Salt tolerance and methionine biosynthesis in *Saccharomyces cerevisiae* involve a putative phosphatase gene

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The progressive salinization of irrigated land poses a threat to the future of agriculture in arid regions. The identification of crucial metabolic steps in salt tolerance is important for the understanding of stress physiology and may provide the tools for its genetic engineering. In the yeast *Saccharomyces cerevisiae* we have isolated a gene, *HAL2*, which upon increase in gene dosage improves growth under NaCl and LiCl stresses. The *HAL2* protein is homologous to inositol phosphatases, enzymes known to be inhibited by lithium salts. Complementation analysis demonstrated that *HAL2* is identical to *MET22*, a gene involved in methionine biosynthesis. Accordingly, methionine supplementation improves the tolerance of yeast to NaCl and LiCl. These results demonstrate an unsuspected interplay between methionine biosynthesis and salt tolerance.

Key words: methionine/phosphatase/salt tolerance/yeast

Introduction

The genetic improvement of salt tolerance is an urgent need for the future of agriculture in arid regions (Downton, 1984; Flowers and Yeo, 1988). Classical genetic methodologies based on crosses between crop plants and salt tolerant relatives have already made some progress (Epstein *et al.*, 1980; Tal, 1985; Wyn Jones and Gorham, 1986). Genetic engineering has the potential to rapidly improve the salinity tolerance of crops (McCue and Hanson, 1990). The methodology to generate transgenic plants is readily available (Potrykus, 1991) but the limiting factor is the isolation of genes with the capability to improve salt tolerance, which we have called halotolerance genes (Gaxiola *et al.*, 1992). A scrutiny of cellular processes involved in the adaptation of micro-organisms and plants to salinity suggests that halotolerance genes could correspond to catalytic or regulatory components of either crucial defence responses (osmolyte synthesis, ion transport, etc.) or metabolic processes (protein synthesis, energy metabolism, etc.) most sensitive to salt stress (Serrano and Gaxiola, 1993). Transgenic tobacco expressing bacterial mannitol-1-phosphate dehydrogenase accumulates the osmolyte mannitol and

has an increased ability to tolerate high salinity (Tarczynski *et al.*, 1993).

A novel approach to the isolation of halotolerance genes and to the identification of cellular processes most crucial to salt tolerance has been the cloning of yeast genes that by overexpression improve growth under salt stress. The first halotolerance gene obtained by this approach, *HAL1*, seems to modulate potassium transport (Gaxiola *et al.*, 1992). We have now isolated a second yeast gene *HAL2*, capable of improving growth under salt stress. This gene is identical to the methionine biosynthetic gene *MET22* and the encoded protein shows homologies to inositol phosphatases.

Results

Isolation of *HAL2*

We have isolated a yeast gene, *HAL2*, which upon increase in gene dosage in centromeric plasmids (1–2 copies per cell; Rose and Broach, 1991) improves yeast growth in media with high concentrations (1–1.5 M) of NaCl (Figure 1A and B). A similar growth improvement was observed in media with 0.1–0.4 M of the more inhibitory salt LiCl. The gene has no effect on normal media and on media with the less inhibitory salt KCl, suggesting that its overexpression specifically counteracts Na and Li toxicities. No further improvement in salt tolerance was obtained when *HAL2* was overexpressed in 2 μ -derived (multicopy) plasmids, existing in 10–40 copies per cell (Rose and Broach, 1991). The growth advantage conferred by *HAL2* in NaCl-containing media is based both in a shorter lag phase and in a faster growth rate (Figure 2).

Although the gene was originally isolated from a genomic library of strain GRF88, the corresponding gene from the tester strain RS-16 has also been isolated by PCR and it conferred the same halotolerance phenotype. Also, the plasmid with the *HAL2* gene conferred salt tolerance to other yeast strains such as BWG1-7A.

HAL2 has homology to inositol phosphatases

HAL2 encodes a polar protein (Figure 1C) of 357 amino acids (39.1 kDa) with predicted pI = 6.14. The *HAL2* protein shows significant homology to a protein family recently identified by Neuwald *et al.* (1991) and which includes animal inositol phosphatases and fungal and bacterial regulatory proteins of unknown enzymatic activity. The two sequence motifs most conserved within the family are clearly present (Figure 3, motifs A and B) and there is good overall homology after alignment. The *HAL2* protein and bovine inositol polyphosphate 1-phosphatase contain an insertion of ~50 amino acids before motif A (Figure 3).

HAL2 is constitutively expressed in yeast

The level of *HAL2* mRNA in strains RS-16 and RS-569 is the same in cells growing in normal medium and in medium

Fig. 1. Isolation and sequence of the *HAL2* gene. (A) Restriction map of 5.8 kb of genomic DNA containing the *HAL2* gene. The coding region and direction of translation are indicated. B, *Bam*HI; Bg, *Bgl*III; E, *Eco*RI; H, *Hind*III; Hp, *Hpa*I. The *Hind*III site at the lower side of the map corresponds to a flanking plasmid site in one of the isolated clones. (B) Halotolerance test for the cloned gene. Strain RS-16 was transformed with the following plasmids: 1, YCp50 (control); 2, plasmid W81 (positive clone from YCp50 library); 3, YCp50 with 1.7 kb fragment extending from *Hpa*I to the *Hind*III at the lower side of panel A. Saturated cultures from three independent transformants of each plasmid were diluted and dropped on normal solid medium (-NaCl) and medium supplemented with 1.4 M NaCl (+NaCl). (C) Nucleotide sequence of 1.9 kb of DNA from *Hpa*I to the outer *Bgl*III site (panel A). The deduced amino acid sequence is indicated. The putative TATA box (TATAAA), the sequence for general amino acid control (TGACTC) and the sequence for specific methionine regulation (TCACGTG) are underlined.

supplemented with NaCl (Figure 4A). Strain RS-569 is a salt-resistant mutant isolated in our laboratory which contains a dominant mutation in a gene designated *HAL4* (R. Gaxiola, unpublished). This mutation does not affect the expression of *HAL2*. The specificity of RNA hybridization was demonstrated by utilizing oligonucleotide probes corresponding to the sense and antisense strands of the coding

Fig. 2. Effects of overexpressing *HAL2* on yeast growth. Open symbols correspond to strain RS-16 transformed with the *LEU2* centromeric plasmid pSB32 (Rose and Broach, 1991) and closed symbols to the same strain transformed with the *Hpa*I-*Hind*III fragment of *HAL2* (Figure 1A) subcloned into pSB32. At time zero saturated cultures were diluted 10 000-fold in uracil-supplemented SD medium (circles) and in the same medium containing 1.2 M NaCl (squares). Growth of the cultures was followed by their absorbance at 660 nm.

region. Only the antisense oligonucleotide hybridized with the RNA band of 1.1 kb (Figure 4B).

The *HAL2*-encoded protein was detected in the soluble fraction of the homogenate with specific antibody (Figure 4C). As predicted by the gene sequence, it exhibits an apparent molecular size of 39 kDa. Increased gene dosage in centromeric and multicopy plasmids produced significant increases in the amount of immunodetected protein (Figure 4D).

HAL2 is required for methionine biosynthesis and identical to *MET22*

In order to gain information about the mechanism of action of *HAL2*, a gene disruption (null mutation) was made by interrupting the coding region of *HAL2* with a piece of DNA containing the *URA3* gene (Figure 5). The only apparent phenotype of *HAL2*-disrupted cells is an auxotrophy for methionine. Therefore this gene seems to participate both in salt tolerance and in methionine biosynthesis. In accordance with this metabolic role, the promoter of *HAL2* contains sequence elements (underlined in Figure 1C) for general amino acid control (TGACTC; Donahue *et al.*, 1983) and for specific methionine control (TCACGTG; Thomas *et al.*, 1989; Korch *et al.*, 1991).

More than 20 genes seem to be involved in methionine biosynthesis in yeast (Thomas *et al.*, 1992). Complementation analysis was achieved by crossing haploid strains with a disrupted *HAL2* gene (*hal2::URA3*) with mutants defective in 16 different methionine genes (Table I). Diploids between *hal2::URA3* and methionine auxotrophs are able to grow in the absence of methionine with one exception: the diploid between *hal2::URA3* and *met22* remains auxotrophic for methionine. This suggests that *HAL2* is identical to *MET22*. This conclusion was reinforced by the cloning of the *MET22* gene by complementation of a *met22* mutant. The complementing plasmid pM22-4 was obtained from Dr H. Cherest (Laboratoire d'Enzymologie, Gif-sur-Yvette, France) and the fragment containing *MET22* was sequenced. The sequence of *MET22* is identical to the *HAL2* sequence shown in Figure 1C.

BOVimp	madpwqecmdy-avtllaggagevvrealknemnimviss-----padlVTATDQKVEKM	53
XELimp	medrwqecmdflavsiarkagsvvcalkedvsimvktsla----padlVTATDQKVEEM	56
BOVipp	msdilqellrvsekaaniaracrqqetlfqllieekkegeknkkfavdfKTLDVLVQEV	60
ScHAL2	maler-ellv---atqavrkaslltkriqsevishkdattitkndnspVTTGDYAAQTI	55
BOVimp	LITSIEKYPshs--FIGEESvaageksiltndnt-----	86
XELimp	IISSEIEKYPshs--FIGEESvaagagstltdnt-----	89
BOVipp	IKENMENKFPglgkklFIGEESneltndlgekiimrlgpteeetvallskvlnngnklasea	120
ScHAL2	IINAIKSNFPddk--VVGEESSsglsdafsvsgilneikandevynknykddflftndqf	113
(A)		
BOVimp	-----WIIDPIDGTTNFVHGfpfvavsigfvvnnk	116
XELimp	-----WIIDPIDGTTNFVHRfpfvavsigfavhkk	119
BOVipp	lakvvhqdvffsdpaldsveinipqdilglW-VDPIDSTYQYIKGSaditpnqgffpsgl	179
ScHAL2	plksledvrqiidfgnyeggrkgrf----WCLDPIIDGTKGFLRGsqf-avclalivdgv	167
BOVimp	mefgviyvsclcdkmytgrkgkgaafcngqklqvshqeditksllvtelgssrtpetvr-ii	175
XELimp	vefgvvyscvcdkmytgrkgkgsfcngqklqvsgqkditksmiitelgsnrnpefiktvs	179
BOVipp	qcvtvligvydiqtgvplmgvinqpfvsqdlhtrrwkgqcywglslgtnihsllppvst	239
ScHAL2	vqlgcicgpnlvlsygaqdlkghefgyifravrglgafyspssdaeawtkihvrhkd	227
BOVimp	lsnierllclpihgirvgtaalm-----CLV	203
XELimp	lsnmerllcpihgiravgtaanm-----CLV	207
BOVipp	rsnseaqsggtqnpsssegscrfsvvistseketikgalshvcgerifraagagykslCVI	299
ScHAL2	tkdmitlegvekghs-----shdeqtaiknknlskslhldsqaky----CIL	271
(B)		
BOVimp	AAGAADAYyemgihc-----WDVAGAGIIVTEAGGVLLDvtggpfdlmsrrviassnkt	257
XELimp	ATCGADAYyemglhc-----WDMAAASVIVTEAGGTILDatggldfmlmscriisassre	261
BOVipp	-LGLADIYifsedttfk---WDSAAHAAILRAMGGGMVDlkeclernpdtgldlpqlvy	354
ScHAL2	ALGLADVYlrpiklsyqeklWDHAAGNVIVHEAGGIHTDamedvpldfngnrtlatkgv	331
BOVimp	-----LAeriaKEIqiipqrdded-----	277
XELimp	-----IAeriaKEIqiiprddgkstn-----	284
BOVipp	hvgnegaagvdqwankgglIAyrseKQIetflsrllqhlavatht	400
ScHAL2	-----IASsgPREIhdlvvstscdviqsrna-	357

Fig. 3. The *HAL2* protein belongs to a family of putative inositol phosphatases. BOVimp, bovine inositol 1-phosphatase (Diehl *et al.*, 1990); XELimp, *Xenopus laevis* inositol 1-phosphatase (Wregett, 1992); BOVipp, bovine inositol polyphosphate 1-phosphatase (York and Majerus, 1990); scHAL2, product of the *S.cerevisiae HAL2 (MET22)* gene. The alignment was optimized by the program of Schuler *et al.* (1991). Blocks of similarities are in upper case letters and boxed. Other residues shared by the four sequences are in lower case letters and boxed. Residues shared by three of the four sequences are in bold letters. The two most conserved motifs (A and B) identified by Neuwald *et al.* (1991) are indicated.

Methionine supplementation improves salt tolerance

The fact that overexpression of a gene involved in methionine biosynthesis improves salt tolerance suggested that the methionine pathway is particularly sensitive to salt stress and that methionine supplementation could improve salt tolerance. This prediction was supported by the experiment of Figure 6. Methionine improves the growth of wild type yeast in media containing either LiCl or NaCl. Methionine supplementation has no significant effect in media with the less toxic salt KCl. Also, methionine has no effect on growth in normal medium without salts. Other amino acids such as cysteine, proline and asparagine were without significant effect. These results were corroborated with two different wild type strains (ATCC42407 and S288C; see Table I) and therefore seem to be of general significance. As predicted, if the effect of *HAL2* on salt tolerance depends on methionine biosynthesis, this gene has no effect on media containing NaCl and methionine.

Discussion

The identity between genes *HAL2* and *MET22* described in the present work demonstrates an unsuspected interplay between salt tolerance and methionine biosynthesis. *MET22* is a gene of unknown function required for sulphate assimilation (Thomas *et al.*, 1992).

The *MET22* protein shows significant homologies to

inositol phosphatases, enzymes known to be inhibited by lithium salts (Hallcher and Sherman, 1980; York and Majerus, 1990). Therefore it is plausible that the *MET22* protein is an enzyme very sensitive to salt inhibition and required for methionine prototrophy. This would explain why either overexpression of the protein or methionine supplementation improve growth under salt stress.

In higher plants application of proline in barley (Lone *et al.*, 1987) and asparagine in *Arabidopsis thaliana* (Lehle *et al.*, 1992) improve salt tolerance. To our knowledge, no information exists about methionine supplementation. Preliminary evidence in our laboratory with *A.thaliana* seedlings indicated that methionine supplementation improves the salt tolerance of shoots despite an inhibitory effect on root growth (H.-U.Gläser, unpublished). Therefore our observation of a connection between methionine and salinity may be of general application.

The three-dimensional structure of human inositol monophosphatase has recently been solved at 0.21 nm resolution and the conserved motifs depicted in Figure 2 seem to participate in binding of both the essential Mg^{2+} cofactor and phosphate (Bone *et al.*, 1992). Therefore, as suggested by Neuwald *et al.* (1991), it is very likely that all the proteins containing these motifs, including the *MET22* protein, are phosphatases. A bacterial gene with homology to inositol phosphatase, *cysQ*, is needed for cysteine synthesis in *Escherichia coli*. It may modulate the pool of 3'-phospho-

Fig. 4. Expression of *HAL2*. (A) Northern blot analysis of total RNA (20 μ g per lane) from strains RS-16 (1 and 2) and RS-569 (3 and 4) grown in normal medium (1 and 3) and in medium supplemented with 1 M NaCl (2 and 4). The hybridization probe was a 2 kb *Bgl*III fragment including most of the *HAL2* gene (Figure 1A). (B) Northern blot analysis with RNA (20 μ g per lane) from strain RS-16 grown in normal medium and probed with *HAL2* oligonucleotides. Lane 1, 'antisense' oligonucleotide; lane 2 'sense' oligonucleotide. In panels A and B the position of the ribosomal RNAs of 3.5 and 1.8 kb is indicated at the left. (C) Western blot analysis of yeast homogenates with antibody against *HAL2* protein. Lanes contained 20 μ g protein from soluble (lanes 1 and 3) and membrane (lanes 2 and 4) fractions immunodecorated with either preimmune serum (lanes 1 and 2) or antiserum (lanes 3 and 4). (D) Overexpression of *HAL2* protein in cells with increased gene dosage. Lane 1, strain RS-16 without plasmids; lane 2, RS-16 with extra *HAL2* genes in centromeric plasmid YCp50; lane 3, RS-16 with extra *HAL2* genes in multicopy plasmid YEp24. Each lane contained 20 μ g of protein of the soluble fraction of the homogenates. In panels C and D the concentration of acrylamide was 10 and 12%, respectively. The position of molecular size markers (kDa) is indicated by arrows at the left and the *HAL2* band by an arrow at the right side.

adenosine-5'-phosphosulfate, a phosphorylated intermediate in sulphate assimilation (Neuwald *et al.*, 1992). A similar function could be proposed for the *MET22* protein. Alternatively, the hydrolysis in inositol phosphates could have an essential role in methionine biosynthesis by unknown mechanisms.

Materials and methods

Isolation of the *HAL2* gene

A genomic library from *Saccharomyces cerevisiae* strain GRF88 in the shuttle plasmid YCp50 (Rose and Broach, 1991) was transformed into yeast strain RS-16 (Table I) and colonies exhibiting improved growth in medium containing 1.5 M NaCl were selected and plasmids analysed as described by Gaxiola *et al.* (1992). Two plasmids with overlapping inserts were isolated. In one (W81), the insert extended ~12 kb left of the genomic *Hind*III site at the right end of Figure 1A. In the other (F5), extensive rearrangements of the plasmid were observed and the insert extended 4 kb

Fig. 5. Disruption of the *HAL2* gene. Southern analysis of the four spores (A–D) of a tetrad derived from an autodiploid of strain RS-16 (Gaxiola *et al.*, 1992) where one copy of the *HAL2* gene has been disrupted by insertion of a 1.1 kb DNA fragment containing the *URA3* gene at the *Eco*RI site (Figure 1A). The growth of the four spores in minimal medium supplemented with leucine and either methionine or uracil is indicated.

left of a flanking *Hind*III site from the plasmid (middle of Figure 1A). In order to locate the halotolerance gene, restriction fragments were subcloned into YCp50, transformed (Ito *et al.*, 1983) into yeast strain RS-16 and tested for halotolerance as described below.

The *HAL2* gene from the RS-16 strain was isolated by PCR (Clackson *et al.*, 1991), utilizing 5'-CTCAAGCTTAACCCCTAGTCCAGAGTGC as 5' primer and 5'-CTCAAGCTTCCCAATTGGGAGATGCCGT as 3' primer, which introduced flanking *Hind*III sites (underlined). The amplified region corresponded to nucleotides 2–1893 of Figure 1C. It was purified (Tautz and Renz, 1983), digested with *Hind*III and subcloned into YCp50 digested with *Hind*III.

Growth and halotolerance tests

Minimal growth medium (SD) contained 2% glucose, 0.7% yeast nitrogen base without amino acids (Difco) and 50 mM MES adjusted to pH 6.0 with Tris. It was supplemented with uracil (30 μ g/ml) and amino acids (30 μ g/ml) as indicated. Before the halotolerance tests, cells were grown for 2 days to saturation (absorbance at 660 nm ~2.7). For testing in solid medium (2% agar) the saturated cultures were diluted 50-fold with water and 3 μ l were dropped on normal medium and on medium supplemented with 1.4 M NaCl. Growth was recorded after 7 days (Gaxiola *et al.*, 1992). For testing in liquid medium 100 μ l of saturated cultures were inoculated in 3 ml medium containing 0.2 M LiCl, 1 M NaCl or 1 M KCl and uracil and amino acids as indicated. Growth was recorded by turbidity measurements after 24 h.

DNA sequencing

Restriction fragments were subcloned into pBluescript plasmids (Short *et al.*, 1988) and both strands sequenced with phage T7 DNA polymerase (Tabor and Richardson, 1987) and walking primers.

Gene disruption

The 2 kb *Bam*HI fragment containing the first part of the *HAL2* gene (Figure 1A) was subcloned into a derivative of plasmid pUC18 (Yanish-Perron *et al.*, 1985) where the *Eco*RI site has been destroyed by *Eco*RI digestion, blunt-end generation with Klenow fragment of DNA polymerase and religation. An 1.1 kb *Hind*III fragment containing the *URA3* gene was obtained from plasmid YEp24 (Rose and Broach, 1991) and cloned with *Eco*RI linkers into the *Eco*RI site of the *Bam*HI fragment of *HAL2* (Figure 1A). The resulting 3.1 kb *Bam*HI fragment was excised from the plasmid and transformed (Ito *et al.*, 1983) into strain RS-784, an autodiploid of strain RS-16 (Gaxiola *et al.*, 1992). The transformed diploid was sporulated and tetrads dissected with a micromanipulator (Sherman and Hicks, 1991).

Table I. Yeast strains

Strain	Genotype	Source or reference
RS-16	<i>MATa ura3-251,328,372 leu2-3,112</i>	Gaxiola <i>et al.</i> (1992)
RS-1051	<i>MATa ura3-251,328,372 leu2-3,112 hal2::URA3</i>	This work
RS-1053	<i>MATα ura3-251,328,372 leu2-3,112 hal2::URA3</i>	This work
RS-784	<i>MATa/MATα ura3-52/ura3-52 leu2-1,112/leu2-3,112</i>	Gaxiola <i>et al.</i> (1992)
BWG1-7A	<i>MATa ade1-100 his4-519 ura3-52 leu2-3,112</i>	G.R.Fink
ATCC42407	<i>MATa suc⁻</i>	R.Lagunas
S228C	<i>MATα gal⁻</i>	G.R.Fink
RS-569	<i>MATα ura3-251,328,372 leu2-3,112 HAL4</i>	R.Gaxiola
YAH106	<i>MATa met1-1</i>	B.-I.Ono
STX6-1A	<i>MATa met2</i>	YGSC ^a
YAH54	<i>MATa met2 leu2-1 lys4</i>	B.-I.Ono
CC346-3B	<i>MATa ura3 met3</i>	Thomas <i>et al.</i> (1992)
YAH89	<i>MATa met4 leu2-1 rad1(ts) ura2</i>	B.-I.Ono
ONO379	<i>MATa met5 ade5 arg4 his7 ilv3 leu2 lys7 rad52-1 trp1</i>	B.-I.Ono
YAH99	<i>MATa met6 leu2-1</i>	B.-I.Ono
XJB3-1D	<i>MATa met6</i>	YGSC ^a
S1896D	<i>MATa met7</i>	YGSC ^a
YAH129	<i>MATa met8-1 leu2-1</i>	B.-I.Ono
ONO380	<i>MATa met10 ade4 his3 leu2-1 rad52-1 trp1 ura4</i>	B.-I.Ono
XS144-S129	<i>MATa met13</i>	YGSC ^a
YAH664	<i>Mata met13 cyh2 his4-166 ilv1-2 ura4-1</i>	B.-I.Ono
YAH63	<i>MATa met14 his6 leu2-1</i>	B.-I.Ono
CC362-2A	<i>MATa leu2 ura3 met16</i>	Thomas <i>et al.</i> (1992)
ONO317	<i>MATa met17</i>	B.-I.Ono
CC363-20B	<i>MATa leu2 ura3 met18</i>	Thomas <i>et al.</i> (1992)
CC370-8C	<i>MATα ura3 met20</i>	Thomas <i>et al.</i> (1992)
CC364-18C	<i>MATα his3 ura3 met22</i>	Thomas <i>et al.</i> (1992)
CC365-1A	<i>MATa his3 ura3 met25</i>	Thomas <i>et al.</i> (1992)

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Southern and Northern analysis

Genomic DNA (Winston *et al.*, 1983) was digested with *Bam*HI and 5 µg were utilized per lane. After electrophoresis and blotting to nylon it was hybridized at high stringency (50% formamide and 0.75 M NaCl at 42°C) with the 2 kb *Bam*HI fragment (Figure 1A) labelled with ³²P by the random priming method (Feinberg and Vogelstein, 1983). Total RNA was extracted (Carlson and Botstein, 1982) from exponentially growing cells (absorbance at 660 nm 0.3–0.4) in normal minimal media and in media supplemented with 1 M NaCl. Samples were separated by electrophoresis in formaldehyde gels, blotted to nylon and hybridized as described above. The probes were either the 2 kb *Bgl*II fragment (Figure 1A) labelled with [α -³²P]dCTP (Amersham) by the random priming method or two oligonucleotide probes corresponding to positions 688–717 (amino acids 72–81, Figure 1C). One oligonucleotide (5'-TGAAGAATCCTCATCAGGATTGAGCGACGC) represents the sense strand and the other (5'-GCGTCGCTCAATCC-TGATGAGGATTCTTCA) the complementary (antisense) strand. Both were end-labelled by polynucleotide kinase and [γ -³²P]ATP (Amersham).

Antibody preparation and Western analysis

A DNA fragment extending from nucleotides 452–1195 of *HAL2* (Figure 1C) was amplified by standard PCR methodology, utilizing 5'-TTTCTGCAGCTCAGAAGACATCAGCAC as 5' primer and 5'-TTTCTGCAGTCCCTTTTCACTCCCTC as 3' primer, which introduced flanking *Pst*I sites (underlined). The 0.7 kb amplified fragment was purified (Tautz and Renz, 1983), digested with *Pst*I and subcloned into plasmid pUEX1 (Bressan and Stanley, 1987) digested with *Pst*I. A fusion of β -galactosidase and the first 240 amino acids of *HAL2* (with eight linker amino acids in between) was injected into rabbits for antibody production. Protein extracts were prepared from exponentially growing RS-16 cells as described (Serrano, 1988). They were fractionated into soluble and membrane fractions by centrifugation for 1 h at 40 000 r.p.m. (Beckman rotor 65). SDS-PAGE in 12% acrylamide gels (Laemmli, 1970) and Western blotting with the alkaline phosphatase system (Blake *et al.*, 1984) were performed by standard procedures.

Complementation test between *hal2::URA3* and methionine auxotrophs

Two strains (RS-1051 and RS-1053) with a disruption of *HAL2* and with different mating types were generated as described above and utilized in

Fig. 6. Methionine supplementation improves salt tolerance. Strain RS-16 was transformed with plasmids YCp50 and pSB32 (Rose and Broach, 1991) to complement its uracil and leucine auxotrophies (Table I) and tested for tolerance to 0.2 M LiCl, 1 M NaCl or 1 M KCl in minimal liquid medium supplemented with the indicated amino acids (30 µg/ml). Results are the average of duplicates differing in < 10% and the experiment was repeated three times with similar results.

crosses with known auxotrophs for methionine (Table I). The conjugation mixtures were formed for 5 h in rich YPD medium (2% glucose, 2% peptone and 1% yeast extract) and then plated in minimal medium without methionine but with other common requirements of the mated strains. Growth was recorded after 24 h.

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