Low-Affinity Orthophosphate Carriers Regulate PHO-genes Expression Independently of Internal Orthophosphate Concentration in Saccharomyces cerevisiae

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Running title : Low-affinity phosphate carriers and phosphate sensing

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Phosphate is an essential nutrient that must be taken up from the growth medium through specific transporters. In Saccharomyces cerevisiae, both high- and low-affinity orthophosphate carriers allow this micro-organism to cope with environmental variations. Intriguingly, in this study we found a tight correlation between selenite resistance and expression of the high-affinity orthophosphate carrier Pho84p. Our work further revealed that mutations in the low-affinity orthophosphate carrier genes (PHO87, PHO90 and PHO91) cause deregulation of phosphate-repressed genes. Strikingly, the deregulation due to pho87Δ, pho90Δ, or pho91Δ mutations was neither correlated to impaired orthophosphate uptake capacity nor to a decrease of the intracellular orthophosphate or polyphosphate pools, as shown by 31P NMR spectroscopy. Thus, our data clearly establish that the low-affinity orthophosphate carriers affect phosphate regulation independently of intracellular orthophosphate concentration through a new signaling pathway which was found to strictly require the cyclin-dependent kinase inhibitor Pho81p. We propose that phosphate-regulated gene expression is under the control of two different regulatory signals: the sensing of internal orthophosphate by a yet unidentified protein and the sensing of external orthophosphate by low-affinity orthophosphate transporters; the former would be required to maintain phosphate homeostasis, while the latter would keep the cell informed on the medium phosphate richness.
INTRODUCTION

Phosphate is an essential nutrient required for biosynthesis of several cellular components such as phospholipids or nucleotides derivatives. Therefore, many organisms have developed mechanisms allowing them to adapt phosphate utilization to phosphate availability. The yeast *Saccharomyces cerevisiae* has been extensively used as a model to study how eukaryotic cells respond to variations of external orthophosphate concentrations. Yeast cells are able to take up orthophosphate from the external environment through at least five orthophosphate carriers catalyzing orthophosphate accumulation (Fig.1): two high-affinity permeases (Pho84p and Pho89p; (1)) and three low-affinity permeases (Pho87p, Pho90p and Pho91p; (2)). Combined deletion of these five genes is lethal but viability can be rescued by overexpression of the *GIT1* gene that encode a glycerophosphoinositol permease which can also take up orthophosphate (2). In the presence of organic phosphate in the medium, secreted acidic phosphatases (Pho3p, Pho5p, Pho11p and Pho12p; (3)) are able to transform it into orthophosphate, which can in turn be transported by the orthophosphate permeases.

In response to high external inorganic orthophosphate concentrations, transcription of several genes is repressed (4,5). Among them are the genes coding for excreted phosphatases (*PHO5, PHO11* and *PHO12*), the high-affinity orthophosphate carriers (*PHO84* and *PHO89*), *GIT1* and a gene encoding a protein required for correct localization of Pho84p in the plasma membrane, *PHO86* (6). The other *PHO*-regulated genes are the *PHO81* family members
\((PHO81, SPL2\) and \(YPL110C\)) encoding potential cyclin inhibitors and several genes coding for various phosphate metabolism enzymes such as the \(PHM\) genes (Fig. 1) that are involved in polyphosphate (polyPi) synthesis or degradation (7).

Differential phosphorylation of the Pho4p transcription factor by the Pho80p/Pho85p cyclin-dependent kinase complex allows transcriptional regulation of the \(PHO\)-regulon in response to variations of external orthophosphate concentration (8). When external concentration of orthophosphate is low, the CDK-inhibitor Pho81p, which is constitutively bound to the Pho80p/Pho85p complex, becomes active resulting in low phosphorylation of Pho4p by the cyclin/CDK complex (9-11). This low-phosphorylated form of Pho4p has a high-affinity for the nuclear importin Pse1p and a low affinity for the nuclear exportin Msn5p and is therefore preferentially located in the nucleus (10,12-14), where Pho4p cooperates with Pho2p, another transcription factor (15), to activate expression of the target genes. When the external concentration of orthophosphate is high, Pho81p effect on Pho85p is lowered. Consequently, Pho4p is hyper-phosphorylated by the Pho80p/Pho85p complex and is therefore mainly located in the cytoplasm, resulting in a decreased expression of the \(PHO\)-regulon.

Although the late molecular events in the \(PHO\) pathway are relatively well characterized, little is known about how the external concentration of orthophosphate is sensed by the cells. Because \(PHO5\) expression is constitutive in a \(pho84\) defective mutant (16), the possibility of a coupling between orthophosphate transport and sensing has been recently investigated (2). However, it was clearly shown that Pho84p is not an inorganic
orthophosphate sensor since derepression of PHOS5 is only a consequence of a reduced orthophosphate uptake in a pho84Δ mutant. Indeed, overexpression of any other orthophosphate carrier restored normal Pho5p levels (2). Consistently, it has been recently shown that expression of PHOS5 was tightly correlated to intracellular orthophosphate concentration as measured by 31P NMR spectroscopy (17). Furthermore, these authors have also established that internal orthophosphate concentration was low in a pho84Δ mutant, confirming thus that Pho84p is most likely to affect phosphate signaling through its uptake capacity (17).

By screening a yeast knock-out collection for resistance to sodium selenite, we found a striking correlation between expression of PHO84 and selenite resistance. Furthermore, we show that mutants affected in the low-affinity orthophosphate transporter genes (PHO87, PHO90 and PHO91) are unable to maintain PHO84 repression under high-orthophosphate conditions. In addition, we demonstrate that derepression of PHO84 in the low-affinity orthophosphate permease mutants does not correlate with an impaired orthophosphate uptake capacity nor with a decreased cytosolic orthophosphate and/or polyPi concentrations.
EXPERIMENTAL PROCEDURES

Yeast Media - YPD and SD media were prepared according to Shermann et al (18). SD casaWA is SD medium supplemented with 0.2% casamino acids (Difco), tryptophan (40 mg/l), and adenine (0.3 mM). The low-orthophosphate media were prepared as previously described (19). Concentrations of orthophosphate in YPD, SDcasaWA and orthophosphate-depleted YPD were measured (20) and found to be respectively 6 mM, 10 mM and less than 0.1 mM, consistently with previously reported values (19). Sodium selenite (SeO3Na2) was purchased from SIGMA (#S-5261) and solutions were always prepared extemporaneously.

Yeast Strain - All strains belong to, or are derived from, a set of 4787 disrupted strains isogenic to BY4742 (Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) purchased from Euroscarf. The single mutant strains used in this study are: phm3Δ (Y16780), pho2Δ (Y13803), pho3Δ (Y13231), pho4Δ (Y15848), pho80Δ (Y11692), pho81Δ (Y15886), pho85Δ (Y12797), pho86Δ (Y11308), pho87Δ (Y15766), pho90Δ (Y11227), pho91Δ (Y15388) and yhr202wΔ (Y12896).

All the double and triple mutants described in Table 1 were constructed from diploid strains obtained by mating. After sporulation and dissection, the double (or triple) mutant spores were identified by PCR using the kanB oligonucleotide (internal to KanMX4) and an oligonucleotide complementary to the promoter of the disrupted gene. The sodium selenite sensitivity, observed in this work for the pho2Δ, pho4Δ, pho81Δ, pho84Δ and pho87Δ mutants, co-segregated with the disruption marker in the meiotic progeny and was
complemented by the wild-type corresponding genes on a centromeric plasmid (data not shown). Furthermore, this sodium selenite sensitivity was also found for \textit{pho4Δ} (EY0130) and \textit{pho81Δ} (EY0138) null-mutants constructed in the W303 genetic background (gift from Dr. E. O’Shea).

\textit{Plasmids} - The \textit{tet-PHO84} plasmid (P2053), allowing overexpression of the \textit{PHO84} gene in the absence of tetracycline, was obtained by PCR amplification of the \textit{PHO84} ORF using oligonucleotides 486 (5’-CCAATGCATCTTTATGCTTGTTGAAG-3’) and 497 (5’-CCGGGATCCAAATGAGTTCCGTCAATAAAG-3’), followed by digestion of the PCR product with \textit{Bam}HI and \textit{Nsi}I, and ligation in pCM189 plasmid (21) opened with \textit{Bam}HI and \textit{Pst}I. The same strategy was used to construct the \textit{tet-PHO89} plasmid (P2079) with oligonucleotides 525 (5’-CGGGATCCAAATGGCTTTACATCAATTG-3’) and 526 (5’-CCGGGATCCATGATTATGTCATTGGTATTC-3’). The \textit{PHO84-LacZ} plasmid (P2284) used for the β-galactosidase assays and harboring \textit{lacZ} gene under the control of \textit{PHO84} promoter was constructed by insertion of a PCR fragment (obtained with oligonucleotides 572 (5’-CGGAATTCGCGTTGGTGCTGTTATAG-3’) and 813 (5’-GAGGGCATGCATCTTTCAATATGAGCAAAATCATTC-3’)) cut with \textit{Eco}RI and \textit{Sph}I in YEp357 (22) opened by \textit{Eco}RI and \textit{Sph}I.

\textit{Growth test} - Yeast strains were grown at 30°C overnight on plates and then replica-plated on fresh medium and grown for six more hours. Cells were then resuspended in sterile water
to an OD$_{600}$ (optical density at 600 nm) = 0.5 and submitted to 1/10 serial dilutions. Drops (5 µl) of each dilution were spotted on freshly prepared plates containing low- or high-orthophosphate SDcasaWA or YPD media and supplemented or not with sodium selenite at different concentrations. Plates were incubated for 48 to 72 hours at 30°C, depending on the medium used.

*Northern Blots* - Yeast strains were grown to an OD$_{600}$ = 1. Total RNAs were isolated using the TRI-Reagent RNA/DNA/PROTEIN Isolation Reagent (EUROMEX). RNA blots were prepared and probed as already described (23). The labeled PCR fragments were amplified using S288C genomic DNA as template and oligonucleotides 676 (5’-GATTGAAACATCCATTTACCTG-3’)+ 715 (5’-CGCGGATCCTTACCAATGTTTAAATCTGTTG-3’), 486 and 497 and 559 (5’-CGGGATCCCCCATGGGCACATATA-3’) + 560 (5’-ATTAACTGCAGCAATCATGATTGTTCTTCGTC -3’) for PHO5, PHO84 and SPL2, respectively. The ACT1 probe was prepared as described previously (23).

βGal assays - βGal assays were performed as described (24) on cells transformed with the PHO84-lacZ fusion plasmid (P2284). Each measurement was done at least twice on two to three independent transformants of two independent transformations. βGal units are defined as $[(OD_{420} \times 1000)/(OD_{600} \times \text{min} \times \text{ml})]$. 
Orthophosphate uptake - Yeast strains were grown in SDcasaWA medium to OD$_{600}$ = 1. Cells harvested by centrifugation, were washed twice in buffer A (50 mM potassium citrate pH 4.5, 4 % glucose) and resuspended in the same buffer at OD$_{600}$ = 1. Uptake measurement was started by addition of KH$_2^{[32P]}$PO$_4$ (70 µl, 10 to 10,000 µM, specific radioactivity 5 to 1,000 Ci/mol) to 630 µl of cells suspension. Cells (200 µl aliquots) were taken at 1, 2 and 3 minutes (period of time during which the uptake was linear, this work and (2)), filtered immediately on 0.8 µm filters (supor®-800, Pall life science) and washed with 3 ml of ice cold 0.5 M KH$_2$PO$_4$ buffer pH 4.5. Radioactivity was measured by scintillation counting. Uptake parameters were determined by a non-linear regression of the saturation curve using the Graph-Pad software.

$^{31}P$ NMR spectroscopy - Yeast strains were grown overnight in 100 ml YPD medium and were harvested at O.D.$_{600}$ = 1. Cells were centrifuged and excess medium was removed to obtain a 500 µl cellular suspension. D$_2$O (100 µl) and methylene-diphosphonate pH 6 (MDP, 1 µmol) were added to the yeast suspension. $^{31}P$ NMR spectroscopy analysis was performed with a Bruker Avance 500 narrow-bore spectrometer equipped with a 5-mm broad-band probe. The $^{31}P$ NMR signal was recorded (512 scans) with the following parameters : 5 µs pulse (~53° flip angle), 0.81 s acquisition time, 0.5 s relaxation delay, 10,080 Hz sweep width, 16 K memory size, Waltz 16 proton decoupling and D$_2$O lock. The free induction
decays were converted by Fourier transformation after applying a 4 Hz line broadening and a 32 K zero filling. Chemical shift scale was calibrated using 0 ppm for 85% phosphoric acid. Peak areas were determined with the Bruker integration routine using the MDP peak as reference. Peak areas were corrected for partial saturation using T1 values for the 31P nuclei in the various compounds as already described (25). To calculate cellular orthophosphate concentration from NMR spectra, duplicates of 200 µl aliquots of cells suspension used for NMR spectroscopy were dried at 80°C for 48 hours to determine cells dry weight. In parallel, cellular volumes were measured with radio-labeled [3H]H2O and [14C]inulin as described (26) and correspond to a mean of 3.85 ± 0.12 µl/mg dry weight. 31P NMR measurements for cytosolic orthophosphate and polyPi (contained in the PP4-n peak, see figure) are given as millimolar of orthophosphate residues.
RESULTS

Screening a collection of yeast knock-out mutants for sodium selenite resistance reveals a link between orthophosphate uptake and selenite sensitivity. In a previous work, we had studied the effect of sodium selenite on yeast cells and isolated two gene-dosage suppressors (27). To identify new yeast genes required for sodium selenite sensitivity, a collection of 4787 yeast haploid knock-out mutants were transferred to solid YPD medium supplemented or not with sodium selenite (5 and 10 mM). After 4 days at 30°C, pho87Δ and yhr202wΔ mutants were clearly more resistant to sodium selenite than the wild-type. Serial dilution on sodium selenite-containing medium further indicated that the pho87Δ mutant was slightly more resistant than yhr202wΔ (Fig. 2A). Since the function of the YHR202w gene product is unknown, we focused our attention on the PHO87 gene which encodes a low-affinity orthophosphate carrier (2,28). We showed that the introduction of a plasmid carrying the PHO87 gene in the pho87Δ strain restored a wild-type sensitivity to sodium selenite of this mutant (data not shown). Mutant strains for the two other low-affinity orthophosphate transporters (Pho90p and Pho91p) also displayed an increased sodium selenite resistance (Fig. 2B). However, resistance of the double or triple mutants was not markedly increased compared to the single mutant strains (data not shown).

To gain further insight into relationships between sodium selenite and orthophosphate assimilation, several mutants involved in various aspects of phosphate metabolism were tested for sodium selenite resistance. Clearly, regulators of the PHO-pathway, pho2Δ, pho4Δ,
pho81Δ and pho86Δ, were highly sensitive to sodium selenite (Fig. 2C), as was the strain deleted for the high-affinity orthophosphate carrier gene PHO84. Importantly, addition of a high concentration (25 mM) of orthophosphate to the growth medium alleviated sodium selenite sensitivity of all the phoΔ mutants and improved resistance of the wild-type strain to the drug (Fig. 2C), thus confirming the clear link between orthophosphate metabolism and sodium selenite sensitivity.

*Sensitivity to sodium selenite correlates with expression of PHO84* - All the sodium selenite sensitive mutants described in the previous section alter the expression or the localization of Pho84p. Indeed, it has previously been shown that PHO84 is not expressed in a pho2Δ, pho4Δ, pho81Δ and pho84Δ single mutant strains (Fig. 3A and (29)) and Pho84p is not correctly localized in the pho86Δ mutant strain (6). The expression of PHO84 was monitored by northern blot on yeast cells grown in high-orthophosphate YPD medium (repression condition) (Fig. 3A). We found that the low basal expression of PHO84 is dependent on the presence of PHO2, PHO4 and PHO81 genes (Fig. 3A), as previously reported (30). Strikingly, the PHO84 transcript was much more abundant in the sodium selenite resistant pho87Δ mutant than in wild-type cells (Fig. 3A). Importantly, PHO84 expression was not affected by selenite itself (Fig. 3A).

We reasoned that, if sodium selenite resistance in the pho87Δ mutant is due to PHO84 overexpression, it should be abolished in a pho84Δpho87Δ double mutant. Indeed, such a double mutant behaved as the single pho84Δ mutant on sodium selenite containing medium
(Fig. 3B). Thus, Pho84p is necessary for the sodium selenite resistance of the pho87Δ mutant, and overexpression of PHO84 in the pho87Δ mutant should be the primary cause for sodium selenite resistance. Indeed, the PHO84 gene placed under control of a tetracycline-repressible promoter led to sodium selenite resistance in the absence, but not in the presence, of tetracycline (Fig. 3C). In contrast, overexpression of Pho89p, the other high-affinity orthophosphate carrier (Fig. 3D), did not result in sodium selenite resistance (Fig. 3C). Therefore, our data reveal a tight and specific correlation between PHO84 expression and sodium selenite resistance.

Finally, several general transcription machinery mutants, such as snf2Δ, gcn5Δ, spt7Δ and spt2Δ, in which the expression of PHO84 was previously reported to be dramatically decreased (31-33) were found to be sensitive to sodium selenite (data not shown), further validating the correlation between PHO84 expression and sodium selenite resistance.

*Expression of PHO-regulated genes is derepressed in the presence of inorganic orthophosphate in the pho87Δ, pho90Δ and pho91Δ mutants* - The data presented in the previous section revealed a role for PHO87 in the regulation of PHO84 expression under high-orthophosphate conditions. To further characterize this effect, the expression of PHO84 was monitored in strains carrying the pho87Δ, pho90Δ and pho91Δ deletions, either alone or combined. In these mutants, levels of PHO84 transcript were unaffected when cells were grown on low-orthophosphate medium, whereas a clear derepression of the PHO84 transcript was observed in the mutant cells under high-orthophosphate conditions (Fig. 4A).
Transcription of another phosphate-regulated gene, SPL2, was also derepressed in the low-affinity carrier mutants, establishing that the derepression mechanism does not specifically affect PHO84 (Fig. 4A). Finally, PHO5 expression monitored in a pho3∆pho90Δ double mutant (PHO5 expression cannot be monitored by northern blot in a PHO3 background due to cross hybridization between the two genes and constitutive expression of PHO3) was much higher than in the pho3Δ single mutant (Fig. 4B). We conclude that mutations in the low-affinity orthophosphate carriers result in derepression of the PHO-regulated genes.

*Phosphate repression is set up normally but cannot be maintained in the absence of the low-affinity orthophosphate transporters* - Our results suggest that the presence of Pho87p, Pho90p and/or Pho91p is required for normal phosphate repression of the PHO-regulated genes. To get a more dynamic vision of the phosphate repression phenomenon, expression of PHO84 in response to orthophosphate addition was monitored more precisely. When wild-type, pho90Δ and pho87Δpho90Δpho91Δ cells were shifted from low-orthophosphate (<100 µM) to high-orthophosphate (5 mM) medium, the amount of PHO84 transcript dropped by more than 95 %. This repression level was maintained after two hours in the wild-type strain while, in the pho90Δ and the pho87Δpho90Δpho91Δ mutants, PHO84 repression was not conserved (Fig. 5). In fact, after two hours, in the triple mutant strain the PHO84 gene expression was similar to the level observed in non-repressible mutants such as pho80Δ and pho85Δ (Fig. 5).
Derepression of PHO84 in the low-affinity carrier mutants does not correlate with decreased orthophosphate uptake - A simple explanation for derepression of PHO84 in the pho87Δ, pho90Δ, or pho91Δ mutants could be that, in the absence of the low-affinity transporters, uptake capacity is lowered, leading to constitutive orthophosphate starvation, as previously reported for pho84Δ mutants (2). Orthophosphate uptake measurements in wild-type, pho84Δ and pho87Δpho90Δpho91Δ mutant strains were performed on cells grown under high-orthophosphate conditions (Fig. 6A). Uptake parameters obtained in these conditions were determined by non-linear regression. Saturation curves obtained for the wild-type and the pho84Δ mutant strains were correctly fitted with a single hyperbola regression with correlation coefficients > 0.99. The kinetic parameters thus determined were: KT = 1.0 ± 0.1 mM and V max = 0.70 ± 0.03 nmol orthophosphate incorporated/min/10^7 cells for the wild-type strain and KT = 0.86 ± 0.17 mM and V max = 0.40 ± 0.02 nmol orthophosphate incorporated/min/10^7 cells for the pho84Δ mutant strain. In contrast, the pho87Δpho90Δpho91Δ triple mutant curve could not be fitted with a single hyperbola but was correctly fitted with a double hyperbola regression curve with the following kinetic parameters: KT1 = 12.0 ± 0.1 µM and V max1 = 0.49 ± 0.01 nmol orthophosphate incorporated/min/10^7 cells and KT2 = 3.6 ± 1.5 mM and V max2 = 1.5 ± 0.7 nmol orthophosphate incorporated/min/10^7. Altogether, these results revealed that maximal uptake velocity (V max) was twice lower in the pho84Δ mutant as compared to wild-type (Fig.6A), in good agreement with previously published results (2)). However, in
the \( \text{pho87}\Delta\text{pho90}\Delta\text{pho91}\Delta \) triple mutant strain, the \( V_{\text{max}} \) measured (\( = V_{\text{max1}} + V_{\text{max2}} = 2 \) nmol orthophosphate incorporated/min/\( 10^7 \)) was higher than that of the wild-type strain, thus indicating that orthophosphate uptake capacity is clearly not diminished but rather increased in this strain (Fig.6A). In the \( \text{pho87}\Delta\text{pho90}\Delta\text{pho91}\Delta \) triple mutant, the \( K_{T1} \) for orthophosphate uptake most probably reflects the strong transcriptional derepression of the \( \text{PHO84} \) gene which encodes a high-affinity permease.

Strikingly, expression of a \( \text{PHO84-lacZ} \) fusion was found more than three times higher in the triple mutant than in the \( \text{pho84}\Delta \) mutant (Fig. 6B), although the triple mutant had much higher orthophosphate uptake capacities (Fig. 6A). Therefore, while in the wild-type and \( \text{pho84}\Delta \) strains the \( \text{PHO84-LacZ} \) expression is correlated to orthophosphate uptake capacity, in the \( \text{pho87}\Delta\text{pho90}\Delta\text{pho91}\Delta \) triple mutant, an orthophosphate uptake capacity comparable to that of the wild-type strain does not allow transcriptional repression of the \( \text{PHO} \)-regulon.

Altogether, these results show that derepression of \( \text{PHO} \)-regulated genes is not correlated to impaired orthophosphate uptake capacities in the absence of Pho87p, Pho90p and/or Pho91p.

Absence of low-affinity orthophosphate transporters affects expression of \( \text{PHO} \)-regulated genes independently of internal orthophosphate concentration - Because orthophosphate uptake might not tightly reflect internal orthophosphate concentration, \( ^{31}\text{P} \) NMR
spectroscopy was used to investigate whether the derepression of \textit{PHO}-regulated genes observed in the \textit{pho87}\textDelta, \textit{pho90}\textDelta, \textit{pho91}\textDelta and \textit{pho87}\textDelta\textit{pho90}\textDelta\textit{pho91}\textDelta mutants could be due to a modification of internal orthophosphate or polyPi concentrations.

We first confirmed that, in our strain background, a much lower internal orthophosphate concentration was measured in wild-type cells grown under low-orthophosphate conditions compared to high-orthophosphate medium (data not shown). A severe decrease of the internal orthophosphate and polyPi concentrations were also observed in the \textit{pho84}\textDelta mutant in high-orthophosphate condition (Fig. 7), as previously reported (17). This severe drop of phosphate pools, that is probably responsible for \textit{PHO}-gene derepression in the \textit{pho84}\textDelta mutant strain, was fully reversed by reintroduction of the \textit{PHO84} gene on a centromeric plasmid (data not shown).

Measurements of intracellular orthophosphate and polyPi pools in the \textit{pho80}\textDelta mutant strain revealed that, in high-phosphate condition, the cytosolic orthophosphate concentration was similar to that of the wild-type, while a large accumulation of polyPi was observed (Fig. 7). Therefore, as expected for such a non-repressible signaling mutant, a disconnection between phosphate pools variation and \textit{PHO}-genes expression was observed.

Finally, phosphate pools measurements in single and triple low-affinity orthophosphate carriers mutants showed that both cytosolic orthophosphate and polyPi concentrations were at least equivalent or higher in these mutants than those measured in wild-type cells when cells were grown in high-orthophosphate medium. These results clearly show that the derepression of \textit{PHO}-regulated genes observed in these mutants is not
correlated to drastic changes in phosphate pools but rather similar to the situation observed in the pho80Δ regulatory mutant in which PHO-genes expression and variation of phosphate pools are disconnected. Together, our results indicate that low-affinity orthophosphate carriers affect PHO84 expression by a mechanism independent of internal orthophosphate concentration.

Low-affinity orthophosphate carriers act upstream of Pho81p in regulation of the PHO-pathway and their effect is independent of intracellular orthophosphate sensing – To further describe the regulatory role of the low-affinity orthophosphate carriers in the PHO-pathway, we intended to place these new regulators in the previously characterized phosphate signal transduction pathway.

First, the pho87Δ, pho90Δ and pho91Δ mutations were individually combined to pho2Δ, pho4Δ or pho81Δ mutations which are required for derepression of PHO-genes (4). Clearly pho2Δ, pho4Δ and pho81Δ were epistatic to the pho87Δ, pho90Δ and pho91Δ mutations (Fig. 8), thus indicating that the product of PHO87, PHO90 or PHO91 genes operate upstream of PHO2, PHO4 and PHO81 in the phosphate transduction pathway.

We then addressed the question of whether pho87Δ, pho90Δ and pho91Δ mutants are still able to respond to intracellular orthophosphate variations. This was done by taking advantage of the fact that a phm3 (vtc4) mutant which is unable to synthesize polyphosphates (4). Consequently, this mutant accumulates orthophosphate in the cytoplasm, and shows lower expression of the PHO-regulon compared to the wild-type strain (17). The phm3Δ
mutant can thus be used to artificially increase cytosolic orthophosphate concentration.

The \textit{pho87}\textsuperscript{Δ} and \textit{pho90}\textsuperscript{Δ} mutations were individually combined to \textit{phm3}\textsuperscript{Δ} mutation to determine whether an increased internal orthophosphate concentration would affect \textit{PHO84} expression in the absence of the low-affinity orthophosphate carriers. \textit{PHO84} expression and cytosolic orthophosphate level were measured in single and combined mutants by northern blot and \textsuperscript{31}P NMR spectroscopy, respectively (Fig. 9). Under high-orthophosphate conditions, the \textit{phm3}\textsuperscript{Δ} single mutant displayed a \textit{PHO84} transcript level lower than the wild-type (Fig. 9 A and 9 B), an increased level of cytosolic orthophosphate (Fig. 9 B) and no detectable polyPi (data not shown) as previously shown (17). Combination of \textit{phm3}\textsuperscript{Δ} with either \textit{pho87}\textsuperscript{Δ} or \textit{pho90}\textsuperscript{Δ} mutations led to \textit{PHO84} expression levels similar to or higher than that of the wild-type strain respectively (Fig. 9 A and 9 B), while cytosolic orthophosphate concentrations were very close to those measured in the \textit{phm3}\textsuperscript{Δ} single mutant (Fig. 9 B) As expected, since Phm3p is essential for polyphosphate synthesis (see Fig.1 and (4,17), polyPi were not detectable in the \textit{phm3}\textsuperscript{Δ}\textit{pho87}\textsuperscript{Δ} and \textit{phm3}\textsuperscript{Δ}\textit{pho90}\textsuperscript{Δ} double mutants (data not shown). Therefore, high internal orthophosphate levels led to tight repression of \textit{PHO84} in the \textit{phm3}\textsuperscript{Δ} mutant, while the same internal orthophosphate levels were unable to maintain the repression of \textit{PHO84} in the \textit{phm3}\textsuperscript{Δ}\textit{pho90}\textsuperscript{Δ} double mutant. These results further confirm that signaling through low-affinity orthophosphate transporter is independent of internal orthophosphate concentration.
DISCUSSION

Our results revealed a tight correlation between \textit{PHO84} expression and sodium selenite resistance. However, sodium selenite toxicity was reversed by orthophosphate even in the absence of Pho84p. Altogether, our results rather indicate that orthophosphate internalization itself is critical for resistance. Importantly, we found that sodium selenite does not compete with either low- or high-affinity orthophosphate uptake systems even in the presence of a ten fold molar excess of selenite (data not shown). Surprisingly, the \textit{pho80Δ} and \textit{pho85Δ} mutants known to constitutively overexpress \textit{PHO84} gene were only slightly more resistant to sodium selenite than the wild-type strain (data not shown). However, we observed that, in the \textit{pho80Δ} and \textit{pho85Δ} mutants, oxidative stress response genes (such as \textit{GLR1}, \textit{GSH1}, \textit{TRR1} and \textit{TRX2}) were not induced in response to sodium selenite (B.P. and B.D.-F. unpublished observation), while these genes are strongly induced in the wild-type strain (27). Thus, the moderate resistance to sodium selenite of the \textit{pho80Δ} and \textit{pho85Δ} mutants could reflect a balance between increased resistance due to \textit{PHO84} overexpression and increased sensitivity due to poor induction of oxidative stress response genes.

How orthophosphate leads to selenite detoxification remains to be clarified. However, our selenite toxicity studies revealed new phenotypes for mutants in the phosphate utilization pathway. In particular, this work provides the first growth phenotype for the single \textit{pho87Δ}, \textit{pho90Δ} and \textit{pho91Δ} mutants. In the future, this growth phenotype could be useful to identify new mutants affecting \textit{PHO84} expression.
Here we show that \textit{PHO87}, \textit{PHO90}, and \textit{PHO91}, which encode low-affinity orthophosphate permeases (2), play an important role in \textit{PHO} genes regulation. Recently, similar results were reported by others, however, in this report the authors observed derepression of \textit{PHO5} in the low-affinity orthophosphate carrier mutants only for intermediary orthophosphate concentrations (34), while we observed the derepression even at high orthophosphate concentration. This discrepancy could be due to strain differences since Harashima and coworkers found much higher polyphosphate levels in their wild-type or \textit{pho84Δ} mutant strains than we did in the BY4742 strain or derived \textit{pho84Δ} mutant (17). To clarify this discrepancy, it would be interesting to measure orthophosphate concentrations in the low-affinity carrier mutants used by Harashima and coworkers (34), under various external orthophosphate conditions. However, despite this difference, it is clear from both reports that \textit{PHO} genes expression is affected in the low-affinity carrier mutants.

The question thus arises as to how the \textit{pho87Δ}, \textit{pho90Δ}, and \textit{pho91Δ} mutations, either alone or in combination, affect \textit{PHO84} expression. Since Pho87p, Pho90p and Pho91p are, presumably, integral membrane proteins, their effect on \textit{PHO} genes transcription is likely to be indirect. In a simple model, the role of Pho87p, Pho90p and Pho91p would principally be to sustain intracellular orthophosphate concentration when orthophosphate concentration is high in the medium and \textit{PHO84} expression is low. Consequently, in these mutant strains, internal orthophosphate concentration would be lower, leading to derepression of \textit{PHO84} despite a high extracellular orthophosphate concentration. Our measurements of internal orthophosphate concentration in the \textit{pho87Δ}, \textit{pho90Δ} and \textit{pho91Δ} mutant strains do not...
support this model because internal phosphate concentration (in the form of either free orthophosphate or polyphosphates) was found equivalent or even higher in the mutants than in the wild-type. Therefore our results demonstrate that the derepression of PHO84 expression in the pho87Δ, pho90Δ and pho91Δ mutants is not the result of an uptake defect that leads to orthophosphate starvation. We rather favor the hypothesis of low-affinity carriers participating to a signaling pathway independently of the previously documented internal orthophosphate signaling pathway.

Clearly, in the wild-type strain there is a tight correlation between internal free orthophosphate concentration and PHO84 expression (17). Consistently, mutations affecting internal free orthophosphate concentration such as pho84Δ or phm3Δ also affect PHO84 expression ((17) and this work). However, in regulatory mutants such as pho80Δ, there is not any correlation between internal orthophosphate concentration and PHO84 expression. Strikingly, the pho87Δ, pho90Δ and pho91Δ mutants behaved more like regulatory mutants than uptake defective mutants. How could those membrane proteins affect PHO84 expression independently of their role in orthophosphate uptake? One attractive possibility would be that Pho87p, Pho90p and Pho91p are both orthophosphate transporters and sensors. Indeed, as other membrane sensors (35), all three proteins have a long hydrophilic tail that could be involved in signaling (36). Strikingly, the N-terminal extremity of Pho91p shows similarities with Pho81p, a major regulator of the Pho85p cyclin-dependent kinase. Furthermore, all three low-affinity transporters, like Pho81p, carry a so called SPX domain (Syg1p, Pho81p and XPR1) that could be involved in G-protein associated signal transduction (37-39).
The reason for having three phosphate membrane sensors, which appear partially functionally redundant, is not yet clear. However, in all experiments pho90 deletion had a stronger effect than deletion of pho87 or pho91, indicating that they are not strictly equivalent. Importantly, this conclusion was only valid for cells in exponential growth, indeed during post-diauxic phase pho87 and pho91 deletions have a greater effect than pho90 mutation (B.P. and B.D.-F. unpublished data). Therefore, each of the three sensors could play its role at a specific stage of growth.

Importantly, we have shown that signaling through Pho87p, Pho90p, Pho91p strictly requires Pho81p. Mutants lacking Pho81p are non-derepressible, indicating that they cannot respond to orthophosphate variations. Consistently, a pho81Δ mutation is epistatic to a pho84Δ mutation (16). Therefore, Pho81p is a critical protein for internal phosphate sensing. Our data further suggest that Pho81p is also critical for signaling through the low-orthophosphate affinity carriers. Pho81p, is a large protein carrying a cyclin inhibitor like domain. Although a minimal inhibitory domain has been characterized (11), other parts of the protein could be important for integration of various signals. Our repression kinetic data (Fig. 4) show that in the pho87Δ, pho90Δ and pho91Δ mutants, phosphate repression can take place in the absence of Pho87p, Pho90p and/or Pho91p, but cannot be maintained. We propose that brusque addition of orthophosphate to the medium results in an intracellular orthophosphate burst leading to repression via the action of intracellular phosphate sensor(s) indicating that the internal phosphate sensing pathway is still functional in the triple mutant. Consistently, the effect of the pho87Δ and pho90Δ mutations was partially compensated by that of the phm3Δ
mutant. Therefore we propose that the low-affinity orthophosphate carriers somehow signal to Pho81p independently of the previously described internal phosphate response pathway. Pho81p would then integrate these two signals and modulate expression of the PHO regulon.

Why should there be an external phosphate signaling pathway in addition to the internal phosphate sensing pathway? The intracellular phosphate concentration cannot vary extensively without affecting essential cellular processes and yeast cells were shown to mobilize polyphosphates when internal orthophosphate concentration drops (40). Therefore, some mutants such as pho84Δ which have a low internal phosphate concentration lack polyphosphates ((4) and this work). It becomes clear that polyphosphates are used by the cell as a phosphate "stock" (40) and consequently internal free orthophosphate concentration might not be adequate to accurately signal phosphate medium richness. Since external orthophosphate concentration is an important parameter for the cell decision to enter or not into a new cell cycle, an attractive hypothesis would be that the low-affinity orthophosphate carriers could somehow sense external orthophosphate concentration and send a mitogenic signal mediated by Pho81p to the cyclin dependent kinase Pho85p.
ACKNOWLEDGEMENTS

We thank Eric Bezançon for his precious technical assistance in $^{31}$P NMR spectroscopy, Dr A. Devin for cell volume measurements, Dr. O’Shea for sending yeast strains, L. Chesneau for participation to some yeast strain constructions, and Dr I. Sagot for critical reading of the manuscript. The work was supported by CNRS UMR 5095/University Bordeaux 2 and by grant 5843 from ARC.
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LEGENDS OF FIGURES

**Fig. 1.** Schematic representation of trans-acting factors controlling the *PHO* regulon. Pi and CDK stand for inorganic orthophosphate and cyclin-dependent protein kinase respectively.

**Fig. 2.** Sodium selenite resistance/sensitivity of several yeast mutant strains and orthophosphate reversion. Serial dilutions of cells in exponential growth-phase were spotted on standard YPD medium containing or not sodium selenite (5 mM) and supplemented or not with potassium phosphate (KPi, 25 mM final concentration) as indicated. Cells were grown at 30°C for 48 hours.

**Fig. 3.** Resistance/sensitivity to sodium selenite is tightly correlated to *PHO84* expression level. A, *PHO84* expression in various mutant strains was monitored by northern blot analysis. Cells were grown in SDcasaWA medium (high-phosphate condition) to an OD$_{600}$ = 1 and submitted (+) or not (-) to 30 minutes sodium selenite treatment (10 mM final concentration). Hybridizations were done independently and were assembled for the figure. B, Serial dilutions of exponentially growing cells spotted onto standard YPD medium containing or not sodium selenite (5 mM). C, Serial dilutions of BY4742 cells transformed with either pCM189 (vector), P2053 (tet-*PHO84*) or P2079 (tet-*PHO89*) plasmids were spotted on SDcasaWA medium supplemented or not with tetracycline (100 mg/l) and containing or not sodium selenite (2.5 mM). D, *PHO84* and *PHO89* expression driven by the Tet-regulable promoter was monitored by northern blot as in Fig. 3A.
**Fig. 4.** Knock-outs of low-affinity orthophosphate transporters result in derepression of

**PHO-regulated genes under high-orthophosphate conditions.** Cells were grown in YPD low-orthophosphate medium supplemented (+) or not (-) with KPi (5 mM final concentration) to an OD$_{600}$ = 1. Northern blot analyses were performed as in Fig. 3A.

**Fig. 5.** Yeast lacking low-affinity orthophosphate carriers are unable to maintain orthophosphate repression. Cells were grown in YPD low-orthophosphate medium to OD$_{600}$ = 1 and orthophosphate (5 mM) was added at time 0. Northern blot analyses were performed as in Fig. 3A on RNA extracted at indicated time after orthophosphate addition. Membranes were hybridized with *PHO84* (left panel) and *ACT1* (central panel) radioactive probes. 

Quantification of the northern blot from Fig. 5 is presented on the right panel as the *PHO84*/*ACT1* ratio. The following symbols were used: Wild-type (opened squares), *pho80Δ* (opened triangles), *pho85Δ* (closed triangles), *pho90Δ* (opened circles) and *pho87Δpho90Δpho91Δ* (closed circles). Quantification results correspond to the mean of three independent northern blot experiments.

**Fig. 6 Derepression of PHO84 is not correlated to an impaired orthophosphate uptake in the pho87Δpho90Δpho91Δ triple mutant.** A, Wild-type (closed squares), *pho84Δ* (opened triangles) and *pho87Δpho90Δpho91Δ* (opened circles) strains were transformed with the pCM189 empty vector. Transformants were grown in SDcasaWA medium (high-phosphate conditions) to OD$_{600}$ = 1, washed with transport buffer, and submitted to uptake
measurement as described in the Experimental Procedures section. Results correspond to the mean of initial rate of uptake measured in, at least, three independent experiments. B, Yeast strains were transformed with the PHO84-lacZ fusion plasmid. Transformants were grown in SDcasaWA medium to OD$_{600} = 1$ and βGal measurements were performed as described in Experimental procedure section.

**Fig. 7** Derepression of PHO-regulated genes in the absence of low-affinity orthophosphate transporters is not linked to impaired internal orthophosphate or polyPi pools. Yeast strains were grown in YPD medium to OD$_{600} = 1$ and internal orthophosphate and polyPi pools were estimated by $^{31}$P NMR spectroscopy as described in the Experimental Procedures section. A, overlapping of $^{31}$P NMR spectra, each one being a representative spectrum for each strain tested. Methylene diphosphonic acid (MDP) was used as a reference. PP$_n$ stands for phosphate residue at the $n$th position in polyphosphate chains. B, orthophosphate (white bars) and polyPi (grey bars) intracellular levels determined by $^{31}$P NMR spectroscopy. Results presented correspond to the mean of three independent experiments for each strain. Polyphosphate levels were expressed in terms of orthophosphate residues determined from the PP$_{4-n}$ peak.

**Fig. 8.** Low-affinity orthophosphate carriers operate upstream of Phop81p, Pho2p and Pho4p in the orthophosphate transduction pathway. Wild-type and mutant strains were transformed
with the \textit{PHO84-lacZ} fusion plasmid. Transformants were grown in S
casaWA medium (high-orthophosphate conditions) to OD$_{600} = 1$ and $^2$Gal measurements were performed as described in Experimental procedure section. Results presented corresponds to the average of at least 3 independent experiments.

\textbf{Fig. 9} \textit{PHO}-regulated gene expression is under the control of both internal orthophosphate and a signal requiring the low-affinity orthophosphate carriers. \textit{A}, Yeast strains were grown in YPD medium (high-orthophosphate conditions) to OD$_{600} = 1$ and northern blot analyses were performed as in Fig. 3A. \textit{B}, Quantification of the northern blot from Fig. 9A (black bars) corresponds to the mean of three independent northern blot experiments. Cytosolic orthophosphate (open bars) was determined by $^{31}$P NMR spectroscopy on cells grown in high-orthophosphate conditions similar to those used for the northern blot analysis of Fig. 9A.
Table 1. Yeast strains constructed in this study.

| Strain   | Genotype                                      |
|----------|-----------------------------------------------|
| Y1498    | MATα   his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pho87::kanMX4 pho91::kanMX4 |
| Y1516    | MATα   his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pho84::kanMX4  |
| Y1533    | MATα   his3-Δ1 leu2-Δ0 ura3-Δ0 pho3::kanMX4 pho90::kanMX4 |
| Y1774    | MATα   his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 pho4::kanMX4 pho91::kanMX4 |
| Y1781    | MATα   his3-Δ1 leu2-Δ0 pho81::kanMX4 pho91::kanMX4  |
| Y1784    | MATα   his3-Δ1 leu2-Δ0 met15Δ0 ura3-Δ0 pho2::kanMX4 pho91::kanMX4 |
| Y1785    | MATα   his3-Δ1 leu2-Δ0 met15Δ0 ura3-Δ0 pho2::kanMX4 pho90::kanMX4 |
| Y1787    | MATα   his3-Δ1 leu2-Δ0 met15Δ0 ura3-Δ0 pho81::kanMX4 pho90::kanMX4 |
| Y1790    | MATα   his3-Δ1 leu2-Δ0 met15Δ0 ura3-Δ0 pho81::kanMX4 pho91::kanMX4  |
| Y1792    | MATα   his3-Δ1 leu2-Δ0 met15Δ0 ura3-Δ0 pho4::kanMX4 pho90::kanMX4 |
| Y1793    | MATα   his3-Δ1 leu2-Δ0 met15Δ0 ura3-Δ0 pho4::kanMX4 pho87::kanMX4 |
| Y1795    | MATα   his3-Δ1 leu2-Δ0 met15Δ0 ura3-Δ0 pho2::kanMX4 pho87::kanMX4 |
| Y1797    | MATα   his3-Δ1 leu2-Δ0 met15Δ0 ura3-Δ0 phm3::kanMX4 pho90::kanMX4 |
| Y2261    | MATα   his3Δ1 leu2Δ0 ura3Δ0 phm3::kanMX4; pho87::kanMX4 |
| Y2272    | MATα   his3Δ1 leu2Δ0 ura3Δ0 phm3::kanMX4; pho87::kanMX4 |


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Fig. 2
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Fig. 3
Figure 4. Immunoblot analysis of protein levels in wild-type (WT) and mutant strains.

Panel A:
- **ACT1**
- **SPL2**
- **PHO84**
- WT
- pho87Δ
- pho90Δ
- pho91Δ

Panel B:
- **ACT1**
- **PHO5**
- **PHO84**
- Pi
- pho3Δ
- pho90Δ
- pho3Δ pho90Δ
**Fig. 6**

A. Graph showing the effect of KPi concentration on nmol Pi incorporated/min/10^7 cells.

B. Bar graph comparing βgal units between WT, pho84Δ, pho87Δ, pho90Δ, and pho91Δ.
Fig. 7

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Fig. 8
Low-affinity orthophosphate carriers regulate PHO genes expression independently of internal orthophosphate concentration in saccharomyces cerevisiae
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*J. Biol. Chem.* published online June 12, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405398200

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