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Direct Selection for P1-Sensitive Mutants of Enteric Bacteria

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A method has been developed to isolate mutants sensitive to coliphage P1 from bacterial genera normally not sensitive to this phage. Plclr100KM was used. This phage is heat inducible and confers kanamycin resistance when present as a prophage (in lysogens). P1-sensitive mutants of *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Erwinia* have been found. This technique provides a well-known genetic system for the study of many bacterial genera that previously had either no such system or only a marginally useful means of genetic manipulation. It also extends the range of possible intergeneric hybrids that may be constructed and studied.

The discovery of techniques for transferring genetic material between different mutants of *Escherichia coli* and between *E. coli* and certain other bacterial genera has allowed a significant expansion of our understanding of the physiology and molecular biology of these organisms. Although genetic transfer via sex factors is useful for mapping the chromosome of *E. coli* and generating stable merodiploids, phage-mediated, generalized transduction, particularly via P1, has allowed a precision of genetic mapping not possible with sex factor techniques (13, 20).

Interesting physiological phenomena have been found in enteric bacteria that have not yet been accessible to genetic manipulation because the bacterium fails to show genetic transfer by standard techniques (11, 17, 19). We present here a simple method, the selection of drug-resistant lysogens, by which many heretofore genetically unapproachable enteric bacteria can be studied by P1-mediated, generalized transduction. With this procedure it is relatively simple to convert entire strain collections to P1 sensitivity.

The expansion of the host range of P1 has made it possible to study and construct intergeneric hybrids that were previously unavailable. We have constructed hybrid strains of *E. coli* carrying *Klebsiella aerogenes* genes (R. Goldberg, manuscript in preparation; B. Tyler, manuscript in preparation) and *Klebsiella pneumoniae* strains carrying *K. aerogenes* genes. These hybrids have allowed us to examine the effects of cytoplasmic factors on physiological and genetic control mechanisms.

MATERIALS AND METHODS

Bacterial strains. Bacteria used in the course of these experiments are listed in Table 1.

Media. LB (11) was the rich medium used in all experiments. Derivative media were supplemented with the following: LBKM, 12.5 μg of kanamycin sulfate per ml; LBC, 5 mM $CaCl_2$. Solid media contained 1 or 2% agar, and petri plates were used within 3 days of pouring. The top agar used contained (per liter of water): tryptone (Difco), 15.5 g; yeast extract (Difco), 8.75 g; NaCl, 0.5 g; 1 N NaOH, 4.25 ml; and agar, 5 g. Phage adsorption media were either LBC or AM (10 mM $MgCl_2$ and 5 mM $CaCl_2$).

Bacterial growth conditions. All bacteria used in this study were grown at 30 or 37 C in LB medium. Kanamycin-resistant lysogens were maintained by growth in LBKM medium.

Isolation of Plclr100KM. Phage Plclr100CM (12) was crossed with phage P1KM by the method of Scott (14). Recombinants able to confer resistance to kanamycin or to both chloramphenicol and kanamycin were selected; they were approximately 3% of the population. Phages Plclr100KMCM and P1KMCM proved to be unstable and readily segregated one or both of the resistance markers from the phage. Plclr100KM was purified by plating for single plaques three times and was then retested for inducibility at high temperature and for specialized transduction of kanamycin resistance.

Growth of phage stocks. Plclr100KM was grown by a modification of the thermal induction method of Rosner (12). An overnight culture of a lysogen grown in LBKM medium at 30 C was diluted 1:100 into LBC medium and grown with vigorous shaking to a density of about 5×10^8 bacteria per ml. The culture was incubated at 40 C for 30 min and then at 37 C for 90 min, or in later experiments at 37 C for 120 min. The latter procedure was more convenient and resulted in similar phage yields. After lysis, the culture

TABLE 1. *Bacterial strains used*

Strain	Relevant genotype	Source
1 *Klebsiella aerogenes* MK1	$P1^R$, $PW52^S$	B.Magasanik (11)
2 *K. aerogenes* MK2	chl^R (deletion of *chl bio hut gal*)	Spontaneous chl^R of MK1
3 *K. aerogenes* KG2	$P1^S$, $PW52^R$	EMS of MK1[a]
4 *K. aerogenes* MK9011	$P1^S$, $PW52^S$	Derivative of MK1
5 *K. aerogenes* MK9271	$P1^S$, *ilvA1*[b] *metB6*[c] *rha-1*	Derivative of MK1
6 *Erwinia amylovora*	$P1^R$	ATCC 15580[d]
7 *Citrobacter freundii*	$P1^R$	ATCC 8090
8 *Enterobacter aerogenes*	$P1^R$	ATCC 13048
9 *Enterobacter cloacae*	$P1^R$	ATCC 13047
10 *K. aerogenes* 1033	$P1^R$, $PW52^R$	B. Magasanik
11 *K. pneumoniae* 57	$P1^R$	CDC 4425-51[e]
12 *K. pneumoniae* 21	$P1^R$	CDC 702-49
13 *Enterobacter liquifaciens*	$P1^R$	ATCC 14460
14 *Pseudomonas putida* PRS1	$P1^R$	M. Wheelis (21)
15 *Salmonella typhimurium* LT-2	$P1^R$	B. Magasanik
16 *S. typhimurium* 15-59	$P1^R$	B. Magasanik
17 *Erwinia carotovora*	$P1^R$	ATCC 495
18 *Escherichia coli* EG47	$P1^S$	Derivative of WD5021 from E. Signer

[a] This strain was isolated by the nibbled colony technique (16; R. Goldberg, manuscript in preparation).
[b] Growth supported by isoleucine (20).
[c] Growth supported by methionine, homocysteine, or cystathionine, but not by B_{12} or *o*-succinyl homoserine (20).
[d] American Type Culture Collection.
[e] Center for Disease Control, Atlanta, Ga.

was treated with chloroform and the debris was removed by centrifugation. The phage were stored over a few drops of chloroform.

Assay of phage titer. Methods for determining the titer of P1 by using *Shigella* have been described (14). It has been difficult to determine P1 titers with *E. coli* because of the small plaque size. Using the top agar described above, we were able to achieve plaque sizes of 0.6 to 0.8 mm on *E. coli*. Titers were determined as follows. *E. coli* strain EG-47 was grown in LB medium to a density of 2×10^8 bacteria per ml and brought to 5 mM $CaCl_2$. A 0.1-ml portion of the appropriate dilutions of the lysate was mixed with 0.2 ml of strain EG-47 and allowed to adsorb at 37 C for 20 min. A 2.5-ml volume of top agar was then added, and the mixture was poured onto fresh LBC plates and incubated at 37 C overnight.

The same procedure was used to determine the titer of KM specialized transducing particles with the following exceptions: (i) adsorption was at 30 C, (ii) 0.1 ml of the phage dilutions was mixed with 0.1 ml of the bacteria, and (iii) 0.1 ml was then spread on an LBKM plate and incubated at 30 C.

Transduction. Specialized transduction of kanamycin resistance was performed as above.

Generalized transduction was performed as follows. A late exponential-phase culture of recipient cells in LB medium was washed and suspended in AM. Plclrl00KB grown on the donor were added at a multiplicity of infection of between 0.1 and 1.0. The phage were allowed to adsorb for 30 min at 30 C, and then 0.1-ml portions were plated on selective plates and incubated at 30 C until colonies appeared, generally 36 to 48 h.

RESULTS

Isolation of P1-sensitive clones. It is normally difficult to select phage-sensitive variants from among a resistant population of bacteria because there is no selectable phenotype. In the past, such variants were found by plating the bacteria on plates that had been previously seeded with phage and searching for "nibbled" colonies (16). We have used a stronger, positive selection procedure, drug resistance conferred by lysogeny, to isolate such mutants.

To isolate drug-resistant lysogens, Plclr-100KM were added to an overnight culture of sensitive bacteria that had been grown in LBC medium. The ratio of phage to bacteria was approximately 2. The mixture was incubated at room temperature for 30 min to allow phage adsorption, then plated on LBKM plates, and incubated overnight at 30 C. This procedure generally yielded between 10 and 100 colonies by the next morning. Most of these proved to be P1 lysogens.

P1 lysogeny was checked by transferring a purified colony onto two LB plates, one of which

was incubated at 30 C and the other at 42 C. Lysogens of P1cl4100KM grow well at 30 C and very poorly at temperatures above 35 C. Lysogeny was confirmed by demonstrating that the kanamycin-resistant, temperature-sensitive cells thus isolated were capable of releasing phage and forming a plaque when seeded into a lawn of indicator bacteria.

Using the technique outlined above we have isolated P1-sensitive derivatives of strains 1 and 6 through 13 listed in Table 1. Table 2 lists typical phage yields from various lysogens upon thermal induction.

In our first attempt, we failed to find a P1 lysogen of *Pseudomonas putida;* we have not pursued this any further. We were also unable to obtain stable lysogens of *Salmonella typhimurium* by this method.

Curing of the prophage. Certain features of P1 lysogens, such as P1-mediated restriction (2, 7) and the temperature sensitivity, interfere with some genetic and physiological studies. The presence of the *clr100* mutation in the prophage has made curing of the lysogen a relatively simple task. The lysogen may be cured by growth overnight in LB medium at 30 C, plating on LB plates, and incubation at 37 C overnight. The number of cells that will grow at high temperature after such treatment varies widely from experiment to experiment, but usually represents a large fraction of the population. The presumptive, cured cells are then purified and shown to be capable of growth at 37 C and to have regained sensitivity to kanamycin.

P1 restriction and modification in K. aerogenes. P1 is known to modify and restrict the deoxyribonucleic acid of phage lambda (2), and the apparent loss of sensitivity to lambda, unmodified by the P1 restriction and modification system, is often used as a means of determining whether P1 lysogeny has been established in *E. coli* (12). *K. aerogenes* is sensitive to phage PW52, a generalized transducing phage (9), and we have examined its sensitivity to P1 restriction and modification.

TABLE 2. *Typical phage yield obtained by thermal induction of P1clr100KM lysogens*

Strain	PFU/ml[a]
Escherichia coli EG47	7×10^9
Citrobacter freundii	6×10^9
Enterobacter liquifaciens	1×10^6
Erwinia amylovora	3×10^5
Klebsiella aerogenes MK9011	5×10^9

[a] Cultures were treated as described in Materials and Methods. PFU, Plaque-forming units.

The efficiency of plating (EOP) of PW52 unmodified by P1 is very low on a P1 lysogen compared to its EOP on a nonlysogen (Table 3). When PW52 is grown on strain MK9011(P1) and then grown on strain MK9011, P1 modification is lost and the phage is again strongly restricted by strain MK9011(P1). The degree of restriction observed is comparable to that seen with phage lambda in *E. coli*. It should be noted, however, that the PW52 modified by both the *K. aerogenes* and P1 modification systems plate more efficiently on the P1 lysogen than on the nonlysogen. This type of interaction between restriction and modification systems has been observed only once before with *hspS* and *hspR*II (15).

Generalized transduction in K. aerogenes. P1-mediated, generalized transduction has been used to map the regions of the *K. aerogenes* genome near a gene involved in glutamine synthesis (*glnA*) and near the histidine utilization (*hut*) operons (S. Streicher et al., manuscript in preparation; R. Goldberg et al., manuscript in preparation). Table 4 shows typical transduction frequencies. These are somewhat variable but usually higher than 10^{-5} transductants per plaque-forming unit for *K. aerogenes*. The two transductions involving strain MK9271 as recipient (Table 5) demonstrate that P1 co-transduction frequencies allow the construction of a linear linkage map.

The gene order derived from the data in Table 5, *ilvA-metB-rha*, is different from the published gene order of the analogous markers in *E. coli* K-12 (*ilvA-rha*-metB) (20). The data presented here and the mapping of several other markers linked to the *ilv-met-rha* region of the *K. aerogenes* chromosome suggest that occurrence of a transposition of the genes in that portion of the *K. aerogenes* chromosome which corresponds to the 74.5- to 77-min region of the *E. coli* chromosome to the region of the *K.*

TABLE 3. *Restriction and modification of phage PW52 by P1 lysogens of K. aerogenes*

Source of phage PW52[a]	Modification type	Efficiency of plating	
		MK9011	MK9011(P1)
K	*Klebsiella*	1	1×10^{-5}
K(P1)	*Klebsiella*, P1	0.6	1
K(P1)·K	*Klebsiella*	1	1×10^{-5}

[a] Abbreviations used are as follows: K, growth on strain MK9011; K(P1), growth on strain MK9011(P1); and K(P1)·K, phage grown on strain MK9011(P1) and cycled through strain MK 9011 before plating.

TABLE 4. *Typical transduction frequency in K. aerogenes*

Donor	Recipient	Selected marker	No. of transductants/10^7 PFU[a]
KG2	MK9271	*ilv*	484
KG2	MK9271	*met*	550

[a] PFU, Plaque-forming units.

TABLE 5. *Construction of a linear map using P1 transduction in K. aerogenes*

Donor	Recipient	Selected marker	Co-transductional frequency (%)		
			ilvA	*metB*	*rha*
KG2	MK9271	*ilv*$^+$		30	5
KG2	MK9271	*met*$^+$	20		60

aerogenes chromosome corresponding to min 79 of the *E. coli* chromosome. (S. Streicher, manuscript in preparation). This transposition results in the linkage of *ilvA* with *rha* and *metB* by P1 transduction of *K. aerogenes.*

DISCUSSION

Generalized transduction mediated by bacteriophage P1 has been widely studied (6, 8) and used to map the *E. coli* chromosome (21) and, to a limited extent, the *Salmonella* chromosome (13). The ability to extend a standardized genetic system offers obvious advantages over having to develop independent systems for specific strains of bacteria.

Previous attempts to isolate phage-sensitive mutants of a given strain have involved negative selection, such as looking for nibbled colonies (16), or the use of fortuitous selectable markers (10). Our procedure provides a much simpler and direct approach, the selection of drug-resistant lysogens of the bacterium. The minimal requirements for the method to be effective are that: (i) the bacterium must be sensitive to a drug for which a specialized transducing phage can be prepared, and (ii) it must be able to grow at a temperature permissive for the phage (<32 C) and at a temperature nonpermissive for the phage (>35 C). We prepared P1clr100KM because the strain regularly used in this laboratory, *K. aerogenes* W70 (9), is naturally resistant to high concentrations of chloramphenicol (4) but is sensitive to kanamycin. For other strains, the P1 phage used should depend on its drug sensitivity. It should be noted that kanamycin resistance can arise in two different ways: by a recessive, chromosomal mutation modifying the bacterial ribosome (1), or by the expression of the dominant, kanamycin-resistance gene carried by P1clr100KM. The former class, those possessing a bacterial mutation, grow much more slowly and can be easily distinguished as small colonies on LBKM plates.

The ability to select drug-resistant lysogens makes it a relatively simple task to convert an entire strain collection to P1 sensitivity. This procedure allows the retention of previously isolated mutants and eliminates the need to reisolate interesting mutants in a sensitive strain.

This technique is also useful for strains that have been sensitive to P1, but may have developed unwanted lesions conferring P1 resistance. Gottesman et al. (5) have used a similar technique to recover markers from *E. coli* strain $15T^-$ where preparation of phage stocks had been difficult because the bacterium harbors several host restriction systems.

We have successfully isolated P1-sensitive ($P1^S$) variants for most strains examined, although we have not tried any gram-positive strains. We have not, however, been able to isolate $P1^S$ mutants of *Pseudomonas putida*. Our results with *Salmonella typhimurium* and *Erwinia carotovora* are also unusual. *Erwinia carotovora* lysogens are easily isolated, but we have not observed phage release from them.

We have not been able to isolate drug-resistant lysogens of *S. typhimurium* strains LT-2 or 15-59. This may be explained by the fact that *Salmonella* appears to be a poor host for P1 either as a lysogen (R. Bender, unpublished data) or lytically (10). *Salmonella* $P1^S$ mutants have been reported (E. P. Ornellas, M. Enomoto, and B. A. D. Stocker, Bacteriol. Proc., p. 207, 1971) to be a subclass of *galE* mutants. This is true also in *K. aerogenes*, where a chlorate-resistant *gal-bio* deletion was found to have become P1 sensitive. This mechanism for achieving P1 sensitivity is not obligatory since all the P1-sensitive strains derived by using P1clr100KM have been *gal*$^+$.

This procedure also provides a means of preparing intergeneric hybrids. In this laboratory we have moved markers from *K. aerogenes* into *E. coli* (R. Goldberg, manuscript in preparation; B. Tyler, unpublished results). Markers have also been moved from *K. aerogenes* to *K. pneumoniae* (S. Streicher, unpublished results). We hope that the observation by Stodolsky (18), that P1 specialized transducing phage can be prepared from any region of the *E.*

coli chromosome, can be generalized. This would allow the preparation of specialized transducing phage from many more bacteria than is presently possible. We hope that the extension of these techniques from the common laboratory strains of enteric bacteria to other strains, many of them of agronomic importance, will facilitate an understanding of their physiology and molecular biology.

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