GENETIC ANALYSIS OF REGULATORY MUTANTS OF ALKALINE PHOSPHATASE OF *E. COLI*

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ABSTRACT

A fine structure map of the *phoR* region of *E. coli*, mutations of which affect the rate of alkaline phosphatase synthesis, was constructed by Hfr × F⁻ crosses. Mutations causing three different phenotypes (previously reported as *phoRa*, *phoRb*, *phoRc* (GAREN and ECHOLS 1962a, b)) are clustered in three closely linked genetic loci. *PhoR* mutants of all three types, including the *phoRb* type not previously tested, are recessive to wild-type *phoR*+. In addition, *phoRa* and *phoRc* complement each other, while *phoRa* and *phoRb* do not. Our results support the hypothesis of MORRIS *et al.* (1974) that *phoRc* mutants represent a cistron (*phoB*) different from *phoR*.

ALKALINE phosphatase (AP) synthesis in *E. coli* is repressed during growth in excess phosphate (Pi) (TORRIANI 1958; HORIUCHI, HORIUCHI and MIZUNO 1959; TORRIANI 1960). Mutations at two loci separated from the structural gene *phoA* (map position = 9.5 min [TAYLOR and TROTTER 1972]) affect the synthesis of AP. One locus has been shown to consist of two cistrons (GAREN and OTSUJI 1964), *phoS* and *phoT* (Map position = 73.5 min [TAYLOR and TROTTER 1972]), which are involved in Pi transport and apparently affect AP synthesis indirectly (WILLSKY, BENNETT and MALMY 1973). Mutations at the other locus, *phoR* (map position = 9.6 min [TAYLOR and TROTTER 1972]) have been found with three phenotypes (GAREN and ECHOLS 1962a,b). The *phoRa* phenotype is constitutive, but not fully derepressible by phosphate starvation. The *phoRb* phenotype is constitutive and fully derepressible. The *phoRc* phenotype is not derepressible; very little AP is made even in limiting Pi (see Table 1).

It has been shown that some *phoR* mutations are recessive to wild type (ECHOLS *et al.* 1961). Therefore, a cytoplasmic factor is indicated. Some *phoR* mutations appear to be suppressed by an amber suppressor, GAREN and GAREN 1963) suggesting that the *phoR* gene product is a protein. Phenotypic analysis of a partially dominant mutation of *phoR* (*phoR723*) implied that its active product is a multimer (PRATT and GALLANT 1972). Recently BRACHA and YAGIL (1973), who independently isolated *phoRc* type mutations, found that the heterozygote *phoRa/phoRc* was both repressible and derepressible. They concluded that because the two mutations complement they belong to different cistrons.

TABLE 1

AP-specific activities

	Specific activity	
	Excess Pi	Limiting Pi
wild type	0.00–0.1	8.6
phoRa	6.0	7.5
phoRb	4.9	16.3
phoRc	0.00–0.01	0.02
phoS, phoT	10	12

The data represent typical values from our results and from ECHOLS *et al.* (1961).

In order to clarify the question of the number and role of the *phoR* cistron(s) controlling alkaline phosphatase synthesis, mapping of the three mutant types was undertaken and complementation and dominance tests were performed.

All the *phoRa* and *phoRb* mutations are referred to as *phoR* while the *phoRc* mutations are referred to as *phoB*, followed by the number of the strain in which they were first observed (for example, *phoR8* is the *phoR* mutation originally in strain C8 (ECHOLS *et al.* 1961)). For simplicity, all Hfr strains used in the mapping will be referred to as Hfr followed by the mutation name (for example, Hfr *phoR8*) and all F^- strains used in the mapping will be referred to as F^- followed by the mutation name (for example, F^-*phoR8* (Table 2)).

MATERIALS AND METHODS

Strains: All bacterial strains used were derivatives of *E. coli* K12. All *phoRa* and *phoRb* mutants were spontaneous (one-step) mutations (ECHOLS *et al.* 1961; GAREN and GAREN 1963) of the prototype Hfr K10, except for Hfr *phoR723*, which was constructed from strain B723

TABLE 2

Bacterial strains used

	Name	Genotype	Origin
Hfr:	K10	*thi*	
	phoR2,8,27,30, 33,34,35	*thi phoRa*	spontaneous mutants of K10: original name C2, C8, etc. (ECHOLS *et al.* 1961)
	phoR6,36	*thi phoRa amber*	spontaneous mutants of K10 (C6, C36 [GAREN and GAREN 1963])
	phoR3	*thi phoRb*	spontaneous mutant of K10 (C3 [ECHOLS *et al.* 1961])
	phoB2,9	*thi phoRc* (*phoS, T*)	mutagen: Hydroxylamine (original name H2, H9; *phoS* or *T* mutation C4)
	phoB3	*thi phoRc*	mutagen: Ethylmethane sulfonate (S3)
	phoB206	*thi phoRc*	mutagen: Nitrosoguanidine (G206)
	phoR723	*phoRa*	P1 transductant of K10 from B723
F^-:	P678	*str thr leu lacY supE*	
	B723	*phoRa purE thy*	mutagen: Nitrosoguanidine (PRATT and GALLANT 1972)
F′:	HD234	*lac*/F′*lac*+ *phoR*+	(PRATT and GALLANT 1972)

(PRATT and GALLANT 1972) by P1 transduction (LENNOX 1955). All *phoRc* mutants were isolated after treatment with mutagenic agents (Table 2).

Media: *Medium 121* (TORRIANI 1968) was routinely used as the synthetic medium. It has the following composition (g/liter): NaCl, 4.68; KCl, 1.5; NH_4Cl, 1.08; Na_2SO_4, 0.35; $MgCl_2$, 0.2; $CaCl_2$, 2.9×10^{-2}; $FeCl_3$, 5×10^{-4}; $ZnCl_2$, 2.7×10^{-4}; Tris (hydroxymethyl) aminomethane, 12.0. The pH was adjusted to 7.5 with HCl. K_2HPO_4, 8.3×10^{-4} M (excess Pi = XP) or 8.3×10^{-5} M (limiting Pi = LP); glucose, 2 mg/ml; amino acids, 100 μg/ml were added after sterilization as required. M63 medium (MILLER 1972) was used in one set of matings. For solid media, 12 g/liter of Special Noble agar (Difco) was added to 121 media. MacConkey's Agar (Difco) was routinely used to distinguish *lac*+ and *lac*- colonies and L broth and L agar (LENNOX 1955) were used as a rich medium.

Alkaline phosphatase assays: For assays of AP activity, each strain was grown in 121 medium containing excess Pi and then diluted 1/100 into the same medium (XP) and into limiting Pi (LP). The XP cultures were harvested after glucose limitation and the LP cultures were harvested 24 hours after Pi limitation. The optical densities (540 nm) of the harvested cultures were recorded and 1 ml was toluenized (1 ml culture + 0.05 ml toluene) at 37° for 20 minutes. Then, 0.3 ml of the toluenized suspension (or an appropriate dilution) was added to 2.7 ml of 1 M Tris (pH 8.2) containing 0.01 M NPP (para-nitrophenol phosphate). The rate of increase of optical density (410 nm) at 37° was then measured. One unit of AP activity is defined as that amount of AP which generates a rate of increase in optical density (410 nm) of 1 per minute. Specific activities are expressed as units of AP activity per ml of toluenized suspension divided by the optical density (540 nm) of the original culture.

Distinguishing alkaline phosphatase phenotype of colonies: Two methods were used to determine whether a colony was synthesizing AP. (1) Plates were sprayed with a solution of 15 mg/ml NPP in 1.0 M Tris (pH 8.0). Colonies synthesizing AP at a high level turn yellow within a few seconds with this treatment. (2) Plates were stained by alpha napthol phosphate and fast blue (MILLER 1972). Five mg of alpha-napthol phosphate (Sigma), dissolved in 0.1 ml dimethyl sulfoxide, was added to 7 ml of 0.12 M Tris (pH 8.0). Then 12 mg of fast blue (Sigma), in 0.2 ml dimethylsulfoxide, was added. The mixture was poured onto the surface of the plate. Colonies synthesizing AP turn blue in about 15 minutes or less; those colonies remaining white or pale yellow were scored as not having AP. This method, which is more sensitive than the spray technique, was used for scoring recombinants.

Mating experiments for mapping of phoR *region*: Phage-T6-resistant mutants (*tsx*) of the Hfr *phoR* strain were selected on L plates with 5×10^9 T6 particles per plate. To transfer the *phoR* mutations into an F- strain, these Hfr *tsx* were mated with the F- strain P678 (*str thr leu lacY tsx*+). T6-resistant, streptomycin-resistant recombinants were selected and scored for *phoR* (by the NPP spray). Among the *str phoR tsx* recombinants, those which were also *thr leu lacY* became the recipients in the mapping experiments.

In the mapping experiments, the series of Hfr *phoR* were mated with the series of F- *phoR*. After 60 minutes of mating at 37°, the cells were suspended in minimal soft agar containing 10^{10} T6 particles and plated on 121 lactose streptomycin with either excess or limiting Pi (depending on the phenotypes of the mutants being crossed). The selected recombinants were *str thr*+ *leu*+ *lac*+ *tsx*.

In the crosses of *phoRc* by *phoRa* or *phoRb*, the selection plates (which contained limiting Pi) were used for replicating onto minimal plates with excess Pi. In these crosses both plates were stained (with alpha-naphthol, fast blue) and wild-type recombinants were found by comparing the staining of each colony on excess and limiting Pi.

In crosses involving two *phoRc* mutants the blue-staining colonies on limiting Pi plates were wild type, while in crosses involving two *phoRa* or *phoRb* mutants wild-type colonies were non-staining (white or pale yellow) on excess Pi plates.

Dominance test of phoRb/phoR+: To obtain a heterozygote *phoRb*/F′ *phoR*+, an F′ *lac*+ *phoR*+ episome (from HD234) was introduced into F- *phoRb* (*str lacY phoR3*) by selection for *lac*+ streptomycin-resistant colonies. These recombinants were tested for AP constitutivity by the NPP spray technique.

Complementation experiments: To obtain a partial diploid homozygous for *phoRa*, an F′ *lac*+ *phoR*+ (from HD234) was introduced into F⁻ *phoRa* (*str lacY phoR8*). The Lac+ streptomycin-resistant recombinants were repressed for AP because *phoR*+ is dominant. In addition, they segregated Lac⁻ clones following a 5 second UV irradiation (45 cm from a germicide lamp), which demonstrated that they contained both the *lac*+ episome and the *lacY*. A culture of one of these F′ recombinants was plated to look for a *phoR8*/F′ *phoR8* homozygote. One clone was found which was *lacY*/F′ *lac*+ (phenotypically *Lac*+, segregated Lac⁻ clones following UV irradiation) and AP constitutive. Since *phoR*+ is dominant this clone must have undergone recombination so that its episome was *lac*+ *phoR8*. This strain (*lacY phoR8*/F′ *lac*+ *phoR8*) was used as a donor in the mating for the complementation test. The recipients were F⁻ *phoR* strains which had been made resistant to Valine (*Val*^{R}) by selection on minimal medium containing 300 μg/ml valine. Each parent was grown in M63 medium with appropriate additions. After 100 minutes at 37°, the mating was interrupted by dilution (1/100). The culture was plated (after 3 hours outgrowth) on plates selective for Lac+ Val^R. Clones were purified and those segregating Lac⁻ followed brief UV irradiation were used as the homozygous partial diploids (*lacY phoR*/F′ *lac*+ *phoR8*) for the complementation assays. The level of complementation was tested by measuring AP-specific activity in XP and in LP media (with appropriate supplements) as described.

RESULTS

In order to verify the classification of the mutants used in these experiments, the AP-specific activities of each Hfr, grown in limiting Pi (LP) and in excess Pi (XP), were determined. The results are reported in Table 3. On the basis of their AP-specific activities, the mutants can be classified into the three groups, *phoRa*, *phoRb*, *phoRc*, described by GAREN and ECHOLS (1962a, b).

TABLE 3

AP-specific activities of Hfr strains

Strain	Growth conditions: Excess Pi	Limiting Pi	Mutant type
phoR+	0.0–0.03	7.6	wild type
phoR2	6.0	6.4*	*phoRa*
phoR3	4.9	16.3	*phoRb*
phoR6	4.9	7.8	*phoRa* (amber)
phoR8	5.9	7.6	*phoRa*
phoR27	5.9	7.9	*phoRa*
phoR30	5.3	7.5	*phoRa*
phoR33	5.3	8.3	*phoRa*
phoR34	6.1	8.4	*phoRa*
phoR35	6.5	7.4	*phoRa*
phoR36	5.7	6.6	*phoRa* (amber)
phoR723	3.9	6.1	*phoRa* (partially dominant)
phoB2	0.0–0.1	0.0–0.1	*phoRc*
phoB3	0.0–0.01	0.0–0.02	*phoRc*
phoB9	0.0–0.1	0.0–0.1	*phoRc*
phoB206	0.0–0.1	0.0–0.1	*phoRc*

Each Hfr strain was grown in triplicate in excess Pi (XP) and in limiting Pi (LP). Each of the three specific activities agreed to within 15% of the average of the three, except for Hfr *phoR2* in LP*, which agreed to within 25%, and those with specific activities of 0–0.1, where accurate comparison was not possible.

I. *Derivation of preliminary map of* phoR *region*

In order to derive a map of the *phoR* locus, F⁻ strains were constructed with each of nine mutations (*phoR2*, *phoR3*, *phoR6*, *phoR8*, *phoR36*, *phoB2*, *phoB3*, *phoB9*, and *phoB206*) as described in MATERIALS AND METHODS. To determine the order of and the distance between two mutations, Hfr × F⁻ crosses between each pair of mutants were done selecting for Str^R Thr⁺ Leu⁺ Lac⁺ $T6^R$ (multiple counterselection was employed against both parents to eliminate reversion and leakiness at *lac*). This selection requires at least one recombination event in the interval between *lac* and *tsx*, which includes the *phoR* region, and therefore amplifies the frequency of recombination between any two mutations in the *phoR* region that do not overlap. The selected recombinants were scored for wild type *phoR*⁺ recombinants with the alpha-napthol phosphate, fast blue staining system (as described in MATERIALS AND METHODS). Each pair of mutants was crossed in both orientations. For example, if the order of markers is *lac phoR8 phoR2 tsx*, cross Hfr *phoR2* × F⁻ *phoR8* should give more *phoR*⁺ recombinants than cross Hfr *phoR8* × F⁻ *phoR2* because a single recombination event between *lac* and *tsx* could produce a *lac*⁺ *phoR*⁺ *tsx* recombinant in the first cross (Figure 1). In the second cross at least three recombination events in the *lac–tsx* interval would be necessary. In this way, the order of each pair of mutations was determined with respect to *lac* and *tsx* and the relative distance was calculated to be the percent of *phoR*⁺ recombinants among the total selected recombinants in the "single exchange" cross.

The frequency of *phoR*⁺ recombinants in the self crosses (for example, Hfr *phoR2* × F⁻ *phoR2*) is less than 0.01% and is most likely due to misscoring of the stained colonies (this frequency is low enough so as not to influence interpretation of other crosses).

In each pair of reciprocal crosses done, the cross in one orientation gives *phoR*⁺ recombinants at a frequency from about 2- to 26-fold greater than in the other orientation. The cross with the higher percentage of *phoR*⁺ recombinants is taken to be the "single exchange" cross. The "triple exchange" crosses gave a significant frequency of *phoR*⁺ recombinants, presumably due to negative interference effects. From the recombination frequencies of the "single exchange" crosses, the map in Figure 2 was derived. The ordering of the positions of those mutations mapped is consistent for all crosses done. In those crosses which showed low recombination frequencies (crosses between *phoRc* mutants), the exact ordering

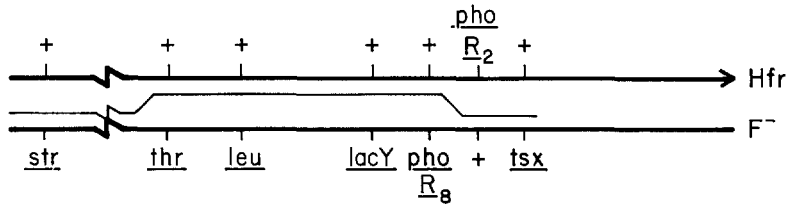

FIGURE 1.—A typical mapping cross. Two *phoRa* mutants are presented as an example: Hfr *phoR2* × F⁻ *phoR8*. Selected recombinants are *str thr*⁺ *leu*⁺ *lac*⁺ *tsx*. These are then scored for *phoR*⁺ as described.

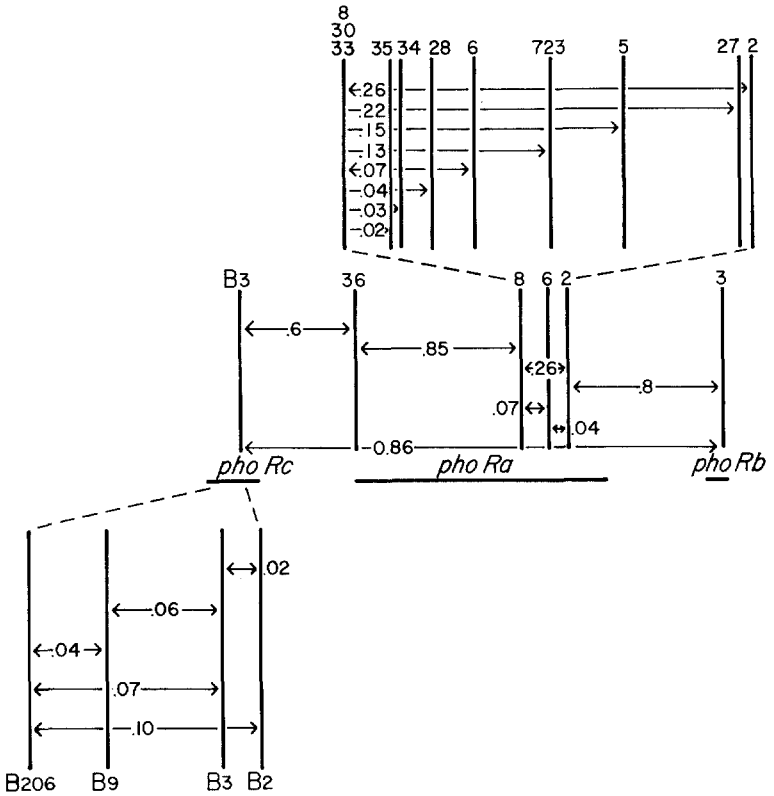

FIGURE 2.—Map of the *phoR–phoB* region. The principal map is located in the center. At the lower left is an enlarged view of the *phoB* cistron (containing the *phoRc* mutations). The upper part is an enlarged view of the *phoR8–proR2* portion (*phoRa*) of the *phoR* cistron. For each cross 3–6 × 10^4 selected recombinants were scored for wild-type *phoR*$^+$.

may not be correct. However, the low recombination frequencies are sufficient to indicate that the *phoRc* mutations are very closely linked.

The position of the amber mutation *phoR36* on the map is probably correct, but *phoR36* gives an unusually large number of apparent *phoR*$^+$ recombinants when crossed with other *phoR mutations*. It does not, however, have an unusually high recombination frequency with *lac* (data not shown). The phenotype of *phoR36* in the P678 background (F$^-$*supE*) is a low level of AP synthesis in both high and low phosphate. It seems likely that the low constitutive AP phenotype causes misscoring of F$^-$*phoR36* recombinants as *phoR*$^+$ on XP plates. The misscoring would increase the apparent recombination frequency between *phoR36* and other *phoR* mutations.

The map (Figure 2), together with the phenotype classification (Table 3), reveals that for each phenotype there is a distinct genetic locus.

II. *Further mapping of the* phoR *region*

Six other *phoRa* mutations were mapped to see if they are located in the locus assigned to *phoRa.*

To map these mutations (*phoR27, phoR30, phoR33, phoR34, phoR35, phoR723*) each Hfr *phoR* was crossed with F⁻ *phoR8*. By comparing wild-type recombination frequencies in these crosses to those in the crosses previously done, it was possible to locate each mutation approximately. Each of these Hfr *phoR* strains gave wild-type recombinants at a frequency less than that given by Hfr *phoR2* when crosses with F⁻ *phoR8*. It can thus be concluded that all six mutations are within the locus assigned to the *phoRa* mutations. A map of their approximate positions is shown in Figure 2 (enlarged view of the *phoR8–phoR2* region).

III. *Dominance test of* phoRb

The *phoRb* mutation *phoR3* was tested to see if it was dominant or recessive to wild-type *phoR*⁺. An F′ *lac*⁺ *phoR*⁺ episome was introduced into F⁻ *phoR3*. Of 125 *lac*⁺ recombinants, 124 were repressed for AP synthesis (white or non-staining with the NPP spray). The one colony which was constitutive (yellow staining) could have been a *lac*⁺ revertant of F⁻ *phoR3* (reversion occurred with a frequency about 10^4 less than recombination), or more likely, a homozygous recombinant, that is, *lacY phoR3*/F′ *lac*⁺ *phoR3* (which could come about by transfer of the F′ *lac*⁺ *phoR*⁺ episome, followed by homozygosis for *phoR3*). Analogous results were obtained with *phoRa* (ECHOLS *et al.* 1961) and *phoRc* (GAREN and ECHOLS 1962b). It can now be concluded that there are recessive *phoR* mutations of all three phenotypes and that the product(s) of the cistron(s) are cytoplasmic.

IV. *Complementation experiments*

Complementation tests among the three mutant types were done using heterozygous partial diploids. The following strains were constructed as described: *lacY phoR3*/F′ *lac*⁺ *phoR8* and *lacY phoB3*/F′ *lac*⁺ *phoR8*. These heterozygous partial diploids were assayed for AP in LP and in XP and tested for heterozygosity of *lac*. Each clone reported was heterozygous for *lac*. The results of the assays (and the control assays on the parental strains) are presented in Table 4.

TABLE 4

Complementation tests of phoR *mutants*

Strain genotype			Specific activity	
Chromosome	Episome	Clone	Excess Pi	Limiting Pi
phoR⁺ (P678)	—	–	<0.1	3.7
phoR3	—	–	3.4	14.7
phoR8	—	–	2.5	7.6
phoB3	—	–	<0.1	<0.1
phoR8	*phoR8*	–	8.2	26.1
phoB3	*phoR8*	a	<0.1	18.0
phoB3	*phoR8*	b	<0.1	16.7
phoB3	*phoR8*	c	<0.1	10.2
phoB3	*phoR8*	d	0.1	17.9
phoR3	*phoR8*	–	6.2	5.7

In the heterozygotes *phoB3*/F′ *phoR8*, complementation is evident both for derepression in LP (specific activities greater than wild-type P678 and for repression in XP (specific activities of the same low level as P678). It is expected that the derepressed level of AP in the partial diploid is higher than in P678 because of the extra copies of the AP structural genes (*phoA*) present in the F′ episome. In the heterozygote *phoR3*/F′ *phoR8*, there is no evidence of complementation acting at the level of repression of AP synthesis. There appears to be some negative complementation acting at the level of induction of AP synthesis in these heterozygotes (as evidenced by the low specific activities in LP as compared to all other partial diploid strains).

DISCUSSION

Fifteen *phoR* mutations, including ten *phoRa*, one *phoRb*, and four *phoRc*, were mapped and found to be clustered in three genetically distinct loci. These mutations could represent as many as three cistrons. But the distance between the two extreme mutations (*phoB206* and *phoR3*) is only about 1–2% of the *lac* to *tsx* interval. On the assumptions that the *lac* to *tsx* distance is 0.8 minutes (TAYLOR and TROTTER 1972), that there are about 3000 average-sized cistrons in the *E. coli* genome (JACOB and WOLLMAN 1961), and that the frequency of recombination in the *lac* to *tsx* interval is constant, the size of the map shown in Figure 2 corresponds to about one cistron. This, of course, does not exclude the possibility of two or three cistrons. Other results, in fact, support the possibility that there are two cistrons rather than three or one.

All of the *phoRa* mutations map very close to each other, except for *phoR36*, for which the distances are enlarged by misscoring as discussed in RESULTS. The only mutant of the *phoRb* type (*phoR3*) maps some distance away from the *phoRa* cluster on the *tsx* side. *PhoR3* probably belongs to the same cistron as the *phoRa* mutations. Like *phoRa*, the *phoRb* mutation is recessive to wild-type *phoR*$^+$, indicating that its gene product is cytoplasmic. In a complementation test between the *phoRb* mutation *phoR3* and a mutation of the *phoRa* type (*phoR8*) positive complementation producing AP repression did not occur. In fact, there appeared to be some negative complementation between these two mutations. Since the product of *phoRa* is presumed to be a multimeric protein (PRATT and GALLANT 1972) because a partially dominant mutation (*phoR723*) maps in the *phoRa* locus, the negative complementation between the *phoRa* and *phoRb* mutations may be the result of aggregation of subunits defective at different positions in the protein. Thus, it is very likely that *phoRa* and *phoRb* are mutations of the same cistron, which should be called *phoR*.

All the *phoRa* mutants have a derepressed level of alkaline phosphatase that is lower than either *phoS*, *T* or *phoRb*. Thus, the *phoR* gene product appears to be required for full derepression of alkaline phosphatase synthesis as well as for its repression. The effect of *phoRa* upon derepression is also evident in the partial diploid *phoRb*/F′ *phoRa* (*phoR3*/F′ *phoR8*), where the derepressed level of AP was observed to be 3–4 times lower than expected. This effect is presumably independent of the effect of *phoRc* mutations upon derepression.

The *phoRc* mutations are clustered apart from the other *phoR* mutations. Positive complementation was observed in a heterozygous partial diploid between a *phoRc* mutation (*phoB3*) and a *phoRa* mutation (*phoR8*). This result, together with the mapping data, suggests, but does not prove, that *phoRc* mutations do not belong to the *phoR* cistron. Since *phoRc* mutations are recessive and fail to derepress AP synthesis, it is likely that the product of the *phoRc* cistron is necessary for AP synthesis. It is therefore reasonable to conclude that the *phoRc* mutations are clustered in a separate cistron. We believe this cistron to be the same one described by MORRIS *et al.* (1974), and think that it should henceforth be referred to as *phoB*, following their suggestion. The *phoB* product acts either as an inducer for the transcription of *phoA* or is essential for the activation of an inducer.

It appears that intracellular Pi is not directly involved in regulation of AP synthesis. Instead, it is likely that some nucleotide (possibly an adenine derivative) is the co-inducer for AP synthesis (WILKINS 1972). Growth in limiting Pi would presumably cause a build-up of the nucleotide co-inducer. Therefore, some interaction among the active gene products of *phoR* and *phoB* and the nucleotide pool of the cell causes full induction of AP synthesis. Repression of AP synthesis is accomplished by the active gene product of *phoR*, possibly with the influence of a co-repressor. Recent experiments suggest that three other periplasmic proteins are coordinately controlled with AP (MORRIS *et al.* 1974). The elucidation of the nature of these interactions, and therefore of the molecular mechanism of control of AP synthesis in *E. coli*, awaits further experimentation.

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