P-type ATPases Mediate Sodium and Potassium Effluxes in Schwanniomyces occidentalis*

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Two genes isolated from Schwanniomyces occidentalis, ENA1 and ENA2, encode P-type ATPases highly homologous to the Na-ATPases of Saccharomyces cerevisiae and complement the Na⁺ sensitivity of an S. cerevisiae mutant strain lacking its own Na-ATPases. The expression of both ENA1 and ENA2 was highly dependent on a high external pH, but whereas a high pH was sufficient for the expression of ENA2, the expression of ENA1 required a high pH and the presence of Na⁺. Disruption of ENA1 rendered the cells less tolerant to Na⁺ than the wild-type strain and decreased their capacity for Na⁺ extrusion. Disruption of ENA2 did not affect Na⁺ tolerance, but decreased both the growth at high pH and K⁺ efflux. We discuss these results and propose that fungal Na-ATPases should be considered alkali cation ATPases. By sequence comparison, we found that fungal Na-ATPases form a homogeneous group that can be distinguished from other cation-pumping P-type ATPases, except from the cta3 Ca-ATPase of Schizosaccharomyces pombe.

In fungal cells, the concentration gradient of Na⁺ across the plasma membrane is normally directed inward, although K⁺ and other nutrients are concentrated several orders of magnitude in response to the membrane potential. This exclusion of Na⁺ is the result of a low Na⁺ permeability of the cells, which probably occurs in all cells adapted to diluted environments, and the eventual cooperation of specific Na⁺ extrusion systems. Because of the low Na⁺ permeability, when the external Na⁺ is low, the cellular steady-state concentration of Na⁺ resulting from Na⁺ influx and growth dilution is much lower than toxic levels, and Na⁺ extrusion is unnecessary. On the contrary, when Na⁺ is abundant, “active” extrusion of Na⁺ is indispensable because Na⁺ influx is high and cannot be compensated by growth dilution. Although under these conditions Na⁺ extrusion plays a central role in ionic homeostasis, in non-animal eucaryotic cells, the knowledge about Na⁺ efflux is almost reduced to phenomenological descriptions, except for the genes cloned in Saccharomyces cerevisiae and Schizosaccharomyces pombe and the biochemical studies on Heterosigma akashiwo. In S. cerevisiae, the major component of Na⁺ efflux is mediated by the Na-ATPase encoded by the ENA1/PMR2 gene (1, 2), existing as a tandem of several repeats of ENA genes (3, 4), which encode at least three isoforms of Na-ATPases (2). In S. pombe, there exists an electroneutral Na⁺/H⁺ antiporter encoded by a tandem of several repeats of the sod2 gene, which is amplified when the cells are grown on Na⁺ (5–7). Finally, in H. akashiwo, Na⁺ efflux is mediated by a Na,K-ATPase (8, 9).

In addition to the small number of non-animal eucaryotic cells in which systems mediating Na⁺ efflux have been determined, this has been done in organisms that are poor representatives of the life in diluted media and not representative of the life in soil. Both S. cerevisiae and S. pombe are normally isolated from fermented plant products (10, 11) and are adapted to low pH environments. In these environments, but not at alkaline pH values (12), an electroneutral Na⁺/H⁺ antiporter, such as that existing in S. pombe (5) and possibly also in S. cerevisiae (13) and Zygosaccharomyces rouxii (14), can mediate uphill Na⁺ loss and may be the normal efflux system. If this is the case, the existence of Na-ATPases in S. cerevisiae may be atypical and occurs only in some strains (4). Furthermore, in the case of environments with alkaline pH values, a Na-ATPase is not the only possible mechanism to mediate uphill Na⁺ efflux because, in Escherichia coli, an electrogenic Na⁺/H⁺ antiporter fulfills this function (15). Thus, the question of whether Na-ATPases are normal mechanisms mediating Na⁺ efflux in fungi remains unanswered.

In addition to Na⁺ extrusion, K⁺ extrusion is also an important component of ionic homeostasis. In S. cerevisiae growing cells, experiments with ⁴²K⁺ (16) and with Rb⁺ (17) have shown that one-third of the total K⁺ taken up is maintained in the cell and that two-thirds are returned to the external medium. This K⁺ efflux cannot occur as a simple diffusion because, according to measurements in Neurospora crassa, the K⁺ diffusion potential is positive to the membrane potential under most conditions. Consistent with this notion, several reports have shown that K⁺ efflux in S. cerevisiae is mediated by a K⁺/H⁺ antiporter (17–19). However, in S. cerevisiae, the disruption of the four ENA genes makes the cells sensitive to K⁺ when the pH is high (1), suggesting that, at least at a high pH, the product of some of these genes is involved in K⁺ efflux.

To obtain more information about the presence of Na-ATPases in fungi and their involvement in Na⁺ and K⁺ effluxes, we have studied Schwanniomyces occidentalis, an ascomycete yeast isolated from soil (20) that is amenable to the techniques of molecular genetics developed in S. cerevisiae (21–23). In S. occidentalis, a highly concentrative K⁺ transporter has been described (24), and now we report that two isoforms of a P-type ATPase mediate Na⁺ and K⁺ effluxes.

EXPERIMENTAL PROCEDURES

Strains, Culture Conditions, and Cation Loading of Cells—The S. cerevisiae strains TE12 (Matα ura3 his3 leu2 trp1 trk1 ena1Δ::LEU2::ena4Δ) (25) and RH16.6 (Matα ura3 his3 leu2 trp1 ena1Δ::LEU2::ena4Δ) (1) have been described previously. A transformant of the RH16.6 strain with plasmid pGB34 (3), which contains the ENA1 gene of S. cerevisiae, was used as a control in some experiments. The strains of S. occidentalis used in this study, ATCC 26076 (wild

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type) and RKA-7 (ade2), have also been described previously (21, 24, 26). Strains were routinely grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) at 28 °C. The K⁺- and Na⁺-free minimal medium used in this study was the arginine phosphate medium (AP medium) described previously (16, 24). This medium was supplemented with 50 mg l⁻¹ and 100 mg l⁻¹ as indicated in each case. The growth capacity of the yeast strains in the presence of NaCl was tested in solid media inoculated with 15-μl drops of serial 10-fold dilutions of cultures at the beginning of the stationary phase. Growth at high pH was tested by inoculating drops of cultures, as before, in solid YPD medium buffered with 20 mM HEPES or TAPS* depending on the pH. For rapid Na⁺ loading in AP medium transferred to 10 mM MES brought to pH 5.5 with Ca(OH)₂, containing 2% glucose, 0.1 mM MgCl₂, 0.5 mM KCl, and 100 mM NaCl, and maintained in this medium for 5–10 min depending on the strain and level of loading. For slow Na⁺ loading, cells were grown overnight in AP medium, pH 5.5, supplemented with the concentrations of KCl and NaCl indicated in each case.

**Cation Contents and Losses—**Cells were collected on Millipore membrane filters, rapidly washed with a 20 mM MgCl₂ solution, acid-extracted, and analyzed by atomic emission spectrophotometry. Cation losses were carried out either in 10 mM MES brought to pH 5.5 with Ca(OH)₂ or in 10 mM TAPS brought to pH 8.0 with Ca(OH)₂; in both cases, the buffer contained 0.1 mM MgCl₂ and 2% glucose. Results are reported as the means of at least four independent experiments. The S. cerevisiae strains were ~20% of the corresponding mean.

**DNA Manipulation and Sequence Analysis—**Manipulation of nucleic acids was done by standard protocols (27) or, where appropriate, following the manufacturer's instructions. For Southern blot hybridization, genomic DNA was digested with restriction enzymes. Then, after Southern blotting, total RNA was extracted (28), fractionated through formaldehydeagarose gels, blotted onto a nylon membrane, and hybridized. Probes were radiolabeled by the random priming method (29). The S. occidentalis genomic library used in this study has been described previously (30). DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. (30) as modified for use with Sequenase (U. S. Biochemical Corp.). DNA sequence data for comparative analysis were obtained from GenBank™/EMBL (release 100). Peptide sequences were obtained from GenBank™/EMBL (release 51) or SwissProt (release 34). Protein comparisons were performed using the BESTFIT, GAP, FastA (updated FastP), and PileUp algorithms or SwissProt (release 34). Protein comparisons were performed using the BESTFIT, GAP, FastA (updated FastP), and PileUp algorithms or SwissProt (release 51). Protein comparisons were performed using the BESTFIT, GAP, FastA (updated FastP), and PileUp algorithms or SwissProt (release 34). Protein comparisons were performed using the BESTFIT, GAP, FastA (updated FastP), and PileUp algorithms or SwissProt (release 51).

**Isolation of S. occidentalis ENA1**—By complementing the TE12 strain of S. cerevisiae, we isolated plasmid pAG34 containing the S. occidentalis ENA1 gene (see “Results”). Cells of the ena1::ADE2 mutant strain (see below) were grown overnight in AP medium, pH 5.5, 15 mM KCl, and 150 mM NaCl and then incubated for 1.5 h in AP medium, pH 8.2, 15 mM KCl, and 150 mM NaCl. Total RNA extracted from these cells (28) was reverse-transcribed into the ORF of the probe except for 30 base pairs at the 3' end. Therefore, an antisense primer (5'-GCTCTAGATCCAGGATACTAAGAATGAC-3') and a sense degenerate primer (5'-GCTCTAGATTAAACAATTCTCCTCATGTC-3') were used. A DNA fragment was inserted into plasmid pCR2.1 as described above. The plasmid was then digested with EcoRI, and the fragment containing the detected ORF was inserted, in the correct orientation, into the yeast expression vector pYPEG15 (32), producing plasmid pAG15.

**Disruption of the S. occidentalis ENA1 and ENA2 Genes—**For disruption of S. occidentalis ENA1, the 4.4-kb EcoRI-BamHI fragment from plasmid pADE (22), containing the gene ADE2, was inserted into the PvuII restriction site of plasmid pAG34, producing plasmid pGB15. This plasmid was then digested with XbaI and transformed into strain RKA-7. For disruption of S. occidentalis ENA2 the 4.4-kb EcoRI-BamHI fragment from plasmid pADE was inserted into plasmid pAG18 after digestion with ClaI and BamHI, producing plasmid pAG11. This plasmid was digested with EcoRV and transformed into the RKA-7 strain. In both cases, transformants were selected in the absence of adenine. DNA samples from Ade⁻ transformants were analyzed by Southern blot hybridization as described above.

**Western Blot Analysis—**Plasma membranes were prepared by the method described for the preparation of the H-ATPase of S. cerevisiae (33). This method yielded a low quantity of membranes when applied to S. occidentalis, 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 electrotransferred to an Immobilon polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) and exposed to rabbit antibodies against S. cerevisiae Ena1p (12). The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) as described previously (34).

**RESULTS**

**Na⁺ Tolerance and Growth at High pH—**At pH 6.5, S. occidentalis showed a notable tolerance to NaCl. In solid YPD medium, which contains >20 mM K⁺, S. occidentalis grew at 1.5 mM NaCl, but not at 2 mM NaCl, and in solid AP medium containing 0.5 mM K⁺, it grew well at 500 mM NaCl and poorly at 750 mM NaCl. S. occidentalis also showed a good adaptation to high pH, better than S. cerevisiae and S. pombe, the two yeast species in which the genes encoding the Na⁺ efflux systems have been isolated. The growth-limiting pH values in YPD medium were 8.5–9.0 for S. occidentalis, 7.0–7.5 for S. cerevisiae, and 6.5–7.0 for S. pombe. Interestingly, at pH 8.5, S. occidentalis was hypersensitive to both NaCl and KCl, growing poorly in YPD medium containing 250 mM NaCl or KCl.

**Na⁺ Loss Is Activated by High pH—**Cells of S. occidentalis grown at pH 5.5 in the absence of Na⁺ did not show Na⁺ extrusion when exposed to Na⁺. As a consequence, a time course experiment of Na⁺ accumulation with cells grown under these conditions followed a straight line, showing no limit to Na⁺ accumulation for more than 30 min (Fig. 1A). Incubation

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1 The abbreviations used are: TAPS, 3-[tris(hydroxymethyl)methyl]-aminopropanesulfonic acid; MES, 2-N-morpholinooethanesulfonic acid; PCR, polymerase chain reaction; kb, kilobase pair(s); ORF, open reading frame.

![Fig. 1. Induction of Na⁺ efflux by incubation at high pH.](image-url)
of the cells for 2 h at pH 8.2 in the absence of Na\(^+\) did not change the initial rate of uptake, but changed the course of accumulation, which almost stopped at a certain Na\(^+\) content (Fig. 1A). This suggested that Na\(^+\) extrusion had been activated by the high pH, and to test this activation directly, cells exposed and not exposed to pH 8.2 were fast-loaded with Na\(^+\), to avoid a possible induction of Na\(^+\) efflux during a long Na\(^+\) loading, and immediately transferred to a medium without Na\(^+\). Cells exposed to pH 8.2 showed a rapid Na\(^+\) loss, whereas in unexposed cells, the loss was very low (Fig. 1B).

To investigate the existence of a Na-ATPase homologous to the ENA1 ATPase of \textit{S. cerevisiae}, the proteins of purified plasma membranes of \textit{S. occidentalis} cells grown at pH 5.5 and exposed for 2 h to pH 8.2 were separated by standard SDS-polyacrylamide gel electrophoresis and then Western-blotted using antibodies against \textit{S. cerevisiae} Ena1p. Immunodetection of a band of \(-120\) kDa, which was not present in the cells not exposed to pH 8.2, suggested that a Na-ATPase may be present in the cells exposed to high pH.

\textit{Isolation, Sequencing, and Expression of the S. occidentalis ENA1 Gene in S. cerevisiae}—To isolate the \textit{S. occidentalis} gene(s) encoding the Na\(^+\) extrusion system, we transformed the TE12 mutant strain of \textit{S. cerevisiae} with a genomic library of \textit{S. occidentalis}. TE12 carries a deletion of the four \textit{ENA} genes and a deletion in the \textit{TRK1} gene, which encodes a high affinity K\(^+\) transporter. This mutant is very sensitive to Na\(^+\) (25) and is a good recipient strain to detect the expression of efflux systems that produce very slight increases in tolerance. After transformation, several clones grew on solid AP medium with 5 mM K\(^+\) and 150 mM Na\(^+\), which is inhibitory for the recipient strain. These clones carried plasmid pAG34, and reintroduction of pAG34 into the TE12 strain reproduced the growth in the restrictive medium.

Plasmid pAG34 and the plasmid in which the library was constructed were then transformed into the RH16.6 strain. This strain is less sensitive to Na\(^+\) than TE12 because it carries a wild-type \textit{TRK1} gene, and it is not restricted by the level of K\(^+\). Extensive testing of Na\(^+\) tolerance in these transformants confirmed that a moderate increase in tolerance was associated with plasmid pAG34 (Fig. 2). In YPD medium, pH 5.5, RH16.6 transformed with pYcDE8 (the plasmid in which the library of \textit{S. occidentalis} was constructed) grew up to 0.2 \(\mu\)M NaCl, whereas RH16.6 transformed with pAG34 grew up to 0.5 \(\mu\)M. Transformation of RH16.6 with a plasmid containing the ENA1 gene of \textit{S. cerevisiae} (pGB34) increased the tolerance up to 1.8 \(\mu\)M NaCl. Although the increase in tolerance produced by pAG34 should be the consequence of an enhancement of Na\(^+\) efflux, this was at the limits of detection, possibly because this plasmid produced only a moderate increase in Na\(^+\) tolerance.

DNA sequence analysis of the 5-kb insert of pAG34 revealed the existence of an ORF of 3165 nucleotides, which could encode a polypeptide of 1055 amino acids, sharing a 58\% sequence identity with \textit{S. cerevisiae} Ema1p (see Fig. 5). Because of this homology, the corresponding gene was designated \textit{S. occidentalis} ENA1. Northern blot analysis showed that \textit{S. occidentalis} ENA1 was expressed as a transcript of \(\sim 3.5\) kb when the cells were exposed simultaneously to NaCl and high pH. At high pH in the absence of NaCl and at pH 5.5 in the presence of NaCl, expression was very low (Fig. 3). Unlike the \textit{ENA1} gene of \textit{S. cerevisiae}, which is induced by osmotic shock (35), the \textit{S. occidentalis} ENA1 transcript was not detected in cells sustaining a slight non-ionic osmotic shock with 0.8 \(\times\) sorbitol or a strong ionic osmotic shock with 1 \(\times\) NaCl.

\textit{Disruption of S. occidentalis ENA1}—In \textit{S. occidentalis}, the \textit{ADE2} gene and an \textit{ade2} mutant strain have been isolated (22). Using the single-step gene disruption procedure (36), an \textit{S. occidentalis ena1::ADE2} mutant strain was obtained from the \textit{ade2} strain. The disruption was confirmed by Southern blot analysis, and this analysis also showed that other copies of the gene did not exist. However, when plasma membranes of the \textit{S. occidentalis ena1::ADE2} cells, grown at pH 8.2 with NaCl, were analyzed by Western blotting with the antibodies against \textit{S. cerevisiae} Ena1p, the 120-kDa immunoreactive band found in the wild-type strain decreased significantly, but did not disappear. These results indicated the existence of a second ATPase with homology to \textit{S. cerevisiae} Ema1p and possibly to \textit{S. occidentalis} Ema1p, but encoded by a gene that was not an identical repeat of \textit{S. occidentalis} ENA1.

The Na\(^+\) tolerance of the \textit{S. occidentalis ena1::ADE2} mutant strain was then tested in AP medium with 0.5 \(\mu\)M K\(^+\), and similar inhibitory effects were found at 75 \(\mu\)M NaCl in the mutant strain and at 750 \(\mu\)M NaCl in the original strain (RKA-7). Although this result indicated that the \textit{ENA1} gene of \textit{S. occidentalis} was important for Na\(^+\) tolerance, the \textit{S. occidentalis ena1::ADE2} strain still showed a notable Na\(^+\) efflux when the cells were exposed to high pH (results similar to those shown in Fig. 1), suggesting the existence of other Na\(^+\) efflux systems in the mutant. To rule out that mRNAs corresponding to these systems had been detected in the expression analysis of \textit{S. occidentalis} ENA1 (Fig. 3), we repeated these Northern blot analyses with the \textit{S. occidentalis ena1::ADE2} strain and found that the hybridization bands disappeared in this null mutant. Further tests of complementation of the TE12 strain with the \textit{S. occidentalis} library failed to produce clones different from \textit{S. occidentalis} ENA1.

\textit{Isolation, Sequencing, and Expression of the S. occidentalis ENA2 Gene in S. cerevisiae}—Because we were interested in Na-ATPases, we tested whether Na\(^+\) efflux in the \textit{S. occidentalis ena1::ADE2} mutant strain was mediated by an electro-
neutral Na\(^+\)/H\(^+\) antiporter, such as that found in S. pombe (5). This test is possible because an electroneutral Na\(^+\)/H\(^+\) antiporter is not functional in a medium with high pH and high Na\(^+\) (12). As materials for the test, we prepared two different types of Na\(^+\)-loaded cells of wild-type and mutant strains: (i) cells grown in Na\(^+\) at pH 5.5 and (ii) cells grown in the same manner and then incubated for 2 h in the same medium at pH 8.2. The cells were then transferred to TAPS, pH 8.0, containing 50 mM NaCl plus 100 mM KCl to inhibit Na\(^+\) influx (12). In the cells maintained at pH 5.5, the wild-type strain showed a slow Na\(^+\) loss, which reverted to a slow uptake in the S. occidentalis ena1::ADE2 mutant strain (Fig. 4A). By contrast, in the cells exposed to pH 8.2, the mutant strain showed Na\(^+\) loss, although at a lower rate than the wild-type strain (Fig. 4B). These results suggested the possibility that a Na-ATPase was induced in the S. occidentalis ena1::ADE2 cells at high pH, although they did not rule out the possibility that it was an electronegenic Na\(^+\)/H\(^+\) antiporter.

To clone the gene encoding a second Na-ATPase, if it existed, we isolated RNA from cells of the S. occidentalis ena1::ADE2 mutant strain exposed to 150 mM NaCl, pH 8.2, for 1.5 h. We then performed reverse transcription-PCR using oligo(dT) and a degenerate primer designed to bind the DNA nucleotide sequence encoding the amino acid sequence DDNNFLSI, which is present in most eucaryotic P-type ATPases, excluding H-ATPases. A 1.0-kb cDNA fragment was repeatedly amplified by this procedure, but not if the cells had always been at pH 5.5, even in the presence of NaCl. Cloning and sequencing of the band showed that it contained an ORF whose translated amino acid sequence showed high homology to S. occidentalis Ena1p. Using this cDNA fragment for colony hybridization of the S. occidentalis library, we isolated plasmid pAG18, whose insert strongly hybridized to the cDNA probe. DNA sequence analysis of the transcript revealed the existence of an ORF of 3216 nucleotides that lacked 30 base pairs at the 3’-end according to the sequence of the 1.0-kb cDNA fragment obtained by reverse transcription-PCR. The complete sequence of ENA2, deduced from the cDNA and the insert in pAG18, could encode a polypeptide of 1082 amino acids, which shared greatest sequence identity with S. occidentalis Ena1p (73%) and lower sequence identity (58%) with S. cerevisiae Ena1p (37). As shown for S. cerevisiae Ena1p (37), both S. occidentalis Ena1p and Ena2p showed a predicted calmodulin-binding region immediately after the twelfth transmembrane region.

Northern blot analysis of the S. occidentalis ena1::ADE2 cells exposed to pH 8.2 showed that S. occidentalis ENA2 expressed a transcript of ~3.5 kb, which was not detected if the cells were not exposed to high pH values. In contrast with the expression of S. occidentalis ENA1 (Fig. 3), the S. occidentalis ENA2 transcript was detected only when the cells had been exposed to a high pH, and its level was insensitive to the presence of 200 mM NaCl at any pH value. As in the case of S. occidentalis ENA1, the transcript of S. occidentalis ENA2 was not detected in cells sustaining an osmotic shock.

To study the function of S. occidentalis ENA2, we obtained a DNA fragment comprising the complete coding region of the gene by PCR and inserted it into the expression vector pYEG15 after the promoter of the PGK1 gene of S. cerevisiae (32), producing plasmid pAG15. Transformation of pAG15 into RH16.6 resulted in a moderate increase in Na\(^+\) tolerance, slightly higher than with plasmid pAG34 containing S. occidentalis ENA1. As in the case of S. occidentalis ENA1, the increase in Na\(^+\) efflux produced by S. occidentalis ENA2 in RH16.6 was hardly detected.

As a further test of the function of the ENA2 ATPase, we disrupted the S. occidentalis ENA2 gene following the same procedure as for the disruption of S. occidentalis ENA1. This disruption did not affect Na\(^+\) tolerance with reference to the wild-type strain (data not shown), but rendered the cells less tolerant to high pH (Fig. 6). Interestingly, this effect was observed when KOH was used to increase the medium pH, but it was not as clear using NaOH. Because the Na\(^+\) content of the basal medium is low (7 mM), the defect seemed to be suppressed by increasing the Na\(^+\) concentration.

S. occidentalis Ena2p Mediates K\(^+\) Efflux—The defective growth of the S. occidentalis ena2::ADE2 mutant strain in YPD medium at high pH without the addition of Na\(^+\) suggested that S. occidentalis Ena2p might mediate K\(^+\) efflux. To test this possibility, we suspended cells of the wild-type and S. occidentalis ena1::ADE2 and ena2::ADE2 strains in TAPS, pH 8.0, containing 20 mM RbCl to inhibit K\(^+\) uptake and determined the K\(^+\) content at different time intervals. Cells grown at pH 5.5 did not show an appreciable K\(^+\) loss, but cells maintained for 2 h at pH 8.2 showed a slight loss. Because in S. cerevisiae the activity of the ENA1 ATPase is inhibited when the internal pH is low (38), we repeated the same experiments in cells induced at pH 8.2, but included 20 mM NH\(_4\)Cl in the buffer to increase the internal pH. In this case, the three strains showed K\(^+\) loss. The wild-type and S. occidentalis ena1::ADE2 mutant strains showed identical K\(^+\) losses, which were higher than the loss in the S. occidentalis ena2::ADE2 strain (Fig. 7). This strongly suggests that the S. occidentalis ENA2 ATPase is involved in K\(^+\) efflux.
which is clearly more related to fungal Na-ATPases than to other fungal, plant, and animal Ca-ATPases. Fungal Na-ATPases were less related to H-ATPases, including fungal H-ATPases, than to the groups described above (data not shown). According to its translated sequence, it has been proposed that a gene of Z. rouxii encodes a Na-ATPase (GenBank™/EMBL accession number D78567). This ATPase clusters with fungal Na-ATPases at a shorter distance from the S. cerevisiae ENA1 ATPase than from the cta3 Ca-ATPase.

**DISCUSSION**

Homology of the translated sequences of the ENA1 and ENA2 genes of S. occidentalis and the ENA genes of S. cerevisiae.

**Fig. 5.** Alignment of the deduced amino acid sequences of the translated coding regions of S. occidentalis ENA1 and ENA2 and S. cerevisiae ENA1. Identical residues are boxed, and the putative transmembrane fragments are underlined. So, S. occidentalis; Sc, S. cerevisiae.

**Fig. 6.** Growth of wild-type, ena1::ADE2, and ena2::ADE2 strains at high pH. Decimal dilution drops of cell suspensions were inoculated in YPD medium at pH 5.5 and 8.6.

**Fig. 7.** K⁺ loss in cells of the wild-type, ena1::ADE2, and ena2::ADE2 strains. Cells of wild-type (●), ena1::ADE2 (○), and ena2::ADE2 (□) strains were grown in AP medium and 1.0 mM K⁺, pH 5.5, and then incubated for 2 h in AP medium and 1.0 mM K⁺, pH 8.2. Cells were transferred to 10 mM TAPS, pH 8.0, containing 20 mM RbCl and 20 mM NH₄Cl, and changes in the internal K⁺ content were followed for 10 min.
Our results of Na⁺ efflux show this clearly in cells grown at pH 5.5 (Fig. 4A). By contrast, when the cells had been grown at or exposed to high pH, Na⁺ efflux was not as clearly dominated by the ENA1 ATPase, at least when the Na⁺ content of the cells was high. However, when the Na⁺ content decreased and reached low concentrations, the ENA1 ATPase seemed again to be the most effective system (Fig. 4B). The existence of Na⁺ efflux systems independent from *S. occidentalis* Ena1p and Ena2p and the functions of these putative systems could not be tested because we could not obtain the *ena1 ena2* double mutant. It is worth noting, however, that the purpose of this work was not to dissect all the Na⁺ transport systems in *S. occidentalis*, but to explore the possible role of Na-ATPases in different fungal species. In this regard, an interesting capacity of the *S. occidentalis* ENA2 ATPase was to mediate K⁺ efflux. This indicates that fungal Na-ATPases should not be considered exclusively Na-ATPases, but alkali cation ATPases, as previously suggested by the conditions of phosphorylation of the *S. cerevisiae* ENA1 ATPase from ATP, which occurs not only in the presence of Na⁺ and Li⁺, but also in the presence of K⁺ and Rb⁺ (42).

The clear effect of pH on the expression of *S. occidentalis* ENA1 and ENA2 (Fig. 3) has been observed previously in the ENA1 gene of *S. cerevisiae* (3). Furthermore, although the expression of *S. cerevisiae* ENA1 is also triggered by other causes such as an osmotic shock or glucose derepression (35), these enhancements are suppressed in acid media, especially in the presence of perment acids such as acetic acid.² The strong dominance of both the external and internal pH for the expression of the Na-ATPases in *S. cerevisiae* and *S. occidentalis* suggests that these ATPases might be involved in pH regulation, mediating a decrease in the cellular pH. This has been proposed previously to explain the increase in tolerance at high pH induced in *S. pombe* by the heterologous expression of the *S. cerevisiae* ENA1 ATPase (12). The defective growth at high pH of the *ena2::ADE2* mutant strain reported here (Fig. 6) is entirely consistent with the results in *S. pombe* and gives further support to the notion that fungal Na-ATPases relieve the alkali load impose by a high external pH. However, the mechanisms involved in this process are not evident, especially if it depends only on K⁺ efflux. If it is mediated by Na⁺ efflux, a decrease in the cellular pH may occur if the ATPase exchanges Na⁺ and H⁺. In this case, the Na⁺ taken up with Na⁺-symported anions (43, 44) is exchanged for H⁺, thus compensating the loss of organic acids imposed by the high external pH. But the mechanism by which K⁺ efflux can decrease the cellular pH is completely unknown at this moment. Certainly, a part of the cellular K⁺ may be exchanged for H⁺, but regaining this K⁺ would restore the alkali load.

Unlike the ENA1 gene of *S. cerevisiae* (35), neither the ENA1 nor ENA2 gene of *S. occidentalis* was expressed when the cells sustained an osmotic shock. At this moment, it is impossible to predict a function for the *S. cerevisiae* ENA1 ATPase when the cells are under osmotic stress, but the present results indicate that Ena1p and Ena2p do not fulfill this function in *S. occidentalis*.

So far, studies on the genetics of K⁺ and Na⁺ transport systems in fungi have been carried out with *S. cerevisiae* and *S. pombe*, which are species normally isolated from high sugar, low pH media (10, 11). In this study, we used *S. occidentalis*, a yeast species normally isolated from soil (20) that is able to grow at pH 8.5. The presence in *S. occidentalis* of P-type ATPases homologous in function to the ENA ATPases of *S. cerevisiae* suggests that this class of ATPases may be extended.

² F. Rubio and A. Rodríguez-Navarro, unpublished results.
among non-animal eucaryotic cells thriving in many different environments. In *Fusarium solani* and *Z. rouxii*, the existence of Na-ATPases has also been proposed on the basis of sequence homology to *S. cerevisiae* Ena1p (GenBank™/EMBL accession numbers U61840 and D78567, respectively). If the predicted function of these ATPases is correct, these new members of the family would give further support to our proposal.

Sequence comparisons of P-type ATPases with different functions cluster fungal Na-ATPases in a group related to sarcoplasmic/endoplasmic Ca-ATPases and to Na,K-ATPases. Fungal Na-ATPases probably diverged in this group earlier than the other components among them. The homology of fungal Na-ATPases to the cta3 Ca-ATPase of *S. pombe* (41) suggests that cta3 diverged directly from fungal Na-ATPases (Fig. 8) or that both types diverged from a proximal common ancestor, which may be a Ca-ATPase. The similarities between Na-ATPases and cta3 indicate that sequence-based distinctions between Ca-ATPases and Na-ATPases in fungi must be made carefully.

REFERENCES

1. Haro, R., García-de Blas, B., and Rodríguez-Navarro, A. (1991) FEBS Lett. 291, 189–191
2. Rodríguez-Navarro, A., Quintero, F. J., and García-de Blas, B. (1994) Biochim. Biophys. Acta 1187, 203–205
3. García-de Blas, B., Rubio, F., Quintero, F. J., Bañuelos, M. A., and Rodríguez-Navarro, A. (1993) Mol. Gen. Genet. 236, 363–368
4. Wieland, J., Nitsche, A. M., Strayle, J., Steiner, H., and Rudolph, H. K. (1995) EMBO J. 14, 3870–3882
5. Jia, Z. P., McCullough, N., Martel, R., Hemmingsen, S., and Young, P. G. (1992) EMBO J. 11, 1631–1640
6. Hohnenberger, K. M., Jia, Z., and Young, P. G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5031–5036
7. Dihov, P., Smith, J. J., Young, P. G., and Fliegel, L. (1997) FEBS Lett. 405, 119–124
8. Wada, M., Sato, H., Kasamo, K., and Fuji, T. (1989) Plant Cell Physiol. 30, 293–928
9. Shono, M., Wada, M., and Fuji, T. (1995) Plant Physiol. (Bethesda) 108, 1615–1621
10. Yarrow, D. (1984) in The Yeast: A Taxonomic Study (Kreger-van Rij, N. J. W. K., ed) 3rd Ed., pp. 379–395, Elsevier Science Publishers B. V., Amsterdam
11. Yarrow, D. (1984) in The Yeast: A Taxonomic Study (Kreger-van Rij, N. J. W. K., ed) 3rd Ed., pp. 414–422, Elsevier Science Publishers B. V., Amsterdam
12. Bañuelos, M. A., Quintero, F. J., and Rodríguez-Navarro, A. (1995) Biochim. Biophys. Acta 1229, 233–238

13. Prior, C., Potier, S., Souciet, J. L., and Sychrova, H. (1996) FEBS Lett. 387, 89–93
14. Watanabe, Y., Miwa, S., and Tamai, Y. (1995) Yeast 11, 829–839
15. Schuldiner, S., and Padan, E. (1993) in Alkaline Cation Transport Systems in Prokaryotes (Bakker, E. P., ed) pp. 25–51, CRC Press, Inc., Boca Raton, FL
16. Rodríguez-Navarro, A., and Ramos, J. (1984) J. Bacteriol. 159, 940–945
17. Ortegía, M. D., and Rodríguez-Navarro, A. (1995) Z. Naturforsch. C 50, 721–725
18. Camarasa, C., Prieto, S., Ros, R., Salmon, J. M., and Barre, P. (1996) Yeast 12, 1301–1313
19. Ramírez, J., Peña, A., and Montero-Lomeli, M. (1996) Biochim. Biophys. Acta 1285, 175–182
20. Phaff, H. J., and Miller, M. W. (1984) in The Yeast: A Taxonomic Study (Kreger-van Rij, N. J. W. K., ed) 3rd Ed., pp. 379–395, Elsevier Science Publishers B. V., Amsterdam
21. Klein, R. D., and Roof, L. L. (1988) Curr. Genet. 13, 29–35
22. Klein, R. D., and Favreau, M. A. (1988) J. Bacteriol. 170, 5572–5578
23. Claros, M. G., Abarca, D., Fernández-Loabo, M., and Jiménez, A. (1993) Curr. Genet. 24, 75–83
24. Bañuelos, M. A., Klein, R. D., Alexander-Bowman, S. J., and Rodríguez-Navarro, A. (1995) EMBO J. 14, 3021–3027
25. Haro, R., Bañuelos, M. A., Quintero, F. J., Rubio, F., and Rodríguez-Navarro, A. (1993) Physiol. Plant. 89, 868–874
26. Deibel, M., Hiebensch, R. R., and Klein, R. D. (1988) Prep. Biochem. 18, 77–122
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Carlson, M., and Botstein, D. (1982) Cell 28, 145–154
29. Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13
30. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
31. Devereux, J., Haebeler, P., and Smithies, O. (1984) Nucleic Acids Res. 12, 387–390
32. Brunelli, J. P., and Pall, M. L. (1993) Yeast 9, 1309–1318
33. Serrano, R. (1988) Methods Enzymol. 157, 533–544
34. Blake, M. S., Johnston, G. J., Russell-Jones, G. J., and Gotschlich, E. C. (1984) Anal. Biochem. 136, 175–179
35. Merquez, J. A., and Serrano, R. (1996) FEBS Lett. 382, 89–92
36. Rothstein, R. (1991) Methods Enzymol. 194, 281–301
37. Rudolph, H. K., Antebi, A., Fink, G. R., Buckley, C. M., Derman, T. E., LeVitre, J., Davidow, L. S., Mao, J., and Moir, D. T. (1989) Cell 58, 133–145
38. Rodríguez-Navarro, A., Sancho, E. D., and Pérez-Lleóteres, C. (1981) Biochim. Biophys. Acta 640, 352–358
39. Fagan, M. J., and Saier, M. H. (1994) J. Mol. Evol. 38, 57–99
40. Møller, J. V., Juul, B., and Maire, M. L. (1996) Biochim. Biophys. Acta 1286, 1–51
41. Ghosal, M., Goffau, A., Halachmi, D., and Eilam, Y. (1990) J. Biol. Chem. 265, 18400–18407
42. Benito, B., Quintero, F. J., and Rodríguez-Navarro, A. (1997) Biochim. Biophys. Acta 1328, 214–225
43. Blasco, G. M., Blasco, E., and Borst-Pauwels, G. W. F. H. (1977) Biochim. Biophys. Acta 467, 65–71
44. Versaw, W. K., and Metzengen, R. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3884–3887