

# A novel P-type ATPase from yeast involved in sodium transport

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The gene *ENA1* was cloned by its ability to complement the  $\text{Li}^+$  sensitivity of a low  $\text{Li}^+$ -efflux strain. The nucleotide sequence of the cloned DNA fragment showed that there are two almost identical genes in tandem, and predicts that they encode P-ATPases. Disruption of both genes originated a strain defective in  $\text{Na}^+$  and  $\text{Li}^+$  effluxes, and sensitive to  $\text{Na}^+$ , to  $\text{Li}^+$  and to alkaline pH. By transformation with *ENA1* the defective effluxes and tolerances were repaired.

Sodium pump, Sodium ATPase, Sodium efflux, Lithium efflux, *Saccharomyces cerevisiae*

## 1. INTRODUCTION

All living cells exclude  $\text{Na}^+$ , and create a  $\text{Na}^+$ -concentration gradient across the membrane. In the naked cells of animals, this gradient is generated by the  $\text{Na}^+$ -pump, ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPase [1], and plays a central role in their physiology, as most uphill transports are  $\text{Na}^+$  coupled [2], and a major  $\text{H}^+$  extruding system is a  $\text{Na}^+$ / $\text{H}^+$  antiport [3]. In contrast, in eucaryotic cells with walls, the membrane potential is generated by a  $\text{H}^+$ -pump ATPase [4], secondary transports are coupled to  $\text{H}^+$  [5], and  $\text{Na}^+$  efflux has never been shown to be a primary process. Here we present evidence that P-ATPases mediate  $\text{Na}^+$ ,  $\text{Li}^+$ , and probably  $\text{K}^+$  effluxes in yeast.

## 2. METHODS

### 2.1 Media, strains, and cation analyses

Standard media and routine genetic methods have been described previously [6]. Arginine-phosphate medium, free of ammonium and alkali cations, has also been described [7]. The yeast strains used in this work were derived from the  $\text{Li}^+$ -tolerant strains (growth at 40 mM  $\text{Li}^+$ , 1 mM  $\text{K}^+$ ) DBY746 (*Mata ura3-52 leu2-3 leu2-112 his3-Δ1 trp1-289*) and DBY747 (*Mata*, isogenic with DBY746), and the  $\text{Li}^+$ -sensitive strain (no growth at 5 mM  $\text{Li}^+$ , 1 mM  $\text{K}^+$ ) GF36 (*Mata*) *Escherichia coli* DH5α [8] was used as the bacterial host for plasmids.

$\text{Li}^+$  and  $\text{Na}^+$  effluxes from the cells were determined by chemical analyses, as described previously [9,10], in 10 mM 3-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)-amino]-1-propanesulphonic acid adjusted to pH 8.0 with  $\text{Ca}(\text{OH})_2$  containing 0.1 mM  $\text{MgCl}_2$ , 10 mM  $\text{KCl}$  and 2% glucose.

### 2.2 Recombinant DNA techniques and DNA sequencing

Standard protocols [8] and manufacturer's instructions were followed for plasmid preparations, restriction enzyme digestion, phosphatase treatment, ligation, transformation, and agarose gel electro-

phoresis. DNA sequencing was performed by the dideoxy-chain termination method of Sanger et al. [11] as modified for use with Sequenase (U.S. Biochemical Corp., Cleveland, OH).

### 2.3 Disruption of *ENA1* and *ENA2*

The 3.9-kb *Bam*HI fragment of pGH1.1 (Fig. 1) was deleted and replaced with the 3.0-kb *Bgl*II fragment of plasmid YEpl3 [12] containing the *LEU2* gene. Then, the *Xba*I-*Pst*I fragment of this construction was used for single-step gene disruption [13] in strain DBY746. DNA samples from  $\text{Leu}^+$  transformants were digested with *Xba*I, then the restriction fragments were separated by electrophoresis in 0.7% agarose gels, transferred to nylon membranes (Hybond-N, Amersham), and hybridized to a  $^{32}\text{P}$ -labeled probe derived from the 2.3-kb *Xba*I fragment of pGH6 (Fig. 1). The probe was radiolabeled by the random primer method, and hybridization was carried out at 42°C in 50% formamide following standard methods [8].

## 3. RESULTS

After failure to obtain  $\text{Na}^+$ -sensitive yeast mutants, the gene encoding a  $\text{Na}^+$  efflux system in yeast was cloned by complementation of  $\text{Li}^+$  sensitivity in a low  $\text{Li}^+$ -efflux strain. This strategy was chosen because such strains are common in yeast [14] and  $\text{Li}^+$  is an analogue of  $\text{Na}^+$  in many transport systems. The selected strain (GF36) was repeatedly backcrossed with DBY747 to obtain the more suitable  $\text{Li}^+$ -sensitive strain RH2, which carried appropriate auxotrophic markers and had good transformation efficiency. This strain did not grow in the presence of 5 mM  $\text{Li}^+$ , at 1 mM  $\text{K}^+$ , and  $\text{Li}^+$  efflux was almost an order of magnitude lower than in the original DBY747. It was also moderately sensitive to  $\text{Na}^+$ , and  $\text{Na}^+$  efflux was lower than in DBY747.

By transformation of RH2 with plasmids of a yeast genomic library constructed in the shuttle vector YCp50 [13] (prepared in the laboratory of A. Jimenez from the  $\text{Li}^+$ -tolerant strain 483), and screening at 40 mM  $\text{Li}^+$ , 1 mM  $\text{K}^+$ , we isolated plasmid pGH1.1 (Fig. 1). The 5.8-kb *Bam*HI-*Bgl*II internal fragment of the insert of

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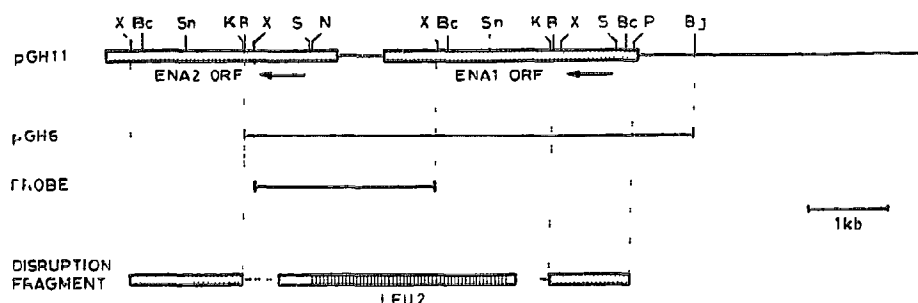


Fig 1 Restriction map of inserts in pGH1 and pGH6, and DNA fragments used for disruption of *ENA1* and *ENA2*, and to probe the disruption (see text). The direction of transcriptions are indicated by the arrows. The position of the restriction sites are indicated for *Bam*HI (B), *Bcl*I (Bc), *Bgl*II (Bg), *Kpn*I (K), *Nco*I (N), *Pst*I (P), *Sac*I (S), *Sna*I (Sn), *Xba*I (X).

pGH1 subcloned in YCp50 (pGH6) was sufficient to confer  $\text{Li}^+$  tolerance to strain RH2, and to increase  $\text{Li}^+$  and  $\text{Na}^+$  effluxes of RH2. Therefore, it was assumed to contain a gene for  $\text{Na}^+$  and  $\text{Li}^+$  effluxes, which we called *ENA1* (for *exitus natru*).

The sequence of the 5.8-kb *Bam*HI–*Bgl*II DNA fragment of pGH1-1 revealed an open-reading frame of 3273 b which could encode a polypeptide of 1091 amino acids,  $M_r$  120 371. This sequence was coincident with that of gene PMR2 reported previously to encode a putative  $\text{Ca}^{2+}$ -ATPase [15], exclusively based on sequence homology.

Downstream of *ENA1* in pGH1-1, at 610 b of the TGA triplet, we found a second open reading frame of 2903 b, which is not closed in pGH1-1. The nucleotide sequence of this second open-reading frame was almost identical to that of *ENA1*, and was designated *ENA2*.

To study the function of *ENA1*, we disrupted both *ENA1* and *ENA2* by a single-step gene disruption [13], as described in section 2. However, because of the homologies between *ENA1* and *ENA2*, integration of the

disruption fragment (Fig. 1) in the chromosome of DB746 could occur in three different forms, disrupting *ENA1*, *ENA2*, or both. By Southern blot analysis of several *Leu*<sup>+</sup> transformants we selected strain RH16.6, in which the 2.3-kb *Xba*I fragment, overlapping *ENA1* and *ENA2*, was missing (Fig. 2). This strain presented a very low  $\text{Li}^+$  tolerance and an almost zero  $\text{Li}^+$  efflux. The tolerance to  $\text{Na}^+$  was also very low, specially at alkaline pH values. At acidic pH values,  $\text{Na}^+$  was less toxic, and the strain presented a slight but significant efflux.

Transformants of RH16.6 (*enal ena2*) with plasmid pGH6 (*ENA1*) tolerated much higher concentrations of  $\text{Li}^+$  than strain RH16.6, at any pH, and also much higher concentrations of  $\text{Na}^+$  when growth was tested at pH 8.0 (Fig. 3). Consistent with the increase in  $\text{Li}^+$  and  $\text{Na}^+$  tolerances, transformants of RH16.6 with pGH6 recovered  $\text{Li}^+$  and  $\text{Na}^+$  effluxes (Fig. 4). None of these effects were observed with plasmids which did not contain *ENA1*.

In addition to the  $\text{Na}^+$  and  $\text{Li}^+$  sensitivity, strain RH16.6 did not grow well at alkaline pH values, and the defect was enhanced by external  $\text{K}^+$ . At low  $\text{K}^+$  (15 mM), RH16.6 did not grow at pH 8.4, but at 500 mM

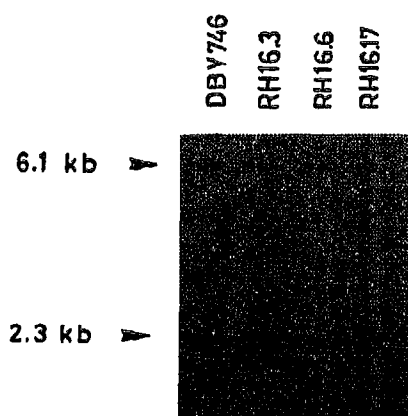


Fig 2 Southern blot analysis of three typical *Leu*<sup>+</sup> clones obtained after transformation of DBY746 for single-step gene disruption of *ENA1* and *ENA2*. In RH16.6 the 2.3-kb *Xba*I fragment was missing indicating that the *Bam*HI fragment of the chromosome had been replaced by the *LEU2* gene as a result of two homologous recombinations, one in *ENA1* and the other in *ENA2*. In the other two clones only *ENA1* or *ENA2* was disrupted.

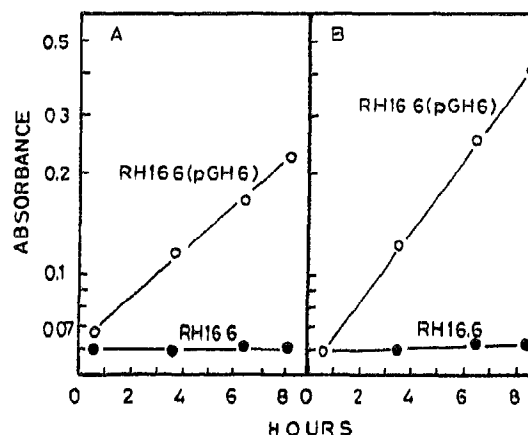


Fig 3 Growth of strains RH16.6 and RH16.6(pGH6) in arginine-phosphate medium with 5 mM  $\text{Li}^+$ , 1 mM  $\text{K}^+$ , pH 6.5 (A), and with 25 mM  $\text{Na}^+$ , 1 mM  $\text{K}^+$ , pH 8.0 (B).

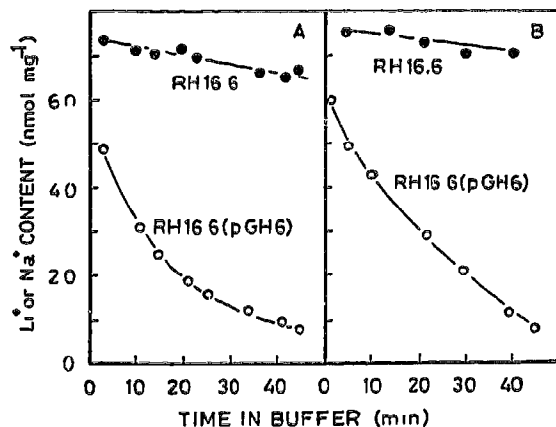


Fig. 4  $\text{Li}^+$  (A) and  $\text{Na}^+$  (B) losses from cells loaded with these cations. Cells were grown overnight in arginine-phosphate medium containing 5 mM  $\text{Li}^+$ , 10 mM  $\text{K}^+$ , or 3 mM  $\text{Na}^+$ , 1 mM  $\text{K}^+$  in RH16.6, and 5 mM  $\text{Li}^+$ , 1 mM  $\text{K}^+$ , or 10 mM  $\text{Na}^+$ , 1 mM  $\text{K}^+$  in RH16.6(pGH6). Then the cells were transferred to  $\text{Li}^+$ - and  $\text{Na}^+$ -free buffer, pH 8.0, and analyzed as described in text.

$\text{K}^+$  the maximum pH tolerated was 7.2. RH16.6(pGH6) grew up to pH 9.1 with 15 mM  $\text{K}^+$  and up to pH 8.8 with 500 mM  $\text{K}^+$ . These results suggest that the product of *ENAI* may also be involved in  $\text{K}^+$  efflux.

#### 4 DISCUSSION

Present results show that the product of *ENAI* is required for  $\text{Li}^+$ ,  $\text{Na}^+$  and probably  $\text{K}^+$  effluxes, although at acidic pH values  $\text{Na}^+$  and  $\text{K}^+$  may have efflux systems independent of this product, possibly  $\text{H}^+$ /cation antiporters [9,10]. The homology of the predicted proteins encoded by *ENAI* and *ENA2* with P-ATPases [15] leaves little doubt that these proteins are cation pumps, whose function is probably to pump  $\text{Li}^+$ ,  $\text{Na}^+$  and  $\text{K}^+$  out of the cell. The rapid stop of  $\text{Li}^+$  efflux when the cells are ATP depleted [9], and the absence of significant defects in RH16.6 (*enal ena2*), except for the functions related to  $\text{Li}^+$ ,  $\text{Na}^+$  and  $\text{K}^+$  effluxes, make it unlikely that the product of *ENAI* pumps another cation whose

concentration regulates the activity of the actual transport system for  $\text{Li}^+$ ,  $\text{Na}^+$  and  $\text{K}^+$ . Therefore, the possibility that *ENAI* (*PMR2*) encodes a  $\text{Ca}^{2+}$  pump [15] has not actually physiological support.

The main function of the product of *ENAI* may be to pump  $\text{Na}^+$ .  $\text{Li}^+$  would be pumped because of its analogy with  $\text{Na}^+$ , and  $\text{K}^+$  might be pumped when there is no  $\text{Na}^+$  in the cytoplasm. This hypothesis of a  $\text{Na}^+$ -pump ATPase may apply not only to yeast but also to plants as suggested previously [16].

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