THE GENETICS OF ALKALINE PHOSPHATASE FORMATION IN *BACILLUS SUBTILIS*

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THE alkaline phosphatase forming system in *Escherichia coli* has been studied extensively by HORIUCHI, HORIUCHI and MIZUNO (1959), TORRIANI (1960), GAREN and LEVINTHAL (1960) and many other workers. Especially from the work of GAREN and his colleagues (TORRIANI and ROTHMAN 1961; ECHOLS, GAREN, GAREN and TORRIANI 1961; GAREN and ECHOLS 1962a,b; GAREN and GAREN 1963), it has become evident that synthesis of the enzyme is controlled by a structural gene and regulator genes, as well as by the concentration of inorganic phosphate.

The present work was undertaken to investigate the phosphatase forming system in *Bacillus subtilis*, using a strain which allowed genetic analysis. Two phosphatases have been studied: alkaline phosphatase and phosphodiesterase. The genetics of alkaline phosphatase formation is described in this paper.

MATERIALS AND METHODS

Strains: A sulfanilamide resistant strain (*Sar*), a tryptophanless mutant (*try* 160) and an arginineless mutant (*arg* 15) of *Bacillus subtilis* Marburg were used. Mutant *arg* 15 was derived from *try* 160, and the *try* was then transformed to *try*⁺.

Media: The medium described by HORIUCHI, HORIUCHI and MIZUNO (1959) was modified for use. Low phosphate medium (LP medium) consisted of ammonium acetate, 3.0 g; MgSO₄ · 7H₂O, 0.25 g; Ca acetate, 0.02 g; MnCl₂, 0.072 mg; sodium lactate (50% solution) 4.0 g; Bacto-peptone (Difco), 10.0 g; L-arginine 0.2 g; L-tryptophan, 0.1 g (only for the *try* mutant); *Tris* (hydroxymethyl)amino methane (pH 6.9) and water, 1 liter. This medium contained 0.18 mM phosphate. High phosphate medium (HP medium) consisted of the components of LP medium and 1.6 mM inorganic phosphate. In addition, the following minimal medium (MM) was used for detecting *arg*⁺ transformants: K₂HPO₄, 7.0 g; KH₂PO₄, 3.0 g; sodium citrate, 0.5 g; (NH₄)₂SO₄, 1.0 g; MgSO₄ · 7H₂O, 0.2 g; glucose, 5 g; water, 1 liter.

Assay of alkaline phosphatase activity: 0.05 ml of 0.02 M Ca acetate and 0.75 ml of *p*-nitrophenyl phosphate (NPP) (5 mg in one ml of 1.0 M *Tris* buffer, pH 9) were added to 0.05 ml of the toluene treated bacterial suspension (about 10⁹ cells in one ml) to incubate the mixture for appropriate period at 35°C. The reaction was stopped by the addition of 4.15 ml of 0.2 N NaOH, and after centrifugation, the optical density of the supernatant was measured at 410 mμ by a Shimadzu QR-50 type spectrophotometer. As the velocity of the reaction is constant, the unit of enzyme activity is expressed as μmoles of *p*-nitrophenol produced by 1 ml enzyme sample per hour.

Determination of alkaline phosphatase positive and negative colonies: A filter paper soaked with NPP was pressed on colonies on a plate. Those which gave yellow color instantly were

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judged as alkaline phosphatase positive and those which did not as negative. The reaction was very sharp.

Transformation experiment: The general procedure of the experiment was the same as described by SARTO and MIURA (1963). Alkaline phosphatase positive transformants were selected on LP agar plates.

RESULTS

Isolation of alkaline phosphatase negative mutants (P^-): Spores harvested from potato slant cultures of *arg* 15 were exposed to X rays and the survivors were plated onto LP agar medium. By using the NPP filter paper, the colonies which did not give yellow color were selected. Fifty-one alkaline phosphatase negative mutants were obtained. Among them, alkaline phosphatase production by ten P^- mutants is presented in Table 1.

Isolation of constitutive mutants (R^-): Spores of *try* 160 and *arg* 15 were exposed to ultraviolet light, X rays or gamma rays (Co^{60}) and the survivors which produced high activities of alkaline phosphatase on HP medium were selected

TABLE 1
Alkaline phosphatase activities of P^- and R^- mutants

Mutation	Strain	APase activity (units/mg dry wt.)	
		Low phosphate	High phosphate
Parental strain	160	220	0.85
P^-	V-11	0.91	1.03
	V-43	0.92	1.03
	Sp-22	1.14	1.15
	Al-1	0.83	1.13
	Sp-25	0.89	1.12
	Sg-3	0.71	1.20
	Sg-13	0.94	1.15
	V-12	0.62	1.01
	V-41	0.77	1.08
	V-13	0.74	1.13
	BC-17	198	2.40
	BC-16	289	1.91
	BC-22	242	138
	BC-14	314	191
R^-	BC-8	211	226
	BC-9	154	228
	BC-5	241	85.9
	BC-3	230	58.8
	BC-6	199	180
	BC-7	155	25.9
	BC-13	176	12.7

(see Table 1). Among the 30 mutants isolated, seven (atypical R^-) produced low activities of alkaline phosphatase on HP medium, but they gave yellow color when tested by the NPP filter paper method.

Dependence and independence of alkaline phosphatase production on concentration of inorganic phosphate: To know the correlation between the concentration of inorganic phosphate in media and the production of alkaline phosphatase, some representative strains were cultivated in media containing different concentrations of inorganic phosphate. The activities were assayed after 14 hours cultivation at 37°C. As the results in Figure 1 indicate, the parental strain of *B. subtilis* possesses high activity of alkaline phosphatase in low concentrations of inorganic phosphate, and low activity of alkaline phosphatase in high concentrations. The production of alkaline phosphatase by P^- mutants (Sp-25 and V-12) is quite low both in low and high concentrations. Typical R^- mutants (BC-6 and BC-9) are able to produce high activities of alkaline phosphatase both in low and high concentrations. The production by an atypical R^- mutant (BC-16) depends greatly on the concentration of inorganic phosphate, but is not so sensitive as the production by wild type. Therefore, we grouped it among the R^- mutants tentatively. The mutant BC-17 exhibited a similar character to BC-16.

Alkaline phosphatase produced by the parental strain: The attempt to prepare a pure sample of alkaline phosphatase from cells of strain *try* 160 is still unsuccessful. This may be because of its close binding with the membrane fraction. It appears, however, that alkaline phosphatase is a single protein, since the enzyme activity is lost as a single mutational event and the cell lysate gives a single band (to the anode side, 14 cm) when tested by zone electrophoresis using the method of SMITHIES (1955) (pH 8.8, 230 volt, 10.4 ma, 15 hours).

Mutual crosses between P^- mutants: Prior to the transformation experiment, P^-arg^+ strains were produced by transformation from representatives of P^-arg

FIGURE 1.—Production of alkaline phosphatase by representative strains in media containing different concentrations of inorganic phosphate. The values of Sp-25 and V-12 are the proportion of alkaline phosphatase produced by Sp-25 and V-12 to that by 160 in a medium containing 0.18 μ mole phosphate per ml.

strains (V-11, Al-1, Sg-3, Sg-13, V-12 and V-41) with P^+arg^+ (wild-type) DNA. No joint transformant was obtained; i.e., the *arg* locus is not closely linked to the *P* loci. Next, P^-arg mutants were treated with P^-arg^+ -type DNAs and P^+ and arg^+ transformants were scored. In parallel, a DNA prepared from a wild-type strain (*Sar*) was given to the same recipients to determine the P^+/arg^+ ratio. This work was used specifically to estimate the efficiency of transfer of P^+ to different P^- recipient strains.

The relative distance between the two P^- mutational sites was calculated by the equation

$$\text{Recombination index} = \frac{P^+/arg^+ \text{ (mutant DNA)}}{P^+/arg^+ \text{ (wild-type DNA)}} \quad (1)$$

(LACKS and HOTCHKISS 1960). In the equation, the P^+/arg^+ (mutant DNA) represents the ratio of P^+ to arg^+ recombinants obtained from a cross between P^-arg^+ donors and P^-arg recipients, and the P^+/arg^+ (wild-type DNA) signifies the ratio of P^+ to arg^+ obtained from the cross between P^+arg^+ (wild-type) donors and P^-arg recipients.

The values given in Table 2 are the average of the recombination indexes calculated from transformation experiments repeated twice or three times. In general, the recombination index is expected to be low if mutational sites of the two strains are located close together on the DNA molecule and to be high if the two sites are located apart. On this criterion, the *P* sites are classified into two groups. One is a cluster consisting of Sg-3, Sg-13, V-12, V-41 and V-13 which are very closely linked to each other, and the recombination index between the most distant mutants is thought to be about 0.03. This cluster will be referred as region *P*-II. Another region (*P*-I) is a cluster consisting of V-11, V-43, Sp-22, Al-1 and Sp-25, which are not so closely linked with each other. When these mutants were plated on LP agar plates, residual growth was observed in contrast to the mutants of region *P*-II.

Crosses between R^- and P^- mutants: DNAs prepared from the wild-type strain ($P^+R^+arg^+$) and from constitutive mutants ($P^+R^-arg^+$) were used to transform

TABLE 2
Reciprocal crosses of P^- mutants

Mutation	Donor	Recipients									
		<i>P</i> -I					<i>P</i> -II				
		V-11	V-43	Sp-22	Al-1	Sp-25	Sg-3	Sg-13	V-12	V-41	V-13
<i>P</i> -I	V-11	0	0	30	25	27	39	40	50	53	59
	Al-1	11	18	0	0	7	32	36	43	47	..
<i>P</i> -II	Sg-3	72	33	53	35	29	0	9	0	0	..
	Sg-13	49	41	18	42	31	0	0	0	0	3
	V-12	54	27	49	42	27	0	0	0	0	3
	V-41	75	42	46	43	29	0	0	0	0	2

Values in the body of the table are recombination index $\times 100$.
.. : not examined.

alkaline phosphatase negative mutants (P^-R^+arg). P^+ and arg^+ transformants were scored. Among the P^+ transformants, about 100 were transferred to HP agar plates to test whether they were repressible with regard to the alkaline phosphatase production (HP-plate test). The number of P^+R^+ type was calculated as

$$P^+R^+ = P^+ \times \frac{P^+R^+}{P^+R^+ + P^+R^-} \quad (2)$$

where P^+ is number of P^+ transformants actually scored, and $P^+R^+/(P^+R^+ + P^+R^-)$ is the frequency of P^+R^+ determined by the HP-plate test. Table 3 presents the actual numbers of arg^+ and P^+ transformants and the frequencies of R^+ types among the P^+ types for mutant V-13.

TABLE 3
Transformation experiments with mutant V-13

Recipient strain	Donor DNA	arg^{+*}	P^{+*}	P^+R^+	P^+R^+/arg^+	Recombination index
				$\frac{P^+R^+}{P^+R^+ + P^+R^-}$		
V-13	Sar	204	307	100/100	1.50	...
	BC-22	1010	642	5/100	0.032	0.02
	BC-14	1950	535	7/100	0.019	0.01
	BC-8	670	154	8/100	0.018	0.01
	BC-17	1377	1513	0/100	0.00	0.00
	BC-16	1230	624	16/200	0.041	0.03
	BC-9	1520	133	83/100	0.073	0.05
	BC-5	1110	873	11/100	0.086	0.06
	BC-3	1709	1126	16/100	0.106	0.07
	BC-6	883	1077	99/100	...	...
	BC-7	1500	2251	100/100	...	...
	BC-13	206	272	104/104	...	...

* Values represent transformants/ml $\times 10^{-2}$.

TABLE 4
Crosses between P^- and R^- mutants

Mutation	Donor	Recipients					
		$P-I$			$P-II$		
		V-43	Al-1	Sp-25	V-12	V-41	V-13
R^-	BC-22	25	20	21	1	1	2
	BC-14	25	20	20	2	2	1
	BC-8	29	25	24	5	3	1
	BC-17	20	16	15	6	5	0
	BC-16	16	15	7	4	6	3
	BC-9	26	31	49	20	7	5
	BC-5	29	11	22	4	7	6
	BC-3	20	16	18	8	8	7
	BC-6*	100	98	100	100	100	99
	BC-7*	100	100	100	100	100	100
	BC-13*	100	100	100	100	96	100

Values in the body of the table are recombination index $\times 100$, except for the last three lines(*), where the frequencies of P^+R^+ ($= \frac{P^+R^+}{P^+R^+ + P^+R^-} \times 100$) are shown instead of the recombination index to indicate relative distance.

Results obtained with other strains are shown in Table 4. The recombination index (LACKS and HOTCHKISS 1960) was calculated with use of the formula:

$$\text{Recombination index} = \frac{P^+R^+/arg^+ \text{ (mutant DNA)}}{P^+R^+/arg^+ \text{ (wild type DNA)}} \quad (3)$$

The P^+R^+/arg^+ (wild-type DNA) is the ratio of P^+R^+ to arg^+ obtained from a cross between $P^+R^-arg^+$ donors and P^-R^+arg recipients. But, when BC-6, P^+R^+/arg^+ (mutant DNA) represents the ratio of P^+R^+ to arg^+ obtained from a cross between $P^+R^-arg^+$ donors and $P^-R^+arg^-$ recipients. But, when BC-6, BC-7 and BC-13 were used as DNA donors, almost all (more than 96%) P^+ type transformants were repressible (P^+R^+), i.e., the P locus is not linked to R loci. For these cases, the frequencies of P^+R^+ ($= P^+R^+/(P^+R^+ + P^+R^-)$) determined by the HP-plate test are shown in the table instead of the recombination index.

As seen in Table 4, where the recombination index is adopted as an indicator of relative distances, eight out of the 11 R^- mutants are closely linked or overlap with region P -II. Three other R^- mutants, BC-6, BC-7 and BC-13, show no linkage relationship with either region P -I or region P -II, suggesting that these mutations occurred on different DNA moieties. It is not yet known whether these three mutational sites are located on the same DNA molecule or not. The region which is closely linked or overlaps with region P -II will be referred as region R -I, and the mutants which are not linked with region P -I and P -II will be designated group R -II mutants.

Reverse mutation at the region P-II and region R-I: The mutants belonging to region P -II gave constitutive strains spontaneously, and the mutants belonging to region R -I gave negative strains (Table 5), but the mutants of region P -I gave no constitutive strain.

DISCUSSION

Figure 2 presents a tentative map of the linkage group of the P^- and R^- mutations. The alkaline phosphatase negative mutants have been classified into two groups, region P -I and region P -II. The P^- mutants were mapped using either Sp-25 or V-13 as recipients. The intracluster distance in region P -I is estimated to be about 0.3 as recombination index, the intracluster distance in region P -II, 0.03, and the intercluster distance between region P -I and P -II, about 0.3. Data of the crosses between P^- and R^- mutants, and of reverse mutations, suggest that region P -II may be identical with region R -I. R -II mutations are linked with neither P -I, P -II nor R -I, but evidence is still poor that the R -II mutants are located on a single segment of DNA.

It has been reported that the formation of the alkaline phosphatase in *E. coli* is under the genetic control of the three genes. One of these, the P gene, specifies the structure of the enzyme molecule. The other two genes, $R1$ and $R2$, regulate the rate of formation of the enzyme (ECHOLS, GAREN, GAREN and TORRIANI 1961; GAREN and ECHOLS 1962a,b). The alkaline phosphatase forming system in *B. subtilis* seems to resemble that of *E. coli*, but the authors have no evidence yet that P -I is actually a structural gene.

TABLE 5

Alkaline phosphatase activities of reverse mutants

Parental mutation	Revertants	APase activity (units/mg dry wt.)	
		Low phosphate	High phosphate
<i>P</i> -II-	V-13-Rev-8	119	1.6
	V-41-Rev-10	219	1.8
	V-12-Rev-10	266	1.9
	V-12-Rev-6	193	2.8
	V-13-Rev-4	246	2.8
	V-12-Rev-7	213	3.3
	V-41-Rev-6	101	12.7
	V-41-Rev-8	278	16.1
	V-13-Rev-11	225	31.4
	V-13-Rev-12	104	139
	V-41-Rev-7	467	217
<i>R</i> -I-	BC-5-Rev-1	1.8	1.3
	BC-14-Rev-1	2.2	1.3
	BC-9-Rev-3	2.2	1.6
	BC-14-Rev-7	2.8	1.3
	BC-9-Rev-2	3.7	1.8
	BC-16-Rev-2	16.0	1.6
	BC-5-Rev-2	16.5	1.7
	BC-16-Rev-1	98.9	1.8
	BC-8-Rev-2	173	1.2
	BC-14-Rev-3	342	1.5
	BC-22-Rev-4	342	1.6
	BC-14-Rev-6	359	1.5
	BC-14-Rev-4	360	1.7

FIGURE 2.—A tentative map of the linkage group of the *P*⁻ and *R*⁻ mutations. It is not yet known whether mutational sites of BC-6, BC-7 and BC-13 are located on the same DNA molecule or not. The values are given in recombination index $\times 100$.

SUMMARY

Alkaline phosphatase negative and constitutive mutants were produced from the Marburg strain of *Bacillus subtilis*. They were crossed with each other by the transformation technique, and the sites of mutation were determined. The negative mutants were classified into two groups, falling in regions *P*-I and *P*-II. Both regions appeared to reside on the same DNA moiety. The constitutive mutants were also classified into two groups, region *R*-I mutants and group *R*-II mutants. Region *R*-I may be identical with region *P*-II. No linkage was found between the group *R*-II mutants and the *P*-I, *P*-II or *R*-I mutants. These observations indicate that the alkaline phosphatase forming system in *B. subtilis* is comparable to that in *E. coli*.

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