VIROLOGY 49, 257–267 (1972)

Genetics of Bacteriophage P22

I. Isolation of Prophage Deletions Which Affect Immunity to Superinfection

RUSSELL K. CHAN AND DAVID BOTSTEIN

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Accepted April 13, 1972

Two kinds of prophage deletions of the *Salmonella* phage P22 were isolated. One type of deletion was isolated by simultaneously selecting for loss of a host gene (*pro*) adjacent to the integrated prophage and for survival at the nonpermissive temperature of a temperature-inducible (tsc_2) lysogen. The second type of deletion was isolated by transduction to tetracycline resistance using high-frequency-transducing lysates obtained by UV-induction of an unusual tetracycline-resistant (tet^R) P22 lysogen.

In the pro^- deletion strains, a continuous block of genes beginning beyond the left end of the prophage and including all or part of *pro*A is deleted. The *c*-linked immunity region (*imm*C) is deleted in all the *pro*-deletion strains. In contrast, in the tet^R deletions a continuous block of genes beginning at the right (*pro*C) end of the prophage is deleted. The m_3-linked immunity region (*imm*I) is deleted in all of the tet^R deletions.

These two sets of prophage deletions confirm the established prophage gene order and have been used to compile a comprehensive deletion map of the P22 prophage which includes many previously unmapped genes.

The bipartite nature of immunity in P22 is confirmed by the observation that both pro^- deletions, which lack *imm*C but retain *imm*I, and tet^R deletions, which lack *imm*I but retain *imm*C, are each sensitive to superinfection by homoimmune nonvirulent P22 phage.

INTRODUCTION

Deletions have proved to be the most reliable and simple tools for genetic mapping of phage since they were first introduced for this purpose by Benzer (1961). In the case of virulent phage, deletions can conveniently be used only in regions of the phage chromosome which are dispensable for growth. However, in the case of a temperate phage which can integrate its genome into the host chromosome, deletions can include any region of the phage genome. Since the host replicates the integrated prophage as if it were a set of bacterial genes, phage functions are not essential to the propagation of the phage genome in this form. Thus, deletions can be used to map any part of the chromosome of a temperate phage, and this method becomes the method of choice for genetic mapping. In this paper we describe two kinds of prophage deletions of the *Salmonella* phage P22 which we have isolated and which we have used to construct a comprehensive map of this phage.

Deletions of the coliphages λ and φ80 have previously been obtained by selecting for deletions of bacterial markers known to lie very near to the prophage in a lysogen (Franklin *et al.*, 1965; Adhya *et al.*, 1968) or by simultaneous selection for the loss of such a bacterial marker and of one or more prophage genes (Shapiro and Adhya, 1969). Since the prophage attachment site of phage P22 on the *Salmonella typhimurium* chromosome is flanked by the genes for proline biosynthesis (Smith and Levine, 1965; Smith

TABLE 1
BACTERIAL STRAINS

Strain	Genotype	Synonyms	Origin
DB21	*su*⁻ prototroph	strain 18	Botstein (1968)
DB45	$su^{+}_{UGA}his$ G200		J. Gots
DB47	*su*⁻*rec*⁻		Botstein and Matz (1970)
DB53	*su*⁻*cys*A1348*his*C527	TR248	J. Roth
DB74	$su^{+}_{am}19cys$A1348*his*C527		Botstein and Matz (1970)
DB88	DB53(L)		This paper
DB109	$su^{+}_{UGA}584su^{+}_{am}19his$C527		Botstein *et al.* (1972)
DB117	DB53(P22$tsc_2^{29}int3sie$A6)		This paper
DB124	DB21(L)		This paper
DB5000	*su*⁻(P22*tet*ᴿ)	Tc-10	T. Watanabe
DB5005	*su*⁻(P22 deletion *tet*ᴿ)	Tc-10-26	T. Watanabe
DB5203	DB21(P22*sie*A1*tet*ᴿ)		This paper
PM476	*su*⁻*leu*D798-*ara*(deletion)*sup*Q234		P. Margolin
PM452	*su*⁻*leu*D798-*ara*(deletion) $fol_{101}his1099trp$A50		P. Margolin

and Stocker, 1966; Itikawa and Demerec, 1968), we have isolated deletions of the prophage by simultaneous selection for proline auxotrophy (*pro*⁻) and loss of prophage genes using a method analogous to that of Shapiro and Adhya (1969).

A second type of prophage deletion was discovered among transductants to tetracycline resistance (*tet*ᴿ) derived from an induced lysate of a special tetracycline-resistant P22 lysogen given to us by Dr. T. Watanabe.

These two kinds of prophage deletions form a complementary set: The *pro*⁻ prophage deletions delete blocks of genes adjacent to the left (*pro*A) side of the prophage whereas the *tet*ᴿ deletions delete blocks of genes near the right (*pro*C) end of the prophage. Using these deletions, mutations occurring nearly anywhere in the phage chromosome can be quickly and accurately mapped.

MATERIALS AND METHODS

Bacterial Strains

All strains (see Table 1) derive from *Salmonella typhimurium* LT2. Unless otherwise indicated, DB74 was used as the permissive strain for *am* mutants; DB45 for UGA mutants. The strains DB21 and DB53 appear to be equally nonpermissive for all nonsense mutants of the phage.

Lysogens were constructed by isolating pure clones (streaked 2 or 3 times) from the center of a plaque. DB117, which carries the prophage P22 $tsc_2^{29}int3sie$A6, was made by complementation on a plate with P22 c_2^5. The genotype of the prophage is evident from the lysogen's phenotype: at 40° it dies (tsc_2) but releases no viable phage (int_3; Smith and Levine, 1967), at 25° it is immune to P22 but sensitive to P22 *vir*3 (sieA6; Susskind *et al.*, 1971).

The origin of the *pro*⁻ alleles *pro*B8, *pro*A15, *pro*B25, *pro*B31 and *pro*C90 is described by Miyake and Demerec (1960).

Phage Strains

The methods of isolation, complementation, and characterization of mutants of phage P22 are described elsewhere (Botstein *et al.*, 1972). The published map of Gough and Levine (1968) was the basis of the characterization of our deletions. Assignments of nonsense mutants into the same complementation groups as one of the published *ts* mutants were in all cases checked by complementation tests at equal multiplicity of infection in liquid culture. No doubtful cases were used for the characterization of deletions.

The tests for immunity and superinfection exclusion are described by Susskind *et al.* (1971).

L phage is described by Bezdek and Amati (1967, 1968). Our strain was originally ob-

tained by induction of LT2 (L). $LcII_{101}$ is a spontaneous clear-plaque mutant isolated by us which fails to complement [using the test of Levine (1957)] $LcII_2$ of Bezdek *et al.* (1970).

P22 tsc_2^{29} *int3sie*A6 was constructed in a series of crosses (see Botstein and Matz (1970) for procedure) from P22 *sie*6 (Rao, 1968), P22 tsc_2^{29} (Levine and Smith, 1964), and P22 *int*3 (called L3 by Smith and Levine, 1967). Note that this phage carries only the *sie*A allele of *sie*6 and thus its lysogen is sensitive to P22 *vir*3 but still excludes the heteroimmune phages MG178 and L (Susskind *et al.*, 1971). Lysogens carrying prophage with the tsc_2^{29} allele are not inducible by ultraviolet irradiation (Wing, 1968).

Media

Liquid media used were LB broth (Levine, 1957), M9-CAA (minimal medium with amino acids: Smith and Levine, 1964). M9-PAM is the same as M9-CAA except that 1% Bacto-Proline Assay Medium (Difco) which contains all the amino acids save proline was substituted for casamino acids. The proline-assay medium is filter-sterilized separately as a 10% (w/v) solution. The formulations for solid media are to be found as follows: λ agar (Signer and Weil, 1968); green indicator agar (Levine and Curtiss, 1961; Susskind *et al.*, 1971). Tetracycline plates were green indicator plates to which 25 μg/ml tetracycline–HCl (Calbiochem) is added. The required amount of drug is weighed aseptically, dissolved in a small volume of sterile water, and added to the agar just before pouring. Dilution media were buffered saline and DF (Susskind *et al.*, 1971).

Isolation of Proline-Requiring Prophage Deletions

All the proline deletion strains were made from strain DB117 which carries the prophage P22 tsc_2^{29} *int3sie*A6. The frequency of deletions was increased (Schwartz and Beckwith, 1969) by mutagenesis with ultraviolet light. Cells were grown to 3×10^8 cells/ml in LB at 25°. Cells (5 ml) were centrifuged and resuspended in buffered saline and irradiated in a 10-cm glass petri dish with a GE germicidal lamp to about 5% survival. The cells were centrifuged and resuspended in 10 ml of fresh LB and grown overnight at 25°.

Selection for survival at 40°. The overnight culture was diluted into LB and grown to 3×10^8 cells/ml at 25°. The cells were diluted 10-fold into 100 ml of LB in a 1-liter Erlenmeyer flask prewarmed to 40°. This suspension was shaken at 40° for 2 hr or until lysis appeared complete. The lysate was filtered through a 47-mm Millipore filter (0.45 μ pore size) to collect the unlysed cells, and the cells were washed with two volumes of saline. The washed cells were then resuspended in 50 ml of M9-PAM in a 1-liter Erlenmeyer flask prewarmed to 40°.

Penicillin selection for proline auxotrophy. The method used is a modification (Roth, 1970) of the method of Gorini and Kaufman (1960). Survivors of the 40° treatment were starved of proline by shaking in M9-PAM for 2 hr at 40°. Potassium penicillin G (Squibb) was added to a final concentration of 10,000 units/ml and the flask was shaken at 40° for two additional hours. Aliquots (5–50 ml each) were collected on Millipore filters, washed with saline, and placed on λ plates to incubate for 16–24 hr. Survival at this point was about 10^{-7}.

Isolated surviving colonies were picked from the filters, and tested for proline auxotrophy (minimal agar plates) and P22 immunity (streak test). Clones chosen for further study required proline, grew normally at 40°, and were sensitive to P22 c_1. They were purified twice by single colony isolation and tested for P22 marker content by methods described below.

Isolation of Tetracycline-Resistant Prophage Deletions

An overnight culture in M9-CAA of DB5000 or DB5203 was diluted into 20 ml M9-CAA and grown at 37° to 3×10^8/ml. Then the cells were transferred to a 10-cm glass petri dish, irradiated with 120 ergs/mm² ultraviolet light and shaken at 30° for 3–4 hr or until complete lysis. A drop of chloroform was added and the lysate was

treated like a regular phage stock (Botstein and Matz, 1970).

Since P22 lysates after induction tend to be tail deficient (Israel, 1967), the purified lysate was incubated with purified P22 baseplate protein (Israel *et al.*, 1967) when it was desired to maximize the titer.

Since the DB5000 or DB5203 lysates contain 10^4-fold more defective particles than plaque-forming units, they were assayed by scoring ability to complement su^- infective centers of a P22 *amber* mutant (Chan, Botstein, Watanabe, and Ogata, in preparation). Then the lysate was used to transduce strain DB21 at low m.o.i. (about 10^{-2} particle per cell). An overnight culture of DB21 in LB was diluted into LB and grown at 37° to 3×10^8/ml. Then 0.2 ml DB21 in LB and 0.2 ml of a dilution of the lysate are mixed and incubated for 15 min at 37°. Dilutions are made in isothermal LB and are spread on tetracycline plates. The plates are incubated at 37° for 16–24 hr.

Tetracycline-resistant colonies were picked and tested for sensitivity to P22 c_2 and P22 *vir*3. Sensitive colonies were purified on tetracycline plates and scored for P22 marker rescue.

An alternative transduction procedure which worked just as well qualitatively was to spread 0.1 ml of a dilution of a DB5000 lysate and 0.1 ml DB21 (about 3×10^8/ml in LB) together on a tetracycline plate.

Table 2 gives the derivation of the tet^R prophage deletion strains.

TABLE 2

ORIGIN OF tet^R PROPHAGE DELETION STRAINS[a]

Strains	Source of P22 tet^R lysate	Sensitive recipient
DB5008-DB5011	DB5000	DB21
DB5057-DB5066	DB5203	DB21
DB5201	DB5005[b]	DB21
DB5208-DB5209	PM476(P22tet^R)	PM452
DB5210-DB5211	DB5203	DB53

[a] These tet^R prophage deletion strains were constructed by transducing a sensitive recipient at a low m.o.i. to tet^R with a UV-induced lysate of the indicated P22 tet^R lysogen.

[b] A stock of P22c^+ grown on DB5005, which is itself a tet^R prophage deletion, was used to transduce tet^R.

Mapping Methods

Several mapping techniques were used. Spot tests are simple and fast and thus well suited for screening large numbers of clones, but they are restricted to those mutants that give unambiguous responses under these conditions. Mutants giving equivocal responses on spot tests must instead be tested by the efficiency of plating method or the permissive rescue method.

Spot tests for marker rescue. The strains to be tested are grown to exponential phase ($\simeq 3 \times 10^8$ cells/ml) in LB at 37°; 0.2 ml of each culture is added to soft agar and poured onto a λ plate. Sterile capillary tubes are used to spot 3 dilutions (10^6, 10^7, 10^8/ml) of each mutant onto the different lawns. The spots are allowed to dry, and the plates are then incubated overnight at 25° or 37°. If *ts* mutants are used, plates are prewarmed to 40° before spotting. Appropriate controls are always carried out. Wild-type c_2 is spotted on all strains. All phages are also spotted onto sensitive su^+ and su^- cells (nonsense mutants) or spots were made on a sensitive strain at 25° and 40° (*ts* mutants). After incubation for 12–16 hr, the plates are scored. The spot of growth on a prospective deletion was compared to the spot on DB21 or DB53. Spots of lysis beyond the small number of discrete plaques occasionally present due to revertants were scored as positive. Occasionally the control spot of P22 c_2 grew poorly and no conclusion could be drawn.

Amber mutants at 37° give the best results; therefore, a standard set of dilutions of *amber* mutants was used to screen prospective deletion strains.

Efficiency of plating (*e.o.p.*). The deletion strains are grown to exponential phase in LB and are used as plating bacteria for the various mutants. Plates in tests of *am* or UGA mutants are incubated at 37°, while tests of *ts* mutants are incubated at 37° or 40° depending on the cutoff temperature for each mutant. Phage are also plated on DB21 or DB53 to measure the reversion rate and on DB45 for UGA mutants or on DB74 for *amber* mutants to measure the titer. We have

observed that virtually all P22 mutants can plate on nonpermissive lysogens of the heteroimmune phage L. Therefore, a positive control for rescue is to plate the mutant on an *su*$^-$ (L) lysogen (DB88 or DB124).

If the e.o.p. normalized to a permissive host was at least 100 times greater than the reversion frequency in the stock, the deletion strain is considered to contain the allele in question. There are very few ambiguous cases by this test.

Permissive rescue. About 0.1 ml of an overnight culture in LB of the strain to be tested is mixed with soft agar and poured onto a plate. Then a spot of a suspension (about 10^8 plaque-forming units per milliliter) of a *ts* mutant is spotted onto the lawn. The plate is incubated at 25° (permissive temperature) for 16–24 hr. A plug of agar from the spot of phage growth is picked with a pasteur pipette and is resuspended in 1 ml of buffered saline containing a few drops of chloroform. Dilutions of this suspension are plated with DB21 at 25° and 40° to determine the ratio of *ts*$^+$ to total phage. If the ratio of *ts*$^+$ to total phage from phage grown on a tested strain is 100-fold greater than the reversion rate, the *ts*$^+$ allele of the *ts* mutant is said to be present in the deletion. A positive control for the ratio *ts*$^+$/total phage comes from phage grown on a L lysogen.

Permissive rescue of *erf* (Botstein and Matz, 1970) can be carried out in the same way since all the deletion strains are *rec*$^+$. The phage are plated on DB21 (*rec*$^+$) and DB47 (*rec*$^-$) to score the *erf* marker. Permissive rescue of *imm*I_P [called I_P by Bezdek and Amati (1968)], one of the regions specifying immunity to superinfection was carried out by growing $LcII_{101}$ one cycle in each deletion strain. Progeny were scored by plating on DB88 (an L lysogen) and on DB53. Only hydrid phage which have rescued *imm*I_P can grow in DB88. (Note that in the *pro*$^-$ deletions *imm*C_P is deleted.)

The method of permissive rescue has the advantage of allowing one to score for the presence of morphological markers by using an appropriately marked *ts* mutant. This enabled us to locate these markers on the deletion map.

Reversion test for deletions. In order to distinguish between proline deletions and point mutations, a test based on the method of Whitfield *et al.* (1966) was used. About 0.1 ml of an overnight culture in LB of the *pro*$^-$ strain was added to minimal top agar. This was poured on a minimal plate containing 2 μg/ml proline. A crystal of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was placed on the center of the plate which was then incubated for 2–3 days at 37°. Point mutants have a ring of revertant colonies which is absent with deletion mutants.

Distinction among pro$^-$ *Mutations Using a proAB*$^+$ *Episome*

*Pro*A$^-$ and *pro*B$^-$, but not *pro*C$^-$, strains should be able to grow in the absence of proline if *pro*A and *pro*B gene products are supplied. *E. coli* E5013 is *trp*$^-$ and carries a *pro*AB$^+$ episome which can be transferred to any *pro*$^-$ strain we wish to test. A lawn of E5013 is spread on minimal M9 glucose plates containing no proline or tryptophan but otherwise supplemented. Dilutions of the strain to be tested are spotted onto the lawn. The plates are incubated at 37° for 24–48 hr. Under these conditions E5013 gave *pro*$^+$ recombinant colonies with *Salmonella typhimurium* strains *pro*A15, *pro*B8, *pro*B25, and *pro*B31, but not with *pro*C90. However, cross-feeding was observed when high concentrations of *pro*C90 were used (Miyake and Demerec, 1960).

RESULTS

Isolation of pro$^-$ *Prophage Deletions*

Deletions of the prophage were first sought by selecting simultaneously for loss of phage markers [in this instance the determinants which cause the death of a lysogen when the c_2 gene product (repressor) is inactivated] and a nearby bacterial gene (in this instance *pro*A). In order to do this, a rather complicated lysogen carrying a prophage with mutations in three different genes, c_2, *int*, and *sie*A, was constructed.

The *sie*A$^-$ mutation inactivates the superinfection exclusion system which normally nonspecifically prevents the functioning of superinfecting phage (Rao, 1968; Susskind *et al.*, 1971). If prophage deletions were to retain the intact superinfection exclusion system, analysis by rescue of markers into a superinfecting phage would not be possible.

A prophage which carries a temperature-sensitive mutation in the c_2 (repressor) gene is induced upon exposure of the lysogen to the nonpermissive temperature (Levine and Smith, 1964). Upon induction, the lysogen is killed and can no longer form a colony. We found that about one in two hundred cells lysogenic for P22 tsc_2^{29} survived when plated nonpermissively. Most of these survivors have totally lost the prophage: they are "cured." In order to eliminate this class of survivors, which would interfere with our selection scheme, the lysogen was made *int*⁻. An *int*⁻ prophage cannot excise itself properly upon induction (Smith, 1968). As expected, this expedient reduced the frequency of survival after induction to about 10^{-6}.

Selection for survival at 40° followed by penicillin selection for proline auxotrophy was carried out on a *Salmonella* strain lysogenic for P22 tsc_2^{29} *int3sie*A6 as described in Materials and Methods; 30–60 % of these survivors required proline for growth. Since deletions of the c_2 (repressor) region are expected to have lost phage immunity, proline-requiring survivors which were sensitive or partially sensitive (by the cross-streak test) were purified and tested for their genetic content by marker rescue tests. It quickly became apparent that the fully P22-sensitive clones had completely lost the prophage: none of the phage markers tested were present.

The partially P22-sensitive proline-requiring survivors of the selection scheme are prophage deletions. The efficiency of plating of wild-type P22 on these strains is normal, but the plaques are small and fuzzy. All these strains are defective in *pro*A or *pro*B but not *pro*C: they became *pro*⁺ upon receiving an episome from an *E. coli* strain which carries *pro*A and *pro*B but not *pro*C. None of the strains reverts to *pro*⁺ even in the presence of nitrosoguanidine (see the Materials and Methods for details). Finally, none of these strains produces detectable phage under a variety of inducing conditions.

To justify our assertion that the *pro*⁻ strains are prophage deletions, it is necessary to show that the missing P22 genes in each defective prophage strain constitute a continuous segment of the P22 prophage map. Such a prophage map based on published data (Levine, 1957; Levine and Curtiss, 1961; Smith and Levine, 1965; Smith and Levine, 1967; Smith, 1968; Gough and Levine, 1968; Bezdek and Amati, 1968; Botstein and Matz, 1970) and on unpublished data appears in Fig. 1.

Figure 1 also shows the extent of the deletions of the prophage in strains DB119, DB120, DB122, DB123, DB136, and DB147. It can be seen that all markers on the *pro*A side of the prophage including the c_2 and c_1 regions are deleted in all these strains. However, the extent of these deletions is variable. The end points of the deletions lie as near to *pro*A as just beyond c_1 and extend as far as $imm\mathrm{I}_\mathrm{P}$.

Figure 1 is based mainly on measurements of the efficiency of plating of *am* mutants on the putative deletion lysogens. These results were unequivocal; either the efficiency of plating was very near the reversion frequency (within a factor of 3) or else it was at least 100-fold greater than the reversion frequency.

In some instances, only *ts* mutants are known in a gene (e. g., genes 18, 21, and 25).

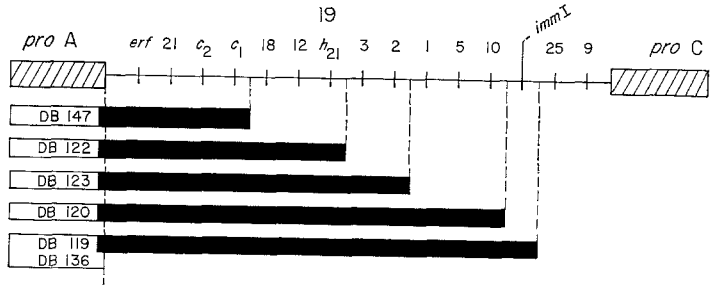

FIG. 1. Map of proline⁻ prophage deletions. The heavy bars indicate the deleted portion of the map.

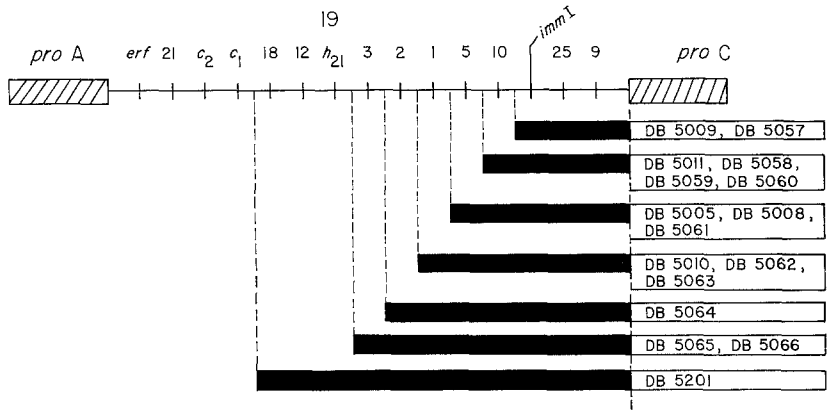

FIG. 2. Map of tetracycline-resistant prophage deletions. The heavy bars indicate the deleted portion of the map.

In these cases the permissive rescue technique was used. The *ts* mutants were grown in the deletion lysogen at permissive temperature and the frequency of ts^+ among the progeny measured. Here again, the results were unequivocal: either the ts^+ values lay within a factor of 3 of the reversion frequency or they were at least 50-fold higher. The presence of $imm\mathrm{I_P}$ was tested by growing phage L ($imm\mathrm{I_L}imm\mathrm{C_L}$) on the deletion lysogens. If phage progeny which grow on both a P22 and an L lysogen arise, $imm\mathrm{I_P}$ must be intact in the deletion lysogen. As shown in Fig. 1, only strains DB119 and DB136 delete any part of $imm\mathrm{I_P}$.

Isolation of tet^R Deletions

DB5000 is a P22 lysogen carrying a tetracycline resistance (tet^R) marker derived from a resistance-transfer episome. A UV-induced lysate of DB5000 transduces tet^R at high frequency. Two main types of tet^R transductants are found: one type, found mainly in transduction at high m.o.i., is similar to DB5000; the other, found mainly in transduction at low m.o.i. (10^{-2} particles/cell), is similar to DB5005. DB5005 is tet^R, sensitive to P22, and produces no viable phage or transducing particles. Marker rescue tests showed that DB5005 lacked a block of P22 genes corresponding to the deletion shown in Fig. 2.

Strains DB5000 and DB5005 were sent to us by Dr. Tsutomu Watanabe. Specific details about the origins of these strains and their properties will be presented in future publications by Dr. Yasuko Ogata, Dr. Watanabe, and ourselves. For the present paper, it suffices to show that transduction at low m.o.i. by an induced lysate of DB5000 and similar strains produces prophage deletions.

Tetracycline-resistant transductants were isolated in strain DB21 by infection at very low m.o.i. with induced lysates of either DB5000 or DB5203. The transductants which were phage-sensitive turned out be missing continuous blocks of phage genes, as shown in Fig. 2.

All the strains shown in Fig. 2 are tet^R, P22-sensitive and fail to produce viable phage after UV induction. They all show a pattern of marker rescue consistent with a set of deletions commencing at the *pro*C end of the prophage and extending for various distances into the prophage. None of these strains requires proline for growth.

Using the incomplete genetic map in Fig. 2, we cannot distinguish among all the tetracycline deletions. However, with the use of additional mutants in the genes shown in Fig. 2, and with the use of mutants in heretofore unmapped genes we shall show in the next section that some of the deletions classed together in Fig. 2 can, in fact, be distinguished. All tet^R deletions have in common the absence of genes 9, 25, and $imm\mathrm{I_P}$. This undoubtedly reflects, at least in part, the selection for phage-sensitive transductants. Since only strains missing

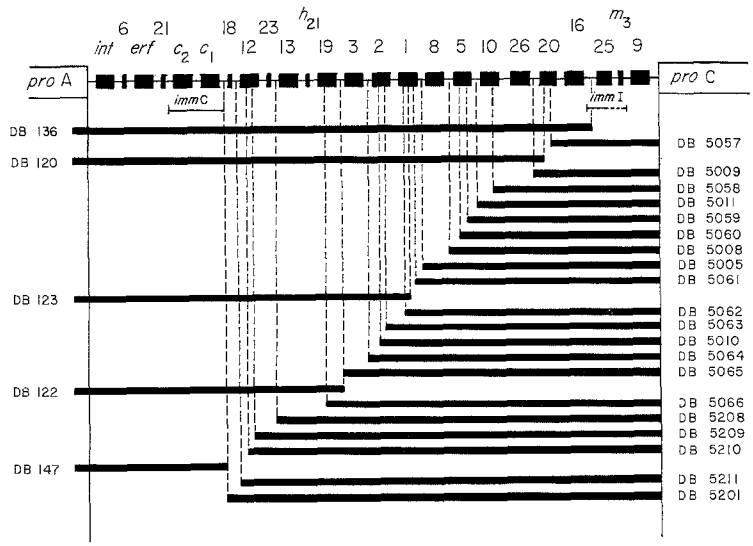

FIG. 3. A deletion map of prophage P22. The heavy bars indicate the deleted portion of the map.

*sie*A$^+$ (Susskind *et al.*, 1971) and either *imm*I$_P$ or *imm*C$_P$ will be sensitive to superinfecting phage, it is not too surprising that the deletions delete the *imm*I$_P$-25-*sie*A region. However, all the deletions we have tested are apparently also missing all of gene 9, the closest known gene to the right end of the prophage. None of the gene 9 mutants tested shows marker rescue from any of the *tet*^{R} deletions.

Map Order

The proline deletions, together with the tetracycline deletions, comprise a complementary set of prophage deletions very well suited for genetic mapping of P22.

We have classified individual P22 mutants and the complementation groups to which they belong into the intervals defined by the deletion strains. Determination of the presence or absence of a particular marker was first attempted with a spot test. Where this result was ambiguous or where more information was desired, efficiency of plating tests were done (*amber* and UGA mutants) or permissive rescue tests were done (*erf* and *ts* mutants). In general the results obtained on the two kinds of deletions agreed with each other and with the standard recombination map of P22. A few anomalous mutants were found which were subsequently interpreted as double mutants and were omitted from the list of mutants considered by this paper.

Figure 3 shows the grouping of all P22 genes into the intervals defined by the two classes of deletions. In the regions covered by the deletions it gives the most complete genetic map of P22 available at this time.

Fine Structure

As shown in Fig. 3, many of the deletions we have isolated have end points in the middle of a gene. Such deletions permit the ordering of alleles within a gene. If one assumes that the end points of deletions are randomly distributed, one can simply screen *tet*^{R} deletions until one is found which distinguishes the two alleles one wishes to order. We have in fact done this for the region near genes 18 and 12. About 100 low m.o.i. *tet*^{R} transductants were screened for the absence of gene 19. Four of these (DB5208, DB5209, 5210, and 5211) were then used to order the various alleles in the desired region. Thus far it appears that *tet*^{R} deletion end points can be found at any arbitrary point.

Another approach to fine structure mapping is to measure the frequencies with which different alleles can be rescued from the same prophage deletion strain. One might expect that the nearer the allele is to the deletion end point, the lower would be the rescue

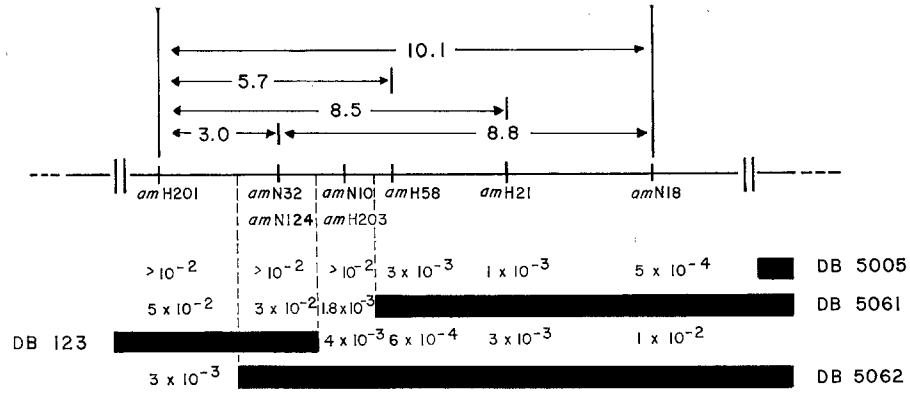

FIG. 4. Fine structure mapping of gene 1. The distances between markers (in percent recombination) are given above the map. The efficiencies of plating on the deletion lysogens (relative to DB74 *su*$^+$) are given below. The heavy bars indicate the deleted portion of the map.

TABLE 3

RELATION OF MARKER RESCUE FREQUENCY TO DISTANCE FROM DELETION END POINT[a]

	Alleles in gene 5 (in map order)							
Deletion	*am*N8	*am*N130	*ug*H4	*am*N122	*am*N114	*am*N113	*am*N3	*am*N13
DB5011	0.4	0.2	0.5	0.7	0.14	0.071	0.03	0.009
DB5059	0.17	0.097	0.066	0.019	0.014	0.0008	0.0006	Deleted
DB5060	0.12	0.064	0.032	0.012	0.0079	Deleted	Deleted	Deleted

[a] Marker rescue frequencies are given as efficiency of plating on the deletion strain relative to the permissive host (DB109) which carries both an *am* and a UGA suppressor.

frequency. If the measurements concern only alleles in a single gene, variation due to complementation should be minimized.

The results of determinations of the frequency of marker rescue of various alleles in gene 1 are shown in Fig. 4. With respect to the *tet*^{R} deletions, the expected gradient of rescue frequency is found. The order of the alleles was determined by two-point crosses in addition to deletion mapping; these data are also shown in Fig. 4. This method has been applied to a number of alleles in gene 5 (Table 3). The gradient of frequencies is clear. The order of the alleles deduced from three different deletions is consistent. This order is the same as that deduced from two- and three-factor crosses and from the deletion map. Table 3 also suggests that different deletions allow rescue of a single allele at different frequencies, and that these frequencies too are related to the distance between the rescued allele and the deletion endpoint. We conclude that rescue of markers from the *tet*^{R} prophage deletions is related to the distance between the allele and the deletion end point and that this relation can be useful in fine-structure mapping.

This conclusion apparently does not apply to the *pro*$^-$ deletions. As shown in Fig. 4, there are exceptions to the rule that closer alleles show lower rescue frequencies. These exceptions are frequent and preclude mapping by this means. The reason for this difference between *pro*$^-$ and *tet*^{R} deletions is not understood.

DISCUSSION

Considerable evidence has accumulated suggesting that the temperate *Salmonella* phage P22 possesses two independent immunity regions. Bezdek and Amati (1968) noted that the progeny of a cross between the heteroimmune phages P22 and L included recombinants which grew equally well on lysogens of either parent. They con-

cluded that there must be two immunity regulator systems—one linked to the *c*-region (Levine and Curtiss, 1961) and one linked to the morphological marker m_3. In this discussion we shall refer to the *c*-linked immunity region as *imm*C and the m_3-linked immunity region as *imm*I. The subscripts following C or I will denote P22 and phage L specificities, respectively (e.g., wild-type P22 is therefore $imm\mathrm{I}_{P22}imm\mathrm{C}_{P22}$).

Our prophage deletions are of two kinds. One set of deletions all extend into the prophage from the left (*pro*A) side and always delete all or part of the c_2 gene, and thus $imm\mathrm{C}_{P22}$. The other set extends into the prophage from the right (*pro*C) end and always deletes all or part of $Imm\mathrm{I}_{P22}$. The first and most striking observation about these deletions is that either kind of prophage deletion renders the deletion lysogen sensitive to superinfection by homoimmune, nonvirulent P22 phage.

This observation confirms and supplements previous evidence indicating the bipartite nature of immunity to superinfection by P22 lysogens. The Bezdek and Amati experiments cited above show that the *specificity* of immunity is determined by two unlinked regions. The combined results of Gough (1968) and Levine and Smith (1964) indicate that two genes synthesize products continuously required for the *maintenance* of lysogeny: stable lysogens of temperature-sensitive mutants in either the c_2 gene or the *mnt* gene are induced by incubation at 37° or 40°. The c_2 gene, of course, maps in *imm*C whereas the *mnt* gene lies in or very near to *imm*I (see accompanying paper, Botstein *et al.*, 1972). Finally, virulent mutations (Bronson and Levine, 1971; Swanson and Botstein, in preparation) are found in or near both the *imm*C and *imm*I regions.

Thus genes responsible for immunity, the maintenance of lysogeny, and the loci which can mutate to virulence are located in two separate regions of the P22 genetic map. This situation is to be compared with the situation in the coliphage λ, where these functions all reside in a single immunity region. Our deletions show that both immunity regions are required to prevent successful superinfection. The viability and stability of the two kinds of deletion strains further suggests that each of the two immunity regions separately controls the expression of genes lying nearby. Deletion of *imm*C (proline$^-$ deletions) removes not only the c_2 gene but all or part of the functions responsible for cell death after heating of a tsc_2 lysogen. Although ordinary P22 phages plate on the *imm*C deletions, the plaques are small and altered in morphology, indicating that the $imm\mathrm{I}^+$ region still has an effect on the superinfecting phage. Deletion of *imm*I (tetracycline deletions) also leaves the cell sensitive to superinfection. Here it is not clear whether the remaining $imm\mathrm{C}^+$ region has any effect. If all the functions responsible for cell death upon induction of a tsc_2 lysogen lie near *imm*C, then the c_2 repressor must be assumed to be working, since the tetracycline deletion strains are viable. The availability of strains carrying only one of the two immunity regions should greatly facilitate analysis of this novel system of control of a temperate phage.

The distribution of end points among the deletions we have obtained is, to a first approximation, random, if one neglects the fact that the nature of our selection procedure specified that all the deletions remove one or the other of the immunity regions. Somewhat more than half of the deletion end points fall within known genes, indicating that if the end points are indeed random, we have identified rather more than half of the P22 genes.

ACKNOWLEDGMENTS

We thank T. Watanabe and Y. Ogata for introducing us to the genetics of tetracycline resistance in *Salmonella* and for their assistance in the form of unpublished data and numerous strains.

We are grateful to Carol Waddell for her skillful and enthusiastic technical assistance.

This work was supported by grant No. VC-18A from the American Cancer Society. R. K. C. is a trainee under a Microbiology Training Grant (GM-00602) to the Department of Biology, Massachusetts Institute of Technology, from the National Institute of General Medical Sciences.

REFERENCES

ADHYA, S., CLEARY, P., and CAMPBELL, A. (1968). A deletion analysis of prophage lambda and adjacent genetic regions. *Proc. Nat. Acad. Sci. U.S.* 61, 956–962.

BENZER, S. (1961). On the topography of the genetic fine structure. *Proc. Nat. Acad. Sci. U.S.* **47,** 403–415.

BEZDEK, M., and AMATI, P. (1967). Properties of P22 and a related *Salmonella typhimurium* phage. I. General features and host specificity. *Virology* **31,** 272–278.

BEZDEK, M., and AMATI, P. (1968). Evidence for two immunity regulator systems in temperate bacteriophages P22 and L. *Virology* **36,** 701–703.

BEZDEK, M., SOSKA, J., and AMATI, P. (1970). Properties of P22 and a related *Salmonella typhimurium* phage. III. Studies on clear-plaque mutants of phage L. *Virology* **40,** 505–513.

BOTSTEIN, D. (1968). Synthesis and maturation of phage P22 DNA. I. Identification of intermediates. *J. Mol. Biol.* **34,** 621–641.

BOTSTEIN, D., and MATZ, M. J. (1970). A recombination function essential to the growth of bacteriophage P22. *J. Mol. Biol.* **54,** 417–440.

BOTSTEIN, D., CHAN, R. K., and WADDELL, C. H. (1972). Genetics of bacteriophage P22. II. Gene order and gene function. *Virology* **49,** 268–282.

BRONSON, M. J., and LEVINE, M. (1971). Virulent mutants of bacteriophage P22. I. Isolation and genetic analysis. *J. Virol.* **7,** 559–568.

FRANKLIN, N. C., DOVE, W. F., and YANOFSKY, C. (1965). The linear insertion of a prophage into the chromosome of *E. coli* shown by deletion mapping. *Biochem. Biophys. Res. Commun.* **18,** 910–923.

GORINI, L., and KAUFMAN, H. (1960). Selecting bacterial mutants by the penicillin method. *Science* **131,** 604–605.

GOUGH, M. (1968). Second locus of bacteriophage P22 necessary for the maintenance of lysogeny. *J. Virol.* **2,** 992–998.

GOUGH, M., and LEVINE, M. (1968). The circularity of the phage P22 linkage map. *Genetics* **58,** 161–169.

ISRAEL, V. (1967). The production of inactive phage P22 particles following induction. *Virology* **33,** 317–322.

ISRAEL, J. V., ANDERSON, T. F., and LEVINE, M. (1967). *In vitro* morphogenesis of phage P22 from heads and base-plate parts *Proc. Nat. Acad. Sci. U.S.* **57,** 284–291.

ITIKAWA, H., and DEMEREC, M. (1968). *Salmonella typhimurium* proline mutants. *J. Bacteriol.* **95,** 1189–1190.

LEVINE, M. (1957). Mutations in the temperate phage P22 and lysogeny in *Salmonella*. *Virology* **3,** 22–41.

LEVINE, M., and CURTISS, R. (1961). Genetic fine structure of the C region and the linkage map of phage P22. *Genetics* **46,** 1573–1580.

LEVINE, M., and SMITH, H. O. (1964). Sequential gene action in the establishment of lysogeny. *Science* **146,** 1581–1582.

MIYAKE, T., and DEMEREC, M. (1960). Proline mutants of *Salmonella typhimurium*. *Genetics* **45,** 755–762.

RAO, R. N. (1968). Bacteriophage P22 controlled exclusion in *Salmonella typhimurium*. *J. Mol. Biol.* **35,** 607–622.

ROTH, J. R. (1970). UGA nonsense mutations in *Salmonella typhimurium*. *J. Bacteriol.* **102,** 467–475.

SCHWARTZ, D. O., and BECKWITH, J. R. (1969). Mutagens which cause deletions in *Escherichia coli*. *Genetics* **61,** 371–376.

SHAPIRO, J. A., and ADHYA, S. L. (1969). The galactose operon of *E. coli* K-12. II. A deletion analysis of operon structure and polarity. *Genetics* **62,** 249–264.

SIGNER, E. R., and WEIL, J. (1968). Recombination in bacteriophage λ. I. Mutants deficient in general recombination. *J. Mol. Biol.* **34,** 261–271.

SMITH, H. O. (1968). Defective phage formation by lysogens of integration deficient phage P22 mutants. *Virology* **34,** 203–223.

SMITH, H. O., and LEVINE, M. (1964). Two sequential repressions of DNA synthesis in the establishment of lysogeny by phage P22 and its mutants. *Proc. Nat. Acad. Sci. U.S.* **52,** 356–363.

SMITH, H. O., and LEVINE, M. (1965). Gene order in prophage P22. *Virology* **27,** 229–231.

SMITH, H. O., and LEVINE, M. (1967). A phage P22 gene controlling integration of prophage. *Virology* **31,** 207–216.

SMITH, S. M., and STOCKER, B. A. D. (1966). Mapping of prophage P22 in *Salmonella typhimurium*. *Virology* **28,** 413–419.

SUSSKIND, M. M., WRIGHT, A., and BOTSTEIN, D. (1971). Superinfection exclusion by P22 prophage in lysogens of *Salmonella typhimurium*. II. Genetic evidence for two exclusion systems. *Virology* **45,** 638–652.

WHITFIELD, H. J., JR., MARTIN, R. G., and AMES, B. N. (1966). Classification of aminotransferase (C gene) mutants in the histidine operon. *J. Mol. Biol.* **21,** 335–355.

WING, J. P. (1968). Integration and induction of phage P22 in a recombination-deficient mutant of *Salmonella typhimurium*. *J. Virol.* **2,** 702–709.