SOME IMPROVED METHODS IN P22 TRANSDUCTION

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ABSTRACT

Recent refinements simplify methods for P22 transduction in Salmonella and allow improved recovery of phage-free transductional clones. The methods include use of: integration- and lysis-defective phage mutants, heat-killed bacteria to eliminate free phage, direct plating of phage and bacteria, replica-plating for detection of phage content of individual clones, improved broth for phage growth, and procurement of high titer phage from P22 lysogens.

WORK in a large number of laboratories has increased our understanding of the physiology of Enteric bacteria and led to construction of a detailed genetic map for *Salmonella typhimurium* (SANDERSON 1972). Generalized transduction with P22 phage (ZINDER and LEDERBERG 1952) has constituted a most important tool in these genetic investigations. Modern protocols for the growth and use of P22 phage in transduction are outlined in numerous research papers and reviewed elsewhere (e.g., CLOWES and HAYES 1968; ROTH 1970). We wish to point out here some improvements in this methodology that make experimentation using P22 phage both more convenient and more reliable.

MATERIALS AND METHODS

Procuring phage-free transductants: In many experiments one desires to prepare P22 phage on a transductional clone in order to confirm the genotype or to transfer a genetic marker to a third strain. Below (*Phage from lysogens*) we describe methods that often allow further genetic manipulation in P22 lysogens. A straightforward approach, however, is to prevent lysogenization in the first place.

Two methods originally were used to obtain non-lysogenic transductional clones after infection of *S. typhimurium* with P22 phage. The first capitalized on the ability to maintain transduction potential of a donor lysate while reducing the phage viability by irradiation with ultraviolet light (cf. GAREN and ZINDER 1955; BENZINGER and HARTMAN 1962). The second method took advantage of the presence of P22-free sub-clones among bacteria infected with H-4 phage (P22 *v1* mutant in ZINDER 1958). Use of H-4 phage most often was coupled with ultraviolet treatment of the phage.

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One major problem with the above methods is that lysis and reinfection of bacteria occur after plating. Consequently, many bacteria are either killed or lysogenized in the two to three days necessary for growth of transductional clones on the test plates. Such reinfections can be greatly reduced by the following method. Transducing phage is preadsorbed for several minutes before plating, as described in the legend to Figure 2. The mixture is then plated in the presence of a 0.1 ml aliquot of a milky (saturated culture 10- to 25-fold concentrated) suspension of sensitive bacteria killed by heating at 100° for one hour. Free phage on the plate are inactivated by adsorption to the somatic antigen of the surplus heat-killed bacteria and thus are prevented from infecting viable bacteria. Addition of heat-killed bacteria is much more effective, and less expensive, than is the addition of high titer anti-P22 immune serum to the plate.

The process of obtaining phage-free transductional clones is enhanced and simplified through the use of integration-defective P22 phage with greatly reduced ability to lysogenize Salmonella. We have used mutant *int-4* while mutants *int-3* and *int-7* have been found just as useful in other laboratories (STRAUS and WYCHE 1974; CORDARO and ROSEMAN 1972). These phage are the L3, L4, and L7 phages of SMITH and LEVINE (1967). For reasons enumerated below (*Transduction frequency*) the double mutant P22, *int-4 cly-2* is the most versatile P22 strain now available for routine laboratory use.

Detection of phage-free clones: In the isolation of phage-free clones, transductional clones must be streaked out for single-colony isolation (see CLOWES and HAYES 1968). Formerly, we chose individual isolated colonies and tested them for P22 sensitivity by the cross-streak method. A lysate of a clear-plaque mutant of P22 phage (H-5 phage, the *v2* mutant in ZINDER 1958) was streaked across the center of a nutrient agar plate. Then suspensions of individual colonies were streaked parallel to each other and vertically across the phage streak. After overnight incubation the streaks were examined for evidence of lysis. This method is not only cumbersome but it fails to detect minor P22 contamination which is often found when *int* phage are used (see below). A more sensitive and efficient method is direct replica-plating of the streak-plate onto a lawn of sensitive indicator bacteria. Lawns are formed by the addition of 2 drops of an overnight nutrient broth culture to 3 ml of 0.75% agar maintained at about 45°; the mixture is poured onto a nutrient agar plate solidified with 1.5% agar. Lawns may be poured and then stored at 4° for several weeks before use. Colonies that fail to exhibit a ring of lysis on the indicator (see Figure 1B, discussed below) are presumptive phage-free clones and are retested by similar replica-plating after a second single-colony isolation. It is sometimes useful to pre-spread the initial streak-out plate with heat-killed sensitive bacteria as described above to inactivate free phage and lessen reinfections occurring on the streak-out plate. Similarly, presumptive phage-free transductional clones often can be detected by direct replica-plating of the original transduction plate onto an indicator lawn if the original plate contains heat-killed bacteria. Presumptive phage-free clones always are rechecked after single-colony isolation and replica-plating as described above.

The replica-plating method both reveals P22-free clones and differentiates P22-containing colonies into two classes (Figure 1). Colonies that engender a very small and often asymmetrical area of lysis on the indicator lawn rarely segregate clones free of phage and are presumed to be composed of true lysogens (Figure 1A). Colonies that give large symmetrical areas of lysis frequently segregate clones free of phage and are presumed to carry P22 *int-4* as a replicating plasmid (Figure 1B). At incubation temperatures greater than 30°, fewer than 0.1% of single-colony isolates from transductional clones obtained with *int-4* are true lysogens. At lower incubation temperatures, lysogens become frequent in some bacterial hosts, but colonies that segregate phage-free clones usually can be found.

Phage from lysogens: Three approaches may be used when transducing phage is desired from a P22-lysogenic strain: (1) curing, (2) use of heterioimmune phage, or (3) direct procurement of phage released by the lysogen.

ZINDER (1958) described a method for the curing of lysogenic bacteria based on the detection of sensitive survivors following ultraviolet irradiation. This procedure is laborious but it works in spite of the weak ultraviolet inducibility of P22 phage. Curing of P22 also has been obtained by superinfection with phages heteroimmune but excluded by P22 (RAO and SMITH 1968). In addition, some of these heteroimmune phages (cf. SUSSKIND, WRIGHT and BOTSTEIN 1971) give

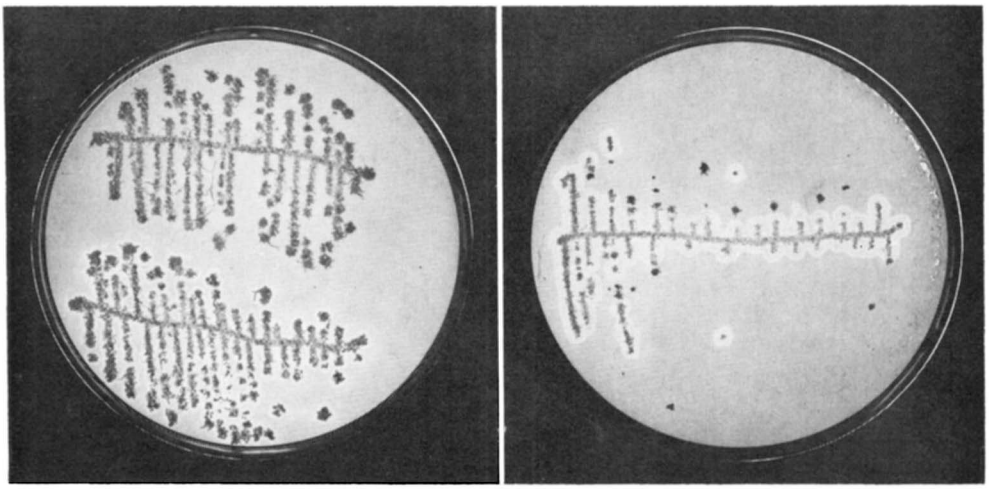

FIGURE 1.—Patterns of lysis elicited by a presumed true P22 lysogen (*A*, lower), for a phage-free clone (*A*, upper), and for a clone presumed to carry P22 as a replicating plasmid (*B*). Streak-plates made from single colonies were replica-plated onto lawns of sensitive indicator bacteria and incubated overnight at 37°. Lawns were prepared by adding 0.1 ml of a fresh overnight culture of sensitive indicator bacteria to 3 ml of melted nutrient soft (0.75%) agar and pouring the mixture onto a fresh nutrient 1.5% agar plate.

variable but sometimes sufficient transducing phage yields upon infection of P22 lysogens. In contrast, another heteroimmune generalized transducing phage, KB1, grows well on P22 lysogens and has a high transducing efficiency (BORO and BRENCHLEY 1971). MCINTIRE (1974) describes an integration-defective variant of KB1 which can be used to prevent the formation of KB1 lysogens. We have found better growth of KB1 in glucose minimal E medium (VOGEL and BONNER 1956) than in nutrient broth. An inoculum of as little as 100 phage per ml in a lightly turbid (about 10^8/ml) log phase culture will yield lysates with titers of 10^{10} KB1/ml. However, at low phage inocula, the resulting lysate contains phage with a wide variety of plaque morphologies, including many clear plaques. Consequently, 10^4 KB1/ml is used as a standard inoculum since this yields a more homogeneous lysate. KB1 transducing fragments appear to be approximately the same size genetically as those carried by P22 since two markers barely co-transducible by P22 are co-transduced at similar frequencies by KB1 (*supD* and *flaA* markers; FANKHAUSER and HARTMAN 1971). Another phage, P1, picks up larger chromosomal fragments than does P22 or KB1 but plates only on particular "rough" derivatives of Salmonella lacking normal lipopolysaccharide (OKADA and WATANABE 1968; ORNELLAS, ENOMOTO and STOCKER 1971). This limits the current utility of P1 in Salmonella transduction since most derivatives have normal lipopolysaccharide and consequently fail to adsorb P1 phage.

A third means of obtaining transducing phage from P22 lysogens is to harvest phage that are spontaneously released from bacteria grown on enriched medium. Low titer (10^7 to 10^9/ml) lysates of P22 can be prepared directly by growth of P22 lysogens beyond culture saturation in the supplemented broth medium described below (*Phage lysates*). The phage can be concentrated after sedimentation of the bacteria by centrifugation. We routinely adjust the NaCl concentration of the supernatant to 0.5 M and add 7% W/V polyethylene glycol 6000 to precipitate the phage (YAMAMOTO *et al.* 1970). After one hour at room temperature the mixture is centrifuged for 10 minutes at 8000 g, decanted, and the phage pellet resuspended in the desired volume of broth or storage buffer. The T2 buffer of HERSHEY and CHASE (1952) constitutes an excellent storage buffer for the phages discussed here.

Phage lysates: Wild-type P22 is easy to grow to high titer in liquid medium, and even higher titers are obtained with P22 mutant *int-4*. We obtain 5×10^{10} to 10^{12} titers in crude lysates when *int-4* is grown on Salmonella in Difco-nutrient broth enriched per 100 ml with 1.5 ml 50-times concentrated E medium (VOGEL and BONNER 1956) plus 3.5 ml 40% glucose (HONG and AMES 1971). P22 *int-4 cly-2* (see below) is grown in the same medium but must be grown at lower temperatures (e.g., 24°) while wild-type P22 and *int-4* can be grown at a variety of temperatures (usually 37°). Additional information on growth of P22 is given by CLOWES and HAYES (1968) and ROTH (1970). We generally use multiplicities 100-fold lower than recommended by these authors to avoid the chance of carry-over of transducing phage particles from one lysate to the next. In the case of *int-4 cly-2*, cultures should be inoculated from lysates grown from single plaques to prevent overgrowth by spontaneously occurring clear-plaque mutants.

P22 infection gives rise to readily scored plaques on most *Salmonella typhimurium* LT-2 sub-lines, although clarity of the plaque does vary somewhat with the indicator strain used (HONG, SMITH and AMES 1971). In addition, we have found as much as a 2-fold difference in plating efficiencies of P22 on different sub-lines, so we routinely use *his-712* or *hisG46* (HARTMAN *et al.* 1971) as our indicator bacteria. Phage are stored over chloroform at 4° in nutrient broth or in T2 buffer (HERSHEY and CHASE 1952).

Transduction frequency: Recovery of transductional clones is enhanced at higher phage multiplicities if the classic pre-adsorption of phage to bacteria (CLOWES and HAYES 1968) is avoided and the phage and bacteria are simply spread together on the surface of the plating medium. Figure 2 shows the results of transduction tests with *his* (HARTMAN *et al.* 1971) and *pts* (CORDARO and ROSEMAN 1972) mutants under conditions of pre-incubation at 37° (open symbols) and direct plating at room temperature (closed symbols). Transduction frequency per infective particle is plotted versus phage multiplicity. The two methods yield comparable numbers of transductants at multiplicities of 2 to about 10 phage per bacterium. At higher multiplicities the transduction frequency per phage decreases drastically in the case of preadsorption, whereas the number of transductants continues to increase in the case of direct plating. The differences between the two methods involve both the adsorption medium and the adsorption temperature. In the pre-incubation method, adsorption takes place in a liquid medium rather than on a solid substrate, and the initial temperature is higher using pre-incubation rather than direct plating. We have shown that each of these components has a significant effect on the ultimate appearance of transductional clones. Consequently, when large numbers of transductants are desired, bacteria and phage should be pipetted directly onto the surface of the transduction plate at room temperature, mixed by spreading, and then incubated at the desired temperature (usually 37°). In addition, the direct plating method is more rapid and consumes less glassware.

Recovery of transductional clones also can be increased, generally about 2-fold for auxotrophic markers, if slight enrichment is provided in the selective medium (cf. HARTMAN, LOPER and ŠERMAN 1960). We enrich VOGEL and BONNER (1956) minimal E medium with 1.25% (V/V) liquid Difco-nutrient broth. The mechanism underlying this stimulation in transduction frequency is unclear.

In particular Salmonella mutants, the decision between lysis and non-lysis in P22 infection is drastically altered so that most bacteria are killed (*cya, crp, pdx* and *rif* mutants: HONG, SMITH and AMES 1971; certain *cys* mutants: J.-S. HONG (personal communication) and J. C. CORDARO (personal communication); and certain *hisS* mutants). This excessive lysis can lead to loss of transductional clones involving these genetic markers. The *cly-2* mutation is a temperature-sensitive phage mutation which greatly reduces the lytic response at 37° (HONG, SMITH and AMES 1971), thus permitting increased recovery of transductants at this temperature. At 25°, however, the lytic response of *cly-2* is approximately that of wild-type phage, and so high titer *cly-2* lysates can be prepared easily at this temperature.

A recombinant P22 phage, containing both the *int-4* and the *cly-2* mutations, was constructed by mixed infection of wild-type LT-2 with *int-4* (moi = 5) and *cly-2* (moi = 0.3). The culture was treated with chloroform after 1 hour shaking at 25°. Of 12 putative recombinants checked, 9 apepared to be double mutants. These produced turbid plaques characteristic of *cly* mutants when plated on *rif-39* bacteria at 37°, and also formed lysogens at low frequency similar to *int* mutants.

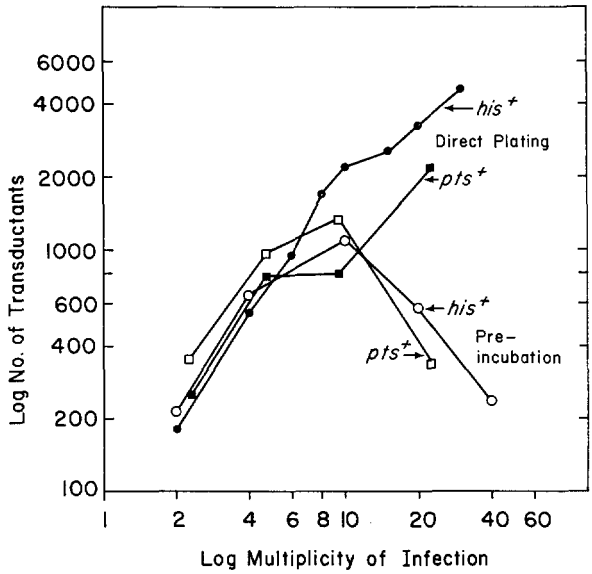

FIGURE 2.—Direct plating versus pre-incubation. For transductions performed by the pre-incubation method (open symbols), aliquots of phage were added to 0.1 ml of a fresh overnight nutrient broth culture of recipient bacteria (about 10^9/ml), incubated at 37° for six minutes, and then 0.1 ml aliquots plated onto selective media. For transductions performed by the direct plating method (closed symbols), aliquots of phage and 0.1 ml of the recipient bacteria were pipetted directly onto the surface of the transduction plate at room temperature and mixed by spreading. In both methods, the plates were incubated at 37° after spreading and then scored for *his*+ or *pts*+ colonies after two days. Transductions to *his*+ (circles) were performed using phage grown on *hisD895* as the donor and *hisG46* (HARTMAN *et al.* 1971) as the recipient on 1.5% agar plates containing E medium (VOGEL and BONNER 1956). Transductions to *pts*+ (squares) were performed using phage grown on *his-2355* (HARTMAN *et al.* 1971) as the donor and SB1690 (*ptsI34 trpB223*) as the recipient on medium A + mannitol + tryptophan plates (CORDARO and ROSEMAN 1972).

Use of *int-4 cly-2* or of lysogenic, immune recipient bacteria is called for in situations where extreme cell lysis lessens transduction frequency or distorts linkage ratios by preferential lysis of particular recombinants. Use of *int-4 cly-2* may be preferable since P22 lysogens exhibit a decreased transduction frequency due to superinfection exclusion (cf. SUSSKIND, WRIGHT and BOTSTEIN 1971). Table 1 shows some examples of the use of *int-4 cly-2*. In crosses 1 to 3 the unselected marker influences the phage decision in lysogenization and results in a decrease in the recovery of bacterial clones containing that marker. In cross 4, where the unselected marker *hisS1520* (ROTH and AMES 1966) is present in the recipient rather than in the donor, no transductants are recovered with *int-4* phage and the background lawn is decreased to a few hundred survivors. When *int-4 cly-2* is used, a background lawn and numerous *hisC*+ transductants are obtained. Phages *int-4* and *int-4 cly-2* yield the same linkages in crosses in which the unselected marker does not influence lysogenization (not shown in Table). Lysates of *int-4 cly-2* tend to contain about 10-fold fewer plaque-forming units than *int-4* lysates. However, an approximately 10-fold higher transduction frequency of *int-4 cly-2* phage compensates. An additional advantage of *int-4 cly-2* phage is the low frequency of lysis, and thus of free phage, among plated recipient bacteria incubated at 37°.

TABLE 1

Transduction with int-4 *and* int-4 cly-2 *phages*

Cross	Donor	Recipient	Selected marker	Second marker	Percent linkage of second marker using: *int-4*	*int-4 cly-2*
1	*cya-408*	*metE338*	*met*+	*cya*⁻	3.6	10
2	*crp-403*	*cysG439*	*cys*+	*crp*⁻	7.6	17
3	*rif-39*	*thi-502*	*thi*+	*rif*⁻	9.2	24
4	wild type	*hisS1520 hisC6330*	*hisC*+	—	0*	100

* No *hisC*+ recombinants were found although about 1000 were expected.

DISCUSSION

Phage P22 has been widely used in bacterial genetics because it is easier to grow and more stable in storage than are most phages. On the other hand, low frequency transduction with wild-type P22 most often results in P22 lysogens, and P22 is only weakly inducible. In addition, killing of transductional clones is observed with wild-type P22 when used under "classic" conditions or when particular genetic markers are present either in the recipient bacteria or are involved in the genetic cross. The supplementary strains and procedures outlined in this report circumvent these major drawbacks and simplify the use of P22 in genetic analysis of Salmonella.

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