Genetic Mapping of the *phoR* Regulator Gene of Alkaline Phosphatase in *Escherichia coli*

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SUMMARY

Genetic mapping of the structural *phoA* and the linked regulatory *phoR* genes for alkaline phosphatase synthesis in *Escherichia coli* was carried out by conjugation. Distal markers were selected and the segregation of proximal markers was determined. The gene order *lac-phoA-phoR-tsx* is proposed.

INTRODUCTION

The synthesis of alkaline phosphatase in *Escherichia coli* is repressed by inorganic orthophosphate (Torriani, 1960). This enzyme is coded by the structural gene *phoA* and its synthesis is controlled by two regulatory genes, *phoR* and *phoS*. A mutation in either of the regulatory genes results in a phenotype constitutive for alkaline phosphatase synthesis. The structural gene and the *phoR* regulatory gene are linked and are located close to the *lacI* gene of the lactose operon. The *phoS* regulatory gene is located in a distant region, between the isoleucine-valine (*ilv*) and tryptophanase (*tnaA*) loci (Garen, 1960; Echols, Garen, Garen & Torriani, 1961; Aono & Otsuji, 1968). The order of the linked *phoA* and *phoR* genes has not yet been determined. This communication describes a series of crosses determining the order and the position of these two loci relative to the linked *lac* and resistance to bacteriophage T6 (*tsx*) markers.

METHODS

Bacterial strains. Table 1 lists the strains used; all are derivatives of *Escherichia coli* K12.

Media. Tris buffered minimal media were used as described by Echols *et al.* (1961. See also Torriani, 1968). These were supplemented with 0.2% of the appropriate sugar, 2% Difco Noble agar and, where necessary, 20 µg./ml. L-amino acid, 1 µg./ml. thiamin and 100 µg./ml. streptomycin. The concentration of KH₂PO₄ in these plates (10⁻³ M) was in excess for the repression of alkaline phosphatase synthesis. For derepression of enzyme synthesis, plates with low concentration (3.3 × 10⁻⁵ M) of KH₂PO₄ were used. Tryptone broth contained, per litre, 10 g. Difco Bacto-Tryptone, 5 g. yeast extract, 5 g. NaCl and 40 mg. NaOH. For plating, 2% agar was added and 0.7% for tryptone soft agar. EMBlac medium as described by Lederberg (1950) was used.

Determination of alkaline phosphatase phenotype. A modification of the method of Levinthal, Signer & Fetherolf (1962) was used. The plates were sprayed with a 1 + 1 (v/v) mixture of α-naphthyl phosphate (Calbiochem; 2 mg./ml. in 1 M Tris-chloride buffer, pH 8.0) and tetrazotized o-dianisidine (Sigma; 10 mg./ml. in water). These

reagents were dissolved and mixed in an ice bath just before spraying. Colonies with a purple coloration indicated enzyme activity. Constitutive colonies stained on media containing KH_2PO_4 at excess or low concentrations; wild type repressible colonies were stained at low phosphate concentration only and *phoA* mutants were unstained on both media.

Table 1. *Characteristics of the Escherichia coli K-12 strains*

Strain	Sex	Genotype*	Source or reference
K 10	Hfr C	wild type	Echols <i>et al.</i> (1961)
C 600	F ⁻	<i>thi thr leu lac tsx-r str-r</i>	F. Jacob
AB 1899	F ⁻	<i>thi thr leu proA lac tsx-r str-r</i> (<i>arg his xyl mtl ara gal lon</i>)	P. Howard-Flanders
NA 1	Hfr C	<i>phoR</i>	K 10 by NG
P 21-NA 1	Hfr C	<i>phoA phoR tsx-r</i>	NA 1 by NG
RE 2	F ⁻	<i>thr leu lac phoA phoR tsx-r str-r</i>	P 21-NA 1 × C 600 by selection for <i>tsx-r</i>

*Non-relevant markers are given in parentheses.

Abbreviations used: *thi* = thiamin, *thr* = threonine, *leu* = leucine, *pro* = proline, *arg* = arginine, *his* = histidine, *lac* = lactose, *xyl* = xylose, *mtl* = manitol, *ara* = arabinose, *gal* = galactose. *tsx-r* = resistance to bacteriophage T6, *str-r* = resistance to streptomycin. *pho* = phosphatase, *lon* = long form. NG = *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Mutant isolation

(a) *phoR* constitutive mutant. Approximately 4×10^7 cells of K 10 were spread on plates containing β -glycerol phosphate as the sole carbon source (Torriani & Rothman, 1961). Pieces of filter paper soaked in a solution of the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at 5 mg./ml. were placed in the centre of the plates. Bacteria from three of the constitutive colonies which grew after 70 hr of incubation at 37° were crossed with strain C 600, and the fraction of *thr*⁺*leu*⁺ and *lac*⁺ recombinant colonies synthesizing alkaline phosphatase constitutively was determined. In one of the crosses (NA 1 × C 600) 50% of the *thr*⁺*leu*⁺ recombinants and 90% of the *lac*⁺ recombinants were constitutive. Strain NA 1 was therefore designated *phoR* (Echols *et al.* 1961).

(b) *phoAphoR* double mutant. Strain NA 1 was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg, Mandel & Chen (1965). After 4 hr of growth to allow segregation, surviving bacteria were plated on minimal medium containing excess KH_2PO_4 . Colonies that did not synthesize alkaline phosphatase were picked, purified and spotted on minimal medium plates containing KH_2PO_4 at a low concentration. Of the 22 colonies tested, two failed to synthesize the enzyme on both media and were designated *phoAphoR* double mutants. The remaining colonies were revertants to the repressible wild type phenotype.

(c) *Resistance to bacteriophage T6*. Approximately 10^8 bacteria were mixed with 2×10^9 phage particles in melted soft tryptone agar (at 43°) and plated on top of tryptone agar. To confirm the phage resistance of bacteria in colonies that grew after incubation, they were cross streaked, after purification, against the phage at 2×10^{10} particles/ml. either on tryptone agar or on EMBlac agar.

Conjugation. The parental strains were grown in tryptone broth. Exponentially growing F⁻ cells (approximately 5×10^8 organisms/ml.) were centrifuged and resuspended in one-fifth of the original volume. Exponentially growing Hfr cells were

added to give a final ratio of 1Hfr:10F⁻ cells. The conjugants were gently shaken in 37° for 1 hr and were then plated on supplemented selective minimal agar. When *tsx-r* recombinants were selected, the conjugants were first incubated with shaking in tryptone broth supplemented with streptomycin, to allow segregation. After three generations of growth they were treated for 25 min. with T6 particles at a multiplicity of 10 before plating for survivors.

RESULTS AND DISCUSSION

Table 2 shows the results of a cross between strains NAI and AB1899. The recombinant colonies on the selective media were sprayed to determine their *phoR* genotype. The fewest exchanges occurred between the *lac* and the *phoR* markers, indicating the closest linkage between them. Since the majority of *phoR*⁺ in either *lac*⁺ or *pro*⁺ recombinants were *tsx-r* (Table 2), it is likely (though not necessary) that the *phoR*

Table 2. Segregation of constitutive colonies among selected recombinants in the cross Hfr NAI *thi*⁺ *thr*⁺ *leu*⁺ *proA*⁺ *lac*⁺ *phoR* *tsx-s* *str-s* × F⁻ AB1899 *thi* *thr* *leu* *proA* *lac* *phoR*⁺ *tsx-r* *str-r*

Selected recombinants	Number of colonies scored	% <i>phoR</i> ⁺ recombinants	% <i>tsx-r</i> amongst <i>phoR</i> ⁺ recombinants
<i>lac</i> ⁺	203	5.4	75
<i>pro</i> ⁺	847	27.0	89.5
<i>thr</i> ⁺ <i>leu</i> ⁺	336	35.7	Not scored
<i>thi</i> ⁺	36	47.2	Not scored

marker is located between *lac* and *tsx*. This, however, becomes evident from results of the cross between strains K10 and RE2. Three kinds of recombinants were obtained and the order of *phoA* and *phoR* loci relative to the linked *lac* and *tsx* markers was determined (Fig. 1, Table 3):

(A) *phoA*⁺*phoR* constitutive recombinants. These recombinants, requiring a cross-over in region c (Fig. 1), were obtained by plating the conjugants on minimal medium containing β-glycerol phosphate as carbon source (Torriani & Rothman, 1961). After two cycles of single colony isolation their *lac* and *tsx* genotype was determined. The results are shown in Table 3A. Since the majority of the *phoA*⁺*phoR* recombinants possessed the *lac*⁻ marker from the Hfr strain and the *tsx-r* marker from the F⁻ strain, the gene order *lac-phoA-phoR-tsx* is most likely.

Fig. 1. The cross between Hfr K10 and F⁻ RE2 shown as a genetic map. The arrow indicates the direction of chromosomal transfer.

(B) *lac*⁺ recombinants. These were obtained on medium with lactose as the carbon source and were sprayed for the determination of their *phoR* phenotype. The constitutive (*phoR*) recombinants must have resulted from cross-overs in regions a and c, and of 731 *lac*⁺ recombinants only 11 (1.5%) were constitutive. (These results agree

with the results obtained by Echols *et al.* 1961, who reported that in the cross between Hfr *thr⁺leu⁺phoA⁺phoR* and F⁻ *thr leu phoA phoR⁺*, of the *thr⁺leu⁺* recombinants 1.8% resulted from a cross-over between the *phoA* and *phoR* loci.) These *lac⁺phoR* recombinants were isolated and after two cycles of single colony purification their *tsx* genotype was determined (Table 3B). The majority (82.2%) were *tsx-r*, and this is also compatible with the gene order *lac-phoA-phoR-tsx*.

Table 3. *The analysis of recombinants from the cross Hfr K10 lac⁺ phoA⁺ phoR⁺ tsx-s str-s × F⁻ RE2 lac phoA phoR tsx-r str-r*

Selected recombinants	Number of colonies scored	Unselected markers	
		Genotype	%
A <i>phoA⁺phoR</i>	100	<i>lac⁺tsx-r</i>	83.0
		<i>lac tsx-r</i>	7.0
		<i>lac⁺tsx-s</i>	10.0
		<i>lac tsx-s</i>	0
B <i>lac⁺phoR</i>	28	<i>tsx-r</i>	82.2
		<i>tsx-s</i>	17.8
C <i>lac⁺tsx-r</i>	96	<i>phoA⁺phoR⁺</i>	29.5
		<i>phoA⁺phoR</i>	22.2
		<i>phoA (phoR)*</i>	48.3

* Due to the mutation of the *phoA* structural gene, the *phoR* genotype cannot be determined, but it is most likely to be mutant rather than wild type.

(C) *lac⁺tsx-r* recombinants. This class of recombinants was selected in order to determine the relative distances between the genes involved. They could result from cross-overs between *lac* and *tsx* in either regions b, c or d (Fig. 1), and the genotype of such recombinants was determined by their ability to synthesize alkaline phosphatase on media with either excess or low phosphate concentrations. The frequency of each recombinant genotype is shown in Table 3C. The fact that all three types of segregants (*phoA⁺phoR⁺*, *phoA⁺phoR*, *phoA phoR*) were obtained conclusively proves the gene order proposed. In addition, assuming that the observed frequency of cross-overs is proportional to the distance between the markers, it is calculated that the relative lengths of regions b, c and d are 2.17:1:1.33, respectively. The ratio of the distances c/(b+c) can be calculated either from selection (C) (22.2/(48.3+22.2) = 0.32) or by dividing the 1.5% *lac⁺phoA⁺phoR* recombinants obtained in selection (B) (above) by the 5.4% *lac⁺phoR⁺* recombinants obtained in Table 2 (1.5/5.4 = 0.28). These two values are in good agreement. However, the assumption concerning the strict proportionality between distance and frequency of crossing-over should be made with caution, since negative interference may have resulted in a higher incidence of cross-overs proximal to the *lac* marker (Maccacaro & Hayes, 1961). The observation that the frequency of *lac phoA⁺phoR* constitutive recombinants (7%) was lower than that of *phoA⁺phoR tsx-s* recombinants (10% — Table 3A) is probably due to spontaneous chromosomal breakage during transfer, since *lac* is a distal marker (Hayes, 1968).

The orientation on the genetic map of the *phoR* regulatory gene is the same as in almost all other operons known in *E. coli* in which the operator or one of the regulator sites are linked to the structural genes (cf. Taylor & Trotter, 1967; Epstein & Beckwith, 1968).

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