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Gene amplification at a locus encoding a putative Na^+/H^+ antiporter confers sodium and lithium tolerance in fission yeast

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We have identified a new locus, *sodium 2* (*sod2*) based on selection for increased LiCl tolerance in fission yeast, *Schizosaccharomyces pombe*. Tolerant strains have enhanced pH-dependent Na^+ export capacity and sodium transport experiments suggest that the gene encodes an Na^+/H^+ antiport. The predicted *sod2* gene product can be placed in the broad class of transporters which possess 12 hydrophobic transmembrane domains. The protein shows some sequence similarity to the human and bacterial Na^+/H^+ antiporters. Overexpression of *sod2* increased Na^+ export capacity and conferred sodium tolerance. Osmotolerance was not affected and *sod2* cells were unaffected for growth in K^+. In a *sod2* disruption strain cells were incapable of exporting sodium. They were hypersensitive to Na^+ or Li^+ and could not grow under conditions that approximate pH7. The *sod2* gene amplification could be selected stepwise and the degree of such amplification correlated with the level of Na^+ or Li^+ tolerance.

Key words: gene amplification/*Schizosaccharomyces pombe*/sodium/tolerance/transport

Introduction

Na^+/H^+ antiporters from bacterial and animal systems function to regulate Na^+ ion concentration and pH. In bacteria they export Na^+ and under high external salt concentrations maintain a Na^+ gradient across the membrane and an acceptable internal level of this ion. The Na^+ provides the driving force for a number of Na^+–substrate cotransport systems; for example, Na^+ cotransport with melibiose, glutamate, proline and serine (MacDonald *et al.*, 1977; Tsuchiya *et al.*, 1977; Stewart and Booth, 1983; Ishikawa *et al.*, 1987). Since they exchange Na^+ for H^+ they can also play a role in intracellular pH regulation (Krulwich, 1986, 1990; Krulwich *et al.*, 1986). In animal cells, an Na^+/H^+ antiport is involved in cell volume control and the regulation of Na^+ concentration and cytoplasmic pH (Grinstein and Rothstein, 1986, Grinstein, 1988). While the antiporter is virtually turned off at neutral pH, it can be activated by various agents such as growth factors, hormones and cytoplasmic acidosis. Recently the bacterial and human Na^+/H^+ antiporters have been cloned and sequenced (Franchi *et al.*, 1986; Goldberg *et al.*, 1987; Karpel *et al.*, 1988; Sardet *et al.*, 1989, 1990; Tse *et al.*, 1991).

An Na^+/H^+ antiporter on the plasma membrane in *Saccharomyces cerevisiae* and *Neurospora crassa* has been suggested to be responsible for Na^+ export from the cell (Rodriguez-Navarro and Asensio, 1977; Rodriguez-Navarro and Ortega, 1982; Rodriguez-Navarro *et al.*, 1981; Ortega and Rodriguez-Navarro, 1986). The driving force for such an antiporter is the H^+ electrochemical potential generated by the H^+-ATPase. We present here the genetic, physiological and molecular analysis of a novel electroneutral Na^+/H^+ antiporter on the plasma membrane in fission yeast, *Schizosaccharomyces pombe*. This antiport shows similarity to the mammalian and bacterial antiports previously described. We show that this antiporter is essential for Na^+ export and that amplification of the gene is sufficient to confer sodium or lithium tolerance on the cell. Our genetic screen to isolate the antiport mutant employed lithium selection. Since it is toxic to the cell at low concentrations, relative to sodium, this eliminates selection for osmotolerance. This gene and mechanism are distinct from the ATPase recently reported to export sodium from *Saccharomyces cerevisiae* at pH values above 7.0 (Haro *et al.*, 1991).

Results

Isolation and characterization of lithium resistant mutants

In *S.pombe* lithium is ~10-fold more toxic than Na^+ on a concentration basis. Since it is usually transported by Na^+ carriers, it provides a screen for cells capable of maintaining low internal sodium or lithium levels without selecting for osmotolerance. The lithium resistance phenotype was dominant and conferred by a locus displaying some genetic instability (see Materials and methods for detailed isolation and genetic analysis of lithium resistant mutants). Growth rate comparisons showed that in addition to the increased LiCl tolerance, lithium resistant mutants were much more tolerant to NaCl than the wild type (Figure 1). For this reason lithium resistant mutants were termed *sod2* (*sod*ium 2) mutants. All the tested strains displayed a pH dependent tolerance, i.e. as the pH of the medium increased they became increasingly sensitive to NaCl. Similar results were obtained in liquid culture: in EMM 0.6 M NaCl was sufficient to arrest growth in wild type, however, *sod2* mutants could grow in up to 1.2 M Na^+ whether Na^+ was added as NaCl or Na_2SO_4.

The KCl, CsCl and NH_4Cl tolerance of *sod2* mutants and of wild type cells was compared. No marked difference in growth response was observed. This indicated that a change specific for NaCl and LiCl tolerance had occurred in the *sod2* strains. A response to the high osmotic potential must also be occurring in order to tolerate high Na^+ concen-

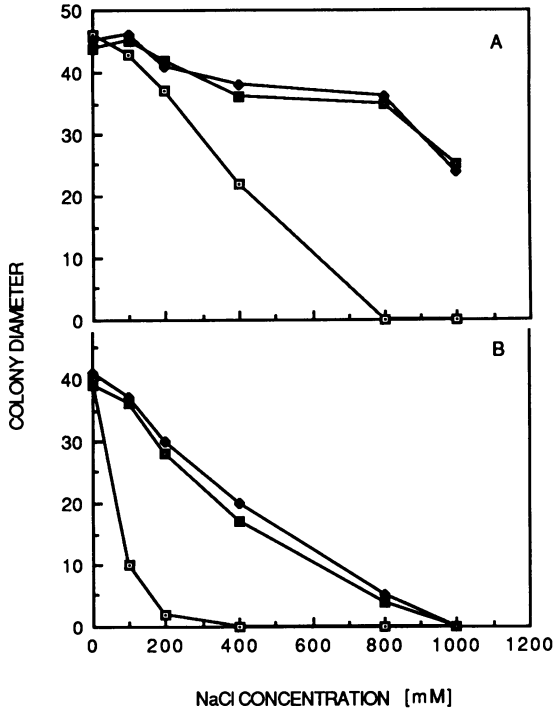

Fig. 1. NaCl tolerance of wild type and *sod2* mutants. Growth response to NaCl was compared between wild type 972 (□) and the *sod2* mutants, *sod2*-1 (◆) and *sod2*-16 (■) at pH 6.0 (**A**) and pH 7.0. (**B**) NaCl was added to EMM at the concentrations indicated and the pH of the medium was adjusted with KOH. Each point represents the average diameter of 10 randomly selected colonies in very low density regions of the plates. The standard error of the mean is ~10%. Incubation was for 96 h at 30°C. A repeat in liquid culture gave similar result.

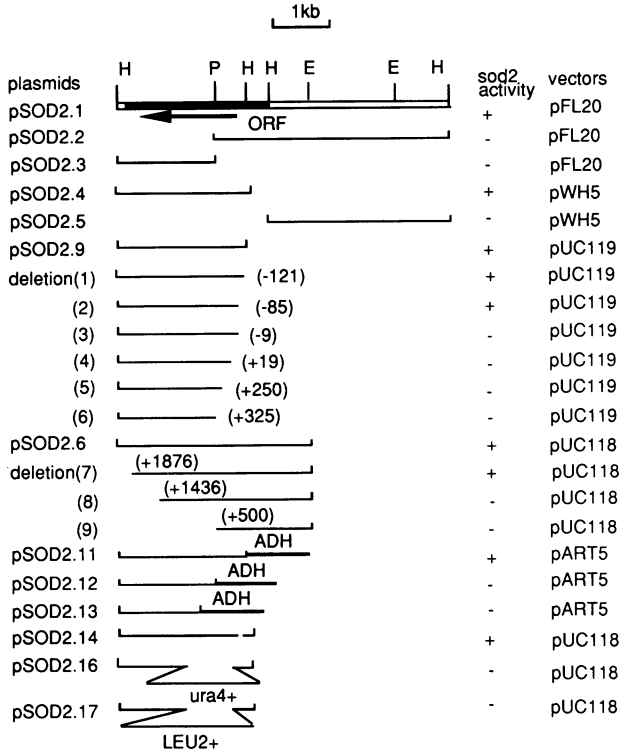

Fig. 2. Restriction map and subcloning of *sod2*. A restriction map of a 6.1 kb *S.pombe* genomic insert in plasmid pSOD2.1 is shown at the top (H = *Hin*dIII, P = *Pvu*II, E = *Eco*RI). The DNA sequence of the filled region was determined as shown in Figure 3. Below the restriction map are various subclones, deletions, overexpression plasmids, disruption mutations and the intronless clone, which were tested for their ability to confer lithium chloride resistance upon wild type cells. Details of their construction are given in Materials and methods. (+) indicates LiCl resistance phenotype; (−) indicates LiCl sensitivity phenotype.

trations. This does not appear to have been affected by the mutation since growth on high concentrations of K^+ was unaffected. Sodium or lithium resistance was likely to have been an ability to maintain low internal concentrations of the ions. This could have been due to either decreased uptake or increased export. This was confirmed by sodium transport experiments: *sod2-1* cells have enhanced Na^+ export capacity (see section ^{22}Na transport physiology).

Cloning, mapping and DNA sequencing of the sod2 locus

The relative instability and potential for stepwise selection of the *sod2* mutants suggested that the lithium resistance may be caused by gene amplification. Based on Na^+ transport experiments the gene product is likely to be or to be able to control an Na^+ and Li^+ export mechanism (see below). It was conceivable that multiple copies of the wild type *sod2* gene would therefore be sufficient to confer resistance by increasing the export capacity. A host strain *ura4-294* was transformed with a pFL20 based *S.pombe* genomic gene bank (Clarke *et al.*, 1986) and the transformants were scored for lithium resistance. A single plasmid, pSOD2.1, was independently recovered twice and shown to confer lithium resistance by retransformation (Figure 2). A derivative of pSOD2.1, pSOD2.11, which by virtue of an *ADH* promoter confes a lithium resistance phenotype (see below) and carries a *LEU2* marker, was integrated into the genome of a *leu1-32* strain (Q241). Random spore analysis of the pSOD2.11 integrant strain (Q803) crossed to a *sod2-1 leu1-32* strain showed no segregation of *sod2* (lithium sensitivity) out of 100 spores tested. This indicated that the isolated plasmid had integrated at or near the *sod2* locus and was consistent with it being chromosomally located rather than an extragenic element.

The location of the *sod2*$^+$ coding sequence in pSOD2.1 was determined by testing the ability of various subclones to confer increased LiCl resistance (Figure 2). These experiments indicated that the *sod2*$^+$ gene was located in a 2.3 kb *Hin*dIII fragment. To localize further and sequence the 2.3 kb *Hin*dIII fragment, a series of nested deletion subclones were generated from pSOD2.6 and pSOD2.9 (Henikoff, 1987). Each of the subclones was used for sequencing and a number of them were tested for *sod2* activity by cotransformation with pWH5 (Wright *et al.*, 1986) (Figures 2 and 3). Based on all of these data the region between −85 bp and +1876 bp was defined as sufficient for *sod2* activity. Two regions of potential open reading frame (ORF) were present: from −222−161 bp and from 195−1482 bp. Deletion mapping suggested that the *sod2* gene did not extend beyond the 2.3 kb *Hin*dIII fragment (−187) but rather that the functional region was from −85−1876 bp (Figure 3). The context of the ATG at position 1 had a conserved A at −3 and a G at +4 as in most mRNAs; the upstream ATG (−222) had a less favoured G at −3 and lacked the G at +4 (Kozak, 1991). The overall match to the initiation consensus sequence was low outside

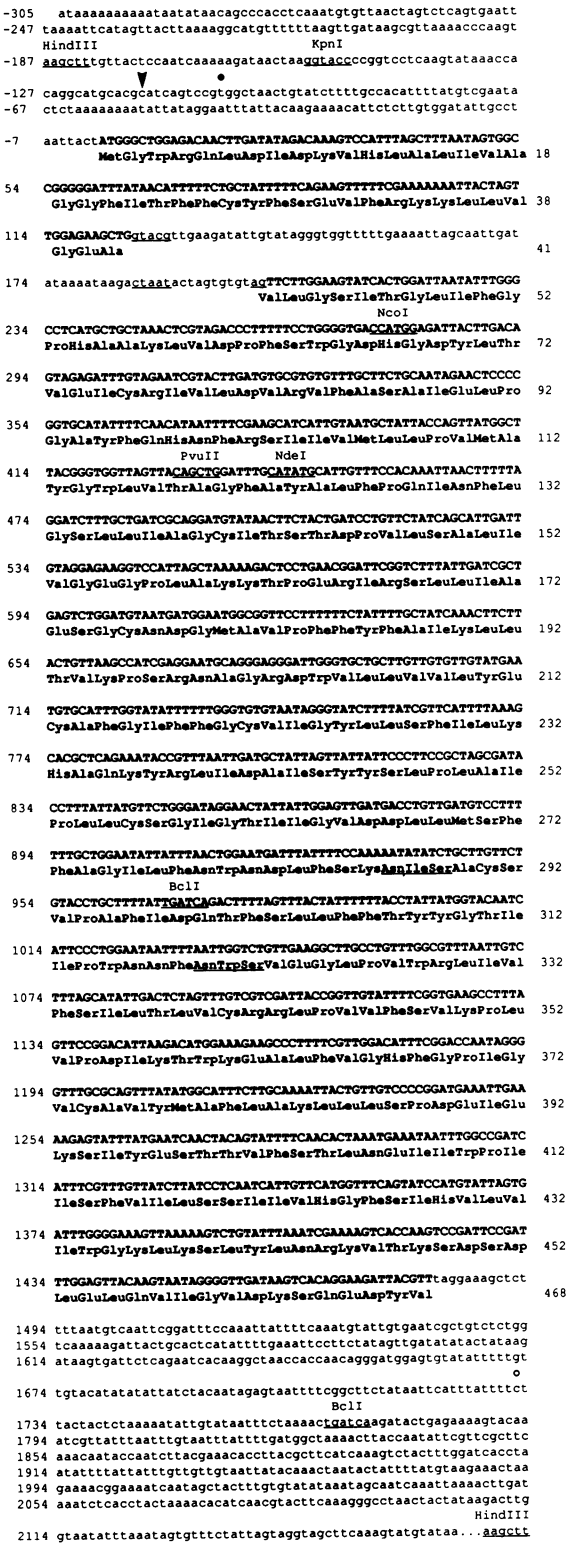

Fig. 3. Nucleotide and predicted amino acid sequence of the *sod2*$^+$ gene. The nucleotide sequence of the *sod2* gene is shown. The coding region is interrupted by a 77 bp intron from position 124−201 bp. The intron consensus sequences are underlined. The transcription start sites revealed by a cDNA clone and S_1 analysis are indicated by filled circle and arrow, respectively. The termination site is indicated by an open circle. The predicted 468 aa sequence is shown in three-letter code below the DNA sequence. Two potential glycosylation sites are underlined. The sequence data reported here have been deposited in the EMBL/Gen Bank/DDBJ nucleotide sequence databases under the accession number Z11736.

of these two strongly conserved residues, however, this is sometimes an attibute of mRNAs which are expressed at low level (Kozak, 1991). Consensus intron splicing sequences (Mertons and Gallwitz, 1987; Russell, 1989) linked the two ORFs with a potential intron extending from 125−201 bp. Within the region required for *sod2* activity defined by the deletion analysis, three ATG codons were located (1, 393 and 615 bp). An *Nde*I site was inserted by oligomutagenesis at each ATG following removal of an internal *Nde*I site at 441 bp in pSOD2.8. An *Nde*I−*Xba*I fragment from each construct was then inserted behind the *ADH* promoter of pART5 (McLeod *et al.*, 1987) yielding plasmids pSOD2.11, pSOD2.12 and pSOD2.13, respectively. When tested for conferring lithium resistance only pSOD2.11 was functional.

The putative intron splicing junctions were confirmed by S_1 analysis and the sequencing of a cDNA clone. A single stranded probe extending from 125 to −187 bp yielded a protected fragment of 239 bp indicating a transcription start site near −115 bp. A probe extending from 625 to −187 bp yielded protected fragments of 424 and 239 bp indicating an intron from 124−202 bp (data not shown). Sequencing of a cDNA clone for *sod2* (prepared from a *sod2-1* cDNA library in pTZ19R) showed that the sequence extended directly from 124−202 bp, confirming the existence of an intron as shown by the S_1 results. The splice junctions are thus 5′-GTACG and 3′-TAG with a branch site of GACTAAT. All three closely match the *S.pombe* consensus sequences (Mertins and Gallwitz, 1987; Russell, 1989). The entire cDNA clone was sequenced. The sequence started at −105 bp and revealed a polyadenylation site at 1732 bp. There was no complete AATAAA polyadenylation signal 5′ to this polyadenylation site (Proudfoot and Brownlee, 1976). A Northern blot of wild type and *sod2-1* RNA showed transcript size to be ~1.8 kb long in both strains. The *sod2-1* cDNA was identical to the wild type genomic clone at all positions except for a translationally silent change (A for T) at position 1292. This confirmed that the lithium resistant *sod2-1* strain was expressing the wild type *sod2* gene product (also, see below).

sod2 hypothetical protein product

The open reading frame starting at 1 bp and ending at 1483 bp (Figure 3) encoded a hypothetical protein of 468 amino acids with a predicted M_r of 52 161. Although the reading frame was open for a further 74 aa upstream to an ATG at −222, the S_1 analysis, deletion analysis and length of the transcript by Northern blot all suggested that the ATG at position 1 was the start of the protein. This sequence did not display strong sequence similarity to any protein in the current databases (Pearson and Lipman, 1988). There was no ATP binding site as seen in various ATP dependent transporters (Walker *et al.*, 1982; Higgins *et al.*, 1988; Gaber *et al.*, 1988; Goffeau *et al.*, 1989; Juranka *et al.*, 1989). There were two potential glycosylation sites at positions 287 and 319. The protein was highly hydrophobic with 54.7% hydrophobic residues. A hydropathy plot (Kyte and Doolittle, 1982) showed it to have twelve putative hydrophobic segments linked by short hydrophilic regions (Figure 4). Nine of these are predicted to be membrane spanning according to Klein *et al.* (1985) and eleven according to Eisenberg *et al.* (1984), domain 2 being slightly below the criterion. The similarity of the 12 transmembrane domain structure to a broad class of transporters (Maiden *et al.*, 1987; Seol and Shatkin, 1991) was striking. The R/K-X-G-R-R/K motif found in many of the metabolite

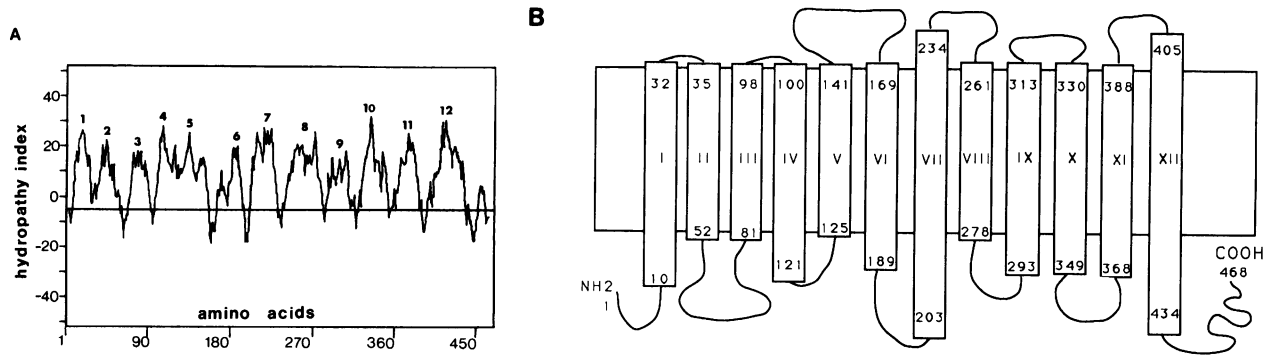

Fig. 4. Hydropathy plot and hypothetical transmembrane structure of the *sod2* gene product. (**A**) Average hydropathicity values were determined for spans of nine residues using the method of Kyte and Doolittle (1982). (**B**) Hypothetical secondary structure model of the *sod2* protein. The model is based on the prediction of the hydropathy profile. The putative transmembrane segments are boxed and the first and the last amino acid of each segment are indicated (see text).

H^+ or Na^+ symports and proteins such as the tetracycline$-H^+$ antiporter (tcr1) was not present (Maiden *et al.*, 1987; Szkutnicka *et al.*, 1989; Yamaguchi *et al.*, 1990; Yoshida *et al.*, 1990; Seol and Shatkin, 1991). There was no sign of internal duplication in the protein. A weak overall similarity was present with the *E.coli* antiport (*ant/nahA*) (Karpel *et al.*, 1988) and with the hydrophobic portion of the 99 kDa mammalian Na^+/H^+ antiport (Sardet *et al.*, 1989; Tse *et al.*, 1991) (Figure 5). 25% of *sod2* residues showed identity to one or the other of the aligned antiports. This alignment required the insertion of a substantial number of gaps and included only the domain 2–10 region of the mammalian antiports. There is considerable sequence similarity in some areas, particularly in the *sod2* V^{38}GEAVLGS^{45} motif and this region also aligns with the Na^+–glucose cotransporter from mammalian cells (V^{16}GgAVLGS^{23}) although the rest of this protein does not show much similarity (Hediger *et al.*, 1987). The G^{22} residue in the glucose transporter is possibly conserved with G^{22} in *putP* and implicated in the latter case as being part of the cation binding site (Yamato *et al.*, 1990).

The sod2 protein was expressed in *E.coli* behind the T7 promoter (Studier and Moffat, 1986). The *E.coli* strains did not grow well even in the presence of the lysE/lysS plasmids to suppress low level expression prior to induction. When induced, only fragments of the protein could be detected and these did not accumulate in the cell. The protein appeared to be highly toxic in this environment presumably through interference with membrane function. Several fragments were isolated from polyacrylamide gels, however, no useful antibodies have been generated so far. A peptide antibody against the carboxyl terminal eight amino acids has been produced, however, it has not proven useful in detecting the protein in the cell.

Amplification of the sod2-1 locus

Based on the instability and behaviour upon stepwise selection, the *sod2* mutation was speculated to involve gene amplification. To formally prove that *sod2*$^+$ has been amplified in the *sod2* mutants, a Southern blot hybridization of *sod2*-1 genomic DNA was performed. When wild type DNA was probed with pSOD2.1 which contained the pFL20 vector (with *S.pombe* sequences *ars1* and *stb* and the *S.cerevisiae URA3* gene) and the 6.1 kb *S.pombe* insert containing the *sod2* gene and flanking sequences, the stoichiometry of the hybridization signals indicated equivalent copy number for the various fragments hybridizing to the pSOD2.1 clone (Figure 6A). In *sod2*-1 DNA *Eco*RI fragments at 1.2 kb (*ars1*) and 1.3 kb (*stb*) and the *Hin*dIII fragment at 1.8 kb (*ura4*) were under-represented relative to other fragments (Figure 6B). These fragments represented single copy genomic sequences of *ars1*, *stb* and the *ura4* gene homologous to the *URA3* gene. The more intense bands represented the amplified 2.3 kb *Hin*dIII fragment which contained the *sod2* and the 0.3 kb and 3.5 kb upstream *Hin*dIII fragments. Since two large fragments (~6 and 15 kb) were present with the amplified stoichiometry, a substantial region flanking *sod2* was amplified with an estimate of at least 20 kb. Amplification units of much larger size have been reported in mammalian cells (Guilotto *et al.*, 1986; Stark *et al.*, 1989). The amplicon copy number estimated would be ~20 times in *sod2*-1 based on relative exposure time for the above blots. DNAs from five other stable *sod2* alleles were Southern blotted and shown to have an amplified 2.3 kb *Hin*dIII fragment. A rough correlation between the degree of amplification by stepwise selection from the wild type 972 cells and the level of LiCl/NaCl resistance was noted (Figure 7). When *sod2*-1 cells were grown in EMM without LiCl for a long period of time they displayed reduced LiCl/NaCl resistance. Also these cells consistently showed decreased amplification when their genomic DNA was analysed. A densitometry scan of this blot yielded an amplification of ~10-fold.

Overexpression of the cloned sod2 gene

Since the basis of resistance appears to be gene amplification, expression of *sod2*$^+$ behind a strong promoter should produce the same effect. A strain (Q803) was therefore constructed to overexpress the *sod2*$^+$ gene by integrative transformation of pSOD2.11 containing the ADH promoter. Southern blotting showed homologous recombination and the presence of a single copy of the *sod2*$^+$ gene. Northern blotting showed that the *sod2*$^+$ gene was highly transcribed in both *sod2*-1 and the overexpressing strain (data not shown). Such strains were highly salt tolerant (Figure 8).

Since an altered expression level confers salt tolerance, the question of inducibility of the gene was addressed. A Northern blot was prepared in order to examine transcript level in wild type cells growing in the absence and presence of 400 mM NaCl. No difference in transcript level was found (data not shown). The *sod2* transcript did not appear to be induced with NaCl.

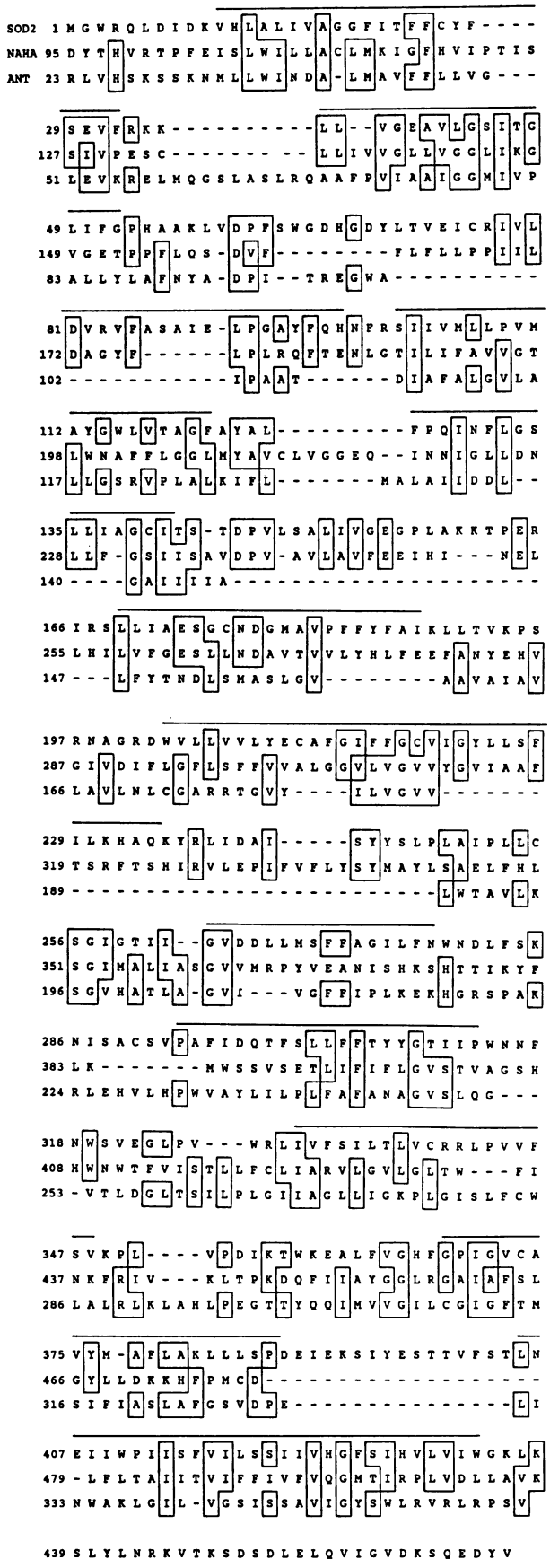

Fig. 5. Sequence alignment for *sod2*, the human (*NaHa*) and *E.coli* (*nahA*) antiporters. The transmembrane domains for *sod2* are indicated by solid lines above the sequence.

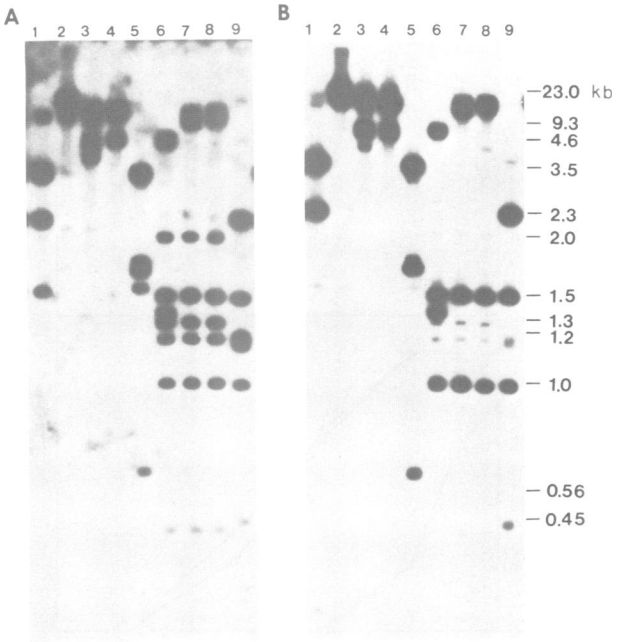

Fig. 6. Southern blot hybridization of *sod2*-1 genomic DNA. Genomic DNA was isolated from wild type 972 (**A**) and *sod2*-1 (**B**), digested with restriction enzymes, blotted to nitrocellulose and hybridized with labeled pSOD2.1. Lanes: 1, *Hin*dIII−*Pst*I; 2, *Pst*I; 3, *Pst*I−*Pvu*II; 4, *Pvu*II; 5, *Pvu*II−*Hin*dIII; 6, *Pvu*II−*Eco*RI; 7, *Eco*RI; 8, *Eco*RI−*Pst*I; 9, *Eco*RI−*Hin*dIII. Autoradiography time for (A) is 20 times longer than that for (B).

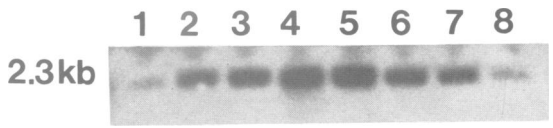

Fig. 7. Gene dosage comparsion for various LiCl tolerant strains. Genomic DNA was isolated from each of the following strains, restriction digested with *Hin*dIII and equivalent quantities (5 μg) electrophoresed then transferred to nitrocellulose and hybridized with a labeled 2.3 kb *sod2 Hin*dIII fragment. Lanes: 1, 972 (wild type); 2, a stable 20 mM LiCl resistant strain stepwise selected from 972; 3, a stable 40 mM LiCl resistant strain stepwise selected from 972; 4, a stable 60 mM LiCl resistant strain stepwise selected from 972; 5, *sod2*-1; 6, a 30 mM LiCl resistant strain resulting from *sod2*-1 grown in EMM without LiCl for 40 days; 7, a 20 mM LiCl resistant strain resulting from *sod2*-1 grown in EMM for 70 days; 8, a 5−10 mM LiCl resistant strain resulting from *sod2*-1 grown in EMM for ~4 months.

Disruption allele of sod2

To construct a *sod2* disruption plasmid, the 0.8 kb *Bcl*I fragment that contained ~40% of the *sod2*$^+$ coding region was excised and replaced by a 1.8 kb *ura4*$^+$ fragment or a 2.2 kb *LEU*$^+$ fragment to create plasmids pSOD2.16 and pSOD2.17 (see Figure 2). Both plasmids contained a non-functional disruption of the *sod2* gene. A *ura4*$^-$ *leu1*$^-$ diploid strain (Q474) was transformed to *ura*$^+$ or *leu*$^+$ with linearized pSOD2::ura4 (pSOD2.16) or pSOD2::LEU2 (pSOD2.17). Stable integrants showed 2:2 segregation of *ura*$^+$ and *ura*$^-$ or *leu*$^+$ and *leu*$^-$ progeny. The presence of the pSOD2::ura4 (or LEU2) allele was confirmed by genomic Southern blot analysis of the haploid *ura*$^+$ or *leu*$^+$ progeny. Haploid cells containing the *sod2* gene disruption were viable, however, the growth of the strain in both rich

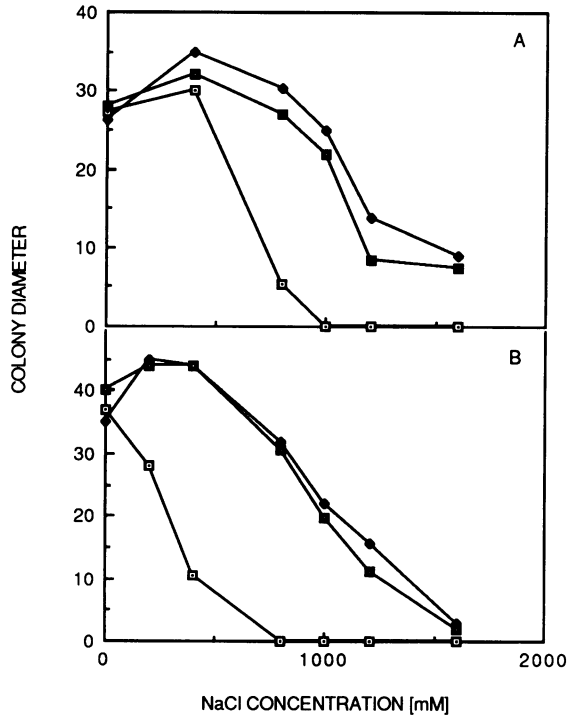

Fig. 8. NaCl tolerance of *sod2−ADH*. NaCl tolerance was compared among wild type 972, *sod2*-1 and the *sod2* overexpressing strain *sod2−ADH* at pH 5.0 (**A**) and pH 6.0 (**B**). Incubation was for 96 h at 30°C. 972 (□); *sod2*-1 (◆); *sod2−ADH* (■).

(YEA) and minimal medium (EMM) was much slower compared with wild type cells. In addition, the cells showed hypersensitivity to NaCl being unable to grow at concentrations above 125 mM.

^{22}Na transport physiology

Sodium uptake was assessed in wild type, *sod2*-1 and *sod2*::*ura4*. Net uptake was lower in the *sod2*-1 strain than in wild type or the gene disruption strain (Figure 9). Sodium uptake could be inhibited by amiloride in all three strains. A concentration of 100 μM was required to block uptake although lower concentrations are known to block cell growth (Jia,Z.-P., McCullough,N., Wong,L. and Young,P.G., unpublished). Whether amiloride was present or not, CCCP stimulated further ^{22}Na uptake in all three strains. These data show that sodium entered the cell by an amiloride inhibitable route and that an alternative influx route was seen in CCCP uncoupled cells. These activities are not related to *sod2* since they are not affected by the *sod2*::*ura4* gene disruption. The CCCP stimulated route is quite likely to be an antiport independent of *sod2*. The CCCP stimulation of uptake was similar to the stimulation of quinolone uptake by CCCP in *Staphylococcus aureus*. In this case it was through inhibition of the H^+ dependent export protein, *norA* (Yoshida *et al.*, 1990).

Sodium export capacity was assayed following ^{22}Na preloading of cells. Export rate was much higher in *sod2*-1 and the overexpression strain than in wild type cells (Figure 10). The *sod2*::*ura4* gene disruption, however, was not capable of exporting sodium showing that this is the only export mechanism for the cell.

Sodium efflux from both wild type and *sod2*-1 cells was affected by pH (Figure 11A). At either pH 6 or pH 4.5, DNP and CCCP substantially inhibited export (Figure 11B and C). DNP gave consistently the same result at concentrations from 1−5 mM. Arsenate did not inhibit export (Figure 11B and C) at concentrations ranging from 200 μM to 1.2 mM. These experiments were also tried both with arsenate preincubation ± glucose with the same result. Arsenate at 100−200 μM was effective in blocking cell growth. KCl at concentrations sufficient to collapse membrane potential (see below) did not block export at either pH.

Glucose strongly stimulated Na^+ uptake (presumably on the K^+ carriers) and this masked the uptake activities shown here. Export was abolished in the *sod2* disruption

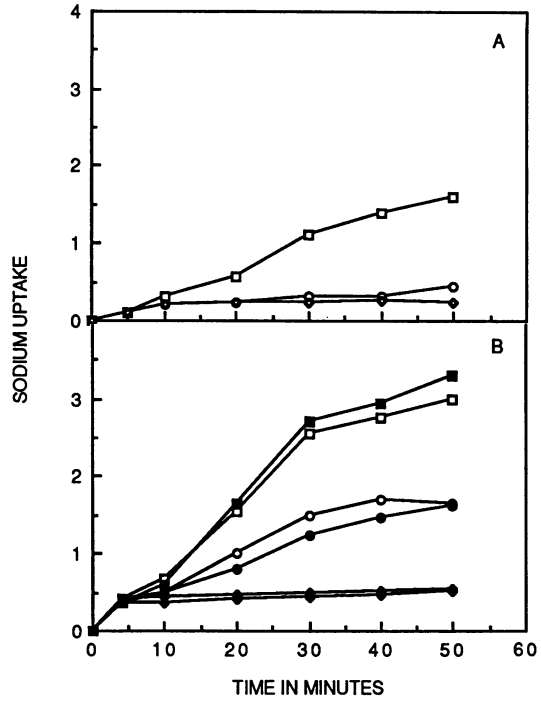

Fig. 9. Sodium uptake in wild type, *sod2*-1 and *sod2*::*ura4* strains. The effect of inhibitors on ^{22}Na uptake was examined for (**A**) *sod2*-1 and (**B**) wild type (solid synmbols), *sod2*::*ura4* (open symbols). No additions (circle), 50 μM CCCP (squares) and 100 μM amiloride (diamonds).

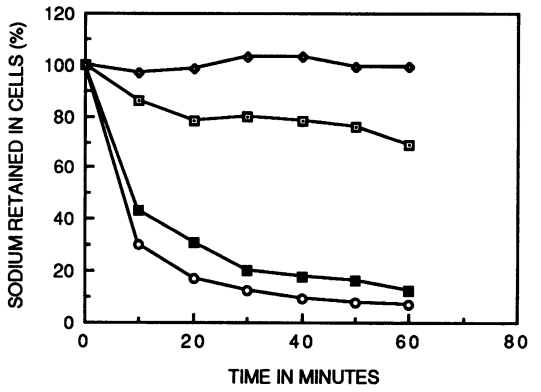

Fig. 10. $^{22}Na^+$ efflux from various strains. Cells were preincubated at 10^8 cells/ml in MES−PIPES−Tris buffer, pH 7, containing 0.5 μCi/ml ^{22}NaCl and 6 mM NaCl for 1 h at 30°C. Cells were then filtered, washed with the same buffer but containing no ^{22}Na, and finally resuspended at the same cell density in MES−PIPES−Tris buffer, pH 4.5, containing 6 mM NaCl. Radioactive Na remaining in the cells is expressed relative to that recorded at T=0 minutes (100%). 972 (□); *sod2*-1 (○); *sod2−ADH* (■); *sod2*::*ura4* (◇).

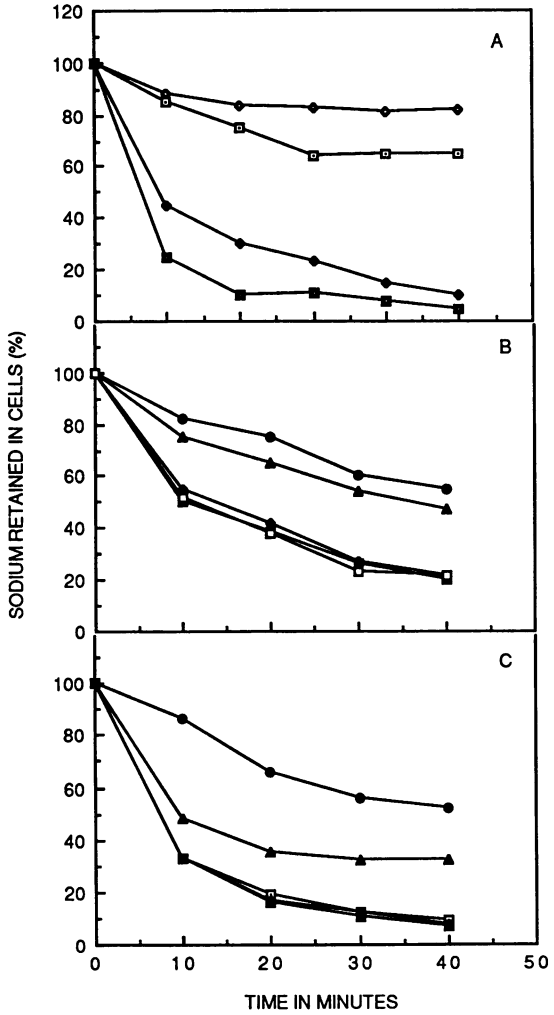

Fig. 11. Inhibition of Na^+ efflux. (**A**) Cells (10^8/ml) of the wild type and *sod2*-1 were incubated in Mes−PIPES−Tris buffer, pH 7.0 containing 6 mM NaCl and 0.5 μCi/ml $^{22}NaCl$ for 1 h at 30°C. Cells were then filtered, washed with the same buffer with ^{22}Na, and finally resuspended at the same cell density in Mes−PIPES−Tris buffer, pH 4.5 or pH 7.0 containing 6 mM NaCl. Radioactive ^{22}Na remaining in cells is expressed relative to that recorded at T=0 minutes. Wild type pH 4.5 (□), pH 7.0 (◇); *sod2*-1 pH 4.5 (■), pH 7.0 (◆). (**B**) ^{22}Na efflux from *sod2*-1 cells at pH 6: no additions (□); 250 mM KCl (■); 200 uM arsenate (◆); 50 uM CCCP (▲); 1 mM DNP (•); (**C**) Same as (B) but at pH 4.5.

in the presence or absence of glucose, again showing that *sod2* is essential for Na export under all conditions.

TPP^+ uptake

TPP^+ (tetraphenylphosphonium) distributes across a membrane in response to membrane potential. [^{14}C]TPP^+ uptake was therefore used to assess the effect of the above treatments on membrane potential. Cells grown in minimal medium (27 mM K^+ and 25 mM Na^+) or low sodium minimal medium (55 mM K^+ and 0.5 mM Na^+) did not accumulate measurable [^{14}C]TPP^+. When cells were grown in Na^+ enriched media (27 mM K^+ and 400 mM Na^+) or were preloaded with Na^+ as for export experiments, the cells readily accumulated TPP^+ with saturation in 3−5 min and then maintained a constant level for at least 1 h. The internal Na^+ presumably provides a counter ion for export for charge balancing with the TPP^+. Under these conditions the reduction in TPP^+ accumulation caused by CCCP

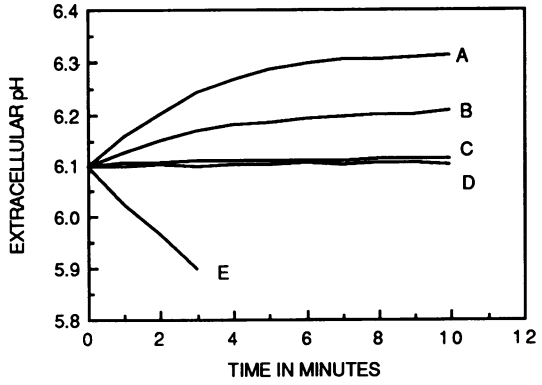

Fig. 12. Influx of H^+ in response to Na^+ efflux. Cells were either preloaded with non-radioactive Na^+ as for efflux experiments or not. They were then washed and resuspended (5×10^8 cells/ml) in 2 mM Tris−citrate buffer with or without glucose. H^+ influx was measured as a rise in external pH. (**A**) *sod2*-1, (**B**) wild type, (**C**) *sod2*::*ura4*, sodium preloaded, no glucose. (**D**) wild type or *sod2*-1, without sodium preloading, no glucose; (**E**) wild type, plus glucose.

50 μM), DNP (100 μM), KCl (250 μM) or KCl + Valinomycin (250 mM and 9 μM, respectively) expressed as a percentage of the control (± Std error n = 3) were 37 ± 6%, 41 ± 4%, 34 ± 2% and 25 ± 3%, respectively. None of the treatments blocked all accumulation. There was no major difference between KCl alone and KCl plus valinomycin. From our ^{22}Na transport experiments, DNP or CCCP inhibited export whereas KCl does not (Figure 11). Since they all had similar effects on membrane potential, we conclude that the primary effect of DNP or CCCP on the transporter is through collapse of the H^+ gradient and that mM membrane potential does not play a major role.

Na^+ dependent H^+ influx

If *sod2* functions as an antiporter then during Na^+ export from the cell, H^+ should be taken up. The direct stimulation of H^+ uptake by sodium export was therefore measured. Under the conditions used for our export studies there was no net H^+ export from the cell unless glucose was added (Figure 12). If wild type or *sod2*-1 cells were preloaded with Na^+ then they took up protons as Na^+ was extruded (Figure 12). This proton uptake was not affected by the presence of 10 mM external K^+. The *sod2* disruption strain was not capable of exporting Na^+ and did not show an influx of protons in response to Na^+ preloading (Figure 12).

Role in pH regulation

pH regulation is the central function of the Na^+/H^+ antiporter in animal cells. Cells deficient in Na^+/H^+ antiport activity fail to grow at neutral and acidic pH in HCO_3^--free media (Pouyssegur, 1985). In bacteria, mutations abolishing Na^+/H^+ antiport activity preclude growth at alkaline pH also suggesting a role in pH regulation (Krulwich, 1986, 1990; Padan *et al.*, 1976; 1981). The pH sensitivity characterized by the two types of mutants could be demonstrated in *sod2* disrupted cells (Figure 13). Wild type cells could grow over a wide range of external pH from pH 3.5−7.0 with an optimum growth at pH 6.0. In contrast, *sod2*::*ura4* cells failed to grow at pH 6.5 and pH 7.0 although growth at acidic pH 3.5−5.5 was normal. This suggests that *sod2* plays a role in intracellular pH regulation at the higher pH. This pH responsiveness of *sod2*

disrupted cells also supports the suggestion that *sod2* is a Na^+-H^+ antiporter.

Discussion

The *sod2* locus was identified based on increased LiCl tolerance. Amplification at the locus provided resistance to the specific toxicity of high external sodium concentrations as well. Lithium selection may thus provide a selection scheme for targeting similar transport systems in other fungi and perhaps higher plants without selecting for osmotolerance mechanisms.

Na^+ transport experiments suggest that the *sod2* gene encodes an electroneutral Na^+/H^+ antiporter or one of its subunits. By its high degree of hydrophobicity and twelve transmembrane segments it is clearly an integral membrane protein belonging to a broad class of permeases, symports and antiports. Sequence alignment with other known antiports suggests low but significant similarity particularly in the domain encompassing Gly^{22} of the human Na^+/glucose transporter, a domain implicated in cation selectivity (Yoshida *et al.*, 1990). Similarity is also quite marked in several other domains although the functional significance is unknown.

The *sod2* gene was specifically amplified in *sod2*-1. The fact that the restriction pattern of the *sod2* gene region and its flanking sequences in both wild type and *sod2*-1 were the same and that the amino acid sequence of *sod2*-1 was unaltered indicates that simple amplification of the $sod2^-$ gene conferred the resistance phenotype. Overproduction of sod2 resulted in increased Na^+ export capacity. The increased Na^+ extrusion accounted for NaCl or LiCl tolerance in both *sod2*-1 and $sod2^+$ overexpressing strains. This appears to be analogous to amplification of the P-glycoprotein locus in drug resistant mammalian cell lines (Gros *et al.*, 1986). Although cases of amplification are rare in yeasts it has been reported for the *CUP* locus of *S.cerevisiae* (Fogel *et al.*, 1984).

Disruption alleles of *sod2* could not export Na^+ and were hypersensitive to sodium and high pH. The fact that the loss of function allele of *sod2* failed to grow at high pH suggested that sod2 could function in pH regulation. These functions of sod2 (Na^+ extrusion and pH regulation) are very similar to those demonstrated for the bacterial and human Na^+/H^+ antiporters (Padan *et al.*, 1981; Krulwich, 1986, 1990; Krulwich *et al.*, 1986; Sardet *et al.*, 1989) and suggest that sod2 has a similar activity and that it resides on the plasma membrane. In plant cells sequestering of Na^+ within the vacuole plays a major role in tolerance. Although vacuolar storage of various ions in yeast has been reported there is no report yet for sodium (reviewed in Klionsky *et al.*, 1990). Whether or not sod2 could play a role in the vacuole is unknown. A definitive test of these possibilities will have to await the purification of the functional protein and the availability of useful antibodies. The cloned *sod2* gene could conceivably encode a regulator of a Na^+/H^+ antiporter. This possibility seems unlikely since inactivation completely abolished sodium export. It also seems unlikely that simple amplification of a regulatory protein would confer such strongly enhanced export capacity. The simplest interpretation is that sod2 represents a novel Na^+/H^+ antiport functionally related to *ant*/*nahA* (Karpel *et al.*, 1988) or the mammalian antiport (Sardet *et al.*, 1989) but with the three proteins showing very limited sequence similarity. Lastly

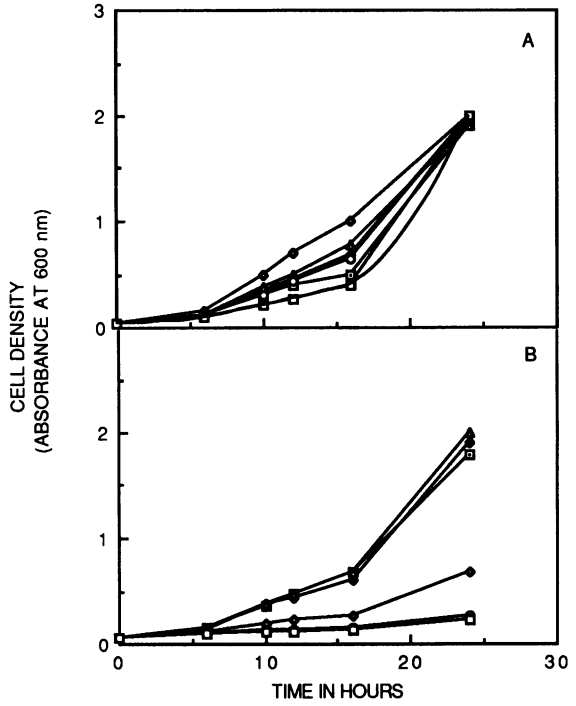

Fig. 13. pH sensitivity of growth. Approximately 10^7 cells of actively growing wild type (**A**) and *sod2*::*ura4* (**B**) were inoculated into liquid low Na^+ EMM at various pH values buffered with Mes, Pipes or Tris. Growth is expressed as turbidity (A_{600}). pH 3.5 (□); 4.5 (◆); 5.5 (△); 6.0 (◇); 6.5 (○); 7.0 (□).

the amiloride sensitive and the CCCP stimulated, amiloride insensitive uptake routes are unaffected in $sod2^-$ suggesting further antiports may be present on the membrane. We are currently defining those components. A second transport protein, sod1, believed to transport amiloride into the cell in this system has been cloned and sequenced; however, gene disruptions at this locus do not affect Na^+ transport (Jia,Z.-P., McCullough,N., Wong,L. and Young,P.G., unpublished observations).

A recent paper (Haro *et al.*, 1991) shows that in *S.cerevisiae*, the P-type ATPase *PMR2* (*ENA2*) can play a role in sodium export, these ATPases function in Na^+ export at high pH (>7.0). When subjected to growth tests the overexpression of the *PMR2* ATPase gave its greatest enhancement of Na^+ resistance at pH 8.0. Even low concentrations of lithium (5 mM) reduced cell growth in ATPase expressing cells at pH 6.5. *S.pombe* can grow only marginally even at pH 7 and is extremely sensitive to Na^+ and Li^+ at this pH. The normal growth conditions for yeast is under acidic conditions. At low pH the *sod2* gene is essential and behaves physiologically to mediate Na^+-H^+ exchange. Failure of *S.pombe* to grow at high pH may in fact reflect the absence of ATPase exporters such as *PMR2*. It would be of interest to know whether *PMR2* disruptions in *S.cerevisiae* are Na^+ and Li^+ sensitive at pH values below 6.0 or whether overexpression of *PMR2* in *S.pombe* (or the equivalent *S.pombe* gene) would allow survival above pH 7.0. These possibilities are being investigated.

Materials and methods

Strains, media, and general genetic and molecular methods

S.pombe was maintained on Edinburgh Minimal medium (EMM) or yeast extract adenine (YEA) using standard methods (Mitchison, 1970; Nurse, 1975). Low sodium minimal medium was made by replacing Na_2HPO_4

by K_2HPO_4 at the same concentration and adjusting the pH to 5.5. The resultant medium contains 250–400 μM Na^+ as determined by flame photometry. General genetic and molecular methods for *S.pombe* have been described elsewhere (Gutz *et al.*, 1974; Beach and Nurse, 1981; Russell, 1989). Yeast cell mutagenesis was with nitrosoguanidine (Gutz *et al.*, 1974; Nurse *et al.*, 1976).

Isolation of LiCl/NaCl resistant mutants

At pH 5.0, 15 mM lithium chloride blocks cell proliferation and 20 mM is lethal. To obtain resistant mutants wild type 972 h^- cells were mutagenized (Nurse *et al.*, 1976) and plated on EMM plus 40 mM LiCl, pH 5.0 at 2×10^6 cells per plate. No time for growth was allowed between mutagenesis and plating. A total of 16 survivors were collected from 100 plates for a frequency of 8×10^{-6}.

The mutant strains had a marked increase in LiCl tolerance which was still pH dependent. At pH 5.0, the mutant cells could grow on 60 mM LiCl. At pH 7 all tested strains were highly sensitive to LiCl: 1 mM LiCl was sufficient to prevent growth of the wild type cells and 3 mM LiCl was sufficient to block mutant cells.

Instability of lithium resistance for some of the mutants was demonstrated by growth in EMM with no LiCl selection. Most of the strains (14 of 16) were relatively stable with < 1% of cells showing lithium sensitivity after 52 h of incubation in EMM. All strains slowly lost their lithium resistance if incubation in EMM was extended for several months. Tetrad analysis of stable mutant strains *sod2*-1 (Q776) and *sod2*-16 (Q790) crossed to wild type strain 975 h^+ showed 2:2 segregation for lithium resistance and sensitivity indicating a single chromosomal mutation. For some tetrads (3 and 4 in 15 tetrads, respectively) the two sensitive strains showed an elevated lithium resistance compared to wild type cells but still much lower than the resistant strain. This can be explained by ectopic recombination from an amplified locus (see Results). For linkage analysis, a strain *sod2*-1 *ade6*-210 h^+ (Q792) was crossed with other stable resistant mutants. Free spore analysis showed that wild type recombinants, i.e. lithium sensitive, occurred with a frequency of < 1:200. This frequency is consistent with close linkage or the stability test observed earlier. Therefore all stable lithium resistant mutants were tightly linked to each other and putative alleles of a single locus of *sod2*. Stable lithium resistant strains were also obtained from the wild type cells by stepwise selection with gradual increase of LiCl concentration and found to be linked to the *sod2* locus. Stepwise selection was performed with several separate cultures. This also suggested a gene amplification mechanism for lithium resistance.

For *sod2* chromosomal mapping, a strain *sod2*-1 *ade6*-704 h^- (Q808) was crossed to *lys1*-131 *leu1*-32 *mat1*-102 (Q238) to form a stable diploid strain. The diploid cells were induced to haploidize by 5-fluorophenylalanine (Gutz *et al.*, 1974). Results showed no segregation of *leu1*-32 *sod2*-1 (lithium resistant *leu1*-32) or *leu*$^+$*sod2*$^+$ (lithium sensitive leu$^+$) in 100 randomly selected haploid colonies tested. Therefore the *sod2* locus was tentatively assigned to chromosome II. The diploid strain *sod2*-1/+ *ade6*-704/+ (Q809) was found to be lithium resistant indicating that the *sod2* mutation is dominant.

DNA sequencing of sod2

The *sod2* gene was isolated as indicated in Results. In order to sequence the 2.3 kb *Hin*dIII fragment containing the *sod2*$^+$ gene, two plasmids, pSOD2.6 and pSOD2.9 were used to create unidirectional deletions using exonuclease III and S_1 nuclease by the method of Henikoff (1987). A single-stranded plasmid template was used for chain termination sequencing reactions as described in Sanger *et al.* (1977) and Biggin *et al.* (1983). The sequence of *sod2*-1 (cDNA) was determined by using denatured double-stranded DNA as templates and various synthetic oligonucleotides as primers. The products of sequencing reactions were resolved on 8% polyacrylamide gels (Maxam and Gilbert, 1977). Fully overlapping DNA sequence in both orientations was obtained.

Plasmid constructions

The structure of various plasmids is shown in Figure 2. A pSOD2.1 plasmid was digested with *Pvu*II and religated to create pSOD2.2. The small *Pvu*II fragment from pSOD2.1 was inserted into the unique *Pvu*II site of pFL20 (Clarke *et al.*, 1986) to create pSOD2.3. Both 2.3 kb and 3.5 kb *Hin*dIII fragments were respectively subcloned into pWH5 (Wright *et al.*, 1986) to produce pSOD2.4 and pSOD2.5. The 3.5 kb *Eco*RI–*Sal*I fragment was subcloned into pUC118/119 to form pSOD2.6 and pSOD2.7. The 2.3 kb *Hin*dIII fragment was inserted into pUC118/pUC119 to produce pSOD2.8 and pSOD2.9. The *Nde*I site at 441 bp within the *sod2* gene in pSOD2.8 was removed to form pSOD2.10, and a new *Nde*I site was created respectively at 1 bp, 392 bp and 614 bp pf pSOD2.10 by oligomutagenesis (Zoller and Smith, 1983). An *Nde*I–*Xba*I fragment from each of three subclones was inserted into pART5 (McLeod *et al.*, 1987) resulting in pSOD2.11, pSOD2.12 and pSOD2.13. The intron sequence was removed from pSOD2.10 by oligomutagenesis using a 22mer overlapping the intron junctions resulting in an intronless plasmid, pSOD2.14. A new *Nde*I site was created at 1 bp of pSOD2.14 and an *Nde*I–*Xba*I fragment was moved to pART5 to create pSOD2.15. The internal 0.8 kb *Bcl*I fragment was excised from pSOD2.8 and replaced with a 1.8 kb HindII fragment bearing the *S.pombe ura4*$^+$ gene or 2.2 kb *Hin*dIII fragment of the *S.cerevisiae LEU2*$^+$ gene resulting in the gene disruption plasmids pSOD2.16 and pSOD2.17.

RNA preparation, S_1 mapping, poly(A)$^+$ isolation

Whole cell RNA was prepared from wild type and mutant cells grown in rich medium. S_1 probes were made from pSOD2.7 by primer extension (Burke, 1984). The probes were annealed with 20 μg of whole cell RNA, digested with S_1 nuclease and separated by gel electrophoresis as described by Hindley *et al.* (1987). The protected DNA fragments were resolved on 6% polyacrylamide gels.

Poly(A)$^+$ RNA was prepared using oligo(dT) cellulose as described by Sambrook *et al.* (1989). A *sod2* cDNA clone was isolated from a cDNA library of *sod2*-1 by colony hybridization.

Growth rate assessment

Salt tolerance was tested on solid media with the addition of various salts (NaCl, LiCl, CsCl, NH_4Cl or $CaCl_2$). Relative growth was assessed by measuring average colony diameter under 10 × objective after inoculation and growth for various times at 30°C. When measured on plates with a low population density the standard deviation for such measurements is ~10% and the results are reproducible. Growth in liquid culture was monitored by cell number at 2 h intervals using a Coulter Counter (Model ZF) following sonication to disrupt aggregates. The results were virtually the same between the plate and liquid assays in all growth response tests.

^{22}Na transport

For Na^+ transport experiments, cells were grown in low Na^+ EMM and harvested at a cell density of 5×10^6 cells/ml, washed with Mes–PIPES buffer (5 mM 1[*N*-morpholino]ethane sulfonic acid, 5 mM piperazine-*N*-*N*'-bis [2-ethane sulfonic acid]), adjusted to the desired pH with Tris (hydroxymethyl) aminomethane. The final cell pellet was resuspended in Mes–PIPES–Tris buffer at ~5×10^8 cells/ml. For Na^+ uptake, cells were diluted to 1×10^8 cells/ml in Mes–PIPES–Tris containing 6 mM NaCl plus 0.5 μCi/ml $^{22}NaCl$ (Amersham) at the desired pH. Incubation was at 30°C with agitation. 500 μl aliquots of the incubation culture were removed, added to 5 ml of 160 ml LiCl, 2.5 mM Tris and 4 mM Hepes pH 7.5, and the cells were collected by filtration on Millipore filters (0.45 μm, HA). After three washes, radioactivity on the filters was assessed with a scintillation counter. For Na^+ efflux studies cells were prepared as before and incubated at 1×10^8 cells/ml in Mes–PIPES–Tris, pH 7.0 buffer with 6 mM NaCl and 0.5 μCi/ml $^{22}NaCl$ for 1 h. The ^{22}Na loaded cells were collected by filtration, washed with the same buffer but with no ^{22}Na and resuspended in the same volume of Mes–PIPES–Tris buffer at the desired pH. The loss of ^{22}Na from the cells was assessed by harvesting 500 μl aliquots at 10 min intervals and the remaining ^{22}Na was measured as above.

TPP$^+$ accumulation

Cells were grown and washed as for sodium efflux experiments. Samples (~5×10^8 cells/ml) were then suspended and incubated with constant shaking in Mes–Pipes–Tris buffer pH 6.0 containing [^{14}C]TPP (1 μM, 0.02 μCi/ml, Amersham) (Eilam *et al.*, 1990). Samples were collected by filtration on glass fibre filters and washed by filtration with 10 ml of Mes–Pipes buffer containing 10 mM KCl. The KCl wash reduced adventitious binding of TPP to the filters. Under these conditions uptake saturated in 3–4 min and then remained constant for up to 1 h. For inhibitor experiments, the inhibitor was added at the same time as the TPP and incubation was for 20 min.

Measurement of proton flux

Proton flux was measured by following the change in pH of the external medium during incubation of cells. Cells were prepared as for sodium transport experiments at 5×10^8 cells/ml. Cells were incubated aerobically with rapid stirring at 25°C in 10 ml of 2 mM Tris–citrate buffer at pH 6.1. Proton movements were followed with an Accumet pH meter 915 connected to a strip chart recorder.

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Note added in proof

The sequence data reported here will appear in the EMBL/GenBank/DDBJ nucleotide sequence databases under the accession number Z11736.

Ivey *et al.* [(1991) *J. Biol. Chem.*, **266**, 23483–23489] report limited sequence homology between *nhaC* from *Bacillus firmus* isolated by plasmid complementation of an *nhaA* deletion strain of *E.coli* NM81 [Padan *et al.* (1989) *J. Biol. Chem.*, **264**, 20297] and the human Na^+/H^+ antiporter. The homology region extends from residue 355 to 522 in the human sequence. This overlaps and matches the latter part of the alignment (domain 12) for *sod2* shown in Figure 5.