<td>6.3-12.1 (5)</td>
</tr>
<tr>
<td>( \gamma )</td>
<td>2.1 (2)</td>
<td>6.2-14.4 (6)</td>
</tr>
</tbody>
</table>

### TABLE 2

The relationship of \textit{Corynebacterium diphtheriae} phages and host strain C4

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Toxigenicity</th>
<th>Sensitivity to Phage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \gamma )</td>
<td>( \beta )</td>
</tr>
<tr>
<td>C4</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C4 (( \gamma ))</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C4 (( \beta ))</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C4/( \beta )</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C4/B</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
plated on differential in vitro medium (Groman, 1953), and of 461 colonies observed 49, or approximately 11 per cent, showed the typical halo of toxigenic colonies. Seventeen toxigenic and 16 nontoxigenic colonies were restreaked for purification and each tested for lysogenicity, phage sensitivity, and toxigenicity as indicated in table 3.

It is apparent that three types of interaction have occurred following superinfection. The presence of C4 (β) indicates that prophage substitution has occurred. Isolation of C4 (γ') and C4 (β') is compatible with a concept of phage recombination, while double lysogenization has been demonstrated with strain C4 (γ) (β). Similar interactions of prophage have been reported by Bertani (1954) with the P system of Shigella phages.

In order to verify the character of the phages carried in these new strains, a sterile filtrate of a culture of each strain was prepared. Strain C4 was then exposed to each filtrate and resistant organisms were isolated and their characteristics determined. Lysogenic cells were recovered which gave reactions identical to those of the parent strains C4 (γ'), C4 (β), and C4 (β') listed in table 3. In addition to the in vitro test, toxigenicity was verified by intradermal inoculation of guinea pigs with these strains and strain C4 (γ) (β).

Double lysogeny of C4 (γ) (β) was proved in the following manner. The supernatant of a broth culture of C4 (γ) (β) was plated on strains C4, C4 (γ), and C4/β. The combined assay of the supernatant on strains C4 (γ) and C4/β yielded more than 90 per cent of the plaques formed on C4, the common indicator strain. One hundred plaques were picked to broth from the C4 plate and tested for activity against C4/β and C4 (γ). Of these, 45 exhibited β host range activity, 53 γ host range activity and 2 the activity of both. Phage from plaques on the C4/β and C4 (γ) plates was active only on the homologous strain. No effort was made to check the toxigenicity locus. From these experiments there is no doubt that at least two types of particles are released by this lysogenic strain. Under the conditions of the experiment the two plaques showing dual host range activity could have arisen from C4 (γ) (β) cells, although the possibility that they arose from heterozygous phage particles remains to be tested.

**Origin of new phage types.** As indicated in the preceding section, two new phage types β' and γ' exhibiting a combination of the characteristics of β and γ phages were recovered. These phages could be mutants of the parent types or they could be recombinant types.

When β or γ phage was plated on C4 (β) and C4 (γ) respectively, no host range mutants were detected among 2.2 × 10⁴ β particles and 4.1 × 10⁴ γ particles. It appears unlikely that either β' or γ' arose by mutation.

There is positive evidence suggesting that both γ' and β' arose as recombinants. Single burst experiments were performed with strain C4 (γ) (β). The total phage in each burst was plated on strain C4, and the majority of the plaques from seven bursts were examined for phage type. Host range and ability to convert to toxigenicity were the markers. In four of seven bursts, at least one parental and one of the recombiant types γ' or β' was present. This high frequency can hardly be explained on the basis of mutation. Further study of this system is in progress.

**DISCUSSION**

The basic facts of conversion to toxigenicity are now reasonably clear. Lysogenization with a phage containing a specific genetic complement results in conversion in the C4-phage β system. It is interesting to note that in the doubly lysogenic strain C4 (γ) (β) toxigenicity is dominant. Efforts to determine whether the order of lysogenization by β and γ phage affects conversion have given no indication of such an effect.

Hewitt (1954a, 1954b) working with the gravis type of C. diphtheriae has concurred in the role of phage in conversion, and has described
results which may be interpreted as phage recombinations involving host range and converting ability. In addition, he has demonstrated the phage specificity in conversion and the fact that lysogenization is not the sole determinant of the process.

To the extent that the C. diphtheriae system has been examined, the interactions of C4 and temperate phages β and γ can be interpreted as paralleling those reported by Bertani (1954) for a Shigella dysenteriae system. These similarities include prophage substitution, phage recombination, and double lysogenization. Furthermore, in two instances C. diphtheriae clones were examined in which two cell types, i.e., cells carrying different prophages, were found, suggesting unsegregated clones similar to those reported by Bertani.

The serological relationship of β and γ phages and their ability to recombine leaves little doubt that these two phages are closely related. The significance of the absence of exclusion is difficult to assess. In a spectrum of relationships between temperate phages, the β-γ relationship represents one more shading between temperate mutants separated by a single mutation on the one hand and completely unrelated temperate phages on the other.

ACKNOWLEDGMENT

We wish to express our appreciation to Mrs. Ruth Mylenbeck and Mrs. Vera Ellingon for their assistance.

SUMMARY

A study was made of the relationship of Corynebacterium diphtheriae phage β, a phage capable of converting strain C4 to toxigenicity, and phage γ, a nonconverting phage. These phages are related serologically, exhibit the ability to recombine, but fail to mutually exclude. It is concluded that β and γ phage are closely related.

Analysis of the types of cells and phages present after exposure of C. diphtheriae strain C4 (γ) to β phage revealed three kinds of phage interactions. These were: (1) prophage substitution, (2) double lysogenization, and (3) recombination. Recombinant types were found in which ability to convert to toxigenicity and host range segregated independently. Thus the discrete genetic character of converting ability in C. diphtheriae phage has been demonstrated.

REFERENCES


GROMAN, N. B. 1953 Evidence for the induced nature of the change from nontoxigenicity to toxigenicity in Corynebacterium diphtheriae as a result of exposure to specific bacteriophage. J. Bacteriol., 66, 184-191.


