TRANSDUCTION TO PENICILLIN AND CHLORAMPHENICOL RESISTANCE IN SALMONELLA TYPHIMURIUM

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Received July 28, 1958

TO explain multistep resistance to antibiotics, DEMEREC (1945, 1948) assumed that there are a number of genes that affect bacterial resistance. If one of them mutates, the bacterial cell acquires resistance to a certain concentration of the antibiotic. The combined effect of mutations in several genes is a high degree of resistance or complete resistance. In penicillin type of resistance the different genes affecting resistance are according to DEMEREC about equally potent, so that the resistance of bacterial individuals in which only one gene has mutated is never very high. In the streptomycin type of resistance, however, the genes for resistance vary greatly in potency: a mutation in a less potent gene will be responsible for a low degree of resistance, a mutation in a highly potent gene for a high degree of resistance.

CAVALLI and MACCACARO (1952) studied the genetic mechanisms of chloramphenicol resistance in *E. coli* K12 by the recombination method. The results were in good agreement with the polygenic theory. The alternative possibility of a system of multiple alleles entailing various degrees of resistance was excluded on the basis that (a) the resistance genes, when crossed together, give rise to a certain proportion of sensitives, and (b) if crossed to marked sensitives, they show different linkages with the markers.

Later DEMEREC *et al.* (1955) obtained evidence of complex loci in *Salmonella typhimurium* by means of the transduction technique. They made tests to determine allelic relations among 25 cystine-requiring mutants and found that transduction to wild type occurred in all but a very few combinations. They concluded that mutational changes occurring at different sites within a gene locus, which extends over a section of chromosome, give rise to nonidentical alleles.

With these findings in mind, I decided to study the genetic mechanisms of multistep resistance by means of the transduction technique. It appeared that theoretically two mechanisms might be expected: a polygenic system; and, in addition, mutations at different sites of the same gene locus.

MATERIALS AND METHODS

Media and drugs: In the experiments to be described below nutrient broth and nutrient agar, soft agar, penicillin G, and chloramphenicol were used.

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Strains: The bacteria used were *Salmonella typhimurium* strain LT-2; a prolineless mutant *proD-129;* three first-step penicillin-resistant mutants; one each second-, third-, and fourth-step penicillin-resistant mutants; and one each first-, second-, and third-step chloramphenicol-resistant mutants. The transducing phage was PLT–22.

Designation of strains: Penicillin-resistant mutants are designated by the abbreviation *pen-r* and chloramphenicol-resistant mutants by *chl-r*. A number attached to the symbol *pen-r* or *chl-r* (for example, *pen-r-1*) identifies the strain. Multistep resistant mutants are identified by two or more numbers (for example, *pen-r-1-2*, a second-step resistant mutant isolated from *pen-r-1*).

Methods: In the first attempts to transduce first-step resistance to penicillin and chloramphenicol, a double-agar-layer technique was employed. The results were encouraging but not satisfactory. An aerated-broth-culture method was therefore worked out. Adsorption took place in a bubbler tube containing 0.5 ml of an overnight broth culture of the recipient strain (about 2×10^9 bacteria per ml) mixed with 0.5 ml of a phage preparation from the donor strain. After six minutes of incubation in a water bath at 37°C, nine ml of broth were added and the tube was aerated in the water bath at 37°C until a density of approximately 2×10^9 bacteria per ml was reached, in order to allow for phenotypic expression of the resistant transductants. When the recipient strain was LT–2 or a relatively fast-growing mutant, this density was obtained in 3–4 hours; when the recipient was *proD-129*, which is slow growing, six or more hours of incubation were necessary. A control tube in which the bacteria were mixed with a homologous phage was treated in the same way as the transduction tube.

An appropriate quantity of the antibiotic was pipetted into one side of an empty Petri dish. An 0.1 ml sample of the culture was taken from the tube and pipetted into the opposite side of the Petri dish. Then 20 ml of melted nutrient agar, adjusted to 45°C, was poured into the Petri dish. The bacteria and antibiotic were well mixed with the agar by moving the plate in different directions for one minute. Three plates each were prepared from the transduction tube and the control tube. The density of the broth cultures in the transduction and control tubes was then checked with a Klett-Summerson apparatus to be sure that there was no significant difference in density between the transduction and control cultures. The plates were kept in the incubator at 37°C for three days, after which the colonies were counted on both series of plates.

Most of the experiments were done in this manner; but eventually the double-agar-layer technique was tried again, because it appeared that at least in some instances that method might be superior. It was realized that the prerequisite for successful transduction of first-step resistance by that technique is a completely even table, since otherwise a gradient plate is obtained that makes an exact colony count impossible. Furthermore, a new detail was introduced into the technique, which proved to be important. After adsorption was carried out in the usual way, four ml of broth were added before plating. This was done, first, because it was desirable to reduce the number of colonies developing from spontaneous mutants

and, second, because bacterial growth in agar is inhibited if the number of bacteria is too great (EAGLE *et al.* 1955). The further procedure was as follows. Samples of 0.1 ml were taken from the cultures and pipetted onto the bottoms of empty plates. Then 20 ml of melted agar were poured into each plate and mixed well with the culture, as described above. After hardening of the agar the plates were incubated at 37°C for three hours. They were then removed from the incubator and five ml of soft agar containing the desired quantity of antibiotic were poured into each plate on top of the first agar layer. The plates were held for two hours at room temperature to allow diffusion of the antibiotic, and then placed again in the incubator; after three days the colonies were counted.

RESULTS

Transduction to penicillin resistance: The aerated-broth-culture method proved to be satisfactory for the transduction of first-step penicillin resistance. The difference in numbers of colonies on the transduction and on the control plates was significant. Better results were obtained with the slow-growing strain *proD-129* as recipient than with strain LT–2. Resistant bacteria are as a rule more slow growing than the sensitive wild type, and the same is true of the transductants. When the faster growing wild type strain is employed as recipient, nontransduced sensitive cells have a selective advantage over the resistant transductants under the conditions of this broth technique. It seems probable that when the recipient strain is a slow-growing one, the difference in growth rate between sensitive and transduced resistant cells is less. The concentration of penicillin employed was 20 units; on this about 50 percent of plated bacteria of a first-step resistant mutant of strain LT–2 are able to survive.

TABLE 1

Numbers of penicillin-resistant colonies observed in transduction experiments LT–2 (×) pen-r-10 *and* proD-129 (×) pen-r-10 (*20 units penicillin per ml of agar*)

Recipient	Donor	Experiment 1		Experiment 2	
		Transd.	Control	Transd.	Control
LT–2	*pen-r-10*	376	97	157	42
proD-129	*pen-r-10*	406	37	501	43

The results of transduction of strains LT–2 and *proD-129*, respectively, by the first-step resistant donor *pen-r-10* are summarized in Table 1. With strain LT–2 as recipient, the transduction plates contained 3.8 times as many colonies as the control plates in one experiment, and 3.7 times as many in another experiment. With *proD-129* as recipient, the number of colonies on the transduction plates was larger by a factor of 10.9 in the first experiment and 11.9 in the second experiment.

The same technique was employed in transductions of the sensitive strain by second-, third-, and fourth-step resistant mutants. In these experiments two concentrations of penicillin were employed: 20 units, corresponding to the resistance

level of the first step, and 60 units, corresponding to that of the second step. The results showed that multistep resistant strains as donors are able to transfer only a degree of resistance corresponding to first-step resistance. The results of transduction on the plates containing 20 units of penicillin per ml of agar are presented in Table 2.

TABLE 2

Results of transduction experiments proD-129 (×) pen-r-1, pen-r-1-2, pen-r-1-2-3, *and* pen-r-1-2-3-4 (*20 units penicillin per ml of agar*)

Donor	Experiment 1		Experiment 2		Experiment 3	
	Transd.	Control	Transd.	Control	Transd.	Control
pen-r-1	69	11	396	63	310	32
pen-r-1-2	233	21	261	23	225	37
pen-r-1-2-3	71	17	64	23	101	27
pen-r-1-2-3-4	59	19	52	23	61	25

Experiments were next made to test whether first-step resistant strains could be transduced to second-step resistance (resistance to 60 units of penicillin) by other first-step resistant strains. Strain *pen-r-5* was found to be transducible to second-step resistance by strain *pen-r-1* or *pen-r-10*. The results are summarized in Table 3. Attempts to transduce strains *pen-r-1* and *pen-r-10* by strain *pen-r-5*

TABLE 3

Results of transduction experiments pen-r-5 (×) pen-r-10 *or* pen-r-1 (*60 units penicillin per ml of agar*)

Donor	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	Transd.	Control	Transd.	Control	Transd.	Control	Transd.	Control
pen-r-10	236	37	113	20	103	25	221	27
pen-r-1	251	40	126	32	149	29	164	26

failed, however, although such transduction was to be expected. This finding suggested that the broth technique is not sensitive enough for the demonstration of transduction in all cases.

New attempts were therefore made to work out a satisfactory double-agar-layer technique, which might be expected to be more sensitive than the broth technique in cases of low transduction frequency and in cases of large difference in growth rate between the sensitive recipient strain and resistant transductants. The improved technique described in the Methods section was employed. Results of transduction to first-step resistance, with *proD-129* as recipient and strains *pen-r-10* and *pen-r-1-2* as donors, were very satisfactory; they are presented in Table 4. With this technique it was also possible to transduce strain *pen-r-10* to

TABLE 4

Results of transduction experiments proD-129 (×) pen-r-10 *and* proD-129 (×) pen-r-1-2, *made with the double-agar-layer technique (20 units penicillin per ml of agar)*

Donor	Transd.	Control
pen-r-10	88	7
pen-r-1-2	58	5

the second step with strain *pen-r-5* as donor (Table 5). Thus this technique proved more sensitive than the broth method.

TABLE 5

Results of transduction experiments pen-r-10 (×) pen-r-5, *made with the double-agar-layer technique (60 units penicillin per ml of agar)*

Donor	Experiment 1		Experiment 2		Experiment 3	
	Transd.	Control	Transd.	Control	Transd.	Control
pen-r-5	35	4	36	3	30	11

Transduction to chloramphenicol resistance: The aerated-broth-culture method was used successfully in transduction to chloramphenicol resistance. In this case, too, strain *proD-129* proved to be a more appropriate recipient than strain LT–2. The experiments were restricted to transduction of strains *proD-129* and LT–2 by donor strains *chl-r-1*, *chl-r-1-2*, and *chl-r-1-2-3*. When the first-step resistant strain *chl-r-1* was the donor, only one concentration of chloramphenicol was used, that is, 8 μg. When second-step (*chl-r-1-2*) and third-step (*chl-r-1-2-3*) resistant strains were donors, two concentrations were employed, namely, 8 μg and 30 μg. On the plates containing 30 μg of chloramphenicol, no colonies developed. Thus with second- and third-step resistant strains as donors it was possible to transfer only a degree of resistance corresponding to first-step resistance. The data for the tranduction plates containing 8 μg of chloramphenicol are given in Tables 6 and 7.

TABLE 6

Numbers of chloramphenicol-resistant colonies observed in transduction experiments proD-129 (×) chl-r-1, chl-r-1-2, *and* chl-r-1-2-3 *(8 μg chloramphenicol per ml of agar)*

Donor	Experiment 1		Experiment 2		Experiment 3	
	Transd.	Control	Transd.	Control	Transd.	Control
chl-r-1	72	16	46	10	66	35
chl-r-1-2	527	19	287	11	248	30
chl-r-1-2-3	159	15	172	17	183	28

TABLE 7

Results of experiments LT–2 (×) chl-r-1, chl-r-1-2, *and* chl-r-1-2-3 (*8 μg chloramphenicol per ml of agar*)

Donor	Transd.	Control
chl-r-1	56	52
chl-r-1-2	64	5
chl-r-1-2-3	60	34

DISCUSSION

Of the two techniques employed for transduction to penicillin or chloramphenicol resistance, the double-agar-layer technique appears to be preferable, although most of the experiments in this study were done by the aerated-broth-culture method. In both procedures the concentration of antibiotic is very important. According to our experience, it is best to choose a concentration which allows about 50 percent survival of the corresponding resistance step. A lower concentration of the antibiotic increases the number of transductants but increases still more the number of spontaneous mutants, which is unfavorable for the experimental results.

In the present study I was interested primarily in the question of whether it is possible to transduce bacteria to first-step penicillin or chloramphenicol resistance. It might be expected *a priori* that there was very little chance of working out an appropriate technique for transduction to first-step resistance, because of the high spontaneous-mutation rate. Results obtained by the techniques described, however, show that it is possible to transfer first-step resistance by transduction. I was interested further in finding out whether it is possible to transfer the full resistance of second-, third-, or fourth-step strains to a sensitive recipient. The results show that it is not possible to produce more than first-step resistance by transduction with phage grown on multistep resistant strains. From this it can be inferred that different genes determining penicillin or chloramphenicol resistance in strain LT–2 are not closely linked. The results indicate that mutations at different sites of the same gene locus do not have a significant role in the building up of multistep resistance.

A third question of interest was whether it is possible to build up multistep resistance to penicillin by the transduction method. It was found that transduction of a first-step resistant strain by another independent first-step resistant mutant results in second-step resistance.

On the basis of our data the following conclusions may be drawn: (a) resistance to penicillin or chloramphenicol in *Salmonella typhimurium* is gene controlled, because it is possible to transfer it to a sensitive recipient by transduction techniques; (b) there is a polygenic system governing such resistance; (c) the different genes for resistance to penicillin or to chloramphenicol are not linked.

SUMMARY

An aerated-broth-culture method as well as a double-agar-layer technique were worked out by which it is possible to transduce sensitive strains of *Salmonella typhimurium* by first-step and multistep penicillin- or chloramphenicol-resistant mutants. Multistep resistant mutants are capable of transducing sensitive strains only to a degree of resistance corresponding to first-step resistance. This means that separate genes controlling resistance to penicillin or to chloramphenicol are not linked. Transduction of first-step penicillin-resistant strains by independent first-step mutants gives rise to second-step resistance. These data confirm the hypothesis that a polygenic system governs multistep resistance.

ACKNOWLEDGMENT

I wish to express my gratitude to DR. M. DEMEREC for helpful advice and criticism during this study.

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