The *KIPHO5* gene encoding a repressible acid phosphatase in the yeast *Kluyveromyces lactis*: cloning, sequencing and transcriptional analysis of the gene, and purification and properties of the enzyme

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A secreted phosphate-repressible acid phosphatase from *Kluyveromyces lactis* has been purified and the N-terminal region and an internal peptide have been sequenced. Using synthetic oligodeoxyribonucleotides based on the sequenced regions, the genomic sequence, *KIPHO5*, encoding the protein has been isolated. The deduced protein, named KIPho5p, consists of 469 amino acids and has a molecular mass of 52 520 Da (in agreement with the data obtained after treatment of the protein with endoglycosidase H). The purified enzyme shows size heterogeneity, with an apparent molecular mass in the range 90–200 kDa due to the carbohydrate content (10 putative glycosylation sites were identified in the sequence). A 16 amino acid sequence at the N-terminus is similar to previously identified signal peptides in other fungal secretory proteins. The putative signal peptide is removed during secretion since it is absent in the mature secreted acid phosphatase. The gene can be induced 400–600-fold by phosphate starvation. Consensus signals corresponding to those described for *Saccharomyces cerevisiae* *PHO4*- and *PHO2*-binding sites are found in the 5' region. Northern blot analysis of total cellular RNA indicates that the *KIPHO5* gene codes for a 1.8 kb transcript and that its expression is regulated at the transcriptional level. Chromosomal hybridization indicated that the gene is located on chromosome II. The *KIPHO5* gene of *K. lactis* is able to functionally complement a *pho5* mutation of *Sacch. cerevisiae*. Southern blot experiments, using the *KIPHO5* gene as probe, show that some *K. lactis* reference strains lack repressible acid phosphatase, revealing a different gene organization for this kind of multigene family of proteins as compared to *Sacch. cerevisiae*.

Keywords: *Kluyveromyces lactis*, acid phosphatase, transcriptional regulation

INTRODUCTION

The acid phosphatase system in yeasts and fungi of different genera varies in the number of genes involved and also in the way these are regulated. In *Saccharomyces cerevisiae*, four genes encoding phosphatases

with an acidic optimum pH have been isolated and sequenced (namely *PHO3*, *PHO5*, *PHO11* and *PHO12*). The proteins are secreted from the yeast cells and are predominantly found between the cytoplasmic membrane and the cell wall. Three of these genes, *PHO5* (which encodes the major acid phosphatase, located on chromosome II), *PHO11* (located on chromosome I) and *PHO12* (located on chromosome VIII), whose products have amino acid sequences very similar to Pho5p – they only differ in four amino acids – are phosphate-repressible and the other one, *PHO3*, which is tightly linked to *PHO5*, is thiamin-repressible (for reviews see Schwein-

Abbreviation: Endo H, endoglycosidase H.

The EMBL accession number for the sequence reported in this paper is Z33995.

gruber, 1987; Vogel & Hinnen, 1990; Johnston & Carlson, 1992).

In the fission yeast *Schizosaccharomyces pombe*, two acid phosphatases encoded by two genes, *pho1* and *pho4*, have been cloned and sequenced. The major acid phosphatase is encoded by *pho1* and its expression is repressed by orthophosphate in the medium as occurs with the *PHO5* gene of *Sacch. cerevisiae*. However, the *pho1* gene can only be induced 2–3-fold in response to phosphate starvation (Elliott *et al.*, 1986), whereas for the *PHO5* gene of *Sacch. cerevisiae* induction ranged from 150-fold (Mizunaga, 1979) to 1000-fold (Oshima, 1982). An acid phosphatase showing 100-fold induction has also been isolated from *Pichia pastoris* (Payne *et al.*, 1995). The *Schiz. pombe pho4* gene is also regulated by thiamin but the overall similarity with the *PHO3* of *Sacch. cerevisiae* is only 27% and no obvious consensus regions can be detected (Yang & Schweingruber, 1990).

Secreted repressible acid phosphatases from fungi have been cloned and sequenced in *Aspergillus niger* (*pacA*; MacRae *et al.*, 1988), *Penicillium chrysogenum* (*phoA*; Haas *et al.*, 1992) and *Aspergillus niger* var *awamori* (*aph*; Piddington *et al.*, 1993). No significant amino acid sequence homologies among the acid phosphatases have been described (Piddington *et al.*, 1993).

The *PHO5* promoter of *Sacch. cerevisiae*, whose expression can be regulated by the supply of inorganic phosphate in the culture medium, has been used for heterologous gene expression. Using this system, several proteins, including hirudin and tissue-type plasminogen activator (t-PA) (Hinnen *et al.*, 1989), have been successfully produced.

The dairy yeast *Kluyveromyces lactis* has been developed as an efficient system for high-level production of foreign proteins (Buckholz & Gleeson, 1991; Fleer *et al.*, 1991; Swinkels *et al.*, 1993). Major advantages of this organism are its impressive secretory capacity, its excellent large-scale fermentative characteristics, its food-grade status, and the availability of both episomal and integrative expression vectors (Chen *et al.*, 1988). In this paper we describe the purification and cloning of a phosphate-repressible acid phosphatase of *K. lactis*, compare the enzyme synthesized and secreted with acid phosphatases produced by *Sacch. cerevisiae* and *Schiz. pombe*, and report a transcriptional analysis and a description of the genomic organization of the acid phosphatases in 2359/152 and 2360/7, the two *K. lactis* strains used in many laboratories.

METHODS

Strains and media. A description of the plasmids and genotypes of the yeast strains used in this study is given in Table 1. Yeasts were maintained on slants of YED medium (1% yeast extract, 1% glucose, 2% agar). Yeast cultures were grown at 28 °C in 1000 ml Erlenmeyer flasks containing 300 ml medium in a gyratory shaker at 250 r.p.m. on minimal liquid medium (MM) [0.7% yeast nitrogen base (Difco), 1% glucose; Wickerham, 1946]. Media were supplemented with uracil, leucine, methionine, arginine and lysine, each at 50 µg

ml⁻¹, as required. As derepression medium we used MM in which the phosphate concentration was lowered to 10 mg l⁻¹ (low-P_i medium). The medium was buffered with 0.05 M citric acid/sodium citrate, pH 4.3.

Enzyme purification. Derepressed cells were harvested from the low-P_i medium by centrifugation and washed twice with 0.01 M citric acid/sodium citrate buffer, pH 4.3. All enzyme purification steps were done at 4 °C unless otherwise stated. To avoid proteolytic degradation, all buffers were supplemented with a mixture of proteinase inhibitors containing PMSF at 3.5 mg ml⁻¹ and pepstatin, antipain, leupeptin and chymostatin at 20 µg ml⁻¹ each.

Packed cells (approximately 90 g wet weight from 42000 ml medium) were resuspended in citric acid/sodium citrate, pH 4.3. Equal volumes of yeast cell suspension and glass beads (0.45–0.50 mm diameter) were broken by mechanical shaking in a Braun MSK homogenizer. This treatment disrupted >98% of the cells as judged by light microscopic examination. The glass beads were removed by washing the broken cell suspension through a coarse sintered-glass filter. Cell debris was removed by centrifugation at 3000 g for 10 min and at 40000 g for 45 min. The supernatant fraction served as the starting material for enzyme purification. This fraction was brought to 30% saturation by the slow addition of powdered ammonium sulphate, stirred gently for 3 h, and centrifuged at 25000 g for 30 min. The resulting supernatant was taken to 60% saturation with ammonium sulphate, stirred for 3 h and centrifuged as before. The supernatant fluid was concentrated and dialysed against the same buffer.

DEAE-Sephacel chromatography. Soluble supernatant was adsorbed onto a 1.3 × 42 cm column bed of DEAE-Sephacel. The column was eluted first with 240 ml 0.01 M Tris buffer, pH 7.5, and then with 100 ml of a linear gradient of 0–0.3 M NaCl in the same buffer. Fractions were collected and those rich in enzyme activity were pooled, concentrated and dialysed against the citric acid/sodium citrate buffer.

Biogel A 5-M chromatography. The material collected from the DEAE-Sephacel column was loaded onto a 2 × 170 cm column of Biogel A 5-M. The proteins were eluted with 2 l 0.05 M citric acid/sodium citrate buffer pH 4.3 with 0.1 M NaCl at a flow rate of 10 ml h⁻¹ and 2.5 ml fractions were collected.

FPLC. The material collected from the Biogel A 5-M column was further purified by FPLC (Pharmacia LKB) with a Mono Q HR 5/5 column (Pharmacia LKB Biotechnology) with an NaCl gradient (30–50%) in Tris/HCl buffer 0.02 M pH 7.6. Fractions were collected and dialysed against 0.05 M citric acid/sodium citrate buffer pH 4.3.

Enzyme activity. Acid phosphatase activity was assayed with *p*-nitrophenyl phosphate (PNPP; Sigma). The assay mixture was composed of 250 µl enzyme sample, 125 µl 0.36 M citric acid/sodium citrate buffer pH 4.3, and 75 µl 0.04 M PNPP solution. The reaction was initiated by the addition of the substrate and terminated by the addition of 750 µl 0.1 M NaOH. One unit of activity was defined as the amount of enzyme which released 1 nmol *p*-nitrophenol in 1 min at 30 °C.

Protein assays. Protein was determined colorimetrically by the Lowry method, with bovine serum albumin as a standard. A₂₈₀ was used to monitor the protein content of column fractions.

Endo β-N-acetylglucosaminidase H (Endo H) treatment. Purified enzyme samples were incubated with Endo H for the

Table 1. Plasmids and strains used

Strain or plasmid	Description or genotype	Source or reference
Plasmids		
pSK1	<i>K. lactis</i> expression vector based on the integrative vector YIp5 of <i>Sacch. cerevisiae</i> with the replication origins pKD1 of <i>K. drosophilum</i> and 2 μ of <i>Sacch. cerevisiae</i> , carrying URA3	Bianchi <i>et al.</i> (1987)
pEF5	2.5 kb <i>Hpa</i> II fragment carrying <i>KIPHO5</i> in pSK1	This work
<i>Sacch. cerevisiae</i>		
ACX1-2A	<i>ura3-52 leu2-112 trp1-1 pho3 pho5</i>	López (1989)
<i>K. lactis</i>		
CBS2359	MAT α	CBS
CBS2360	MAT α	CBS
2359/152	MAT α <i>metA1-1</i>	Goffrini <i>et al.</i> (1989)
2360/7	MAT α <i>lysA</i>	Goffrini <i>et al.</i> (1989)
MD2/1	MAT α <i>uraA lysA argA k₁⁰ k₂⁰ rag2</i> pKD1 ⁺	Goffrini <i>et al.</i> (1989)
2359/152F	MAT α <i>metA1-1 ura3-20</i>	This work
<i>E. coli</i>		
HB101	F ⁻ <i>hsdS20 recA13 ara14 proA2 lacY1 galK2 rpsLB20(Sm^r) xyl-5 mtl-1 supE44 λ⁻</i>	Boyer & Roulland-Dussoix (1969)
MC1061	<i>hsdR mcrB araD139 Δ(<i>araABCleu</i>)769 ΔlacX74 galU galK rpsL thi</i>	Meissner <i>et al.</i> (1987)
DH5 α	<i>supE44 ΔlacU169 (ϕ80 <i>lacZ</i>ΔM15) <i>hsdR17 recA endA gyrA96 thi-1 relA1</i></i>	Hanahan (1983)
MV1190	F': <i>traD36 proAB lacI^q lacZ</i> Δ M15 Δ (<i>lac-proAB</i>) <i>thi supE Δ(<i>srl-recA</i>), 306::Tn10(Tet^r)</i>	Bio-Rad

desired times at 37 °C in 0.05 M acetic acid/sodium acetate buffer, pH 5.6, containing 1 mM PMSF and 10 μ M pepstatin.

Electrophoresis, electroblotting and immunological detection. Slab gel electrophoresis was performed essentially as described by Laemmli (1970). Nondenaturing PAGE was carried out in a similar way, except that SDS was omitted. Electrotransfer of proteins to nitrocellulose membranes and immunological reactions were performed according to Towbin *et al.* (1979) and Erickson *et al.* (1982).

Protein stains. Gel slabs were stained for protein by the silver staining method of Morrissey (1981) and for enzyme activity as described by Dimond *et al.* (1983).

Carbohydrate content. Samples were assayed for carbohydrate content by the phenol/sulphuric acid method of Dubois *et al.* (1956) with mannose as standard.

Cyanogen bromide digestion. Protein samples (10 μ g) were incubated with 100 μ l 5% 2-mercaptoethanol in water for 10 min at ambient temperature. Then 200 μ l 70% formic acid containing approximately 4 mg cyanogen bromide ml⁻¹ was added and the sample was incubated overnight in the dark at ambient temperature. It was then dried in a Speed-Vac. The blots were extracted by sequential addition of three aliquots of 100 μ l acetonitrile for 10 min each, after which the eluted material was removed. The three extracts were combined, dried in the Speed-Vac and dissolved in 100 μ l 0.1% trifluoroacetic acid. The peptides were separated on an Applied

Biosystems microbore HPLC and were sequenced on an Applied Biosystems model 473A instrument.

DNA manipulations. Total DNA from *K. lactis* was prepared as described for filamentous fungi by Raeder & Broda (1985). The media and procedures used for yeast transformation have already been described (Sherman *et al.*, 1977; Becker & Guarente, 1991; Sánchez *et al.*, 1993).

Restriction enzyme digestions and DNA ligations were performed according to the recommendations of the manufacturers. Plasmid DNA was isolated from *Escherichia coli* by standard procedures (Sambrook *et al.*, 1989). DNA fragments to be used as probes were labelled by random priming with digoxigenin-dUTP (Boehringer Mannheim) and used according to the instructions of the manufacturer.

RNA preparations and Northern analysis. RNA was prepared from exponentially growing cultures on YED and MM by the method of Percival-Smith & Segall (1984). Prehybridization, hybridization and primer extension were performed as described by Sambrook *et al.* (1989). A synthetic oligonucleotide (5'-3') complementary to nucleotide positions +22 to +49 of the *KIPHO5* gene sequence was employed. In all Northern analysis experiments, the RNA concentration was normalized using hybridization of the *ACT1* transcripts from *K. lactis*.

PCR amplifications. PCR experiments were performed using *Taq* DNA polymerase as recommended by the supplier (Perkin Elmer Cetus). A 900 bp fragment was amplified by oligomers

1 [5'-GCN CCN AT(TAC) TCN AA(AG) GA(TC) AA(TC) GGN ACN-3'] and 2 [5'-NGG (AG)TT NCC NGC NCC (TC)CT (TC)CT (AG)TA (AC)AA-3']. The PCR conditions used to amplify *K. lactis* DNA were as follows: 10 ng total DNA from strain 2359/152 was mixed with 50 pmol of each primer in a final reaction volume of 50 µl and subjected to 30 amplification cycles (95 °C, 1 min; 42 °C, 1 min; 72 °C, 1 min).

Sequence analysis of the *KIPHO5* locus. The DNA restriction fragment harbouring the *KIPHO5* gene fragment was subcloned into the pBluescript plasmids (SK⁺ and KS⁺, Stratagene) and a nested set of closely spaced deletions was created using exonuclease III (Henikoff, 1987). Templates were sequenced on both strands with Sequenase enzyme (Boehringer) using the dideoxynucleotide chain-termination sequencing reactions (Sanger *et al.*, 1977). The products of the sequencing reactions were resolved on buffer gradient polyacrylamide-urea sequencing gels (Biggin *et al.*, 1983) and exposed to Kodak XAR-5 X-ray film. DNA and protein sequences were analysed using the DNASIS and PROSIS programs (Pharmacia-LKB, Hitachi). The deduced amino acid sequence of the *KIPHO5* protein product was compared with the SWISS-PROT database using the FASTA program (Pearson & Lipman, 1988). Alignments of protein sequences were done with CLUSTAL programs (Higgins & Sharp, 1988).

RESULTS

Derepression of acid phosphatase

Three strains of *K. lactis* (2359/152, 2360/7 and MD2/1) were grown in MM to the exponential phase. A basal level of acid phosphatase activity between 1 and 3 units per 10⁷ cells was detected. Cells were harvested, resuspended at a concentration of 2.5 × 10⁷ cells ml⁻¹ in buffered low-P_i medium, and growth and acid phosphatase activity were determined in intact cells and in the supernatant. The results are shown in Fig. 1. No derepressible acid phosphatase activity was found in

Fig. 1. Effect of phosphate starvation on acid phosphatase derepression. Cells were grown to the exponential phase in MM (P_i concentration 1 g l⁻¹) and then resuspended (2.5 × 10⁷ cells ml⁻¹) in buffered low-P_i medium (P_i concentration 10 mg l⁻¹). Samples were removed at the times indicated and acid phosphatase activity was measured in washed cells: ▲, *K. lactis* 2359/152; ■, *K. lactis* 2360/7; ●, *K. lactis* MD2/1. OD₆₀₀: △, *K. lactis* 2359/152; □, *K. lactis* 2360/7; ○, *K. lactis* MD2/1.

strains 2360/7 or MD2/1. However, in strain 2359/152 an increase in acid phosphatase activity was detected after 2 h growth; this reached a maximal value of about 600 times the basal level after 8 h. Most of the phosphatase activity (90–92 %) was associated with washed cells and a small amount (8–10 %) was found in the supernatant. Our results are in agreement with those described for *Sacch. cerevisiae* (Schurr & Yagil, 1971), *Pichia pastoris* (Payne *et al.*, 1995) and *Yarrowia lipolytica* (López & Domínguez, 1988). In view of these findings, to purify the enzyme, *K. lactis* 2359/152 cells were collected after 8 h incubation in buffered low-P_i derepression medium.

Purification of the enzyme

Breakage of cells as described in Methods followed by centrifugation produced a distribution of activity as follows: 30 % in the cell wall (3000 g pellet); 5 % in cell membranes (40000 g pellet) and 65–70 % in the soluble fraction. We decided to use the last fraction as starting material. The purification steps used were similar to those described for other yeast glycoproteins such as invertase in *Sacch. cerevisiae* (Babcinski, 1980) or *Schiz. pombe* (Moreno *et al.*, 1990) or repressible acid phosphatase in *Y. lipolytica* (López & Domínguez, 1988). The results are shown in Table 2. SDS-PAGE of the MonoQ-FPLC-eluted fractions showed by silver staining only a single diffuse band due to the sugar moiety (Fig. 2a, lane 2), a characteristic of yeast glycoproteins (Domínguez *et al.*, 1991; Moreno *et al.*, 1990), with an estimated molecular mass between 90 and 200 kDa.

The covalent association between carbohydrate and protein was confirmed by applying the purified enzyme to a column of concanavalin A-Sepharose. All the activity was retained and could be eluted with 1 M α-methylmannoside. Furthermore, the same band detected in the gels by silver staining could be detected by enzyme activity or by periodic acid/Schiff staining, again indicating the glycoprotein nature of the enzyme (not shown). After exhaustive digestion with Endo H, only one protein band, of molecular mass 52 kDa, appeared (Fig. 2a, lane 3), confirming that the heterogeneity of the enzyme is due to its carbohydrate content. This result was further confirmed by immunoblotting. Rabbit antibodies raised against the purified protein moiety of the *Y. lipolytica* acid phosphatase (Domínguez *et al.*, 1991) reacted with both the complete (Fig. 2b, lane 1) and the deglycosylated protein (Fig. 2b, lane 2). Cross-reactivity of these antibodies against the acid phosphatase of *Sacch. cerevisiae* has also been reported by us (López & Domínguez, 1988), indicating a high similarity between the protein moiety of many yeast repressible acid phosphatases.

The pure enzyme showed an asymmetric pH-activity profile, with maximal activity at pH 4.3 in citric acid/sodium citrate buffer (not shown). Thermostability experiments indicated a high degree of lability for the *K. lactis* acid phosphatase: 25 % and 100 % of the activity was lost after 30 min incubation at 30 °C and 37 °C

Table 2. Purification of an acid phosphatase from derepressed cells of *K. lactis* 2359/152

Purification step	Total protein (mg)	Total activity (units)	Specific activity	Purification (-fold)	Yield (%)	Carbohydrate (%)
Crude extract	6930	1247 600	180	1	100	—
Supernatant	1673	842 000	500	3	68	—
(NH ₄) ₂ SO ₄ (60%)	53	97 600	1850	10	8	—
DEAE-Sephacel	3	90 000	33 900	188	7	50
Biogel A 5-M	2	70 000	36 800	204	6	47
Mono-Q (FPLC)	1	51 200	44 130	245	4	45

Fig. 2. (a) SDS-PAGE of purified repressible acid phosphatase from *K. lactis* (KIPHO5p). Lane 1, size standards; lane 2, KIPHO5p after FPLC Mono Q exclusion; lane 3, KIPHO5p after treatment with Endo H. (b) Blot of (a) with antiserum against the 60 kDa polypeptide of the acid phosphatase of *Y. lipolytica*.

respectively (not shown), in agreement with the results described for the acid phosphatase of other yeasts (Domínguez *et al.*, 1991).

A Lineweaver–Burk plot for purified repressible acid phosphatase was constructed with enzyme assays performed at pH 4.3. An apparent K_m value of 3×10^{-4} M for *p*-nitrophenyl phosphate as substrate was determined.

Molecular cloning of the KIPHO5 gene

The purified protein (digested with Endo H) was separated by SDS-PAGE and electroblotted onto Pro-Blott membrane. A 167 pmol sample of the purified protein (10 µg) was subjected to Edman degradation to obtain the first 15 N-terminal residues of the mature product, NH₂-APIKDNQTVCYALN. Another sample was digested with cyanogen bromide and the peptides separated by reverse-phase HPLC; one of them (that eluting at 21.4 min) was analysed for nine residues. A sequence of NH₂-FYRRGAGNP was obtained. Degenerate oligonucleotides based on both peptides were synthesized and used as primers in PCR to amplify the region of the yeast genomic DNA flanked by them. A

major band, approximately 0.9 kb in length, was detected upon electrophoresis of the PCR products. After sequencing, this fragment showed strong homology with the *PHO5* gene of *Sacch. cerevisiae*. Southern analysis was performed on digested total DNA of *K. lactis* and probed with the PCR fragment (not shown). An approximately 2500 bp (*HpaII*–*HpaII*) fragment was used to obtain a minigenomic DNA library that was assayed for positive clones with the homologous probe. From 1023 colonies assayed, seven positive clones were detected. The insert (*HpaII*–*HpaII*) was sequenced on both strands, revealing an ORF of 1407 bp (Fig. 3). The gene was designated *KIPHO5*.

Analysis of KIPHO5 flanking sequences

The nucleotide sequence surrounding the putative initiation codon conformed to the consensus for translation initiation in yeasts, with a conserved A at positions –1 and –3 (Cigan & Donahue, 1987; Kozak, 1987). A TATA element in the 5' untranslated region of the *KIPHO5* gene was found at position –134 relative to the A of the initiation codon, preceded (at –214) by a CAAT box (see Fig. 3). In yeast TATA boxes have been found at positions varying from –30 to –300 upstream from the translation start (Breathnach & Chambon, 1981; Kim *et al.*, 1986).

Vogel *et al.* (1989) identified two upstream activating sequences involved in transcriptional regulation of the phosphate-repressible *Sacch. cerevisiae* acid phosphatase gene *PHO5*. The yeast gene sequence CACGT(G/T) is the target for binding a putative *PHO4* regulatory factor. We found such a sequence in the 5' flanking region of the *K. lactis* acid phosphatase gene at –430 and –192 relative to the first nucleotide of the ATG start codon (Fig. 3). Also, a region with a symmetric axis –ACTTTCTAAGAAAGT– between the two *PHO4*-binding sites could be defined (positions –319 to –305). Both the spacing and the symmetry resemble those described for the *PHO2*-binding site in *Sacch. cerevisiae* (Vogel *et al.*, 1989).

In yeast, the TAA ... TAGT/TATGT ... TTT transcription termination motif (Zaret & Sherman, 1982) is found at the 3' end of most genes so far published. These motifs were also observed at the 3' end of the *KIPHO5* gene (Fig. 3, open circles).

Translation of the *KIPHO5* ORF would yield a 469 amino acid polypeptide, moderately hydrophilic, with a predicted molecular mass of 52 522 Da and an isoelectric point of 4.17. A codon bias value of 0.23 was calculated (according to Bennetzen & Hall, 1982) which corresponds to a protein of low expression.

The protein carries 10 potential sites for N-glycosylation and, similarly, 12 glycosylation sites are found in the *Sacch. cerevisiae* acid phosphatases (Arima *et al.*, 1983; Bajwa *et al.*, 1984). Currently we do not know whether all these acceptor sites bear oligosaccharide chains N-glycosidically attached to the asparagine residue, although Riederer & Hinnen (1991) have described that in *Sacch. cerevisiae* all 12 sequons are glycosylated.

Northern blotting using the 1500 bp *Bam*HI–*Bgl*II fragment as probe revealed a single band of about 1·8 kb in RNA from *K. lactis* 2359/152 (see Fig. 5). This observation suggests that in our conditions the *Bam*HI–*Bgl*II fragment does not include another transcriptional unit. *KIPHO5* transcription start sites were determined by extending a ³²P-labelled synthetic primer with reverse transcriptase following hybridization to poly(A)⁺ RNA. Two major and three minor cDNA products were obtained, indicating that the initiation of transcription preferentially takes place at the C and G residues –34 and –40 bases upstream from the predicted start of translation (Fig. 4). Three faint bands also appeared at –35, –46 and –53, taking ATG as position +1.

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Fig. 4. Transcript mapping by primer extension. A synthetic oligonucleotide complementary to the sense strand of the *KIPHO5* gene between +24 and +48 was labelled at the 5' end (10^5 c.p.m., 0.125 pmol) and annealed to 3.5 μ g poly(A)⁺ RNA. The primer was then elongated with murine reverse transcriptase (Amersham) and the extended products (lane 2) were resolved on a sequencing gel alongside the sequencing ladder of the noncoding strand (lanes A, C, G, T) obtained using the same oligonucleotide as a primer. Lane 1, control (tRNA).

Fig. 5. Northern blot analysis of *KIPHO5* mRNA levels as a function of the derepression time. Total RNA was isolated from cells grown under derepression conditions in low- P_i medium (see Fig. 1). Samples of 15 μ g RNA taken after 0, 2, 4, 6 and 8 h (lanes 1, 2, 3, 4 and 5, respectively) and after 8 h in high- P_i medium (lane 6) were hybridized with a *KIPHO5* fragment as probe (top) or with an *ACT1* gene fragment (actin probe, bottom) kindly provided by M. Wesolowski-Louvel.

Expression of *KIPHO5* during induction conditions

We have shown that the production of acid phosphatase by *K. lactis* must be derepressed. We used a *Bam*HI–*Bgl*II fragment of 1500 bp as probe in Northern blot analysis to measure the time course of *PHO5* expression in low- P_i medium (Fig. 5). Only one band appeared and this revealed a temporal pattern of expression similar to those described for *Sacch. cerevisiae*. As shown in Fig. 5, *PHO5* transcripts were absent when the cells were first shifted to the low- P_i medium (time 0) and expression of the *PHO5* gene was first detected 4 h after the transfer to the low- P_i medium (lane 3). The amount of transcripts reached a maximum level between 6 and 8 h (lanes 4 and

5), coinciding with maximum enzyme activity (see Fig. 1). No activity could be detected in cells grown in normal MM (lane 6). To control the integrity of the RNA isolated under different conditions, the same blot was hybridized to a fragment containing the actin gene; the corresponding transcripts were visible at approximately equal intensities in all the gel lines.

Expression of *KIPHO5* in *Sacch. cerevisiae*

If structural homology is indeed indicative of functional homology, the functional *PHO5* homologue from *K. lactis* should be expressed in *Sacch. cerevisiae*. An *Hpa*II–*Hpa*II DNA fragment of 2.5 kb (see Table 1), which contains the entire *PHO5* gene, was cloned in pSK1 (Bianchi *et al.*, 1987) giving rise to pEF5 (Table 1). This plasmid, together with pSK1 (as control), was used to transform the *Sacch. cerevisiae* strain ACX1-2A (*ura3-25 leu2-112 trp1 pho3 pho5*), selected by us after ascus digestion of a diploid strain derived from a cross between AH220 and TD29 (López, 1989).

The desired transformants were selected on the basis of their expected *Ura*⁺ phenotype. From an estimated 10 *Ura*⁺ transformants obtained with pSK1, none was *Pho*⁺, as could be expected. Of 10 *Ura*⁺ transformants obtained with pEF5, all were *Pho*⁺, indicating that the *PHO5* gene from *K. lactis* was functionally expressed in *Sacch. cerevisiae* and thus complementing the *pho5* mutation. Acid phosphatase activity was also repressible by orthophosphate. To confirm our results, plasmid segregation experiments were carried out as previously described (Sánchez *et al.*, 1993). In all cases (50 transformants selected randomly), loss of the *Ura*⁺ character was accompanied by loss of the *Pho*⁺ character, confirming the functionality of the *PHO5* gene of *K. lactis* in *Sacch. cerevisiae*.

Mapping of the *KIPHO5* locus in several *K. lactis* strains

In standard *Sacch. cerevisiae* strains several repressible and one constitutive acid phosphatase have been described to be located on different chromosomes (Steensma *et al.*, 1989). To see whether a multigene family of acid phosphatases scattered on different chromosomes also exists in *K. lactis*, the *KIPHO5* locus was mapped in the standard strain CBS2359 (Workshop *Biology of Kluyveromyces* II, Rome, 1989).

First, we examined Southern blots of yeast chromosomes separated by CHEF (Wesolowski-Louvel & Fukuhara, 1995) using the cloned *KIPHO5* as probe. Only one band (even after long autoradiographic exposures) located on chromosome II appeared (Fig. 6), indicating that either several (all?) acid phosphatases are located on this chromosome or that in *K. lactis* only one repressible acid phosphatase exists.

To confirm these experiments, and since no repressible acid phosphatase could be detected in *K. lactis* strains 2360/7 and MD2/1, we performed Southern blot experiments using the same probe on genomic DNA

Fig. 6. CHEF gel and hybridization determining the location of *KIPHO5* on the electrophoretic karyotype. (a) CHEF gel illustrating separation of chromosomes for strain 2359/152; chromosomes IV and III are not resolved. (b) An identical gel blotted and hybridized with the *Bam*HI–*Bgl*II fragment of *KIPHO5* as probe.

Fig. 7. Southern blot analysis of the *KIPHO5* locus in three *K. lactis* strains. Genomic DNA prepared as described in Methods was digested with *Cla*I (lanes 1, 4 and 7), *Bam*HI (lanes 2, 5 and 8) or *Eco*RI (lanes 3, 6 and 9). After transfer to Hybond N membrane filters (Amersham), the DNA was probed with the *Bam*HI–*Bgl*II fragment. Size standards are indicated on the left.

prepared as described in Methods (Fig. 7). The DNA was digested with *Cla*I (lanes 1, 4 and 7), *Bam*HI (lanes 2, 5 and 8) or *Eco*RI (lanes 3, 6 and 9). Surprisingly, the same fragments were recognized in all the strains with either *Cla*I or *Bam*HI and only one extra fragment of

5.5 kb appeared in strain 2359/152. As no *Eco*RI restriction site was found in all 3555 bp extended *Hpa*II–*Bam*HI (–771 bp upstream of the ATG and 1377 bp after the TAA) fragments sequenced by us (not shown), we propose that this fragment encodes the repressible acid phosphatase. Thus, the only rational explanation for all our results is the existence of another constitutive acid phosphatase in the three *K. lactis* strains analysed and also located on chromosome II.

DISCUSSION

Because of its distinctive physiological properties, *K. lactis* has become an important alternative to the classical *Sacch. cerevisiae* and we therefore decided to study the phosphate assimilation system in this yeast. Our first results showed that two strains used in many laboratories – *K. lactis* 2359/152 and *K. lactis* 2360/7 – show different behaviour in low- P_i medium. No repressible acid phosphatase could be detected in strain 2360/7 while a repressible activity, which increased with incubation time in a similar way to the results described for *Sacch. cerevisiae* or *Y. lipolytica* (Bostian *et al.*, 1980; López & Domínguez, 1988), was found in strain 2359/152. The same results were obtained with strains CBS2359 and CBS2360 (unpublished results). Although several acid phosphatase genes have been isolated from yeasts and fungi (Arima *et al.*, 1983; Bajwa *et al.*, 1984; Elliot *et al.*, 1986; Yang & Schweingruber, 1990; MacRae *et al.*, 1988; Haas *et al.*, 1992; Piddington *et al.*, 1993) no significant homology has been detected between the proteins (Piddington *et al.*, 1993). Thus, in order to clone the gene we decided to purify the protein. The purified protein gave a single smeared wide band on SDS-PAGE, similar to those described for acid phosphatases from other yeast and for yeast secretory glycoproteins in general. The molecular mass estimated by SDS-PAGE was 90–200 kDa. Treatment with Endo H produced a sharp band of 52 kDa, again of comparable size to the deglycosylated band of the acid phosphatase of *Sacch. cerevisiae* (Bostian *et al.*, 1980). Characteristics such as K_m values, heat denaturation and isoelectric point were similar to those described for other yeast acid phosphatases. Additionally, the enzyme shows cross-reactivity with antibodies obtained against the protein moiety of the repressible acid phosphatase from *Y. lipolytica* (Domínguez *et al.*, 1991), indicating that all these proteins share common epitopes.

We obtained the sequences of the N-terminus and of an internal peptide, designed degenerate oligonucleotides, and isolated by PCR a DNA fragment that after sequencing was used as probe to isolate genomic clones from a mini yeast genomic DNA library. The sequences of selected clones identified a complete ORF and surrounding sequences. The ORF, designated *KIPHO5*, contains the original peptide sequences and encodes a protein, KIPho5p, whose predicted size agrees well with its apparent size in SDS-polyacrylamide gel (469 aa, 52500 Da). The DNA sequence predicts a signal peptide of 16 amino acid with an Ala-Ala processing site. The

next 15 amino acids – 17 to 31 – correspond exactly to those obtained after sequencing the N-terminal portion of the mature enzyme, indicating that the wild-type KIPho5p is synthesized as a precursor that is proteolytically processed to produce the mature form as has been shown for *Sacch. cerevisiae* acid phosphatase (Haguenauer-Tsapis & Hinnen, 1984). The enzyme also has ten glycosylation sites. Comparison of the protein sequence with the ScPho5p shows that the best alignment was found in the four C-terminal glycosylation sites. This observation supports the conclusion of Riederer & Hinnen (1991) that the oligosaccharides found at the C-terminus have the most prominent influence on protein folding. KIPho5p shows strong similarity with ScPho5p and ScPho3p and, to a lesser extent, with SpPho1p and PpPho1. Our results are in agreement with the evolutionary relationship described for *Sacch. cerevisiae* and *K. lactis* (Barns *et al.*, 1991).

In KIPho5p, the consensus signature RHGXXRP, described by Piddington *et al.* (1993) for all – *E. coli*, yeast, fungal, rat and human – acid phosphatases, is found between amino acids 76 and 82. Currently, site-specific mutagenesis is being carried out to confirm this aspect. The signals for translation, transcription, termination and polyadenylation agree with the consensus for yeast motifs in general (Cigan & Donahue, 1987; Kozak, 1987; Zaret & Sherman, 1982).

Transcription of *KIPHO5* is strongly regulated in response to the level of inorganic phosphate. By primer extension in the promoter we identified five specific initiation sites. All of them bear the consensus motif RRYRR (R = purine; Y = pyrimidine) or TC(G/A)A described by Hahn *et al.* (1985). Furthermore, two of them are major transcription initiation sites and lie between 55 and 110 bp downstream of the functional TATA element, as has been described by Rudolph & Hinnen (1987) for *Sacch. cerevisiae*.

Rudolph & Hinnen (1987) have described regulatory regions in the *Sacch. cerevisiae* *PHO5* promoter that act as phosphate-controlled upstream activation sites (UASps). Two of them [CACGT(G/T)] are also found in the 5' flanking region of the *KIPHO5* promoter. Our previous results (unpublished) support the idea that those regions are in fact the binding site for the regulatory protein Pho4, in agreement with the results described for *Sacch. cerevisiae* by Rudolph & Hinnen (1987) but in contrast with those reported for the same yeast by Bergman *et al.* (1986) or for the *Penicillium chrysogenum* *phoA* gene by Haas *et al.* (1992).

In *Sacch. cerevisiae* it has been described that Pho2 binds to a region located between the two UASs. We located a region in the *KIPHO5* promoter with a symmetrical axis (see Results) and are now carrying out experiments to explore the possible role of this region.

We cloned a 2.5 kb fragment of *K. lactis* DNA containing the whole *KIPHO5* gene and the promoter region (–771 bp upstream of the ATG) in an autonomous plasmid, pSK1, able to function as a shuttle vector

between *K. lactis* and *Sacch. cerevisiae*. After transforming a strain of *Sacch. cerevisiae* lacking repressible acid phosphatase activity we observed the recovery of this activity. Also, the acid phosphatase activity is regulated by the amount of P_i in the culture medium (repressed in normal MM and derepressed in low- P_i medium). Our results indicate that the *PHO5* gene from *K. lactis* is functionally expressed in *Sacch. cerevisiae* and that in the *KIPHO5* promoter there are regulatory elements that respond to the regulatory proteins Pho4 and Pho2.

Identification of the physical location of the *KIPHO5* gene has allowed us to examine the organization of the acid phosphatase gene family in *K. lactis*. Using chromosomal hybridization we mapped the *KIPHO5* gene on chromosome II of strain CBS2359. In contrast with the results described for *Sacch. cerevisiae* (Steensma *et al.*, 1989), we were unable to find any other signal. A similar result was obtained with strain CBS2360 (not shown). However, after Southern blot experiments we found differences between the two strains. With *Clal* and *BamHI* the same bands appeared, whilst with *EcoRI* a different pattern emerged.

In view of our results – derepression conditions, gene sequencing, chromosome mapping and Southern blotting – we believe that strain 2360/7 has only one acid phosphatase, located on chromosome II. Strain 2359/152 has a constitutive and a repressible acid phosphatase, both also located on chromosome II. We know that the constitutive acid phosphatase is either located distant from the *KIPHO5* gene, towards the 3' region (we have sequenced a further 6 kb) or upstream of the promoter. This is therefore opposite to the results described for *Sacch. cerevisiae* (Bajwa *et al.*, 1987). Experiments to isolate the constitutive *KIPHO* gene are currently under way in our laboratory.

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