STUDIES OF MONO- AND POLYLYSOGENIC *CORYNEBACTERIUM DIPHTHERIAE*[1]

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Although the phenomenon of lysogenic conversion in *Corynebacterium diphtheriae* was described by Freeman in 1951 there have been only limited studies of the lysogenic complex of this species. Thibaut and Frédéricq (1952) gave a brief description of ultraviolet induction of seven lysogenic *gravis* strains, Barksdale and Pappenheimer (1954) described the ultraviolet induction of a *mitis*-like *C. diphtheriae*, and in work relating to lysogenic conversion Groman and Eaton (1955) demonstrated that double lysogeny and prophage substitution occurred in a similar strain. In the present work a comparative study has been made of induced phage production by two monolysogenic strains carrying serologically related phages and of a third strain lysogenized with both of these phages. The bacterial strain and the phages employed were studied previously in relation to the conversion phenomenon (Groman and Eaton, 1955).

MATERIALS AND METHODS

Phage and bacterial strains. The serologically related temperate phages β and γ and lysogenic strains C4(β), C4(γ) and C4(γ)(β) were employed in this study. Beta phage converts C4 to toxigenicity while γ phage does not. Except where noted phage titrations were performed with streptomycin resistant mutants of strains C4 and/or C4/β as indicators. The one step growth curves were performed with streptomycin sensitive C4. The host ranges of the two phages, the sensitivity patterns of the bacterial strains, and the origins of the lysogenic strains were previously described (Groman and Eaton, 1955).

Materials and techniques. In most experiments a proteose peptone salt medium (PPS) composed of 2 per cent proteose peptone (Difco) and 0.5 per cent sodium chloride was used as a growth and suspending medium. Heart infusion broth (HIB) (Difco) was used as a diluent for bacterial and phage titrations, and in some instances as a growth medium.

Methods of determining phage sensitivity, toxigenicity, and numbers of phage and bacteria remain unchanged (Groman and Eaton, 1955). When streptomycin was used in the plaque count system it was added to the overlay in a final concentration of 100 μg per ml. An incubation temperature of 37 C was used throughout.

RESULTS

Phage production during growth of C4(β), C4(γ) and C4(γ)(β). The relationship between the growth of C4(β) in PPS medium and the production of β phage is given in figure 1. The relationship between the growth of C4(γ) and γ phage production is similar. In both instances the phage:bacteria ratios varied between 1:1000 to 1:100 over the range of 10^5 to 10^8 bacteria per ml. Under the conditions of these experiments the rate of phage production exceeded the bacterial growth rate during the logarithmic phase. Why this is so is a matter of speculation. It is possible that the rate of induction increases in relation to some other variable, or alternatively the tendency for the bacteria to adhere after division may increase causing an apparent increase in the phage:bacteria ratio. In some experiments Tween 80 was added to the medium to minimize bacterial clumping and to prevent readsorption of phage (Groman and Bobb, 1955) but the results were similar.

The relationship between phage production and the growth of C4(γ)(β) is illustrated in figure 2. The rate of total phage production (phage/bacteria/generation) by the doubly lysogenic strain is much higher than that of the singly lysogenic strains. This is evidenced in the total phage:bacteria ratios which varied over the range 1:25 to 1:1. As with the singly lysogenic strains there was a tendency for the ratio to increase with increased bacterial growth.

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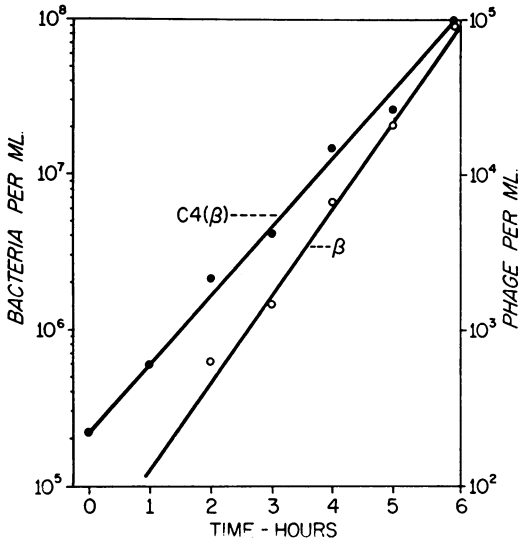

Figure 1. Production of phage by C4(β). Cultures were grown on a shaker to a concentration of approximately 10^8 cells/ml at which time the cells were in the logarithmic phase. The cells were washed twice in PPS broth and diluted to a suitable concentration in the same medium. The cultures were placed on the shaker and aliquots were withdrawn at hourly intervals for assay of phage and bacteria. Indicator strain C4 was used for phage titrations.

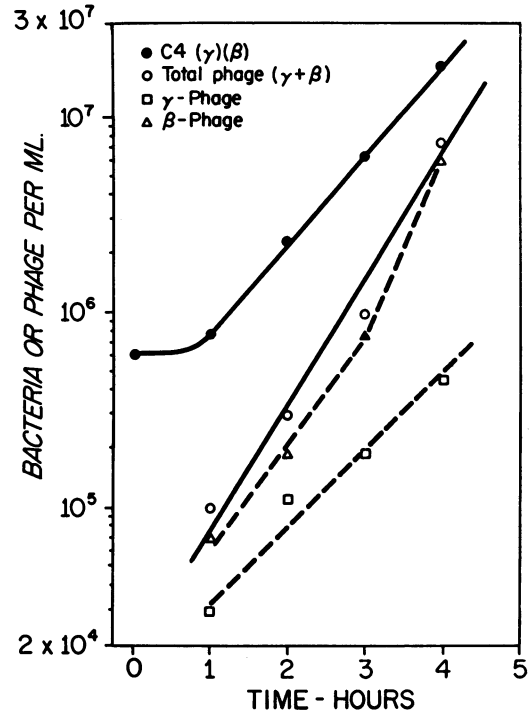

Figure 2. Production of phage by C4(γ)(β). The experimental conditions were similar to those given under figure 1. Total phage was determined on C4, γ phage on C4/β and β phage was determined by difference. The plating efficiency for γ phage was identical on C4 and C4/β.

Although the rate of phage production by C4(γ)(β) was reasonably consistent from experiment to experiment, the rates of production of the individual phages were subject to much variation. In most instances β phage titers, as measured by host range, were five to ten fold higher than those of γ phage. However, in some experiments the ratio of the two approached unity. One factor that might contribute to this inconsistency is the relatedness of β and γ and their ability to recombine after induction, a point to be discussed later. Although the experiments were performed in a consistent manner, subtle factors influencing both induction and recombination may have varied.

Induction of C4(β), C4(γ) and C4(γ)(β). The ultraviolet induction response of related singly and doubly lysogenic strains was studied with two purposes in mind. The first, to compare the induction properties of C4(β) and C4(γ) as a further test of the relationship of β and γ phages; the second, to determine whether the higher induction rate suggested by the growth experiments was reflected in ultraviolet induction of the doubly lysogenic strain. In previous studies on the induction of diphtheria strains by ultraviolet light (Thibaut and Frédéricq, 1952; Barksdale and Pappenheimer, 1954) a maximum of 10 per cent of the cells were induced.

The induction and inactivation data for C4(β) and C4(γ) and the inactivation data for their bacterial and phage components are given in figure 3. Since their behavior was similar the data for the two lysogenic strains were combined as were the data for the two phages. These similarities further substantiate the hypothesis that β and γ phage are related. The maximum induction achieved for C4(β) and C4(γ) was 10 to 15 per cent of the original bacterial population. Cells of C4(β) grown in PPS medium instead of HIB exhibited similar induction levels. The induction curves are qualitatively similar to those reported for other organisms except that a plateau of maximum induction is evident during the 1 to 3 min period of irradiation. The data

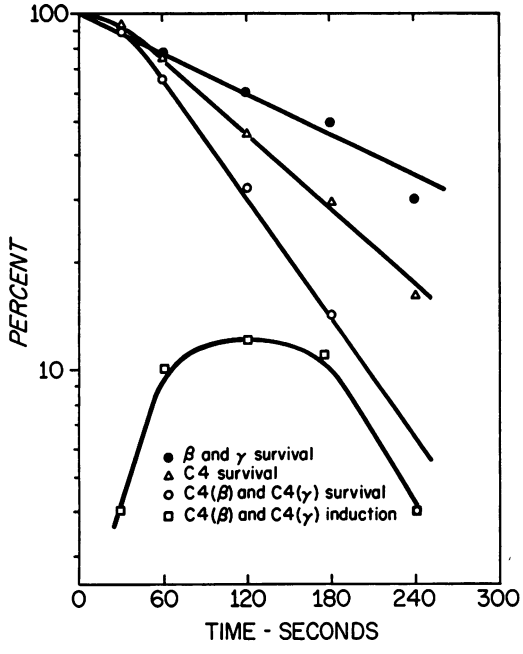

Figure 3. Ultraviolet induction of monolysogenic *Corynebacterium diphtheriae*. Log phase cultures grown in HIB were washed once in PPS medium and resuspended in this medium to a concentration of 1 to 3 × 10^7 cells/ml. Five ml of this suspension was pipetted into a standard petri dish, and the cells were constantly agitated during irradiation with a GE 15 watt germicidal lamp fixed at a distance of 15 inches from the plate. Approximately 350 ergs/cm²/sec were delivered as determined with an ultraviolet dosimeter (Latarjet *et al.*, 1953). Infectious centers were determined by plating aliquots with indicator strain C4 immediately after irradiation. Streptomycin was not added to the overlay. Plate counts for surviving bacteria were made simultaneously. Experiments were performed in subdued light to minimize photoreactivation. Ultraviolet inactivation data for bacterium C4 and phages β and γ were obtained under conditions identical with those described for induction.

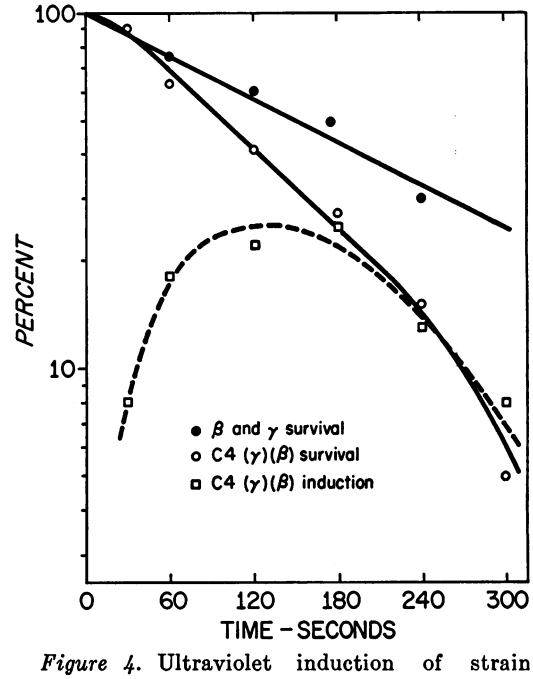

Figure 4. Ultraviolet induction of strain C4(γ)(β). The experimental conditions were identical with those described under figure 3.

confirm the observations of Barksdale and Pappenheimer (1954) with C4(β_1).

Sodium thioglycolate and sodium thiomalate were also tested as inducing agents for C4(β). Thioglycolate increased phage production in a variable manner over the concentration range M/2 to M/200, but its effectiveness was much less than that of ultraviolet light. Thiomalate depressed phage production. The addition of histamine, or manganous, cuprous and ferrous ions in concentrations used by previous workers (Lwoff and Siminovitch, 1952) did not potentiate thioglycolate induction.

The induction and inactivation data from a representative experiment with C4(γ)(β) are given in figure 4. The maximum induction levels ranged from 20 to 40 per cent of the cells exposed, which confirmed the higher rate of induction suggested by the growth experiments. As with the monolysogenic strains a plateau of induction appeared during the 1 to 3 min period although a slight peak often appeared at 2 min. The percentage induction of the doubly lysogenic strain is approximately twice that of the singly lysogenic relatives at comparable dosages. This is taken to indicate that the probability of induction is additive when two phages are carried.

One-step growth curves of β and γ phage. One step growth curves of β and γ phage were studied by infecting strain C4 with each of these phages or by inducing strains C4(β) and C4(γ) with ultraviolet light. Strain C4(γ)(β) was also studied after induction with ultraviolet light. A comparison of the data from a series of experiments is given in table 1.

There are no significant differences in the latent periods of infected or induced cells. However, there is a consistent difference in the rise period which is about 45 to 50 min longer for induced

C4(β) and C4(γ). In this respect the rise period of strain C4(γ)(β) is intermediate to the monolysogenic strains. The burst size range given in table 1 represents the best estimates from one-step growth experiments obtained by infecting C4 with β or γ phage. In the case of β phage produced by C4(β) and total phage production by C4(γ)(β) estimates of average burst size from single burst experiments also fall within this range. Burst size estimates from ultraviolet-induced cultures proved least accurate due to fluctuation of the infectious center count during the latent period. Nevertheless the results of these experiments confirm the general conclusion that the burst sizes of the mono- and polylysogenic strains are similar.

Phage production by C4(γ)(β). The similarity of the ultraviolet induction response of C4(β) and C4(γ) raised the question of whether both phages were induced simultaneously in strain C4(γ)(β). An examination of single bursts of C4(γ)(β) provided an affirmative answer to this question and simultaneously confirmed a suggestion made previously (Groman and Eaton, 1955) namely, that recombination occurs between β and γ phage. The data are given in table 2.

The bursts listed in table 2 have been grouped

TABLE 1

One step growth curve characteristics of Corynebacterium diphtheriae phages

	β Phage		γ Phage		C4(γ)(β) Induced
	C4 infected	C4 (β) induced	C4 infected	C4 (γ) induced	
Latent period, min....	60	60	60	60	60
Rise period, min.....	45	90	40	90	70
Burst size.....	20–35				

The methods outlined by Adams (1950) were used in the infection experiments. PPS medium was employed. For the induction experiments, cells were grown in HIB, removed during the logarithmic phase, washed twice in PPS medium and resuspended in this medium to a concentration of 10^8/ml. After irradiating for 2 min under conditions previously outlined, cultures were diluted 1:100 in PPS broth, assayed immediately for infectious centers, and then placed on the shaker. At subsequent intervals infectious centers were again measured.

TABLE 2

Phage types released in single bursts of C4(γ)(β)

Burst No.	Total Phage per Burst	Phage Type				Plaque Type*	
		β	β'	γ'	γ	D	D
		hβ tox$^+$	hβ tox$^-$	hγ tox$^+$	hγ tox$^-$	tox$^+$	tox$^-$
1	47	15	7	11	6	4	4
2	59	4	37	4	6	4	4
3	34	9	5	3	1	10	6
4	28	4	4	2	2	15	1
5	24	4	2	2	1	4	11
6	43	12	7	5	8	7	4
7	27	1	5	5	13	1	2
8	12	1	5	0	3	3	0
9	16	1	1	0	14	0	0
10	17	2	2	3	0	8	2
11	16	2	0	2	4	3	5
12	30	16	6	0	0	5	3
13	19	3	0	2	0	10	4
14	29	0	25	0	4	0	0
15	20	0	0	20	0	0	0
16	8	0	0	8	0	0	0
Total.....	429	74	106	67	62	74	46
Per cent total.....		17	25	16	14	17	11

One ml aliquots containing less than 10 cells (unirradiated) were suspended in PPS medium, incubated for four hours and then plated with C4 plus streptomycin. Of 84 plates 18 exhibited from 8 to 60 plaques. Based on a Poisson distribution 16 plates would be expected to contain single bursts and two plates two bursts. The two plates with the largest number of plaques were arbitrarily discarded and all the plaques on the remaining 16 plates were tested for the host range (hβ or hγ) or C4 converting ability (tox$^+$ or tox$^-$) of their phages. To test for these markers material was withdrawn from each plaque with an inoculating needle and suspended in 2.5 ml of PPS broth. Host range was tested using C4/β and C4(γ) as indicator strains. Then 0.5 ml of log phase C4 cells was added to each tube of broth to give a final concentration of 2.5×10^8 bacteria per ml and the cultures were incubated until the bacteria reached approximately 10^8 cells per ml. Each culture was then tested for toxigenicity by the "in-vitro" streak plate method. The efficiency of this procedure was established in control experiments as 100 per cent.

* D plaques are those which yielded material giving positive host range tests on the two indicator strains specific for β and γ phage.

according to the number of phage types present in each burst excluding the D category. The D plaques are so designated because material taken from the original plaques gave positive host range tests on the two indicator strains specific for β and γ. The data show that from one to four types of phage were released in each burst and that in all but three bursts (14, 15, and 16) the host range and conversion markers of both parents are represented. Over-all, the frequency of parental and recombinant type phages is about equal. It is concluded that in most bursts both prophages are induced within a short time of one another and that recombination occurs between β and γ.

The inclusion of bursts 12 and 13 with the group exhibiting all four parental markers rests on an analysis of the phage types present in a limited number of D plaques. Material from D plaques was replated on indicator strain C4 in the presence of streptomycin and a number of these plaques were tested for the phage type present. The data given in table 3 show that all four parental markers are present in D tox$^+$ plaques. Recombination during plaque formation may account for the presence of the four phage types. The absence of any tox$^+$ markers in the two D tox$^-$ plaques indicates that there was no suppression of the tox$^+$ marker by our testing procedures. The absence of certain parental markers in bursts 14, 15, and 16 suggests that the markers may have been eliminated preceding induction

TABLE 3

Phage types found in single plaques exhibiting dual host range

Plaque No.	Original Plaque Type	Phage Types Found After Replating Plaque Material					
		β	β'	γ'	γ	D tox$^+$	D tox$^-$
		hβ tox$^+$	hβ tox$^-$	hγ tox$^+$	hγ tox$^-$		
1	D tox$^+$	2	3	5	5	0	0
2	D tox$^+$	4	6	1	5	0	0
3	D tox$^+$	2	0	0	21	2	1
4*	D tox$^+$	13	1	4	11	3	1
5	D tox$^-$	0	33	0	3	0	0
6	D tox$^-$	0	15	0	1	0	0

*D tox$^+$ plaque selected from number 3 and replated.

The host range and conversion markers were scored as outlined under table 2.

(Appleyard, 1954). This possibility is supported by the isolation of singly lysogenic C4(β) and C4(γ') from cultures of C4(γ)(β).

At the present time the significance of the D plaques is not known. The percentage of D plaques varied from 28 to 7.3 per cent in two single burst experiments and was 2 per cent in a sample of a log phase culture of C4(γ)(β). This latter yield is consistent with the finding of other investigators for heterozygosis (Doermann, 1953) but it seems unlikely that the large number of D plaques found in the other experiments represent heterozygotes. In the absence of other evidence phage clumping must be considered an equally likely explanation for the D plaques.

DISCUSSION

The intense study devoted to temperate phage and the lysogenic state of the gram-negative organisms *Pseudomonas pyocyaneus*, *Escherichia coli*, and *Shigella dysenteriae* has been only partially matched by similar efforts with gram-positive organisms. However, studies of the *Bacillus megaterium* system (Lwoff, 1953) and the present and previously cited studies of lysogenic *Corynebacterium diphtheriae* suggest that the phage-bacteria relationships are basically similar in gram-positive organisms. The production of mixed yields in bursts of normally induced polylysogenic C4(γ)(β) and the observation of bacterial segregants C4(β) and C4(γ') in cultures of this organism parallel the findings of Appleyard (1954) and Jacob and Wollman (1954) with the *E. coli* K12-λ phage system. The low 10 to 15 per cent level of ultraviolet induction of the monolysogenic strains studied is consistent with the finding for other Corynebacterium strains (Thibaut and Frédéricq, 1952; Barksdale and Pappenheimer, 1954). However, the polylysogenic strain responds more favorably to induction. In addition to the 20 to 40 per cent induction found with C4(γ)(β), we have observed ultraviolet induction levels as high as 60 per cent with a second polylysogenic strain. In no instance has the 100 per cent induction level attained with other bacterial species been reached.

In the present study the relationship between β converting and γ nonconverting phage has been substantiated. In addition to a serological relationship (Groman and Eaton, 1955) recombination between these phages has been estab-

lished and similar induction patterns have been demonstrated in the study of their lysogenic derivatives C4(β) and C4(γ). The inability of either β or γ phage to immunize its lysogenized host against infection by the other (Groman and Eaton, 1955) is not the usual finding with closely related temperate phages. However, Bertani (1957) has reported an even more striking example of the lack of cross-immunizing ability with mutants of P2 phage, which seem to be allelic with respect to their positions on the bacterial chromosome. Since these findings indicate that cross-immunizing ability is not an inviolate criterion of relatedness the serological similarity and genetic compatibility of β and γ phage are taken as good evidence of their relationship. Finally it should be noted that the recombination data presented in this paper clearly establish that lysogenic conversion to toxigenicity is mediated by a discrete genetic unit of phage.

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SUMMARY

Phage production by lysogenic *Corynebacterium diphtheriae* strains C4(β), C4(γ) and C4(γ)(β) was determined. Phage:bacteria ratios for both monolysogenic strains varied from 1:100 to 1:1000 while the ratios for the polylysogenic strain varied from 1:1 to 1:25. In all cases, the relative proportion of phage increased as the bacterial population increased. The ultraviolet inducibility of the monolysogenic strains was found to be identical over a wide dosage range with a maximum induction level of 10 to 15 per cent. Induction levels of 20 to 40 per cent were found for the polylysogenic strain and its general induction level was twice that of the monolysogenics over the entire dosage range. Burst sizes for the three strains fell within the range 20 to 35.

Single bursts of C4(γ)(β) generally yielded both phages and their recombinants. The demonstration of recombination provides proof that lysogenic conversion to toxigenicity is mediated by a discrete genetic unit of the phage.

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