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Restoration of inositol prototrophy in the fission yeast *Schizosaccharomyces pombe*

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The biosynthesis of inositol requires only two enzymes, inositol-1-phosphate synthase (encoded by *INO1*) and an inositol monophosphatase, but the regulation of inositol biosynthesis is under multiple controls and is exquisitely regulated. In the budding yeast *Saccharomyces cerevisiae*, mutations in any of 26 different genes lead to inositol auxotrophy. The fission yeast *Schizosaccharomyces pombe*, however, is a natural inositol auxotroph. An investigation has been initiated to examine the possible reasons that might have led to inositol auxotrophy in *Sch. pombe*. Complementation with a genomic library of an inositol prototrophic yeast indicated that a *Pichia pastoris INO1* gene alone could confer inositol prototrophy to *Sch. pombe* and that the gene was absent in *Sch. pombe*. To investigate possible reasons for the loss of *INO1* gene in *Sch. pombe*, an attempt was made to disrupt inositol homeostasis in *Sch. pombe* by overproduction of intracellular inositol, but this did not lead to any discernible adverse effects. The sources of inositol in the natural environment of *Sch. pombe* were also examined. As the natural environment of *Sch. pombe* contains significant amounts of phytic acid (inositol hexaphosphate), an investigation was carried out and it was discovered that *Sch. pombe* can utilize phytic acid as a source of inositol under very specific conditions.

Keywords: *Schizosaccharomyces pombe*, inositol auxotrophy, *INO1*, phytic acid

INTRODUCTION

Inositol is essential for the growth of all eukaryotic cells. It is a precursor of phosphatidylinositol (PI), a major membrane phospholipid, and also a precursor in the synthesis of sphingolipids and glycosylphosphatidylinositol (Steiner & Lester, 1972). Phosphorylated metabolites of inositol play an important role in the signal transduction pathway (reviewed by Majerus *et al.*, 1986).

The biosynthesis of inositol involves the conversion of glucose 6-phosphate to inositol 1-phosphate through a set of complex reactions involving a coupled oxidation/reduction, stearic rearrangement and cyclization (Eisenberg *et al.*, 1964) and is carried out by a single enzyme, inositol-1-phosphate synthase (Donahue & Henry, 1981). The structural gene for this enzyme is *INO1* and it has been isolated from yeasts (Klig & Henry, 1984; Klig *et al.*, 1991), protozoa, plants and mammals (Majumdar *et al.*, 1997). In the second reaction, inositol monophosphatase dephosphorylates inositol 1-phosphate to give free inositol (Murray & Greenberg, 1997). Although most eukaryotic organisms have the capacity to synthesize their own inositol, a few are naturally auxotrophic for inositol. *Saccharomyces carlsbergensis*, *Kloekera apiculata* and *Schizosaccharomyces* spp. show an absolute requirement for inositol (Ridgway & Douglas, 1958; McVeigh & Bracken, 1955).

A genetic investigation into inositol biosynthesis in *Saccharomyces cerevisiae* has revealed a complex pathway controlling inositol biosynthesis (reviewed by Greenberg & Lopes, 1996). Apart from mutations in *INO1* (Dean-Johnson & Henry, 1989), mutations in several other loci also lead to inositol auxotrophy (Table 1). Mutations in several other genes, however, lead to inositol overproduction and an inositol excretion phenotype (reviewed by Greenberg & Lopes, 1996). The involvement of such a large number of genes indicates that inositol biosynthesis is a highly regulated process.

Abbreviations: EMM, Edinburgh Minimal Medium; PI, phosphatidylinositol.

The GenBank accession number for the sequence reported in this paper is AF078915.

Table 1. Genes required for inositol prototrophy in *Sac. cerevisiae*

Function	Gene	Reference
Transcription factors	*INO2*, *INO4*, *SWI1*, *SWI2*, *SWI3*, *HAC1*, *SPT7*	Greenberg & Lopes (1996); Nikawa *et al.* (1997)
RNA polymerase II subunits	*RPB1*, *RPB2*, *RPB4*, *SRB2*	Greenberg & Lopes (1996); Archambault *et al.* (1996)
TATA-binding protein	*SPT15*	Greenberg & Lopes (1996)
Co-activators for transcription	*ADA1*, *ADA5*, *BSD2*, *SUB1*	Kagiwada *et al.* (1996); Knaus *et al.* (1996); Horiuchi *et al.* (1997); Roberts & Winston (1996)
Enzymes	*INO1*, *IRE1*, *TRL1*, *UBC4*, *UBC5*, *DOA4*	Greenberg & Lopes (1996); Dean-Johnson & Henry (1989); Sidrauski *et al.* (1996); Henry & Patton-Vogt (1998)
Integral membrane proteins	*SAC1*, *SCS2*	Greenberg & Lopes (1996); Whitters *et al.* (1993)
Unknown function	*SCS3*, *CSE1*	Greenberg & Lopes (1996); Hosaka *et al.* (1994)

We are interested in the fission yeast *Schizosaccharomyces pombe* and the possible reasons why it has evolved as a natural inositol auxotroph. Analysis of membrane phospholipids in *Sch. pombe* indicate that under inositol-supplemented conditions, PI levels in the membrane are comparable to that of *Sac. cerevisiae* (Fernandez *et al.*, 1986). In these cells, inositol is taken up from the growth medium via inositol transporters (Niderberger *et al.*, 1998). In the absence of inositol, *Sch. pombe* cells, however, undergo growth stasis and eventually die.

Earlier studies on the natural inositol auxotrophy of *Sch. pombe* have demonstrated an increased resistance to death in the absence of inositol in *Sch. pombe* as compared to *Sac. cerevisiae* (Fernandez *et al.*, 1986). In addition, the turnover products of PI were shown to be inositol in *Sch. pombe* (rather than glycerophosphoinositol) and can be reutilized in *Sch. pombe* (Angus & Lester, 1975; Fernandez *et al.*, 1986). These properties have been shown to confer an increased ability on *Sch. pombe* to survive under adverse inositol conditions. In this study, however, we have reinvestigated slightly different aspects of inositol auxotrophy in *Sch. pombe*. We have attempted to examine possible reasons for inositol auxotrophy in these yeasts and to isolate genes from inositol prototrophic organisms that can confer inositol prototrophy in *Sch. pombe*. In addition we have examined the consequences of disturbing inositol homeostasis in this yeast and have examined whether phytic acid, which is present in abundance in the natural environment of *Sch. pombe*, can be utilized as a source of inositol by this yeast.

METHODS

Chemicals and reagents. All chemicals were of analytical grade and were obtained from local companies or Sigma. Phytic acid (sodium salt) was obtained from Sigma. Media components were from Hi Media and Difco. MES (monohydrate) was from USB. Restriction enzymes and modifying enzymes were from New England Biolabs, Boehringer Mannheim and Bangalore-Genei. Oligonucleotides were obtained from Ransom Hill Biosciences. The DIG DNA-labelling and detection kit was from Boehringer Mannheim and the DNA sequencing kit (Sequenase version 2.0) was from USB. [α-^{35}S]dATP, with a specific activity of 1·0 Ci mmol^{-1} (37 GBq mmol^{-1}), was obtained from Bhabha Atomic Research Centre, India. The DNA prep kit was from Qiagen.

Yeast strains, media and growth conditions. The yeast strains used in this work were *Sch. pombe* ABP2 (*h*$^+$ *ura1-161*), ABP13 (*h*$^-$ *ade6-210 ura4Δ-18*), ABP20 (*h*$^+$ *ade6-216 leu1-32 ura4Δ-18*) and ABP320 (*h*$^+$ *arg3Δ-4 ura4Δ-18*), and *Sac. cerevisiae* ABC719 (FY250) (*MATα trp1 his3 ura3 leu2 ino1::HIS3*).

Sch. pombe was routinely grown at 30 °C under aerobic conditions in Yeast Extract Supplements (YES) medium (0·5 % yeast extract, 3·0 % glucose and supplements adenine, leucine, uracil and arginine at 50 mg l^{-1} each, as per requirements). Edinburgh Minimal Medium (EMM) was prepared as described by Alfa *et al.*, (1992) except that sodium glutamate (0·5 %) was used as a nitrogen source and supplements were used for defined medium growth. EMM − Ino was identical to EMM except that vitamin stock was prepared by omitting inositol. EMM is deficient in thiamine, therefore EMM + thiamine was prepared by adding 0·5 μM thiamine to EMM. EMM − Arg was EMM without arginine. EMM (high phosphate) was the regular EMM containing 12·64 mM Na_2HPO_4. To make EMM (low phosphate), the concentration of Na_2HPO_4 was reduced from 12·64 to 0·1 mM (Dhamajia *et al.*, 1987). To prepare low and high pH minimal media, EMM was supplemented with 100 mM MES (as buffering agent) and the pH was adjusted with HCl or NaOH. Plates were prepared by adding 2 % agar. Filter-sterilized phytic acid was added where indicated to a final concentration of 0·5 mM. LiCl- and NaCl-containing plates were prepared by adding different concentrations of LiCl and NaCl to EMM. *Sac. cerevisiae* was grown in YPD and SD, as well as in YES and EMM in which it grew well.

Plasmid constructions. The different subclones that were constructed for complementation analysis and sequencing purposes were constructed in *Sch. pombe* shuttle vectors pSP1 and pSP2 (Cottarel *et al.*, 1993). Plasmid pSPIN6 was constructed by cloning a 3·3 kb *Bam*HI fragment of the *Pichia pastoris INO1* gene into the *Bam*HI site of pSP2 (Cottarel *et al.*, 1993). Plasmid pSPIN6 was digested with *Sal*I and religated to obtain plasmid pSPIN7 (i.e. a 2·2 kb *Bam*HI–*Sal* I fragment in pSP2). pSPIN14 was obtained by cloning a 3·3 kb *Bam*HI fragment into the *Bam*HI site of pUR18N. Plasmid pnmt-INO1 was constructed by cloning a 3·3 kb *Bam*HI fragment of the *P. pastoris INO1* gene into the *Bam*HI site downstream of the thiamine-repressible *nmt1*$^+$ promoter in the expression

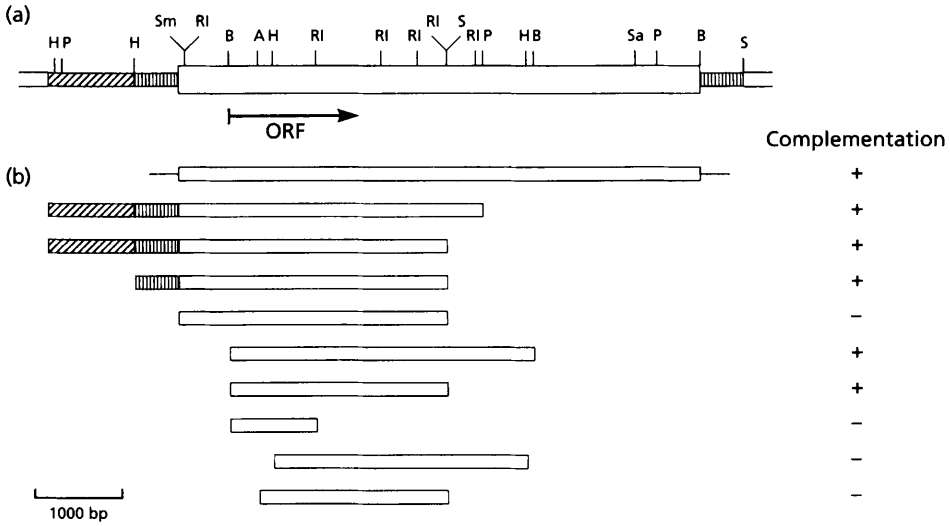

Fig. 1. (a) Restriction map of the *INO1* gene of *P. pastoris*. The insert with flanking vector sites has been shown. Unshaded region, insert; vertically hatched region, *tet* gene of vector; diagonally hatched region, vector. A, *Asp*718; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; RI, *Eco*RI; S, *Sal*I; Sa, *Sac*I; Sm, *Sma*I. (b) Subcloning and complementation data. Only the insert region has been shown for each subclone. The largest fragment corresponds to the original clone containing the 6 kb insert.

vector paR33d (*arg3*$^+$ as selection marker) (Waddell & Jenkins, 1995). All plasmids were maintained in *Escherichia coli* DH5α. *E. coli* cells were cultured in LB medium at 37 °C and 100 μg ampicillin ml^{-1} was added for selection of plasmids.

Transformation of *Sch. pombe*. *Sch. pombe* transformations were carried out by the lithium acetate method as described previously (Chaudhuri *et al.*, 1997).

Isolation of genomic DNA and plasmid DNA from yeast. Plasmid DNA and genomic DNA were isolated from *Sch. pombe* by the glass bead lysis method as described for *Sac. cerevisiae* (Kaiser *et al.*, 1994).

Recombinant DNA methods. Plasmid DNA isolation and DNA manipulation were carried out according to standard procedures (Sambrook *et al.*, 1989).

The DNA sequence was determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) using Sequenase version 2.0. The sequence was obtained by using subclones and primers T_3 and T_7. From this sequence, primers were constructed to obtain a complete sequence on both strands.

Southern blot analysis was carried out using a non-radioactive DIG DNA labelling and detection kit (Boehringer Mannheim), according to the manufacturer's protocols. A 0·8 kb *Eco*RI fragment of the *P. pastoris INO1* gene was used as a probe and hybridization was done at 64 and 50 °C.

Growth experiments. A single colony from a freshly grown culture was inoculated into EMM and grown until it reached late exponential phase. Cells were harvested, washed twice with EMM−Ino and inoculated in EMM containing the desired supplements to an initial OD_{600} ~0·1. Cultures were incubated at 30 °C on a shaker and after regular intervals growth was monitored by measuring OD_{600}.

Inositol excretion assay. Transformants were grown overnight in EMM plus supplements but lacking arginine or EMM plus supplements but lacking uracil. Cells were washed, resuspended in the same volume of water and 10 μl of this suspension was spotted on an EMM−Ino plate containing limiting adenine (10 mg l^{-1}) on which 10^6 *Sch. pombe* ABP13 cells containing an *ade6-210* mutation were spread. The plates were incubated at 30 °C. After 2 d the presence of a red ring around the spotted cells indicated they were excreting inositol and allowing *Sch. pombe* ABP13 to grow. The diameter of the ring was a measure of the extent of inositol excretion.

Phospholipid estimation. The phospholipid composition of *Sch. pombe* cells grown under different conditions was determined. *Sch. pombe* cells were grown in the presence of inositol, in inositol-deprived medium and by overexpressing *INO1*. For inositol deprivation, cells were first grown in EMM for few hours, washed twice with EMM−Ino and re-suspended in EMM−Ino medium. For *INO1* overexpression *Sch. pombe* cells containing pnmt-INO1 were grown in EMM−Ino and thiamine was not added for derepressed conditions. Lipids were extracted from cells according to Folch's procedure (Folch *et al.*, 1957). Phospholipids were separated on silica-impregnated G-60 plates by two-dimensional TLC. The first dimension solvent contained chloroform/methanol/25% ammonia solution (65:25:5, by vol.) and the second dimension solvent contained chloroform/methanol/acetone/acetic acid/water (40:8:16:8:3·5, by vol.). After chromatography spots were labelled with iodine vapours and the spots corresponding to the various phospholipids were cut out very carefully. Quantitative estimation of phospholipids was done according to Wagner's protocol (Wagner *et al.*, 1962).

RESULTS AND DISCUSSION

Cloning and analysis of a gene that confers inositol prototrophy to *Sch. pombe*

As the sequencing of the *Sch. pombe* genome is still incomplete, whole genome comparisons with an inositol prototrophic yeast such as *Sac. cerevisiae* are still partial. We therefore decided to attempt to isolate genes that

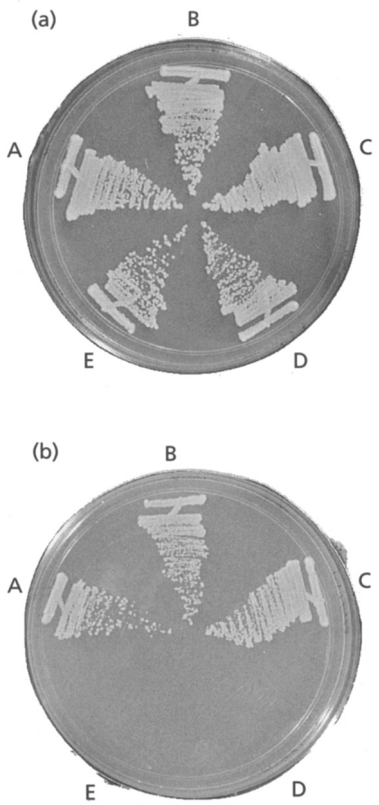

Fig. 2. Growth of *Sch. pombe* transformants on minimal medium with (a) or without (b) inositol. *Sch. pombe* ABP20 was transformed with (A) pSPIN-1, (B) pSPIN-6, (C) pSPIN-7, (D) pSPIN-14 or (E) pSP2.

might confer inositol prototrophy in *Sch. pombe* and give some clues about inositol auxotrophy. We used libraries from inositol prototrophic organisms (a human cDNA library constructed in a *Sch. pombe* expression vector (Superti-Furga *et al.*, 1996) and a genomic library of *P. pastoris*, constructed in a *Sch. pombe* cloning vector (Gould *et al.*, 1992). Transformants were selected on minimal medium lacking inositol. No complementing transformants were obtained with the human cDNA library. However, with the *P. pastoris* genomic library several transformants were obtained that allowed growth on medium lacking inositol. These transformants were confirmed by plasmid loss. Isolation of the plasmids and restriction mapping showed that there were three different plasmids that could confer inositol prototrophy. However, all three plasmids contained overlapping regions as seen from restriction mapping, suggesting the presence of a common gene in all the plasmids. One of these clones, with an insert of approximately 6 kb, was picked up for further study. Subclones were constructed in the *Sch. pombe* vectors pSP1 and pSP2 (Cottarel *et al.*, 1993), making use of the available restriction sites, and checked for complementation on minimal medium containing inositol (Fig. 1). The smallest complementation fragment was a 2·2 kb *Bam*HI–*Sal* I fragment (Fig. 2).

Sequencing of the minimal complementing fragment revealed an ORF encoding 525 aa. When we examined the GenBank and EMBL databases using the BLAST program (Altschul *et al.*, 1990), the ORF product was shown to have significant homology to the *Candida albicans* and *Sac. cerevisiae* INO1 proteins, indicating that we had cloned the *P. pastoris INO1* gene. This was also confirmed by multiple sequence analysis of different yeast INO1 proteins (data not shown). Interestingly, the *Bam*HI–*Sal* I fragment contained only 60 bp of the *P. pastoris INO1* promoter which was sufficient to permit expression of this gene in *Sch. pombe* and to allow the strains bearing this plasmid to behave as inositol prototrophs. However, when we cloned the same fragment into pUR18N (to yield pSPIN14), no inositol prototrophy was conferred (Fig. 2). This indicated that the vector region upstream of the *Bam*HI site in vector pSP2 was possibly behaving as a cryptic promoter, allowing sufficient expression of the *INO1* gene in this vector.

The cloning of the *P. pastoris INO1* gene complementing inositol auxotrophy in *Sch. pombe* suggested that the gene was probably absent in *Sch. pombe*. *Sch. pombe* extracts have previously been shown to lack inositol-1-phosphate synthase activity (Fernandez *et al.*, 1986). An alternative possibility would be that *INO1* could be present, but that the gene is not expressed. We carried out Southern blotting using the *P. pastoris INO1* gene as a probe. However, we could not see any band even at a lower hybridization temperature of 50 °C at which bands could be seen for *C. albicans* and *Sac. cerevisiae* (data not shown). This suggested that the gene was absent in *Sch. pombe*. As an *INO1* gene with no significant stretches of homology would also fail to show in Southern blotting, confirmation of the absence of the gene will only be possible after completion of the *Sch. pombe* genome sequencing project.

Effect of *INO1* overexpression in *Sch. pombe*

The apparent absence of the *INO1* gene in *Sch. pombe* made us look for possible reasons for this absence. Such questions are difficult to address experimentally. However, we considered the possibility that the fine regulatory control that existed for the *INO1* gene in *Sac. cerevisiae*, for some reason, did not evolve in *Sch. pombe*. In such a situation *INO1* gene expression would be deleterious for the cells under some conditions. To test this possibility we decided to overexpress the *P. pastoris INO1* gene in *Sch. pombe* and examine the subsequent phenotypes.

The *P. pastoris INO1* gene was cloned downstream of the strong, thiamine-repressible *Sch. pombe nmt1*$^+$ promoter (Maundrell, 1990). The resulting plasmid was transformed into *Sch. pombe*. In the presence of thiamine, *Sch. pombe* cells containing this plasmid failed to synthesize inositol-1-phosphate synthase and inositol was required extraneously for growth. In the absence of thiamine, however, *Sch. pombe* cells containing pnmt-INO1 grew on minimal medium lacking inositol

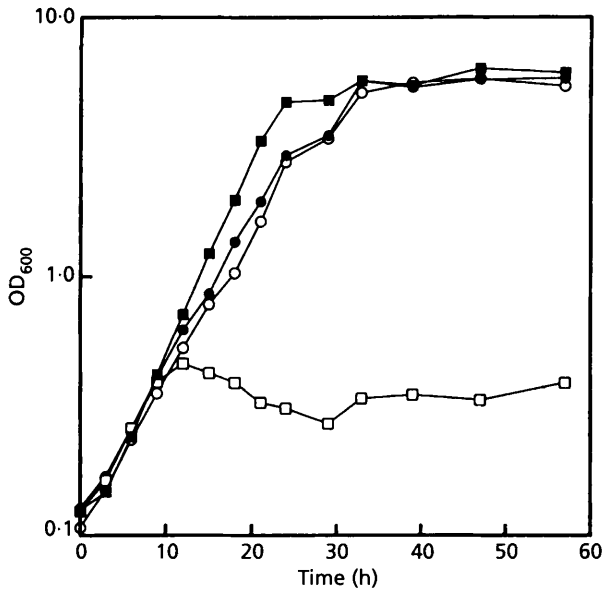

Fig. 3. Growth of *Sch. pombe* ABP 320 containing pnmt-INO1 in EMM with thiamine + inositol (■j), thiamine and no inositol (□), inositol and no thiamine (●) and no inositol and no thiamine (○).

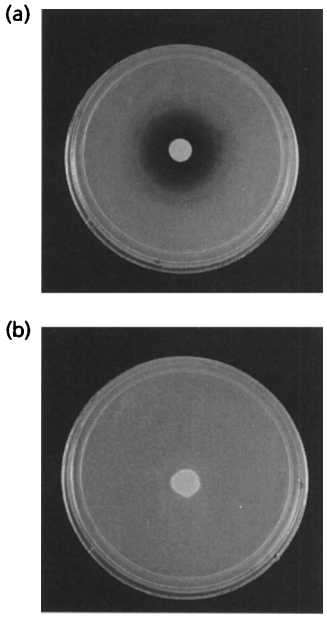

Fig. 4. Excretion of inositol from *Sch. pombe* cells bearing pnmt-INO1 (a) or pSPIN7 (b).

(EMM − Ino) (Fig. 3). Overexpression of the *INO1* gene in *Sac. cerevisiae* leads to overproduction of inositol and thus excretion of inositol in the medium (Greenberg *et al.*, 1982). In *Sch. pombe* cells, when the *INO1* gene was overexpressed, we also checked whether the cells excreted inositol. Inositol excretion was observed in cells containing pnmt-INO1 under derepressing conditions, reflecting an excess of inositol within the cell (Fig. 4).

Table 2. Phospholipid composition of *Sch. pombe* strain ABP320 grown under different conditions

Phospholipid estimation was done as described in Methods. The values given are from a representative experiment. PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

Inositol (μM)	Phospholipid (%)					
	PI	PS	PC	PE	PG	CL
50	20·60	6·10	57·59	14·25	0·42	0·57
0	2·25	14·79	59·88	16·08	1·10	0·97
0 (pnmt-INO1)	15·39	6·54	55·77	19·39	1·92	1·00

These cells were examined for differences in morphology but no differences could be detected (data not shown). We also examined growth of *Sch. pombe* containing pnmt-INO1 at different growth temperatures (22, 30 and 37 °C), but we did not observe any effects on *INO1* overexpression (data not shown).

The second enzyme in inositol biosynthesis is inositol monophosphatase. *Sac. cerevisiae* contains several inositol monophosphatases (Murray & Greenberg, 1997) but the precise role of each is yet to be determined. The ability of the *INO1* gene alone to confer inositol prototrophy suggested that such an enzyme activity was also present in *Sch. pombe*. Recently, an inositol monophosphatase activity has been demonstrated in *Sch. pombe* (Kippert, 1997). Inositol monophosphatase is sensitive to lithium (Hallcher & Sherman, 1980). We therefore examined if the dependence of *Sch. pombe* cells on endogenous synthesis of inositol might make it more lithium-sensitive. However, we detected no increase in the sensitivity of these cells to either lithium or sodium over a range of concentrations (data not shown) and one cannot rule out the possibility that some other non-specific phosphatase might also be involved in the conversion of inositol 1-phosphate to inositol in *Sch. pombe* cells expressing the *INO1* gene.

One of the major requirements of the cell for inositol is in the synthesis of membrane phospholipids. We examined if inositol overproduction within *Sch. pombe* would affect its membrane lipid composition in any way. We estimated the phospholipid content of *Sch. pombe* cells grown in the presence of inositol, in inositol-deficient medium and in cells containing pnmt-INO1 which were grown in the absence of thiamine. The phospholipid composition of *Sch. pombe* cells grown in the presence and absence of inositol (Table 2) was similar to that reported by Fernandez *et al.* (1986). In the *INO1*-overexpressed cells, PI levels were comparable to those in *Sac. cerevisiae* when grown in the absence of inositol and, in fact, decreased slightly. No severe imbalance in membrane composition was seen and these data indicate that *Sch. pombe* has evolved a mechanism

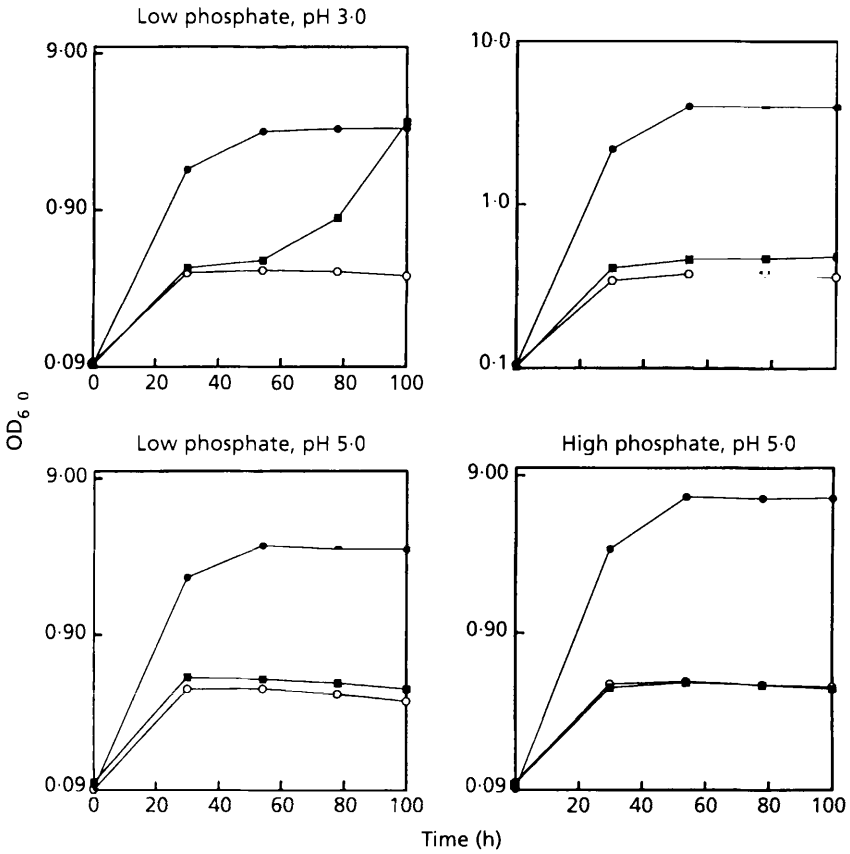

Fig. 5. Growth of *Sch. pombe* ABP2 at pH 3·0 and pH 5·0 in EMM (low phosphate), EMM (high phosphate) containing 500 μM sodium phytate (■), no inositol and no phytic acid (○) and inositol (●).

(such as excretion) to cope with internal disturbances of inositol homeostasis.

Phytic acid as a source of inositol for *Sch. pombe*

The natural environment of *Sch. pombe* is fruit juices and syrups (Phaff & Macmillan, 1978). These contain a significant amount of phytic acid (inositol hexaphosphate). We therefore decided to examine if *Sch. pombe* might be able to utilize phytic acid as a source of inositol and whether this might be one of the possible reasons for its evolution as an inositol auxotroph. The utilization of phytic acid would require the secretion of a phosphatase that could release inositol from phytic acid. *Sch. pombe*, like *Sac. cerevisiae* is able to secrete acid phosphatases (Dibenedetto, 1972; Boer & Steyn-Parve, 1966). Furthermore, the active site region of acid phosphatases of *Sac. cerevisiae* and *Sch. pombe* shows significant homology to that of phytases (Wodzinski & Ullah, 1996).

Two major acid phosphatases are known to be present in *Sch. pombe*. The bulk of the acid phosphatase is phosphate-repressible. We examined the growth of *Sch. pombe* in the presence of phytic acid under low and high phosphate conditions. *Sch. pombe* could not use phytic acid as a source of inositol under these conditions (data not shown). As *Sch. pombe* is known to tolerate a more acidic pH and the acid phosphatase of *Sch. pombe* functions in a very narrow pH range with a lower pH optimum (Dibenedetto, 1972), we repeated the experiment at pH 3·0 and pH 5·0, and under both low and high phosphate conditions. Only at low pH and low phosphate conditions was *Sch. pombe* able to utilize phytic acid as a source of inositol (Fig. 5). *Sac. cerevisiae ino1*Δ could not utilize phytic acid as a source of inositol under these conditions, although it could under higher pH conditions (data not shown). This could be one of the possible reasons for the differences in the behaviour of the two yeasts and for the evolution of *Sch. pombe* as an inositol auxotroph. However, elucidation of this will require more investigation.

We have attempted in this report to reinvestigate an unusual phenotype, natural inositol auxotrophy, observed in wild-type *Schizosaccharomyces* spp. but not seen in the majority of other yeasts or even higher eukaryotic systems. With the release of complete genome sequences of a large number of organisms, molecular explanations need to be sought for evolutionary reasons for difference in behaviour. We hope that the analysis we have presented in this report will greatly stimulate analyses in these directions.

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