JOURNAL OF BACTERIOLOGY, Feb. 1973, p. 727-738

Vol. 113, No. 2
Printed in U.S.A.

Isolation and Characterization of Acid Phosphatase Mutants in *Saccharomyces cerevisiae*

AKIO TOH-E, YOSHINAMI UEDA, SEI-ICHIRO KAKIMOTO, AND YASUJI OSHIMA
Department of Fermentation Technology, Osaka University, Suita-shi, Osaka, Japan

Received for publication 31 October 1972

Saccharomyces cerevisiae strain H-42 seems to have two kinds of acid phosphatase: one which is constitutive and one which is repressible by inorganic phosphate. The constitutive enzyme was significantly unstable to heat inactivation, and its K_m of 9.1×10^{-4} M for *p*-nitrophenylphosphate was higher than that of the repressible enzyme (2.4×10^{-4} M). The constitutive and the repressible acid phosphatases are specified by the *phoC* gene and by the *phoB*, *phoD*, or *phoE* gene, respectively. Results of tetrad analysis suggested that the *phoC* and *phoE* genes are linked to the *lys2* locus on chromosome II. Since both repressible acid and alkaline phosphatases were affected simultaneously in the *phoR*, *phoD*, and *phoS* mutants, it was concluded that these enzymes were under the same regulatory mechanism or that they shared a common polypeptide. The *phoR* mutant produced acid phosphatase constitutively, and the *phoR* mutant allele was recessive to its wild-type counterpart. The *phoS* mutant showed a phenotype similar to that of a mutant defective in one of the *phoB*, *phoD*, or *phoE* genes. However, the results of genetic analysis of the *phoS* mutant clearly indicated that the *phoS* gene is not a structural gene for either of the repressible acid and alkaline phosphatases, but is a kind of regulatory gene. According to the proposed model, the *phoS* gene controls the expression of the *phoR* gene, and inorganic phosphate would act primarily as an inducer for the formation of the *phoR* product which represses phosphatase synthesis.

The existence of repressible phosphatases (EC 3.1.3.1 and EC 3.1.3.2) in *Escherichia coli* (10), *Saccharomyces cerevisiae* (16, 19), *Neurospora crassa* (13, 14), and *Euglena gracilis* (2) has been reported. Among these, the regulatory mechanism of alkaline phosphatase formation in *E. coli* was most extensively studied by Garen and his colleagues. They indicated that the enzyme formation was controlled by the structural gene and two regulator genes, R1 and R2, as well as by inorganic phosphate (6, 7). However, the regulatory mechanism of phosphatase formation has not been clearly elucidated in eukaryotic microorganisms because of the lack of an efficient method for the isolation of phosphatase mutants. Recently, Toh-e and Ishikawa (20), however, reported the genetic analysis of acid and alkaline phosphatase formation in *N. crassa* by developing an efficient procedure for the isolation of nuclease mutants, based on the ability to utilize nucleic acid as a sole source of phosphate, and for the isolation of phosphatase mutants by a diazo coupling method which has been described by Dorn (4). They suggested that one of the nucleases in the mycelial extract, i.e., nuclease N_3, might be an inducing factor for the production of repressible phosphatases in *N. crassa* (20).

Schurr and Yagil (17), using the diazo coupling method, recently isolated and characterized mutants of *S. cerevisiae* which lack the repressible acid phosphatase. They observed that these mutants were also defective in the repressible alkaline phosphatase and that constitutive mutants could produce both enzymes in media containing enough phosphate for the repression of both enzymes in the wild-type strain. They suggested that the formation of both enzymes might be controlled by a common regulatory mechanism or that these two enzymes might share a common polypeptide.

We have isolated many strains of the *S. cerevisiae* mutant for acid phosphatase by the

diazo coupling method, and genetic and biochemical analyses of these mutants have been carried out. In the present paper, we report that there seem to be two kinds of acid phosphatases, constitutive and repressible, in the wild-type strain of *S. cerevisiae*, and we suggest the possibility of a new regulatory gene which might control the expression of the regulator gene for repressible acid phosphatase synthesis.

MATERIALS AND METHODS

Organisms. A heterothallic haploid strain, H-42 (*a gal4*), which was obtained from a diploid strain of *saké* yeast, *S. cerevisiae* strain KM-46 (15), by random spore isolation, was used as the wild-type strain for acid phosphatase formation. All phosphatase mutants were derived from H-42 or its derivatives. Another haploid strain, T-1074-77D (*α lys2 gal1*), a mutant for *phoC*, was selected from our breeding stocks for yeast genetics. The gene symbols *lys* and *gal* indicate the requirement for L-lysine and the inability to ferment galactose, respectively.

Media. The nutrient medium contained 40 g of glucose, 10 g of polypeptone, 5 g of yeast extract, 5 g of KH_2PO_4, and 2 g of $MgSO_4 \cdot 7H_2O$ per liter of tap water. Synthetic minimal medium, in which 2 g of recrystallized asparagine per liter was the sole source of nitrogen, was prepared according to Burkholder (3). Phosphate-free medium was prepared by the substitution of 1.5 g of KCl per liter for 1.5 g of KH_2PO_4 per liter in the minimal medium. The original minimal medium was used as the high phosphate medium (high-Pi medium), and phosphate-free medium supplemented with 30 mg of KH_2PO_4 per liter was used as the low phosphate medium (low-Pi medium). If necessary, 0.2 mM L-lysine hydrochloride was added to these synthetic media. For the preparation of solid media, 20 g of agar per liter was added. The sporulation medium contained 5 g of anhydrous sodium acetate and 15 g of agar per liter of tap water.

Isolation of mutants. All mutants were selected by inspecting colonies in the petri dishes according to the method described by Dorn (4). Cells to be subjected to mutagenesis were grown in nutrient medium for 24 hr at 30 C without shaking. Then each culture broth, containing about 10^7 cells per ml, was diluted 100-fold with sterilized water and a 10-ml portion was transferred to a sterilized petri dish 9 cm in diameter. Mutagenesis was performed by ultraviolet irradiation for 80 sec with an 18-w Toshiba germicidal lamp at a distance of 15 cm with stirring by a magnetic stirrer. Under these conditions, 1 to 10% of the cells survived after being spread on a nutrient plate. Each 0.1-ml sample was spread on a plate containing high-Pi or low-Pi medium, according to the mutants to be isolated. After 3 to 5 days of incubation at 30 C, each colony which developed was overlaid with melted soft agar (50 C) containing a staining solution consisting of 5 mg of α-naphthylphosphate per ml, 50 mg of Fast blue salt B per ml, and 1% agar in 0.05 M acetate buffer (*p*H 4.0). In the presence of acid phosphatase activity, the colonies were stained to a dark red within 30 to 60 min at 30 C, whereas in its absence the colonies remained white. Since the cells were not killed by the staining, mutants could easily be isolated directly from this plate; if necessary, each isolate was purified by repeated spreading on a nutrient agar plate. For the diagnosis of phosphatase activity of the tetrad segregant, the same procedure was applied on the replicated plates. An adenine-requiring mutant such as *ade1* was not useful for the present study, because it produces colonies colored pink to dark red, and it seems to produce a substance which reacts with Fast blue salt B.

Methods for genetic analysis. Diploid hybrids were obtained by the mass mating method described by Lindegren and Lindegren (11). For the sporulation of diploid hybrids, after cultivation for 1 or 2 days in nutrient medium at 30 C, one loopful of cells was smeared on sporulation medium and incubated for another 2 days at 30 C. The cell walls of the asci were digested by treatment with snail gut juice, and four-spored asci were dissected with the aid of a micromanipulator.

Preparation of cell extract. For the extraction of acid phosphatase, the strains to be tested were grown in nutrient medium to full growth at 30 C without shaking. The cells were harvested, washed, and resuspended in the same volume of sterilized water; 1 ml of cell suspension was inoculated in a 500-ml Sakaguchi flask containing 100 ml of the indicated medium. Incubation was carried out at 30 C with shaking. Cells grown under the indicated conditions were harvested and washed with 0.01 M acetate buffer (*p*H 4.0). All subsequent preparations were carried out below 4 C. Cell concentration was measured turbidimetrically at 660 nm. A 4-ml amount of heavy cell suspension (optical density at 660 nm, approximately 100) was shaken with 20 g of glass beads (0.45 to 0.55 mm in diameter) for 15 sec by use of a Braun cell homogenizer (model MSK). To the mixture of the disrupted cell suspension and glass beads was added 4 ml of 0.01 M acetate buffer (*p*H 4.0), and the liquid layer was decanted and saved. This procedure was repeated three more times. Insoluble material was removed from the pooled washings by centrifugation at 17,000 × *g* for 20 min. The resultant supernatant fluid was used as an enzyme solution, if not otherwise noted. For the extraction of both acid and alkaline phosphatases, 5 mM tris(hydroxymethyl)-aminomethane-maleate (Tris-maleate) buffer (*p*H 6.5) was used instead of 0.01 M acetate buffer (*p*H 4.0) in the above procedure. Protein concentration was determined according to Lowry et al. (12), with bovine serum albumin as the standard.

Determination of phosphatase activity. Phosphatase activity was assayed according to the method described by Torriani (21). The reaction mixture (2 ml) for the acid phosphatase assay consisted of 0.64 mg of *p*-nitrophenylphosphate per ml and enzyme in 0.05 M acetate buffer (*p*H 4.0). The reaction was carried out at 35 C for 10 min; the reaction was stopped by the addition of the same volume of 10% trichloroacetic acid. Insoluble material was removed by centrifugation at 2,000 × *g* for 10 min. Then 2 ml of the supernatant fluid was added to 2 ml of a

saturated solution of Na_2CO_3. When intact cells were used as enzyme, cells were removed by centrifugation after the addition of a saturated solution of Na_2CO_3. Liberated *p*-nitrophenol was determined spectrophotometrically at 420 nm. Alkaline phosphatase activity was assayed by the same method described above, except that the reaction mixture contained 0.05 M Veronal buffer (*p*H 9.0) and 5 mM $MgSO_4 \cdot 7H_2O$ instead of 0.05 M acetate buffer (*p*H 4.0). One unit was defined as the amount of enzyme which liberates 1 μmole of *p*-nitrophenol per min under the conditions described above.

Chemicals. *p*-Nitrophenylphosphate was purchased from Wako Pure Chemical Industries, Ltd., and Fast blue salt B was from Merck & Co., Inc. Snail gut juice (suc d'helix pomatia stabilise standardisé) was obtained from the Industrie Biologique, Française.

RESULTS

Acid phosphatase species in S. cerevisiae. Schmidt et al. reported that the acid phosphatase in *S. cerevisiae* was derepressed in a medium in which the concentration of inorganic phosphate was kept low (16). The same relationship between acid phosphatase formation and the concentration of inorganic phosphate in the medium was observed in our wild-type strain, H-42. Strain H-42 was grown in a medium containing various amounts of inorganic phosphate at 30 C with shaking, and the time course of phosphatase activity was determined during the cultivation. Results indicated that the acid phosphatase activity increased as the concentration of inorganic phosphate was reduced, as shown in Fig. 1. Since both acid phosphatase formation (Fig. 1a) and cellular growth (Fig. 1b) in the medium containing 30 mg of KH_2PO_4 per liter were satisfactory, this medium was used as the low-Pi medium for giving a derepressed condition for phosphatase formation. In the low-Pi medium, the highest level of acid phosphatase activity was constantly maintained during 15 to 24 hr of cultivation. Strain H-42 also produced a small but significant amount of acid phosphatase in high-Pi medium (standard minimal medium containing 1.5 g of KH_2PO_4 per liter), and production was more efficient in the nutrient medium.

Several enzymatic characteristics of these acid phosphatase activities present in cells grown on nutrient medium or low-Pi medium were compared with crude extracts prepared from cells of the wild-type strain, H-42, grown in these media. Each extract was dialyzed against 0.01 M acetate buffer (*p*H 4.0) at 0 C overnight and used as the enzyme solution. It was evident that the acid phosphatase produced in the low-Pi medium was markedly resistant to heat inactivation at 55 C compared with that produced in the nutrient medium (Fig. 2). A kinetic study was also performed with *p*-nitrophenylphosphate as substrate. The

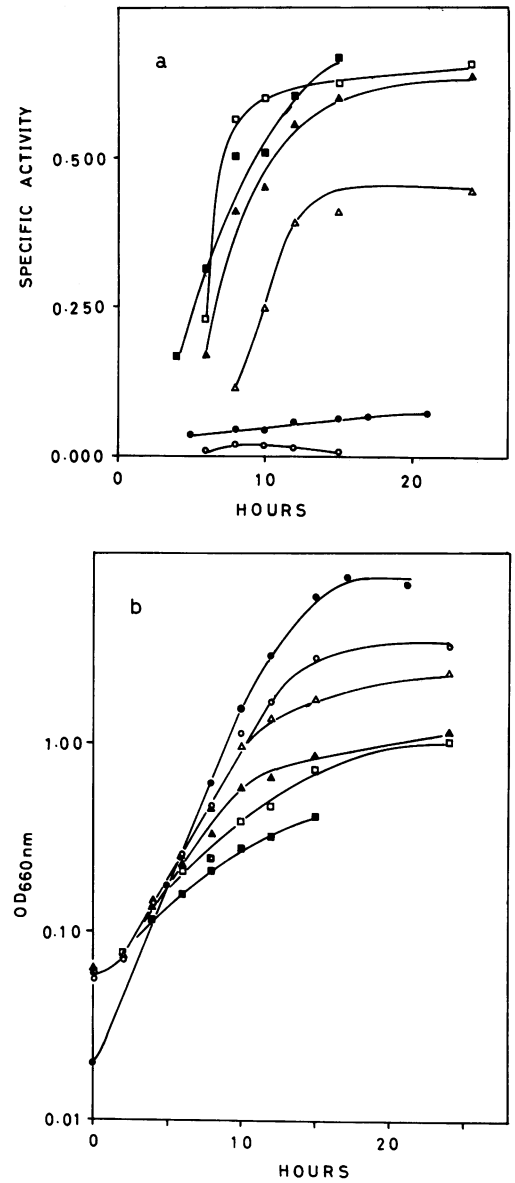

FIG. 1. *Effect of the concentration of inorganic phosphate on the formation of acid phosphatase. The wild-type strain, H-42, was grown in various media containing different amounts of inorganic phosphate at 30 C with shaking. Acid phosphatase activity was determined with a suspension of intact cells as the enzyme source. Specific activity was expressed as units per unit of optical density at 660 nm. (a) Time course of enzyme formation. (b) Time course of growth. Symbols: ○, 1,500 mg; △, 30 mg; ▲, 15 mg; □, 3 mg; ■, 0 mg of KH_2PO_4 per liter in Burkholder's synthetic minimal medium; ●, nutrient medium.*

K_m values were calculated from the Lineweaver-Burk plots shown in Fig. 3. It was found that the K_m of the acid phosphatase produced in the high-Pi medium was 9.1×10^{-4} M, whereas the acid phosphatase produced in low-Pi medium had a lower K_m of 2.4×10^{-4} M. The same K_m values for the respective acid phosphatases were also obtained when a suspension of intact cells was used as the enzyme source. These results tend to support the idea that acid phosphatases produced under conditions of limited or of sufficient amounts of inorganic phosphate in the medium were different molecular species and corresponded to the repressible and constitutive acid phosphatases, respectively.

It is evident that almost all of the activity of the acid phosphatase in the extract prepared from H-42 cells grown in low-Pi medium was due to the activity of the repressible acid phosphatase, because the K_m of the acid phosphatase produced in low-Pi medium by O8-M3, which were derived from H-42 by mutation and does not show acid phosphatase activity in cells grown in nutrient medium, showed the same value as that from H-42 grown in low-Pi medium. In addition, no significant difference was observed between these two enzymes with respect to the other enzymatic characteristics tested, such as optimal *p*H (3.5 ~ 4.0) and the effect of metal ions and ethylenediaminetetraacetate on their activity.

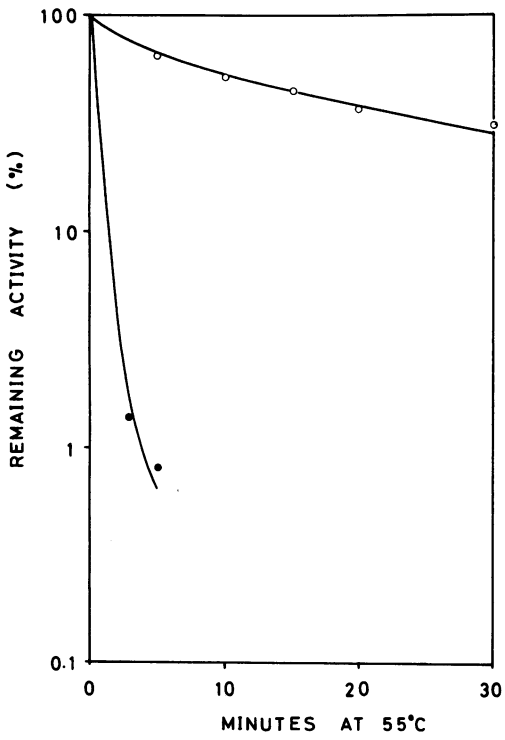

FIG. 2. *Effect of heat treatment on acid phosphatase activity. The wild-type strain, H-42, was grown to the early stationary phase in nutrient medium (●) and low-Pi medium (○) at 30 C with shaking. Extracts were prepared from these cells in 0.01 M acetate buffer (pH 4.0) and dialyzed against the same buffer at 0 C overnight. An extract containing about 1 unit of acid phosphatase per ml was preincubated at 55 C for various periods and then rapidly cooled in an ice bath, and the remaining activity was determined. Activity without preincubation was scored as 100% and the experimental values were expressed as percentages of this value.*

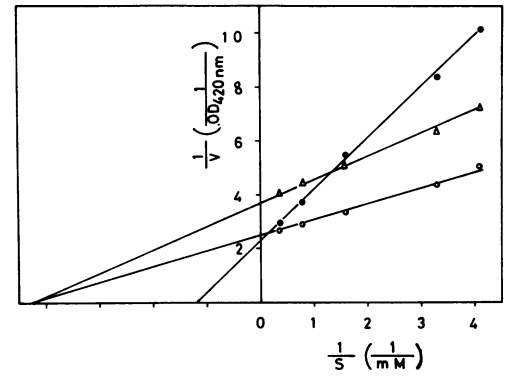

FIG. 3. *Lineweaver-Burk plots of acid phosphatase with p-nitrophenylphosphate as substrate. Extracts were prepared from cells of the wild-type strain, H-42, grown to the early stationary phase in nutrient medium (●) and low-Pi medium (○), and from cells of the mutant, O8-M3, grown in low-Pi medium (△). The velocity was expressed as the change in absorbance measured at 420 nm per 10 min of incubation.*

Isolation of mutants lacking constitutive acid phosphatase. Freshly cultivated cells of the strain H-42 were irradiated with ultraviolet light, plated on high-Pi medium, and incubated for 3 to 5 days at 30 C. The colonies which developed on the surface of each plate were stained by overlayering with the soft agar of the diazo coupling reagent. Colonies which were not stained were picked up. To test the formation of the constitutive acid phosphatase, the wild-type strain H-42 and mutant strains O19-M20, O19-M32, and a tetrad segregant from hybrid P-22 (i.e., P-22-14D, which has the same genotype for inability to produce constitutive acid phosphatase as O8-M3) were grown in the nutrient and low-Pi media. At appropriate intervals during the cultivation, the acid phosphatase activity of each strain was determined with a cell suspension as an enzyme source. These mutant clones showed no constitutive acid phosphatase activity in the

nutrient medium (Fig. 4), but they retained the repressible acid phosphatase activity. The maximal specific activities of the repressible acid phosphatase were, however, slightly different among these mutants. Another strain which does not have constitutive acid phosphatase, T-1074-77D (*α lys2 gal1*), was selected from our stock cultures for genetic study.

Two strains, O8-M3 and T-1074-77D, were found to lack the constitutive acid phosphatase and were subjected to genetic analysis. Each strain was crossed to the wild-type strain (H-42 or its derivative) which had the constitutive acid-phosphatase. The mutant present in O8-M3, designated *phoC-1*, had segregated 2:2 for constitutive versus absence of the constitutive acid phosphatase in all 76 tetrads tested. The diploid heterozygous for *phoC-1* produced the constitutive acid phosphatase. The allele *phoC-1* is, hence, recessive. Segregations for the mutation in T-1074-77D leading to the absence of the constitutive acid phosphatase, designated as *phoC-2*, were essentially 2:2 for the presence or absence of the constitutive acid phosphatase on high-Pi medium in 29 of 34 asci tested. However, one or two acid phosphatase-forming clones in each of five asci showed an ambiguous color development. The ambiguous color development might be attributable to a certain difference in genetic background between the parental strains and might not be due to an abnormal meiotic event, because it was observed that the *lys2* marker showed 2+:2− segregation in each ascus. When a segregant from this cross bearing *phoC-2* was backcrossed to H-42 (*PHOC*), the *phoC-2* allele again segregated 2:2 in a total of 16 asci. Diploids heterozygous for *phoC-2* also make acid phosphatase constitutively. Thus, the allele *phoC-2* is recessive.

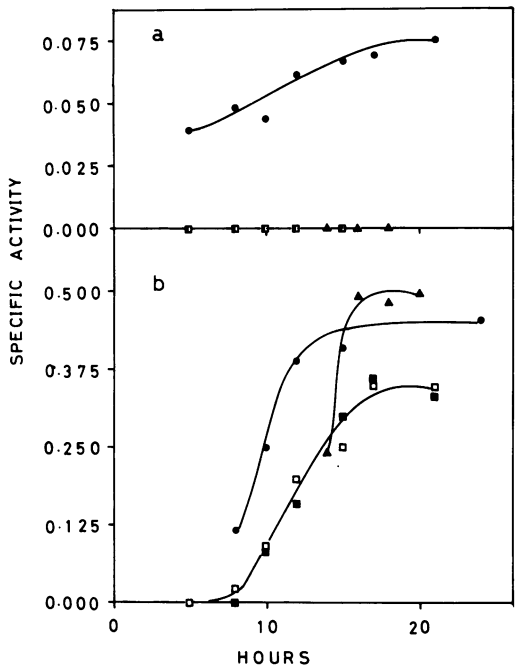

FIG. 4. *Comparison of the formation of acid phosphatase in the wild-type strain, H-42, and mutant strains. Each strain was grown in nutrient medium (a) and low-Pi medium (b). Acid phosphatase activity was determined with a suspension of intact cells as the enzyme source. Specific activity was expressed as units per unit of optical density at 660 nm. Symbols: ●, H-42; ▲, P-22-14D; ■, O19-M20; and □, O19-M32. P-22-14D has the phoC-1 genotype derived from the mutant O8-M3.*

To check the allelism of *phoC-1* and *phoC-2*, a cross was made to produce a *phoC-1/phoC-2* diploid. It did not produce acid phosphatase constitutively. No segregant of this diploid able to make acid phosphatase constitutively has occurred in the 11 asci so far examined. Thus, two mutations, *phoC-1* and *phoC-2*, have occurred in the same cistron. Additional mutant alleles at this locus have been obtained and characterized as above.

Tetrad distribution from the diploids heterozygous for *lys2* and *phoC-2* indicated that these two genes are linked. Pooled data on the numbers of parental ditype, nonparental ditype, and tetratype asci were 47, 0, and 9, respectively. These values indicate a map distance of 11 strains (18) between *lys2* and *phoC-2* on chromosome II (9).

Mutants lacking the activity of the repressible acid phosphatase. From the *a phoC-1* and *α phoC-1* strains, many mutants lacking repressible acid phosphatase activity were isolated on low-Pi medium. Several mutants were crossed with one of the original *phoC-1* strains according to their mating types. Each hybrid was sporulated and dissected, and it was observed that every ascus showed 2+:2− segregation with respect to the formation of the repressible acid phosphatase. From the segregants of each hybrid, both *a* and *α* mating type clones which lacked the repressible acid phosphatase activity were collected. By use of the complementation test, these mutants were divided into four groups. This experiment also indicated that each mutant gene is recessive to the corresponding wild-type gene, because the diploid hybrid between different classes of mutations was able to produce the repressible acid phosphatase. The number of mutants so far obtained is listed in Table 1, according to the results of the complementa-

TABLE 1. *Classification of mutants for the production of repressible acid phosphatase*

Genotype	Original *phoC-1* strain	No. of mutants isolated	Total no. of mutants obtained
phoB	O8-M3	1	4
	P-22-14D	3	
phoD	O8-M3	1	6
	P-22-14D	5	
phoE	O8-M3	1	61
	P-22-14D	60	
phoS[a]	O8-M3	1	16
	P-22-14D	15	

[a] The *phoS* mutants showed the same phenotype as other mutants lacking repressible acid phosphatase activity, but their genetic behavior was significantly different; *phoS* presumably works as a kind of regulatory gene, as discussed later in the text.

tion tests. These gene loci, controlling the formation of the repressible acid phosphatase, were designated as *phoB*, *phoD*, *phoE*, and *phoS*. Mutants belonging to *phoE* were most frequently isolated. Some of these mutant strains were tested for the formation of acid and alkaline phosphatases in cells cultivated in high-Pi and low-Pi media. The results shown in Table 2 clearly indicate that the acid phosphatase activity of all mutants was decreased several-fold below that of the parental strain. The activity of the alkaline phosphatase, however, did not show a significant difference between mutant and parental strains, except for the *phoD* and *phoS* mutants, in which the activities of both acid and alkaline phosphatases in low-Pi medium were significantly decreased simultaneously. Although mutants classified as *phoS* showed a phenotype similar to that of the *phoB*, *phoD*, and *phoE* mutants, it was found that the *phoS* gene had a certain regulatory function in phosphatase formation, as will be described later. It is noteworthy that when one of the *phoE* mutants, *a phoC-1 phoE*, was crossed with the *α PHOC PHOE* strain and the resulting diploid hybrid was subjected to tetrad analysis, all asci (20 asci) showed the parental ditype tetrad, with respect to the traits controlled by the *phoC* and *phoE* genes. This result indicates that the *phoE* locus is linked to the *phoC* locus on chromosome II. The *phoB*, *phoD*, and *phoS* genes segregated independently of one another, and also independently of *phoC*, *gal1*, *lys2*, and mating type.

To confirm that the constitutive acid phosphatase lacking in the *phoC* mutants could not be derepressed in low-Pi medium, a cross was made between the *a PHOC PHOD* (H-42) and the *α phoC phoD* (O25-M57) strains. The resulting diploid hybrid, P-61, was sporulated, and four-spored asci were dissected. Acid phosphatase formation was tested in the cells of each tetrad clone of the tetratype ascus cultivated in nutrient and low-Pi media. Since the amount of the acid phosphatase activity due to the production of the repressible enzyme is far greater than that from the constitutive enzyme in low-Pi medium, it was easy to classify each segregant, by use of the staining method, to determine whether the activity of acid phosphatase was due to the *PHOC* or *PHOD* genotype. The results of a typical experiment are shown in Fig. 5. It is clear that the *PHOC* allele is indispensable for the production of acid phosphatase in nutrient medium and that the *PHOD* allele is required for production of the repressible acid phosphatase. The constitutive acid phosphatase controlled by the *phoC* gene could not be derepressed even in low-Pi medium. These results, in addition to the arguments described earlier, may allow the conclusion that the activities of constitutive and repressible acid phosphatases are attributable to different enzyme species.

TABLE 2. *Comparison of the formation of repressible acid and alkaline phosphatases among mutant strains*

Genotype	Strain[b]	Enzyme activities[a]			
		Acid phosphatase		Alkaline phosphatase	
		High-Pi	Low-Pi	High-Pi	Low-Pi[b]
+ (*phoC-1*)[c]	P-22-14D	0.009	2.41	0.084	0.316
phoB	O16-M3	0.004	0.010	0.076	0.208
	O21-M4	0.006	0.330	0.075	0.278
phoD	O16-M21	0.005	0.011	0.089	0.088
	O25-M57	0.006	0.020	0.067	0.077
	O26-M68	0.010	0.018	0.069	0.076
phoE	O17-M6	0.003	0.160	0.072	0.200
	O21-M10	0.004	0.331	0.075	0.280
	O22-M1	0.004	0.279	0.076	0.283
phoS	O22-M2	0.008	0.008	0.078	0.090
	O22-M11	0.009	0.007	0.089	0.095
phoR	P-32-2B	0.268	2.78	0.133	0.450
	P-49-1C	0.169	3.45	0.264	0.435

[a] Specific activities of acid and alkaline phosphatase are expressed as units per milligram of protein.

[b] Each strain was grown in high-Pi or low-Pi medium at 30 C for 19 to 22 hr with shaking. Extracts were prepared with 5 mM Tris-maleate buffer (*p*H 6.5).

[c] The original *phoC-1* strain used for isolation of mutants lacking repressible acid phosphatase.

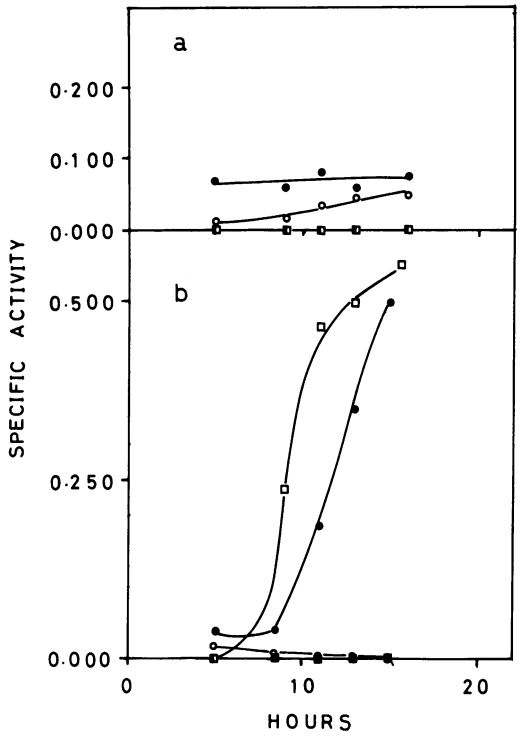

FIG. 5. *Time course of the formation of acid phosphatase in tetrad clones having the PHOC PHOD, PHOC phoD, phoC PHOD, and phoC phoD genotypes. These tetrad clones were obtained from a diploid hybrid (P-61) of α phoC phoD × a PHOC PHOD combination. Each clone was grown in nutrient medium (a) or low-Pi medium (b), and the formation of acid phosphatase was determined at appropriate intervals. Acid phosphatase activity was determined with a suspension of intact cells as the enzyme source. Specific activity is expressed as units per unit of optical density at 660 nm. Symbols:* □, *P-61-1A (phoC PHOD);* ○, *P-61-1B (PHOC phoD);* ■, *P-61-1C (phoC phoD); and* ●, *P-61-1D (PHOC PHOD).*

Isolation of constitutive mutants from a strain having the phoC-1 genotype. Many mutants which showed the constitutive phenotype with regard to acid phosphatase formation were induced from a strain of the *phoC-1* genotype, such as O8-M3 (*a*) or P-22-14D (*α*), by ultraviolet irradiation. These constitutive mutants could have occurred as a result of one of four possible mechanisms, as summarized in Table 3. The first possibility is that the constitutive production of acid phosphatase is attributable to a true reversion or another mutation which has occurred in the *phoC* locus and works as an intragenic suppressor. The second is that an extragenic suppressor mutation effective for the original *phoC-1* allele has occurred. The third possible mechanism is that the constitutive production of the repressible acid phosphatase might have resulted from a mutation which had occurred in a regulator gene. The fourth possibility is that of a mutation which exerts a function similar to that of the O^c mutation in a bacterial system. These mutations could be distinguished by examining the acid phosphatase formation in the diploid hybrid obtained by a cross between such a constitutive mutant and the standard *phoC-1* and *PHOC* strains and by tetrad analysis of these hybrids. In the case of the first possibility, the constitutive character of the mutant is dominant and the diploid hybrid made between this mutant and the *PHOC* strain should show essentially 4+:0− segregation in each ascus with respect to the constitutive formation of acid phosphatase. If a mutation caused an extragenic suppressor for the *phoC-1* allele and if this suppressor was dominant, a diploid with the *phoC-1* strain should produce some amount of acid phosphatase constitutively. The diploid hybrid obtained by a cross between this type of mutant and the *PHOC* strain should show 4+:0−, 3+:1−, and 2+:2− segregation with respect to the constitutive formation of acid phosphatase. If a mutation occurred as a result of the third possibility, the constitutiveness is recessive and the diploid hybrid obtained by a cross between the *phoC-1* strain should not produce the acid phosphatase on nutrient or high-Pi media. The diploid hybrid between this type of mutant and the *PHOC* strain should show 4+:0−, 3+:1−, and 2+:2− segregation for the formation of acid phosphatase in high-Pi medium. If a mutation occurred as a result of the fourth possibility, the diploid hybrid should produce the acid phosphatase constitutively and the constitutive trait of this mutant would be dominant. The diploid hybrid obtained by a cross between this mutant and the *PHOC* strain should segregate a clone which is unable to produce acid phosphatase constitutively. Although the genetic characteristics of the mutants of this class are quite similar to those of the mutants of the second class, it may be possible to distinguish them by examining the enzymatic characteristics of the acid phosphatase produced in high-Pi medium and by genetic analysis of hybrids obtained by a cross between such a mutant and the *phoC phoB*, *phoC phoD*, or *phoC phoE* strains.

Since the constitutiveness is recessive only in the third class of mutant, constitutive mutants were divided into two groups, dominant and recessive, by examining the phenotype of the diploid hybrids obtained by crossing each mu-

TABLE 3. *Representation of the hypothesis for establishing the genetic nature of constitutive mutation from the phoC-1 mutant strain*

Hypothesis	Possible genotype of constitutive revertant	Phenotype of diploid hybrid in high-Pi medium[a]	Possible segregation pattern in high-Pi medium	No. of mutants isolated
1. Reversion or intragenic suppressor at the *phoC* locus	*PHOC* or *phoC-1 phoC-SUP*[c]	× *PHOC* : +[b] × *phoC-1* : +	4+:0− 2+:2−	6
2. Extragenic suppressor to the *phoC-1* allele	*phoC-1 SUP*	× *PHOC* : + × *phoC-1* : +[d]	4+:0−, 3+:1−, 2+:2− 2+:2−	0
3. Mutation in the regulator gene for the repressible enzyme	*phoC-1 phoR*	× *PHOC* : + × *phoC-1* : −	4+:0−, 3+:1−, 2+:2− 2+:2−	19
4. Mutation at the operator for the repressible enzyme	*phoC-1 phoO*[c]	× *PHOC* : + × *phoC-1* : +	4+:0−, 3+:1−, 2+:2− 2+:2−	1

[a] Each mutant was crossed with the standard *PHOC* or *phoC-1* strain. Each hybrid obtained was tested for the ability to produce acid phosphatase constitutively and was subjected to tetrad analysis after sporulation.

[b] Phenotype on high-Pi medium by the staining method; + and − indicate the ability and inability to produce acid phosphatase in this medium, respectively.

[c] *phoC-SUP* is an intragenic suppressor for the *phoC-1* allele.

[d] A constitutive phenotype is expected if the suppressor is dominant.

tant with the *phoC-1* strain. Dominant constitutive mutants were then classified according to the above criteria. Among several constitutive mutants, O12-M15 (*a*), O27-M66 (*α*), and O15-M2 (*a*) were used in further studies as representative; the first two were dominant and the last one was recessive. These mutants were crossed with the original *phoC-1* strains, and the resulting diploid hybrids were sporulated and dissected. All asci showed 2+:2− segregation with respect to the formation of acid phosphatase in high-Pi medium. From the above crosses involving O12-M15 and O15-M2, constitutive segregants were isolated; they were crossed to the wild-type strain, H-42 (*PHOC*), and the resulting diploids were subjected to tetrad analysis. Mutant O27-M66 was crossed directly to H-42. Essentially all asci (33 of 34 asci) showed 4+:0− segregation with respect to the constitutive formation of the acid phosphatase in the cross involving the O12-M15 derivative. Thus, constitutivity in O12-M15 is due to reversion or intragenic suppression at *phoC*. In the case of the cross involving the O15-M2 derivative, 4+:0−, 3+:1−, and 2+:2− segregations appeared with respect to the formation of acid phosphatase on high-Pi medium, as shown in Table 4. Since constitutivity in O15-M2 is recessive and since the repressible segregants were segregated in the cross to the wild-type (*PHOC*) strain, we have tentatively concluded that constitutivity in O15-M2 is due to mutation in a regulator gene, designated *phoR*. The data in Table 4 also indicated that constitutivity in O27-M66 is due either to extragenic suppression for *phoC-1* or to *O*^{c}-type mutation for the repressible acid phosphatase production, since the repressible clones were segregated from the constitutive hybrid and constitutivity was dominant. In further testing, O27-M66 was crossed with O16-M3 (*a phoC-1 phoB*), and the diploid hybrid was sporulated and dissected. If the constitutivity in O27-M66 is attributable to the second possibility illustrated in Table 3, 2+:2− segregation will be expected in each ascus on high-Pi medium. The diploid hybrid, however, showed a 2+:2− (1 ascus) and a 1+:3− (12 asci) segregation with respect to the formation of acid phosphatase in high-Pi medi-

TABLE 4. *Tetrad segregations in crosses between PHOC and the constitutive mutants from the phoC-1 strain*

Combination of hybrid		Segregation in asci (+:−)[b]				
Mutant[a]	*PHOC*	4:0	3:1	2:2	1:3	0:4
O12-M15*	H-42	34	1	0	0	0
O15-M2*	H-42	2	18	5	0	0
O27-M66	H-42	3	12	0	0	0

[a] The constitutive mutants from the *phoC-1* strain. Strains marked by an asterisk were replaced by their derivative in the practical crosses.

[b] The formation of acid phosphatase was tested on high-Pi medium by the staining method.

um; all asci showed a 2+:2− segregation in low-Pi medium. This result suggests that the mutation in O27-M66 belongs to the fourth class of the mutation described in Table 3 and that the *phoB* locus was not linked to the supposed *phoO* locus. Further characterization of this mutant is now underway. The constitutive mutants so far obtained were classified according to these criteria, and the number of mutants belonging to each class is listed in the last column of Table 3.

It was found that the formation of both acid and alkaline phosphatases was significantly affected by the mutation classified as *phoD* and *phoS*, as shown in Table 2. This observation suggests that these repressible phosphatases may be under the same controlling mechanism or that both enzymes share a common factor. Therefore, the question of whether the repressible acid and alkaline phosphatases are also controlled by the *phoR* gene was investigated. Two independently isolated *phoR* mutants were grown in high-Pi and low-Pi media, and the activity of both acid and alkaline phosphatases was assayed. The results presented in Table 2 clearly indicate that the *phoR* mutants produce a significant amount of alkaline phosphatase as well as acid phosphatase in high-Pi medium. Furthermore, full derepression was observed only when the cells were cultured on low-Pi medium. The *phoR* mutants showed a higher activity for both enzymes in low-Pi medium than did the wild-type (*phoC-1*) strain.

Role of the phoS gene. If a gene or a gene system which controls the synthesis of an active repressor is contained in the regulatory system of the acid phosphatase formation, some of the mutants which produce the active repressor constitutively will show the same phenotype as a mutant with respect to the structural gene(s). Furthermore, a double mutant for such a gene and for *phoR* should produce the acid phosphatase constitutively. This possibility was examined by crossing the mutant lacking the repressible acid phosphatase with the *phoR* strain. Tetrad segregants from each cross were tested for acid phosphatase formation on a plate containing high-Pi or low-Pi medium by the staining method. Two types of patterns in tetrad segregations from these crosses were observed (Table 5). One of the segregation patterns is characterized by a 2+:2− segregation of the acid phosphatase formation in low-Pi medium; some of these acid phosphatase producers are constitutive. This type of segregation would be expected if the *phoR phoB*, *phoR phoD*, and *phoR phoE* genotypes could not produce the acid phosphatase in the presence or absence of inorganic phosphate in the medium. Another type of segregation was observed in the hybrid, P-54, from the cross between the *phoR* and *phoS* mutants and is characterized by more than two spore cultures which produce acid phosphatase on low-Pi medium in each ascus; two of them were always constitutive, as shown in Table 5. This type of segregation was most adequately explained by the presence of another type of regulatory gene, *phoS*. To confirm the above hypothesis, one of the tetratype asci, consisting of P-54-5A, -5B, -5C, and -5D spore cultures, was selected for further study, and the genotype of each strain was determined (Table 6). (Since all of the strains discussed below contained *phoC-1*, this genetic symbol has been omitted in the following descriptions.) In this tetratype ascus, four genotypes should be possible, namely, *phoR PHOS*, *PHOR phoS*, *phoR phoS*, and *PHOR PHOS*; the first two are parental types and the last two are recombinants. Judging from the phenotypes of the segregants, P-54-5A, -5B, and -5D showed the parental phenotypes, and among them it can be easily supposed that the genotype of P-54-5B should be *PHOR phoS* because it does not produce acid phosphatase on either high-Pi or low-Pi medium. Another clone, P-54-5C, behaved like the wild-type clone and should be recombinant having the *PHOR PHOS* genotype. Therefore, either the P-54-5A or the -5D clone has the genotype corresponding to *phoR PHOS* and the other has the genotype *phoR phoS*. Since the *phoR* and *phoS* alleles are recessive to the corresponding wild-type allele, as mentioned above, the *phoR PHOS* strain can be distinguished from the *phoR phoS* strain by examining the phenotype of diploids obtained by crossing each of these strains to the *PHOR phoS* strains; the *phoR PHOS/PHOR phoS* diploid should produce acid phosphatase in low-Pi medium but not in high-Pi medium. On the other hand, the *phoR phoS/PHOR phoS* diploid should not produce the enzyme in either low- or high-Pi media. These possibilities were tested by backcrosses of the tetrad clones, P-54-5A or -5D, to the original *phoS* strain, O16-M6. The ability of these diploid hybrids to produce the acid phosphatase was examined on high-Pi and low-Pi media by the staining method. Results shown in Table 6 clearly indicate that the diploid obtained by a cross between P-54-5A and O16-M6 could not produce the acid phosphatase either on high- or low-Pi media. On the other hand, the diploid obtained by crossing P-54-5D to O16-M6 could

TABLE 5. *Tetrad segregations in crosses between the phoC phoR strain and the phoC phoB, phoC phoD, phoC phoE, or phoC phoS strain*[a]

Hybrid no.	Combination	Segregation in asci[b]				
		On low-Pi On high-Pi	+ + + + + + - -	+ + + - + + - -	+ + - - + + - -	+ + - - + - - -	+ + - - - - - -
P-44	P-32-2B[c] × O16-M3 (*phoC phoB*)		0	0	2	19	2
P-45	P-32-2B × O16-M21 (*phoC phoD*)		0	0	4	6	9
P-75	P-32-2B × O17-M6 (*phoC phoE*)		0	0	3	8	3
P-54	P-32-2B × O16-M6 (*phoC phoS*)		10	39	17	0	0

[a] Four mutant strains, O16-M3, O16-M21, O17-M6, and O16-M6, were used as the representatives of the *phoB*, *phoD*, *phoE*, and *phoS* mutants, respectively.

[b] Each tetrad segregant was tested for its ability to produce acid phosphatase in high-Pi and low-Pi media by the staining method.

[c] P-32-2B has the *phoC phoR* genotype.

TABLE 6. *Determination of the genotype of each tetrad clone in one of the tetratype asci from diploid P-54*

Segregant	Mating type	Acid phosphatase formation				Expected genotype
		In haploid segregant[a]		In diploid hybrid[b]		
		High-Pi	Low-Pi	High-Pi	Low-Pi	
P-54-5A	*a*	+[c] (0.16)[d]	+ (0.41)	-	-	*phoR phoS*
P-54-5B	*a*	- (0.00)	- (0.00)	NT[e]	NT	*PHOR phoS*
P-54-5C	*a*	- (0.00)	+ (0.31)	NT	NT	*PHOR PHOS*
P-54-5D	*α*	+ (0.17)	+ (0.43)	-	+	*phoR PHOS*

[a] Phenotype of each tetrad segregant in high-Pi or low-Pi medium.

[b] P-54-5A and -5D were constitutive and they were backcrossed with O16-M6 (*a phoC PHOR phoS*).

[c] Phenotype determined by the staining method.

[d] Specific activity of acid phosphatase (units per unit of optical density at 660 nm) determined with a suspension of intact cells as the enzyme source. Each clone was grown in high-Pi or low-Pi medium for 20 hr at 30 C with shaking.

[e] Not tested.

produce the enzyme, but only in low-Pi medium. Therefore, the genotypes of P-54-5A and -5D were labeled *phoR phoS* and *phoR PHOS*, respectively. It should be emphasized that the *phoR phoS* genotype results in a constitutive formation of the acid phosphatase. To make a quantitative comparison of the formation of the acid phosphatase among these strains, each strain was cultivated in low- or high-Pi medium at 30 C for 20 hr with shaking. The activity of the acid phosphatase was assayed with a suspension of intact cells as the enzyme source; the specific activity was expressed as units per unit of optical density at 660 nm. The results presented in Table 6 indicate that the enzyme formation of each strain reflects the characteristics shown by the staining method. In addition, both of the constitutive strains showed a higher acid phosphatase activity in low-Pi medium than in high-Pi medium.

DISCUSSION

It has been reported in other organisms, such as *E. coli* (8) and *N. crassa* (20), that derepression of phosphatase has resulted from the de novo synthesis of the enzyme. It seems reasonable to assume that that is also the case in *S. cerevisiae*. The haploid strain H-42 produced acid phosphatases in both the repressed and derepressed conditions. These phosphatases showed a difference in their K_m values for *p*-nitrophenylphosphate and in their heat stability. It was suggested by these observations that these phosphatases were different species. This suggestion was also supported by genetic studies of certain mutants lacking one of these acid phosphatases. The genetic locus controlling the formation of the constitutive acid phosphatase was designated as *phoC*, and it was found that the *phoC* locus is linked with the *lys2* locus on chromosome II (9).

The mutants lacking repressible acid phosphatase were divided into four classes; mutations at two of the loci, *phoD* and *phoS*, led to inability to derepress alkaline as well as acid phosphatases. The mutations at the *phoB* and *phoE* loci, however, did not have any significant effect on the activity of the alkaline phosphatase. The *phoA*$^-$ mutants described by Schurr and Yagil (17) were also defective in both acid and alkaline phosphatases. These authors suggested the existence of a common regulatory mechanism or a common polypeptide to explain the pleiotropic effect of these mutations. Since mutations at the *phoD* and *phoS* loci showed a similar pleiotropic effect on these enzymes, they might correspond to the *phoA*$^-$ mutants. However, as will be discussed later, the *phoS* gene is not a structural gene for the repressible acid phosphatase. It is possible to speculate that the *phoD* gene might act in the manner suggested by Schurr and Yagil (17) or might have the positive function of derepressing these phosphatases. To date, however, it is still unclear which mutation is in the structural gene(s).

Genetic analyses of the constitutive mutants derived from the *phoC* mutant showed that the constitutive synthesis of acid phosphatase was the result of several distinct genetic events, such as the reversion to *PHOC*, mutations occurring in the regulator gene (*phoR*), and mutations occurring in an unknown gene other than *phoC* and *phoR*. Since the *phoR* mutant allele was recessive to the corresponding wild-type allele, and the acid phosphatase produced by the *phoR* mutant grown in high-Pi medium was similar to the repressible acid phosphatase with respect to heat stability and the K_m value for *p*-nitrophenylphosphate (*unpublished data*), the simplest interpretation of the function of the *phoR* gene is that it specifies the repressor molecule for the synthesis of the repressible acid phosphatase. It was also found that the *phoR* mutation exerts a pleiotropic effect on both acid and alkaline phosphatases, as described by Schurr and Yagil (17).

In bacteria, the site on which repressor acts is well characterized as an operator locus (1); it is localized at one end of the structural gene and binds the repressor molecule reversibly. Such a controlling site may exist in eukaryotic cells, although its presence has not been confirmed as yet, except for a report on the *c-GAL4* complex in a galactose-utilizing system in *S. cerevisiae* by Douglas and Hawthorne (5). In this respect, it would be interesting to study the nature of a mutant which preserves the *phoC PHOR* genotype and shows constitutive synthesis of the acid phosphatase. We succeeded in isolating such a mutant from the *phoC-1* strain; its characterization is now underway.

The observation that the *phoC phoR phoS* strain produced acid phosphatase in both high- and low-Pi media indicates that the *phoS* gene is not a structural gene but, rather, a kind of regulatory gene. This result also eliminates the possibility that the product of the *phoS* gene might positively control the synthesis of the repressible acid phosphatase directly, because, if this were the case, the *phoC phoR phoS* strain would not produce acid phosphatase in high- and low-Pi media. The assumption that the *phoS* gene has a regulatory function is consistent with the fact that the *phoS* mutant could not derepress both acid and alkaline phosphatases simultaneously. The phenotype of the *phoC phoR phoS* strain suggests that the *phoS* gene controls the synthesis of repressible phosphatases via the expression of the *phoR* gene. At present, the simplest interpretation of the data from tetrad analysis of the diploid P-54 and the phenotype of the *phoS* mutant is that: (i) the *phoS* mutant has a normal structural gene(s) for both acid and alkaline phosphatases, and is defective in regulatory gene other than *phoR*, (ii) the *phoS* gene controls the expression of the *phoR* gene via a certain cytoplasmic substance somehow involved with inorganic phosphate, and (iii) the *PHOR* product represses the synthesis of the repressible acid and alkaline phosphatases regardless of the presence or absence of inorganic phosphate. According to this model, the phenotype of the *phoS* and the *phoR phoS* mutants can be explained as follows: (i) the *phoS* mutant produces the repressor for the structural gene(s) constitutively and cannot derepress these phosphatases, and (ii) although the *phoR phoS* double mutant produces the repressor for the structural gene(s) constitutively, the repressible phosphatases can be produced because of an inactive repressor resulting from a mutation occurring in the *phoR* gene.

Assuming that the *phoS* mutation results in a constitutive synthesis of the *phoR* product and that inorganic phosphate only affects the expression of the *phoR* gene, the extent of the acid phosphatase produced by the *phoR phoS* double mutant in low-Pi medium should be the same as that produced by the *phoR* mutant in high-Pi medium. However, as shown in Table 6, the *phoR phoS* double mutant produced a larger amount of the acid phosphatase in low-Pi medium than the *phoR* mutant did in high-Pi medium. This fact indicates that inor-

ganic phosphate also affects the formation of acid phosphatase in ways other than the expression of the *phoR* gene.

LITERATURE CITED

1. Beckwith, J. R., and D. Zipser (ed.). 1970. The lactose operon. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. Blum, J. J. 1956. Observation of the acid phosphatases of *Euglena gracilis*. J. Cell Biol. **24**:223-234.
3. Burkholder, P. R. 1943. Vitamin deficiencies in yeasts. Amer. J. Bot. **30**:206-211.
4. Dorn, G. 1965. Genetic analysis of the phosphatases in *Aspergillus nidulans*. Genet. Res. **6**:13-26.
5. Douglas, H. C., and D. C. Hawthorne. 1966. Regulation of genes controlling synthesis of the galactose pathway enzymes in yeast. Genetics **54**:911-916.
6. Garen, A., and H. Echols. 1962. Properties of two regulating genes for alkaline phosphatase. J. Bacteriol. **83**:297-300.
7. Garen, A., and H. Echols. 1962. Genetic control of induction of alkaline phosphatase synthesis in *E. coli*. Proc. Nat. Acad. Sci. U.S.A. **48**:1398-1402.
8. Garen, A., and C. Levinthal. 1960. A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. I. Purification and characterization of alkaline phosphatase. Biochim. Biophys. Acta **38**:470-483.
9. Hawthorne, D. C., and R. K. Mortimer. 1968. Genetic mapping of nonsense suppressors in yeast. Genetics **60**:735-742.
10. Horiuchi, T., S. Horiuchi, and D. Mizuno. 1959. A possible negative feedback phenomenon controlling formation of alkaline phosphomono-esterase in *Escherichia coli*. Nature (London) **183**:1529-1530.
11. Lindegren, C. C., and G. Lindegren. 1943. A new method for hybridizing yeast. Proc. Nat. Acad. Sci. U.S.A. **29**:306-308.
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
13. Nyc, J. F. 1967. A repressible acid phosphatase in *Neurospora crassa*. Biochem. Biophys. Res. Commun. **27**:183-188.
14. Nyc, J. F., R. J. Kadner, and B. J. Crocken. 1966. A repressible alkaline phosphatase in *Neurospora crassa*. J. Biol. Chem. **241**:1468-1472.
15. Oda, M., and K. Wakabayashi. 1954. Yeasts isolated from the *saké-moromi* (mash of *saké*) fermented by *kimoto*. J. Ferment. Technol. **32**:289-294.
16. Schmidt, G., G. Bartsch, M. C. Lamont, T. Herman, and M. Liss. 1963. Acid phosphatase of baker's yeast: an enzyme of the external cell surface. Biochemistry **2**:126-131.
17. Schurr, A., and E. Yagil. 1971. Regulation and characterization of acid and alkaline phosphatase in yeast. J. Gen. Microbiol. **65**:291-303.
18. Shult, E. E., and C. C. Lindegren. 1956. Mapping methods in tetrad analysis. I. Provisional arrangement and ordering of loci preliminary to map construction by analysis of tetrad distribution. Genetica **28**:165-176.
19. Suomalainen, H., M. Linko, and E. Oura. 1960. Changes in the phosphatase activity of baker's yeast during the growth phase and location of the phosphatases in the yeast cell. Biochim. Biophys. Acta **37**:482-490.
20. Toh-e, A., and T. Ishikawa. 1971. Genetic control of synthesis of repressible phosphatases in *Neurospora crassa*. Genetics **69**:339-351.
21. Torriani, A. 1960. Influence of inorganic phosphate in the formation of phosphatases by *Escherichia coli*. Biochim. Biophys. Acta **38**:460-479.