ELSEVIER

Biochimica et Biophysica Acta 1229 (1995) 233–238

BBA Biochimica et Biophysica Acta

Functional expression of the ENA1(PMR2)-ATPase of *Saccharomyces cerevisiae* in *Schizosaccharomyces pombe*

María A. Bañuelos, Francisco J. Quintero, Alonso Rodríguez-Navarro *

Departamento de Microbiología, Escuela Técnica Superior de Ingenieros Agrónomos, E-28040 Madrid, Spain

Received 31 October 1994; accepted 13 January 1995

Abstract

Na^+ efflux and Na^+ tolerance depend on a putative P-type ATPase encoded by the gene *ENA1(PMR2)* in *Saccharomyces cerevisiae* and on a putative Na^+/H^+ antiporter encoded by the gene *sod2* in *Schizosaccharomyces pombe*. This report shows that a *sod2::ura4* mutant of *S. pombe* transformed with the *ENA1* gene of *S. cerevisiae* expressed the ENA1 protein, and recovered Na^+ efflux and Na^+ tolerance. The efflux of Na^+ in the wild strain of *S. pombe* was sensitive to the transmembrane Na^+ and H^+ gradients, whereas in the *sod2::ura4* mutant transformed with *ENA1* it was independent of these gradients. The data give further support to the notion that *ENA1* and *sod2* encode Na^+ transporters and not regulators of the process of Na^+ export; they show also the physiological consequences of exporting Na^+ through an Na^+-ATPase or an Na^+/H^+ antiporter.

Keywords: Sodium pump; Sodium ATPase; ATPase, Na^+- ; (*Saccharomyces cerevisiae*); (*Schizosaccharomyces pombe*)

1. Introduction

Na^+ is a minor component of living cells, maintained less concentrated inside the cells than in the external medium by specific efflux systems in the plasma membrane. In fungi, two genes involved in Na^+ efflux have been isolated: the *ENA1(PMR2)* gene of *Saccharomyces cerevisiae*, encoding a putative P-type ATPase [1,2], and the *sod2* gene of *Schizosaccharomyces pombe*, encoding a putative electroneutral Na^+/H^+ antiporter [3]. Although the proteins encoded by these genes have not been isolated, and direct evidence of their functions has not been obtained, the physiological data obtained with null mutants indicate that they are the real transporters, mediating the transfer of Na^+ through the membrane, and not regulators of this process [1–3]. The null mutants in the *ENA* genes of *S. cerevisiae* and in the *sod2* gene of *S. pombe* are completely defective in Na^+ and Li^+ effluxes, suggesting that ENA-ATPases and sod2 antiporters do not coexist in these species. The existence of two different strategies for exporting Na^+ is unknown, but the low effectivity of an electroneutral antiporter at neutral pH may be postulated. Now we have studied the Na^+ and Li^+ tolerances, and the Na^+ exports when *S. pombe* expresses the genes *sod2* or *ENA1*. The results show the ineffectiveness of an Na^+/H^+ antiport in comparison with an Na^+-ATPase to protect the cells from Na^+ when the external pH is close to neutrality. They also give further support to the notion that *ENA1(PMR2)* and *sod2* encode Na^+ efflux systems [1–3].

2. Materials and methods

2.1. Media, strains, cation analyses, and recombinant DNA techniques

Standard media and routine microbiological procedures have been described previously [4]. The wild strain of *S. pombe* (strain 556) and the *sod2::ura4* mutant were grown in YE medium [4], and the transformants of these strains with pSP34 (see below) in SD medium [4] without leucine. Tests of Na^+ and Li^+ tolerance were performed in YE medium supplemented with NaCl or LiCl; at the concentrations reported in Table 1 the doubling time was 2-fold that observed in the absence of Na^+ and Li^+; at twice the reported concentrations, no appreciable growth was ob-

Abbreviations: Mes, 4-morpholineethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Taps, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-1-propanesulphonic acid.

* Corresponding author. Fax: +34 1 3365757.

SSDI 0005-2728(95)00006-2

Table 1
Na^+ and Li^+ tolerances of strains of *S. pombe* with different genotypes

Strains	Na^+ (mM)	Li^+ (mM)
sod2::ura4	50	1
sod2::ura4(pSP34)	300	90
556 (wild strain)	200	5
556(pSP34)	250	50

Plasmid pSP34 contains the gene ENA1. Tests as described in the text.

served. The capacity to form colonies at different pH values was tested in YE medium buffered with 20 mM Mes, Hepes or Taps, according to the pH. The strain RH16.6 (*ena1Δ::LEU2::ena4Δ*) of *S. cerevisiae* transformed with the *ENA1*-containing plasmid pGB34 [2] was grown in SD medium without uracil [4].

Na^+ losses were determined by chemical analyses of the cells, as described previously [1,2,5,6]. In *S. pombe*, cells were loaded with Na^+ by growing them in YE medium supplemented with NaCl: 10 mM in the *sod2::ura4* strain, 100 mM in the *sod2::ura4*(pSP34), and 70 mM in the wild type; Na^+-loaded cells were then transferred to YE for pH 5.5, YE supplemented with 10 mM Mes for pH 6.5, 10 mM Hepes for pH 7.5 or 10 mM Taps for pH 8.5 (pH adjusted with KOH), and with 50 mM KCl, 50 mM NaCl when indicated. At intervals, samples of cells were removed, washed and analyzed. Experiments were performed with cells in the mid-exponential growth phase at 0.2–0.6 mg (dry weight) ml^{-1}. Control experiments of Na^+ loss with *S. cerevisiae* were performed essentially as with *S. pombe*, except that arginine phosphate medium [5,6], with 0.5 mM K^+ and 100 mM Na^+, was used for loading the cells with Na^+. In *S. cerevisiae* the losses were followed in 10 mM Mes, Hepes or Taps containing 50 mM KCl, 50 mM NaCl, 2% glucose, 2 mM $CaCl_2$ and 0.1 mM $MgCl_2$. The internal pH in cells exposed to pH 8.0 was determined by the distribution of [7-^{14}C]benzylamine, adding this compound: 10 mM, 1500 Bq ml^{-1}, directly to the 20 mM Taps YE medium. Cellular water was determined in packed cells using ^3H_2O and D-[U-^{14}C]sorbitol, as described previously [5].

All experiments were repeated three or four times. The Na^+ content of Na^+-loaded cells varied less than 20% from batch to batch. Figures show the results of one experiment, which is representative of all the others.

Standard protocols [4,7] and manufacturer's instructions were followed for plasmid preparation, restriction-enzyme digestion, phosphatase treatment, ligation, transformation, and agarose gel electrophoresis.

2.2. Construction of pSP34

The coding region of the *ENA1* gene was cloned in the correct orientation on the *Bgl*II site of the yeast expression vector pMA91 [8], between the 5′ and 3′ non-coding regions of the *PGK1* gene. To achieve this construct, two *Bgl*II commercial linkers (Boehringer Mannheim, Mannheim, Germany) were first introduced into the *Xmn*I (position −11) and *Mlu*I (position +238 beyond the end of the coding region) sites of *ENA1*. The resulted gene was then digested with *Bgl*II and cloned in pMA91.

2.3. Antibodies

Polyclonal antibodies were raised against the product of expression in *Escherichia coli* of an in-frame fusion of the *cro-lacZ* gene with the coding region for amino acids 27–386 of the ENA1 protein. The 1.1 kb *Eco*RI-*Bam*HI fragment of *ENA1* was ligated to the expression vector pUEX2 [9] digested with *Eco*RI and *Bam*HI. The resulting plasmid was then transformed into *E. coli*. Purification of the fusion protein from the inclusion bodies, and rabbit immunization were performed as described in [10]. IgGs were purified from the antisera [11] using the hybrid protein obtained in *E. coli*.

2.4. Western blot analysis

Plasma membranes were prepared by the method described for the preparation of the H^+-ATPase of *S. cerevisiae* [12]. This method yield a low quantity of membranes when applied to *S. pombe*, but no other problem was found with this procedure. After standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (total monomer concentration 8%, cross-linking 2.7%) the plasma membrane proteins were transferred to an Immobilon polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA) at pH 11.0 [13]. The membrane was then exposed to the rabbit IgGs described above, and the bound antibodies detected with alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA, USA), as described previously [14].

3. Results

3.1. Functional expression of ENA1 in S. pombe

When plasmid pSP34, containing the gene *ENA1*, was transformed into the wild type or into the *sod2::ura4* null mutant strain of *S. pombe*, all Leu^+ transformants increased their Li^+ and Na^+ tolerances. At pH 5.5 *ENA1* increased 90-fold the Li^+ tolerance of the *sod2::ura4* mutant, and 10-fold in the wild strain (Table 1). The increase in the tolerance to Na^+ was less dramatic, but still important, 6-fold, in the *sod2::ura4* mutant strain. Interestingly, transformants of the wild strain, carrying the *sod2* and the *ENA1* genes, were less tolerant to Na^+, and specially to Li^+, than transformants of the *sod2::ura4* mutant strain.

Increasing the pH from 5.5 to 6.5 decreased slightly the tolerances of the *sod2::ura4* strain to Na^+ and Li^+ (from

50 to 40 mM, and from 1 to 0.8 mM, respectively), probably reflecting a slightly lower discriminatory capacity of the uptake system between K^+ and Na^+ or Li^+. A synergic effect of Na^+ or Li^+ with the pH could also explain the decrease in tolerance, because the *sod2::ura4* strain grew slowly at pH 6.5. However, because the *sod2::ura4* mutant transformed with *ENA1* also showed a slightly lower Na^+ and Li^+ tolerances at pH 6.5, but good growth, the hypothesis of a lower discriminatory capacity seems more likely. In the wild type the increase of one pH unit decreased 5-fold the tolerance to Na^+, and very slightly the tolerance to Li^+. To understand the different effect of the pH on the Na^+ and Li^+ tolerances in the wild type, it is worth noting that Na^+ toxicity in all strains occurred at approximately 100 nmol Na^+ ml^{-1} (cell water), and Li^+ toxicity at approximately 8 nmol Li^+ ml^{-1}. Although the exact concentration of Na^+ and Li^+ in the cytoplasm in these conditions are not known because these cations are partially accumulated into the vacuole [15,16], it is clear that Na^+ toxicity in the wild type at pH 5.5 (200 mM Na^+) occurred with an Na^+ gradient directed inward, whereas the Li^+ gradient in similar conditions was null or directed outward.

To confirm that the gene *ENA1* was expressed in *S. pombe*, preparations of membranes of *sod2::ura4* and *sod2::ura4* (pSP34) strains were Western blotted using specific antibodies for ENA1. The *sod2* null mutant did not react with the antibodies whereas the *ENA1* transformants showed the band of the ENA1 protein (Fig. 1).

3.2. Characteristics of Na^+ efflux in ENA1 transformants

The different effect of the external pH on the Na^+ tolerance of the wild type and the *sod2::ura4* strain transformed with *ENA1* indicated that the mechanisms of Na^+ efflux operating in these strains were different. Clearly, if *sod2* encodes an electroneutral Na^+/H^+ antiporter [3] and *ENA1* an Na^+-ATPase [1,2], both located in the plasma membrane, the pH dependence of the Na^+ effluxes in the strain expressing *sod2* and in the strain expressing *ENA1*

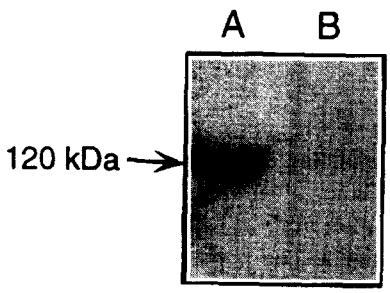

Fig. 1. Western blot analysis of plasma membrane preparations of the *S. pombe* strains *sod2::ura4* transformed with *ENA1* (A), and *sod2::ura4* (B). Aliquots of purified plasma membranes containing 50 μg of protein were separated by SDS-PAGE, electrotransferred to nitrocellulose and immunodecorated with antibodies against the protein ENA1 as described in the text.

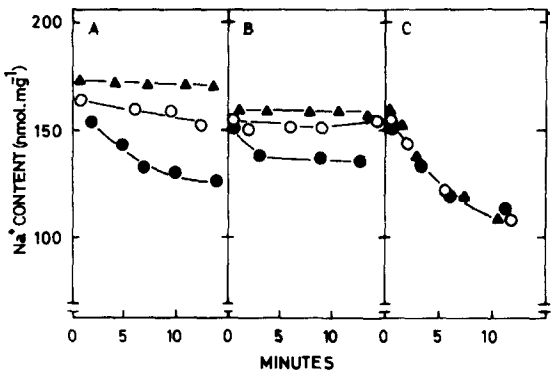

Fig. 2. Loss of Na^+ from Na^+-loaded cells of the *S. pombe* strains *sod2::ura4* (A) and (B), and *sod2::ura4* transformed with *ENA1* (C). In (A) the external medium did not contain Na^+; in (B) and (C) it contained 50 mM KCl and 50 mM NaCl. Experiments at pH 5.5 (●), 7.5 (○) and 8.5 (▲). The cells were loaded with Na^+ and then transferred to the testing media as described in the text.

should be greatly different. For example, an Na^+/H^+ antiport cannot mediate uphill Na^+ loss at a neutral or alkaline pH, because in these conditions there is no driving force for the antiport. In contrast, an Na^+-ATPase should mediate uphill Na^+ loss even at a high external pH. Therefore, we tested the effect of the external pH on the loss of Na^+ in the *sod2::ura4* strain, in the *sod2::ura4* strain transformed with pSP34, in the wild strain and in the wild strain transformed with pSP34. The cells of the four strains were loaded with Na^+ as described in Materials and Methods section (the loaded cells contained approximately 150 nmol Na^+ mg^{-1}, and 2 μl of water mg^{-1}), and afterwards they were transferred to buffered YE medium, in the absence or in the presence of 50 mM NaCl and 50 mM KCl. In the experiments with 50 mM Na^+ the presence of 50 mM K^+ inhibited Na^+ influx, uncovering the efflux of Na^+ occurring when the external concentration of Na^+ was maintained roughly at the same concentration than in the cytoplasm of the cells (considering that Na^+ and Li^+ accumulate in the vacuole [15,16], the actual concentration of Na^+ in the cytoplasm of these cells is probably lower than the figure obtained dividing the Na^+ content by the total water content). In the *sod2::ura4* strain the loss of Na^+ was effective only when, simultaneously, the Na^+ gradient was directed outward (absence of external Na^+), and the H^+ gradient was directed inward (pH 5.5) (Fig. 2A; in the presence of external Na^+, at pH 5.5, as presented in Fig. 2B, we found in all experiments a small loss lasting only 2 or 3 min); this loss is probably mediated by a spurious Na^+ efflux system, because a real Na^+ efflux system should mediate efflux when the Na^+ gradient is directed inward as in Fig. 2C and Fig. 3B. The *sod2::ura4* strain transformed with *ENA1* showed pH-independent Na^+ loss at 50 mM external Na^+ (Fig. 2C), and it is worth observing that this loss was effective in the absence of a driving force for an Na^+/H^+ antiport (50 mM external Na^+ and pH 8.5). In the wild strain, the loss

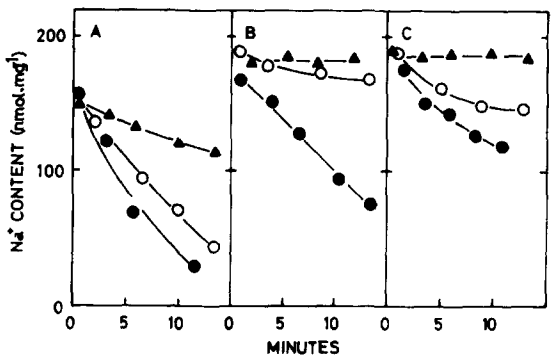

Fig. 3. Loss of Na^+ from Na^+-loaded cells of the wild-type strain of *S. pombe* (A) and (B), and the wild-type transformed with *ENA1* (C). In (A) the external medium did not contain Na^+; in (B) and (C) it contained 50 mM KCl and 50 mM NaCl. Experiments at pH 5.5 (●), 6.5 (○) and 8.5 (▲). The cells were loaded with Na^+ and then transferred to the testing media as described in the text.

of Na^+ was effective when the Na^+ gradient was directed outward (absence of external Na^+) at the three pH levels tested (Fig. 3A); but in the presence of external Na^+, the loss of Na^+ depended on an H^+ gradient directed inward (Fig. 3B). In cells expressing both the ENA1 system and the endogenous system (i.e. the wild strain transformed with *ENA1*), in the presence of external Na^+, the rate of the Na^+ loss was pH-dependent (Fig. 3C), in contrast with the *sod2::ura4* mutant transformed with *ENA1* (Figs. 2C and 3C); remarkably, the loss was null at pH 8.5.

As a control, the experiments of Na^+ loss described above were repeated with *S. cerevisiae* (an *ena1Δ::LEU2::ena4Δ* strain transformed with the *ENA1* containing plasmid pGB34 [2]). Consistent with the results obtained in Fig. 2C, the Na^+ loss in the presence of Na^+ was not inhibited by a high external pH (Fig. 4).

3.3. pH range of growth

Disruption of *ENA1* in *S. cerevisae* [1] and *sod2* [3] in *S. pombe* decreased the upper limits of the pH ranges at

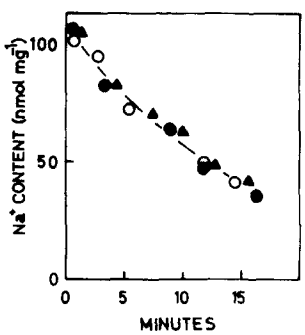

Fig. 4. Loss of Na^+ from Na^+-loaded cells of the *ena1Δ::LEU2::ena4Δ* strain of *S. cerevisiae* transformed with the gene *ENA1*. The external medium contained 50 mM KCl and 50 mM NaCl, at pH 5.5 (●), 7.5 (○) and 8.5 (▲). The cells were loaded with Na^+ and then transferred to the testing media as described in text.

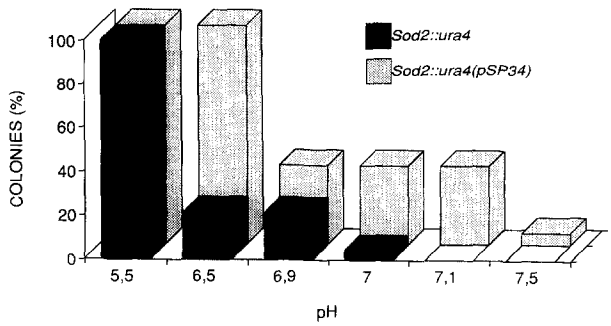

Fig. 5. Decrease in the number of colonies as a response to the increase of the pH of the medium. Samples with the same number of cells from a 24 h cultures in SD medium were spread on the surface of buffered-YE media, and incubated.

which growth occurs in these species. The capacity of *ENA1* to complement the defective growth of the *sod2::ura4* strain at limiting pH values was investigated in solid media buffered at different pH values. The capacity to form colonies in these media was found highly reproducible, whereas the tests in liquid media [1,3] produced inconsistent results, dependent partially on the size of the inoculum. The reasons for the inconvenience of the tests in liquid media are unknown, but it may be related to the formation of clumps of cells, in which the pH may decrease because of the high capacity of the cells to produce organic acids. The increase of the pH of a solid medium increased the time necessary for the formation of colonies, and decreased the proportion of the cells able to form colonies. Tests with strains *sod2::ura4*, and *sod2::ura4* transformed with *ENA1* showed clearly that *ENA1* improved the resistance to a high pH (Fig. 5). These results suggested that the ENA1 system may alleviate the alkaline load imposed by the high external pH, but this could not be confirmed by the accumulation of ^{14}C-benzylamine in cells exposed at pH 8.0. In four hour, strains *sod2::ura4* and *sod2::ura4* transformed with *ENA1* showed identical time courses of the counts (an initial rapid accumulation was followed by a constant decrease), and consequently identical increases in the cellular pH values.

4. Discussion

The gene *ENA1* of *S. cerevisiae* complemented the *sod2::ura4* mutation of *S. pombe*, inducing Na^+ and Li^+ tolerances (Table 1). Furthermore, *ENA1* produced a much higher protection from Li^+ than *sod2*, fiftyfold higher, and at least as high as the *sod2-1* mutation [3]. Whether this large effect was due to the overexpression of ENA1 or to the intrinsic properties of ENA1 is unknown at this moment.

The Na^+ and Li^+ tolerances induced by *ENA1* in the *sod2::ura4* mutant may occur in two different ways: either *sod2* and *ENA1* encode Na^+ efflux systems, and *ENA1*

replaces the efflux system absent in the *sod2::ura4* mutant, or *ENA1* encodes a protein that restores the regulatory function defective in the *sod2::ura4* mutant, and allows the endogenous Na^+ efflux system to recover its function. The probability that *sod2* encodes an Na^+/H^+ antiport and not a protein with a regulatory function has been discussed previously [3], and present results are consistent with this notion. If the products of *sod2* and *ENA1* fulfil regulatory functions, the Na^+ effluxes in the wild strain and in the *sod2::ura4* strain transformed with *ENA1* would be mediated by the same system; consequentially, they should present the same dependence of ionic gradients (i.e. ΔpH and ΔpNa), which is not the case. In the wild type the loss of Na^+ in the absence of a significant transmembrane Na^+ gradient required an acidic pH (Fig. 3B), as expected for an electroneutral Na^+/H^+ antiporter, whereas in the *sod2::ura4* strain transformed with *ENA1* the loss of Na^+, in the same conditions, did not required an inward directed H^+ gradient (Fig. 2C). Furthermore, the pH independence of the ENA1-dependent Na^+ efflux in *S. pombe*, in the absence of a transmembrane Na^+ gradient, reproduced the findings in *S. cerevisiae* (Fig. 2 and Fig. 4), suggesting that, with the transfer of *ENA1*, *S. pombe* acquired the Na^+ efflux system of *S. cerevisiae*. Therefore, it is highly probable that ENA1 and sod2 are the real Na^+ transporters mediating the efflux of Na^+ in *S. cerevisiae* and in *S. pombe*.

The conclusion that ENA1 is an Na^+ transporter, its capacity of transport in the absence of favourable Na^+ and H^+ gradients across the membrane, and the sequence analysis of the putative ENA1(PMR2) protein, as deduced from the sequence of the *ENA1(PMR2)* gene [17], gives further support to previous reports suggesting that ENA1 is an Na^+-ATPase [1,2]. This suggestion should be confirmed by the biochemical analysis of the ATPase, but this confirmation has been impossible so far. ENA1-ATPase protein almost free of H^+-pump ATPase has been prepared from plasma membranes, endoplasmic reticulum, and in vesicles obtained from *sec6* mutants of *S. cerevisiae*, but in all cases without ATPase activity (F.J. Quintero, B. Benito, and A. Rodríguez-Navarro, unpublished results).

In the wild strain of *S. pombe* transformed with *ENA1* both the Na^+/H^+ antiporter and the Na^+-ATPase probably coexist. The lower tolerance of this transformant with reference to the *sod2::ura4* strain transformed with *ENA1* (Table 1) implies that the presence of the ATPase and the antiporter is detrimental with reference to a strain with the sole presence of the ATPase. The most likely explanation for this is that the antiporter can reverse the exchange, accumulating Li^+ or Na^+ inside the cells, when the H^+ gradient is directed outward, and sufficient to compensate the thermodynamic barrier of the inward movement of Li^+ or Na^+. The reverse exchange also explains the null net loss of Na^+ in the wild strain transformed with *ENA1*, at pH 8.5 with 50 mM of external Na^+, in contrast with the notable loss of Na^+ in the *sod2::ura4* strain transformed with *ENA1* under the same conditions (Figs. 2C and 3C). However, the uptake of Na^+ through the antiporter might occur only under special circumstances, because Na^+ uptake was not observed at pH 8.5 in Fig. 3B, although in these conditions the prevailing ΔpH could drive Na^+ uptake.

There are two apparent inconsistencies in our results: (i) at pH 5.5, the *sod2::ura4* strain transformed with *ENA1* was more tolerant to Na^+ and Li^+ than the wild strain (Table 1) but the loss of Na^+ was less effective (Figs. 2C and 3B, observe the differences in the scales), in contradiction with previous observations both in *S. cerevisiae* and *S. pombe* suggesting that higher tolerances are supported by higher effluxes [2,3], and (ii) transformation with *ENA1* decreased the Na^+ loss of the wild strain at pH 5.5 (Figs. 3B and 3C). Both unexpected results may be due to the overexpression of *ENA1* in *S. pombe* (pSP34 is a high expression vector) because they are very similar to what is found with the overexpression of *ENA1* in *S. cerevisiae*, which produces high Li^+ tolerance and low Li^+ efflux. In the overexpression of *ENA1* a large amount of ENA1 protein is in the endoplasmic reticulum (F.J. Quintero and A. Rodríguez-Navarro, unpublished results), as when *S. cerevisiae* expresses a heterologous ATPase [18]. As a consequence of its accumulation in the endoplasmic reticulum, the ATPase may pump Li^+ or Na^+ into some kind of vesicles, decreasing the actual Li^+ or Na^+ concentrations in the cytoplasm. This would decrease the efflux, but increase tolerance.

P-type ATPases pump a single ion or exchange two different ions. The Ca^{2+}-ATPase [19] and the H^+-ATPase [20,21] belong to the first group, whereas the Na^+, K^+-ATPase [22] and the H^+, K^+-ATPase [23] belong to the second. The ENA1-ATPase probably mediates Na^+/H^+ and K^+/H^+ exchanges, pumping H^+ into the cells. Three observations support this notion: (i) disruption of the *ENA* genes decreases the limit for growth at high pH, specially in the presence of high K^+ [1]; (ii) the gene *ENA1* is specifically induced by high pH [2]; and (iii) *ENA1* restored the defective growth of the *sod2::ura4* mutant of *S. pombe* at high pH. However, the role of the ENA1 and sod2 systems in the control of the cytoplasmic pH cannot be deduced from these observations, and at this moment that role is obscure. In both *S. cerevisiae* and *S. pombe* the upper limit of the pH range for growth is low (7.5–7.8), and both species are able to produce a high amount of organic acids. In these conditions the return of H^+ through the Na^+ efflux system may be of little importance. Furthermore, a pathway of Na^+ uptake independent of the uptake occurring in exchange with the H^+ pumped by the H^+-pump ATPase has not been reported, and this pathway is absolutely necessary if the uptake of H^+ coupled to Na^+ efflux plays a role in the decrease of the internal pH. Our results of ^{14}C-benzylamine accumulation also failed to show a clear role of the ENA1-ATPase in decreasing the internal pH of cells exposed to a high pH.

Acknowledgements

We thank P.G. Young for the generous gift of the *sod2::ura4* strain. This work was supported by research grant PB92-0907 from Dirección General de Investigación Científica y Técnica.

References

[1] Haro, R., Garciadeblas, B. and Rodríguez-Navarro, A. (1991) FEBS Lett. 291, 189–191.
[2] Garciadeblas, B., Rubio, F., Quintero, F.J., Bañuelos, M.A., Haro, R. and Rodríguez-Navarro, A. (1993) Mol. Gen. Genet. 236, 363–368.
[3] Jia, Z.P., McCullough, N., Martel, R., Hemmingsens, S. and Young P.G. (1992) EMBO J. 11, 1631–1640.
[4] Guthrie, C. and Fink G.R. (eds.) (1991) Methods Enzymol. 194.
[5] Ramos, J., Haro, R. and Rodríguez-Navarro, A. (1990) Biochim. Biophys. Acta 1029, 211–217.
[6] Rodríguez-Navarro, A. and Ramos, J. (1984) J. Bacteriol. 159, 940–945.
[7] Sambrook, J., Fristch, E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Press, NY.
[8] Mellor, J., Dobson, M.J., Roberts, N.A., Tuite, M.F., Emtage, J.S., White, S., Lowe, P.A., Patel, T., Kingsman, A.J. and Kingsman, S.M. (1983) Gene 24, 1–14.
[9] Bressan, G.M. and Stanley, K.K. (1987) Nucleic Acids Res. 15, 10056.
[10] Monk, B.C., Montesinos, C., Ferguson, C., Leonard, K. and Serrano, R. (1991) J. Biol. Chem. 266, 18097–18103.
[11] Chiong, M., Lavandero, S., Ramos, R., Aguillón, J.C. and Ferreira, A. (1991) Anal. Biochem. 197, 47–51.
[12] Serrano, R. (1988) Methods Enzymol. 157, 533–544.
[13] Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038.
[14] Blake, M.S., Johnston, K.H., Rusell-Jones, G.J. and Gotschlich, E.C. (1984) Anal. Biochem. 136, 175–179.
[15] Perkins, J. and Gadd, G.M. (1993) FEMS Microbiol. Lett. 107, 255–260.
[16] Haro, R., Bañuelos, M.A., Quintero, F.J., Rubio, F. and Rodríguez-Navarro, A. (1993) Physiol. Plant. 89, 868–874.
[17] Rudolph, H.K., Antebi, A., Fink, G.R., Buckley, C.M., Dorman, T.E., LeVitre, J., Davidow, L.S., Mao, J. and Moir, D.T. (1989) Cell 58, 133–145.
[18] Villalba, J.M., Palmgrem, M.G., Berberián, G.E., Ferguson, C. and Serrano, R. (1992) J. Biol. Chem. 267, 12341–12349.
[19] Inesi, G., Sumbilla, C. and Kirtley, M.E. (1990) Physiol. Rev. 70, 749–760.
[20] Goffeau, A. and Gree, N.M. (1990) in Monovalent Cations in Biological Systems (Pasternak, C.A., ed.), pp. 155–169, CRC Press, Boca Raton.
[21] Serrano, R. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 61–94.
[22] Horisberg, J.-D., Lemas, V., Kraehenbühl, J.-P. and Rossier, B.C. (1991) Annu. Rev. Physiol. 53, 565–584.
[23] Rabon, E.C. and Reuben, M.A. (1990) Annu. Rev. Physiol. 52, 321–344.