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Virulent Mutants of Phage P22

II. Physiological Analysis of P22 virB-3 and Its Component Mutations[1]

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The virulent mutant of phage P22, *virB-3*, consists of two mutations: *K5*, which maps in the c_2 repressor gene, and *Vx*, which maps between c_2 and c_3. Although neither P22 *K5* nor P22 *Vx* is virulent, each of these mutants can express gene functions not expressed by other nonvirulent phages in the presence of prophage immunity. In mixed superinfection of a lysogen with P22 *virB-3*, only a small fraction of the yield consists of c^+, c_1, or c_2 phage even though the virulent grows normally. This is the phenomenon of replication inhibition. In contrast, P22 *K5* and to a lesser extent, P22 *Vx* show escape from replication inhibition. However, neither P22 *K5* nor P22 *Vx* alone replicates extensively in a lysogen. These mutants complement in *trans* for phage DNA synthesis and phage production. This result indicates that both P22 *K5* and P22 *Vx* express some of the functions required for phage replication in immune conditions. In addition, P22 K5 kills lysogens at low multiplicities, and lysis is observed when these complexes are treated with chloroform.

P22 *virB-3* is repressed by the c_2 gene product made by a c^+ phage in mixed infection of a sensitive host. There is an inverse relationship between the burst size and the multiplicity of infection of the c^+ phage. P22 *virB-3* represses its own growth at high multiplicities even though the *K5* mutation maps in the c_2 gene and confers a clear plaque phenotype to phage P22. Introduction of a second c_2 mutation into the P22 *virB-3* genome abolishes this multiplicity effect. These findings demonstrate that P22 *virB-3* is sensitive to its own repressor and to that of a coinfecting phage bearing a c_2^+ allele. We have called this effect *multiplicity repression*. The residual sensitivity of P22 *virB-3* to repressor suggests that at least one of its component mutations is of the operator constitutive type.

INTRODUCTION

The temperate bacteriophage P22 is able to establish lysogeny in *Salmonella typhimurium*. Lysogens are immune to superinfection by phage P22 (Zinder, 1958) and have the potential to lyse and produce progeny of the prophage type on induction (Boyd, 1951). The integrated prophage also causes the appearance in the host cell of a new somatic antigen (Robbins and Uchida, 1962; Young *et al.*, 1964) and exclusion of superinfecting genomes of P22 and heteroimmune phage (Rao, 1968; Walsh and Meynell, 1967). Maintenance of lysogeny is dependent upon functional products of the c_2 (Levine and Smith, 1964) and *mnt* (Gough, 1968) genes.

Mutants of P22 have been isolated which produce phage progeny on superinfection of a P22 lysogen (Bronson and Levine, 1971). These virulent mutants have been classified into three groups: (1) *VirA* mutants, which map at or very near the *mnt* locus (Fig. 1); (2) *VirB* mutants, which contain two mutations mapping in the clear region; and (3)

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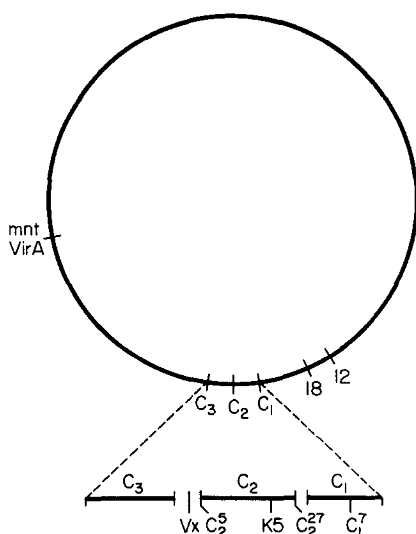

FIG. 1. Circular linkage map of phage P22 with clear region in detail.

VirC mutants, which are comprised of the determinants of both *VirA* and *VirB* mutants. The *VirB* mutant, P22 *virB-3*, has been analyzed in detail (Bronson and Levine, 1971). One of its component mutations, *Vx*, maps between the c_2 and c_3 genes (Fig. 1). The other mutation, *K5*, maps in the c_2 repressor gene (Fig. 1).

Phage P22 is sensitive to replication inhibition (Levine *et al.*, 1970), first described for phages λ and P2 by Thomas and Bertani (1964). In mixed superinfection of a lysogen by P22 *virB-3* and a nonvirulent P22 c_1 mutant, virulent phage progeny represent greater than 98% of the yield. The burst size of the nonvirulent phage is less than its input. The P22 *virB-3* DNA associates with the phage replication complex and undergoes normal replication and maturation, whereas little association of the input nonvirulent phage DNA with the complex is demonstrable (Botstein, 1968; Levine *et al.*, 1970). The inability of the virulent mutant to complement nonvirulent DNA into the active replication machinery suggests that repressor exerts a direct physical effect in replication inhibition.

This report demonstrates that P22 *K5* and to a lesser extent P22 *Vx* show escape from replication inhibition. In addition, both mutants express gene functions required for phage DNA replication in a lysogen. However, P22 *virB-3* is sensitive to high levels of c_2 repressor coded for by its own genome or by that of a coinfecting phage. These findings are discussed in relation to the mechanism of virulence of P22 *virB-3*.

MATERIALS AND METHODS

Bacteria and phage. Strain 18, a derivative of *Salmonella typhimurium* LT2 cured of prophage PB1 (Zinder, 1958) was used as the sensitive host. Strain 210, which is strain 18 lysogenic for P22 *sie1 ts2.1 ts12.1* (Rao, 1968) was used as the immune host. Prophage containing the *sie* mutation do not exclude superinfecting phage P22 (Rao, 1968; Walsh and Meynell, 1967). A galactose-negative strain was used as the indicator on EMB galactose agar plates (Levine, 1957). P22 *virB-3* has a plating efficiency of 0.7 on the immune host relative to the sensitive host (Bronson and Levine, 1971). Nonvirulent P22 phage form plaques only on the sensitive host. The clear mutant phages P22 c_1, P22 c_2, and P22 c_3 have been described by Levine (1957). Phage bearing the *mnt-1* mutation, originally called v_1 by Zinder (1958), are unable to establish stable lysogeny.

Media. L broth (Levine, 1957), supplemented M9 medium (Smith and Levine, 1964), soft agar for top layers (Levine, 1957), EMB galactose agar (Levine, 1957), indicator agar (Levine, 1957), and tryptone agar (Bronson and Levine, 1971) have been described.

Phage infections. Overnight cultures of the above strains were diluted 1:100 into LB or supplemented M9 medium and grown to a concentration of 10^8 cells/ml with aeration at 37°. The log phase cells were infected at 37° at various multiplicities. Superinfections of lysogenic strain 210 were performed at a total multiplicity of infection of 10 or less to avoid titration of repressor (Rao, 1968). After 5 min adsorption, the infected cells were diluted 1:10 in growth medium and antiserum (K = 2) for 5 min to inactivate unadsorbed phage. Samples were then diluted into growth tubes containing either LB or supplemented M9 medium. At 90 min after infection, chloroform was added to

the growth tubes, and the lysates were assayed on the appropriate indicators. In one-step growth experiments, samples were assayed for phage at various times after infection. The burst size is defined as the number of plaque-forming phage particles produced at 90 min after infection divided by the number of infected cells. The frequencies of killing and of infectious center formation were determined by plating diluted samples of infected cells on EMB galactose agar plates at 10 min after infection. Surviving cells give rise to purple colonies, whereas infectious centers are identified as plaques.

Measurement of the rate of DNA synthesis. The rate of DNA synthesis was estimated by incorporation of [^{3}H]thymidine into acid-insoluble material during a 1-min pulse as described by Smith and Levine (1964).

RESULTS

P22 VirB-3 Is Sensitive to Repressor

P22 *virB-3* shows normal kinetics of phage production on infection of either a sensitive or lysogenic host at 37° at low multiplicities. The latent period is about 30 min, and the burst size taken at 90 min after infection at a multiplicity of infection (m.o.i.) of 5, ranges around 100 (Table 1, infection 1; Table 2, infection 1). The prophage is not induced, suggesting that P22 *virB-3* does not inactivate the prophage repressor.

The above data suggest that P22 *virB-3* has a reduced sensitivity to repressor. If this is the case, higher levels of repressor than that made by a single prophage may inhibit development of P22 *virB-3*. This can be tested by coinfection of a sensitive host with P22 *virB-3* and varying multiplicities of wild-type P22 c^{+} phage. If P22 *virB-3* is sensitive to repressor, its burst size should decrease as the m.o.i. of P22 c^{+} is increased. Increasing the m.o.i. of a c_2 mutant which does not make repressor should not cause a reduction in burst size.

Sensitive strain 18 was infected with P22 *virB-3* at a constant multiplicity of 5 and a second phage at multiplicities ranging from 0 to 50. As the m.o.i. of P22 c^{+} increased, the burst size of the mixed infection decreased (Table 1, infections 5–8). However, raising the m.o.i. of P22 $c_2{}^{5}$ or P22 $c_2{}^{27}$ did not decrease the burst size (Table 1, infections 9–12). Thus, P22 *virB-3* phage development is sensitive to the c_2 repressor.

Phage bearing the *mnt-1* mutation make a defective *mnt* gene product resulting in inability to form stable lysogens (Zinder, 1958). Prophage bearing a temperature-sensitive mutation in the *mnt* gene is induced if the temperature is raised (Gough, 1968). Thus, functional *mnt* gene product is required for the maintenance of lysogeny and has properties characteristic of a repressor. Nevertheless, in mixed infection with P22 *virB-3*, a decreased burst size was observed when the m.o.i. of *mnt-1* was raised (Table 1, infections 13 and 14). These results suggest that functional *mnt* gene product is not required for inhibition of P22 *virB-3*. A decreased burst size with increased m.o.i. of phage supplying c_2 repressor will hereafter be referred to as *multiplicity repression.*

VirB-3 Can Repress Its Own Development

P22 *virB-3* contains a mutation, *K5*, which maps within the c_2 gene (Bronson and Levine, 1971). Both P22 *virB-3* and P22 *K5* make clear plaques on sensitive strain 18, and P22 *K5* complements as a c_2 mutant (Bronson and Levine, 1971). It was, therefore, surprising that P22 *virB-3* itself shows multiplicity repression. As the m.o.i. of P22 *virB-3* was raised from 5 to 50, the burst size decreased from 104 to 1.1 (Table 1, infections 1–4). Thus, P22 *virB-3*, a virulent phage containing a c_2 mutation, can inhibit its own development. These results suggest that P22 *virB*-3 makes functional repressor, and represses itself in the same way it is repressed by c^{+} phage. Two possible explanations for this self-repression by P22 *virB-3* were considered: (1) in the double mutant P22 *virB-3*, the *Vx* mutation in some way suppresses the defective c_2 phenotype expressed by the *K5* allele. (2) the c_2 gene of P22 *K5* codes for a product with some repressor activity. At high multiplicities of infection, enough of this product is made to cause multiplicity repression. To distinguish between these hypotheses, the double mutant P22 $Vxc_2{}^{5}$ and the single mutant P22 *K5* were tested for multiplicity repression.

TABLE 1

MULTIPLICITY REPRESSION IN SENSITIVE STRAIN 18[a]

Infection	Phage	M.o.i.	Burst size	Infection	Phage	M.o.i.	Burst size
1	*virB-3*	5	104	15	*K5*	5	63
2		10	34	16		10	24
3		20	5.4	17		20	5.7
4		50	1.1	18		50	1.7
5	*virB-3* × c^+	5:5	47	19	vxc_2^5	5	200
6		5:10	26	20		50	99
7		5:20	7.4	21	Vxc_2^5K5	5	210
8		5:50	2.2	22		50	75
9	*virB-3* × c_2^5	5:5	74	23	$VxK5c_2^{27}$	5	275
10		5:50	140	24		50	180
11	*virB-3* × c_2^{27}	5:5	89	25	Vxc_2^5K5 × c^+	5:5	100
12		5:50	66	26		5:50	2.4
13	*virB-3* × *mnt − 1*	5:5	37	27	$VxK5c_2^{27}$ × c^+	5:5	130
14		5:50	6.5	28		5:50	1.9

[a] All infections were performed at 37°. At 90 min after infection, the cultures were treated with chloroform and assayed for plaque-forming particles. The burst size is defined as the number of plaque-forming particles produced divided by the number of infected cells.

TABLE 2

MULTIPLICITY REPRESSION IN LYSOGENIC STRAIN 210[a]

Infection	Phage	M.o.i.	Burst Size
1	*virB-3*	5	82
2		10	31
3		50	4.5
4	*virB-3* × c^+	5:5	94
5		5:50	68
6	*virB-3* × *Vx*	5:5	45
7		5:50	6.9
8	*virB-3* × *K5*	5:5	73
9		5:50	9.7
10	*virB-3* × Vxc_2^5	5:5	100
11		5:50	77

[a] All infections were performed at 37°. At 90 min after infection, the cultures were treated with chloroform and assayed for plaque-forming particles. The burst size is defined as the number of plaque-forming particles produced divided by the number of infected cells.

If the *Vx* mutation suppresses the *K5* phenotype, the single P22 *K5* mutant should not exhibit multiplicity repression. P22 Vxc_2^5 would be expected to exhibit self-repression at high multiplicities if *Vx* suppression is gene specific. If, on the other hand, phage carrying the single *K5* mutation make repressor, then P22 *K5* should show multiplicity repression, whereas P22 Vxc_2 should not. The data clearly show that infection by P22 *K5* resulted in multiplicity repression to about the same extent as observed with P22 *virB-3* (Table 1, infections 15–18). Multiplicity repression was not observed in the infections by P22 Vxc_2^5 (Table 1, infections 19–20). These findings suggest that *K5* is a mutation in the c_2 gene which confers a clear plaque phenotype to phage P22 but does not eliminate all c_2 repressor activity.

Since neither P22 c_2^5 nor P22 c_2^{27} cause multiplicity repression, introduction of either the c_2^5 or the c_2^{27} mutation in *cis* to *K5* would be expected to result in destruction of any repressor activity and thereby abolish multiplicity repression. To test this, the triple virulent mutants P22 Vxc_2^5K5 and P22 $VxK5c_2^{27}$ were constructed. Neither of these phages in single infection showed the multiplicity repression characteristic of P22 *K5* and P22 *virB-3* (Table 1, infections 21–24). However, both P22 Vxc_2^5K5 and P22 $VxK5c_2^{27}$ were repressed in mixed infections with high multiplicities of wild-type c^+ phage (Table 1, infections 25–28). These results demonstrate that the introduction of an additional c_2 mutation in *cis* can abolish the self-repression of P22 *virB-3*,

and that the resultant triple virulent mutants are still sensitive to multiplicity repression induced by a second phage.

Multiplicity Repression in a Lysogen

P22 *virB-3* undergoes multiplicity repression in single superinfection of lysogenic strain 210 (Table 2, infections 1–3). However, mixed superinfection of P22 *virB-3* and wild-type P22 c^+ does not result in multiplicity repression when the m.o.i. of the latter phage is increased (Table 2, infections 4 and 5). This is in striking contrast to mixed infection of the sensitive host by these phages (Table 1, infections 5–8) and indicates that wild-type phage do not contribute sufficient repressor to inhibit development of P22 *virB-3*.

In contrast to wild-type phage, both P22 Vxc^+ and P22 *K5* caused multiplicity repression upon mixed superinfection of the lysogenic host with P22 *virB-3* (Table 2, infections 6–9). P22 Vxc_2^5, a phage which does not make functional c_2 gene product did not repress P22 *virB-3* (Table 2, infections 10 and 11). These results indicate that P22 Vxc^+ and P22 *K5* produce repressor in the lysogenic host, and suggest that this property is associated with the constitutive nature of the *Vx* and *K5* mutations.

Escape from Replication Inhibition

The inability of wild-type P22 c^+ to cause multiplicity repression in a lysogen may be a consequence of replication inhibition. In mixed superinfections of lysogenic strain 210 with P22 *virB-3* and either wild-type P22 c^+, c_1, or c_2 phage at an m.o.i. of 5 each, the nonvirulent phage represented only a few percent of the phage yield in each case (Table 3, infections 2, 4, 6, and 8). The burst sizes of the nonvirulent phages in mixed superinfection with P22 *virB-3* showed little or no increase over the burst sizes observed on single superinfection (Table 3, infections 1–8). Progeny of the prophage type represented less than 0.5% of the phage yield for each superinfection. These results demonstrate that the nonvirulent phages are repressed even though the virulent phage replicated normally in a lysogen. When these phages mixedly infected strain 18 at the same multiplicities, in each case over 25% of the phage yield consisted of the nonvirulent phage (Table 3, infection 1–8), ruling out competition with the virulent phage as the reason for the low yields of nonvirulents in the lysogenic host.

Like c^+, c_1, and c_2 phages, P22 *K5* is nonvirulent and produces a burst size that is large on the sensitive host and small on the lysogenic host (Table 3, infection 9). However, in mixed superinfection of strain 210 with P22 *virB-3*, P22 *K5* represented 44% of the phage yield (Table 3, infection 10), a greater than 15-fold increase in burst size over that found on single superinfection. This burst size of P22 *K5* was equal to that observed in mixed infection of the sensitive host. We conclude from these data that P22 *K5* escapes replication inhibition.

P22 Vxc^+ and the double mutants P22 Vxc_2^5 and P22 Vxc_2^{27} also gave small burst sizes on the lysogenic host (Table 3, infections 11, 13, and 15). In mixed superinfection with P22 *virB-3* the burst size of each of these mutants was increased (Table 3, infections 11–16). The burst size and percent of yield of P22 Vxc_2^5 and P22 Vxc_2^{27} in mixed superinfections was about three times that of P22 c_2^5 and P22 c_2^{27} in the corresponding superinfections (Table 3, cf. infection 12 with infection 6 and infection 14 with infection 8). However, the percentages of P22 Vxc_2^5 and P22 Vxc_2^{27} in the yields from mixed superinfection of the lysogenic host are still much less than those found in lysates of mixed infections of the sensitive host. The burst size of P22 Vxc^+ was 10-fold greater in mixed superinfection than in single superinfection (Table 3, cf. infection 16 with infection 15) and three times greater than that found for wild-type P22 c^+ in the corresponding mixed superinfection (Table 3, cf. infection 16 with infection 2). We conclude that the *Vx* mutation has a small effect on relieving replication inhibition of a nonvirulent phage.

The capacity to cause multiplicity repression in the lysogenic host is correlated with escape from replication inhibition. The *Vx* and *K5* mutations appear to confer both of these properties simultaneously to phage

TABLE 3

REPLICATION INHIBITION WITH PHAGE P22[a]

Infection	Phage	Sensitive Strain 18			Lysogenic Strain 210		
		Total burst size	Nonvirulent burst size	% Nonvirulent	Total burst size	Nonvirulent burst size	% Nonvirulent
1	c^+	90			0.4		
2	c^+ × *virB-3*	45	12	27	94	2.2	2.3
3	c_1^7	200			2.6		
4	c_1^7 × *virB-3*	96	40	42	90	1.4	1.5
5	c_2^5	205			3.3		
6	c_2^5 × *virB-3*	74	28	38	110	2.4	2.2
7	c_2^{27}	104			1.1		
8	c_2^{27} × *virB-3*	89	42	47	74	4.5	6.1
9	*K5*	93			2.0		
10	*K5* × *virB-3*	84	32	38	73	32	44
11	Vxc_2^5	230			2.5		
12	Vxc_2^5 × *virB-3*	98	37	37	100	7.3	7.3
13	Vxc_2^{27}	235			3.6		
14	Vxc_2^{27} × *virB-3*	69	40	58	98	16	16
15	Vxc^+	15			0.6		
16	Vxc^+ × *virB-3*	9.5	1.4	15	45	6.9	13

[a] Single infections were performed at an m.o.i. of 5, mixed infections at an m.o.i. of 5 for each phage. The clear plaques with a small ring of surviving bacteria in the center characteristic of *virB-3* are easily distinguishable from turbid plaques made by c^+ phage and the completely clear plaques made by *K5*, c_1, and c_2 phage.

P22. In contrast, wild-type P22 c^+ is replication inhibited and does not cause multiplicity repression in a lysogen.

K5 and Vx Complement in a Lysogen

When the *K5* and *Vx* mutations are in the *cis* position, as in the double mutant P22 *virB-3*, good phage yields result on superinfection of a lysogenic host at low multiplicities. We asked whether the *K5* and *Vx* mutations in the *trans* position could promote phage production in a lysogen. Although neither P22 *K5* nor P22 *Vx* alone produced significant bursts on superinfection of the lysogenic strain 210 (Table 4, infections 1–4), mixed superinfection by these phages resulted in phage yields at the level found for P22 *virB-3* (cf. Table 4, infections 5–7 with Table 2, infections 1 and 2). Virulent recombinants represented less than 1% of the phage yield.

In mixed infection of sensitive strain 18 by *K5* and *Vx*, the P22 *Vx* markers represented approximately half the phage yield (Table 4). However, mixed superinfection of the lysogenic host under the same conditions resulted in only about 20% of the phage yield carrying the *Vx* markers (Table 4). This result is consistent with the finding that P22 *K5* shows complete excape from replication inhibition, whereas P22 *Vx* shows only partial excape.

A number of c_1, c_2, and c_3 mutants were tested to see whether they could complement either P22 *K5* or P22 *Vx* in a lysogen and were not able to do so. These data indicate that *K5* and *Vx* are specific mutations in the clear region which confer to phage P22 constitutive expression of diffusible gene products necessary for growth.

DNA Synthesis in Superinfected Lysogens

Superinfection of lysogenic strain 210 by P22 *virB-3* resulted in a rate of DNA synthesis pattern (Fig. 2A) similar to that found by Smith and Levine, 1964) for P22 c_1 infection of a sensitive host. There was a brief drop in the rate of DNA synthesis immediately after infection followed by a rapid increase in rate above the level of the uninfected control, reaching a peak at about 20 minutes after superinfection. The rate of

TABLE 4

COMPLEMENTATION IN A LYSOGEN BETWEEN P22 *K5* AND P22 *Vx*[a]

Infection	Phage	Sensitive strain 18			Lysogenic strain 210		
		Total burst size	*Vx* burst size	% *Vx* markers	Total burst size	*Vx* burst size	% *Vx* marker
1	*K5*	24			2.2		
2	Vxc^+	1.7			1.2		
3	Vxc_2^{27}	230			3.9		
4	Vxc_2^5	170			4.4		
5	$K5 \times Vxc^+$	10	4.2	42	34	6.9	19
6	$K5 \times Vxc_2^{27}h_{21}$	56	29	52	44	9.2	21
7	$K5h_{21} \times Vxc_2^5$	38	19	49	18	4.0	22

[a] The total m.o.i. in all infections was 10. In mixed infections the m.o.i. was 5 for each phage. The large clear plaques made by P22 *K5* are easily distinguishable from the small turbid plaques made by P22 Vxc^+. To distinguish between P22 *K5* and P22 Vxc_2^5 or P22 Vxc_2^{27}, one of the parental phage carried the h_{21} plaque morphology marker, which confers no selective advantage or disadvantage.

DNA synthesis then declined sharply as the bacteria lysed and phage progeny were released.

Although P22 *K5* is not subject to replication inhibition, superinfection of strain 210 by P22 *K5* did not lead to a greatly increased rate of DNA synthesis. The rate of DNA synthesis at no time exceeded the rate in the uninfected control (Fig. 2B). P22 Vxc_2^{27} showed a pattern of DNA synthesis similar to that of P22 *K5* on superinfection of the lysogenic host (Fig. 2C). Mixed superinfection by P22 *K5* and P22 Vxc_2^{27} resulted in a rate of DNA synthesis pattern similar to P22 *virB-3* (Fig. 2D). Since P22 *K5* and P22 *Vx* complement to make DNA in a lysogen, these results indicate that diffusible products controlled at both the *K5* and *Vx* mutational sites are necessary for extensive phage DNA replication.

Cell Killing and Lysis of a Lysogen

When lysogenic strain 210 was superinfected by P22 *virB-3* at an m.o.i. of 5, about 98% of the cells were killed. The optical density of the culture increased until 30 min after superinfection, at which time lysis occurred (Fig. 3). Strain 210 was superinfected with P22 c_2^5, P22 Vxc_2^5 or P22 *K5* at an m.o.i. of 5 to determine the effect of the individual mutations on cell killing and lysis. Less than 1% of the cells superinfected by P22 c_2^5 were killed. The optical density increased with time at the same rate as the uninfected control (Fig. 3). Superinfection by P22 Vxc_2^5 resulted in a similar increase in optical density of the culture, and less than 4% of the cells were killed (Fig. 3). In contrast, superinfection by P22 *K5* killed 46% of the cells. The optical density of the culture increased until 30 min after superinfection, after which time the optical density remained approximately constant until 60 min after superinfection (Fig. 3). Between 60 and 90 min, the optical density increased slightly. Although 46% of the cells were killed, only 3% gave rise to infectious centers, and the burst size was only 2.0. When the P22 *K5* superinfected culture was treated with chloroform at 90 min, the optical density dropped, giving the appearance of cell lysis. In contrast, cells superinfected by P22 c_2^5 or P22 Vxc_2^5 gave no indication of lysis when treated with chloroform. These results indicate that the *K5* mutation but not the *Vx* mutation confers the ability to kill upon superinfection of a lysogen. Furthermore, since lysogens superinfected by P22 *K5* are lysed by treatment with chloroform, it is likely that the *K5* mutation confers constitutive expression of some late gene function(s).

DISCUSSION

The ability of P22 *virB-3* to grow in an immune host without inducing prophage indicates that its component mutations, *K5*

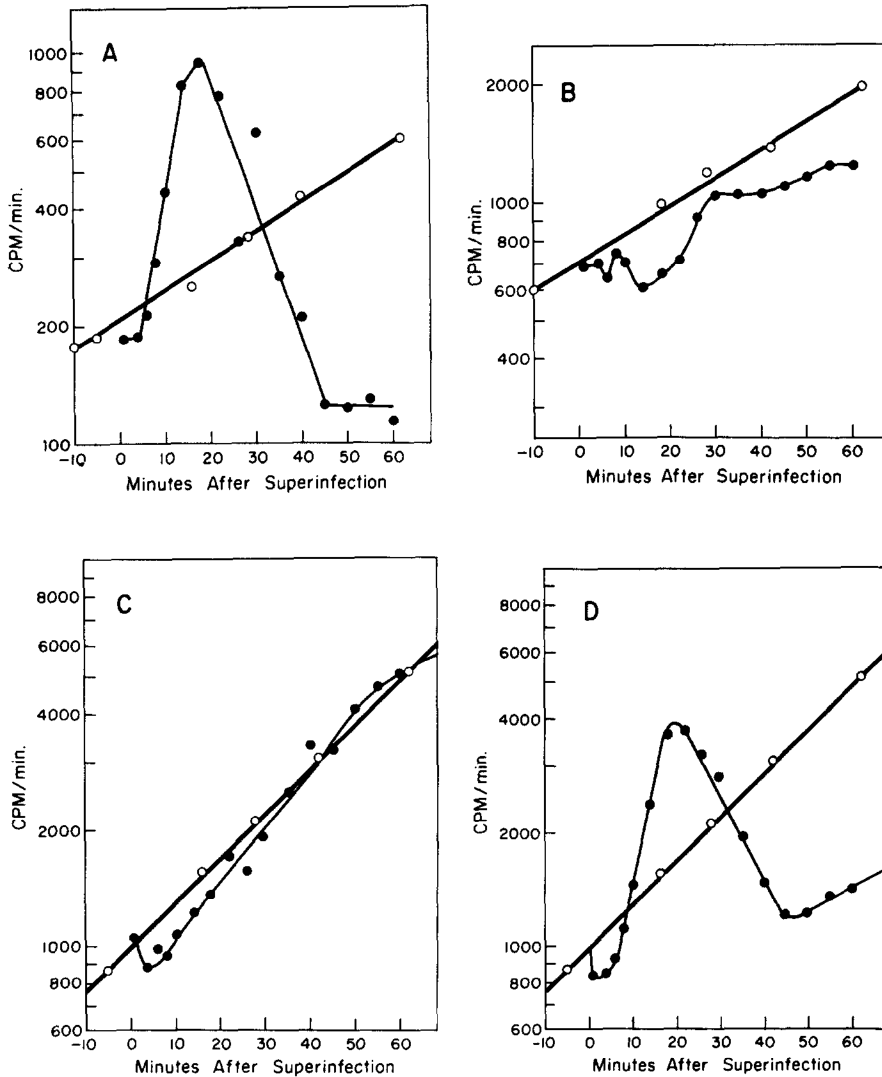

FIG. 2. Rate of incorporation of [^{3}H]thymidine into DNA after superinfection of lysogenic strain 210 at 37°C. One-minute pulses of [^{3}H]thymidine were administered as described by Smith and Levine (1964). (A) P22 virB-3, m.o.i. = 5. (B) P22 *K5*, m.o.i. = 5. (C) P22 Vxc_2^{27}, m.o.i. = 5. (D) P22 *K5*, m.o.i. = 5 + P22 Vxc_2^{27}, m.o.i. = 5. ○—○, Uninfected control culture; ●—●, superinfected cells.

and *Vx*, relieve this phage of repressor control. The constitutive nature of the *K5* and *Vx* mutations was demonstrated in several ways:

1. Although neither P22 *K5* nor P22 *Vx* replicates extensively in a lysogen, they complement in *trans* for phage DNA synthesis and production of progeny phage. We conclude that both P22 *K5* and P22 *Vx* can express functions involved in replication of phage P22 in the presence of repressor.

2. P22 *K5* and, to a lesser extent, P22 *Vx* escape replication inhibition. This *cis* function is not expressed by other nonvirulent phage.

3. Superinfection by P22 *K5* results in killing of lysogens and expression of some late gene function(s). These effects have not been demonstrated for P22 *Vx*.

The constitutive expression of phage functions by P22 *virB-3* is not absolute. Growth of P22 *virB-3* is increasingly inhibited as the

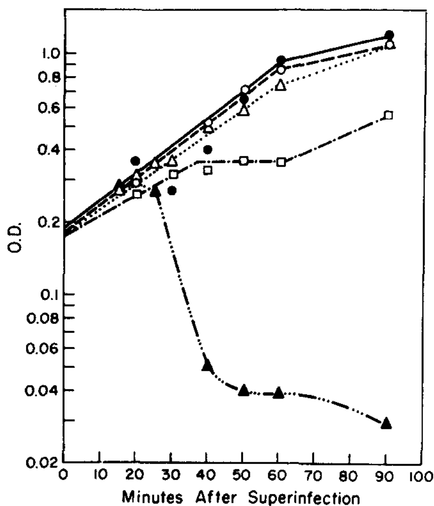

FIG. 3. Effect of superinfection of strain DB103 on cell growth as measured by increase in optical density (O.D.). Log phase cultures of the lysogenic host were superinfected at an m.o.i. of 5, shaken at 37°, and periodically assayed for O.D ○-----○, uninfected control; ▲··—▲, P22 *virB-3*; ●——●, P22a $c_2{}^5$; △····△, P22 $Vxc_2{}^5$; □-·-·□, P22 *K5*.

multiplicity of coinfecting wild-type phage is raised. We have given the name *multiplicity repression* to the phenomenon of a decreased burst size with increased multiplicity. Multiplicity repression is due to the action of the c_2 repressor and is not dependent on a functional *mnt* gene product.

P22 *virB-3* also exhibits multiplicity repression in single infection. This finding was unexpected, since one of its component mutations, *K5*, maps in the c_2 gene. Introducing either the $c_2{}^5$ or $c_2{}^{27}$ mutation in *cis* to P22 *virB-3* eliminates self-induced multiplicity repression. This indicates that the *K5* mutation does not completely destroy c_2 repressor activity. We conclude that P22 *virB-3* synthesizes active c_2 repressor and exhibits a reduced but finite sensitivity to the action of this repressor.

Two c_2 mutants other than P22 *K5* also exhibit multiplicity repression (unpublished data). By this test we can classify P22 c_2 mutants into two groups: those which retain some repressor activity and those which have no detectable repressor function. More data must be accumulated to determine a possible relationship between mutational site and multiplicity repression. However, it should be noted that the two c_2 mutations, $c_2{}^5$ and $c_2{}^{27}$, shown to destroy all repressor activity define the c_2 gene by mapping at its extremes (Levine and Curtiss, 1961).

Multiplicity repression resembles a multiplicity effect described for phage P22 in which reduction and the lysogenic response are favored by high multiplicities of wild-type P22 c^+ phage (Boyd, 1951; Levine, 1957), but it is unclear whether or not the two effects are mediated by the same mechanism. In this context it should be noted that P22 *virB-3* does not form stable lysogens (unpublished data). Clear mutants of the closely related heteroimmune phage L have been described which show an effect similar to multiplicity repression (Bezdek *et al.*, 1970).

The ability of P22 *K5* and P22 *Vx* to express gene functions in an immune host and the sensitivity of *virB-3* to c_2 repressor give some clues as to the nature of the *K5* and *Vx* mutations. The ability to complement in *trans* suggests that P22 *K5* and P22 *Vx* are constitutive for two different segments of the P22 genome normally under repressor control, and may be analogous to the mutations involved in phage λ virulence. The lambda *c*I repressor, analogous to the P22 c_2 repressor, blocks transcription of the λ genome by binding to two operator sites which map on each side of the *c*I gene and control the adjacent operons (Ptashne and Hopkins, 1968; Taylor *et al.*, 1967). A virulent λ mutant bearing operator constitutive (O^c) mutations at both of these sites has been described (Ptashne and Hopkins, 1968). These O^c mutations, v_2 in the left-hand operator and v_1v_3 in the right-hand operator, decrease but do not abolish the affinity of λ DNA for repressor *in vitro* (Ptashne and Hopkins, 1968). Sly and Rabideau (1969) suggested that residual repressor binding to λv_2 DNA prevents fully constitutive expression of the adjacent operon. P22 *virB-3* may be sensitive to multiplicity repression for a similar reason. *Vx* maps to the left of the c_2 gene, and *K5* maps in c_2 to the right of *Vx*. If *K5* and/or *Vx* are O^c mutations leaving P22 DNA with some residual affinity for repressor at the

operator sites, high concentrations of the c_2 gene product could create a binding equilibrium favoring repression. It should be stressed that only one of the component mutations of P22 *virB-3* need be an O^c mutation of this type for multiplicity repression to obtain. The other mutation may be of the new promoter type as has been suggested for the c_{17} mutation which is a component of a virulent mutant of phage λ (Sly and Rabideau, 1969). c_{17} maps at some distance from the λ operator sites and creates constitutivity of the right-hand operon, suggesting release from operator control.

Escape from replication inhibition is a prerequisite for phage development in the presence of repressor. It follows that at least one component mutation should confer this *cis* property to P22 *virB-3*. P22 *K5* completely escapes from replication inhibition, whereas P22 *Vx* gives only partial escape. It has been demonstrated that the physical basis of replication inhibition is the direct block by prophage repressor on the association of input nonvirulent DNA with the P22 replication complex (Levine *et al.*, 1970). This complex, called intermediate I by Botstein (Botstein, 1968), consists of parental phage DNA, newly synthesized DNA, and other cell constituents. P22 *K5* genomes, in accord with their escape from replication inhibition, associate with intermediate I in a lysogen to a greater extent than other nonvirulent genomes (unpublished data). Although this association is dependent on the *trans* function of gene *25* (Levine *et al.*, 1970), a *cis* function is required in addition. Three hypotheses are advanced to explain this *cis* function: (1) The site on the phage genome for binding of phage DNA to the replication machinery is blocked by bound repressor. Loss of affinity for repressor as a consequence of an O^c mutation would result in escape from replication inhibition. (2) Binding to intermediate I is a consequence of general transcription. Constitutive transcription by a genome bearing an O^c or a new promoter mutation would be required for association with intermediate I in a lysogen. (3) Transcription of a critical region of the input genome is required for binding to the replication machinery as has been suggested for phage lambda (Dove *et al.*, 1969). The mechanisms suggested in (1) and (3) require events at unique sites on the P22 genome. Although both *K5* and *Vx* have effects on replication inhibition, these mutations map at different loci (Fig. 1). Therefore, mechanism 1 requires the assumption that there is more than one site of binding to intermediate I on the P22 genome. Mechanism 3 requires the assumption that both *K5* and *Vx* mutations confer constitutive transcription of a critical region of the genome. No further assumptions are required for mechanism 2. The *K5* mutation which confers complete escape from replication inhibition to the P22 genome may be either a new promoter or an O^c mutation which is insensitive to repressor. The resultant strong transcription of the constitutive operon could lead to binding of the P22 *K5* genome to the replication complex. The partial sensitivity of P22 *Vx* to replication inhibition could be due to residual binding of repressor at the *Vx* site. *Vx* might be a leaky O^c or a weak new promoter mutation which confers weakly constitutive transcription to the genome.

Mutations which confer escape from replication on phage λ, v_1v_3 and c_{17} (Ptashne and Hopkins, 1968; Sly and Rabideau, 1969) have additional similarities to *K5*. Two genes required for phage DNA synthesis map to the right of each mutation: genes *18* and *12* of phage P22 (Levine and Schott, 1971) and genes *O* and *P* at analogous positions in phage λ (Sly and Rabideau, 1969). Like λv_1v_3 and λc_{17} (Sly and Rabideau, 1969), P22 *K5* shows constitutive killing. Despite these similarities, the λ mutants have some properties not associated with P22 *K5*. Both λv_1v_3 and λc_{17} are constitutive for genes *O* and *P*, although transcription of genes to the left of *c*I is under normal repressor control (Sly and Rabideau, 1969). These λ mutants replicate extensively in a lysogen, suggesting that *O* and *P* functions are sufficient for λ phage DNA synthesis. Strong virulent mutants are formed when a *c*I mutation is introduced in *cis* to v_1v_3 or c_{17}. In contrast to these λ mutants, introduction of a mutation in the repressor gene in *cis* to

K5 does not result in the formation of a virulent mutant (Bronson and Levine, 1971). Furthermore, P22 *K5* alone does not replicate extensively in an immune host. A function(s) supplied by P22 *Vx* is required for phage DNA synthesis. If by analogy with phage λ we assume that the products of genes *18* and *12* are sufficient for replication of phage P22, it appears that P22 *K5* is not constitutive for these genes. This would suggest that a constitutive function supplied by P22 *Vx* can activate P22 *K5* to transcribe genes *18* and *12* in the presence of repressor. Alternatively, P22 *K5* alone may be constitutive for *18* and *12*, but the functions of these genes are not sufficient for extensive phage replication. The constitutive function(s) supplied by P22 *Vx* may be required in addition.

The inability of wild-type c^+ phage to cause multiplicity repression of P22 *virB-3* in a lysogen suggests that the superinfecting replication inhibited genome cannot express the c_2 gene function in the presence of immunity. Both P22 *K5* and P22 *Vx* induce multiplicity repression in a lysogen and escape from replication inhibition. This correlation between multiplicity repression and escape from replication inhibition suggests that superinfecting genomes must associate with the P22 replication complex in order to transcribe the c_2 gene. Wild-type P22 c^+ inhibits growth of *VirA* mutants in a lysogen, suggesting that they can synthesize repressor (Swanson and Botstein, personal communication). This result is complicated by the apparent ability of *VirA* mutants to inactivate or inhibit synthesis of the prophage repressor. The amount of repressor synthesized by P22 c^+ in a lysogen may be sufficient to repress *VirA* but not *VirB* mutants. Although λ c^+ phage are subject to replication inhibition, the *c*I repressor is expressed by λc^+ in a lysogen and inhibits the growth of a virulent mutant (Sly and Rabideau, 1969). It is unclear just how much repressor, if any, is synthesized by wild-type phage P22 in mixed superinfection with P22 *virB-3*.

In summary, we have demonstrated that the *K5* and *Vx* mutations confer virulence to *P22 virB-3* by reducing the sensitivity of P22 DNA to the prophage repressor. P22 *virB-3* remains sensitive to high levels of the c_2 repressor and has the unusual property among virulent mutants of being able to repress its own development at high multiplicities. Both P22 *K5* and P22 *Vx* express functions involved in phage DNA synthesis in the presence of repressor. In addition, P22 *K5* is constitutive for cell killing and some late function(s).

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