JOURNAL OF VIROLOGY, May 1971, p. 559-568

Vol. 7, No. 5
Printed in U.S.A.

Virulent Mutants of Bacteriophage P22

I. Isolation and Genetic Analysis

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Received for publication 21 December 1970

Mutants of phage P22 which form plaques on a P22 lysogen have been isolated. These virulent mutants have been classified into three groups. (i) *VirA* mutants arise spontaneously in wild-type stocks and form very small turbid plaques on a P22 lysogen. The single mutation responsible for *VirA* virulence maps near the *mnt* locus, one of the immunity regions of phage P22. (ii) *VirB* mutants do not arise spontaneously and have been isolated only from mutagenized P22 stocks. *VirB* mutants form small, clear plaques on a P22 lysogen. One of the *VirB* mutants, *virB-3*, was analyzed in detail. The *virB-3* mutant is comprised of two mutations: *K5*, which maps within the c_2 gene, and *Vx*, which maps in the region between the c_2 and c_3 genes. Phages carrying either the *K5* or *Vx* mutation are not virulent in themselves but mutate to *VirB* virulence at a frequency of 10^{-5} to 10^{-6}. It is concluded that *K5* and *Vx* are mutations at specific sites which together confer the ability to undergo phage development in the presence of repressor. (iii) *VirC* mutants are defined by a large clear plaque morphology when plated on a P22 lysogen. *VirC* mutants are comprised of the determinants of both *VirA* and *VirB* virulence.

Phage P22, a temperate phage of *Salmonella typhimurium*, can elicit either of two responses upon infection of a sensitive cell. In the lytic response, phage progeny are produced and are released when the bacteria lyse. In the lysogenic response, the bacteria survive infection and show a stable hereditary alteration as a result of prophage integration into the host chromosome (16, 17). The prophage renders the lysogenic cell immune to superinfection by phage P22 (19). The lysogenic cell can be induced to produce phage progeny upon exposure to ultraviolet light or the antibiotics mitomycin and streptonigrin (7, 8). Prophage with a temperature-sensitive mutation in the c_2 gene can be heat induced (11). Upon exposure to high temperature, cells carrying tsc_2 prophage lose their immunity and the prophage undergoes the lytic cycle of development. Thus, the c_2 gene codes for one of the immunity substances or repressors of phage P22. Phages carrying temperature-sensitive mutations in a second immunity gene, *mnt* (Fig. 1), have been described which also show heat induction although the kinetics are different from tsc_2 heat induction (4).

A replication complex for P22 deoxyribonucleic acid (DNA) synthesis has been described (1). This complex, intermediate I, is an association of parental phage DNA, newly synthesized phage DNA, and other cell constituents. In a previous paper we have shown that, upon superinfection of P22 lysogens with the homoimmune phage, the input phage DNA does not associate with intermediate I (9). That this inhibition is due to the action of repressor was shown by good association of the superinfecting DNA with the complex after induction with ultraviolet light.

The control of DNA replication and phage development was further studied by the isolation of virulent mutants of P22. Such mutants replicate in the presence of repressor and undergo the the normal sequence of phage development in a lysogen. This paper describes the isolation and genetic analysis of such mutants. A preliminary report of this work has been presented (M. Bronson and M. Levine, Bacteriol. Proc., p. 201, 1970).

MATERIALS AND METHODS

Bacteria and phage. Strain 18, a derivative of *S. typhimurium* LT2, cured of the PBI phage, was used as the sensitive host (19). Strain 210 is strain 18 carrying a mutant prophage, *sie-1 ts 12.1, ts 2.1* and was used as the lysogenic host. In the prophage state, wild-type P22 excludes superinfecting phage; *sie* mutants (15, 18) do not exclude. Both wild-type phage and *sie* mutants immunize the lysogen. The conditional lethal mutations, *ts 2.1* and *12.1*, are from the Levine collection. Mutants carrying these alleles form plaques on strain 18 at 25 C but not at 37 C. Strain 200 is strain 18 carrying a wild-type P22 prophage.

The wild-type turbid c^+, the clear mutants c_1, c_2,

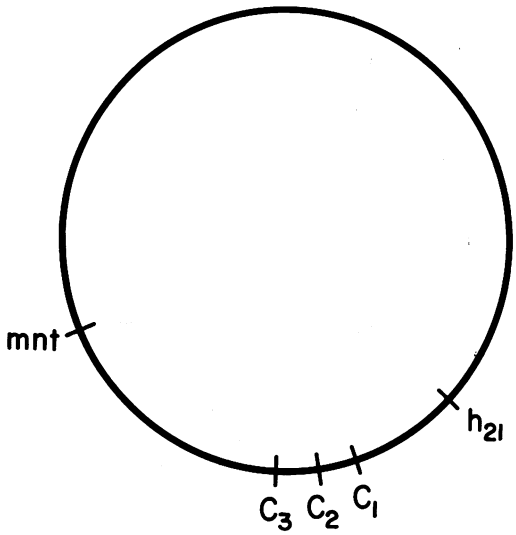


FIG. 1. *Circular map of phage P22.*

and c_3 (6, 10), and the heat-inducible mutants tsc_2^{29} (11) and *tsmnt-1* (4) were previously described.

Media. L broth (LB), buffered saline, supplemented M9 medium, indicator agar, and soft agar for top layers have previously been described (6). Phage were also plated on tryptone agar: tryptone, 10 g; NaCl, 5 g; agar, 12 g; and water, 1 liter.

Infection and phage crosses. An overnight culture of strain 18 was diluted 1:100 in LB and incubated at 37 C with continuous aeration until the cells reached a concentration of 10^8/ml. Phage were added at a total multiplicity of infection (MOI) of 20 (10 each). After a 5-min adsorption period, the culture was diluted 1:10 into LB containing antiserum (final K = 2) for 5 min to inactivate unadsorbed phage. Samples were then diluted in tubes containing LB. At 90 min after infection, the growth tubes were shaken with chloroform, and the lysates were assayed on the proper indicators.

RESULTS

Selection of virulent mutants. Virulent mutants of phage P22 are defined as phage which form plaques on a P22 lysogen, strain 210. The following procedure was used to select for such mutants. High concentrations of mutagenized or untreated P22 stocks were plated on tryptone agar seeded with strain 210. The plates were incubated at 37 C overnight. The small number of plaques which appeared were picked into buffered saline and plated on tryptone agar seeded with sensitive strain 18. Isolated plaques were picked and plated in duplicate on tryptone agar plates seeded with strain 18 or with strain 210. Those phage isolates which again formed plaques on both strain 18 and strain 210 were purified again from plaques on strain 18 and grown to high titer for further study.

The frequency of virulent mutants in a phage preparation was determined by the number of plaques formed on the lysogenic strain 210 relative to the number formed on sensitive strain 18. Virulent mutants were found spontaneously in various P22 stocks at a rate of 10^{-8} or less (Table 1). Spontaneous virulent mutants showed only one type of plaque morphology on strain 210. The plaques were very small and turbid. Virulent mutants with this phenotype were designated as *VirA*. The *VirA* mutants derived from c^+ stocks made large plaques with bullseye turbid centers, typical of *mnt* mutants (4) on strain 18. The *VirA* mutants derived from clear mutants made large clear plaques on strain 18.

Stocks of wild-type phage P22 mutagenized with 1-methyl-3-nitro-1-nitrosoguanidine and with 2-aminopurine (gifts of M. Gough) were also used as a source of virulent mutants. The frequency of virulent mutants was 2×10^{-6} for the nitrosoguanidine-treated stocks and 5×10^{-6} for the aminopurine-treated stocks (Table 1).

Three plaque types were observed when the mutagenized phage stocks were plated on strain 210. Small turbid plaques characteristic of *VirA* mutants showed the bullseye turbid phenotype on strain 18 made both by *mnt* mutants and by the spontaneous *VirA* mutants. Small clear plaques were characteristic of a second class of virulent mutants designated *VirB*. These mutants made large clear plaques with a small turbid center on strain 18. A third class of virulent mutants which made large clear plaques on strain 210 was desig-

TABLE 1. *Frequency of virulent mutants*

Phage stock	Frequency of virulent mutants	Type
Wild type	10^{-9}	*VirA*
c_2^3	10^{-10}	
c_2^5	10^{-8}	*VirA*
c_2^8	$<10^{-8}$	
c_2^{19}	$<10^{-8}$	
c_2^{23}	$<10^{-10}$	
c_2^{27}	$<10^{-8}$	
tsc_2^{29}	10^{-8}	*VirA*
$c_2^{71}c_3^{32}$	10^{-8}	*VirA*
$c_2^6c_3^{32}$	10^{-8}	*VirA*
tsmnt-1	10^{-9}	*VirA*
tsc_2^{29} *tsmnt-1*	$<10^{-8}$	
Nitrosoguanidine mutagenized wild type	2×10^{-6}	*VirA, VirB, VirC*
Aminopurine mutagenized wild type	5×10^{-6}	*VirA, VirB, VirC*

nated *VirC*. These mutants made large clear plaques without a turbid center on strain 18.

Plating efficiency of virulent mutants. All three types of virulent mutants plated well on the P22 *sie* lysogen, strain 210 (Table 2). *VirB* and *VirC* mutants showed an efficiency of plating (EOP) of 0.7 on strain 210 compared to strain 18 at 37 C. *VirA* mutants had an EOP of 0.4. None of the virulent mutants plated on strain 200 which carries a sie^+ prophage. Thus, although virulent mutants overcome the effect of prophage repressor, they are still sensitive to superinfection exclusion.

Genetic analysis of virulent mutants. To determine the mutations involved in virulence, the virulent mutants were backcrossed with wild-type P22. Progeny from these crosses were analyzed for plaque morphology different from that of the parental phage.

Mapping of VirA mutants. Phages carrying the tsc_2^{29} mutation were used as a source of virulent mutants. The heat-inducible mutant, tsc_2^{29}, makes turbid plaques at 25 C and semiclear plaques at 37 C on strain 18. Plaques of the *VirA* phenotype arise spontaneously from our tsc_2^{29} stock at a low frequency (Table 1). One of these *VirA* mutants, tsc_2^{29}*virA-9*, was subjected to genetic analysis.

The tsc_2^{29}*virA-9* mutant makes large clear plaques on strain 18 at 37 C which are distinguishable from the semiclear plaques of tsc_2^{29}. Two plaque types differing from the parental plaque types were observed when tsc_2^{29}*virA-9* was backcrossed with wild-type P22. One recombinant showed the semiclear plaque morphology of tsc_2^{29}, and the other type showed the bullseye turbid plaque morphology typical of *mnt* mutants. Recombinants with the *mnt* phenotype formed the small turbid plaques typical of *VirA* mutants on strain 210. The mutation(s) carried by this phage was designated as V_9. Recombinants with the plaque morphology typical of tsc_2^{29} did not make plaques on strain 210. It appears that the V_9 mutation confers virulence to tsc_2^{29}*virA-9* and that the tsc_2^{29} mutation is not required for virulence. Recombination data suggested that the V_9 mutation is not closely linked to the clear region of P22. Several other *VirA* mutants derived from various stocks were analyzed as above. In each case the *VirA* phenotype was associated with mutants exhibiting the *mnt* phenotype on the sensitive host.

Phages carrying the V_9 mutation were backcrossed with wild-type P22. If V_9 consisted of more than one mutation, progeny with plaque morphology different from that of either parent might be expected to result from the backcross. Only the parental phenotypes were observed among 11,000 progeny plaques. This result is consistent with the idea that *VirA* virulents are the result of a single mutation.

Although *VirA* mutants have the same plaque morphology on strain 18 as *mnt* mutants, the latter are not in themselves virulent. If the *VirA* and *mnt* sites are closely linked, wild-type recombinants would be rare among the progeny of a cross between *tsmnt-1* and V_9. The heat-inducible mutant, *tsmnt-1*, forms wild-type plaques at 25 C and plaques that appear identical to V_9 at 37 C. Of 10,000 progeny from the above cross, only two formed the wild-type turbid plaque when plated on strain 18 at 37 C. This result indicates that V_9 maps very close to *tsmnt-1*, about 0.04 units away. Several other mutations conferring *VirA* virulence were mapped in this way, and each mapped very near to *tsmnt-1* and V_9.

Analysis of a VirB mutant. A typical *VirB* mutant, *virB-3*, was selected for genetic analysis. The frequency of turbid plaque recombinants was extremely low in crosses between *virB-3* and various clear mutants, indicating that *virB-3* carries at least one mutation which maps in or near the clear region. In an attempt to define its component mutations, the *virB-3* mutant was backcrossed with wild-type P22, and the lysate was plated on strain 18. Only one recombinant phenotype was noticed: a clear plaque morphology without the turbid center of *virB-3* plaques. These plaques occurred with a frequency of 0.69%. The formation of a new recombinant type among the progeny of the backcross demonstrated that *virB-3* differs from wild-type P22 by at least two mutations, and the low frequency of the clear plaque recombinants suggested that two mutations in *virB-3* map in or near the clear region.

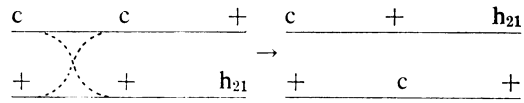
One explanation for the apparent absence of a second recombinant type could be that both recombinants have the same clear plaque morphology. To test this possibility, *virB-3* was backcrossed with wild-type P22 carrying the h_{21} plaque-morphology marker, which maps seven

TABLE 2. *Plating efficiency of virulent mutants*

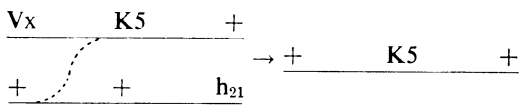
Mutant	Type of virulent	Plating efficiency[a]		
		Strain 18	Strain 210	Strain 200
c_2^5		1.0	10^{-8}	$<10^{-10}$
virA-9	*VirA*	1.0	0.4	$<10^{-10}$
virB-3	*VirB*	1.0	0.7	$<10^{-9}$
virC-1	*VirC*	1.0	0.7	$<10^{-9}$

[a] All platings were made at 37 C.

units to the right of the clear region. If both recombinants had the same morphology, clear plaques showing the h_{21} phenotype should arise with the same frequency as those showing the h_{21}^+ phenotype:

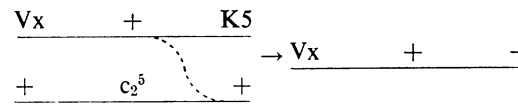


This is clearly not the case. Of 5,575 progeny plaques, 32 clear recombinants were observed: 25 h_{21}^+ and 7 h_{21}. None of the clear recombinants was virulent. These data suggested that the clear plaque morphology of these recombinants is due to a component mutation of *virB-3*, designated *K5*. Virulence requires a second mutation, designated *Vx*. The *Vx* recombinants do not make clear plaques. Furthermore, the preferential association of the clear plaque morphology with the h_{21}^+ phenotype indicated that *K5* maps 1.14 recombination units to the right of *Vx*:



Mapping of the K5 mutation. Phages carrying the *K5* mutation complemented c_1 and c_2 mutants on the standard spot test for lysogeny (6) but did not complement c_2 mutants. This result suggested that *K5* is a mutation in the c_2 gene. The *K5* mutation was mapped in detail by three-factor crosses with other clear mutants. The frequency of turbid recombinants and the segregation of the outside marker, h_{21}, were followed among these recombinants (Fig. 2). The *K5* mutation mapped to the right of all c_2 mutations tested except for c_2^{27} (Table 3). Thus, *K5* maps within the c_2 gene between known c_2 mutations (Fig. 3). No plaque with a morphology different from that of the parental phage was observed among 4,000 individual plaques made by the progeny of a *K5* × wild-type P22 backcross. This result and the mapping data are consistent with *K5* being a single mutation.

Isolation of the Vx mutation. Phages carrying the *Vx* mutation would not have been recognized among progeny of the *virB-3* backcross if they form plaques similar to those of wild-type P22. On this assumption, a genetic selection procedure for *Vx* phage was devised based on recombination data indicating that the *Vx* mutation maps further to the left of *K5* (1.14 units) than does c_2^5 (0.21 units; Table 3). The *virB-3* mutant was crossed with a phage carrying the c_2^5 mutation. Turbid recombinants carrying the *Vx* mutation should arise by a single crossover between *K5* and c_2^5. Formation of wild-type recombinants would require a second crossover between *Vx* and c_2^5. The majority of turbid recombinants should, therefore, carry the *Vx* mutation.



Of 12 turbid recombinants tested, 8 showed a small plaque morphology on strain 18. These recombinants bred true, exhibiting the small turbid plaque morphology through several cycles of growth and were assumed to carry the *Vx* mutation. These *Vx* phage did not make plaques on strain 210. Although their morphology is similar, wild-type plaques can be recognized by virtue of their larger size among a known background of *Vx* plaques.

K5 and Vx phages contain the component mutations of virB-3. If *K5* and *Vx* phages contain all the components of *VirB* virulence, they should recombine to form the original *virB-3* mutant. The lysate from a mixed infection of *K5* and *Vx* in the sensitive strain was plated on strain 18 for total progeny and on strain 210 for virulent recombinants. Progeny which formed small clear plaques on the lysogen were found at a frequency of 6.5×10^{-3} (Table 4). In contrast, only about 10^{-5} of the progeny formed plaques on strain 210 when either *K5* or *Vx* alone infected strain 18 (Table 4). The calculated distance between *K5* and *Vx* from these data is 1.30 map units, in good agreement with the distance calculated from the backcross of *virB-3* and wild-type P22. These results indicate that *K5* and *Vx* phages, though not virulent in themselves, contain the component mutations of *virB-3*.

Mapping of the Vx mutation. Wild-type P22 and *Vx* phages were mixedly infected in strain 18, and the progeny were plated on the sensitive indicator. No plaques with a morphology different from that of either parental phage were observed among 2,000 individual plaques analyzed. This result is consistent with *Vx* being a single mutation.

The *Vx* mutation was mapped by three-factor crosses with various clear mutants. Wild-type recombinants were analyzed for segregation of the h_{21} marker or the h_{21}^+ marker (Fig. 4). The *Vx* mutation mapped 1.29 units to the right of c_3^{77}, the right-most c_3 mutation known, and 0.18 units to the left of c_2^5, the left-most c_2 mutant known (Table 5, reference 9). These data are consistent with *Vx* being a single mutation which maps in the region between the c_2 and c_3 genes (Fig. 3).

Mapping of VirC mutants. A typical *VirC*

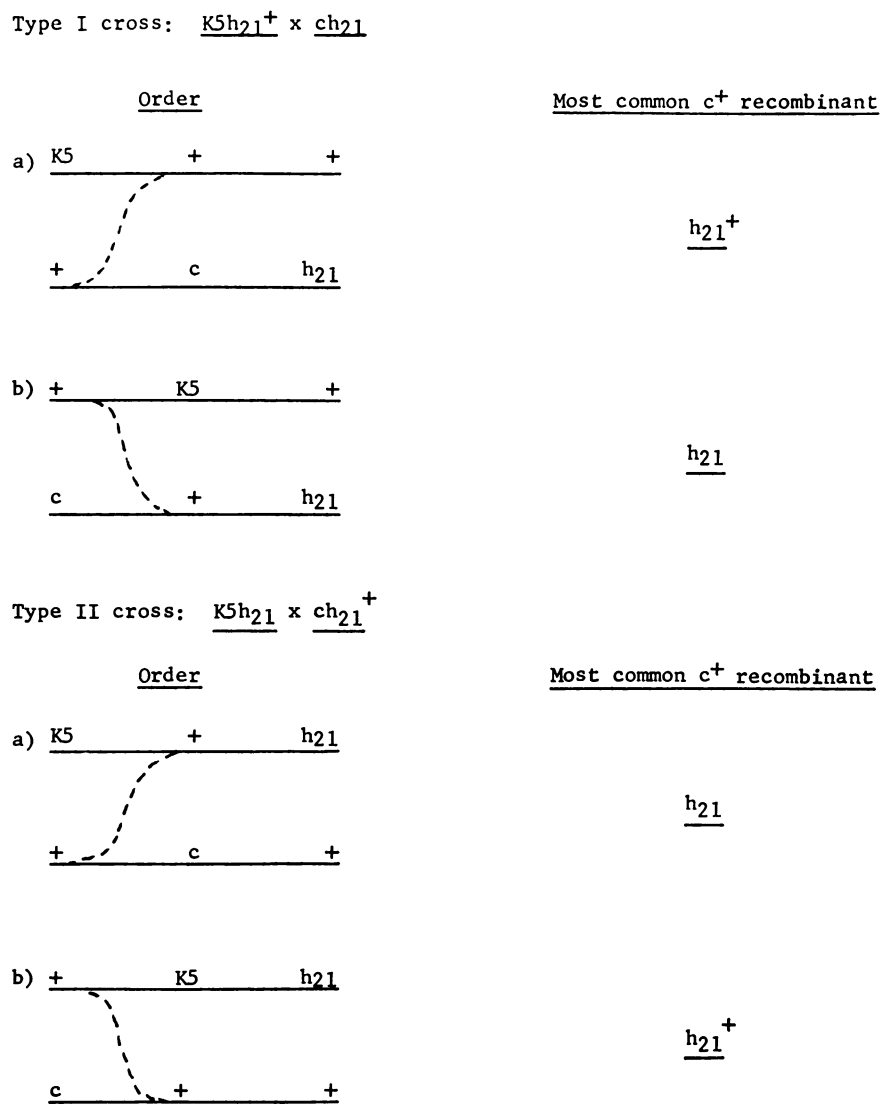
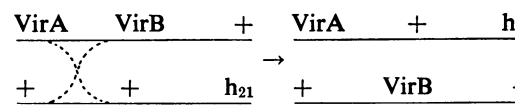


FIG. 2. *Determination of map position of K5.*

mutant, *virC-1*, makes large clear plaques on both strain 18 and strain 210. The *virC-1* mutant was backcrossed with P22 c^+h_{21}. Two classes of recombinant phenotypes were observed when the progeny were plated on strain 18: large bullseye turbid plaques typical of *VirA* mutants and large clear plaques with a small turbid center typical of *VirB* mutants. The former class made typical *VirA* plaques when plated on a P22 lysogen, and the latter made typical *VirB* plaques on the lysogen. Of 2,300 plaques analyzed from the backcross, 81 *VirA* recombinants carried the h_{21} marker, whereas 23 *VirA* phage carried the h_{21}^+ marker. Of 311 *VirB* recombinants, 231 showed the h_{21}^+ plaque morphology and 80 had the h_{21} phenotype. It is evident that the h_{21} marker segregated preferentially with the *VirA* phenotype, and the h_{21}^+ marker segregated with the *VirB* phenotype. This result suggests the following order of markers in the above cross:



The calculated map distance between the *VirA* and *VirB* determinants from the above data is 18.0 map units. These results are consistent with

TABLE 3. *Mapping of K5 mutation*

Cross	Total plaques	$c^+h_{21}^+$	c^+h_{21}	Recombination frequency (%)	Order[a]
$K5+ \times c_2^5h_{21}$	23,500	4	19	0.21	$c_2^5 - K5$
$K5h_{21} \times c_2^8+$	23,000	10	3	0.13	$c_2^8 - K5$
$K5h_{21} \times c_2^{19}+$	21,000	16	7	0.18	$c_2^{19} - K5$
$K5+ \times c_2^{19}h_{21}$	26,400	6	14	0.14	$c_2^{19} - K5$
$K5h_{21} \times c_2^{27}+$	28,000	7	28	0.23	$K5 - c_2^{27}$
$K5+ \times c_2^{27}h_{21}$	45,080	65	18	0.66	$K5 - c_2^{27}$
$K5h_{21} \times tsc_2^{24}+$[b]	16,540	23	15	0.52	$tsc_2^{24} - K5$
$K5h_{21} \times tsc_2^{25}+$[b]	21,715	11	3	0.42	$tsc_2^{25} - K5$
$K5h_{21} \times tsc_2^{27}+$[b]	18,090	25	15	0.67	$tsc_2^{27} - K5$
$K5h_{21} \times tsc_2^{28}+$[b]	24,310	19	11	0.56	$tsc_2^{28} - K5$
$K5h_{21} \times tsc_2^{29}+$	25,500	22	7	0.15	$tsc_2^{29} - K5$
$K5+ \times tsc_2^{29}h_{21}$	23,000	8	15	0.14	$tsc_2^{29} - K5$
$K5h_{21} \times tsc_2^{30}+$[b]	14,990	23	11	0.85	$tsc_2^{30} - K5$
$K5h_{21} \times tsc_2^{31}+$[b]	21,200	20	14	0.70	$tsc_2^{31} - K5$
$K5h_{21} \times tsc_2^{33}$[b]	16,980	20	7	0.45	$tsc_2^{33} - K5$

[a] The order of loci is derived according to the information in Fig. 2.
[b] Not all of the $c+$ recombinants observed in these crosses were scored for the h_{21} and $h_{21}+$ alleles.

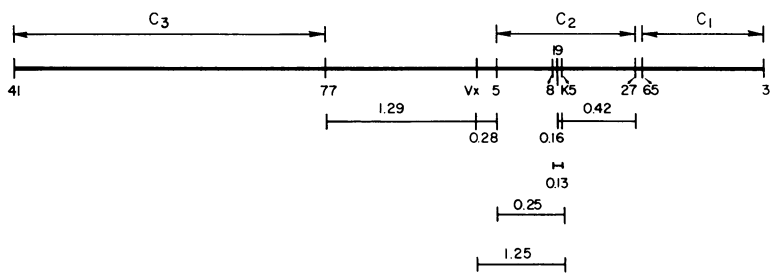


FIG. 3. *Linkage map of the clear region of phage P22. The order and distance are based on the three-factor crosses described in the text.*

TABLE 4. *Frequency of virulent mutants among progeny of phage infections*

Infection	Frequency of virulent mutants
Vx	10^{-5}
K5	10^{-5}
$K5 \times Vx$	6.5×10^{-3}
$c_1^3 \times Vx$	$<5 \times 10^{-5}$
$c_2^8 \times Vx$	$<10^{-4}$
$c_2^{27} \times Vx$	10^{-6}
$c_3^{11} \times Vx$	$<2 \times 10^{-5}$
$c_3^{41} \times Vx$	$<5 \times 10^{-5}$
$c_3^{77} \times Vx$	$<2 \times 10^{-4}$
tsmnt-1 $\times$ *Vx*	$<2 \times 10^{-4}$
$c_1^7 \times K5$	2×10^{-6}
$c_2^5 \times K5$	$<10^{-4}$
$c_2^8 \times K5$	10^{-5}
$c_2^{27} \times K5$	10^{-5}
$c_3^{11} \times K5$	10^{-5}
tsmnt-1 $\times$ *K5*	$<5 \times 10^{-5}$

the previous mapping of *VirA* mutants near the *mnt* locus and *VirB* mutants in the clear region.

The *VirB* mutant derived from *virC-1* was analyzed genetically and appeared to be made up of two mutations similar or identical to *K5* and *Vx*. This result confirmed the site of *VirB* virulence to be in the clear region.

The *VirC* phenotype of the original *virC-1* mutant was reconstructed when the *VirA* and *VirB* mutants derived from *virC-1* were crossed. Several other *VirC* mutants were backcrossed with wild type. In each case recombinant *VirA* and *VirB* phenotypes were observed. These results indicate that *VirC* mutants make large clear plaques on a lysogen, because these virulents contain the determinants of both *VirA* and *VirB* virulence.

Characteristics of K5 and Vx mutants. The *K5* mutant forms plaques exhibiting the *VirB* phenotype on strain 210 at a frequency of 10^{-5} to 10^{-6}. Plaques exhibiting the *VirA* or *VirC* phenotypes are not observed at this high frequency in *K5* stocks. Several of the spontaneous *VirB* mutants were purified and plated on strain 18. All showed a phenotype similar to *virB-3*. One of these new mutants was analyzed genetically by backcrossing with wild-type P22. In addition to the original *K5* mutation, this virulent was found

to contain a second mutation which mapped at a site very close to *Vx*.

The *Vx* phage also spontaneously mutated to *VirB* virulence at a rate of 10^{-5} to 10^{-6}. Phages of the *VirA* and *VirC* classes do not arise with this frequency in *Vx* stocks. Several *VirB* mutants from the *Vx* stocks were isolated and purified. They were phenotypically similar to *virB-3*. Four of these mutants were backcrossed with wild-type P22. Each mutant appeared to contain in addition to *Vx* a new clear mutation which was phenotypically similar to *K5*. None of these four

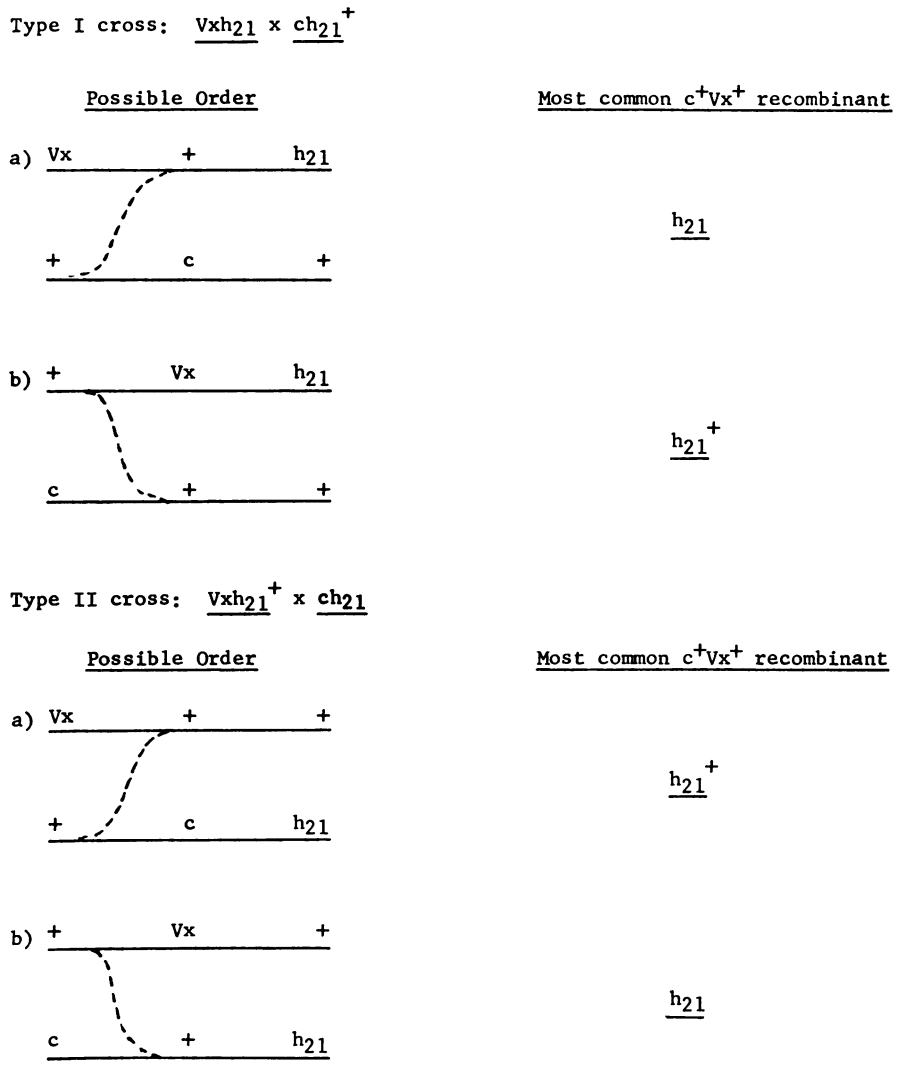


FIG. 4. *Determination of map position of Vx.*

TABLE 5. *Mapping of Vx*

Cross	Total plaques	Wild-type h_{21}^+	Wild-type h_{21}	Recombination frequency	Order[a]
$Vxh_{21} \times c_2^5+$	15,800	6	12	0.23	$Vx - c_2^5$
$Vx+ \times c_2^5h_{21}$	7,176	11	5	0.44	$Vx - c_2^5$
$Vxh_{21} \times c_3^{77}+$	6,225	33	6	1.29	$c_3^{77} - Vx$

[a] The order of loci is derived according to the information in Fig. 4.

independent clear mutants formed turbid recombinants when crossed with *K5*. Apparently, all four of these phage contain a mutation at the *K5* site. These results indicate that there are two sites involved in *VirB* virulence, one at or very near *K5* and one near *Vx*. Virulent mutants can arise spontaneously in either *Vx* or *K5* stocks as a result of a second mutation at or near the other site involved in virulence.

Although *K5* and *Vx* recombine with each other to form *VirB* mutants, these phages do not form virulent mutants when crossed with other phages. When either *K5* or *Vx* was crossed with other clear mutants or with *tsmt-1*, only about 1 in 10^5 progeny phage formed plaques on a lysogen (Table 4, infections 4 to 16). This frequency is no greater than that found for infection of either *K5* or *Vx* alone (Table 4), and approximates the spontaneous mutation rate to *VirB* virulence from *K5* and *Vx* stocks. Although c_2 mutants mapping on either side of *K5* recombine with *Vx*, virulent mutants are not formed. As mentioned in a previous section, progeny from a cross between *K5* and *Vx* phages contain 0.65% *VirB* mutants (Table 4). These results indicate that *K5* is a mutation at a specific site within the c_2 gene which is required for the *VirB* class of virulence.

DISCUSSION

Three types of virulent mutants of phage P22 have been isolated by selecting for phages which form plaques on a P22 *sie* lysogen. The *VirA* class virulents arise spontaneously with very low frequency in wild-type stocks of P22 and form very small turbid plaques on the lysogenic indicator. Mutants of the *VirB* class have been isolated from mutagenized stocks but have not been found spontaneously. The *VirB* mutants form larger and clearer plaques on a lysogen than *VirA* mutants. Virulent phages of the *VirC* class have been found only in mutagenized stocks. They form clear plaques which are much larger than *VirB* plaques on the lysogenic indicator. Several *VirC* mutants were backcrossed with wild-type P22. In each case the *VirC* mutant was shown to be comprised of the determinants of both *VirA* and *VirB* virulence.

All three types of virulent mutants plate with a high efficiency on a P22 *sie* lysogen but do not form plaques on the sie^+ lysogen. Thus, the mutations which confer insensitivity to P22 prophage immunity do not overcome the superinfection exclusion directed by the prophage.

The *VirA* class virulents have a plaque morphology on the sensitive strain identical to that of *mnt* mutants of P22 and map close to the *mnt* locus. The product of the *mnt* gene has been shown to be necessary for maintenance of repression (4). However, *mnt* mutants are not in themselves virulent nor do they mutate to virulence at a higher rate than wild type. It is possible that *VirA* mutants have an operator constitutive or promoter site mutation conferring insensitivity to the *mnt* gene product. Alternatively, the mutations involved in *VirA* virulence could lie within the *mnt* gene. In this case, the *mnt* gene product made by *VirA* mutants could be dominant to the wild-type *mnt* gene product by contributing a nonfunctional subunit to a repressor complex. Similar mutants have been isolated by Botstein (*personal communication*).

A typical *VirB* mutant, *virB-3*, was analyzed in some detail. This phage was backcrossed with wild-type P22 and found to be comprised of two mutations, *K5* and *Vx*. The *K5* phage makes large clear plaques on the sensitive strain and complements as a c_2 mutant. Thus, the *K5* mutant does not make functional c_2 repressor upon infection. The *Vx* mutant makes small turbid plaques on a sensitive strain. Neither *K5* nor *Vx* phage make plaques on a P22 lysogen.

Mutants of the *VirB* class arise spontaneously from both *K5* and *Vx* stocks at frequencies of 10^{-5} to 10^{-6}. These mutants have not been found spontaneously in other than *K5* or *Vx* stocks. Since the probability of finding a spontaneous *VirB* mutant may be as low as 10^{-12}, this is not surprising. Mutants of the *VirC* class contain the determinant of *VirA* virulence in addition to the determinants of *VirB* virulence and should be even more rare than *VirB* mutants in unmutagenized stocks.

The *K5* mutation maps within the c_2 gene, probably on the right side of that gene, since only one c_2 mutant is known which maps to the right of *K5*. The *Vx* mutation maps at a site just to the left of the c_2 gene. No new plaque morphology phenotypes were observed when either *K5* or *Vx* was backcrossed with wild-type P22. This result plus the fact that *K5* and *Vx* mutate to *VirB* virulence at high rates suggest that *K5* and *Vx* are single mutations.

Since *K5* and *Vx* recombined to form *VirB* mutants, they were confirmed as the component mutations of *virB-3*. Other clear mutants and *tsmnt-1* recombine with *K5* and *Vx* but do not form virulent mutants. We conclude that *Vx* and *K5* are mutations at specific sites in the clear region of P22 which are required for *VirB* virulence.

The clear region genes of phage λ and phage P22 appear to be analogous (5, 6). It has recently been demonstrated that λ phage development is regulated by the action of repressor at two operator sites, one on each side of the c_1 repressor gene (14). When repression is lifted, transcription is

initiated at the two promotor sites controlled by the operators. Transcription starting at the promoter to the left of the c_1 gene proceeds in a leftward direction from one DNA strand, whereas rightward transcription proceeds on the other DNA strand starting at the promoter to the right of the c_1 gene. The mechanism of regulation of P22 phage development may be similar to that described for λ phage.

The *Vx* mutation maps to the left of the c_2 repressor gene, a position analogous to that of the v_2 mutation of phage λ (14). The v_2 mutation has been shown to be at an operator site by virtue of decreased binding of repressor to λv_2 DNA relative to wild-type λ DNA. Phage carrying the λv_2 mutation show expression of genes mapping to the left of the clear region in the presence of repressor as expected for an operator constitutive mutation at a site controlling leftward transcription. The *Vx* mutation may act in a similar manner in P22 as either an operator constitutive mutation or by creating a new promoter site.

Understanding the mechanism of action of the *K5* mutation is complicated by the mapping of this mutation within the c_2 gene rather than at a site distinct from the c_2 gene. If *K5* were an operator constitutive mutation, the repressor molecule would have a binding site within the same gene that codes for it. Miller et al. (12) have shown that the operator site for the lactose operon lies between the natural promoter site and the structural genes of the operon. Repressor control can be bypassed by the establishment of new promoter sites which map between the operator site and the structural genes controlled by the operator (2, 13). If the *K5* mutation created a new promoter which allows constitutive expression of genes to the right of the clear region, the operator would be expected to map to the left of *K5*. Since *K5* appears to map on the right side of the c_2 gene, the operator controlling rightward transcription would lie within the c_2 gene or at a site to the left of this gene. Such mutations have not been found among virulent mutants of phage λ. Mutations that allow constitutive expression of genes to the right of the clear region have been described (2, 13, 14). These mutations map to the right of the λ repressor gene, c_1, rather than within the repressor gene as in the case of the P22 *K5* mutation. The $\lambda v_1 v_3$ mutations map in the operator region for control of rightward transcription, whereas λc_{17} and the ri^c mutants map to the right of this region.

If transcription of the c_2 gene itself is leftward and transcription of genes to the right of the clear region is rightward, the two operator-promoter regions may overlap. In such a case the *K5* mutation could allow constitutive expression of genes to the right while destroying or inhibiting the promoter for leftward transcription of the c_2 gene. Both *K5* and c_2^{27}, the only c_2 mutation mapping to the right of *K5*, would not lie within the structural gene for c_2 but would map in the c_2 promoter region to the right of the c_2 gene. The *K5* mutation but not the c_2^{27} mutation would simultaneously confer the property of constitutive rightward transcription. The fact that all known tsc_2 mutations have been mapped to the right of *K5* and c_2^{27} is consistent with this interpretation (Table 3, lines 7 to 15). If a tsc_2 mutant, which is presumably temperature sensitive for the c_2 protein itself, mapped to the right of *K5*, it would indicate that the *K5* mutation actually lies within the structural gene for c_2 repressor. A model in which leftward transcription of the repressor gene of phage λ is inhibited by rightward transcription across the c_2 promoter site has been proposed (3).

Another interpretation of the mechanism of action of *K5* involves a model in which functional c_2 repressor consists of subunits. This model assumes that the operator region controlling transcription to the left of the clear region binds a different site on the repressor than the operator region controlling transcription to the right of the clear region. A c_2 gene containing the *K5* mutation would code for a defective subunit which when combined with the wild-type subunit, made by the prophage, results in a repressor complex defective at the site which binds to the right-hand operator. Thus, *K5* would exhibit constitutive expression of genes to the right but would not be virulent because the left-hand operator still binds repressor. Other c_2 mutants may not be involved in virulence, because they code for a defective subunit which combines with the wild-type subunit to form a fully functional repressor complex. Data to be presented in a later paper make this hypothesis unlikely.

ACKNOWLEDGMENT

This work was supported by Public Health Service grants GM-15419-04 and NIH-5-T01-GM00071 from the National Institute of General Medical Sciences.

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