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Identification of the Genetic Locus for the Structural Gene and a New Regulatory Gene for the Synthesis of Repressible Alkaline Phosphatase in *Saccharomyces cerevisiae*

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Two lines of evidence showed that the *PHO8* gene encodes the structure of repressible, nonspecific alkaline phosphatase in *Saccharomyces cerevisiae*: (i) the enzyme produced by a temperature-sensitive *pho8* mutant at the permissive temperature (25°C) was more thermolabile than that of the wild-type strain, and (ii) the *PHO8* gene showed a gene dosage effect on the enzyme activity. The *pho8* locus has been mapped on chromosome IV, 8 centimorgans distal to *rna3*. A new mutant carrying the *pho9* gene was isolated which lacks repressible alkaline phosphatase, but has the normal phenotype for the synthesis of repressible acid phosphatase. The *pho9* gene segregated independently of all known *pho*-regulatory genes and did not show the gene dosage effect on repressible alkaline phosphatase activity. The *pho9/pho9* diploid hardly sporulated and showed no commitment to intragenic recombination when it was inoculated on sporulation medium. Hence the *pho9* mutant has a phenotype similar to the *pep4* mutant, which was isolated as a pleiotropic mutant with reduced levels of proteinases A and B and carboxypeptidase Y. An allelism test indicated that *pho9* and *pep4* are allelic.

In a previous communication (20), we observed that a cellular extract of *Saccharomyces cerevisiae* contains two species of alkaline phosphatase: a nonspecific alkaline phosphatase (EC 3.1.3.1) and a specific *p*-nitrophenylphosphatase. The nonspecific enzyme is repressible by inorganic phosphate in the medium, whereas the specific *p*-nitrophenylphosphatase is synthesized constitutively. The repressible alkaline phosphatase (namely, the nonspecific alkaline phosphatase) shows, however, a significant level of activity even under repressed conditions. This basal activity was found to be severely reduced in a *pho8* (formerly *phoH*) mutant, and the enzyme failed to be derepressed.

Repression or derepression of the repressible alkaline phosphatase occurs in response to signals of the presence or absence of inorganic phosphate in the medium (16, 20, 23). The signals are transmitted by the function of a regulatory system specified by the *PHO4* (formerly *PHOD*), *PHO80* (*PHOR*), *PHO81* (*PHOS*), and *PHO85* (*PHOU*) genes. This same regulatory system combined with the *PHO2* (*PHOB*) gene product controls the synthesis of acid phosphatase (EC 3.1.3.2) (23, 25). It was proved that the *PHO2* function is not involved in the regulation of alkaline phosphatase synthesis (20). The function of those regulatory genes in the synthesis of repressible acid and alkaline phosphatases have been discussed elsewhere (11) and can be summarized as follows (Table 1). A few molecules of a positive factor specified by *PHO4* are produced constitutively. Under repressed conditions, the *PHO4* gene product aggregates with a complex of the *PHO80* and *PHO85* gene products (negative factor) and hence is unable to activate transcription of *PHO5* (formerly *PHOE*, a structural gene of the repressible acid phosphatase [19]). Under derepressed conditions, the *PHO81* product aggregates with the *PHO80* or *PHO85* product or both, thereby releasing the positive factor, which can then combine with *PHO2* protein to activate *PHO5* transcription. The binding of the *PHO81* product to the negative factor is regulated by the effector, inorganic phosphate or a metabolite thereof. In the absence of the effector, the *PHO81* product is bound with the negative factor; in its presence, the *PHO81* product is unbound. (The numerical gene designations used in this communication are consistent with current yeast genetic nomenclature [11, 13]. The correspondence of the numerical designation with the previous letter designation, as well as the function of each gene, are given in Table 1 along with the genes specific for acid phospha-

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TABLE 1. Genes and their possible functions in the phosphatase system[a]

Gene	Former designation	Probable function of gene or its product
PHO1	*PHOA*	Required for synthesis of both acid and alkaline phosphatases (described by Schurr and Yagil [16])
PHO2	*PHOB*	Specific factor for expression of *PHO5* and inorganic phosphate permease gene
PHO3	*PHOC*	Constitutive acid phosphatase; structural gene
PHO4	*PHOD*	Positive factor
PHO5	*PHOE*	Repressible acid phosphatase; structural gene
PHO6	*PHOF*	Required for *PHO3* expression
PHO7	*PHOG*	Required for *PHO3* expression
PHO8	*PHOH*	Repressible, nonspecific alkaline phosphatase; structural gene
PHO9	*PHOI*	Specific factor for *PHO8* expression
PHO80	*PHOR*	Negative factor
PHO81	*PHOS*	Mediator
pho82[b]	*phoO*	Region with positive factor defining negative factor interaction site
pho83[b]	*phoP*	Regulatory site contiguous to *PHO5*
PHO84	*PHOT*	Component of inorganic phosphate transport system
PHO85	*PHOU*	Negative factor

[a] Gene symbols are given in wild-type form.
[b] Mutations in the locus are, in general, dominant or semidominant over the wild-type counterpart.

tase synthesis, the inorganic phosphate transport system, or both. In the description of gene symbols, the dominant wild-type alleles are represented by capital letters and the recessive mutant alleles are indicated by lower-case letters, whereas the *PHO82* [*PHOO*] and *PHO83* [*PHOP*] mutant alleles, which lie respectively in the *PHO4* locus [18] and adjacent to the *PHO5* locus [22] and cause the constitutive production of the enzyme activity, are represented by capital letters because they are dominant over their wild-type counterparts.)

This communication reports that the *PHO8* locus is the structural gene for the repressible alkaline phosphatase and is mapped on chromosome IV at a site 8 centimorgans distal to *rna3*. A new mutation, *pho9*, was isolated which lacks the repressible alkaline phosphatase activity but is not concerned with the synthesis of repressible acid phosphatase. The *pho9* gene is complementary with the *pho8* gene and is allelic with *pep4*, a mutation which was isolated on the basis of its proteinase-less phenotype and has been proved to be pleiotropic, reducing the level of proteinases A and B, carboxypeptidase Y (8), and RNase, along with that of alkaline phosphatase activity (7).

MATERIALS AND METHODS

Yeast strains. The *S. cerevisiae* strains used in this study are listed in Table 2. All strains, except for 1164, 1165, X963-18C, and 20B-12, were selected from our stock cultures or constructed in this study. They were marked with the *pho3-1* (formerly *phoC-1*) mutation (19, 23) to eliminate the constitutive acid phosphatase activity. Two strains (1164 and 1165) were kindly supplied by R. B. Wickner of the National Institutes of Health, Bethesda, Md., one (X963-18C) was obtained from C. C. Lindegren while he was at Southern Illinois University, Carbondale, and another (20B-12) was from E. W. Jones of the Carnegie Mellon Institute, Pittsburgh, Pa. The nomenclature of genetic symbols recommended by Plischke et al. (13) is used throughout this work, except for the conventional symbols of **a** and α for mating types.

Media. Nutrient medium was prepared by dissolving 20 g of glucose, 20 g of polypeptone (Daigo Eiyo Chemicals), 10 g of yeast extract (Daigo Eiyo Chemicals), 2 g of KH_2PO_4 and 0.4 g of adenine in deionized water to a volume of 1 liter. This medium was used as nutrient high-phosphate (nutrient high-P_i) medium. Nutrient low-phosphate (nutrient low-P_i) medium was prepared by the method of Rubin (15) with a minor modification: 10 g of yeast extract (Difco) and 20 g of peptone (Difco) were dissolved in deionized water to a volume of 1 liter, and to this was added 10 ml of 1 M $MgSO_4$ and then 10 ml of concentrated aqueous ammonia. The solution was mixed well and left to stand at room temperature for 30 min. The precipitate formed was removed by filtration with Whatman no. 1 filter paper. The filtrate was supplemented with 0.4 g of adenine and 20 g of glucose after adjustment to pH 5.8 by dropwise addition of concentrated HCl, and the whole solution was autoclaved for sterilization. Minimal high-P_i and minimal low-P_i media were prepared by modification of the Burkholder medium as described previously (23). To test auxotrophic markers, omission media were prepared from a basal medium of Yeast Nitrogen Base without amino acids (Difco). Sporulation was conducted with a medium containing

TABLE 2. List of yeast strains

Strain	Genotype	Source
P-28-24C	a *pho3-1*	Our stock culture
F16C	α *pho3-1*	a to α mutant of P-28-24C
AX4-4A	a *pho3-1 trp5*	Segregant from repeated crosses of *trp5* mutant of P-28-24C with F16C
AX43-7C	a *pho3-1 leu1-ELF52*	Segregant from repeated crosses of *leu1* mutant of P-28-24C with F16C
AX45-21A	a *pho3-1 leu1-ELF52*	Segregant from AX43-7C × F16C cross
AX45-29A	α *pho3-1 leu1-ELF52*	Segregant from AX43-7C × F16C cross
AX48-2C	α *pho3-1 ade10*	Constructed from *ade10* mutant of P-28-24C by repeated crosses and tetrad analyses of crosses with F16C and P-28-24C
Ax49-4B	α *pho3-1 his4*	Constructed from *his4* mutant of P-28-24C by repeated crosses and tetrad analyses of crosses with F16C and P-28-24C
AX51-3C	α *pho3-1 his7*	Constructed from *his7* mutant of P-28-24C by repeated crosses and tetrad analyses of crosses with F16C and P-28-24C
AX54-10C	α *pho3-1 ade10*	Constructed from *ade10* mutant of P-28-24C by repeated crosses and tetrad analyses of crosses with F16C and P-28-24C
APT2-4B	α *pho3-1 pho8-2 arg6*	Constructed from *arg6* mutant of P-28-24C by repeated crosses and tetrad analyses of crosses with KO-14D (19, 20), AL20-3C (20), and F16C
KYC73	a *pho3-1 arg6*	Segregant from APT2-4B × P-28-24C cross
AL1-9C	α *pho3-1 pho8-1*	Segregant from *pho8-1* mutant of P-73-3B (19, 20) × P-28-24C cross
AL7-3A	α *pho3-1 pho8-3*	Segregant from *pho8-3* mutant of P-28-24C × P-73-3B cross
AL9-2A	α *pho3-1 pho8-4*	Segregant from *pho8-4* mutant of P-28-24C × P-73-3B cross
AL14-1C	α *pho3-1 pho8-5*	Segregant from *pho8-5* mutant of P-28-24C × P-73-3B cross
AL20-2A	α *pho3-1 pho8-2*	Constructed from *pho8-2* mutant of P-73-3B by repeated crosses and tetrad analyses of crosses with P-28-24C and F16C
AL20-3C	a *pho3-1 pho8-2*	Constructed from *pho8-2* mutant of P-73-3B by repeated crosses and tetrad analyses with P-28-24C and F16C (19, 20)
AL30-1A	a *pho3-1 pho8-11 leu1-ELF52*	Segregant from *pho8-11* mutant from AX43-7C × F16C cross
AL30-2C	α *pho3-1 pho8-11 leu1-ELF52*	Segregant from *pho8-11* mutant from AX43-7C × F16C cross
AL32-3D	α *pho3-1 pho8-13 leu1-ELF52*	Segregant from *pho8-13* mutant from AX43-7C × F16C cross
AL51-1D	a *pho3-1 pho8*(Ts)*-18*	Constructed by repeated crosses of *pho8*(Ts)*-18* mutant of AX43-7C to F16C
AL51-6B	α *pho3-1 pho8*(Ts)*-18*	Constructed by repeated crosses of *pho8*(Ts)*-18* mutant of AX43-7C to F16C
AL70-3A	α *pho3-1 pho8-29 trp5*	Segregant from *pho8-29* mutant of AX4-4A × F16C cross
AL71-1A	α *pho3-1 pho8-30 trp5*	Segregant from *pho8-30* mutant of AX4-4A × F16C cross
AL81-3B	α *pho3-1 pho8-37 trp5*	Segregant from *pho8-37* mutant of AX4-4A × F16C cross
AL82-2A	α *pho3-1 pho8-38 trp5*	Segregant from *pho8-38* mutant of AX4-4A × F16C cross
AL101-1D	a *pho3-1 pho9-1 trp5*	Segregant from *pho9-1* mutant of AX4-4A × F16C cross
AL101-2A	α *pho3-1 pho9-1 trp5*	Segregant from *pho9-1* mutant of AX4-4A × F16C cross
AL203-9A	α *pho3-1 pho9-1 trp5*	Segregant from AL101-2A × P-28-24C cross
AL203-9D	a *pho3-1 pho9-1 trp5*	Segregant from AL101-2A × P-28-24C cross
AL211-12B	a *pho3-1 pho8-2 arg6*	Segregant from APT2-4B × P-28-24C cross
AL212-4D	a *pho3-1 pho9-1 ade10*	Segregant from AX54-10C × AL203-9D cross
AL212-6C	α *pho3-1 pho9-1 ade10*	Segregant from AX54-10C × AL203-9D cross
AL218-11A	α *pho3-1 pho9-1*	Segregant from AX49-4B × AL203-9D cross
AL224-4A	a *pho3-1 leu1-ELF52*	Segregant from AL218-11A × AX45-21A cross
AL224-4D	a *pho3-1 pho9-1 leu1-ELF52*	Segregant from AL218-11A × AX45-21A cross
AL225-2D	α *pho3-1 pho9-1 leu1-SA54*	Segregant from *leu1-SA54* mutant of P-28-24C × AL218-11A cross

TABLE 2—*Continued*

Strain	Genotype	Source
AL225-4A	α *pho3-1 leu1-SA54*	Segregant from *leu1-SA54* mutant of P-28-24C × AL218-11A cross
1164	**a** *aro1D pet14 rna3 ade8 trp4 mal SUP2*	R. B. Wickner
1165	α *aro1D pet14 rna3 ade8 trp4 ade1 SUP2*	R. B. Wickner
X963-18C	α *met2 his8 ade8*	Lindegren's stock
20B-12	α *pep4-3 trp1 gal2 SUC*	E. W. Jones
DH1	**a** *pho3-1 PHO8⁺* / α *pho3-1 PHO8⁺*	Constructed by P-28-24C × F16C cross
DH2	**a** *pho3-1 pho8*(Ts)*-18* / α *pho3-1 PHO8⁺*	Constructed by AL51-1D × F16C cross
DH3	**a** *pho3-1 pho8*(Ts)*-18* / α *pho3-1 pho8*(Ts)*-18*	Constructed by AL51-1D × AL51-6B cross
DI1	**a** *pho3-1 PHO9⁺ leu1-ELF52* / α *pho3-1 PHO9⁺ leu1-SA54*	Constructed by AL224-4A × AL225-4A cross
DI2	**a** *pho3-1 pho9-1 leu1-ELF52* / α *pho3-1 PHO9⁺ leu1-SA54*	Constructed by AL224-4D × AL225-4A cross
DI3	**a** *pho3-1 pho9-1 leu1-ELF52* / α *pho3-1 pho9-1 leu1-SA54*	Constructed by AL224-4D × AL225-2D cross

0.5% of potassium acetate and 2% agar. Agar media were prepared by the addition of 2% agar. Cells were cultivated at 30°C unless otherwise noted, and cell growth was monitored by reading optical density of the culture at 660 nm.

Isolation of mutants. Mutagenesis of a haploid strain was induced with ethyl methane sulfonate by the method of Fink (5).

Construction of tetraploids. Triploids were constructed from a regular **a**/α diploid and a haploid strain by forced mating and selection on an appropriate selection medium, when the diploid and haploid strains had the appropriate complementary auxotrophic traits. When the diploid strain was prototrophic, it was mated with an auxotrophic haploid strain by the RD auxotroph mating method as described by Gunge and Nakatomi (6), and hybrid colonies were selected on lactate-ethanol minimal medium, which is composed of minimal high-P_i medium with the glucose replaced by 15 g of sodium lactate and 30 ml of ethanol (95%) per liter. Since all the triploids constructed by these methods were prototrophs, the RD auxotroph mating method was adopted for construction of a tetraploid from a triploid and an auxotrophic haploid strain.

Detection of phosphatase activity of colonies. Acid phosphatase activity of colonies on agar media was detected by a staining method based on the diazo coupling reaction (21). The staining method for alkaline phosphatase activity of colonies described previously (20) was followed, with one modification: the soft agar containing *p*-nitrophenyl-phosphate was buffered with 0.5 M Tris-hydrochloride buffer (pH 9.0) containing 25 mM $MgSO_4$ instead of 0.1 M Veronal buffer (pH 9.0) containing 5 mM $MgSO_4$.

Enzyme assay. The activity of acid phosphatase was assayed by using intact cells as the enzyme source and *p*-nitrophenylphosphate as the substrate, as described previously (23). The alkaline phophatase activity was assayed by using *p*-nitrophenylphosphate as the substrate and cell extract or cells permeabilized to the substrate as the enzyme source, as described previously (20). Cells were permeabilized by treatment with toluene and ethanol as described (20), according to the method of Serrano et al. (17). All enzyme assays were performed at 30°C unless otherwise noted. One unit of acid or alkaline phosphatase was defined as the amount of enzyme which liberates 1 μmol of *p*-nitrophenol per min under the assay conditions. When α-naphthylphosphate was used as substrate, liberated inorganic phosphate was assayed according to the method of Chen et al. (2), and one unit of enzyme activity was defined as the amount of enzyme which liberates 1 μmol of inorganic phosphate per min.

Preparation of cell extract. Harvested cells were washed once with 25 mM Tris-hydrochloride buffer (pH 8.5) containing 1 mM $MgSO_4$ (the standard buffer) and suspended in an appropriate amount of the same buffer. The cells were broken by passing once through a French Pressure Cell (Ohtake, model 5501) at 500 kg/cm^2 or by two periods of shaking with 0.5-mm glass beads in a Braun cell homogenizer (model MSK) at full speed (3,400 rpm) for 15 s each. All subsequent operations were carried out below 4°C. Insoluble material was removed by centrifugation at 17,000 × *g* for 10 min, and the supernatant was used as crude extract. Protein concentration was determined by the method of Lowry et al. (10) with bovine serum albumin as the standard.

Fractionation of alkaline phosphatase. Streptomycin

was added to the crude cell extract to a final concentration of 1%. Insoluble material was removed by centrifugation at 17,000 × *g* for 10 min, and the supernatant was dialyzed against the standard buffer containing 0.1 M NaCl overnight at 4°C. A 10- to 20-ml portion of the dialyzed extract, containing approximately 100 to 150 mg of protein, was applied to a DEAE-Sephadex A-50 column (2 by 30 cm) equilibrated with the standard buffer containing 0.1 M NaCl. Gradient elution was effected with a mixing chamber containing 300 ml of the standard buffer with 0.1 M NaCl and a reservoir containing 300 ml of the standard buffer with 0.5 M NaCl. Effluent was collected in 5-ml portions. NaCl concentration of the effluent was determined by measuring electric conductivity with a conductivity meter (CD-35M II type, Emuesu Kiki). The protein concentration of the effluent was estimated by reading optical density at 280 nm.

Partial purification of repressible alkaline phosphatase. Crude extract was treated with 1% streptomycin and centrifuged as described above. For the wild-type strain, the supernatant was fractionated by salting out with $(NH_4)_2SO_4$ (90% saturation); the precipitate was then collected and dissolved in the minimum amount of the standard buffer, and the solution was dialyzed against the standard buffer at 4°C overnight. The salting out with $(NH_4)_2SO_4$ significantly reduces the total enzyme activity of the cell extract (to below 50% of the initial level) even with the wild-type strain, and the enzyme activity of a temperature-sensitive *pho8* mutant was, in general, about 50% lower than that of the wild-type strain. Thus to retain a sufficient level of enzyme activity in the cell extract of the mutant, the supernatant was dialyzed without salting. A 20- to 50-ml portion of dialysate, containing 60 to 200 mg of protein, was applied to a DEAE-cellulose column (Whatman DE32, 2 by 30 cm) equilibrated with the standard buffer. The column was washed with the same buffer, and then gradient elution was performed as described above, except that the mixing chamber contained 300 ml of the standard buffer. Two peaks of alkaline phosphatase activity were detected by using *p*-nitrophenylphosphate as substrate. The fractions of the later peak, which corresponds to the repressible alkaline phosphatase, were pooled, and protein was precipitated with $(NH_4)_2SO_4$ (90% saturation). The precipitate was dissolved in 4 ml of the standard buffer and applied to a Sephadex G-100 column (2 by 50 cm) equilibrated with that buffer, and protein was eluted with the same buffer. The fraction containing the highest enzyme activity was used to test the thermostability of repressible alkaline phosphatase activity. The specific activity of the enzyme preparation was 20- to 70-fold higher than that of the crude cell extract.

RESULTS

Isolation of mutants bearing *pho8* (Ts) and *pho9*. Tryptophan (AX4-4A) and leucine (AX43-7C) auxotrophic mutants from our stock culture were subjected to ethyl methane sulfonate mutagenesis and spread on nutrient plates after appropriate dilution. The plates were incubated at 35°C for 2 days. Colonies appearing on the plates were replicated to minimal low-P_i plates supplemented with tryptophan or leucine. The replicated plates were incubated at 35°C for another day. Alkaline phosphatase activity of each colony was detected by the staining method after treatment with chloroform vapor. We isolated 28 colonies from the plates inoculated with AX4-4A and 14 from those with AX43-7C; these colonies failed to show alkaline phosphatase activity. All of the isolates were crossed with a wild-type strain, either F16C or AX45-29A or both. All of the mutations were found to be recessive, since the diploids showed the wild-type phenotype with respect to alkaline phosphatase activity. Tetrad analysis of the diploids has shown a 2+:2− segregation at 35°C of the same phenotype in the 6 to 14 asci so far tested for each cross. This fact indicates that each mutant bears a single chromosomal mutation. The original mutants or haploid segregants from the above crosses bearing a mutant allele were then crossed with the *pho8-2* mutant APT2-4B, which was obtained in the previous study (20). The resultant diploids were tested for their ability to develop alkaline phosphatase activity at 35°C by the staining method. All the mutants except one (AL101-1D) failed to complement the *pho8* mutation. Thus, we concluded that 41 of the 42 isolates are *pho8* mutants. All of the 46 *pho8* mutants so far isolated (41 newly isolated and 5 previously isolated [20]) were spotted on duplicate minimal low-P_i plates, one of which was incubated at 35°C and the other at 25°C. None of the *pho8* mutants developed repressible alkaline phosphatase activity at 35°C, and only one of them, K44 from AX43-7C, showed the activity at 25°C.

To confirm the temperature sensitivity of the mutation in K44, strain AL51-1D, which carries the same mutant allele as K44, was crossed with the standard *pho8* strains (AL1-9C, AL7-3A, AL9-2A, AL14-1C, and AL20-2A), and the resulting diploids were dissected. So far, no recombinants have appeared in the tetrads of 50 asci (9 to 12 tetrads for each cross; data not shown). Thus, we concluded that this temperature-sensitive mutation occurred in the *pho8* locus, and we designated the mutant allele *pho8*(Ts)-*18*.

A haploid segregant, AL101-1D, from the cross between F16C (wild type) and the exceptional mutant which complemented the *pho8* mutation was further crossed with four *pho8* mutants (AL7-3A, AL9-2A, AL14-1C, and AL20-2A) having different alleles, and the diploids were subjected to tetrad analysis. In 6 to 11 asci so far tested for each of the four crosses, the tetrad distribution of alkaline phosphatase activity clearly indicated that this mutation is unlinked with *pho8* (Table 3). Linkage analyses between the same mutation and other known *pho* regulatory genes, i.e., *pho2*, *PHO82-pho4*,

pho80, *pho81*, and *pho85*, also indicated that the mutation occurred at a new locus (Table 3). (Linkage to the *pho3*, *pho5*, and *pho84* [formerly *phoT*; 24] loci was not test[illegible] because the former two genes encode the structure of acid phosphatases [19] and the latter one is specific for inorganic phosphate transport; thus they are not concerned directly with the regulation of repressible alkaline phosphatase.) We designated this mutation *pho9-1*.

The specific activity of the alkaline phosphatases of the *pho8-2*, *pho8*(Ts)-*18*, *pho9-1*, and wild-type strains grown on high-P_i and low-P_i media are listed in Table 4. The wild-type strain showed a significant basal level of alkaline phosphatase activity under repressed (high-P_i) conditions, whereas the *pho8* and *pho9* mutants did not.

Thermolabile enzyme from the *pho8*(Ts)-*18* mutant. To test whether the *PHO8* gene is a structural gene or a regulatory gene for the synthesis of repressible alkaline phosphatase, the thermostability of the repressible alkaline phosphatase activity of the *pho8*(Ts)-*18* mutant was compared with that of the wild-type strain by using partially purified enzyme preparations. The enzyme preparation of the *pho8*(Ts)-*18* mutant (AL51-1D) was obtained from the crude cell extract without the $(NH_4)_2SO_4$ pretreatment, because the cell extract easily lost the enzyme activity by the salting process, and that of the wild-type strain (P-28-24C) was prepared with the pretreatment. The enzyme activity of the *pho8*(Ts)-*18* mutant was much more rapidly inactivated at 60°C than that of the wild-type strain (Fig. 1A). A mixture of the two enzyme preparations initially lost its residual activity rapidly, like the *pho8*(Ts)-*18* enzyme, and later slowly, like the wild-type enzyme.

To study the effect of in vivo mixing on thermostability of the wild-type and *pho8*(Ts)-*18* enzymes, diploids DH1 (homozygous for *PHO8*$^+$), DH2 [heterozygous for *pho8*(Ts)-*18*/*PHO8*$^+$], and DH3 [homozygous for *pho8*(Ts)-*18*] were constructed as described in Table 2. Crude extract was prepared from the cells of each diploid strain grown in nutrient low-P_i medium at 25°C for 15 h. Since the cell extracts contain two activities which cleave the phosphomonoester bond of *p*-nitrophenylphosphate, namely, specific *p*-nitrophenylphosphatase and nonspecific repressible alkaline phosphatase, α-naphthylphosphate was used as substrate because it is inert to the former activity. The cell extract of DH1 retained about 70% of the activity of the untreated control after treatment at 60°C for 15 min, whereas the extract of DH3 retained only about 10% of the initial activity. The enzyme activity in the extract of DH2 was inactivated in two phases: a fast inactivation comparable to that of DH3, followed by slow inactivation comparable to that of DH1 (Fig. 1B). This observation indicates that there are two types of alkaline phosphatase in the *pho8*(Ts)-*18*/*PHO8*$^+$ diploid, and that the *pho8*(Ts)-*18* lesion affects the thermostability of repressible alkaline phosphatase rather than the production of inhibitory material for the enzyme at 35°C.

TABLE 3. Tetrad distributions in combinations of *pho9* with one of the authentic *pho8*, *pho2*, *PHO82-pho4*, *pho80*, *pho81*, and *pho85* mutations[a]

Gene pair	Tetrad distribution[b]		
	PD	NPD	T
pho9-pho8-2[c]	1	5	3
pho9-pho8-3[c]	2	6	3
pho9-pho8-4[c]	0	4	7
pho9-pho8-5[c]	0	2	4
pho9-pho2[d]	3	2	15
pho9-PHO82-pho4[d]	3	5	12
pho9-pho80[d]	1	2	2
pho9-pho81[d]	1	2	11
pho9-pho85[d]	3	1	6

[a] Acid and alkaline phosphatase activities of each tetrad segregant were determined by the respective staining methods for the colonies grown on nutrient low-P_i or high-P_i medium at 30°C.

[b] Each ascus was classified as parental ditype (PD), nonparental ditype (NPD), or tetratype (T) ascus, according to the segregation of the pair of *pho* genes.

[c] See text and Table 2 for the details of the combination of the crosses.

[d] The *pho9-1* strains (AL101-1D and AL203-9A) were crossed with various authentic *pho* mutants selected from our stock culture (not listed in Table 2).

TABLE 4. Alkaline phosphatase activity of the *pho8* and *pho9* mutants[a]

Strain	Genotype	Sp act (units/ml per OD_{660}[b]) in medium:	
		High P_i	Low P_i
P-28-24C	Wild type	0.027	0.081
AL211-12B	*pho8-2*	0.009	0.011
AL203-9A	*pho9-1*	0.008	0.011
AL51-1D (35°C)[c]	*pho8*(Ts)-*18*	NT[d]	0.012
AL51-1D (25°C)	*pho8*(Ts)-*18*	NT	0.044
P-28-24C (35°C)	Wild type	NT	0.068
P-28-24C (25°C)	Wild type	NT	0.089

[a] Cells were cultivated in nutrient high-P_i or nutrient low-P_i medium for 8 h at 30°C unless otherwise indicated. Alkaline phosphatase activity was assayed at 30°C unless otherwise noted, using cells permeabilized with a mixture of 4% ethanol and 1% toluene as enzyme source and *p*-nitrophenylphosphate as substrate.

[b] OD_{660}, Optical density at 660 nm.

[c] The temperature in parentheses is that for cultivation; the enzyme was assayed at 25°C.

[d] NT, Not tested.

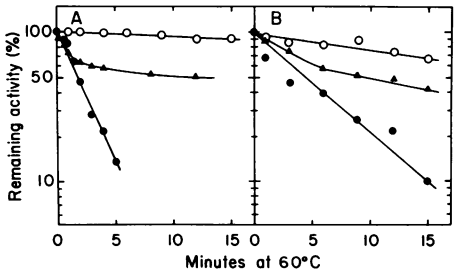

FIG. 1. Thermal inactivation of repressible alkaline phosphatases specified by the *pho8*(Ts)-*18* mutant allele and the wild-type allele. (A) Enzymes were partially purified from cells of the haploid *pho8*(Ts)-*18* mutant and the wild-type strain grown for 20 h by shaking in minimal low-P_i medium at 25°C. Each enzyme preparation or the mixture of the two preparations was kept at 60°C for the indicated period and then immediately cooled in an ice bath, and the remaining enzyme activity was assayed with *p*-nitrophenylphosphate as substrate at 25°C. Symbols: ●, *pho8*(Ts)-*18* (AL51-1D); ○, wild type (P-28-24C); and ▲, mixture containing approximately equal activities of both the enzyme preparations. (B) Crude cell extracts were prepared from diploid strains having the *pho8*(Ts)-*18*/*pho8*(Ts)-*18*, *pho8*(Ts)-*18*/*PHO8*$^+$, or *PHO8*$^+$/*PHO8*$^+$ genotype grown in nutrient low-P_i medium at 25°C for 15 h with shaking. The crude extracts were treated with 1% streptomycin, dialyzed against the standard buffer, and subjected to the thermal inactivation experiments at 60°C as described above. The remaining enzyme activity was assayed with α-naphthylphosphate as substrate at 25°C. Symbols: ● *phos8*(Ts)-*18*/*phos8*(Ts)-*18* (DH3); ○ *PHO8*$^+$/*PHO8*$^+$ (DH1); and ▲, *pho8*(Ts)-*18*/*PHO8*$^+$ (DH2).

Dosage effect of *PHO8*$^+$ on the enzyme activity. The structural gene for an enzyme should show a gene dosage effect on the enzyme activity. We examined the effect of *PHO8*$^+$ gene dosage on the repressible alkaline phosphatase activity in tetraploid strains. Simplex, duplex, triplex, and quadruplex tetraploid clones with respect to the *PHO8*$^+$ and *pho8-2* alleles were constructed from strains P-28-24C (**a** *PHO8*$^+$), F16C (α *PHO8*$^+$), AL20-3C (**a** *pho8-2*), AL20-2A (α *pho8-2*), KYC73 (**a** *PHO8*$^+$ *arg6*), and AL211-12B (**a** *pho8-2 arg6*) by the RD auxotroph mating method as described in Materials and Methods. On shaking these tetraploids in nutrient low-P_i at 30°C for 8 h, the specific activity of alkaline phosphatase for *p*-nitrophenylphosphate as substrate increased in proportion to the number of *PHO8*$^+$ alleles (Fig. 2A). Since the component strains used in the tetraploid breeding are thought to be isogenic for specific *p*-nitrophenylphosphatase, we could ignore the contribution of this enzyme to the different levels of alkaline phosphatase activity of the tetraploids. Thus, the *PHO8* gene showed a dosage effect on the activity of repressible alkaline phosphatase. This is consistent with the expectation that the *PHO8* gene is the structural gene encoding repressible alkaline phosphatase.

Mapping of the *pho8* locus. Tetrad analyses revealed a linkage between *pho8* and some of the genes on chromosome IV. In crosses of *pho8* mutants (AL30-1A, AL30-2C, AL32-3D, AL70-3A, AL71-1A, AL81-3B, and AL82-2A) and *ade8* strains (X963-18C, 1164, and 1165), we found a ratio of parental ditype to nonparental ditype to tetratype tetrads (PD/NPD/T ratio) of 218:13:157. From these data, the distance between *pho8* and *ade8* on chromosome IV was calculated at 30 centimorgans by the equation of Perkins (12). The tetrad distribution in crosses of *pho8* and *rna3* using strains 1164 and 1165 as *rna3* standards showed a PD/NPD/T ratio of 213:1:35, from which the distance between the genes was calculated at 8 centimorgans. Similarly, the distance between *ade8* and *rna3* was calculated at 17 centimorgans on the basis of the tetrad distribution (PD/NPD/T ratio) of 133:1:58; with the data of the common crosses of the *pho8-ade8* and *pho8-rna3* combinations, the gene order on the right arm of chromosome IV is *ade8-rna3-pho8*.

Characterization of *pho9* mutation. To characterize the enzyme species affected by the *pho9* mutation, the cell extract of a *pho9* mutant, AL101-1D, grown in nutrient low-P_i was fractionated by DEAE-Sephadex A-50 column chromatography as described in Materials and Methods. Only one peak of enzyme activity was observed, at the NaCl concentration of 0.1 M

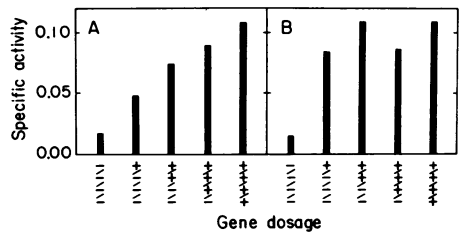

FIG. 2. Alkaline phosphatase activity of tetraploid strains bearing various combinations of (A) *pho8-2* mutant and wild-type alleles and (B) *pho9-1* mutant and wild-type alleles. Cells of each tetraploid were grown in nutrient low-P_i medium at 30°C for 8 h, at which time the culture attained an optical density at 660 nm of 1.2 to 1.5 units and showed the highest level of enzyme activity. Cells were harvested, and alkaline phosphatase activity was assayed using cells permeabilized by treatment with a mixture of 4% ethanol and 1% toluene as enzyme source and *p*-nitrophenylphosphate as substrate. Specific activity is expressed as units per milliliter per optical density unit at 660 nm. + and − symbols of abscissa indicate the wild-type and mutant alleles, respectively.

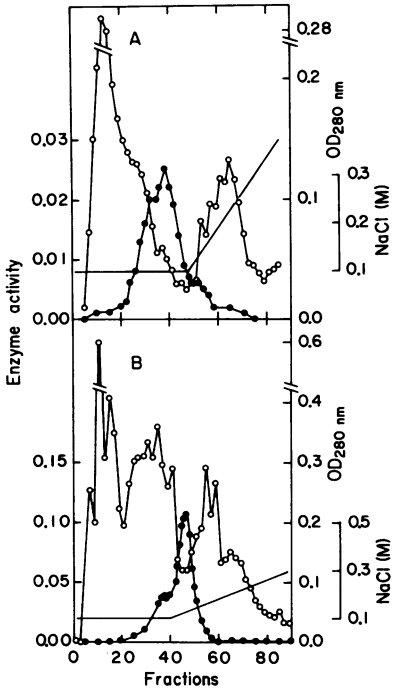

FIG. 3. DEAE-Sephadex A-50 column chromatography of the alkaline phosphatases of (A) *pho9-1* and (B) *pep4-3* mutants. Crude extracts were prepared from cells of strains AL101-1D (*pho9-1*) and 20B-12 (*pep4-3*) grown by shaking in nutrient low-P_i medium at 30°C overnight, and were subjected to column chromatography as described in Materials and Methods. Enzyme activity (●) in each fraction was assayed with *p*-nitrophenylphosphate as substrate and expressed as units per milliliter of effluent. Protein content (○) in each fraction was assayed spectrophotometrically at 280 nm. The solid line indicates the gradient of NaCl.

(Fig. 3A). This peak of enzyme activity must be that of specific *p*-nitrophenylphosphatase, because the crude extract of the mutant bearing *pho9* did not contain the activity to hydrolyze α-naphthylphosphate (data not shown), and the repressible alkaline phosphatase activity should be eluted at the NaCl concentration of 0.2 M (20). These facts indicate that the *pho9* mutation gives rise to a deficiency in repressible alkaline phosphatase activity, as does the *pho8* mutation, but does not affect the specific *p*-nitrophenylphosphatase.

Since the syntheses of both repressible acid and alkaline phosphatases are controlled in part by a common mechanism (11, 16, 20, 23), it is interesting to investigate the synthesis of repressible acid phosphatase in the *pho9-1* mutant. No differences in acid phosphatase activity were observed between colonies of strain AL212-4D (*pho9-1*) grown on nutrient high-P_i or low-P_i plates and those of strain P-28-24C (wild type) when tested by the staining method (data not shown). This was confirmed by examining the time courses of appearance of repressible acid phosphatase activity in the same strain (Fig. 4). These facts clearly indicate that the *PHO9* gene is effective in the synthesis of repressible alkaline phosphatase but not acid phosphatase.

Double mutants having the *pho9* mutation and the *pho80*, *pho85*, or *PHO82* mutations, which confer constitutive synthesis of repressible acid and alkaline phosphatases, showed the same phenotype as the *pho9* mutant with respect to synthesis of repressible alkaline phosphatase. Therefore, the *pho9* mutation is epistatic to the whole regulatory system consisting of *pho80*, *pho85*, *PHO82-pho4* (18, 20), and *pho81* (*PHO81* gene is hypostatic to the *pho80* and *pho85* mutations [23]) genes in the synthesis of the enzymes.

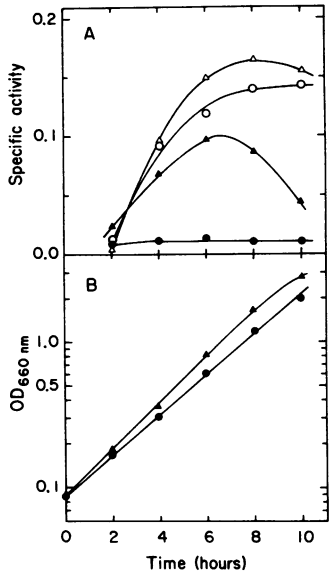

FIG. 4. Time courses of the production of repressible acid and alkaline phosphatases by the *pho9-1* and wild-type strains in nutrient low-P_i medium at 30°C. During the cultivation, samples were taken at intervals, and acid phosphatase activity (A, open symbols) was determined using intact cells as the enzyme source and *p*-nitrophenylphosphate as substrate. Alkaline phosphatase activity (A, closed symbols) was determined using cells permeabilized to the substrate by treatment with a mixture of 4% ethanol and 1% toluene as the enzyme source and *p*-nitrophenylphosphate as substrate. Specific activity for both the enzymes was expressed as unit per milliliter per optical density unit at 660 nm. (A) Symbols: ○, ●, AL212-4D (*pho9-1*); △, ▲, P-28-24C (wild type). (B) Cell growth of the mutant (●) and wild-type (▲) strains was monitored by reading optical density of the culture at 660 nm.

To test the dosage effect of the *PHO9*$^+$ gene on the enzyme activity, tetraploid strains bearing simplex, duplex, triplex, and quadruplex combinations of the *PHO9*$^+$ and *pho9-1* alleles were constructed from strains P-28-24C (**a** *PHO9*$^+$), F16C (α (*PHO9*$^+$), AL101-1D (**a** *pho9-1 trp5*), AL101-2A (α *pho9-1 trp5*), AL212-4D (**a** *pho9-1 ade10*), KYC73 (**a** *PHO9*$^+$ *arg6*), and AX45-21A (**a** *PHO9*$^+$ *leu1*) by the forced mating and RD auxotroph mating methods, and their specific activities were determined. The result (Fig. 2B) contrasts strikingly with that for the *PHO8* gene (Fig. 2A) and indicates that the *PHO9* gene lacks a dosage effect on the synthesis of repressible alkaline phosphatase. It further suggests that the *PHO9* gene does not code for the structure of repressible alkaline phosphatase, but rather functions in another way for expression of the *PHO8* gene.

Sporulation deficiency of the *pho9/pho9* diploid. During our study, we observed that diploid cells of *pho9/pho9* genotype did not sporulate. To confirm this, we constructed four diploid strains of *pho9-1/PHO9*$^+$ genotype by crossing AL203-9D (**a** *pho9-1 trp5*) with AX48-2C (α *PHO9*$^+$*ade10*), AX49-4B (α *PHO9*$^+$ *his4*), AX51-3C (α *PHO9*$^+$ *his7*), or AX54-10C (α *PHO9*$^+$ *ade10*). All the diploids could sporulate, and four-spored asci were dissected. Each haploid segregant was crossed with a haploid *pho9-1* strain (AL203-9A, AL203-9D, AL212-4D, or AL212-6C), and the resultant diploids were tested for their ability to sporulate. Although none of the *pho9-1/pho9-1* diploids could sporulate, in 30 asci tested so far, all the *PHO9*$^+$*/pho9-1* diploids could (data not shown).

To see whether the deficiency of sporulation in the *pho9-1/pho9-1* diploids is caused by a defect at or before the premeiotic DNA replication, diploids DI1 (*PHO9*$^+$*/PHO9*$^+$), DI2 (*PHO9*$^+$*/pho9-1*), and DI3 (*pho9-1/pho9-1*) were constructed as described in Table 2. All of the diploids were heteroallelic for the *leu1-ELF52* and *leu1-SA54* alleles. From the frequencies of Leu$^+$ colonies, we examined the intragenic recombination of the *leu1* locus in these diploids during sporulation (Table 5). In strains DI1 and DI2, the frequency of Leu$^+$ colonies increased about 100-fold after incubation for 10.5 h. In DI3, however, the frequency showed no increase after at least 72 h. These results indicate that no commitment to intragenic recombination is made in the *pho9-1/pho9-1* diploid and suggests that the defect in sporulation is at or before premeiotic DNA synthesis (3, 4, 14).

The *pho9* mutation is allelic with the *pep4* mutation. Recently we have learned that one of the 59 proteinase mutants isolated by Jones (8) is pleiotropic, with reduced levels of proteinases A and B, carboxypeptidase Y, and RNase of 8 to 10% of wild-type levels and of alkaline phosphatase of 25 to 30% of wild type (7). All five enzyme deficiencies cosegregated with that mutation, i.e., *pep4*. All five affected enzyme activities are located in the vacuole, although alkaline phosphatase is also found in the cytoplasm (26). We were also informed that the *pep4/pep4* diploid cannot sporulate (27; E. W. Jones, personal communication). Hence, the *pep4* mutation has an effect strikingly similar to that of *pho9* on the synthesis of alkaline phosphatase and sporulation. To confirm the similarity, we compared the *pho9* and *pep4* mutations. A test strain, 20B-12 (α *pep4-3 trp1 gal2 SUC*), was provided for the study by E. W. Jones. The profile of the cell extract of the *pep4-3* mutant on DEAE-Sephadex A-50 column chromatography (Fig. 3B) was similar to that of the *pho9-1* mutant (Fig. 3A): only one peak of alkaline phosphatase activity was observed by using *p*-nitrophenylphosphate as substrate, and the enzyme activity of this peak failed to hydrolyze α-naphthylphosphate. These results indicate that the *pep4*-bearing mutant lacks the repressible alkaline phosphatase but has specific *p*-nitrophenylphosphatase, as does the *pho9-1* mutant.

A heterozygous *pep4-3/pho9-1* diploid constructed by crossing 20B-12 and AL224-4D (**a** *pho9-1 leu1-ELF52*) showed no activity of repressible alkaline phosphatase and hardly sporu-

TABLE 5. Frequency of appearance of Leu$^+$ clones during incubation on sporulation medium in a diploid cell population having the *leu1-ELF52/leu1-SA54* and either the *PHO9*$^+$*/PHO9*$^+$, *pho9-1/PHO9*$^+$, or *pho9-1/pho9-1* genotype[a]

Strain	Genotype	Number of Leu$^+$ clones per 10^6 viable cells at time:		
		0 h	10.5 h	72 h
DI1	$\frac{leu1\text{-}ELF52\ PHO9^+}{leu1\text{-}SA54\ PHO9^+}$	8.5	720	NT[b]
DI2	$\frac{leu1\text{-}ELF52\ pho9\text{-}1}{leu1\text{-}SA54\ PHO9^+}$	10	860	NT
DI3	$\frac{leu1\text{-}ELF52\ pho9\text{-}1}{leu1\text{-}SA54\ pho9\text{-}1}$	22	21	8.9

[a] Diploid cells grown on nutrient agar at 30°C for 1 day were transferred onto sporulation medium and incubated at 30°C. Cells were scraped from the sporulation medium at 0, 10.5, and 72 h after inoculation and suspended in sterile water. After appropriate dilution, the suspensions were spread on minimal plates to count the Leu$^+$ prototrophs and on nutrient plates to count viable cells. Plates were incubated at 30°C for 3 days.

[b] NT, Not tested.

lated. Rare asci (33 asci) produced by the diploid were dissected and analyzed. No wild-type recombinant with repressible alkaline phosphatase activity was found in any tetrad. These results indicate that the *pep4* and *pho9* genes are allelic and that the *pep4-3* and *pho9-1* mutant alleles are defects in the same cistron.

DISCUSSION

The simplest interpretation for the observations that the *pho8*(Ts)-*18* mutation affects the thermolability of repressible alkaline phosphatase activity and that the *PHO8* gene has a dosage effect on the same enzyme activity is that the *PHO8* gene encodes the structure of repressible alkaline phosphatase. In contrast to *PHO8*, the *PHO9* gene shows no dosage effect on the specific activity of the enzyme, whereas the *pho9* mutation gives rise to the same phenotype as the *pho8* mutation with respect to the synthesis of repressible alkaline phosphatase. How then does the *PHO9* gene exert its function? This has been argued elsewhere (11). It was proposed that the *PHO9* gene product works as a specific factor for the expression of *PHO8*, in the same manner that the *PHO2* gene product participates in the expression of *PHO5*, the structural gene for repressible acid phosphatase (19), and an unidentified structural gene for inorganic phosphate permease (unpublished data) as a specific regulatory factor. In this scheme, the *PHO2* and *PHO9* genes convey the signals of the presence or absence of the effector in the medium from the regulatory circuit consisting of the *PHO81*, *PHO80*, *PHO85*, and *PHO4* gene products to the respective structural genes. It is interesting that *PHO9* (i.e., *PEP4*) is pleiotropic with respect to the synthesis of various cellular activities, most of them localized in vacuole, whereas *PHO2* is pleiotropic with respect to two enzymes for phosphorus metabolism located on the cell envelope.

This hypothesis concerning the function of *PHO2* is consistent with the recent observation of Bostian et al. (1), from assays of mRNA specific for acid phosphatase, that the appearance of enzyme is controlled at the transcriptional level. The participation of some of the *pho* genes, i.e., *PHO2*, *PHO4*, and *PHO80*, in the regulation of *PHO5* transcription was further supported by Kramer and Andersen (9). Although these authors failed to detect mRNA derived from the expression of putative structural gene(s) for acid phosphatase in *pho2* and *pho4* mutants grown in low-P_i medium by examining cDNA of mRNA species hybridizable with cloned DNA sequences containing a supposed structural gene of acid phosphatase, they did detect the mRNA in the *pho80* mutant regardless of whether cells were cultivated in high-P_i or low-P_i medium.

Recently, however, Hemmings et al. (7) have suggested a different function of *PHO9*. They have characterized a *pep4-3* (i.e., *pho9*) mutation that prevents maturation of a larger precursor of carboxypeptidase Y, a vacuolar enzyme in *S. cerevisiae*. They argue that the *PEP4* (i.e., *PHO9*) gene product participates in a common processing event for the proenzymes of the affected enzymes—at least for five vacuolar enzymes: proteinases A and B, carboxypeptidase Y, RNase, and nonspecific alkaline phosphatase (7, 26)—while alkaline phosphatase is also located in the cytoplasm. In this case *PHO9* would exert its function at the post-translational stage. Whether the *PHO9* gene exerts its function at the transcriptional stage as argued previously (11) or at the post-translational stage remains to be investigated.

The *pho9-1*/*pho9-1* diploid neither sporulated nor committed itself to intragenic recombination. These findings suggest that the sporulation of the *pho9*/*pho9* diploid is arrested at or before premeiotic DNA synthesis, because the commitment to recombination is detected at the time of onset of premeiotic DNA synthesis (3, 4, 14). However, the defect in repressible alkaline phosphatase activity is not directly concerned with the deficiency in meiosis, because the *pho8*/*pho8* cells could sporulate (data not shown).

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