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Two new genes, *PHO86* and *PHO87*, involved in inorganic phosphate uptake in *Saccharomyces cerevisiae*

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Abstract The *PHO84* gene in *Saccharomyces cerevisiae* encodes a P_i transporter, mutation of which confers constitutive synthesis of repressible acid phosphatase (rAPase), in medium containing repressible amounts of P_i, and an arsenate-resistant phenotype. We selected an arsenate-resistant mutant showing the constitutive synthesis of rAPase on nutrient plates containing 4.5 mM arsenate. This mutant has double mutations designated as *pho86* and *pho87*. The mutant transcribes *PHO84* even in the repressible condition but has a severe defect in P_i uptake. The constitutive rAPase$^+$ phenotype of the *pho86 pho87* mutant was partially suppressed by an increased dosage of the *PHO84* gene. The *PHO87* gene was found to be identical with *YCR524*, according to the published nucleotide sequence of chromosome III, which encodes a protein of 923 amino-acid residues with a highly charged N-terminal half followed by a C-terminal half consisting of 12 membrane-spanning segments as in Pho84p. These and the other findings suggest that the Pho86p and Pho87p proteins collaborate with Pho84p in P_i uptake.

Key words Arsenate resistance · Phosphatase regulation · P_i-transporter · Yeast

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Introduction

Two systems have been shown to be involved in the active transport of inorganic phosphate (P_i) into *Saccharomyces cerevisiae* cells from the medium (Tamai et al. 1985). One has a low *K*m value (8.2 μM) and the other a high *K*m value (770 μM) for external P_i. The *PHO84* gene most probably encodes a P_i transporter for the low *K*m system (Bun-ya et al. 1991). Its transcription is regulated by P_i through the *PHO* system for the regulation of repressible acid phosphatase (rAPase; EC 3.1.3.2) encoded by *PHO5* (Yoshida et al. 1989 a, b).

To investigate the P_i transport system in *S. cerevisiae*, we attempted to isolate arsenate-resistant mutants because, like vanadate (Willsky et al. 1985), arsenate is an analog of P_i in many cellular reactions and thus inhibits cellular metabolism and cell growth (Johnson 1971). A vanadate-resistant mutant of *Neurospora crassa* has been isolated and shown to have a mutation in a gene encoding a component of the high-affinity phosphate transport system (Bowman et al. 1983). Similar mutants of *Escherichia coli*, having an altered phosphate transporter, have been isolated by selection for arsenate resistance (Bennett and Malamy 1970). Although vanadate-resistant mutants of *S. cerevisiae* have been isolated, none of them had an alteration in phosphate transport (Willsky et al. 1985; Kanik-Ennulat and Neff 1990).

The present report describes the isolation of mutants showing resistance to 4.5 mM arsenate together with a rAPase$^+$ phenotype in high-P_i medium. These mutations were recessive to the wild-type counterpart and all the mutants were found to have a *pho84* mutant allele, except one that had two mutations designated *pho86* and *pho87*. The *pho86 pho87* double mutant transcribed the *PHO84* gene constitutively. The *PHO87* gene was found to be identical with *YCR524* (Thierry et al. 1990), encoding a putative membrane protein. These and other observations suggest that the

Pho86p and Pho87p proteins are involved in P_i uptake in collaboration with Pho84p.

Materials and methods

Organisms, plasmids and DNA fragments. The *S. cerevisiae* strains used are listed in Table 1. Except for three strains, MA3C, MB126 and MB158, all the others have the *pho3-1* genotype to eliminate the activity of other acid phosphatases. *E. coli* JA221 (Clarke and Carbon 1978) and MV1184 (Vieira and Messing 1987) were used for the manipulation of DNA, and LE392 (Maniatis et al. 1982) was used for the preparation of λ phage. Plasmids pUC118 and pUC119 (Vieira and Messing 1987) were used in the preparation of single-stranded DNAs and for the sequencing and manipulation of DNA. The plasmid vectors for the *S. cerevisiae* host were YCp50 as a low-copy number vector and YEp24 as a high-copy number vector (Parent et al. 1985). Two low-copy number plasmids, p373 bearing the *PHO84* and *URA3* genes (Bun-ya et al. 1991) and pAC879 ligated with a *GAL1p-PHO81* construct (Ogawa et al. 1995), were described previously. A high-copy number plasmid bearing the *PHO84* gene, pAC613, was constructed by filling in the *Eco*O109I end of a 2.6-kb *Eco*O109I-*Xho*I fragment of *PHO84* DNA prepared from pMB15 (Bun-ya et al. 1991) and inserting it into the *Sma*I-*Sal*I gap of YEp24 marked with *URA3*. pMB212, a high-copy number plasmid with the same architecture as pAC613 but with an inactive *pho84* gene, was constructed by restriction and filling in of the *Cla*I site in the *PHO84* open reading frame. A DNA fragment bearing the *PHO87* gene (i.e., *YCR524*) was obtained from an ordered DNA clone bank covering chromosome III of *S. cerevisiae* constructed by Yoshikawa and Isono (1990) with a λ phage vector. Plasmid pMB215 bearing the *YCR524* open reading frame was constructed by inserting a 6.0-kb *Eco*RI fragment (see Fig. 4) of the above *YCR524* DNA.

Media, and genetic and analytical methods. Nutrient YPAD (nutrient high-P_i), nutrient low-P_i, synthetic high-P_i (containing 11.0 mM P_i), low-P_i (0.22 mM P_i) and Ura test media for *S. cerevisiae*, and the LB and M9 media for *E. coli* together with most genetic and analytical methods, including rAPase assay of the cell suspension or by staining colonies, were as described previously (Bun-ya et al. 1991). Reported methods were used for assays of P_i uptake (Ueda and Oshima 1975) and mutagenesis with ethylmethane sulfonate (Lindegren et al. 1965) of *S. cerevisiae*. For transcription of the *GAL1p-PHO81* construct, YPAGal medium containing 2% of galactose, instead of glucose, in YPAD medium was prepared. For scoring the arsenate-resistant phenotype of *S. cerevisiae*, cells were cultivated in YPAD liquid medium at 30 °C for 1 day and plated onto YPAD plates containing 4.5 mM arsenate after appropriate dilution. The proper concentration of arsenate in the test medium was determined by several trials as described below. Colonies that developed on the arsenate plates were scored after incubation for 3–5 days at 30 °C.

Table 1 Yeast strains used

Strain	Genotype[a]	Source or reference
AX66-10D	*MAT***a** *pho3-1 leu2 lys1 ura3*	Our stock
F16C	*MATα pho3-1*	Our stock
KYC163	*MAT***a** *pho3-1 pho84-1 arg6 his6*	Our stock
KYC164	*MATα pho3-1 pho84-1 trp1*	Our stock
MA3C	*MAT***a** *ade2 his3 leu2 trp1 ura3*	Our stock
MB126	*MATα pho2::LEU2*[b] *pho84-1 his leu2*	Our stock
MB144	*MATα pho3-1 pho80::HIS3*[c] *PHO84-1 leu2 ura*	Our stock
MB158	*MATα pho4-1 pho84-1 his leu2*	Our stock
MB182	*MATα pho3-1 pho86-1 pho87-1 leu2 trp1 ura3*	A tetrad segregant of a diploidized clone of NS213 by introduction of the *HO* gene
MB247	*MAT***a** *pho3-1 pho87-1 leu2*	A segregant from a F16C × NS213 cross
MB248	*MATα pho3-1 leu2*	A segregant from a F16C × NS213 cross
MB249	*MATα pho3-1 pho86-1 lys2 ura3*	A segregant from a F16C × NS213 cross
MB250	*MAT***a** *pho3-1 pho86-1 pho87-1 lys1 ura3*	A segregant from a F16C × NS213 cross
NA87-11A	*MATα pho3-1 pho5-1 his3 leu2-3,112 trp1 can1*	Our stock
NS30-7B	*MAT***a** *pho3-1 pho87-1 leu2 lys1*	A segregant from a NS104 × F16C cross
NS30-7C	*MATα pho3-1 pho86-1 ura3*	A segregant from a NS104 × F16C cross
NS104	*MAT***a** *pho3-1 pho86-1 pho87-1 leu2 lys1 ura3*	This study
NS213	*MAT***a** *pho3-1 pho86-1 pho87-1 leu2 trp1 ura3*	A segregant from a NS30-7B × NS30-7C cross
NS214	*MAT***a** *pho3-1 pho86-1 pho87-1 leu2*	A segregant from a NS30-7B × NS30-7C cross
NS215	*MATα pho3-1 pho86-1 ura3*	A segregant from a NS30-7B × NS30-7C cross
O106-M30	*MAT***a** *pho3-1 PHO81*[c]*-1 gal4 his5 leu1*	Our stock
P-28-24C	*MAT***a** *pho3-1*	Our stock
P143-3A	*MAT***a** *pho3-1 pho2-1 arg6*	Yoshida et al. 1989 a, b
P144-2D	*MATα pho3-1 pho4-1 arg6*	Yoshida et al. 1989 a, b
P145-2B	*MATα pho3-1 pho81-1 arg6*	Yoshida et al. 1989 a, b
P146-8B	*MATα pho3-1 pho80-1 arg6*	Yoshida et al. 1989 a, b
SH1547	*MATα pho3-1 pho81-1 pho84-1 trp1*	Our stock
SH3614	*MAT***a** *pho84::HIS3 pho4::LEU2 his3-532 leu2 trp1 ura3-1,2*	Our stock

[a] The genetic symbols used are as described by Mortimer et al. (1992)
[b] A disrupted allele of *pho2* by insertion of a *LEU2* DNA
[c] A disrupted allele of *pho80* by insertion of a *HIS3* DNA

Results

The *pho84* mutant is resistant to arsenate

In a previous study, we observed that the *pho84* mutant is unable to incorporate P_i (Ueda and Oshima 1975). If the *pho84* mutant does not incorporate arsenate as P_i, it might be able to grow on medium with arsenate. We examined this possibility with the *pho84-1* mutant (KYC164) and various other authentic *pho* mutants, P143-3A (*pho2-1*), P144-2D (*pho4-1*), P145-2B (*pho81-1*), O106-M30 (*pho81^c-1*) and P146-8B (*pho80-1*), as well as with P-28-24C (wild-type). The cells were cultivated at 30 °C in YPAD medium overnight and spread on YPAD plates containing various amounts of arsenate and the plates were incubated at 30 °C for 3–5 days. All the *pho* mutants except *pho84* showed significantly reduced colony formation on YPAD plates with arsenate (Table 2). We confirmed that the arsenate resistance of the *pho84* mutant is caused by the *pho84* mutation by tetrad analysis of a diploid constructed by a KYC164 (*MAT*α *pho84*) × AX66-10D (*MAT***a** *PHO84*$^+$) cross. All 30 tetrads examined showed a 2 + :2 − segregation for rAPase activity in high-P_i medium and for arsenate resistance, and the rAPase$^+$ phenotype co-segregated with the arsenate-resistant phenotype.

To examine epistasis and hypostasis relationships of the above *pho* regulatory mutations to the *pho84* mutation in arsenate resistance, we spread cells of strains MB126 (*pho2*::*LEU2 pho84-1*), MB158 (*pho4-1 pho84-1*), MB144 (*pho80*::*HIS3 pho84-1*), and SH1547 (*pho81-1 pho84-1*) on YPAD plates containing 4.5 mM arsenate, and incubated the plates at 30 °C for 5 days. We found that only strain MB144 could form colonies on the plates. Thus, the *pho2*, *pho4*, and *pho81* mutations confer the arsenate-sensitive phenotype even when the cells have the *pho84* mutation. Since the *pho2*, *pho4*, and *pho81* mutations confer the rAPase$^-$ phenotype, we investigated whether the arsenate-resistant phenotype is correlated with rAPase production by examination of tetrad segregants of a diploid constructed by a NA87-11A (*MAT*α *pho5-1*) × KYC163 (*MAT***a** *pho84-1*) cross. In five tetrads tested, the diploid showed a 2 resistant:2 sensitive segregation for arsenate resistance, irrespective of the rAPase phenotype. This indicates that rAPase activity is not a prerequisite for the arsenate resistance shown by the *pho84* mutation. These results also suggest that the arsenate sensitivity of these double mutants is due to a defect, or loss, of *PHO81* expression because *PHO81* transcription requires Pho4p (encoded by *PHO4*) and Pho2p function (Ogawa et al. 1993). To test this possibility, pAC879 (bearing the *GAL1p-PHO81* construct and *URA3*$^+$) was introduced into cells of strain SH3614 (Δ*pho4::LEU2* Δ*pho84::HIS3 ura3*). We confirmed that the Ura$^+$ transformant could transcribe *PHO81* from the *GAL1*p-*PHO81* construct in YPAGal but not in YPAD (data not shown). However, the cells showed an arsenate-sensitive phenotype on both YPAD and YPAGal media. We recently reported that the *PHO81* gene expressed by the *GAL1*-promoter could not confer a rAPase$^+$ phenotype on galactose high-P_i medium (Ogawa et al. 1995). This suggests that Pho81p function is inhibited in a high-P_i condition. Therefore, we also examined the same transformant for its arsenate resistance in galactose low-P_i medium. This experiment did not give any conclusive result because the transformant hardly grew in the low-P_i medium due to the Δ*pho84* allele, as observed previously (Bun-ya et al. 1992). However, the fact that the *pho84* mutant could express the *PHO5* gene in high-P_i medium suggests that Pho81p is active in the Δ*pho84* cells even in high-P_i medium. These facts further suggest that Pho81p function, or the product of another unidentified gene whose expression is regulated by the *PHO* regulatory system, is indispensable for arsenate resistance.

Isolation of arsenate-resistant mutants

To isolate new mutations in the P_i transport machinery, we subjected cells of a *MAT***a** wild-type strain,

Table 2 Growth phenotypes of various *pho* mutants

Strain	Relevant genotype	Relative growth[a] at the indicated arsenate concen. (mM)						
		0	0.5	1.0	2.0	4.5	8.0	25
P-28–24C	Wild-type	++	++	++	+	−	−	−
KYC164	*pho84-1*	++	++	++	++	++	++	+
P143-3A	*pho2-1*	++	++	++	+	−	−	−
P144-2D	*pho4-1*	++	++	++	+	−	−	−
P145-2B	*pho81-1*	++	++	++	+	−	−	−
O106-M30	*PHO81^c-1*	++	++	±	−	−	−	−
P146-8B	*pho80-1*	++	+	±	−	−	−	−
NS30-7C	*pho86-1*	++	++	++	++	+	+	−
NS30-7D	*pho87-1*	++	++	++	−	−	−	−
NS213	*pho86-1 pho87-1*	++	++	++	++	++	++	+

[a] Growth was scored as follows: ++, growth after 3 days; +, growth after 4–5 days; ±, barely detectable growth, and −, no growth

AX66-10D, to EMS mutagenesis and the cells were then spread on YPAD plates containing 4.5 mM arsenate after appropriate dilution. The plates were incubated at 30 °C for 5–7 days, and the approximately 200 arsenate-resistant colonies which appeared on the plates were tested for rAPase activity by staining. Six colonies (clones NS101 to NS106) showed the rAPase$^+$ phenotype, and these were purified by repeated spreading on YPAD plates. When these six mutants were crossed with F16C (*MATα* wild-type), none of the resultant diploids showed the rAPase$^+$ phenotype on YPAD plates and all were sensitive to 4.5 mM arsenate. Thus all the mutations were recessive to the wild-type counterpart.

Tetrad analysis of the diploids showed a 2 rAPase$^+$:2 rAPase$^-$ segregation in YPAD, and the rAPase$^+$ segregants showed the arsenate-resistant phenotype in 5–7 tetrads tested for each cross. But the NS104 × F16C diploid showed a 2:15:2 ratio of ascus types with 2 rAPase$^+$:2 rAPase$^-$, 1 rAPase$^+$:3 rAPase$^-$, and 0 rAPase$^+$:4 rAPase$^-$ segregations in YPAD in 19 asci tested. These results indicate that the NS104 mutant has at least two recessive mutations, while each of the other five mutants has a single chromosomal mutation.

To determine whether the mutations are allelic to *pho84*, these mutants were crossed with the *MATα pho84-1* mutant, KYC164, and the resultant diploids were tested for rAPase activity on YPAD plates. All the diploids except NS104 showed the rAPase$^+$ phenotype. This result indicates that NS104 has a mutation(s) other than *pho84* but that each of the other five mutants has an allele of the *pho84* mutation.

To determine the number of mutations in NS104, an ascus showing a 0 rAPase$^+$:4 rAPase$^-$ segregation was selected from the asci of the NS104 × F16C cross. Tetrad segregants from such an ascus, NS30-7, i.e., spore-clones of 7A, 7B, 7C, and 7D, were crossed with either NS214 (*MAT***a**) or MB182 (*MATα*), both showing the same phenotypes as NS104, and the resultant diploids were subjected to tetrad analysis. If each of the tetrads of the NS30-7 ascus has a single mutation, tetrads from these diploids should show a 2 rAPase$^+$:2 rAPase$^-$ segregation in YPAD medium. The results were consistent with this prediction for 6–16 asci examined in each of these crosses. Thus, these tetrad clones should each have a single mutation for the rAPase$^+$ phenotype in YPAD medium, and for arsenate resistance. If the original mutant, NS104, has two mutations, each two-spore clone from the NS30-7 ascus should have the same mutation, but a different one from that of the other two spores. These mutations were designated as *pho86* and *pho87*.

To distinguish the genotypes of the four tetrads of the NS30-7 ascus, we carried out tetrad analyses of six possible combinations of crosses between these tetrad clones and those with some of the rAPase$^-$ segregants of diploids constructed by crosses of the original mutants with MB182 or NS214. Although detailed data are not shown, we were able to conclude that NS30-7C and NS30-7D have a mutation assigned as *pho86-1*, whereas NS30-7A and NS30-7B have another mutation, *pho87-1*. We also confirmed that MB182 and NS213 have double mutations, *pho86-1 pho87-1*, by examination of the rAPase and arsenate-resistant phenotypes of diploids constructed by crosses with tetrad clones of the NS30-7 ascus.

Characterization of the *pho86* and *pho87* mutations

We examined the sensitivities to arsenate of single and double mutants of *pho86* and *pho87* using cells of NS30-7C (*pho86-1 PHO87*$^+$), NS30-7B (*PHO86*$^+$ *pho87-1*), and NS213 (*pho86-1 pho87-1*). The *pho86 pho87* double mutant showed the same high level of survival in YPAD medium containing 25 mM arsenate as the *pho84* mutant (Table 2). In contrast, the *pho87* mutant was as sensitive to arsenate as wild-type cells, and the *pho86* mutant showed intermediate resistance. The *pho86 pho87* double mutant exhibited significant rAPase activity, whereas the *pho87* mutant had the same low level of rAPase activity as the wild-type strain (Fig. 1). The *pho86* mutant synthesized rAPase in synthetic high-P_i medium, but its activity was less than that of the *pho86 pho87* double mutant. In contrast with *pho84*, which had a significantly lower growth rate than the wild-type strain in synthetic low-P_i medium (Bun-ya et al. 1992), these mutants grew as fast as the wild-type strain in synthetic high-P_i and low-P_i media.

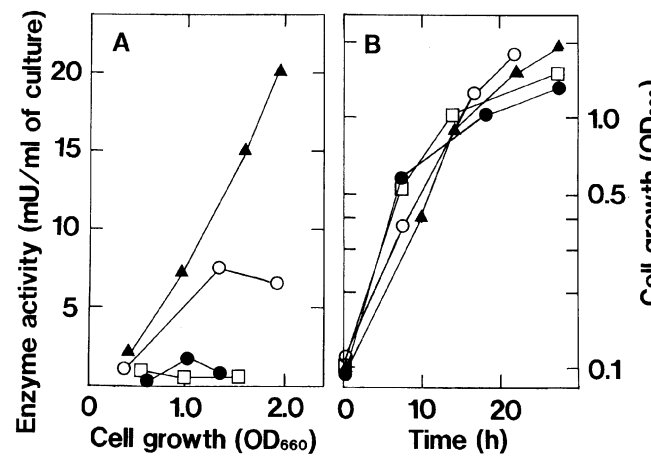

Fig. 1A, B Time courses of acid-phosphatase synthesis in the wild-type strain, and *pho86-1*, *pho87-1* and *pho86-1 pho87-1* mutants. Cells cultivated in synthetic high-P_i medium with shaking at 30 °C for 24 h were harvested, washed, and suspended in the same volume of sterilized water. Then 1 ml of the cell suspension were inoculated into 100 ml of synthetic high-P_i medium and the cultures were shaken at 30 °C. Acid-phosphatase activity (**A**) as a function of cell growth (**B**; D_{660} of the culture) was determined with the cell suspension as the enzyme source. Strains and genotypes: ●, MB248 (*PHO86*$^+$ *PHO87*$^+$); ○, MB249 (*pho86-1 PHO87*$^+$); □, MB247 (*PHO86*$^+$ *pho87-1*); and ▲, MB250 (*pho86-1 pho87-1*)

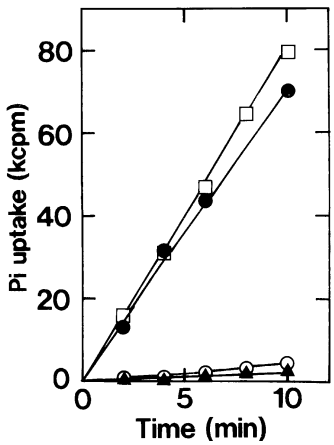

Fig. 2 P_i uptake by cells of the *pho86-1 pho87-1* double mutant. Cells were shaken at 30 °C in nutrient low-P_i medium. The cells were collected when growth reached a D_{660} of 1.0, washed and inoculated into synthetic low-P_i medium to give a cell concentration of $D_{660} = 0.1$. The radioactivity of the medium was adjusted to 8.6×10^5 cpm per ml with $^{32}P_i$. The amount of P_i absorbed by the cells was expressed as cpm of ^{32}P radioactivity per ml of cell suspension with a $D_{660} = 0.1$. Strains and genotypes: ●, MB248 (*PHO86*$^+$ *PHO87*$^+$): ○, MB249 (*pho86-1 PHO87*$^+$): □, MB247 (*PHO86*$^+$ *pho87-1*); and ▲, MB250 (*pho86-1 pho87-1*)

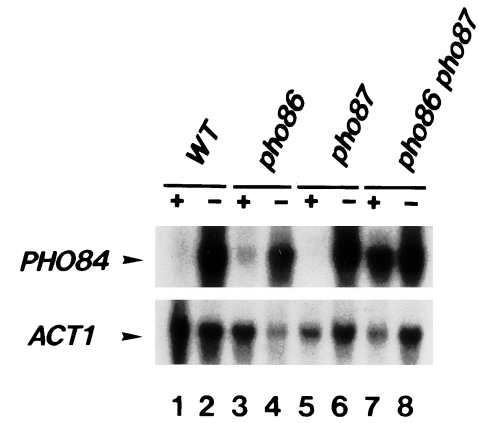

Fig. 3 Detection of the *PHO84* transcript in the wild-type strain and the *pho86*, *pho87* and *pho86-1 pho87-1* mutants by Northern-blot hybridization. Samples of 10 μg of total RNAs prepared from cells of P-28-24C (*PHO86*$^+$ *PHO87*$^+$; *lanes 1 and 2*) and NS30-7C (*pho86-1*; *lanes 3 and 4*), NS30-7B (*pho87-1*; *lanes 5 and 6*) and NS213 (*pho86-1 pho87-1*; *lanes 7 and 8*) grown on nutrient high-P_i (*lanes 1, 3, 5 and 7*) or low-P_i (*lanes 2, 4, 6 and 8*) medium were charged in slots. A ^{32}P-labeled 564-bp *Bgl*II-*Xho*I fragment of the *PHO84* DNA and that of a 1.0-kb *Hin*dIII-*Xho*I fragment of *S. cerevisiae* encoding the *ACT1* gene were used as the probes for detection of the *PHO84* and *ACT1* transcripts, respectively. The specific radioactivities of the probes were 1.0×10^8 cpm per μg of DNA. The 25 *s* and 18 *s* rRNAs, visualized by staining with ethidium bromide (data not shown), were used as size markers as described by Philippsen et al. (1978)

We also confirmed that the *pho86 pho87* double mutant (NS213) showed significantly lower P_i uptake activity in low-P_i medium than the wild-type strain (Fig. 2), like the *pho84* mutant (Bun-ya et al. 1992). The *pho86* mutant also showed reduced P_i uptake activity, whereas the *pho87* mutant had the same level as the wild-type strain. Thus, the *PHO86* gene exerts an important role in P_i transport, whereas the *PHO87* gene does not. The *pho87* mutation therefore confers high arsenate resistance only in combination with the *pho86* mutation.

The rAPase constitutive phenotype of the *pho86 pho87* double mutation was partially suppressed by increased dosage of the *PHO84* gene

To determine whether the rAPase$^+$ phenotype of the *pho86 pho87* mutant is suppressed by an increased dosage of the *PHO84* gene, we transformed NS213 (*pho86-1 pho87-1 ura3*) cells to Ura$^+$ with a plasmid of low copy number, p373, bearing the *PHO84* gene and marked with *URA3*. Four Ura$^+$ transformants isolated at random were tested for rAPase production in synthetic high-P_i medium. All these Ura$^+$ transformants showed the rAPase$^+$ phenotype. However, when we introduced pAC613, a high-copy number plasmid bearing the *PHO84* gene, into NS213 cells, the Ura$^+$ transformants showed significantly lower rAPase activity (8.7 ± 1.39 milli-units per D_{660}/ml of culture; standard deviation calculated from triplicated determinations) than that of control transformants with YEp24 (43.6 ± 2.10 milli-units per D_{660}/ml), which gave the same level of activity as transformants with p373. To examine whether the suppression was due to an increased dosage of the *PHO84*$^+$ gene, we measured rAPase activity of the same NS213 cells harboring plasmid pMB212 (which has the same structure as pAC613 but has a 2-bp insertion into the *PHO84* coding region) in synthetic high-P_i medium and found much higher rAPase activity ($21.1 + 1.93$ milli-units per D_{660}/ml) than that of pAC613. These results suggest that the increased rAPase activity of the *pho86 pho87* double mutant in high-P_i medium is caused by a reduction of Pho84p function.

PHO84 is transcribed in the *pho86 pho87* mutant

Since the rAPase$^+$ phenotype of the *pho86 pho87* mutant was suppressed by an increased dosage of *PHO84*, transcription of the genomic *PHO84* gene might be reduced in the double mutant. To examine this possibility, we prepared total RNAs from NS213 (*pho86 pho87*) cells cultivated in nutrient low-P_i or high-P_i medium for 12–16 h and subjected them to Northern analysis with a ^{32}P-labeled fragment of *PHO84* DNA as a probe. The results showed that the *PHO84* gene was transcribed substantially in *pho86 pho87* cells, irrespective of whether the cells were cultivated in high-P_i or low-P_i medium, while the *pho86* or *pho87* single mutants showed a significant reduction of *PHO84* transcription in high-P_i medium (Fig. 3). These observations, together with the fact that the P_i transport

activity was markedly reduced in the *pho86 pho87* mutant (Fig. 2), strongly suggest that the Pho86p and Pho87p proteins are involved in P_i uptake in collaboration with the Pho84p protein.

The *PHO87* gene encodes a putative membrane protein

During these analyses, we noticed close linkage between the *pho87* and *MAT* loci on chromosome III. Furthermore a NS214 (*MAT***a** *pho86-1 pho87-1*) × NS215 (*MATα pho86-1*) cross showed a tetrad distribution of 38:0:0 for the PD:T:NPD ratio of the **a** mating type and arsenate-resistant phenotypes in 38 asci tested. The distance between the *pho87* and *MAT***a** loci was calculated from the above data by the Perkins' equation (1949) to be less than 1.3 centimorgans.

For cloning the *PHO87* gene, various DNA fragments of the *MAT* region from the ordered DNA clone bank (Yoshikawa and Isono 1990) were subcloned into a YCp50 vector and a 4.7-kb *Eco*RV_1-*Eco*RI_2 fragment suppressing the rAPase$^+$ phenotype of the *pho86 pho87* double mutant on YPAD was selected (Fig. 4). We determined the nucleotide sequence of each approximately 200-bp region at the left and right ends of the above fragment and found that the sequences were identical to those of the 4.7-kb *Eco*RV_1-*Eco*RI_2 region characterized by Thierry et al. (1990). This region contains only one ORF, *YCR524*, of 2769 bp. Thus, *PHO87* might be identical with *YCR524*.

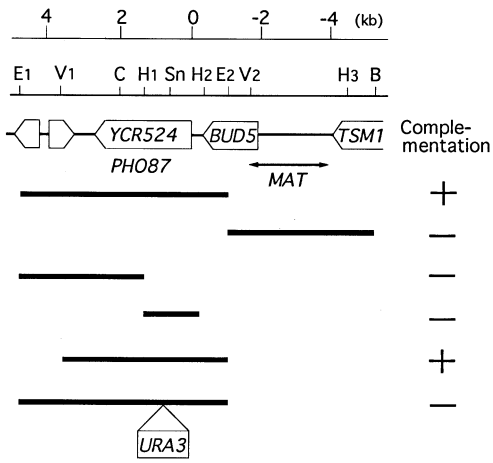

Fig. 4 Restriction map of a DNA region at the *PHO87* locus and results of complementation tests with various sub-fragments. *Open arrows* indicate the approximate position and direction of *YCR524* (Thierry et al. 1990), *BUD5* (Chant et al. 1991), and *TSM1* (Ray et al. 1991) relative to the *MAT* locus. The open arrow originally assigned as *YCR524* was reassigned as *PHO87* by the present study. The *thick lines* indicate yeast DNA fragments ligated in the YCp50 vector. Disruption allele of *YCR524* was constructed by inserting DNA fragment containing the *URA3* gene into the *Sna*BI site (see text for details). The symbols + and − indicate ability and inability to complement the rAPase$^+$ phenotype of the *pho86-1 pho87-1* double mutant, NS213, in high-P_i medium. Abbreviation of restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Sn, *Sna*BI and V, *Eco*RV

To confirm this, we inserted a 1.2-kb *Hin*dIII fragment of the *URA3* DNA, after blunt-ending the fragment, into the *Sna*BI site in the 6.0-kb *Eco*RI_1-*Eco*RI_2 fragment of the *YCR524* DNA (Fig. 4) and the composite DNA fragment was inserted into the *Eco*RI site of pUC118. The genomic *YCR524* locus of a diploid strain (*MAT***a**/*MATα* and homozygous for *PHO84*$^+$ *PHO86*$^+$ *PHO87*$^+$; constructed by autogamous diploidization of MA3C by introduction of a plasmid bearing the *HO* gene) was disrupted with the 7.2-kb *Eco*RI_1-*Eco*RI_2 fragment containing the *URA3* DNA prepared from the above plasmid by the method of one-step gene disruption (Rothstein 1983). The Ura$^+$ transformants showed normal sporulation and a 2 + :2 − segregation in asci for the Ura phenotype, but all the spore-clones showed a wild-type phenotype for rAPase (data not shown). One of the Ura$^+$ spore clones was crossed with NS213 (*pho86-1 pho87-1*) and the resultant diploid was subjected to tetrad analysis. A 2 + :2 − segregation was observed for the rAPase phenotype on YPAD high-P_i medium and arsenate resistance in all of eight asci tested, and these phenotypes co-segregated, indicating that the *PHO87* and *YCR524* genes are identical. We crossed one of the segregants showing arsenate resistance and constitutive rAPase synthesis with MB250 (*pho86 pho87*) and found that as expectedly, the resultant diploid showed arsenate resistance and constitutive rAPase production.

According to the nucleotide sequence determined by Thierry et al. (1990), the *YCR524*/*PHO87* ORF encodes a protein with 923 amino-acid residues. A hydropathy plot of the amino-acid sequence of the deduced Pho87p protein by the method of Kyte and Doolittle (1982) showed a highly charged region from the N-terminal to the 461th amino-acid residue, followed by a C-terminal half with almost regular alterations of hydrophilic and hydrophobic segments (Fig. 5). Based on the algorithm of Eisenberg (1984), Pho87p consists of 12 putative membrane-spanning segments, each consisting of 21 amino-acid residues. The putative Pho84p protein has two blocks of six membrane-spanning segments, separated by a segment of charged amino acids (Bun-ya et al. 1991). The putative Pho87p protein also has two blocks of six transmembrane domains separated by a segment of charged amino acids (9 of 19 amino-acid residues). Potential *N*-linked glycosylation sites (Lehle and Bause 1984) are found at amino-acid positions 162, 202, 274, and 872 from the N-terminal, and potential threonine substrate sites for cyclic AMP-dependent protein kinase at amino-acid positions 78 and 99. We detected some similarity between the predicted amino-acid sequence of Pho87p at the hydrophilic N-terminal region (from amino-acid position 1 to 362) and that (from amino-acid position 1 to 196) of Pho81p (Ogawa et al. 1993) (Fig. 6). Pho81p is a member of the *PHO* regulatory

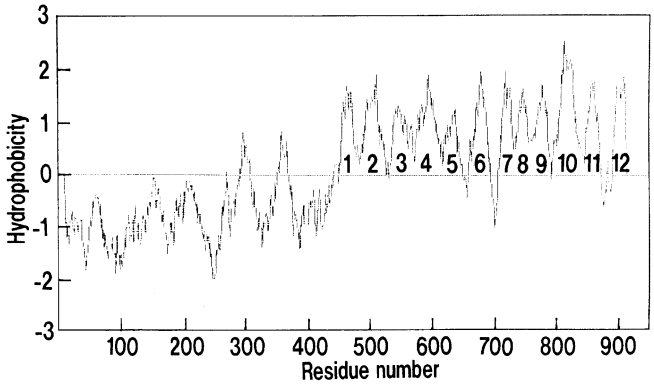

Fig. 5 Hydrophobicity profile of the predicted Pho87p protein. Hydropathy values for a window of 21 amino-acid residues were averaged, assigned to the middle residue of the span, and plotted with respect to position along the amino-acid sequence. The numbers refer to putative membrane-spanning domains predicted by the algorithm of Eisenberg (1984)

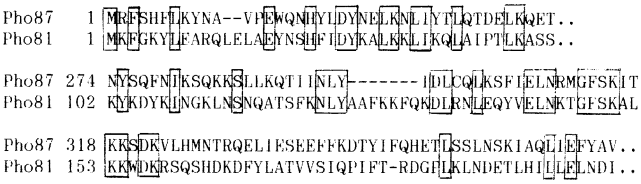

Fig. 6 Sequence similarities of amino acids between the predicted Pho87p and Pho81p proteins. The two amino-acid sequences are aligned to obtain maximum fitting. Identical residues of Pho87p (= *YCR524*; Thierry et al. 1990) with Pho81p (Ogawa et al. 1993) are *boxed*. *Numbers* at the left indicate positions of the first amino-acid residues in the respective lines. *Dashes* indicate gaps introduced to optimize the alignment

circuit and *pho81* mutations, in general, confer a rAPase$^-$ phenotype on the cells (Yoshida et al. 1989 b). However, an increased dosage of the *PHO87* gene could not complement the *pho81* mutation (data not shown). Pho81p consists of 1179 amino-acid residues and has six repeats of an ankyrin-like motif in the central region (from amino-acid position 424 to 656) of the protein. Although the N-terminal domain of Pho81p has been suggested to modulate Pho81p activity negatively through intramolecular interaction with the C-terminal domain of Pho81p (Ogawa et al. 1995), it is not known at present whether the homologous regions between Pho81p and Pho87p that we detected play some role in the uptake and signal transduction of P_i.

A 9-bp motif, 5′-GCACGTGGG-3′ or 5′-GCACGTTTT-3′, is known to function as the binding site for Pho4p, a specific positive regulator for the transcription of genes in the *PHO* regulon (Ogawa et al. 1994). In the promotor region of *PHO87*, we found one copy of the above motif, 5′-GCACGTGGG-3′, at nucleotide position -132 from the ATG codon. Therefore, by Northern-blot hybridization using a ^{32}P-labeled 0.8-kb *Hin*dIII$_1$-*Cla*I fragment of the *YCR524*/*PHO87* ORF (Fig. 4) as a probe we investigated whether *PHO87* transcription is regulated by external P_i. RNA samples of a wild-type strain cultivated in low-P_i and high-P_i (YPAD) media gave a single hybridization band of approximately 3.3 kilobases, whereas no significant differences were observed in the amounts of the *PHO87* transcript in the cells cultivated in high-P_i and low-P_i media (data not shown).

Discussion

The toxic inorganic-ion-resistant phenotype caused by mutation has been explained by several mechanisms. One is de-toxification of the ion, as in the case of a *CUP1*^{r} mutant in *S. cerevisiae* (Fogel and Welch 1982). This was shown to be accomplished by an increase in the copy number of the *CUP1* gene. A second mechanism is a defect in an ion-uptake system. The vanadate (an analog of inorganic phosphate)-resistant mutation of *N. crassa*, caused by a defect in the high-affinity P_i transport system (Bowman et al. 1983), is an example of this type. Another possibility is the acquisition of a pumping out system for the ion.

Wild-type strains of *S. cerevisiae* are sensitive to arsenate. The *pho84* single mutation and the *pho86 pho87* double mutation both conferred an arsenate-resistant phenotype, as described. This might result from a defect in either arsenate uptake or the penetration mechanism, because these mutants are defective in P_i uptake. The increased resistance caused by the *pho84* mutation was reduced by a *pho2*, *pho4*, or *pho81* mutation, which resulted in the loss of expression of the *PHO5* and *PHO84* genes. Another class of mutants, the *PHO81*^{c} and *pho80* mutants showing constitutive expression of the *PHO5* and *PHO84* genes, were more sensitive to arsenate than were the *pho2*, *pho4*, and *pho81* mutants (Table 2). Based on these observations, we speculate that Pho81p (or an unidentified protein in the *PHO* regulon) is required for arsenate resistance. Thus, the sensitivity or resistance of *S. cerevisiae* to arsenate is affected by several different mechanisms.

The *pho86 pho87* double mutant could transcribe *PHO84* irrespective of whether the cells were grown in low-P_i or high-P_i medium (Fig. 3), and the rAPase$^+$ phenotype of the double mutant was partially suppressed by an increased dosage of the *PHO84* gene. These observations strongly suggest that the Pho86p and Pho87p proteins are involved in the P_i uptake mechanism in collaboration with the Pho84p P_i-transporter. The *pho86* single mutation resulted in a substantial reduction of P_i uptake by the cells. This indicates that Pho86p is directly associated with the Pho84p P_i transport system. The *PHO86* gene was cloned recently and its deduced amino-acid sequence suggested that Pho86p is also a membrane protein (our unpublished results). In contrast, the phenotype of the

pho87 single mutant and a disruptant of it did not differ significantly from that of the wild-type cells, while the *pho87* mutation conferred increased resistance to arsenate and the rAPase$^+$ phenotype in the *pho86 pho87* double mutant. One possible explanation of these findings is that Pho87p is concerned with another P_i transport system independent of Pho84p. This was supported by the fact that the putative Pho87p protein has 12 transmembrane domains (Fig. 5), like the Pho84p P_i-transporter. Thus, Pho87p may function in the low-affinity P_i uptake system, while no similarities were found between the amino-acid sequences of Pho84p and Pho87p. These arguments suggest that Pho84p and Pho86p (and possibly Pho87p) form a complex on the cell membrane. Another protein, Gtr1p, might also be involved in the above P_i-transporter complex as it has been shown to be directly involved in Pho84p function (Bun-ya et al. 1992).

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