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Mutations in *LIS1* (*ERG6*) gene confer increased sodium and lithium uptake in *Saccharomyces cerevisiae*

Ajith A. Welihinda [a], Andrew D. Beavis [b], Robert J. Trumbly [a,*]

[a] *Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, OH 43699, USA*
[b] *Department of Pharmacology, Medical College of Ohio, Toledo, OH 43699, USA*

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Abstract

A *Saccharomyces cerevisiae* mutant, *lis1–1*, hypersensitive to Li^+ and Na^+ was isolated from a wild-type strain after ethylmethane sulfonate mutagenesis. The rates of Li^+ and Na^+ uptake of the mutant are about 3–4-times higher than that of the wild-type; while the rates of cation efflux from the mutant and wild-type strains are indistinguishable. The *LIS1* was isolated from a yeast genomic library by complementation of the cation hypersensitivity of the *lis1–1* strain. *LIS1* is a single copy, nonessential gene. However, the deletion of *LIS1* from the wild-type results in a growth defect in addition to the cation hypersensitive phenotype. The order of increasing cation uptake rates of the wild-type and mutant strains, *LIS1* < *lis1–1* < *lis1-Δ1::LEU2*, correlates perfectly with the degree of cation hypersensitivity, suggesting that the cation hypersensitivity is primarily due to increased rates of cation influx. *LIS1* encodes a membrane associated protein 384 amino acids long. Data base searches indicate that *LIS1* is identical to *ERG6* in *S. cerevisiae* which encodes a putative *S*-adenosylmethionine-dependent methyltransferase in the ergosterol biosynthetic pathway. Cell membranes of *lis1* (*erg6*) mutants are known to be devoid of ergosterol and have altered sterol composition. Since membrane sterols can influence the activity of cation transporters, the increased cation uptake of the *lis1* mutants may stem from an altered function of one or many different membrane transporters.

Key words: Sodium ion transport; Lithium ion transport; Hypersensitivity, Na^+ and Li^+; Ergosterol mutant; Yeast

1. Introduction

Yeast, like all living cells, maintain K^+ at a high internal concentration (150 mM), while the concentrations of other monovalent cations including Na^+ and Li^+ are kept low. A plasma membrane H^+-ATPase plays a key role in this process by pumping protons out and creating a proton gradient across the membrane, which provides the driving force for the transport of alkali cations catalyzed by a number of different transporters. Two K^+ transporters which differ from one another by their affinities for K^+ have been identified in *S. cerevisiae* [1] and shown to be coded for by the genes *TRK1* [2] and *TRK2* [3]. Another non-specific cation transporter [4] also exists which is coded for by a mutant form of *HOL1*. Though transport pathways specific for Na^+ and Li^+ have not been described in yeast, both Na^+ and Li^+ can be taken up via K^+ transport systems [5,6].

As a means to investigate factors controlling monovalent cation transport in yeast, we have searched for mutations which render cells hypersensitive to Li^+ and Na^+ and examined those for altered transport properties. In this paper, we show that mutations in the *LIS1* gene result in a cation-hypersensitive phenotype which exhibits elevated rates of cation influx. Sequence analysis indicates that *LIS1* is identical to *ERG6*, which encodes a putative methyltransferase [7] which catalyzes the conversion of zymosterol to fecosterol.

Abbreviations: EMS, ethylmethane sulfonate; SAM, *S*-adenosylmethionine; ORF, open reading frame; kb, kilobase(s); bp, base pair(s); kDa, kilodalton(s); SDC, synthetically defined complete; YEPD, yeast extract 1%/peptone 2%/dextrose 2%; *LIS1*, gene responsible for Li^+ and Na^+-hypersensitivity; Lis1p, gene product of *LIS1* gene; *ERG6*, gene which encodes a methyltransferase in the ergosterol biosynthetic pathway.

* Corresponding author. Fax: +1 (419) 3827395.

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2. Materials and methods

2.1. Media, strains, and plasmid constructions

The *Escherichia coli* strain XL1 Blue (Stratagene) was used for propagation of plasmids. *S. cerevisiae* strains used in this study are listed in Table 1. The genetic methods and standard media were previously described [10]. Sodium-free medium was essentially similar to Wickerham's chemically defined media [11] except $[NH_4]_2MoO_4$ was added in place of Na_2MoO_4 and potassium concentration was adjusted to 5 mM by adding KCl. Sodium was removed from agar prior to addition to Na-free medium by washing in 1 M and then 0.05 M Tris-HCl (pH 6.0) followed by three washings in double distilled water [3]. Plasmid pAW7 was constructed by ligating a 2.1 kb fragment resulting from the *Xba*I and *Sal*I digestion of pAW3 into the same sites of pRS316 [9]. Plasmid pAW11 was constructed by ligating the 5.1 kb *Sph*I fragment of pAW3 into the same sites of YCp50 [12]. To create pAW14, the *Sph*I–*Ase*I fragment (2.5 kb) containing *LIS1* was first subcloned into the *Sph*I and *Nde*I sites of pT7Blue(R) (from Novagen) and the fragment containing *LIS1* was excised using *Hin*dIII and *Bam*HI and recloned into the same sites of pRS316. Plasmid pAW12 was made by subcloning the *Sph*I–*Ase*I fragment (2.5 kb) containing *LIS1* into the *Sph*I and *Nde*I sites of pJDH118 [13]. Plasmid pAW15 carrying *lis1–2::URA3* was created by inserting *URA3* on a *Xba*I fragment (1.1 kb) into an internal *Xba*I site of *LIS1*. Plasmid pAW17 was constructed by subcloning the *Bam*HI–*Hin*dIII fragment of pAW14 containing *LIS1* into the same sites of the vector pRS426 [14]. The *lis1-Δ1::LEU2* mutation on plasmid pAW18 was created by inserting the *LEU2* gene carried on 1.8 kb *Nhe*I–*Sma*I fragment into the *Apa*I and *Xba*I sites within *LIS1* on pAW12, after the *Apa*I site had been made blunt with T4 DNA polymerase. Plasmid pAW19 was made by subcloning the 1.1 kb *Sca*I–*Nhe*I fragment of *LIS1* into *Sma*I and *Xba*I sites of pATH20 [15].

Yeast strains RTY755, RTY756 and RTY757 containing either a deletion or an insertion within the *LIS1* gene were generated by the method of one step gene disruption [16]. RTY234 cells were transformed with gel-purified *Pvu*II fragments of the above mentioned constructs containing the disrupted *lis1* and selected for leucine or uracil prototropy. Strains RTY757 and RTY795 were generated by transforming RTY462 and RTY465, respectively, with the *lis1-Δ1::LEU2* deletion construct. RTY234 cells were transformed with pAW17 to obtain the multi-copy *LIS1* strain RTY759. Strains RTY761 and RTY762 were made by crossing RTY564 with RTY462 and RTY757, respectively. Strain RTY796 was obtained by transforming RTY234 strain with a restriction fragment containing the *LEU2* gene and selecting for leucine prototrophy.

2.2. Isolation of lithium-sensitive mutants

RTY234 cells were mutagenized with ethylmethane sulfonate (EMS) as described by Lawrence [17]. Cells were plated on SD complete (SDC) medium and replica plated onto SDC plus 50 mM lithium acetate (adjusted to pH 7 with LiOH) and Li^+-sensitive mutants were recovered and purified on SDC medium to homogeneity.

2.3. Li^+ and Na^+ uptake assays

To cultures grown to mid log phase in SDC medium, either LiOAC or NaOAC was added to a final concentration of 20 mM. Aliquots of 15 ml were removed at

Table 1
Yeast strains

Strain	Genotype	Source
RTY234	a *his4–519 leu2–3,-112 ura3–52*	[8]
YPH102	α *ade2–101 leu2-Δ1 lys2–801 his3-Δ200 ura3–52*	[9]
YPH274	a/α *ade2–101 leu2-Δ1 lys2–801 his3-Δ200 ura3–52* / *ade2–101 leu2-Δ1 lys2–801 his3-Δ200 ura3–52*	[9]
RTY564	a *his4–519 leu2–3,112 ura3–52 lis1–1*	this study
RTY755	a *his4–519 leu2–3,112 ura3–52 lis1-Δ1::LEU2*	this study
RTY756	a *his4–519 leu2–3,112 ura3–52 lis1–2::URA3*	this study
RTY757	α *ade2–101 leu2-Δ1 lys2–801 his3-Δ200 Δlis1::LEU2*	this study
RTY761	a/α *leu2–3,112 ura3–52 lis1–1 his3-Δ200 his4–519* / *leu2-Δ1 ura3–52 LIS1* + +	this study
RTY762	a/α *leu2–3,112 ura3–52 lis1–1 his4–519 his3-Δ200* / *leu2Δ1 ura3–52 lis1-Δ1::LEU2* + +	this study
RTY795	a/α *ade2–101 leu2-Δ1 lys2–801 his3-Δ200 ura3–52 LIS1* / *ade2–101 leu2-Δ1 lys2–801 his3-Δ200 ura3–52 lis1-Δ1::LEU2*	this study
RTY796	a *his4–519 leu2–3,-112 ura3–52 LEU2*	this study

various time points, centrifuged at 3000 rpm at 4°C for 3 min in an IEC Centra-8R centrifuge (International Equipment Co.) and washed once with double-distilled ice-cold water. The cell pellet resulting from each sample was weighed, resuspended in 1.2 ml of extraction buffer (100 mM NH_4OAc, 4 mM CsCl, pH 4.55), boiled for 5 min, and centrifuged. The Li^+ and Na^+ content of the supernatant was measured by atomic absorption spectroscopy (Perkin-Elmer 5000 Atomic Absorption Spectrophotometer) using Li^+ and Na^+ specific lamps. The cation content of each sample was calculated as mmol/kg of cells. To express intracellular cation concentration in mM, the values were divided by 0.55 l kg^{-1}, the intracellular volume per kg of cells [18].

2.4. Li^+ efflux assay

To log phase cultures of RTY234 and RTY564, LiOAc was added to 100 mM and 20 mM, respectively, and incubated for 2 h. Cells loaded with Li^+ were harvested as above and resuspended in SDC medium. Aliquots of the cultures were removed at 15-min intervals and intracellular Li^+ concentrations were determined as above.

2.5. Cloning of LIS1

The *LIS1* gene was cloned by electroporating [19] the strain RTY564 (see Table 1) with a genomic library in YCp50 carrying the *URA3* selectable marker. Approximately 22400 transformants were screened for complementation of the mutant phenotype of Li^+ sensitivity. Plasmids from eight independent *LIS1* transformants were propagated in *E. coli* XL1 Blue strain, then retransformed into RTY564 to reconfirm their complementation ability. Restriction analysis revealed that all eight plasmids contained the same insert. Several subclones were made from the original plasmid pAW3 and the plasmid containing the shortest genomic fragment which complemented the mutation, pAW14, was retained for further analysis (see Fig. 3).

2.6. DNA sequence analysis of LIS1

A nested set of exonuclease III deletion derivatives of pAW14 was generated by the method of Henikoff [20] using the Erase-a-Base kit from Promega Corporation. DNA sequence analysis was performed according to Sanger et al. [21] using Sequenase (United States Biochemical Corp.) and [α-^{35}S]dATP (Amersham). Double-stranded sequencing was carried out on miniprep DNA prepared according to the method of Sal et al. [22]. Sequence of the second strand was achieved by making nested deletions in the reverse orientation.

2.7. Construction of trpE::LIS1 gene fusion

An in-frame gene fusion was created between *E. coli trpE* gene and *LIS1* by ligating the *Sca*I–*Nhe*I fragment of *LIS1* into the *Sma*I and *Xba*I sites of the pATH20 vector [15]. The resulting plasmid, pAW19, encoded a 72 kDa fusion protein containing 338 amino acids in the carboxy terminus of *LIS1* fused to the TrpE protein. Isolation of the hybrid protein and subsequent purification was carried out according to Koerner et al. [15].

2.8. Preparation of antiserum

Approximately 50 μg of gel-purified TrpE-Lis1 hybrid protein was emulsified either with Inject Alum or Freund complete adjuvant and injected into rabbits subcutaneously and intramuscularly. Similarly, booster doses were given in either Inject Alum or Freund incomplete adjuvant after 7, 11, and 15 weeks. Antiserum was collected 3 weeks after the third injection. *Lis1* antibodies were affinity purified by incubating with antigen adsorbed to nitrocellulose paper and by subsequent elution with 0.1 M glycine and 0.1 M NaCl (pH 2.5) as described by Smith and Fisher [23].

2.9. Hybridizations

Nucleic acid hybridizations were carried out as described in Sambrook et al. [24]. The glass bead method of Hoffman and Winston [25] was adapted to isolate yeast genomic DNA. Total RNA was isolated as described by Schmitt et al. [26]. Restriction fragments for probes were purified by gel electrophoresis using the Gene Clean kit from Bio 101. Probes were labelled with [^{32}P]dATP using the Prime-A-Gene kit from Promega.

2.10. Western blots

Yeast cells were broken as described by Williams et al. [27] and proteins were extracted by boiling with an equal volume of 2 × Laemmli buffer [28] for 3 min. The supernatant was recovered and the proteins were quantified by the method of Zaman and Verwilghen [29]. The proteins were then separated by SDS-polyacrylamide gel electrophoresis in 10% acrylamide gels [28] and transferred electrophoretically to nitrocellulose membranes. Western blotting was performed by standard procedures [30] using alkaline phosphatase conjugated goat anti-rabbit secondary antibodies (Bio-Rad Laboratories).

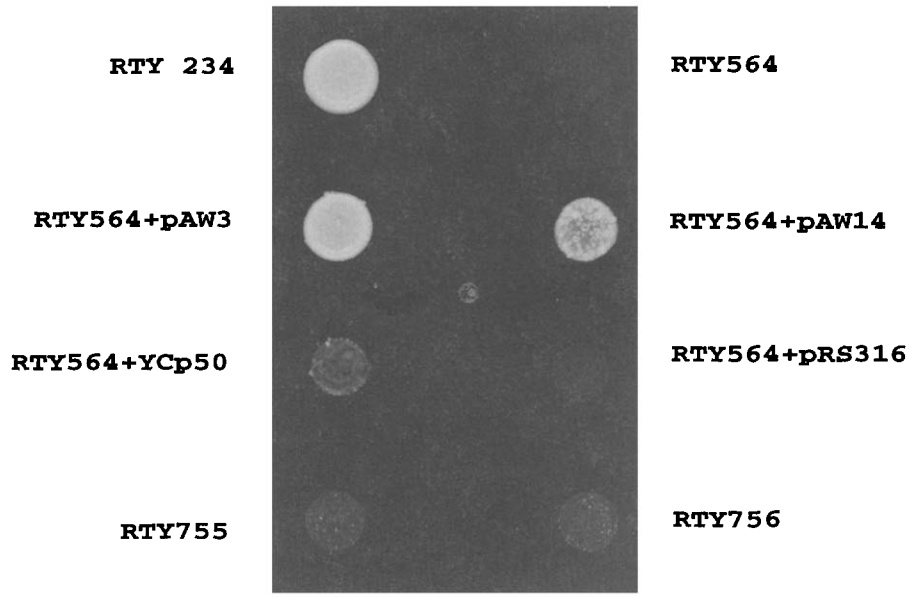

Fig. 1. Effect of Li^+ on the growth of wild-type and *lis1* mutant strains. Cells were grown to the same density in liquid SDC medium or medium without uracil and spotted onto SDC plates containing 50 mM LiOAc. Strains: RTY234 (*LIS1*), RTY564 (*lis1–1*), transformants of RTY564 harboring plasmids pAW3 (original genomic clone), pAW14 (subclone containing *LIS1* gene on a *Sph*I–*Ase*I fragment; see Fig. 3), YCp50 (vector control) and pRS316 (vector control), RTY755 (*lis1-Δ1::LEU2*) and RTY756 (*lis1–2::URA3*).

3. Results

3.1. Li^+- and Na^+-hypersensitive mutants

Preliminary experiments showed that growth of wild-type strains is almost completely inhibited by 200 mM Li^+ but only moderately inhibited by 50 mM Li^+, consequently we chose the latter concentration to screen for lithium-hypersensitive mutants. By screening 7000 colonies resulting from EMS mutagenesis, we isolated 13 independent yeast mutants which could not grow in the presence of 50 mM LiOAc. However, 50 mM KOAc had no growth inhibitory effects on any of these mutants. Only one of the mutants, RTY564, was sensitive to both 50 mM LiOAc (Fig. 1) and 50 mM NaOAc. In addition, this strain was more sensitive to acetate salts than to chloride salts and was the most stable on SDC media with respect to reversion to the wild-type lithium-insensitive phenotype. When RTY564 was crossed with wild-type strain RTY462, the result-

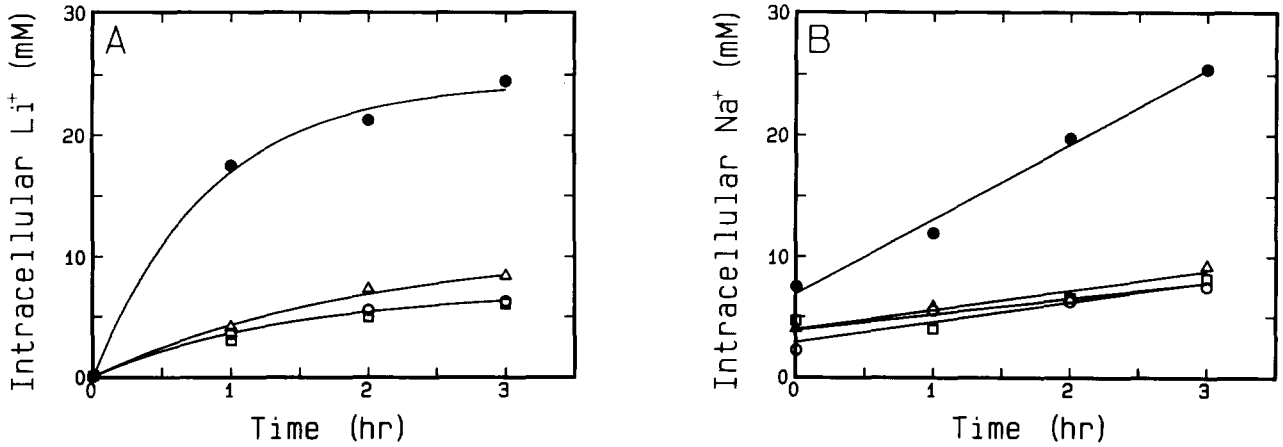

Fig. 2. Uptake of Li^+ (A) and Na^+ (B) by wild-type, *lis1* mutants and transformants. Cells were grown to mid-log phase in SDC medium and either LiOAc or NaOAc was added to a final concentration of 20 mM. After continued incubation at 30°C, samples of the cultures were harvested by centrifugation and intracellular Li^+ and Na^+ content was determined by atomic absorption spectroscopy. ○, RTY234 (*LIS1*); ●, RTY564 (*lis1–1*); □, the transformants of RTY564 harboring plasmids pAW3 (genomic clone); △, RTY564 carrying plasmid pAW14 (subclone containing complete *LIS1* gene).

ing diploid was able to grow on 50 mM LiOAc equally well as the wild-type, indicating that Li^+ hypersensitivity in the mutant strain *lis1–1* is recessive. Subsequent analysis of the segregation pattern revealed a 2 *lis1*:2 *LIS1* pattern among the meiotic segregants demonstrating that Li^+-hypersensitive phenotype in *lis1–1* strain is due to a mutation in a single gene.

3.2. Increased Li^+ and Na^+ uptake in lis1–1 strain

As the Li^+ hypersensitivity in the mutant strain could be due to either decreased efflux or increased uptake, we investigated both possibilities. Li^+ and Na^+ uptake were assayed in exponentially growing and stationary phase cultures of wild-type and *lis1–1* strains using atomic absorption spectroscopy. In exponentially growing *lis1–1* cells the uptake rates of both ions were about 3–4-times faster than those determined for the wild-type (Fig. 2); however, in the stationary phase, the uptake rates in both strains were negligible. The efflux rates of these ions determined for the *lis1–1* strain and the wild-type were essentially indistinguishable (data not shown). These data suggest that the Li^+ and Na^+ hypersensitivity of the *lis1–1* strain is due to an increased rate of cation uptake rather than decreased efflux.

To investigate the basis for the difference in cation sensitivity in acetate and chloride salts in the *lis1–1* strain, we determined the Li^+ uptake rates in LiOAc and LiCl media. In the presence of acetate, the uptake rate was about 2.5-times faster than in the presence of chloride. A similar difference was also observed with the wild-type strain.

3.3. Cloning of the LIS1 gene

The *LIS1* gene was cloned on the basis of its ability to complement the Li^+ and Na^+ hypersensitive phenotype in the *lis1–1* cells. The *lis1–1* cells harboring the plasmid pAW3, which was isolated eight times independently, showed a Li^+-insensitive wild-type phenotype whereas the vector alone did not complement the mutant phenotype (Fig. 1). Retransformation of pAW3 propagated in *E. coli* into the *lis1–1* strain resulted in a Ura^+ Lis^+ phenotype. Moreover, plasmid loss experiments demonstrated that Ura^+ and Li^+-insensitive phenotypes were linked in the transformants.

The region complementing the Li^+-hypersensitivity was further defined by constructing subclones of the 10-kb insert of pAW3 in the vector pRS316. The resulting subclones were transformed into a *lis1–1* strain and tested for their ability to confer growth on 50 mM LiOAc. As shown in Figs. 1 and 3, the ability to complement *lis1–1* resides in a 2.5-kb *Sph*I–*Ase*I fragment present in pAW14. The plasmids pAW3 and pAW14 also restored the rates of Na^+ and Li^+ uptake

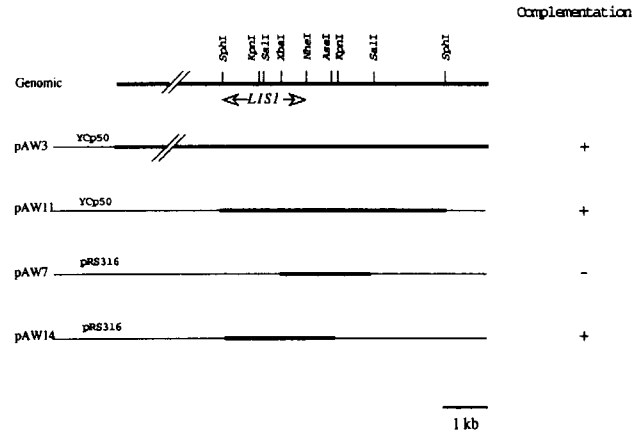

Fig. 3. Restriction map and localization of the *LIS1* gene. Isolation of the genomic clone (pAW3) and the construction of the subclones pAW7 (2.1 kb *Xba*I–*Sal*I fragment in pRS316) pAW11 (5.1 kb *Sph*I–*Sph*I fragment in YCp50) and pAW14 (2.5 kb *Sph*I–*Ase*I fragment in pRS316) are described in Materials and methods. *LIS1* ORF is marked. Complementation means the ability of the plasmid to restore growth on SDC medium containing 50 mM LiOAc.

in the mutant to the wild-type values (as shown in Fig. 2).

3.4. DNA sequence analysis of the LIS1 gene

The nucleotide sequence of the *LIS1* gene was determined by DNA analysis of the exonuclease III deletion derivatives of the plasmid pAW14. Analysis of the sequence by the start/stop method revealed a single long open reading frame (ORF) of 1152 bp (Fig. 4). Fickett's analysis [31] for coding regions also revealed that this region had 98% coding probability in all six reading frames. This is consistent with Northern blot analysis which showed a 1.35 kb RNA species with a *LIS1*-specific probe (Fig. 5). A potential TATA box [32] and a transcription initiation site [33,34] were found 110 and 35 bases upstream of the first ATG. A consensus sequence for a transcription termination [35] was found between nucleotides 1518 and 1550. Assuming that translation initiates at the first ATG, the sequence predicts an unmodified 43560 Da protein 384 amino acids long. The *LIS1* protein is not predominantly hydrophobic and does not contain any membrane-spanning domains. However, there are three fairly hydrophobic regions towards the C-terminus. Interestingly, using the computer program PROSITE, we identified a perfect match to the consensus sequence for an ATP/GTP binding site (GXXXXGKS; Fig. 4), which is also present in the Ras family of proteins [36], elongation factors [36], and proteins involved in active transport [37]. Using this program we also have found a good PEST sequence [38] at the C-terminus from amino acid 268 to 384. Searching both DNA and protein data bases (using blast network service at NCBI) with *LIS1*

sequence revealed 99% nucleotide and amino acid identity between *LIS1* and *ERG6* [39]. The discrepancies are 9 mismatches in the 5′-flanking region, absence of nucleotides AAG (position 1417–1419) which results in a shorter protein lacking glutamic acid[381], and one mismatch in the 3′-flanking region. A significantly high homology with methylase encoded by *EryG* in *Saccharopolyspora erythraea* [40] was observed. A perfect amino acid sequence identity between *LIS1* and two conserved motifs of *S*-adenosylmethionine (SAM)-dependent methyltransferases also became evident (Fig. 6). Homology searches done with flanking sequences of *LIS1* disclosed the identity of its neighboring genes; *PDR4* (= *YAP1*) on the 5′ and *SPT5* on the 3′ ends of *LIS1*. An additional partial ORF (774 bp) reported along with *PDR4* sequence [44] overlap with N-terminal coding region of *LIS1*. The map positions of *PDR4* [45], *SPT5* [46], and *ERG6* [47] have been determined and they reside very close to the centromere on chromosome XIII. Since *ERG6* mutants show increased resistance to nystatin [7] and increased sensitivity to cycloheximide [47], we tested both *lis1-1* and *lis1-Δ::LEU2* strains for such phenotypes. As expected, *lis1* mutants grew well in SDC-containing nystatin (4 μg/ml) whereas the wild-type did not. On SDC-containing cycloheximide (0.05 μg/ml), the mutants failed to grow while the wild-type showed good growth. Gaber et al. [47] have demonstrated that *erg6* mutants do not have C^{24}-methylated sterols, arguing that *ERG6* is indeed a methyltransferase. In view of these findings, we conclude that *LIS1* is identical to *ERG6* and encodes a putative SAM-dependent methyltransferase.

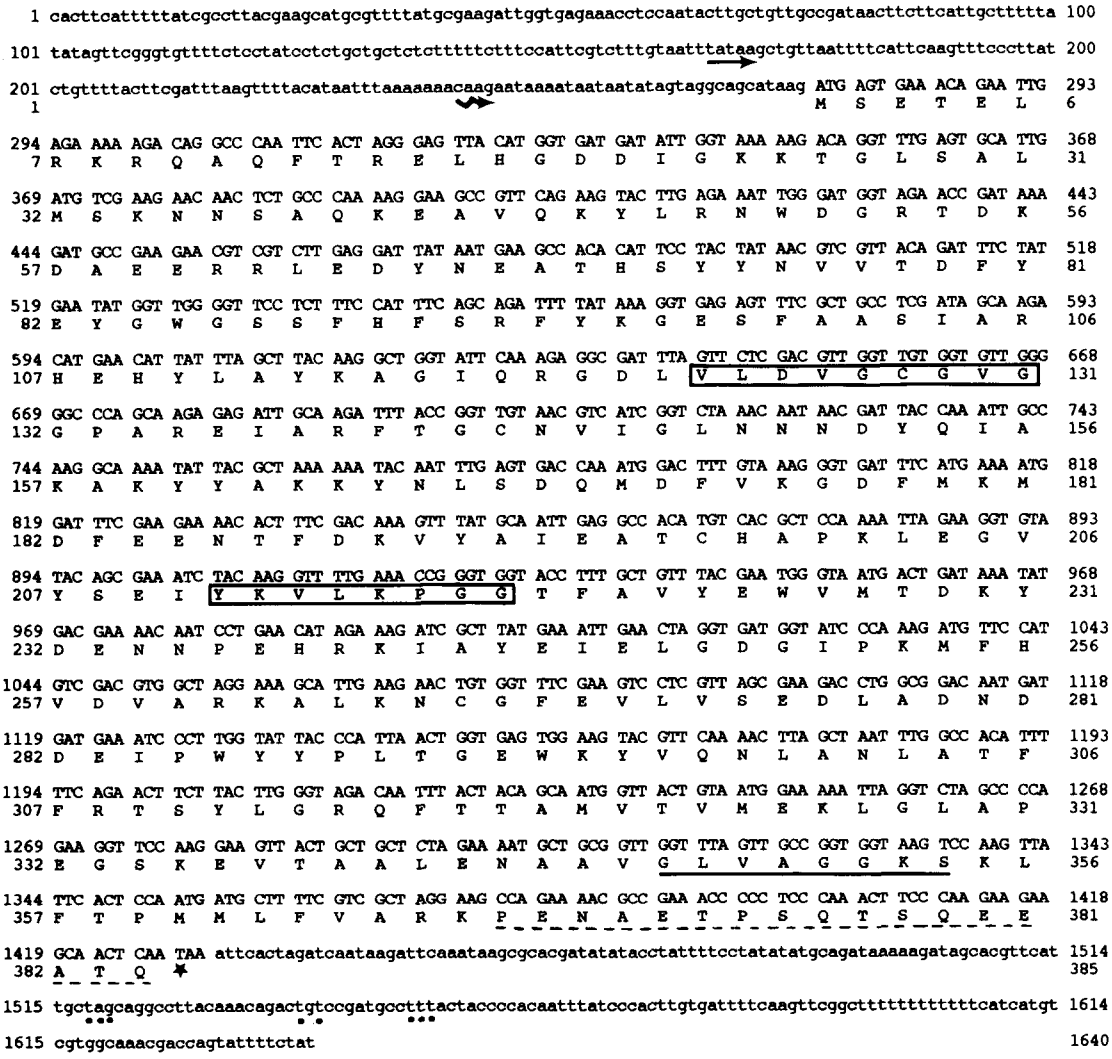

Fig. 4. Nucleotide sequence of the *LIS1* gene and the predicted amino acid sequence of its gene product. Upper and lower numbers on both sides represent the nucleotide and amino acid numbers, respectively. The termination codon is indicated by an asterisk. The motifs conserved in *S*-adenosylmethionine-dependent methyltransferases are boxed. The nucleotide binding motif (P loop) is underlined. The PEST sequence is marked by a dotted line. TATA box and transcription initiation site are indicated by straight and wavy arrows respectively. Proposed consensus for termination signals are denoted by underdots.

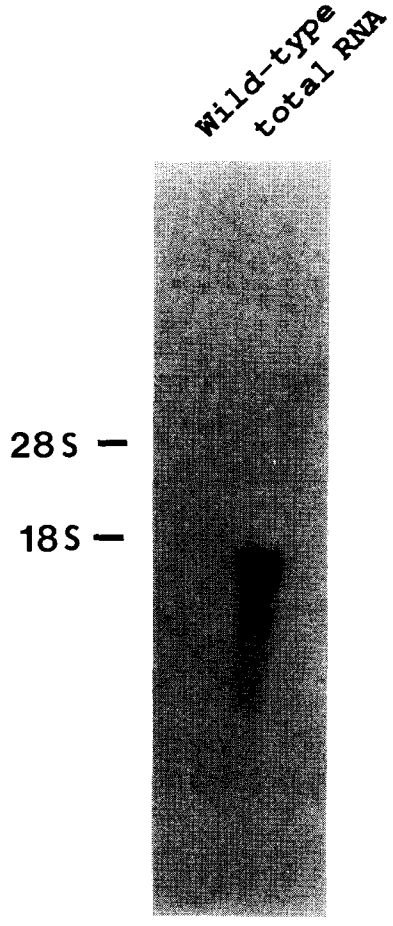

Fig. 5. Northern analysis of mRNA from RTY234 (*LIS1*). RNA was separated on a formaldehyde gel and transferred to nitrocellulose [24] then probed with *LIS1*-specific probe (1.5 kb *Sph*I–*Nhe*I fragment; see Fig. 7A). Hybridization and washings were done under conditions of high stringency [24]. Position of 18S and 28S ribosomal RNA bands are shown.

LIS1 (*ERG6*)	*S. cerevisiae*	^{123}**VLDVG**CG**VGG**^{132}	^{211}YKVL**KPGG**^{218}
DHHB MT (41)	*E. coli*	^{61}**VLDVG**CG**GG**I^{70}	^{146}AQLV**KPGG**^{153}
CR MT (42)	*S. peucetius*	^{183}**VLDVG**G**GKGG**^{192}	^{271}AEALE**PGG**^{278}
MT (40)	*S. erythraea*	^{85}**VLDVG**CG**LGA**^{94}	^{174}EFVL**KPGG**^{181}
A/IA MT (43)	*H. sapiens*	^{81}**ALDVG**CG**SGI**^{90}	^{169}IDQL**KPGG**^{176}

Fig. 6. Regions of sequence similarity of *LIS1* gene product with other SAM-dependent methyltransferases. Amino acid sequence positions are in superscripts. Conserved residues are in bold phase letters. DHHB MT, 3,4-dihydroxy-5-hexaprenylbenzoate methyltransferase; CR MT, carminomycin 4-O-methyltransferase; MT, methyltransferase; A/IA MT, D-aspartyl/L-isoaspartyl methyltransferase.

3.5. Cloned *LIS1* is allelic to *lis1* in RTY564

In order to find out whether or not cloned *LIS1* and the mutated gene in the strain RTY564 were the same, we performed the following crosses. RTY564 was crossed with the wild-type strain RTY462 (Table 1). The resulting diploids (RTY761) were insensitive to 50 mM LiOAc. However, the diploids (RTY762) resulting from the cross between RTY564 and RTY757 (*lis1-Δ1::LEU2*) were all sensitive to 50 mM Li^+. This lack of complementation between the null and the point mutant demonstrates that we have cloned the authentic *LIS1* gene. Furthermore, all the meiotic segregants of the diploid RTY762 were sensitive to 50 mM Li^+, confirming the authenticity of the *LIS1* gene.

3.6. *LIS1* is a single copy non-essential gene

We carried out Southern blots on RTY234 DNA digested with eight different restriction enzymes whose sites were not present within the *LIS1*. The *LIS1*-

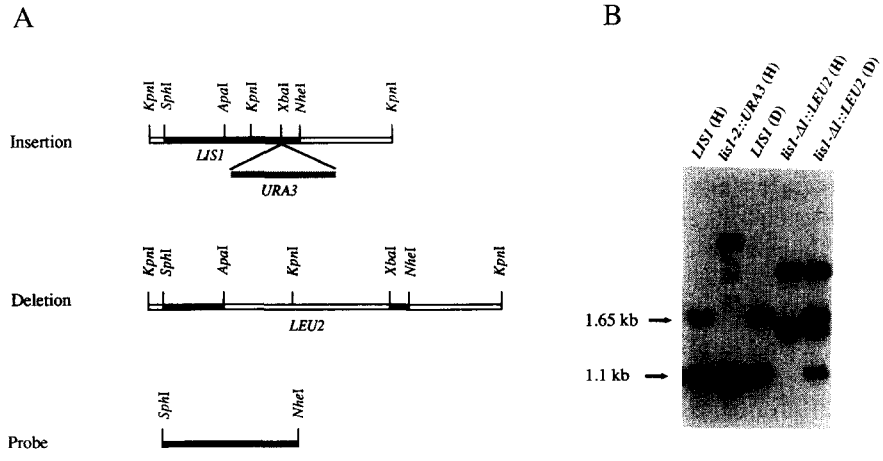

Fig. 7. *LIS1* gene disruption. (A) Genomic structure of *lis1–2::URA3*, *lis1-Δ1::LEU2* and the probe used for Southern blots. The filled-in bar corresponds to probe sequence. (B) Southern analysis of *lis1–2::URA3* and *lis1-Δ1::LEU2* strains. H and D denote haploid and diploid respectively. Genomic DNAs were digested with *Kpn*I. Hybridization and washings were done under conditions of high stringency [24].

specific probe recognized only one band in each case (data not shown), indicating that *LIS1* is present as a single copy per haploid genome. In order to find out other possible roles of *LIS1* in yeast biology, we constructed two strains carrying null alleles of *LIS1*. In RTY755, the *LIS1* open reading frame was disrupted by replacing the 627 bp *Apa*I–*Xba*I fragment with the *LEU2* gene. This construction removed amino acid residues 133 to 340 of *LIS1*. In the other null mutant, RTY756, *URA3* was inserted into the unique *Xba*I site in the 3′end of the *LIS1* coding region. Southern (Fig. 7) and Western (Fig. 9) analysis confirmed that the above mutants contained the disrupted *LIS1* gene. Both null mutants are viable, demonstrating that *LIS1* is not an essential gene for cell viability. However, the null mutants had slower growth rates compared to the wild-type on solid SDC and YEPD, and in liquid media *Δlis1* cells grow as clumps. The degree of cell aggregation increased with increasing Li^+ or Na^+ in the medium.

To examine whether the slower growth rate of the null mutant on SDC and YEPD media was due to an inherent growth defect or due to Na^+ in these media, we compared the growth of the wild-type and null mutant in Na-free synthetic medium. Since *leu2* strains grow slower than *LEU2* strains even in media containing leucine, RTY796, a leucine prototroph of RTY234, was used as the wild-type strain. The growth curves for these strains are shown in Fig. 8A and B. The wild-type grew 26% faster than the null mutant indicating a growth defect in the latter. In Na-free medium 20 mM LiOAc inhibited the growth of the null mutant by 71.4% (Fig 8B, open circles) whereas in SDC medium the same concentration of LiOAc inhibited almost completely (data not shown). Similar results were observed when cell numbers were used in place of A_{600} in the plots. The difference in sensitivity of the two strains to Li^+ is shown in Fig. 8C. Notice that the doubling time of the null mutant is twice that of the wild-type in the presence of 20 mM LiOAc. IC_{50} for the wild-type and the null mutant are 47 mM and 14.7 mM LiOAc, respectively.

Cation uptake rates in the deletion mutant were then compared with those of *lis1–1* and wild-type strains using 15 mM acetate salts. Since the cation uptake was growth-rate-dependent and the growth rate of the null mutant was about 5-times slower than that of wild-type, we normalized cation uptake to the respective growth rates. Under these conditions, *Δlis1* had the highest rate of cation uptake (3.4-times the wild-type) followed by *lis1–1* (1.8-times the wild-type). The reduction of fold difference in cation uptake between the mutants and the wild-type can be attributed to the low concentration of external Li^+ (15 mM) used in these experiments.

In the presence of 200 mM or more LiOAc, the wild-type cells grow unusually large and have enlarged vacuoles which occupy most of the cell. Similar results were recently obtained by Perkins and Gadd [48] who

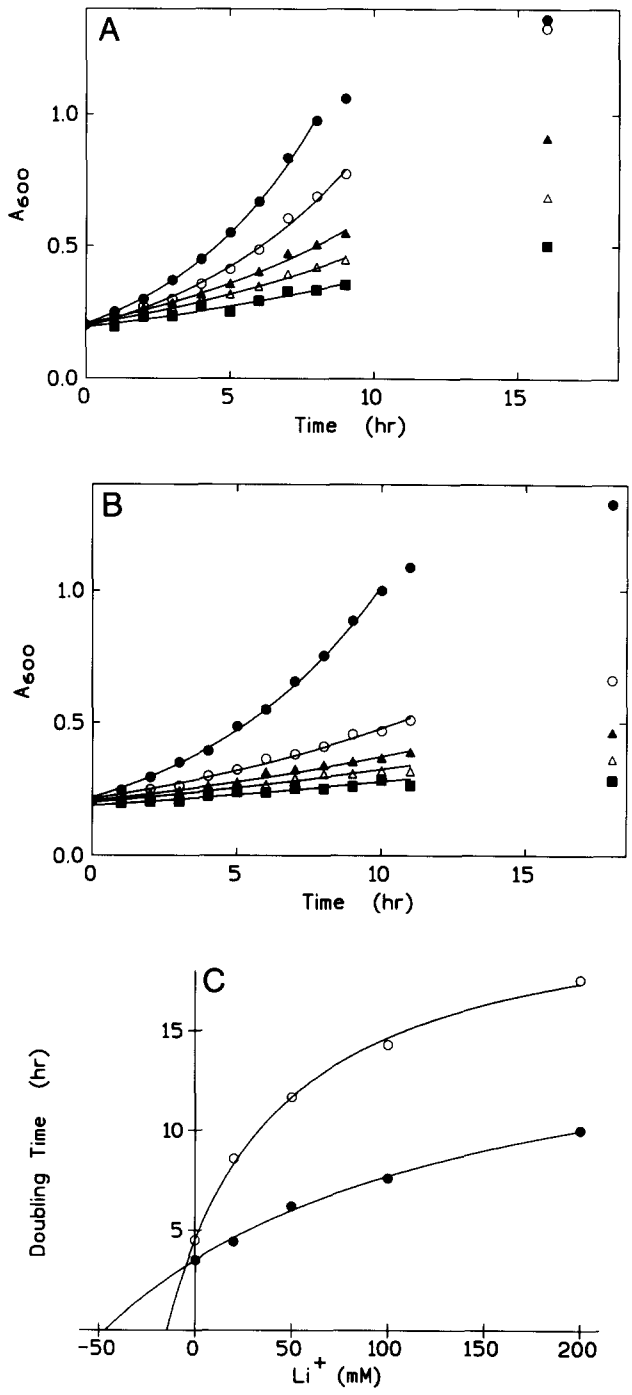

Fig. 8. Effect of Li^+ on the growth of (A) RTY796 (*LIS1*) and (B) RTY755 (*lis1-Δ1::LEU2*) in Na^+-free medium. Cultures of strains RTY234 and RTY755 were grown in Na^+-free medium to an A_{600} of 0.2 and divided into five flasks. LiOAc was added to final concentrations of ●, 0; ○, 20; ▲, 50; △, 100; and ■, 200 mM followed by continued incubation at 30°C. Aliquots were taken at the time points indicated and A_{600} was measured. (C) The difference in the sensitivity of ●, RTY796 (*LIS1*) and ○, RTY755 (*lis1-Δ1::LEU2*) to Li^+.

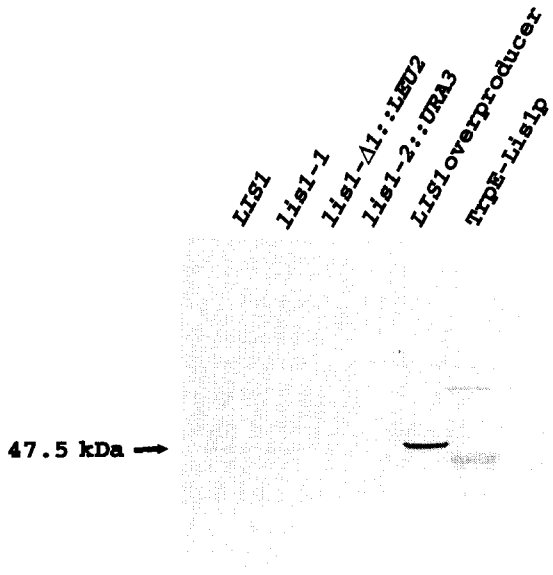

Fig. 9. Identification of the *LIS1* gene product by Western blotting. Samples containing 6.5 μg protein from yeast or 0.26 μg protein from *E. coli* cell homogenate were separated on SDS-PAGE, transferred to nitrocellulose and probed with affinity-purified antibodies for TrpE-Lis1 hybrid protein.

demonstrated that this phenomenon is due to compartmentalization of Li^+ in the vacuole. The *lis1–1* and *Δlis1* mutants showed enlarged vacuoles, and bigger cells at LiOAc concentrations as low as 50 mM and 20 mM, respectively (data not shown), consistent with the finding that deletion mutants have higher cation uptake rates compared with *lis1–1* strain. In order to find out the possible effects of overproduction of *LIS1* on ion transport, we overexpressed *LIS1* on a multi-copy plasmid in RTY234. Overproduction of *LIS1* gene product was confirmed by Western blots (Fig. 9). The overproducing strain, RTY759, was not sensitive to Li^+ or Na^+ and the cation uptake rates of RTY234 and RTY759 were indistinguishable (data not shown). Overexpression also did not render cells tolerant to higher levels of these cations.

3.7. Identification of the LIS1 gene product

Western blot analysis of total yeast proteins was performed using affinity-purified antibodies raised against TrpE-Lis1 hybrid protein. As shown in Fig. 9, a protein band of molecular mass 47.5 kDa was detected by the TrpE-Lis1 antibody. Moreover, this protein was more abundant in the *Lis1* overproducing strain, RTY759, and was absent in RTY755 (*lis1-Δ1::LEU2*) and RTY756 (*lis1-2::URA3*) strains. In order to find out whether Lis1p is a membrane-associated protein, we fractionated cell homogenate into soluble and crude membrane fractions. As detected by the Western blots, Lis1p associates with the membrane fraction (data not shown). Moreover, Lis1p could not be extracted from the membranes with 1 M NaCl.

4. Discussion

The *lis1–1* strain was initially isolated by genetic selection designed to identify the genes that are involved in monovalent cation transport in *S. cerevisiae*. To avoid selection for osmotolerant mutants lithium was used in the genetic screens, since it exerts growth inhibitory effects at much lower concentrations than K^+ and Na^+. The *LIS1* gene was cloned on the basis of its ability to complement the cation hypersensitive phenotype of *lis1–1* cells. The following genetic evidence supports the conclusion that the cloned gene is allelic to the mutated gene in *lis1–1* cells: (i) both the insertion and the deletion mutations of the *LIS1* gene result in phenotypes common to *lis1–1* cells. Moreover, these phenotypes are more pronounced in the deletion strain than the *lis1–1* strain. (ii) There is lack of complementation between RTY757 (*lis1-Δ1::LEU2*) and RTY564 (*lis1–1*) and all the spores resulting from this cross are sensitive to Li^+.

Several lines of evidence suggest that *LIS1* is identical to *ERG6*, which is the structural gene for *S*-adenosylmethionine:Δ^{24}-sterol *C*-methyltransferase in *S. cerevisiae* [7]. (i) Mutants of both *LIS1* and *ERG6* share common phenotypes such as hypersensitivity to cations and cycloheximide, increased resistance to nystatin, and decreased transformation efficiency. (ii) *LIS1* is located very close to the centromere of chromosome XIII, a region where *ERG6* has previously been mapped [47]. (iii) There is 99% sequence identity between *LIS1* and *ERG6* at both the nucleotide and the amino acid levels [39]. (iv) The *LIS1* sequence contains DVGXGXGX (putative SAM-binding site) and KPGG motifs common to methyltransferases. (v) Immunolocalization of the *LIS1* gene product to membrane fractions. Sterol biosynthesis is known to occur in the endoplasmic reticulum and the Δ^{24}-sterol methyltransferase activity has been localized to microsomal and lipid particles [49].

Decreased cell viability among *erg6* mutants upon exposure to hypertonic cation solutions has been observed by Bard et al. [50]. Since these authors exposed cells to hypertonic (2 M) solutions, it is not clear from their data whether the loss of cell viability was a result of osmotic shock or alteration of membrane permeability. However, using electron spin resonance analysis Kleinhans et al. [51] have provided evidence that the *erg2 erg6* double mutant is more permeable to Ni^+ than the wild-type. Using conventional transport assays, we have clearly demonstrated that it is the cation influx and not the efflux that is altered in the *lis1–1* cells. The order of increasing cation influx rates, *LIS1*

< lis1–1 < lis1-Δ1::LEU2, correlates perfectly with the degree of cation hypersensitivity, arguing that the cation hypersensitivity is primarily due to increased rates of cation influx.

In agreement with the findings of Gaber et al. [47], our results show that the *LIS1* (*ERG6*) is a single copy gene which is not essential for growth. Our observations agree with those by Hardwick and Pelham [39] in that the *LIS1* null mutant grows slower than the wild-type on standard yeast media. However, this finding is inconsistent with the results of the study by Gaber's group [47] which showed no growth defect in the null mutant. By comparing the growth rates in Na^+-free medium, we clearly demonstrated that the null mutant has a growth defect in addition to cation hypersensitivity. Perhaps transport systems for key nutrients such as amino acids and sugars are also defective in the mutant. Gaber et al. [47] have provided evidence for such a possibility by demonstrating a reduced tryptophan uptake in *erg6* null mutants. However, in our case the key nutrient in question cannot be tryptophan, since all the strains used in our study are Trp^+.

We found that cation uptake was dependent upon the growth phase. In log phase and stationary phase cultures, the membrane potentials are reported to be −127 mV and −68 mV [6], respectively. Since the cation uptake is driven by the membrane potential, the negligible uptake observed in the stationary phase may be related to the decreased membrane potential. The difference in the amount of the H^+-ATPase in log phase and stationary phase cultures, 50% and 25% of the total plasma membrane protein respectively [52], could account for the difference in the membrane potentials.

Our results show that *lis1* mutants are more sensitive to acetate salts than chloride salts of alkali cations. In aqueous solutions, LiOAc dissociates to yield HOAc. When cells are exposed to such extracellular permeant weak acids, an intracellular acidification has been observed in *S. cerevisiae* [53] and *Neurospora crassa* [54]. Such an acidification activates the H^+-ATPase, causing hyperpolarization of the membrane [53] which then enhances K^+ influx in *N. crassa* [55]. Similarly, in *S. cerevisiae*, a correlation between intracellular acidification and the subsequent enhancement of K^+ uptake has been reported [52]. Since K^+ and Li^+ are transported via the same pathways [5,6], this would explain the differences in transport rates and toxic effects seen between LiOAc and LiCl.

Mutations in *LIS1* result in a number of different phenotypes, all apparently related to membrane permeability. What is more striking is the differential permeabilities and sensitivities imposed by these mutations. For example, increased cation uptake (this study), decreased tryptophan uptake [47], increased sensitivity to cycloheximide [47] and increased resistance to nystatin [7]. As *lis1 (erg6)* mutants lack ergosterol [7,47] and have altered sterol composition [7], the simplest model to explain the altered permeability would be lesions (holes) in the membranes which result in a general 'leak'. However, the differential effects on permeability and the absence of any increase in cation efflux in the mutants argue against such a model. As the membrane sterols influence a number of cation transporters in vitro [56,57], it is reasonable to believe that a similar effect may occur in vivo. If that is the case, then the H^+-ATPase is quite possibly affected by the altered sterol composition created by the mutations in *LIS1*. Since H^+-ATPase is the primary pump, any change in its activity would in turn affect all secondary transport processes. The activity of the proton pump is tightly regulated. Its activation under different physiological conditions such as decreased intracellular pH and glucose metabolism appears to be regulated at the post-transcriptional level [58]. In the case of glucose metabolism, phosphorylation is the mechanism of regulation [59]. Portillo et al. [60] have shown that the carboxy-terminus of the H^+-ATPase contains a negative regulatory domain. Perhaps in the *lis1* mutants, the altered sterol composition affects the overall arrangement of H^+-ATPase in the membrane in such a way that these regulatory mechanisms are no longer functional, leading to hyperactivation of the pump. It is also possible that an altered sterol composition could affect the function of a number of different membrane transporters, leading to such phenotypes as decreased tryptophan uptake [47] and increased cation uptake.

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