Functional expression of the *Schizosaccharomyces pombe* Na⁺/H⁺ antiporter gene, *sod2*, in *Saccharomyces cerevisiae*

KAREN M. HAHNENBERGER*†, ZHENGPING JIA‡, AND PAUL G. YOUNG§¶

*Molecular Devices Corporation, Sunnyvale, CA 94089; ‡Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, Toronto, ON M5G 1X5, Canada; and §Departments of Biology and Pathology, Queen's University, Kingston, ON K7L 3N6, Canada

ABSTRACT   In the fission yeast, *Schizosaccharomyces pombe*, tolerance to high sodium and lithium concentrations requires the functioning of the sod2, Na⁺/H⁺ antiporter. We have directly measured the activity of this antiporter and demonstrated reconstitution of the activity in gene deletion strains. In addition, we have shown that it can be transferred to, and its antiporter activity detected in, the budding yeast, *Saccharomyces cerevisiae*, where it also confers sodium and lithium tolerance. Proton flux through the *S. pombe* Na⁺/H⁺ antiporter was directly measured using microphysiometry. The direction of transmembrane proton flux mediated by this antiporter was reversible, with protons being imported or exported in response to the external concentration of sodium. This bidirectional activity was also detected in *S. cerevisiae* strains expressing *sod2* and expression of this gene complemented the sodium and lithium sensitivity resulting from inactivation of the *ENA1/PMR2* encoded Na⁺-exporting AT-Pases. This suggests that antiporters or sodium pumps can be utilized interchangeably by *S. cerevisiae* to regulate internal sodium concentration. Potent inhibitors of mammalian Na⁺/H⁺ exchangers were found to have no effect on sod2 activity. The proton flux mediated by sod2 was also found to be unaffected by perturbation of membrane potential or the plasma membrane proton gradient.

Maintaining low intracellular sodium concentrations is a problem common to almost all cell types because high internal sodium is generally toxic. In animal cells, the Na⁺,K⁺-ATPase directly exports sodium and establishes an inwardly directed sodium gradient to drive secondary transport systems. This enzyme is not present in plants and fungi and instead a primary proton gradient is established by the H⁺-ATPase. Secondary transport systems use the energy in the proton gradient to carry nutrients into the cell. Sodium is removed by specialized export systems (1, 2).

Recently the export mechanism for intracellular sodium in fission and budding yeasts has been described and rather surprisingly two quite distinct mechanisms have evolved. In *Schizosaccharomyces pombe* tolerance to external sodium is dependent upon the *sod2* gene, which encodes a protein with homology to bacterial and mammalian Na⁺/H⁺ antiporters (3). Na⁺ or Li⁺ export from these cells is via this carrier and dependent upon the proton gradient established by the H⁺-ATPase. Deletion of the gene results in Na⁺ and Li⁺ hypersensitivity. In *Saccharomyces cerevisiae*, a family of genes encoding Na⁺-ATPases is responsible for sodium efflux (1, 4). Disruption of these P-type sodium pumps in *S. cerevisiae* results in a phenotype very similar to disruption of *sod2* in *S. pombe*; the cells become hypersensitive to Na⁺ and Li⁺ and cannot export these ions from the cell. Overexpression of the gene causes an increase in Na⁺ and Li⁺ tolerance. To date, no

antiporter activity has been detected in budding yeast and no Na⁺-ATPase in fission yeast.

We have performed an analysis of Na⁺/H⁺ antiporter activity in *S. pombe* by measuring the rate of transmembrane proton flux in wild-type and *sod2* mutant strains. A pH sensing silicon chip based microphysiometer was used to directly monitor the rate of acid excretion by the cells (5, 6). This allowed us to easily examine the activity of the antiporter in the fission yeast as well as to detect the functional expression of the gene following heterologous expression in *S. cerevisiae*. The activity of the *sod2* antiporter is reversible and the direction of proton movement is controlled by the relative extracellular concentrations of Na⁺ and H⁺. Overexpression studies demonstrate directly that the increased flux on this carrier correlates with increased Na⁺ and Li⁺ tolerance. In addition, we show that expression of the *sod2* antiporter in a strain of *S. cerevisiae* lacking the sodium exporting Na⁺-ATPases results in restoration of sodium tolerance coupled with directly measurable bidirectional movement of protons in response to sodium gradients. These results, as well as recent data demonstrating complementation of an *S. pombe sod2* null mutation by expression of the *PMR2/ENA1* Na⁺-ATPase gene from *S. cerevisiae* (7), show that either of these very different mechanisms for regulating intracellular sodium concentration are sufficient to provide sodium or lithium tolerance to these cells. These effects are ion specific and quite independent of the parallel osmotic adjustment that a cell must make in dealing with high concentrations of external solutes of any kind (8, 9).

MATERIALS AND METHODS

Strains, Media, and Construction of Plasmids. *S. pombe* and *S. cerevisiae* strains were grown at 30°C in SD medium (10) containing appropriate nutritional supplements. For expression of *sod2* in *S. pombe*, strain *sod2::ura4* (3), an *NdeI/BamHI* fragment containing the coding region but lacking the intron sequence was cloned under control of the cauliflower mosaic virus 35S promoter (11) in vector p35S resulting in plasmid p35S-*sod2*. p35S is a derivative of pREP3 (12) in which the nmt1 promoter and transcription termination sequences have been replaced with the cauliflower mosiac virus 35S promoter and transcription termination sequences. For expression of *sod2* in *S. cerevisiae*, the *NdeI/BamHI* fragment was cloned under transcriptional control of the promoter for phosphoglycerate kinase in plasmid A241 (13) resulting in plasmid A241-*sod2*. Plasmids were introduced into *S. pombe* and *S. cerevisiae* using the alkali cation transformation method in kit form (Bio 101).

Abbreviations: DNP, 2,4-dinitrophenyl; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TMA, tetramethylammonium; DES, diethylstilbestrol.
¶To whom reprint requests should be addressed at: Department of Biology, R.432 Earl Hall, Queen's University, Kingston, ON K7L 3N6, Canada.
†Present address: Bioscience Research and Development, Hewlett–Packard Co., 3500 Deer Creek Road, Palo Alto, CA 94304.

Growth Assays. The *sod2* gene was expressed in *S. cerevisiae* strain RH16.6 (*ena1D::LEU2::ena4D*), which lacks the P-type ion transporting ATPases *ENA1*, *ENA2*, *ENA3*, and *ENA4* and that is defective for sodium and lithium export (1, 4). Cultures of strain RH16.6 transformed with A241 or A241-*sod2* were grown in minimal medium lacking uracil in the presence or absence of various concentrations of LiCl, sorbitol, or KCl. At various time points, aliquots of the cultures were removed and growth measured by optical density at 660 nm.

Microphysiometer Analysis of Na^+/H^+ Antiporter Activity. Na^+/H^+ antiporter activity was measured with a Cytosensor microphysiometer (Molecular Devices). This instrument measures the extracellular acidification rate of cells immobilized in a microvolume flow chamber in contact with a pH sensitive silicon sensor (5, 14). Approximately 2.5×10^6 yeast cells were immobilized in Agarose Cell Entrapment Medium (Molecular Devices) between two microporous membranes. Flow chambers at 30°C containing the immobilized cells were perfused at a rate of 100 μl/min through the 3 μl chamber with a buffer solution at pH 6.3 composed of 10 mM Hepes and tetramethylammonium (TMA) chloride at either 150 mM or 300 mM. In some experiments, the buffer solution also contained 0.1% BSA. Acidification rates were measured every 100 s by interrupting the flow of buffer for 10 s. After stable acidification rates were achieved, a valve switch was done so that the chambers were perfused with the same buffer but with an equal concentration of NaCl substituted for the TMA. After recovery of the extracellular acidification rates to a new baseline, the buffer perfusing the chambers was switched back to the original solution containing TMA in place of NaCl. Extracellular acidification rates are expressed as a percentage of the basal rates before exposure to NaCl. Negative values indicate alkalinization rather than acidification of the extracellular environment.

Effects of known inhibitors of mammalian Na^+/H^+ antiports (amiloride and derviatives) and compounds that collapse the plasma membrane proton gradient [2,4-dinitrophenyl (DNP) and carbonylcyanide *m*-chlorophenylhydrazone (CCCP)] or inhibit the H^+-ATPase diethylstilbestrol (DES) on

antiporter activity were determined by addition of these compounds to the buffer perfusing the flow chambers for 15–20 min before and then during the exposure to NaCl. All microphysiometer experiments were repeated at least twice and representative data are shown.

RESULTS

Bidirectional Activity of the *S. pombe* Na^+/H^+ Antiporter Encoded by *sod2*. Previous studies of *sod2* activity in *S. pombe* demonstrated that cells preloaded with Na^+ import protons as Na^+ is exported, but were not able to show H^+ export in response to Na^+ uptake (3). In the present study, microphysiometry (5) has been used to sensitively measure changes in transmembrane proton flux in *S. pombe* in response to changes in the external concentration of sodium.

Activity of the Na^+/H^+ antiporter in *S. pombe* was detected by incubating cells in buffer containing the sodium substitute TMA and then exposing them to buffer with sodium chloride replacing the TMA. To measure the relatively small transmembrane proton flux due to the Na^+/H^+ antiporter above the proton pumping activity of the plasma membrane H^+-ATPase, pma1 (2, 15), the cells were incubated in Hepes buffer at pH 6.3 containing TMA but lacking glucose. Activity of the H^+-ATPase is very low under these conditions and extracellular acidification rates are close to zero.

Exposure of wild-type strain Sp223 (*ade6-216, leu1-32, ura4-294*) and mutant strains *sod2-1* and *sod2::ura4* (3) to 300 mM NaCl is shown in Fig. 1. Shifting the wild-type strain from 300 mM TMA to 300 mM NaCl resulted in an increase in acidification rate, indicating export of protons as sodium is taken up by the cells. This increase was transient, with the acidification rate returning to baseline after approximately 10 min. Upon removal of the NaCl and a return to TMA, the cells showed a transient extracellular alkalinization, indicating uptake of protons as sodium was exported. Strain *sod2-1* contains a genomic amplification of the *sod2* locus with a copy number of approximately 20 per genome (3). This strain, which overexpresses the Na^+/H^+ exchanger, exhibited a much larger increase in acid-
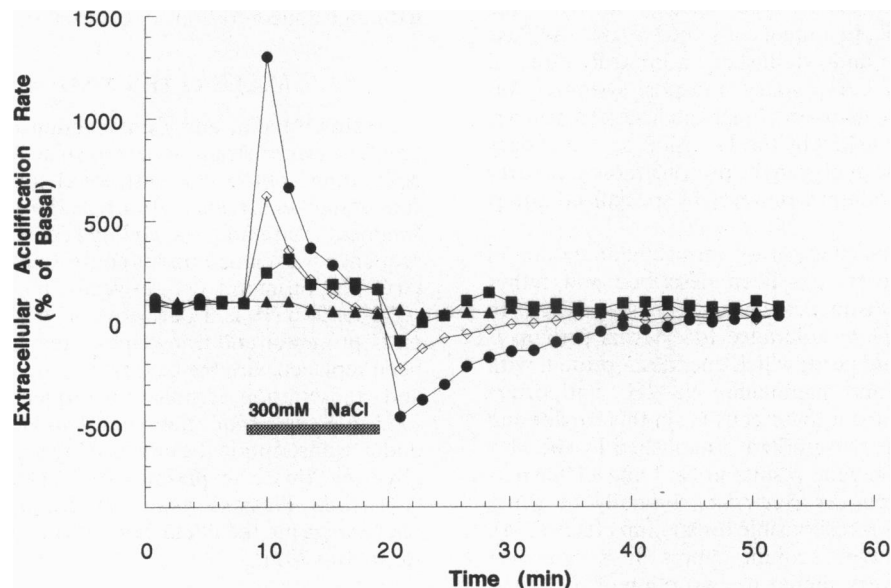

FIG. 1.   Microphysiometry demonstrates bidirectionality of the *S. pombe* antiporter in response to sodium concentration gradients. Sensor chambers contained equal numbers of cells of wild-type strain Sp223 (squares), *sod2-1* (circles), *sod2::ura4* (triangles), or *sod2::ura4* cells in which the *sod2* gene was overexpressed on plasmid p35S-sod2 (diamonds). Chambers were perfused with 10 mM Hepes, 0.1% BSA, and 300 mM TMA chloride at pH 6.3. When stable acidification rates were obtained, a valve switch was performed so that the cell chambers were exposed to the same buffer solution containing 300 mM NaCl in place of TMA. After approximately 12 min, a second valve switch was performed so that the chambers were again perfused with the TMA-containing buffer. Normalized acidification rates are plotted versus time (in minutes) and the period of exposure to the NaCl containing-buffer is indicated by the bar.
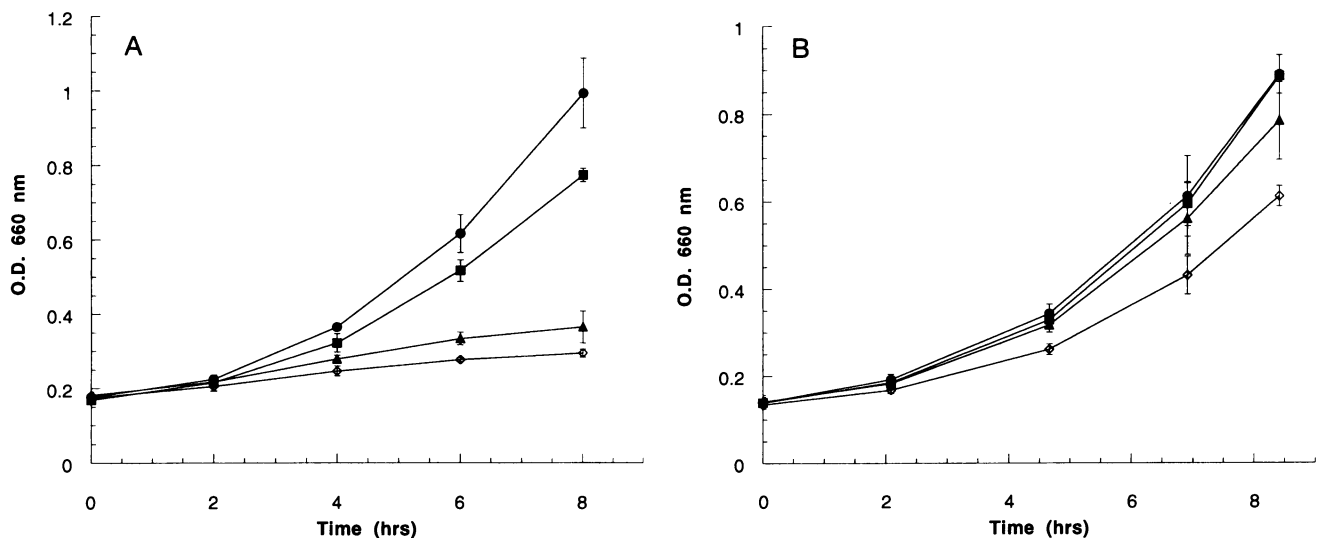
Cell Biology: Hahnenberger *et al.*

Proc. Natl. Acad. Sci. USA 93 (1996)   5033

FIG. 2.   Functional expression of *S. pombe sod2* in *S. cerevisiae*. Cultures of RH16.6 transformed with the expression vector A241 (*A*) or A241-*sod2* (*B*) were grown in minimal medium containing various concentrations of LiCl. At various times, aliquots of the cultures were removed and the optical density (O.D.) at 660 nm was determined. The average optical density and range from two experiments are plotted versus time (in hours). LiCl concentrations were as follows: no LiCl (circles), 10 mM LiCl (squares), 50 mM LiCl (triangles), and 100 mM LiCl (diamonds).

ification rate upon exposure to 300 mM NaCl than did the wild-type strain. Similarly, upon removal of the NaCl, *sod2-1* showed a transient alkalinization of the extracellular environment that was significantly higher than the alkalinization exhibited by the wild-type strain. Strain *sod2::ura4* contains a disruption of the *sod2* gene and displays hypersensitivity to NaCl and ·an inability to export sodium (3). Analysis of *sod2::ura4* by microphysiometry showed no change in the rate of extracellular acidification in response to either addition or withdrawal of NaCl, indicating that the changes in acidification rates observed for the wild-type strain and the *sod2* overexpressor are due to activity of the Na$^+$/H$^+$ antiporter.

To confirm that the changes in proton flux detected by microphysiometry were a result of the bidirectional activity of the Na$^+$/H$^+$ exchanger encoded by *sod2*, the *sod2* gene was cloned under control of the cauliflower mosaic virus ^{35}S promoter and transcription termination sequences. The resulting plasmid, p^{35}S-*sod2*, was transformed into the disruption mutant strain, *sod2::ura4*. Several of the transformants were analyzed by microphysiometry and all showed a dramatic increase in acidification rate upon exposure to 300 mM NaCl and a corresponding alkalinization upon its removal (Fig. 1), similar to that observed for the overexpressing strain *sod2-1*. In contrast, *sod2::ura4* transformed with the vector alone exhibited no change in proton flux in response to the external concentration of sodium (data not shown). This provides further confirmation that the overexpression of the *sod2* Na$^+$/H$^+$ antiporter is sufficient to confer antiport activity above the level seen in wild-type cells.

Functional Expression of *sod2* in *S. cerevisiae*. In *S. pombe* it has been demonstrated that the Na$^+$/H$^+$ exchanger encoded by *sod2* provides the sole mechanism for export of sodium from the cell (ref. 3; and above). In *S. cerevisiae*, however, sodium export is accomplished by a family of cation transporting P-type ATPases encoded by a tandem array of genes alternatively called *ENA1* (4, 16) or *PMR2* (17, 18). The *S. pombe sod2* gene was cloned under the control of the constitutive promoter for phosphoglycerate kinase and introduced into *S. cerevisiae* strain RH16.6, which contains a deletion of the *ENA1-ENA4* genes. RH16.6 lacks any sodium or lithium efflux activity and exhibits increased sensitivity to these ions (4).

Because *sod2* is necessary for growth in the presence of Na$^+$ or Li$^+$ in *S. pombe* (3), the function of *sod2* in strain RH16.6 in lithium tolerance was assessed by measuring growth rates of

transformants in the presence of increasing concentrations of lithium. Transformants containing the empty vector A241 exhibited a decrease in growth rate at concentrations of lithium as low as 10 mM, while culturing of these cells in media containing LiCl at 50 mM or 100 mM led to an almost complete inhibition of growth (Fig. 2*A*). In contrast, strain RH16.6 transformed with the *sod2* expression plasmid, A241-*sod2*, showed a greatly enhanced resistance to lithium. With 10 mM LiCl present in the medium, no effect on growth rate was observed and, at a concentration of 100 mM, only a slight decrease in growth rate resulted (Fig. 2*B*). Growth assays showed that the RH16.6 transformants expressing *sod2* also displayed enhanced resistance to sodium, and that even in a wild-type *S. cerevisiae* strain, expression of *sod2* resulted in an increase in resistance to both lithium and sodium (Table 1).

In *S. cerevisiae*, an increase in the osmolarity of the external environment induces an osmosensitive signal transduction pathway mediated by the *HOG1* (*sty1* in *S. pombe*) protein kinase (8, 19). To determine if the increased resistance to lithium and sodium exhibited by strain RH16.6 expressing *sod2* was due to an increased response to high osmolarity, growth rates of the transformants were measured in the presence of various concentrations of sorbitol or KCl. It was found that increasing concentrations of both sorbitol and KCl inhibited the growth rates of transformants containing the vector and the *sod2* expression plasmid to a similar extent. This indicates

Table 1.   Expression of *sod2* in *S. cerevisiae* results in enhanced resistance to sodium and lithium

Strain	Minimum inhibitory concentration, mM	
	Na+	Li+
RH16.6/A241	500	15
RH16.6/A241-*sod2*	2000	500
INVSC2/A241	1000	250
INVSC2/A241-*sod2*	2000	1000

Approximately 10^4 cells of strain RH16.6 (*ena1 ena2*) or INVSC2 (wild type) containing the expression plasmid A241 or A241 containing the *sod2* gene were aliquoted into microtiter wells in minimal medium containing serial dilutions of NaCl or LiCl. Microtiter plates were incubated at 30°C and growth in the wells was scored after 48 hr.
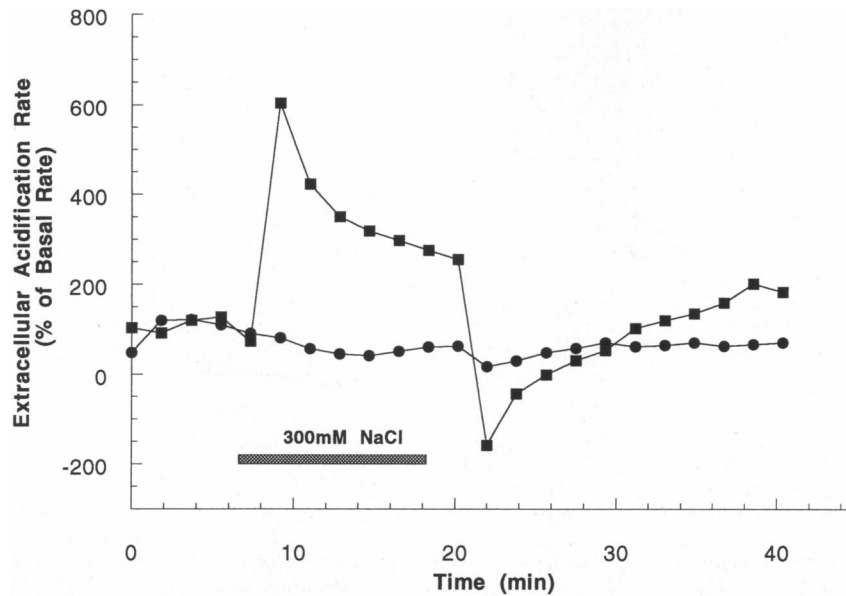
FIG. 3. Bidirectional activity of *sod2* expressed in *S. cerevisiae*. Sensor chambers containing equal numbers of cells of RH16.6 transformed with A241 (circles) or with A241-*sod2* (squares) were alternately perfused with 10 mM Hepes and 300 mM TMA at pH 6.3 and the same buffer containing 300 mM NaCl in place of TMA. Normalized extracellular acidification rates are plotted versus time (in minutes) and the period of exposure to NaCl is indicated by the bar.

that expression of the *S. pombe* Na$^+$/H$^+$ antiporter in a strain of *S. cerevisiae* lacking the Na$^+$-ATPase gene family restores lithium and sodium efflux activity without affecting the response to osmotic stress.

Functional expression of the *sod2* gene expressed in RH16.6 was also directly measured using microphysiometry. Exposure of cells to 300 mM NaCl resulted in the characteristic increase in the rate of proton export whereas withdrawal of the sodium was accompanied by a transient alkalinization of the external environment (Fig. 3). As a control, cells of strain RH16.6 transformed with the vector A241 showed no change in proton flux in response to the external concentration of sodium. In numerous experiments, the equilibration time following so-

dium addition was characteristically of longer duration than that seen in *S. pombe* overexpressing *sod2*.

Effect of Amiloride on *sod2* activity in *S. pombe*. Amiloride and derivative compounds are potent inhibitors of the mammalian Na$^+$/H$^+$ exchanger encoded by the *NHE1* gene (20) and are believed to exert their effect through competition for the external sodium binding site. The effect of these compounds on the activity of the *S. pombe* Na$^+$/H$^+$ exchanger was determined using microphysiometry. Cells of the overexpressing strain, *sod2-1*, were exposed to 300 mM NaCl in the presence of 100 μM amiloride, 5-(*N*-methyl-*N*-isobutyl)-amiloride (MIA), or 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) (Fig. 4). The increase in acidification rates upon
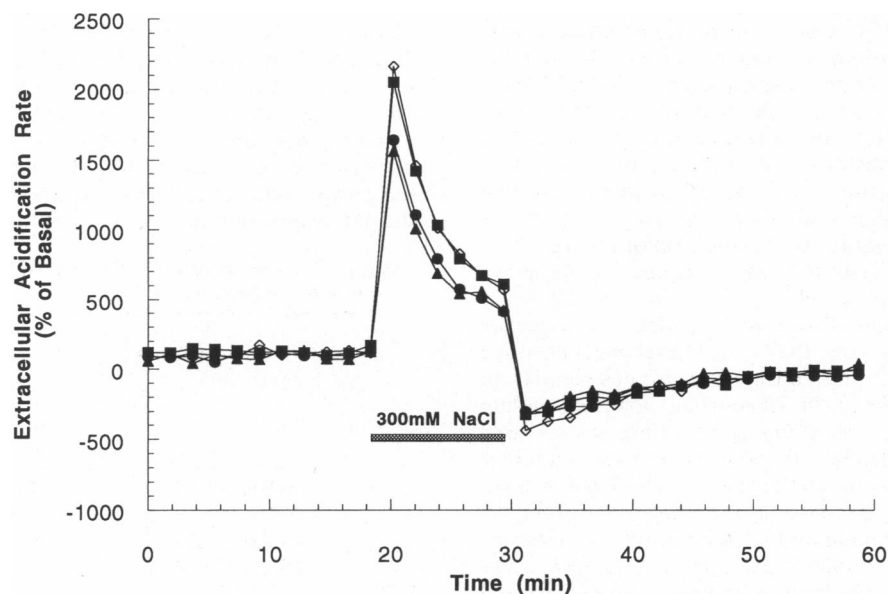

FIG. 4. Activity of the *S. pombe* antiporter is not inhibited by amiloride and derivatives. Chambers containing *sod2-1* cells were perfused with 10 mM Hepes, 0.1% BSA, and 300 mM TMA at pH 6.3 in the absence (circles) or presence of 100 μM MIA (squares), 100 μM EIPA (triangles), or amiloride (diamonds). Cells were then exposed to the same buffer containing 300 mM NaCl in place of TMA. After a 12-min exposure to NaCl (indicated by the bar) the cells were exposed to the TMA-containing buffer. Normalized acidification rates are plotted versus time in minutes.

exposure to NaCl and the corresponding alkalinization upon removal of sodium did not differ between the untreated control cells and those treated with amiloride, MIA or EIPA. This is in marked contrast to results obtained with Chinese hamster ovary-K1 cells in the microphysiometer, showing that the large pulse of extracellular acidification observed upon exposure of Chinese hamster ovary cells to 130 mM NaCl is almost completely abolished by 100 μM amiloride (5). These results show that, unlike mammalian Na^+/H^+ exchangers, the activity of the *S. pombe* Na^+/H^+ antiporter is not inhibited by amiloride or its derivatives.

Effect of Disruption of the Plasma Membrane Proton Gradient on *sod2* Activity. Cells of strain *sod2-1* were exposed to 300 mM NaCl on the microphysiometer in the presence of 50 μM CCCP, 1 mM DNP, or 100 μM DES (Fig. 5). CCCP and DNP would be expected to collapse the proton gradient and membrane potential, whereas DES is an inhibitor of the plasma membrane H^+-ATPase. No effect on proton flux measured in the microphysiometer was observed with any of these compounds. This suggests that membrane potential does not affect the activity of the *S. pombe* Na^+/H^+ antiporter. It would also suggest that perturbation of the transmembrane proton gradient does not have an effect on *sod2* activity under these conditions. However, in the microphysiometer analysis, no carbon source was present and the pH of the buffer was very close to intracellular pH. Under these conditions the proton gradient would be expected to be minimal, so that agents that disrupt it might not be expected to have any effect in the presence of the high Na^+ gradient (300 mM NaCl).

DISCUSSION

Na^+/H^+ antiporters exchange Na^+ and H^+ ions, with the direction of exchange being determined by the gradients of the two ions across the cell membrane. In mammalian cells, the predominant function of the Na^+/H^+ exchanger is to regulate intracellular pH by transporting protons out of the cell in exchange for sodium (21). In contrast, the *S. pombe* Na^+/H^+ antiporter encoded by *sod2* functions to regulate the internal concentration of sodium, rather than protons, and previous

studies have measured the influx of protons as sodium is exported from cells expressing this antiporter (3). In this study, the technique of microphysiometry has been used to measure the rate of transmembrane proton movement in *S. pombe* mediated by the *sod2*-encoded antiporter. The microphysiometer analysis demonstrated that the activity of this antiporter is bidirectional, importing or exporting protons in response to the external or internal concentration of sodium. The level of expression of *sod2* correlated directly with the magnitude of the change in proton flux in response to changes in external sodium concentration; *sod2-1* exhibited dramatic changes in the rates of extracellular acidification and alkalinization whereas the changes were much smaller for a wild-type strain. No changes in proton flux were observed for the null mutant, *sod2::ura4*.

The mechanism for sodium efflux in *S. cerevisiae* is through the activity of a family of P-type ATPases (4) displaying some differential activity toward Na^+ or Li^+ (18). Inactivation of the genes encoding these Na^+-ATPases results in hypersensitivity to Li^+ and Na^+, similar to the phenotype seen in *S. pombe* strains in which *sod2* has been disrupted (3). Recently, it has been shown that the *ENA1* gene from *S. cerevisiae*, which encodes one of these sodium pumps, complements the sodium efflux defect in the *S. pombe sod2* disruption mutant (7). This study demonstrates that expression of *sod2* in an *S. cerevisiae* strain lacking any Na^+-ATPase activity (RH16.6), restores the ability of these cells to export sodium and greatly increases their resistance to both Na^+ and Li^+ in the medium. As measured by microphysiometry, these *S. cerevisiae* transformants exhibit changes in bidirectional transmembrane proton flux in response to sodium identical to those observed for *S. pombe*. Under growth conditions where glucose is present, the Pma1 H^+-ATPase generates a strong inwardly directed proton gradient and this ensures that sodium movement is outward and that sodium cannot leak into the cell via this carrier.

It was found that expression of *sod2* in a wild-type strain of *S. cerevisiae* also enhanced the tolerance for Na^+ to the same extent as in RH16.6, and it enhanced Li^+ resistance to a level 2-fold higher than for RH16.6. This is in contrast to results obtained for expression of *PMR2/ENA1* in *S. pombe*, which
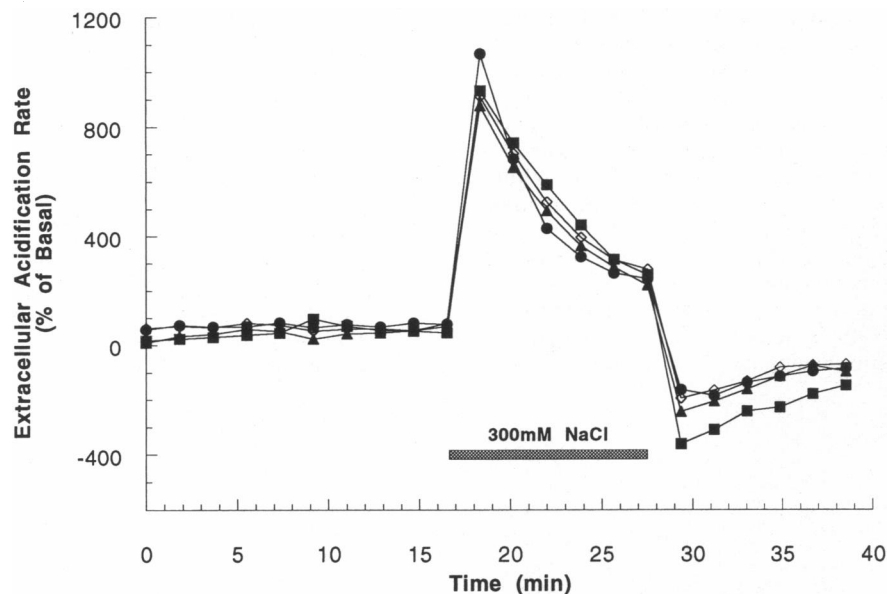
FIG. 5. *sod2* activity is not affected by compounds which collapse the transmembrane proton gradient or an inhibitor of the plasma membrane H^+-ATPase. Chambers containing *sod2-1* cells were perfused with 10 mM Hepes, 0.1% BSA, and 300 mM TMA at pH 6.3 in the absence (circles) or presence (squares) of 50 μM CCCP, 1 mM DNP (triangles), or 100 μM DES (diamonds). Cells were then exposed to the same buffer containing 300 mM NaCl in place of TMA. After a 12-min exposure to NaCl (indicated by the bar) the cells were exposed to the TMA-containing buffer. Normalized acidification rates are plotted versus time in minutes.

indicated that expression of the Na^+-ATPase in a wild-type strain is not as effective and leads to a lower resistance than observed for transformation in a *sod2::ura4* background (7). No compelling explanation of this effect is available although possibly some increased influx of sodium through the antiporter under conditions of relatively weak proton gradients may occur. However, under normal growth conditions with glucose present, a strong proton gradient should prevent this and export through the antiport would be enhanced. It is conceivable that the two proteins simply interfere with each other when both are present in the *S. pombe* membrane at the same time, especially when the heterologous expression is on a strong promoter.

A characteristic of mammalian $Na^+/H+$ exchangers is their inhibition by amiloride and its derivatives. Analysis of amiloride resistant NHE isoforms and point mutants have defined the putative amiloride binding site as residues 164-173 (22). Activity of the *S. pombe* Na^+/H^+ antiporter was found to be unaffected by amiloride, EIPA, or MIA, which is the most potent of the analogs tested. This may reflect a lack of conservation of the amino acid residues which comprise the amiloride binding region. Comparisons of sod2 and NHE1 show that the region in the *S. pombe* antiporter corresponding to the amiloride binding site present in the human Na^+/H^+ exchanger (^{163}DVFFLFLLPPI^{173} in NHE1, likely aligning with ^{81}DVRVFASAIELPG^{93} in *sod2*) differs markedly in sequence and this may explain the failure to inhibit. The sodium binding site of sod2 has not been identified.

Earlier work had shown a partial inhibition of $^{22}Na^+$ efflux from fission yeast when the H^+ gradient was collapsed with CCCP or DNP (3). The present study saw no effect. The major difference apart from more sensitive instrumentation was a much higher sodium concentration in the present case (150–300 mM versus 6 mM previously). The higher sodium concentration was clearly sufficient to give a net flux of sodium into the cell even in the presence of some H^+ gradient and the effect of the proton ionophores was negligible under these physiological conditions.

Recently, a gene encoding a Na^+/H^+ antiporter has been identified in the distantly related, extremely halotolerant yeast *Zygosaccharomyces rouxii* (23). The zsod2 putative protein displays strong sequence similarity to, and substantial domains of identity with, sod2. As in *S. pombe*, the *Z. rouxii* antiporter is involved in sodium efflux and disruption of the gene results in reduced tolerance for sodium. There is also evidence that an additional copy of the gene may exist and this may explain the extreme sodium tolerance seen in this yeast. The primary function of the Na^+/H^+ antiporter in each of these yeasts is to regulate the intracellular concentration of sodium. Within sequences made available through the *S. cerevisiae* genome sequencing project an apparent homolog of sod2/zsod2 has recently appeared (Genpept SCX11RA 18). No information is yet available regarding its function.

Available data serve to point out that there are two mechanisms for Na^+ or Li^+ tolerance in yeasts; the antiporter-dependent export seen in *S. pombe* and *Z. rouxii* and the P-type Na^+-ATPase dependent mechanism seen in *S. cerevisiae*. It remains to be seen whether an antiporter in *S. cerevisiae* or cryptic Na^+-ATPase in *S. pombe* can also serve this function. What is clear at the present time is that if such cryptic genes exist then their expression or physiological regulation is such that they cannot compensate for the loss of the major sodium export route in either species.

1. Rodriquez-Navarro, A., Quintero, F. J. & Garciadeblas, B. (1994) *Biochim. Biophys. Acta* **1187**, 203–205.
2. Serrano, R. (1985) *Plasma Membrane ATPase of Plants and Fungi* (CRC, Boca Raton, FL).
3. Jia, Z., McCullough, N., Martel, R., Hemmingsen, S. & Young, P. G. (1992) *EMBO J.* **11**, 1631–1640.
4. Haro, R., Garciadeblas, B. & Rodriquez-Navarro, A. (1991) *FEBS Lett.* **291**, 189–191.
5. McConnell, H. M., Owicki, J. C., Parce, J. W., Miller, D. L., Baxter, G. T., Wada, H. G. & Pitchford, S. (1992) *Science* **257**, 1906–1912.
6. Kurtz, S., Luo, G. X., Hahnenberger, K. M., Brooks, C., Gecha, O., Ingalls, K., Numata, K. & Krystal, M. (1995) *Antimicrob. Agents Chemother.* **39**, 2204–2209.
7. Banuelos, M. A., Quintero, F. J. & Rodriquez-Navarro, J. (1995) *Biochem. Biophys. Acta* **1229**, 233–238.
8. Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E. & Gustin, M. C. (1993) *Science* **259**, 1760–1763.
9. Maeda, T., Takekawa, M. & Saito, H. (1995) *Science* **269**, 554–558.
10. Sherman, F., Fink, G. R. & Hicks, J. B. (1983) *Methods in Yeast Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY).
11. Gmunder, H. & Kohli, J. (1989) *Mol. Gen. Genet.* **220**, 95–101.
12. Maundrell, K. (1993) *Gene* **123**, 127–130.
13. Tang, W., Ruknudin, A., Yang, W.-P., Shaw, S.-Y., Knickerbocker, A. & Kurtz, S. (1995) *Mol. Biol. Cell* **6**, 1231–1240.
14. Hafeman, D. G., Parce, J. W. & McConnell, H. M. (1988) *Science* **240**, 1182–1185.
15. Goffeau, A., Coddington, A. & Schlesser, A. (1989) in *Molecular Biology of the Fission Yeast*, eds. Nasim, A., Young, P. G. & Johnson, B. F. (Academic, San Diego), pp. 397–429.
16. Garciadeblas, B., Rubio, F., Quintero, F. J., Bañuelos, M. A., Haro, R. & Rodriguez-Navarro, A. (1993) *Mol. Gen. Genet* **236**, 363–368.
17. Rudolph, H. K., Antebi, A., Fink, G. R., Buckley, C. M., Dorman, T. E., LeVitre, J., Davidow, L. S., Mao, J. I. & Moir, D. T. (1989) *Cell* **58**, 133–145.
18. Wieland, J., Nitsche, A. M., Strayle, J., Steiner, H. & Rudolph, H. K. (1995) *EMBO J.* **14**, 3870–3882.
19. Millar, J. B. A., Buck, V. & Wilkinson, M. G. (1995) *Genes Dev.* **9**, 2117–2130.
20. Kleyman, T. R. & Cragoe, E. J. (1988) *J. Membr. Biol.* **105**, 1–21.
21. Fliegel, L. & Frolich, O. (1993) *Biochem. J.* **296**, 273–285.
22. Counillon, L., Franchi, A. & Pouysségur, J. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 4508–4512.
23. Watanabe, Y., Miwa, S. & Tamai, Y. (1995) *Yeast* **11**, 829–838