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Na^+-ATPases and Na^+/H^+ antiporters in fungi

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1. Introduction

The sodium ion is abundant and K^+ scarce in most natural environments, but in living cells Na^+ is at a low concentration and K^+ at a fairly high concentration. Because living cells are not completely impermeable to Na^+, the low Na^+ concentration in the cell requires the continuous extrusion of the ion, usually against a concentration gradient. In 1954, Edward J. Conway [1] demonstrated that yeast cells are capable of actively and specifically extruding Na^+, by a system independent of that mediating K^+ uptake. Since then, many authors have investigated on the system or systems mediating the efflux of Na^+ in fungi [2,3]. In yeast many observations on the transport of Na^+ and Li^+ have complemented the pioneering kinetic description made by Conway, some of them suggesting that Na^+ efflux might play a role in the control of internal pH. In a brief description: (i) the system presents a low affinity for Na^+ and Li^+, and the effluxes follow first-order kinetics [4,5], (ii) the effluxes of Na^+ and Li^+ are inhibited by the decrease of the cytoplasmic pH [6,7], (iii) the efflux of Li^+ is proportional to the ATP content of the cell [7], and (iv) in some conditions the system behaves as a Na^+/H^+ antiporter [7]. In *Neurospora crassa* two processes account for Na^+ efflux, one is constitutive and independent of the presence of external K^+, and the other is induced by growing the cells in Na^+, and depends on the external K^+ [8].

Although this general description of the efflux of Na^+ in yeast and in *N. crassa* had been known for many years, the difficulty in isolating the systems from the plasma membrane limited the progress in the field until the genes encoding the system were isolated.

2. The PMR2/ENA1-ATPase of *Saccharomyces cerevisiae*

The gene *ENA1* was isolated because it complemented the Na^+ and Li^+ sensitivity of a wild strain that presented low Na^+ and Li^+ effluxes. Subsequently it was found that disruption of this gene rendered the cells sensitive to Na^+ and Li^+, and decreased the capacity of the cells to export Na^+ and Li^+ [9]. The gene *PMR2* had been isolated previously by identifying yeast DNA fragments that crosshybridized with the **i** and **j** region of the gene *PMA1* [10]. *PMR2* and *ENA1* are the same gene and the putative protein that they encode is a P-type ATPase, according to the predicted amino acid sequence. Originally, *PMR2* was thought to be a Ca^+-ATPase because, excluding the ten conserved regions of P-type ATPases, maximum homology was found with Ca^+-ATPases [10]. Although this is correct, fragments of homology with Na^+, K^+-ATPases can also be found, making it difficult to deduce the function from studies of homology with animal ATPases. The maximum homology of the PMR2/ENA1 is with the cta3-ATPase of *Schizosaccharomyces pombe*. Physiological studies suggest a Ca^+ transport function for the cta3-ATPase [11,12], but a similar evidence of Ca^+ transport activity has not been found for the PMR2/ENA1-ATPase. Furthermore, five out the six amino acids considered necessary for Ca^+ transport in the M_4, M_5, M_6 and M_8 membrane fragments of Ca^+-ATPases [13] are conserved in cat3 and only two (Thr 799 and Glu 908) in PMR2/ENA1.

ENA1 is the first gene in a tandem of four repeats (at least in strains DBY746/747 and W303.1A/B) [14]. Although the DNA sequence of the four repeats is not complete (a 3.8 kb fragment extending from the 3′ end of *ENA3* to the 5′ end of *ENA4* has not been sequenced), the known sequence and the restriction analysis of the DNA fragment including the entire tandem

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Table 1
Li^+ tolerance of strains with different repeats of the ENA *genes*

Genes	Li^+ tolerance (mM)
ENA1ENA2ENA3ENA4	30
ENA1 [a]	20
ENA2 [a]	0.5
ENA4 [b]	0.5
ENA2ENA3ENA4 [b]	15

Saturated cultures were diluted 50-fold with water, and 5 μl were dropped on solid (2% agar) arginine phospate medium containing 1 mM KCl, and different concentrations of LiCl [14]. Growth was recorded after 7 days. Strain DBY746, derivatives obtained making disruptions in the *ENA* genes, and deletion strain *ena1Δ::LEU2::ena4Δ* transformed with plasmids Ycp50 containing different genes.
[a] In plasmid.
[b] Constructed by gene disruption.

indicate that there are two types of 5′ non-coding regions in these genes: the *ENA1* type, only in *ENA1*, and the *ENA2* type, repeated also in *ENA3* and *ENA4*. The coding regions are almost identical, and the putative proteins encoded by these genes differ only in thirteen amino acids (Fig. 1) [14]. The differences in the 5′ non-coding regions of the genes in the tandem result in a differential expression of these genes. The expression of *ENA1* is induced by Na^+, Li^+ and high pH, and the induction is regulated by calcineurine, a regulatory phosphatase with a pleiotropic effect on salt tolerance in yeast [15]. The other three genes present a weak constitutive expression [14]. As a consequence of the differences in the gene expressions: *ENA1* confers a good protection towards Na^+ and Li^+ toxicity, but a single gene with the *ENA2* type promoter confers only a poor protection. However, the tandem *ENA2-ENA3-ENA4* brings about a protection similar to that conferred by *ENA1* (Table 1). It seems that amplification of those genes with the *ENA2* type promoter has compensated their weak expression. Finally, the differences in the amino acid sequence between the four genes in the tandem have an unknown significance because the protective effects of the ENA1 and ENA2 ATPases are identical. A chimeric gene in which the 5′ non-coding region of *ENA2* was replaced by the 5′ non-coding region of *ENA1* has the same effect as *ENA1* [14].

Overexpression of the ENA-ATPases produces high Na^+ and Li^+ tolerance by a complex process. Substitution of the 5′ non-coding region of the *PGK1* gene for the 5′ non-coding regions of *ENA1* or *ENA2*, and transformation of the resulting genes into a *ena1Δ::LEU2::ena4Δ* strain produced strains with a remarkable Li^+ tolerance (5-fold higher than in the wild type, and tenfold higher than in a strain containing only *ENA1*), but with a low Li^+ efflux, at least when measured in short term experiments. This dissociation of Li^+ tolerance and Li^+ efflux had not been previously observed in extensive studies of the *ENA* genes [14], and suggested a mechanism of tolerance that did not involved a rapid efflux of the cation, although the cell contained a substantial amount of Li^+. Investigating such mechanism we found that a significant amount of the ENA proteins could be immunologically detected in the endoplasmic reticulum (Quintero, F.J. and Rodríguez-Navarro, A., unpublished results), as when yeast cells express an heterologous ATPase [16]. Therefore, the dissociation of Li^+ tolerance and Li^+ efflux may occur because Li^+ accumulates in vesicles of the endoplasmic reticulum, with the result of decreasing significantly the Li^+ concentration in the cytoplasm. Whether the contents of these vesicles is eventually evacuated by exocitosis is now under study.

3. The sod2 antiport of *S. pombe*

By selecting Li^+ resistant mutants the *sod2* locus of *S. pombe* was identified. Regarding Na^+ and Li^+ tolerance and Na^+ efflux, the effect of *sod2* is very similar to the effect of *ENA1* in *S. cerevisiae*, provided that the external medium is acidic [16]. The sequence of the putative protein encoded by *sod2* has a weak overall similarity with the *Escherichia coli* antiport, and

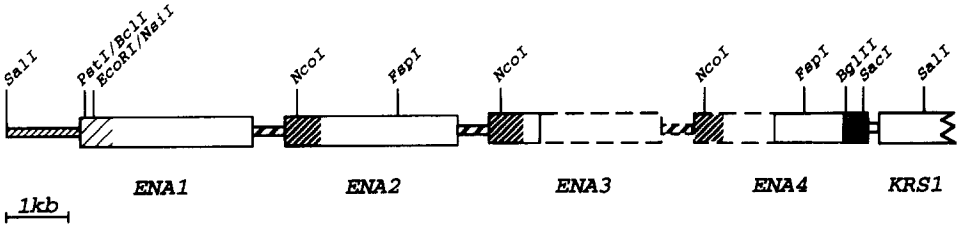

Fig. 1. Schematic representation of the physical map of the *ENA* genes. The wide bars represent the coding regions, and the narrow bars the non-coding regions. The fragments of bars with dashed lines indicate the non sequenced fragments. Hatched areas represent fragments of different sequences. The 5′ end of the coding region of *ENA1* is different in 41 nucleotides (encoding differences in 12 amino acids) with respect to *ENA2* and *ENA3*; according to the restriction sites the 5′ end of the coding region of *ENA4* is like *ENA2*. The 3′ end of the coding region of *ENA4* is different in 35 nucleotides (encoding differences in 14 amino acids) with respect to *ENA1* and *ENA2*. The 3′ end of *ENA3* is like *ENA1* according to the restriction sites. The *Fsp*I site in *ENA2* and *ENA4* results in a alanine residue in the putative ENA2 and ENA4 proteins instead of a threonine residue in ENA1 and, probably, in ENA3.

with the 99 kDa mammalian Na^+/H^+ antiport [16]. These homologies suggest that *sod2* encodes a Na^+/H^+ antiport. The mediation of a Na^+/H^+ antiport in the efflux of Na^+ in *S. pombe* is also consistent with the inhibitory effect of the high external pH on Na^+ efflux, and with the association of H^+ influx and Na^+ efflux [16].

In striking similarity with *ENA1*, the *sod2* locus of *S. pombe* contains several repeats of the gene *sod2*. Mutants of *S. pombe* involving gene amplification in this locus can be isolated by selecting strains resistant to NaCl and LiCl [16].

4. Na^+-ATPases and Na^+/H^+ antiporters

Although the biochemistry of the systems encoded by the *ENA1* gene of *S. cerevisiae* and *sod2* of *S. pombe* has not been described, the physiology of strains carrying wild-type genes and mutant genes strongly suggests that they encode a Na^+-ATPase, and a Na^+/H^+ antiporter respectively [9,14,16]. The question then is how these systems are distributed in fungi. Antibodies against the ENA1 protein reveal that a similar protein does not exist in *S. pombe* (Quintero F.J., Bañuelos, M.A. and Rodríguez-Navarro, A., unpublished results), and the lack of a Na^+-ATPase, with the capacity to pump Na^+ in the absence of ΔpH, explains the high sensitivity of *S. pombe* to Na^+ and Li^+ when the pH is close to 6.5. However, *S. pombe* is an acidophilic organism that grows poorly at pH 7.0 [16], and that does not face the necessity to pump Na^+ when the external pH is high. It is worth mentioning that the low capacity of *S. pombe* to grow at high pH is not due to the lack of a gene like *ENA1*, because transformation of *ENA1* into *S. pombe* allows the cells to pump Na^+ outward even at pH 8.5, but it does not improve significantly the growth at high pH (Bañuelos, M.A. and Rodríguez-Navarro, A., unpublished results). *Schwanniomyces occidentalis*, another yeast that grows in a broad range of pH values, has a gene highly homologous to the *ENA1* gene of *S. cerevisiae*, which can restore Na^+ and Li^+ tolerance in a *ena1Δ::LEU2 ::ena4Δ* strain of *S. cerevisiae* (Bañuelos, M.A. and Rodríguez-Navarro, A., unpublished results).

The existence of a gene encoding a Na^+/H^+ antiport in *S. cerevisiae* has not been reported. However, disruption of the four *ENA* genes of *S. cerevisiae* does not completely eliminate Na^+ and Li^+ effluxes [9], and in certain conditions Na^+/H^+ exchanges can be detected in wild strains [7]. A likely possibility is that a K^+/H^+ antiporter, which does not discriminate between K^+ and Rb^+ [17], can also exchange Na^+ and H^+. Acidophilic species may use only this system, whereas species capable of growing at high pH have Na^+-ATPases.

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