A temperature-sensitive mutant of the yeast plasma membrane ATPase obtained by in vitro mutagenesis

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The yeast plasma membrane ATPase is a proton pump essential for cell growth. We have further analyzed the physiological role of the enzyme by constructing a temperature-sensitive mutant. The cloned gene was treated with hydroxylamine and introduced into a haploid strain in which the constitutive promoter of the ATPase gene had been replaced by a galactose-dependent promoter. One transformant exhibited thermosensitive growth on glucose but not on galactose. Under non-permissive conditions the mutant is defective in proton efflux and amino acid uptake and it stops growing either unbudded or with an elongated bud. These results constitute the first genetic evidence for the chemiosmotic role of the enzyme suggested by biochemical and physiological studies.

(Saccharomyces cerevisiae) *Proton pump* *Active transport* *Mutagenesis* *Cell cycle*

1. INTRODUCTION

The yeast plasma membrane ATPase is a proton pump which seems to drive active nutrient transport and to mediate growth responses through the modulation of intracellular pH [1]. The ATPase gene has recently been cloned and sequenced and a large deletion was found to be lethal [2]. The study of the essential physiological role of this enzyme has been complicated by the lack of inhibitors specific enough for in vivo experiments [1]. To circumvent this difficulty we have constructed a temperature-sensitive mutant by functional replacement of the wild-type chromosomal gene by an in vitro mutagenized gene in a plasmid. The work with this mutant described here supports the essential role of the plasma membrane ATPase for yeast growth and constitutes the first genetic evidence for the participation of the enzyme in proton and amino acid transport.

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2. MATERIALS AND METHODS

2.1. *Strains and growth media*

Saccharomyces cerevisiae strain RS-73 is a haploid strain where the constitutive promoter of the plasma membrane ATPase gene has been replaced by the galactose-dependent promoter of the yeast GAL 1 gene (Cid, A., Perona, R. and Serrano, R., submitted). It was derived from strain BWG1-7A (MATa, ade1, his4, leu2, ura3) by homologous recombination between the chromosomal ATPase gene and an integration plasmid containing the URA3 gene for selection and a galactose-dependent promoter in front of a truncated ATPase gene. After integration the chromosome contained a constitutive truncated gene and a complete gene under galactose control.

Yeast cells were grown on synthetic medium containing 2% galactose or glucose, 0.7% yeast nitrogen base without amino acids (Difco), 0.15 mM adenine, 0.4 mM histidine and 1 mM leucine. Leucine was omitted for strains transformed with LEU2 plasmids.

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2.2. *Mutagenesis and selection of mutants*

A *Hind*III fragment of 5 kb containing the ATPase gene and flanking sequences [2] was subcloned into the autonomous plasmid pSB32. This is a pBR322 derivative containing the LEU2 gene for selection and a yeast centromere and origin of replication. The recombinant plasmid (pRS14) was mutagenized by treatment with 0.5 M hydroxylamine and 1 mM EDTA (pH 6 with HCl) for 3 h at 70°C. The hydroxylamine was removed by precipitation with ethanol. This treatment reduced the frequency of transformation of bacteria and yeast to 15 and 25% of control, respectively. From 10 μg mutagenized plasmid about 9000 yeast transformants were obtained by the method of Ito et al. [3] on galactose plates without leucine. Temperature sensitivity was tested by replicating the colonies with toothpicks on twin glucose plates incubated at 25 and 38°C. Thermosensitivity was also tested on galactose plates.

2.3. *Proton efflux, amino acid uptake and ATPase assay*

Yeast cells were grown on glucose medium without leucine at 25°C to late exponential phase (absorbance at 660 nm about 0.8) and starved in water for 6 h at 25°C. Proton efflux was measured by recording pH changes triggered by glucose as described [4]. For amino acid uptake the cells were resuspended in sodium phosphate buffer (50 mM, pH 6.5), preincubated for 10 min with 2% glucose and then for 10 min with 1 mM of either L-[U-^{14}C]leucine or L-[U-^{14}C]histidine (Amersham, 2 Ci/mol). The cells were diluted with cold water, collected by filtration and counted. For assay of the plasma membrane ATPase activity membranes were prepared by homogenization with glass beads and differential centrifugation and assays performed as in [5].

3. RESULTS AND DISCUSSION

The strategy for obtaining a temperature-sensitive mutant of the yeast plasma membrane ATPase was based on a strain (RS-73) in which the constitutive promoter of the ATPase gene has been replaced by a galactose-dependent promoter. As the ATPase is essential [2] this strain can only grow on galactose media in order to induce the expression of the gene. A normal (constitutive) ATPase gene was subcloned into a plasmid, mutagenized, and introduced into strain RS-73. The transformants were tested for thermosensitive growth on glucose medium, where only the mutagenized gene on the plasmid is expressed. It functionally replaces the chromosomal ATPase gene which is dependent on galactose for expression. This strategy for functional gene replacement is more convenient than the physical replacement by two recombinations usually employed [6].

Eight out of 9000 transformants were unable to grow on glucose at 38°C but could grow on galactose at this temperature. This was expected from a thermosensitive mutation on the plasmid ATPase gene because the non-mutagenized chromosomal ATPase gene is expressed on galactose but not on glucose media. These thermosensitive clones were grown at 25°C on glucose and proton efflux was measured at either 25 or 38°C. Only one of the clones (strain RS-131) exhibited thermosensitive proton efflux (fig. 1). In the control strain transformed with a non-mutagenized plasmid (RS-122) the rate of proton efflux increased when transferred to 38°C (from 20 to 30 nmol · min^{-1} · mg cells $^{-1}$). On the other hand, in the thermosensitive mutant (RS-131) this rate decreased to 10 and

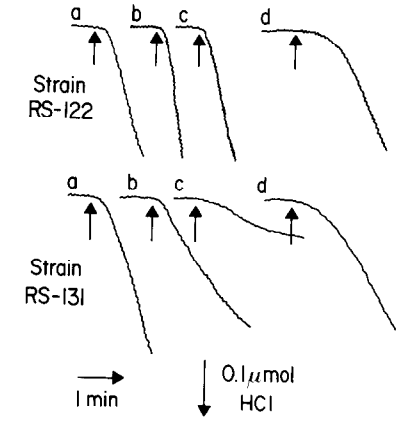

Fig.1. Proton efflux in strains RS-122 (control) and RS-131 (thermosensitive mutant) at 25 and 38°C. The arrow indicates the time of addition of glucose. Traces 'a' correspond to measurements performed at 25°C just after starving the cells (see section 2). Then the cells were incubated for 45 'b' and 90 min 'c' at 38°C and proton efflux measured at this temperature. After 100 min at 38°C the cells were incubated for 120 min at 25°C and profon efflux measured at this temperature 'd'.

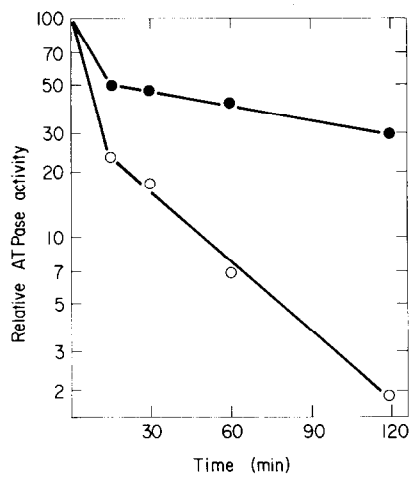

Fig.2. Thermal stability of the plasma membrane ATPase in membrane preparations from strains RS-122 (control, ●) and RS-131 (thermosensitive mutant, ○). Membranes were incubated at 38°C in a medium containing 2% glycerol, 1 mM Tris-HCl (pH 7.5), 0.05 mM EDTA and 0.05 mM dithiothreitol. Protein concentration was 0.2 mg/ml and at the time indicated samples were taken to measure the residual plasma membrane ATPase activity at 38°C.

4 nmol · min^{-1} · mg $cells^{-1}$ after 45 and 90 min at 38°C. A partial recovery of activity was observed after incubating the cells for 2 h at 25°C. These results suggest a reversible change in the activity of the mutant proton pump at the non-permissive temperature. The other thermosensitive clones not exhibiting thermosensitive proton efflux may have a proton pump thermosensitive during its synthesis but not after assembly in the membrane. They were not further studied for the present work.

Membranes were prepared from cells grown at 25°C on glucose and the plasma membrane ATPase activity was found to be much more labile at 38°C in the thermosensitive mutant RS-131 than in the control strain RS-122 (fig.2). The uptake of leucine and histidine by whole cells was also much more inactivated during incubation at 38°C in RS-131 than in RS-122 (table 1). This should be expected if the proton gradient generated by the thermosensitive plasma membrane ATPase is the driving force for amino acid uptake by proton symport [1]. [The long incubation at 38°C in the experiment of table 1 explains the decrease in proton efflux in RS-122. This was not observed in the experiment of fig.1, where the incubation at 38°C was much shorter (1.5 vs 6 h).]

These results constitute the first genetic evidence for the participation of the plasma membrane ATPase in proton pumping and active nutrient transport under physiological conditions. Up to now the evidence for this important role of the enzyme was based on the in vitro capability of the enzyme for proton transport and on the in vivo sensitivity of proton efflux and active transport to ATPase inhibitors [1]. However, the proton transport measured in vitro need not correspond to a physiological activity and the ATPase inhibitors were not specific enough to discard side effects on the cells. However, the results with the mutant described here strongly support the chemiosmotic role of the enzyme suggested by previous biochemical and physiological studies.

The morphology of the thermosensitive mutant after growth arrest at 38°C is shown in fig.3. The cells were grown in glucose medium at 25°C to an

Table 1

Effect of temperature on proton efflux and amino acid uptake in strains RS-122 (control) and RS-131 (thermosensitive mutant).

Strain	Temperature (°C)	Proton efflux (nmol/min per mg cells)	Leucine uptake (nmol/10 min per mg cells)	Histidine uptake (nmol/10 min per mg cells)
RS-122	25	43	0.36	0.46
	38	23	0.27	0.45
RS-131	25	46	0.53	0.66
	38	8	0.06	0.28

The cells were preincubated in water for 6 h at either 25 or 38°C and then proton efflux and amino acid uptake were measured at the respective temperatures. The result of a typical experiment is shown. The values correspond to the average of duplicates differing less than 10%

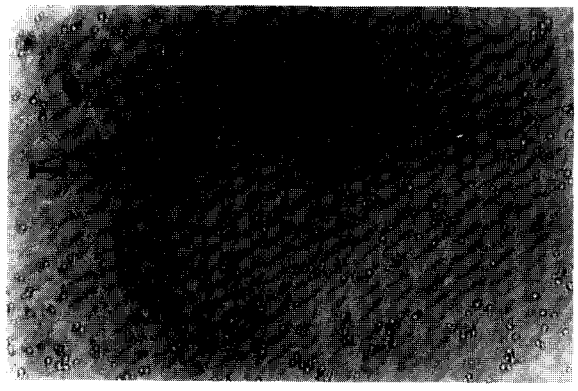

Fig.3. Morphology of the thermosensitive mutant after growth arrest at 38°C. Bar, 50 μM.

absorbance at 660 nm of 0.1. Then the culture was changed to 38°C. Growth was unaffected for two duplications (about 7 h) and then slowed to reach a final absorbance of 0.6 after 10–12 h at 38°C. The picture was taken after 12 h at 38°C and a similar morphology was observed 14 h later. Most of the cells appear either unbudded or with an elongated, abortive bud. This suggests a double block in the cell cycle: a defect in DNA synthesis would explain the abortive buds and the unbudded cells could reflect a defect on the start of the cell cycle [7]. As the ATPase is required for nutrient uptake and the start of the cell cycle is controlled by the availability of nutrients [8], a plausible hypothesis is that a defect in plasma membrane ATPase activity at 38°C causes arrest at the start because a decrease in the proton gradient reduces nutrient uptake. On the other hand, it has been observed in synchronized yeast that an increase in intracellular pH precedes the initiation of DNA synthesis [9] and, as in the case of animal cells [10], may be the signal for it. As this increase in intracellular pH is probably caused by an activation of the plasma membrane ATPase [1], it is also plausible that a defect of the enzyme at 38°C prevents the initiation of DNA synthesis.

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