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Altered Na^+ and Li^+ Homeostasis in *Saccharomyces cerevisiae* Cells Expressing the Bacterial Cation Antiporter NhaA

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The bacterial $Na^+(Li^+)/H^+$ antiporter NhaA has been expressed in the yeast *Saccharomyces cerevisiae*. NhaA was present in both the plasma membrane and internal membranes, and it conferred lithium but not sodium tolerance. In cells containing the yeast Ena1-4 (Na^+, Li^+) extrusion ATPase, the extra lithium tolerance conferred by NhaA was dependent on a functional vacuolar H^+ ATPase and correlated with an increase of lithium in an intracellular pool which exhibited slow efflux of cations. In yeast mutants without (Na^+, Li^+) ATPase, lithium tolerance conferred by NhaA was not dependent on a functional vacuolar H^+ ATPase and correlated with a decrease of intracellular lithium. NhaA was able to confer sodium tolerance and to decrease intracellular sodium accumulation in a double mutant devoid of both plasma membrane (Na^+, Li^+) ATPase and vacuolar H^+ ATPase. These results indicate that the bacterial antiporter NhaA expressed in yeast is functional at both the plasma membrane and the vacuolar membrane. The phenotypes conferred by its expression depend on the functionality of plasma membrane (Na^+, Li^+) ATPase and vacuolar H^+ ATPase.

The homeostatic mechanisms which maintain intracellular ion concentrations within the range compatible with biochemical processes are essential for living cells (14, 36). These mechanisms, composed of membrane transporters and regulatory components, are relatively well understood in bacteria (1) and animal cells (14, 36) but in the case of fungal and plant cells they have only started to be elucidated (31). Sodium plays an important role in animal cells, which are adapted to live with an extracellular salt concentration of approximately 150 mM (14). The Na^+/K^+ ATPase found in all animals couples the sodium transport out of the cell to potassium influx utilizing ATP as a driving force (19, 34, 36). In addition to controlling several physiological functions (such as osmoregulation, water and salt balance, membrane potential, and K^+ homeostasis), this Na^+/K^+ exchange pump establishes a Na^+ gradient across the plasma membrane. The dissipation of the Na^+ gradient through secondary transport systems (Na^+ symporters and Na^+ antiporters) can be used for the uptake of nutrients (sugars and amino acids), the expulsion of metabolic end products, the regulation of internal pH via the Na^+/H^+ antiporters, or the modulation of Ca^{2+} homeostasis via Na^+/Ca^{2+} antiporters (34, 36).

In contrast to animals, sodium is not essential for most fungi and plants (21), organisms in which a Na^+/K^+ ATPase is not present. In these organisms, evolution took another course and a plasma membrane H^+ ATPase generates the proton gradient which drives secondary active H^+ cotransport (29). With the exception of halophytic species (the native flora of saline soils), which grow and develop optimally at high salt concentrations (21), most plants and fungi cannot tolerate high NaCl concentrations in soils and water (8, 31). The toxic effects of salinity on cells may be mediated by osmotic inhibition of water absorption, specific and nonspecific effects of high Na^+ and Cl^- concentrations, and nutritional imbalance (8, 31, 43).

One of the major deleterious effects of high salinity is caused by Na^+ accumulation in the cytoplasm, where many metabolic activities are sensitive to Na^+ inhibition (31). Expression of heterologous sodium efflux transporters could be a useful approach to improve salt tolerance in sensitive organisms such as nonhalophytic fungi and plants. However, the complexities of higher organisms, with several intracellular compartments and, in the case of plants, with interconnected organs, put a note of caution on the anticipated results. As a first step for exploring the capability of this approach to alter ion homeostasis, we have expressed the bacterial cation antiporter NhaA in the yeast *Saccharomyces cerevisiae*. This combination of recipient cell and sodium transporter offers special advantages for the interpretation of results. NhaA is a well-characterized sodium and lithium extrusion system composed of a single polypeptide which operates as an electrogenic antiporter ($2H^+$ exchanged for each Li^+ or Na^+) (25). Yeast is a useful model system because (i) it shares basic bioenergetic mechanisms with plant cells (31), (ii) it has been extensively studied as a host of heterologous membrane proteins (32), and (iii) its transport mechanisms at the plasma membrane and vacuolar membrane are relatively well characterized at the molecular level (16, 30).

Many strains of the yeast *S. cerevisiae* contain a major sodium and lithium extrusion P ATPase encoded by the *ENA1-4*/*PMR2* gene cluster (12, 41). Lithium is a sodium analog with higher toxicity and can be used as a growth inhibitor, at lower concentrations than sodium, to reduce osmotic effects (11, 31). We demonstrate in the present work the capability of the bacterial NhaA secondary transporter to replace the yeast *ENA1-4* pump in terms of lithium but not sodium tolerance. In addition, by using mutants deficient in vacuolar H^+ ATPase,

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TABLE 1. Yeast strains used

Strain	Genotype	Source or reference[a]
DBY746	MATα *his3-Δ1 leu2-3,112 trp1-289 ura3-52*	YGSC
W303-1A	MAT**a** *ade2-1 can1-100 his3-11,15 leu2-3,112 tpr1-1 ura3-1*	40
RH16.6	DBY746 *ena1-4/pmr2::LEU2*	12
K633	W303-1A *ena1-4/pmr2::HIS3*	4
RS-1140	W303-1A *ena1-4/pmr2::HIS3 tfp1::LEU2*	
RS-1142	W303-1A *ena1-4/pmr2::HIS3 LEU2*	
RS-1144	W303-1A *tfp1::LEU2*	
RS-1146	W303-1A *LEU2*	
RS-997	DBY746(pRS-699)	
RS-1045	DBY746(pRS-824)	
RS-1043	RH16.6(pRS-699)	
RS-1041	RH16.6(pRS-824)	
RS-1175	RS-1146(pRS-699)	
RS-1176	RS-1146(pRS-824)	
RS-1177	RS-1144(pRS-699)	
RS-1178	RS-1144(pRS-824)	
RS-1170	RS-1142(pRS-699)	
RS-1171	RS-1142(pRS-824)	
RS-1179	RS-1140(pRS-699)	
RS-1180	RS-1140(pRS-824)	

[a] Unless otherwise indicated, the source for strains was this study. YGSC, Yeast Genetic Stock Center, Berkeley, Calif.

we provide evidence for the role of this proton pump in the altered ion homeostasis conferred by NhaA.

MATERIALS AND METHODS

Yeast strains and culture conditions. Standard methods for yeast culture and manipulation were used (9). The *Saccharomyces cerevisiae* strains used in the present work are described in Table 1. The standard growth medium contained 2% glucose, 0.7% yeast nitrogen base without amino acids (Difco), 50 mM succinic acid adjusted to pH 5.5 with Tris, 50 μg of adenine/ml, 100 μg of tryptophan/ml, 100 μg of histidine/ml, and 100 μg of leucine/ml. Solid medium contained 2% bacteriological-grade agar. For media at pH 7.2, the succinate-Tris buffer was replaced by 50 mM MOPS [3-(*N*-morpholino)propanesulfonic acid] adjusted to pH 7.2 with Tris. Media were supplemented with NaCl and LiCl as indicated.

Construction of the yeast vacuolar mutants. Plasmid pPK8, containing a 5-kb *Xba*I fragment with the *tfp1::LEU2* null allele of the catalytic subunit of vacuolar H^+ ATPase (13, 33), was a gift of T. H. Stevens, University of Oregon, Eugene. It was digested with *Xba*I and utilized to transform (15) yeast strain W303-1A and its *ena1-4/pmr2::HIS3* derivative K633 (Table 1). To check the vacuolar ATPase phenotype, Leu^+ transformants were replicated to plates buffered at either pH 5.5 or 7.2. Disruption of vacuolar ATPase causes conditional lethality at neutral pH (24, 42). Disruption of the *TFP1* gene was confirmed by amplification of a 0.53-kb fragment of the gene using as sense primer 5′-GGACTATC AAGTGGCAATTTACTC and as antisense primer 5′-CCCATGACCTTATTA CCAACCTC. Transformation was also made with the 2.3-kb *Xho*I-*Sal*I *LEU2* fragment of plasmid YEp13 (3) to generate control strains without leucine auxotrophy.

Construction of expression plasmid pRS-824. The open reading frame of the bacterial Na^+/H^+ antiporter gene *nhaA* was amplified from plasmid pGM36 (17) using as sense primer 5′-GCGCTCGAGATGAAACATCTGCATC and as antisense primer 5′-GCGCTCGAGTCAAACTGATGGACG, containing *Xho*I sites (underlined) before the start and stop codons, respectively. The GTG start codon of the *nhaA* gene (37) was changed to ATG to ensure that the yeast cells would recognize methionine as the first encoded amino acid. In order to minimize amplification errors, the reaction included a relatively large amount of plasmid pGM36 (2 μg/ml) and only 15 temperature cycles. The amplified fragment of 1.2 kb (about 20 μg/ml) was extracted with phenol-chloroform, precipitated with ethanol, digested with *Xho*I, and purified free of residual plasmid by preparative electrophoresis and extraction with GeneClean (Bio 101, La Jolla, Calif.). The purified 1.2-kb *Xho*I fragment was ligated to the yeast expression plasmid pRS-699 linearized with *Xho*I. pRS-699 is a yeast multicopy plasmid of 7.05 kb containing the *URA3* marker and the strong constitutive promoter of the *PMA1* gene before the *Xho*I cloning site (32). Three recombinant plasmids with the correct orientation (*Bam*HI fragments of 6.5 and 1.75 kb) were tested for lithium tolerance after transformation into yeast strain RH16.6 (see below) and produced similar lithium tolerance. One of them (pRS-824) was selected to transform all the different yeast strains (see Table 1).

Salt tolerance tests. Cultures were pregrown in solid medium without salt because expression of NhaA was found to decrease viability in stationary phase more in liquid cultures than in solid cultures. After 2 days of growth, one small loop of cells was diluted in water and the absorbance at 660 nm (measured with Spectronic 20D; Milton Roy, Rochester, N.Y.) was adjusted to 1 (2×10^7 cells/ml). After dilutions of 1/4, 1/16, and 1/50 were made, about 1 μl was spotted with an 8 by 6 stainless steel replica plater (Sigma, St. Louis, Mo.) on plates containing the indicated concentrations of salt. The growth of the intermediate dilution (about 10^3 cells) is shown in the figures.

Sucrose gradient fractionation and Western blot analysis. Yeast membranes were isolated, fractionated by isopycnic centrifugation in linear sucrose gradients (16 to 49% sucrose [wt/wt]), and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide gels) and Western immunoblotting with antibodies against the plasma membrane marker Pma1p as previously described (38). Antibodies against the C terminus of NhaA (6) were utilized to localize the bacterial antiporter in the different fractions. Purification of the NhaA protein from overproducing bacterial cultures was performed as previously described (37). Protein concentration was determined by the method of Bradford (2) with the protein assay reagent from Bio-Rad (Hercules, Calif.).

Measurement of intracellular ion concentrations and efflux of lithium. Cultures were grown to exponential phase (absorbance at 660 nm, approximately 0.5) with the indicated concentrations of salt, and aliquots of 10 ml were centrifuged (2 min at $2{,}000 \times g$) and then washed three times by resuspension and centrifugation with ice-cold 10 mM $MgCl_2$ containing sorbitol at the same osmotic concentration as the salt present in the growth medium. Washed cells were resuspended in 1 ml of 10 mM $MgCl_2$ without sorbitol. After the cell concentration was determined by the absorbance at 660 nm of a 10-fold dilution, intracellular ions were extracted by addition of concentrated HCl to a 0.1 M final concentration and incubation for 10 min at 95°C. After removal of cell debris by centrifugation, potassium, sodium, and lithium concentrations in the supernatant were determined with an atomic absorption spectrometer (Varian) in flame emission mode. Intracellular water (1.4 μl/ml per absorbance unit) was estimated as previously described (5).

For the determination of lithium efflux, cultures were grown to exponential phase (absorbance at 660 nm of about 0.3) with either 2 or 50 mM LiCl, washed twice with 10 mM $MgCl_2$ by vacuum filtration on nitrocellulose circles (Millipore HATF08250), and resuspended in the same volume of fresh medium without LiCl. After incubation for the times indicated in the figures, samples were processed for the determination of intracellular lithium as described above.

RESULTS

The yeast expression plasmid pRS-699, a multicopy episome with the strong *PMA1* promoter (32), was utilized to express

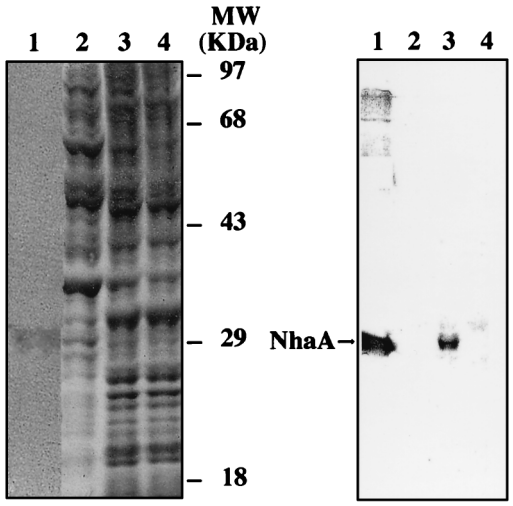

FIG. 1. Immunological evidence for expression of NhaA in yeast membranes. Cellular homogenization, centrifugation to resolve soluble and membrane fractions, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blot analysis were performed as described in Materials and Methods. Lanes 1, purified NhaA protein (2 μg); lanes 2, soluble fraction from strain RS-1045 (expressing NhaA) (30 μg of protein); lanes 3, membrane fraction from strain RS-1045 (expressing NhaA) (30 μg of protein); lanes 4, membrane fraction from control strain RS-997 (not expressing NhaA) (30 μg of protein). (Left) Coomassie blue-stained gel. (Right) Western immunoblot with antibody against the NhaA protein.

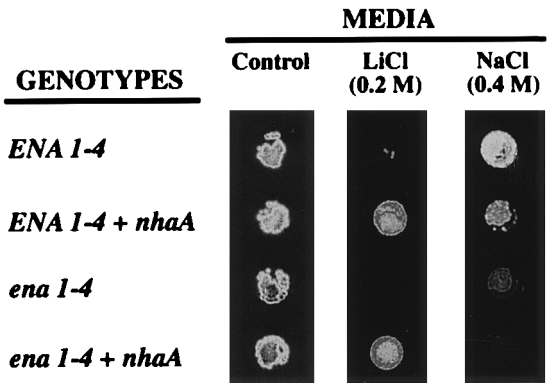

FIG. 2. Lithium tolerance (but not sodium tolerance) is conferred by expression of NhaA in yeast. Strains RS-997 (*ENA1-4*), RS-1045 (*ENA1-4 nhaA*), RS-1043 (*ena1-4*), and RS-1041 (*ena1-4 nhaA*) were tested for salt tolerance as described in Materials and Methods. Plates contained either no salt (control) or 0.2 LiCl or 0.4 M NaCl as indicated. Similar results were obtained in three independent experiments.

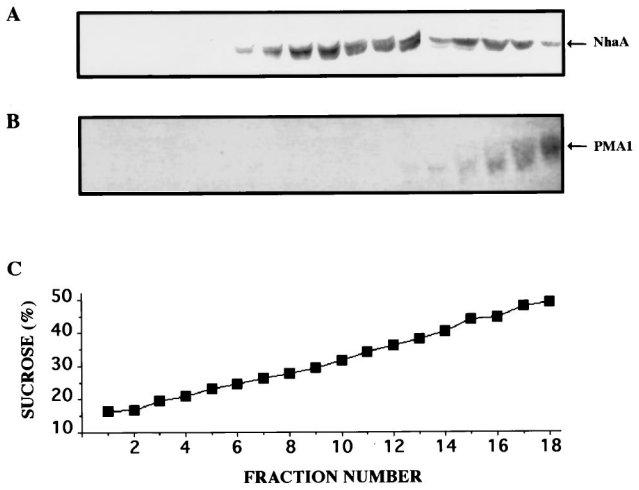

FIG. 3. Dual subcellular localization of NhaA expressed in yeast. Membranes from strain RS-1045 (expressing NhaA) were fractionated by equilibrium sucrose gradient centrifugation as described in Materials and Methods. The top of the gradient is fraction 1. Aliquots (15 μl) of the fractions were analyzed by Western blotting with antibodies against either NhaA (A) or the plasma membrane marker Pma1 (B). The sucrose concentration of every fraction is also shown (C).

the *Escherichia coli nhaA* gene in the yeast *S. cerevisiae*. As indicated in Fig. 1, the bacterial NhaA antiporter could be expressed in yeast membranes with an electrophoretic mobility similar to that of the NhaA protein purified from bacteria (about 30 kDa). No NhaA protein was detected in the soluble fraction of the yeast homogenate. We estimate the NhaA protein to represent about 3% of total yeast membrane protein (0.5% of total yeast protein), a level comparable to that obtained with the plant plasma membrane H^+ ATPase (38).

Yeast cells expressing NhaA exhibited no significant growth difference with respect to control cells when tested in normal medium (Fig. 2). However, in medium with a highly toxic LiCl concentration, the expression of NhaA in both *ENA1-4* and *ena1-4* cells dramatically improved yeast growth (Fig. 2). This suggested that NhaA expressed in yeast was functional as a Li^+/H^+ antiporter and was apparently more active for lithium extrusion than the endogenous yeast *ENA1-4* ATPase. One puzzling result was that the expression of NhaA did not increase sodium tolerance. On the contrary, yeast cells expressing NhaA were slightly more sensitive to NaCl than controls (Fig. 2).

In order to investigate the mechanisms of salt tolerance conferred by NhaA, we determined the intracellular levels of Na^+ and Li^+ in cells growing in the presence of these cations (Table 2). The concentration of salt was adjusted to allow for equivalent growth rates with all strains. In medium with NaCl, expression of NhaA produced no significant changes in the intracellular sodium level of *ENA1-4* cells (grown with 0.5 M NaCl) and a small decrease (13%) in *ena1-4* cells (grown with 0.3 M NaCl). This could explain the absence of a sodium tolerance phenotype upon expression of NhaA. On the other hand, in medium with LiCl, two different responses were observed: in cells with *ENA1-4* ATPase, grown with 50 mM LiCl, the intracellular Li^+ was increased about threefold upon expression of NhaA, while in *ena1-4* cells grown with 2 mM LiCl, the intracellular Li^+ was decreased about threefold by NhaA expression. Since in both cases lithium tolerance was improved by NhaA expression (Fig. 2), a simple correlation between tolerance and total intracellular concentrations of toxic cations was unlikely.

TABLE 2. Potassium, sodium, and lithium contents of yeast cells grown in liquid medium supplemented with different concentrations of Na^+ or Li^+

Ion concn in medium	Genotype[a]	Ion concn in cells (mM)[b]		
		K^+	Na^+	Li^+
0.5 M Na^+	*ENA1-4*	328 ± 25	90 ± 9	
	ENA1-4 nhaA	318 ± 22	113 ± 13	
50 mM Li^+	*ENA1-4*	329 ± 8		7.7 ± 0.5
	ENA1-4 nhaA	268 ± 8		24 ± 2
0.3 M Na^+	*ena1-4*	189 + 21	402 + 39	
	ena1-4 nhaA	222 ± 19	350 ± 15	
2 mM Li^+	*ena1-4*	406 ± 42		13.0 ± 1.8
	ena1-4 nhaA	311 ± 22		3.9 ± 0.6

[a] Strains used were RS-997 (*ENA1-4*), RS-1045 (*ENA1-4 nhaA*), RS-1043 (*ena1-4*), and RS-1041 (*ena1-4 nhaA*).

[b] Results are means ± standard errors of results from at least three independent experiments.

Transmission electron microscopy and selective permeabilization of the yeast plasma membrane have been used to demonstrate that a large fraction of cellular cations are compartmentalized into the vacuole (16, 27). The operation of NhaA as transporter of toxic cations into the vacuole was a plausible explanation for the discrepancy between salt tolerance and total concentration of intracellular cations. Growth inhibition by toxic cations depends on their cytoplasmic concentrations (31) and their accumulation into the vacuole may distort measurements of total intracellular cations.

The subcellular distribution of NhaA was investigated by equilibrium sucrose gradient centrifugation (Fig. 3). In these gradients the plasma membrane (identified by the anti-Pma1p immunological marker) equilibrates at the highest density and internal membranes, including the vacuole, equilibrate at intermediate densities (38). A dual localization of NhaA was apparent, with approximately 30% of the protein at the plasma membrane and 70% in internal membranes. These results were compatible with the operation of NhaA at the vacuolar compartment. Unfortunately, the available antibodies against NhaA do not recognize the protein in formaldehyde-fixed cells utilized for immunofluorescence localization (38a).

In order to further investigate the activity of NhaA in intra-

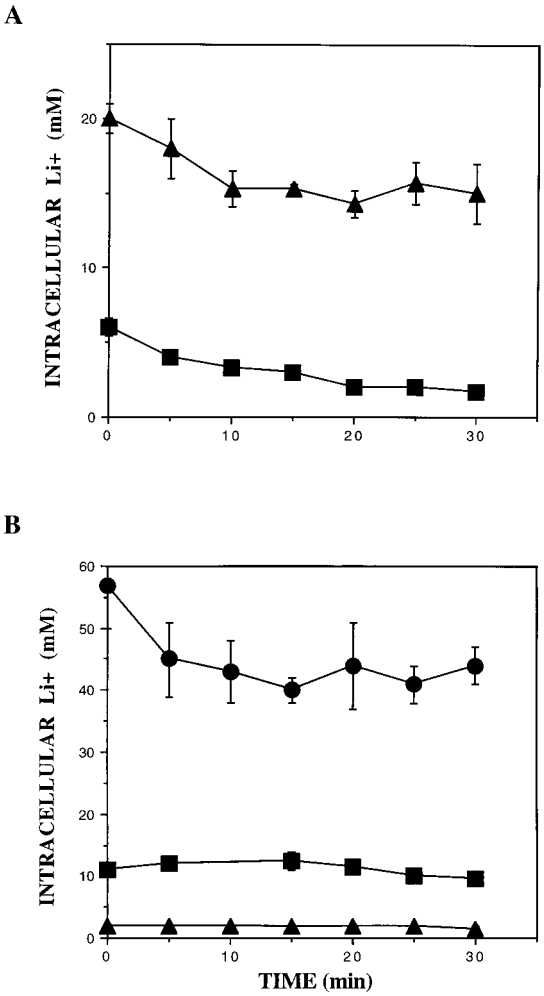

FIG. 4. Effect of the expression of NhaA on lithium accumulation and efflux kinetics. (A) Cells from strain RS-1045 (*ENA1-4* NhaA) (triangles) and RS-997 (*ENA1-4*, control) (squares) were grown on medium with 50 mM LiCl and washed, and lithium efflux was measured as described in Materials and Methods. (B) Cells from strain RS-1041 (*ena1-4* NhaA) (triangles) and RS-1043 (*ena1-4*, control) (squares) were grown on medium with 2 mM LiCl and washed, and lithium efflux was measured as described in Materials and Methods. Circles correspond to strain RS-1041 grown on medium with 50 mM LiCl. Values are averages from three experiments and the error bars indicate standard deviations.

cellular compartments, we performed efflux studies using control and NhaA-expressing cells. Efflux kinetics may differentiate between a fast-efflux cytoplasmic pool and a slow-efflux vacuolar compartment (39). As indicated in Fig. 4A, most of the intracellular lithium in control *ENA1-4* yeast cells grown with 50 mm LiCl exits from the cells in 20 min. Expression of NhaA resulted in a greater level of intracellular lithium, with most of it constituting a slow-efflux pool. These results are compatible with an NhaA-mediated accumulation of lithium within the yeast vacuole. In *ena1-4* cells grown with 2 mM LiCl no lithium efflux was observed and expression of NhaA greatly reduced lithium accumulation (Fig. 4B). After growth with 50 mM LiCl, *ena1-4* cells expressing NhaA exhibited two kinetic pools of intracellular lithium; a small fraction effluxed within 20 min but most of the intracellular lithium constituted a slow-efflux pool. Control *ena1-4* cells without NhaA cannot grow with 50 mM LiCl.

The role of the yeast vacuole in cation accumulation mediated by NhaA was investigated by testing the effect of mutations in the vacuolar H^+ ATPase. The vacuolar proton pump should be necessary to energize cation transport mediated by a cation/H^+ antiporter, (16, 30). As indicated in Fig. 5, a null mutation in the catalytic subunit of the vacuolar H^+ ATPase dramatically decreased intracellular accumulation of lithium in NhaA-expressing cells. Therefore, the NhaA antiporter seems to transport lithium into the vacuolar compartment.

The salt tolerance phenotypes conferred by expression of NhaA were reinvestigated in the mutant without functional vacuolar H^+ ATPase. As indicated in Fig. 6, the lithium tolerance conferred by NhaA in *ENA1-4* yeast cells was dependent on a functional vacuolar H^+ ATPase. Therefore, this tolerance is explained by lithium accumulation at the energized vacuole mediated by the Li^+/H^+ exchange activity of NhaA. Expression of NhaA produced no sodium tolerance in *ENA1-4* yeast cells, independently of the presence of a functional vacuolar H^+ ATPase. In the *ena1-4 tfp1* double mutant, devoid of both plasma membrane (Na^+, Li^+) ATPase and vacuolar H^+ ATPase, the expression of NhaA produced a clear tolerance to both lithium and sodium (Fig. 6). As described above, in the *ena1-4* mutant with functional vacuolar H^+ ATPase, NhaA conferred lithium tolerance but sodium sensitivity.

DISCUSSION

It is remarkable that despite large differences in the mechanisms of membrane biogenesis between bacteria and eukaryotic cells (28), we could achieve the functional expression of an integral membrane protein from *E. coli*, the (Na^+, Li^+/H^+) antiporter NhaA, in the yeast *S. cerevisiae*. The *E. coli* glycerol channel GlpF can partially substitute for the yeast glycerol channel Fps1 (20) but we are not aware of previous reports on the functional expression in yeast of bacterial ion transporters.

The bacterial NhaA antiporter seems to be active at both the plasma membrane and the vacuolar membrane of the recipient yeast cells. Vacuolar membranes have been proposed as the default destination for membrane proteins in yeast (35). In yeast containing a plasma membrane (Na^+, Li^+) ATPase, the

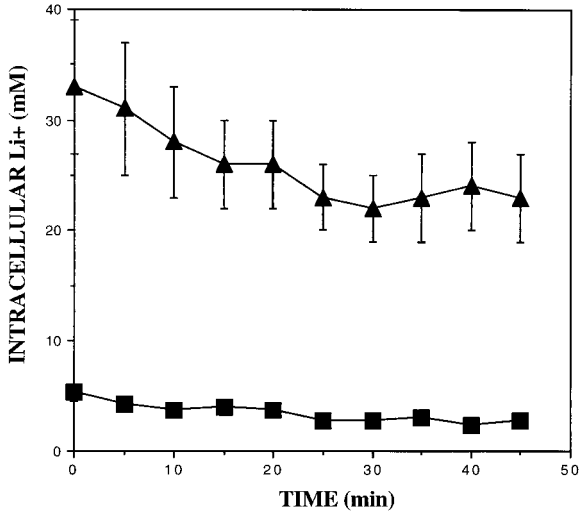

FIG. 5. Effect of a functional vacuolar H^+ ATPase on lithium accumulation and efflux kinetics. Cells from strains RS-1178 (*ENA1-4 tfp1 nhaA*) (squares) and RS-1176 (*ENA1-4 TFP1 nhaA*) (triangles) were grown on medium with 50 mM LiCl and washed, and lithium efflux was measured as described in Materials and Methods. Values are averages from three experiments and the error bars indicate standard deviations.

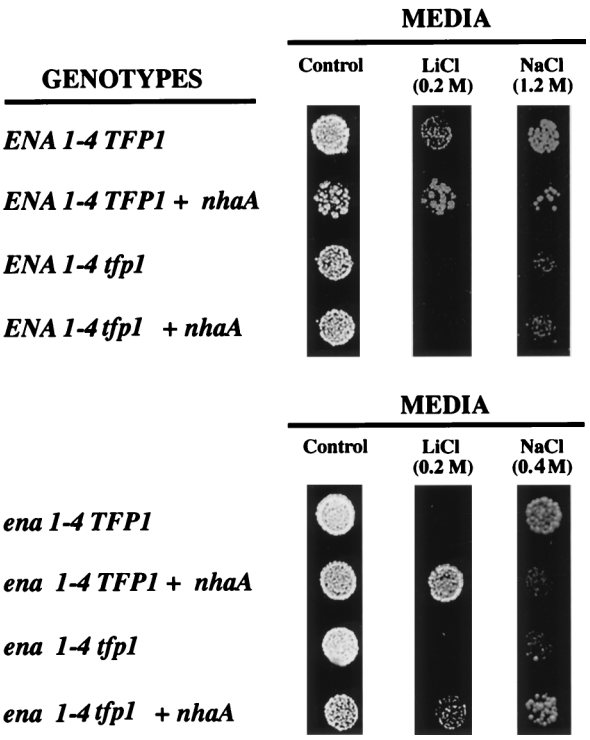

FIG. 6. Lithium and sodium tolerance in yeast without functional vacuolar H^+ ATPase. Strains RS-1175 (*ENA1-4 TFP1*), RS-1176 (*ENA1-4 TFP1 nhaA*), RS-1177 (*ENA1-4 tfp1*), and RS-1178 (*ENA1-4 tfp1 nhaA*) (top) and strains RS-1170 (*ena1-4 TFP1*), RS-1171 (*ena1-4 TFP1 nhaA*), RS-1179 (*ena1-4 tfp1*), and RS-1180 (*ena1-4 tfp1 nhaA*) (bottom) were tested for salt tolerance as described in Materials and Methods. Plates contained either no salt (control) or 0.2 M LiCl, 0.4 M NaCl, or 1.2 M NaCl as indicated. Similar results were obtained in three independent experiments.

lithium tolerance conferred by expression of NhaA depends on a functional vacuolar H^+ ATPase and is correlated with increased lithium accumulation into a compartment of slow efflux. The most plausible explanation for these results is that lithium transport at the plasma membrane mediated by the *ENA1-4* ATPase is very active and cannot be improved by expression of NhaA. On the other hand, lithium accumulation at the yeast vacuole (probably mediated by the *NHX1* antiporter [23]) is limiting for lithium tolerance, and such accumulation can be improved by a heterologous antiporter such as NhaA.

The lithium tolerance conferred by NhaA in the *ena1-4* mutant correlates with reduced levels of intracellular lithium and is still observed in the double mutant *ena1-4 tfp1*, which is devoid of both plasma membrane (Na^+, Li^+) ATPase and vacuolar H^+ ATPase. These results suggest that NhaA is also active at the yeast plasma membrane and that it can substitute for the (Na^+, Li^+) ATPase. It has recently been reported that sod2, the plasma membrane Na^+/H^+ antiporter of the yeast *Schizosaccharomyces pombe*, can also substitute for the *S. cerevisiae* (Na^+, Li^+) ATPase (10). It remains a puzzle why *S. cerevisiae* has an (Na^+, Li^+) ATPase as a major cation efflux system while bacteria and other yeasts such as *S. pombe* employ an antiporter mechanism.

Despite the clear phenotype of lithium tolerance conferred by NhaA, this bacterial antiporter could not increase the sodium tolerance of yeast. This was difficult to explain because sodium transport is limiting for sodium tolerance in *S. cerevisiae* (31) and the purified NhaA protein can transport both sodium and lithium (25). Interestingly, NhaA conferred sodium tolerance to *S. cerevisiae* mutants devoid of both plasma membrane (Na^+, Li^+) ATPase and vacuolar H^+ ATPase. Also, in these double mutants we could detect sodium efflux mediated by NhaA (data not shown). Apparently, the activity of NhaA in energized vacuolar membranes is deleterious for the cell in the presence of sodium but not lithium. We have no clues about the mechanisms involved but this phenomenon points to important differences between lithium and sodium homeostasis in the vacuolar compartment. As this compartment includes not only the large vacuole but also the Golgi apparatus and intermediary vesicles, the toxic effects of increased sodium accumulation may occur at specific subcompartments and not necessarily at the large central vacuole. The potassium dependency of some membrane assembly and secretory functions (26) may provide targets for sodium toxicity in the vacuolar compartment.

The sod2 antiporter from *Schizosaccharomyces pombe* can be functionally expressed in *S. cerevisiae* and it gives an additive tolerance phenotype to both sodium and lithium (10). One possible explanation for the different results in terms of sodium tolerance obtained with NhaA and sod2 is that the latter antiporter could be confined to the plasma membrane and be absent from the vacuolar compartment, where sodium accumulation may be deleterious. The fact that sod2 is electroneutral (10) while NhaA is electrogenic (25) could also contribute to the observed phenotypic differences.

The manipulation of sodium transport in transgenic fungi and plants could be a useful approach to improve salt tolerance in these organisms (31). However, the present results with the yeast model system and the bacterial NhaA antiporter suggest that a better understanding of intracellular ion homeostasis is needed before this manipulation can produce useful results. In particular, the effect of sodium accumulation in the vacuolar compartment should be further investigated. Transgenic plants with a reduced level of vacuolar H^+ ATPase have been generated by antisense methodologies (7), and they could be useful in characterizing the role of the plant vacuole in the phenotypes conferred by heterologous sodium transporters. In addition, strategies for the targeting of heterologous sodium transporters to either the plasma membrane or the vacuolar membrane should be developed. Fusions with vacuolar pyrophosphatase have been employed for the targeting of soluble proteins to the plant vacuolar membrane (18), and this approach could also be applied in the case of heterologous membrane proteins. The observation that the heterologous expression of ion transporters perturbs the yeast secretory pathway (22, 38) adds further complications to the genetic engineering of ion homeostasis in eukaryotic cells.

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