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Aquaporins in *Saccharomyces*

GENETIC AND FUNCTIONAL DISTINCTIONS BETWEEN LABORATORY AND WILD-TYPE STRAINS*

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Aquaporin water channel proteins mediate the transport of water across cell membranes in numerous species. The *Saccharomyces* genome data base contains an open reading frame (here designated *AQY1*) that encodes a protein with strong homology to aquaporins. *AQY1* from laboratory and wild-type strains of *Saccharomyces* were expressed in *Xenopus* oocytes to determine the coefficients of osmotic water permeability (P_f). Oocytes injected with wild-type *AQY1* cRNAs exhibit high P_f values, whereas oocytes injected with *AQY1* cRNAs from laboratory strains exhibit low P_f values and have reduced levels of Aqy1p due to two amino acid substitutions. When the *AQY1* gene was deleted from a wild-type yeast and cells were cultured *in vitro* with cycled hypo-osmolar or hyper-osmolar stresses, the *AQY1* null yeast showed significantly improved viability when compared with the parental wild-type strain. We conclude that *Saccharomyces cerevisiae* contains at least one aquaporin gene, but it is not functional in laboratory strains due to apparent negative selection pressures resulting from *in vitro* methods.

Aquaporin water channel proteins have been characterized in animals, plants, and insects and are composed of two subgroups; one is permeable only to water (orthodox aquaporins) and a second is permeable to water, glycerol, and other small uncharged molecules (aquaglyceroporins) (for review, see Ref. 1). The three-dimensional structure of human red cell AQP1 at 6 Å resolution revealed the putative aqueous pore (2); however, the structure-function relationships and the physiological roles of other members of the aquaporin family are still poorly understood (for review, see Ref. 3).

Aquaporins have more recently been recognized in bacteria and other microorganisms where their physiological roles are now being explored. *Dictyostelium discoideum* contains *wacA,* a related gene with a developmentally regulated pattern of expression; however, disruption of the gene did not reveal phenotypic differences in spore formation or osmotic challenge (4). In addition to *glpF*, the well recognized glycerol facilitator, *Escherichia coli* contains *aqpZ,* a second related gene with a monocistronic organization (5) encoding a functionally defined aquaporin (6). When a wild-type *E. coli* parental strain and an *aqpZ* null mutant were directly compared, the latter showed reduced growth when cultured in hypo-osmolar medium and under conditions of maximum growth rate (7). A highly related gene, *smpX*, has been identified in *Synechocystis* (8), but its function and null phenotype have not been defined.

Because of powerful genetic and molecular approaches, *Saccharomyces cerevisiae* has been extensively used for studies of osmoregulation (9) and thus may be a good microorganism for studies of possible physiological roles of aquaporins. The *S. cerevisiae* genome (10) is now accessible through the *Saccharomyces* genomic data base and has been found to contain open reading frames (ORFs)[1] related to the aquaporins (11). *FPS1* has been characterized previously as a glycerol facilitator homolog (12, 13), and the deduced amino acid sequence of a second ORF, *YFL054*, is highly homologous (Fig. 1). Additional ORFs were found in the genome with DNA sequences more closely related to aquaporin water channels than to bacterial glycerol facilitators or mammalian aquaglyceroporins (Fig. 1). The ORF *YPR192* is located on chromosome XVI (here designated *AQY1* for aquaporin from yeast). The contiguous ORFs YLL052 and YLL053 reside on chromosome XII and may correspond to fragments of an aquaporin gene (here designated *AQY2*).

Laboratory yeast strains are the result of interbreeding wild-type *S. cerevisiae* and related species, as well as extensive and sometimes incompletely documented growth selection and genetic modifications of the initial strain (14). Many laboratory strains of *S. cerevisiae* were derived from the parental strain, S288C. For example, the strains used for the European Union Yeast Genome Sequencing Program (*Saccharomyces* genomic data base) were isogenic to S288C (10, 15, 16) but may contain genetic modifications induced by selection, growth, and storage conditions. To explore the potential significance of aquaporins in microorganisms, we have genetically evaluated the putative water channel gene *AQY1* from *S. cerevisiae*, functionally characterized its water transport capacities, and directly evaluated the null phenotype. Our studies reveal that wild-type[2] and laboratory strains contain coding differences within *AQY1* that result in marked alterations of function.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions—The yeast strains used in this study are listed in Table I. Cells were routinely grown in YPD media (1% yeast extract and 2% bactopeptone (Difco) with 2% dextrose) and were plated on YPD media supplemented with 1.8% bactoagar. Selection for geneticin resistance was made with YPD liquid media or plates supplemented with 200 mg/liter of G418 sulfate (Life Technologies,

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF053981.

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[1] The abbreviations used are: ORF, open reading frame; PCR, polymerase chain reaction; bp, base pair(s); MOPS, 4-morpholinepropanesulfonic acid.

[2] Unless otherwise stated, the Σ1278b strain was used as our standard wild type; wild-type strains GRF5 and NRRL-Y-12632 were also studied. Strain FY86 was used as our standard laboratory strain.

Inc.). One laboratory strain FY86 (*S. cerevisiae*, *MATα*, *ura3–52*, *his3Δ200*, *leu2Δ1*) and three wild-type strains, NRRL-Y-12632 (*S. cerevisiae*, *MATα*, Northern Regional Research Laboratories), GRF5 (*S. norbensis*, *MATα*), and Σ1278b (*S. cerevisiae*, *MATα*), were kindly provided by J. Boeke. Alignments were made with sequences from the yeast genome data base (10, 15).

PCR Amplifications—Oligonucleotide primers used in this study are listed in Table I. All PCR reactions were performed with the Expand High Fidelity System (Boehringer Mannheim) with 10 × buffer 2 containing $MgCl_2$. Reactions contained 0.2 μM primers, 160 μM dNTPs, 1 × buffer 2, and 50 ng of DNA template. Polymerase (0.75 μl per 50-μl reaction) was added after denaturation for 5 min at 95 °C. Except where stated, 30 cycles were performed with the following conditions: denaturation for 1 min at 94 °C, annealing for 1 min at a temperature specific for each pair of primers, elongation for 1 min at 72 °C. To complete the final strand, the last step was allowed to run 11 min at 72 °C. *AQY1* was amplified from genomic DNA from strains FY86, NRRL-Y-12632, GRF5, and Σ1278b using the primers SCAQP1–5′ and SCAQP1–3′ (and also SCAQP1–5′ and LAQY1–3′ for Σ1278b). To create a stronger translation initiation (17) for expression of Aqy1p in *Xenopus* oocytes, the second codon (TCT) after the start codon was modified to AGT in the primer SCAQP1–5′. Annealing was done at 63 °C for both pairs of primers. To construct the plasmid pX912104, a 229-bp fragment was amplified from p912103 with the primers AQY1-*Ava*II and AQY1-LT, annealing at 65 °C. The kanamycin resistance cassette *loxP-KanMX-loxP* was amplified from the plasmid pUG6 kindly provided by N. Shani (18) using the primers 5′KOKanY1 and 3′KOKanY1 after denaturation for 5 min at 95 °C followed by five cycles of amplification: 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C. This was followed by 20 cycles: 1 min at 94 °C, 1 min at 65 °C, 1 min at 72 °C. To complete the final strand, the last step ran 11 min at 72 °C. To generate a genomic Southern probe, a 650-bp fragment was amplified from Σ1278b genomic DNA using primers SCAQP103 and SCAQP104 specific to the S288C *AQY1* 5′-flanking region with annealing at 63 °C.

Plasmid Construction—Plasmids used in this study are listed in Table I and were generated with standard cloning methods (19). Constructs were checked by restriction digestion and double strand sequencing. The SCAQP1–5′/SCAQP1–3′ PCR products amplified from genomic DNA of strains FY86, NRRL-Y-12632, GRF5, and Σ1278b were cloned into pCR2.1 (Invitrogen) yielding pFY86103, pGN18102, p799102, and p912102. The SCAQP1–5′/LAQY1–3′ PCR product from strain Σ1278b was cloned into pCR2.1 yielding p912103. For expression of Aqy1p in oocytes, *Bgl*II-*Bgl*II fragments containing the *AQY1* ORF from pFY86103, pGN18102, and p912103 were cloned into the *Bgl*II site of the pXβG-ev1 expression vector (20), yielding pXFY86103, pXGN18101, and pX912103. *AQY1* mutant constructs were created as follows: pX912104 was created by ligating the 706-bp *Bgl*II-*Ava*II fragment from p912103 and the 229-bp *Ava*II-*Bgl*II PCR fragment from pFY86103 into the *Bgl*II site of pXβG-ev1. pX799101 and pX912101 were obtained by subcloning the 938-bp *Bgl*II-*Bgl*II fragments from p799102 and p912102 into the *Bgl*II site of pXβG-ev1 encoding the $Aqy1p_{Ch2}$. To test the role of the residues Asn-47, Val-121, and Pro-255 in Aqy1p activity, three mutations (D47N, M121V, T255P) were created in pXFY86103 using the Chameleon double-stranded site-directed mutagenesis kit (Stratagene). The selection primer was *Sca*I-*Mlu*I, and the mutation primers (Table I) contained mutations AGA to AAA for Aqy1p D47N, ATG to GTG for Aqy1p M121V, and GCC to GCA for Aqy1p T255P. All combinations were then made by ligating the different *Hin*dIII-*Alw*nI and *Alw*nI-*Xba*I fragments from the chosen constructs into the *Hin*dIII-*Xba*I sites of pXβG-ev1, resulting in the constructs pXFY86DN, pXFY86MV, pXFY86TP, pXFY86DNMV, pXFY86DNTP, pXFY86MVTP, and pXFY86DNMVTP.

Expression in Xenopus Oocytes and Osmotic Water Transport Assays—All plasmids were linearized by *Xba*I, and capped cRNAs were synthesized *in vitro* as described (20). Defolliculated stage V-VI oocytes from *Xenopus laevis* were injected with 50 nl of water or 25 ng of sample cRNA and incubated 3–4 days in 200 mosM Barth's solution at 18 °C. Oocytes were individually transferred to 70 mosM modified Barth's solution; swelling was monitored by videomicroscopy, and P_f was determined (21).

AQY1 Gene Disruption—*AQY1* was deleted from strain Σ1278b by the one-step gene replacement method (22) using the *loxP-KanMX-loxP* module, geneticin resistance cassette (18). After transformation, cells were incubated 4 h at 30 °C in YPD, washed once in distilled deionized H_2O, and plated on YPD plates plus 200 μg/ml G418. After 2 days at 30 °C, the plates were replicated on fresh YPD plates plus G418 to eliminate contaminants. The correct integration of the Kanamycin resistance cassette was tested by genomic Southern blot. 10 μg of genomic DNA from five geneticin-resistant clones (MELY11–15) and wild-type Σ1278b were cut by *Hin*cII and *Eco*RV + *Spe*I. After electrophoresis and overnight capillary transfer, the nylon membrane was probed with the [α^{32}-P]dCTP radiolabeled PCR-generated probe specific to the 650-bp 5′-untranslated region of *AQY1*.

Antibody—A polyclonal anti-Aqy1p antibody was raised against a synthetic N-terminal peptide Aqy1p (NH_2-SNDSNDTDKQHTRLDPT-GVDD-COOH). The peptide was cross-linked by its primary amine groups to the keyhole limpet hemocyanin and used to immunize New Zealand White female rabbits (Covance Research Products Inc., Denver, PA). Polyclonal antibodies to Aqy1p were affinity purified over Affi-Gel-15 (Bio-Rad) containing the cross-linked Aqy1p peptide.

Electrophoresis and Immunoblotting—Membranes from water-injected or cRNA-injected oocytes were prepared, and sample pellets of five oocytes were resuspended in 2 μl per oocyte of 5 × Laemmli buffer + dithiothreitol (6 mg/ml) for 30 min at 37 °C (20). Yeast total membranes were prepared from 300 ml of YPD cultures at $A_{600\ nm}$ of 2.0. The cells were washed with 45 ml of ddH_2O water and pelleted at 1,500 × *g* for 5 min at 4 °C. The pellets were resuspended with 10 ml of cold resuspension buffer (50 mM MOPS-KOH, pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.015 mM leupeptin, 30 mM benzamidine, 0.05 mg/ml aprotinin, 0.013 mg/ml pepstatin A, and 0.26 mg/ml phenylmethylsulfonyl fluoride). The cells were lysed with three passes through a French press (18,000 p.s.i.) at 4 °C and spun at 12,000 × *g* for 10 min at 4 °C. The membrane-containing supernatant was spun at 100,000 × *g* for 1 h at 4 °C. The membrane pellet was resuspended with 20 ml of 1 × Laemmli buffer + dithiothreitol (6 mg/ml) and incubated at room temperature for 30 min before loading 25 μl per lane. Samples were separated by SDS-polyacrylamide gel electrophoresis (23) in a 15% gel and blotted to Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad) in transfer buffer (20 mM Tris, pH 7.4, 10 mM sodium acetate, 1.0 mM EDTA, 0.02% SDS). Membranes were probed with the affinity-purified anti-Aqy1p antibody overnight at 4 °C. Then, horseradish peroxidase conjugated to donkey anti-rabbit IgG (Amersham Pharmacia Biotech) was added to a dilution of 1:10,000. Bands were visualized using ECL^+Plus reagent (Amersham).

Phenotypic Analyses—Studies routinely employed 200 ml of YPD cultures of Σ1278b and MELY11 inoculated with single colonies from fresh YPD or YPD/G418 plates. The cells were incubated at 30 °C until $A_{600\ nm}$ reached 0.1–0.4 (15–16 h). The correlation between $A_{600\ nm}$ and the number of cells for the two different strains, as well as the initial and final number of living cells, was calculated by counting colonies on five YPD or YPD/G418 plates. For the growth curve experiments, 50-ml cultures of Σ1278b or MELY11 at 0.05 $A_{600\ nm}$ were started in 20% YPD, YPD, or YPD + 1 M NaCl. At time zero, 100 μl of each culture was diluted 100 times in YPD, and five 20-μl aliquots of Σ1278b or MELY11 were plated on YPD or YPD/G418 plates. OD was determined, and aliquots were diluted 200 times (after incubation periods of 2 and 4 h) and 1,000 times (after incubation periods of 6 and 8 h) and plated. After 8 h, the cells grown in 20% YPD were spun down at 1,500 × *g* for 5 min and resuspended in fresh 20% YPD to restore nutrients. Cultures were incubated a total of 27 h, and the final $A_{600\ nm}$ was determined. For the hypo-osmolar growth and hyper-osmolar wash cycles, 7.5 OD units of a preculture in YPD were spun at 1,500 × *g* for 5 min at room temperature. After removing supernatants, the pellets were washed once with 25 ml of YPD + 1 M NaCl, resuspended in 25 ml of 20% YPD, and shaken for 1 h at 30 °C. Five more cycles were undertaken with 20% YPD and YPD + 1 M NaCl. The cells were finally resuspended in 25 ml of 20% YPD. Aliquots were diluted 1000 times after one, two, and four cycles and 2000 times after six cycles. From each dilution, 20 μl were plated on five YPD plates (Σ1278b and MELY11) or five YPD/G418 plates (MELY11). For the hyper-osmolar growth and hypo-osmolar wash cycles, the experiments were conducted similarly, except the yeast cells were grown for 1 h in YPD + 1 M NaCl and washed in sterile distilled, deionized H_2O. Aliquots were diluted 100 times after one and two cycles and 3 times after four and six cycles before plating.

Sequence Alignments and Phylogenetic Analysis—Nucleotide and deduced polypeptide sequences of *AQY1* and *AQY2* were used from the *Saccharomyces* genome data base corresponding to laboratory strain S288C (*AQY1*, GenBank accession no. 786301) and *AQY2* (nos. 136025 and 51360256). Alignments were refined by two successive runs; generation of phylogenetic trees were performed with CLUSTALW 1.7 (24), and polypeptide sequences of human or rat aquaporins used were hAQP0 (266537), hAQP1 (267412), hAQP2 (728874), hAQP3 (2497938), hAQP4 (1351967), hAQP5 (1703358), rAQP6 (2497939), rAQP7 (2350843), rAQP8 (2358277), hAQP9 (AB008775), *Arabidopsis* homologs γTIP (135860) and PIP1a (1175010), *E. coli* homologs AqpZ (2506860) and GlpF (417065), and yeast homologs Fps1p (1706896) and

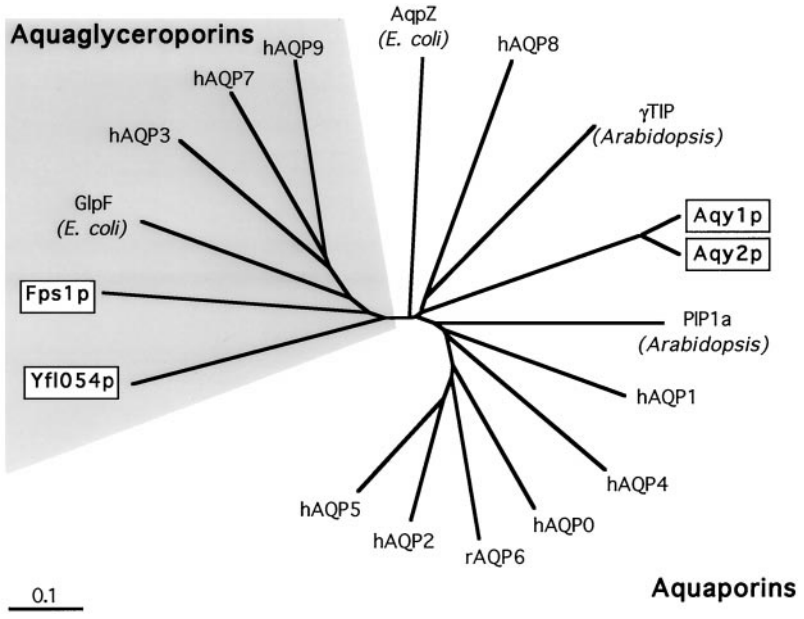

FIG. 1. **Phylogenetic analysis of aquaporin homologs from *Saccharomyces* compared with known mammalian homologs (AQP0–9), *E. coli* homologs (AqpZ and GlpF), and selected *Arabidopsis* homologs.** Two subsets are revealed: homologs permeable to water (aquaporins, *unstippled area*) and homologs permeable to water, glycerol, and other small molecules (aquaglyceroporins, *stippled area*). The *bar* represents the genetic distance of the branch length (24).

Yfl054p (1175958). Transmembrane segments were predicted with the PHDtm program (25).

RESULTS

DNA Sequence Analysis of Putative Aquaporins—The complete genomic DNA sequence from the *S. cerevisiae* laboratory strain S288C (10) was found to contain four genes related to the aquaporin family of membrane water channels. The known gene *FPS1* encodes a protein related to bacterial glycerol facilitators (12), and a second related gene *YFL054* was identified. These homologs are genetically close to the aquaglyceroporins, the group of mammalian proteins that transport water, glycerol, and other small uncharged molecules (Fig. 1; see review in Ref. 1). In contrast, the new ORF *YPR192* (here designated *AQY1*) occupies a branch on the phylogenic tree between two *Arabidopsis* members of the aquaporin group of water-selective transporters (Fig. 1) and two ORFs YLL052 and YLL053, which may correspond to fragments of an aquaporin gene (*AQY2*). To facilitate genetic and functional studies, *AQY1* was isolated using PCR primers designed from the yeast genome data base (Table I), and genomic DNA was obtained from multiple laboratory and wild-type strains of *Saccharomyces* (Table I).

Preliminary functional analyses of Aqy1p from the *Saccharomyces* laboratory strain FY86 failed to demonstrate enhanced water permeability (see below), so the *AQY1* gene was sequenced from five different laboratory and wild-type strains. Like known aquaporins, the deduced amino acid sequence of Aqy1p from all strains contains the two highly conserved tandem NPA motifs (Asn-Pro-Ala), six predicted bilayer-spanning domains, and cytoplasmic N and C termini (Fig. 2*A*). The strains did not contain identical sequences, and two variant ORFs were identified. The first variant is represented by strains FY86, S288C, and NRRL-Y-12632, which all contain 918-bp sequences with a series of eight adenines beginning at position 872 leading to translation from the reading frame beginning with G293 (Fig. 2*B*, *arrows*). The deduced 305-residue polypeptides from FY86 and S288C are identical, and each has a calculated molecular mass of 32.7 kDa. Two residues that are highly conserved among mammalian and plant aquaporins were found to be substituted in both of these strains: V121M and P255T (Fig. 2). In addition, the deduced amino acid sequence from multiple laboratory strains differed from the wild-type strain NRRL-Y-12632 at four positions: N47D, F101S, P255T, and P256L.

A second variant of *AQY1* ORF was found in two wild-type strains, Σ1278b and GRF5 (Fig. 2*B*), which both share a C terminus distinctly different from the first variant ORFs. These *AQY1* genes encode 327-amino acid polypeptides with a calculated molecular mass of 35.4 kDa (Σ1278b) and structural domains and residues highly conserved among mammalian and plant aquaporins, including Val-121 and Pro-255. The deduced amino acid sequences were otherwise identical except for three polymorphisms: R42K, V53A, and P308S. The major difference between these Aqy1 polypeptides and those with the first variant is the C-terminal domain. In the 984-bp ORF, a series of seven adenines beginning at position 872 (instead of eight adenines) lead to translation from the reading frame beginning with Val-293 (Fig. 2*B*, *arrows*). Thus, when compared with FY86, S288C, and NRRL-Y-12632 strains, the Aqy1p from wild-type strains Σ1278b and GRF5 each possess a longer C terminus with an 18-residue hydrophobic stretch, which may represent a seventh transmembrane segment.

Functional Analysis of Water Permeability—Functional demonstration of yeast aquaporin homologs was established by calculating the coefficients of osmotic water permeability, P_f (21), from rates of hypo-osmotic swelling measured from *Xenopus* oocytes injected with cRNA synthesized from *AQY1* constructs prepared from multiple laboratory strains or wild-type strains of *Saccharomyces* (described in Tables I and II). Oocytes expressing Aqy1p from laboratory strain FY86 failed to show an increase in P_f (Table II, Fig. 3*A*). In contrast, oocytes expressing Aqy1p from wild-type strain Σ1278b (Table II) exhibited more than a 10-fold increase in the P_f (127.6×10^{-4} cm/s) when compared with the water-injected oocytes (10.5×10^{-4} cm/s, Fig. 3*A*). Consistent with the lack of a cysteine preceding the second NPA motif (28), no inhibition of P_f was achieved by preincubating oocytes in up to 1 mM $HgCl_2$ (128×10^{-4} cm/s). To determine whether all cRNA injections resulted in Aqy1p expression, an antibody was raised to a synthetic peptide corresponding to 20 amino acids of the Aqy1p N terminus. Immunoblot analysis revealed that oocytes exhibiting increased P_f contained significant amounts of the 35-kDa polypeptide. In contrast, oocytes injected with cRNAs that conferred no increase in P_f were found to have a correspondingly weak 34-kDa band accompanied by an even fainter band of higher molecular weight (Fig. 3*B*).

The most notable sequence difference in Aqy1p between lab-

TABLE I
Yeast strains, plasmids, and oligonucleotides

Strain	Genotype	Source, reference
Σ1278b	*S. cerevisiae, MATα*	J. Boeke
FY86	*S. cerevisiae, MATα, ura3–52, his3Δ200, leu2Δ1*	J. Boeke
NRRL-Y-12632	*S. cerevisiae, MATα*	J. Boeke; North. Reg. Res. Labs.
GRF5	*S. norbensis, MATα*	J. Boeke
MELY11	Σ1278b *MATα, aqy1::loxP-KanMX-loxP*	This study
Genomic database	*S. cerevisiae* S288C derivatives	Winston *et al.*, 1995; Dujon, 1994
Plasmid	**Description**	
p912102	Truncated $AQY1_{\Sigma 1278b}$ ORF cloned into pCR2.1	
p912103	Full $AQY1_{\Sigma 1278b}$ ORF cloned into pCR2.1	
pX912101	Chimera #2 of $AQY1_{\Sigma 1278b}$ ORF cloned into pXβG-ev1	
pX912103	$AQY1_{\Sigma 1278b}$ ORF cloned into pXβG-ev1	
pX912104	Chimera #1 of $AQY1_{\Sigma 1278b}$ ORF cloned into pXβG-ev1	
pFY86103	$AQY1_{FY86}$ ORF cloned into pCR2.1	
pXFY86103	$AQY1_{FY86}$ ORF cloned into pXβG-ev1	
pXFY86DN	pXFY86103 containing the D47N mutation	
pXFY86MV	pXFY86103 containing the M121V mutation	
pXFY86TP	pXFY86103 containing the T255P mutation	This study
pXFY86DNMV	pXFY86103 containing the D47N-M121V mutation	
pXFY86DNTP	pXFY86103 containing the D47N-T255P mutation	
pXFY86MVTP	pXFY86103 containing the M121V-T255P mutation	
pXFY86DNMVTP	pXFY86103 containing the D47N-M121V-T255P mutation	
p799102	$AQY1_{GRF5}$ ORF cloned into pCR2.1	
pX799101	Chimera #2 of $AQY1_{GRF5}$ ORF cloned into pXβG-ev1	
pGN18102	$AQY1_{NRRL\text{-}Y\text{-}12632}$ ORF cloned into pCR2.1	
pXGN18101	$AQY1_{NRRL\text{-}Y\text{-}12632}$ ORF cloned into pXβG-ev1	
pUG6	loxP-KanamycinMX-loxP cassette	N. Shani; Güldener *et al.*, 1996
pXβG-ev1	5′ and 3′ UTRs of the *Xenopus* β-globin gene	Preston *et al.*, 1992
pCR2.1	TA cloning vector	Invitrogen
Primer	**Sequence**	
ScAQP1–5′	5′ GGGAGATCTACCATGAGTTCGAACGATTCGAAGATAC 3′	
ScAQP1–3′	5′ GGGAGATCTAGGAGGGAAAAATTAGACTTCAGCC 3′	
LAQY1–3′	5′ GGGAGATCTCAATTAATCTTACTAGTTTTTGCC 3′	
AQY1-AvaII	5′ CCCGGTCCTTGGGTGCTGCTGTCG 3′	
AQY1-LT	5′ CCCAGATCTTTAGACTTCAGCCACAGCAGAGGAACTGCTGGTTTCACCTTTTTTTTGAGCTTTTTCCTTGG 3′	
AQY1D47N	5′ CGCAGCGATAAAGTGGTTTCTCAGGGTGTCCC 3′	
AQY1M121V	5′ CACAGCGAAAGCGACACAGCAGGATTC 3′	
AQY1T254P	5′ CTAAAATGGATCCTAACAGCGGGCCAATCCAATAAATC 3′	
ScaI-MluI	5′ CTGTGACTGGTGACGCGTCAACCAAGTC 3′	
SCAQP103	5′ CTTAAAACATACTTTCTCCCGTCC 3′	
SCAQP104	5′ CCGGGATCCGTTATAGTTAATTACATGTTACTTTATG 3′	
5′KOKanY1	5′ GGTGCTGTCTGTCAATACGGCACATAAAGTAACATATAATTAACTATAACCCAGCTGAAGCTTCGTACGC 3′	
3′ KOKanY1	5′ TATAACATTAAGTGCTAGTGAGCGAGAAATAAAGAAAAGGAGGGAAAAAATAGGCCACTAGTGGATCTG 3′	

oratory and wild-type strains is at the C terminus (Fig. 2). To establish the functional importance of this variation, we constructed two DNAs encoding chimeric proteins (Table II, Fig. 2). The first chimera, $Aqy1p_{Ch1}$, is a fusion of Σ1278b wild-type Aqy1p (M1-K292) with the C terminus of FY86 laboratory strain Aqy1p (G293-V305). The second chimera, $Aqy1p_{Ch2}$, resulted from the serendipitous fusion of the wild-type Aqy1p (M1-L310) and a unique, non-naturally occurring C-terminal sequence encoded by the 3′-untranslated region of the *Xenopus* β-globin gene (DLVTTKPASRTPEWSL-COOH) producing a 326-residue polypeptide. Although equivalent amounts of cRNA were injected into oocytes, both oocytes expressing the chimeric proteins exhibited higher P_f values than oocytes expressing the wild-type protein: $Aqy1_{Ch1}$ 218 × 10^{-4} cm/s, $Aqy1p_{Ch2}$ 178 × 10^{-4} cm/s (not shown), and $Aqy1p_{WT}$ 127.6 × 10^{-4} cm/s (Fig. 3*A*).

To explore other possible explanations for reduced water permeability by oocytes expressing Aqy1p from laboratory strains, a series of site-directed mutants was prepared with each of the amino acid substitutions replaced individually or in pairs by the corresponding residues in Aqy1p from the wild-type strains Σ1278b and GRF5: D47N, M121V, and T255P (Fig. 2*A*). Oocytes expressing $Aqy1p_{LS}$ modified with single substitutions D47N, M121V, or T255P did not exhibit a detectable increase in the P_f when compared with the water-injected oocytes or oocytes expressing the unmodified $Aqy1p_{LS}$ (Fig. 3*A*). This failure to enhance P_f was correlated with a low level of expression of the mutant proteins (Fig. 3*B*). Similar results were observed with the $Aqy1p_{LS}$ modified with double substitutions D47N/M121V or D47N/T255P. However the double substitution M121V/T255P and the triple substitution D47N/M121V/T255P generated high P_f values (193 and 176 × 10^{-4} cm/s, Fig. 3*A*) with correspondingly large levels of 34-kDa polypeptide (Fig. 3*B*). Thus, mutant forms of Aqy1p with reduced P_f have correspondingly low levels of expressed protein.

Sensitivity to Osmotic Stress by Laboratory and Wild-type Strains—The identification of *AQY1* with two amino acid substitutions that impair protein biogenesis or function in multiple laboratory strains of *Saccharomyces* suggests that the yeast are under selective pressure not to express functional aquaporins during laboratory procedures. Membranes from a laboratory strain of yeast (FY86) were directly compared with a wild-type strain (Σ1278b) by immunoblotting with anti-Aqy1p (Fig. 4*A*). Although the lab strain contained a weakly detectable band of ~35 kDa, the wild-type yeast contained a much stronger signal. To determine whether deletion of the *AQY1* gene from Σ1278b cells would produce a similar selective advantage, it was removed by the gene replacement technique (18). Genomic DNAs from five geneticin-resistant clones were digested with *Hin*cII and *Eco*RV + *Spe*I and probed with the

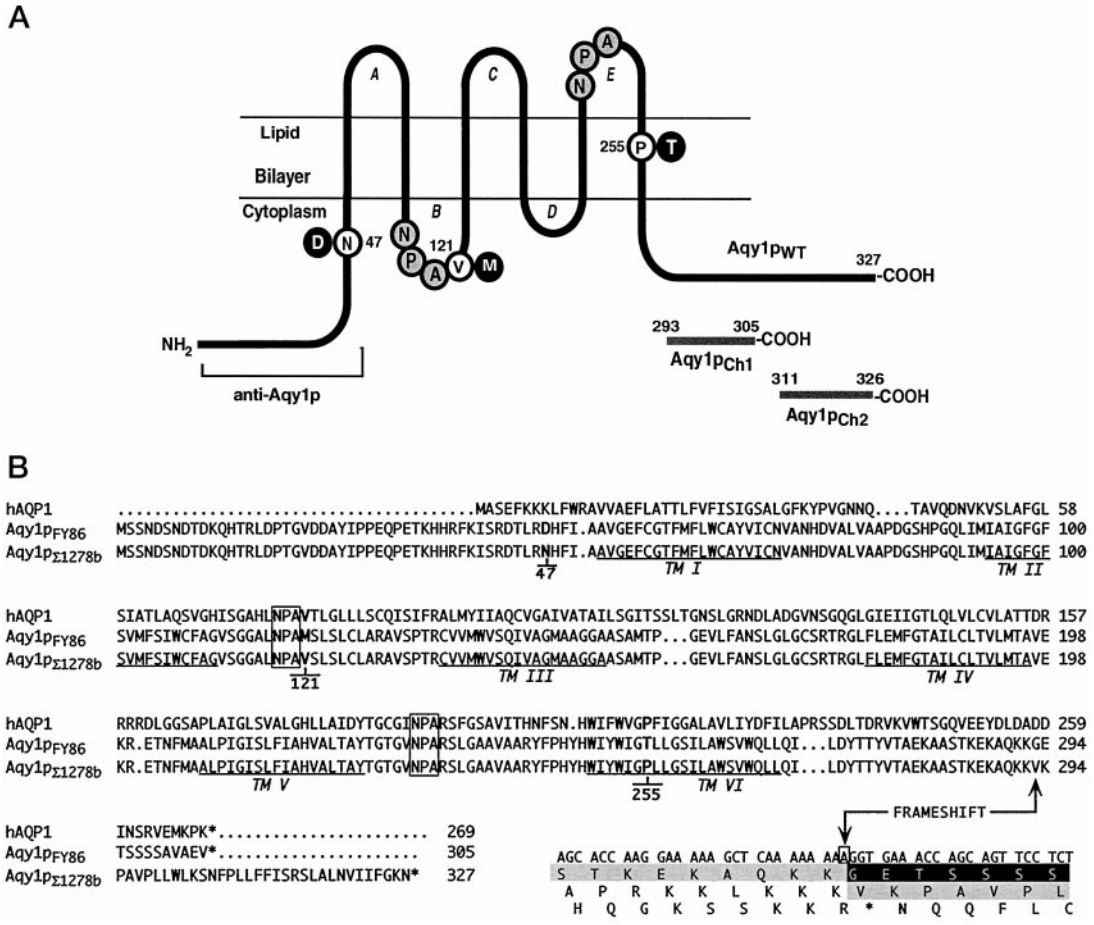

FIG. 2. **Schematic representation of the membrane topology of Aqy1p$_{WT}$ and primary sequences of wild-type and laboratory Aqy1ps.** *A*, locations of site-directed mutations and chimeric alterations are indicated. *Filled circles* represent amino acids in the FY86 laboratory strain Aqy1p, and *open circles* represent the corresponding residues in wild-type Σ1278b Aqy1p. *B*, sequence alignments of Aqy1p from indicated yeast strains and human AQP1. Complete deduced amino acid sequences indicate different residues in Aqy1p (47, 121, 255). Transmembrane domains are denoted (*TM*), and site of shifted reading frames is identified (*arrows*). Nucleotide sequences encoding the Aqy1p C terminus include a seven-adenine sequence in wild-type Σ1278b strain (*gray box*) and GFR5 strain (not shown). The presence of an eighth adenine at this site in the laboratory FY86 strain results in a frameshift for the C-terminal sequence (*black box*).

TABLE II
Yeast aquaporin proteins studied in this report

	Strain source
Aqy1p$_{WT}$	*AQY1*$_{\Sigma 1278b}$ gene product
Aqy1p$_{LS}$	*AQY1*$_{FY86}$ gene product
	C-terminal chimeric proteins
Aqy1p$_{Ch1}$	Aqy1p$_{WT}$ (M1-K292) with C terminus of Aqy1p$_{LS}$ G293-V305
Aqy1p$_{Ch2}$	Aqy1p$_{WT}$ (M1-L310) with non-natural C terminus (DLVTTKPASRTPEWSL)
	Amino acid substitutions in Agy1p$_{LS}$
DN	D47N
MV	M121V
TP	T255P
DN/MV	D47N, M121V
DN/TP	D47N, T255P
MV/TP	M121V, T255P
DN/MV/TP	D47N, M121V, T255P

PCR-amplified fragment specific to the S288C *AQY1* 5′-flanking region (Fig. 4*B*). Hybridization signals of 630 bp after the *Hin*cII digestion and 2270 bp after *Eco*RV + *Spe*I digestion were detected in four clones (MELY11–14), confirming the recombination between the *loxP-KanMX-loxP* cassette and *AQY1*. *AQY1* deletion clones were also confirmed by PCR using the *AQY1* ORF-specific primers SCAQP1–5′ and SCAQP1–3′ (not shown).

To test the phenotype of the *AQY1*-deleted yeast, the growth rate of parental wild-type strain Σ1278b and clone MELY11 were compared in hypo-osmolar media (20% YPD), iso-osmolar media (YPD), and hyper-osmolar media (YPD + 1 M NaCl). In all growth conditions, wild-type and MELY11 cells exhibited similar growth rates (Fig. 5*A*). Wild-type and MELY11 cells were similarly sensitive to hyper-osmolarity, as their growth rates were equivalently reduced in presence of 1 M NaCl (Fig. 5*A*). Likewise, they both exhibited equivalently brisk growth rates in YPD and 20% YPD. Wild-type and MELY11 cells were then subjected to a series of osmotic stress-growth cycles. Growth in hypo-osmolar buffer with hyper-osmolar washes produced a clear advantage for the MELY11 cells, with incremental differences becoming more pronounced after six cycles (Fig. 5*B*). Equivalent results were achieved in nine successive experiments. The converse experiment was performed by culturing the cells in hyper-osmolar media followed by hypo-osmolar washes. After the first cycle, the number of surviving cells had greatly declined, but the MELY11 cells exhibited a 4-fold survival advantage (Fig. 5*C*). Although the number of surviving cells further declined after each cycle, the MELY11 cells were found to be relatively spared with 6.4-, 6.8-, and 9.2-fold greater survival after two, four, and six cycles (Fig. 5*C*). Equivalent results were achieved in eight successive experiments. To confirm that the plating on geneticin did not affect the survival rates, MELY11 cells were plated on YPD plates

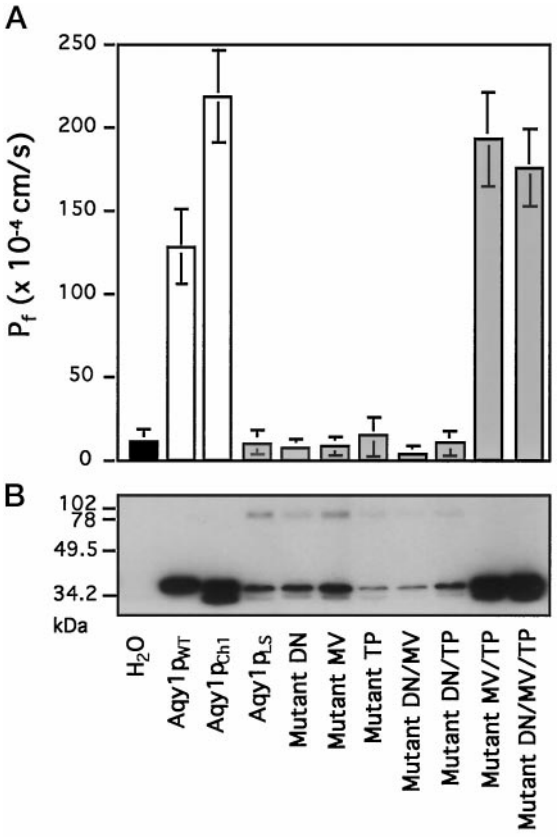

FIG. 3. **Correlation of osmotic water permeability (P_f) measurements and expression levels of the different Aqy1 polypeptides (Table II).** *A*, oocytes were injected with water (control) or 25 ng of the indicated cRNA (synthesized from denoted plasmid) and incubated 3 days before measurement of P_f. Aqy1p_{WT} (pX912103), Aqy1p_{Ch1} (pX912104), Aqy1p_{LS} (pXFY86103), mutant DN (pXFY86DN), mutant MV (pXFY86MV), mutant TP (pXFY86TP), mutant DN/MV (pXFY86DNMV), mutant DN/TP (pXFY86DNTP), mutant MV/TP (pXFY86MVTP), and mutant DN/MV/TP (pXFY86DNMVTP). *B*, anti-Aqy1p immunoblot of whole membranes corresponding to *panel A* (five oocytes per lane).

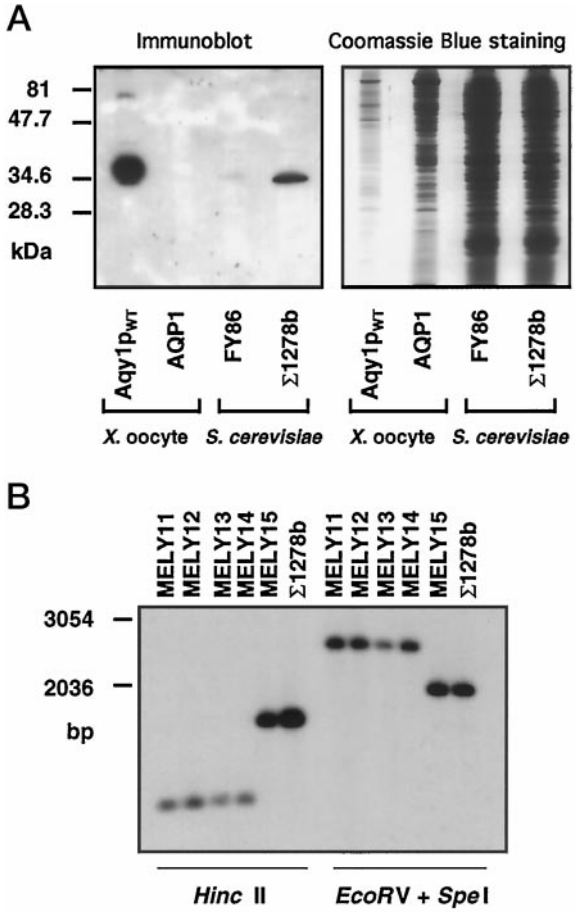

FIG. 4. **In vivo expression of Aqy1p and *AQY1* null-mutant construction.** *A*, in the *left panel*, anti-Aqy1p immunoblot of oocyte membranes contain *S. cerevisiae* Aqy1p or human AQP1, or yeast membranes from lab strain (FY86) or wild-type (Σ1278b). In the *right panel*, Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis is shown of the same samples. *B*, Southern blot of the *AQY1* after disruption in parental wild-type yeast strain Σ1278b. Genomic DNA from five clones (MELY11–15) or parental cells were digested with *Hinc*II or *Eco*RI + *Spe*I and incubated with a 650-bp ^{32}P-labeled probe specific for the S288C *AQY1* 5′-untranslated region. Expected sizes for the *Hinc*II digestions were 630 bp (*AQY1* disruption) and 1290 bp (wild type); for *Eco*RI + *Spe*I digestions, expected sizes were 2270 bp (*AQY1* disruption) and 1655 bp (wild type).

without or with the antibiotic, and survivals were similar in the presence of geneticin (Fig. 5, *B* and *C*). Thus, we conclude that increased survival under these conditions is significantly enhanced by deletion of *AQY1*.

DISCUSSION

The recent availability of completely sequenced genomes has greatly facilitated recognition of genes homologous to known sequences; however our experience illustrates some complexities encountered when taking this information to the laboratory bench. Computer screening of the genome from *S. cerevisiae* (10) revealed that the widely expressed aquaporin family of membrane water channels is represented in yeast. As expected, we found the gene *FPS1*, which is known to encode a glycerol transporter with sequence similarities to aquaglyceroporins (12), and a second sequence-related ORF was found, which most likely also encodes a glycerol transporter (Fig. 1). Another sequence-related ORF was recognized in the *S. cerevisiae* genome, and our studies indicate that *AQY1* represents an aquaporin gene.

Surprisingly, different laboratory and wild-type strains of *S. cerevisiae* were found to have specific sequence differences with functional consequences. *AQY1* from two laboratory strains and one wild-type strain encode a 305-residue polypeptide that fails to confer increased osmotic water permeability when expressed in *Xenopus* oocytes. In contrast, *AQY1* from two other wild-type strains encodes a 327-residue polypeptide that is functional in oocytes. To our knowledge, these observations represent the first demonstration of a functional aquaporin from yeast and are supported by preliminary studies made independently by other scientists (26).

The failure of Aqy1p from laboratory strains to transport water is not due to the C terminus. The single nucleotide insertion before codon 293 of *AQY1* from laboratory strains results in a frameshift with termination after codon 305. Although we initially suspected this to be an explanation for the lack of function, creation of an Aqy1p chimeric molecule with the laboratory strain C terminus on wild-type Aqy1p did not cause reduced activity but actually resulted in a significant 70% increase in P_f. In addition, creation of a chimeric Aqy1p with the wild-type C terminus replaced by a non-natural peptide sequence also increased the P_f. Although not yet conclusive, these observations suggest that the C terminus of the wild-type sequence containing hydrophobic stretches with spaced, charged residues may serve as a negative regulator of Aqy1p.

Differences within the coding region of Aqy1p from laboratory strains revealed genetic explanations for the lack of function. Three amino acid substitutions were found when the

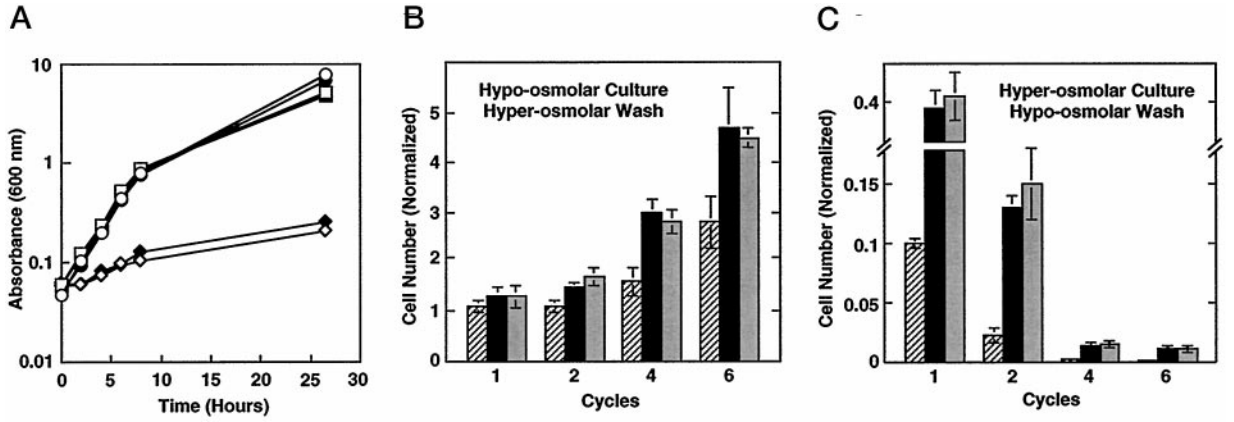

FIG. 5. **Phenotypic analyses of MELY11 *(AQY1* disrupted) or parental wild-type Σ1278b yeast.** *A*, growth curve in 20% YPD (*circles*), YPD (*squares*), and YPD + 1 M NaCl (*diamonds*); *filled symbols* represent parental cells, and *open symbols* represent clone MELY11. *B*, cycled hypo-osmolar culture, hyper-osmolar wash. After preculturing in YPD, the cells were resuspended in 20% YPD, shaken for 1 h at 30 °C, washed in YPD + 1 M NaCl, and resuspended for the next cycle in 20% YPD. The number of cells was normalized to the starting number; *diagonally hatched columns* represent parental wild-type yeast; *black columns* represent MELY11; *lightly stippled columns* represent MELY11 plated on geneticin plates. *C*, cycled hyper-osmolar culture, hypo-osmolar wash. After preculturing in YPD, the cells were resuspended in YPD + 1 M NaCl, shaken for 1 h at 30 °C, washed in distilled-deionized H_2O, and resuspended for the next cycle in YPD + 1 M NaCl. The number of cells was normalized to the starting number (*columns* as in *B*).

Aqy1p sequences of wild-type and laboratory strains were compared, and two led to loss of water transport activity when expressed in oocytes. The V121M substitution lies within loop B (Fig. 2) and may occlude the channel possibly destabilizing the protein, since in the hourglass model it is proposed that loops B and E fold back in the membrane bilayer forming a single aqueous pathway (27). The P255T substitution occurs at the site of a critical bend in the sixth membrane-spanning α-helix (3) and therefore may induce a conformational change impairing stability of the protein. Immunoblot analysis of oocytes expressing these mutant forms of Aqy1p indicated that cRNA-injected oocytes with low P_f values contained only low levels of the protein and some higher molecular weight aggregates (Fig. 4*B*). Since translation should be similar for all injected *AQY1* cRNAs, the low level of protein indicates that the Aqy1p from the laboratory strains may be misfolded and unstable in the oocytes. Although this may possibly represent an artifact of the oocyte system (30), these results are consistent with the reduced level of endogenous Aqy1p in the laboratory yeast strain FY86 compared with the wild-type strain Σ1278b (Fig. 4*A*). Studies of mutant forms of AQP1 (28) and AQP2 (29) demonstrated the failure to traffic to the outer membrane because of apparent misfolding.

Our studies demonstrate that Aqy1p is not essential for life, and standard laboratory conditions exert negative selection pressure on yeast expressing the protein. This is particularly well supported by identification of two separate mutations in *AQY1* leaving minimal chance for spontaneous reversal. When compared by survival in conditions representing exaggerations of laboratory procedures, we found that osmotic stress during growth cycles was much better tolerated by *AQY1* null yeast than by the parental wild-type yeast.

Our studies do not define the physiological role of Aqy1p in a natural environment; however, a role in osmoregulation seems likely. Current models describing how cells sense osmotic stress do not delineate the very first step in the process, which is rapid flow of water among intracellular compartments. It is known that in response to a hyper-osmotic stress, yeast cells lose cytoplasmic water; this is followed by an efflux of water from the vacuole to the cytoplasm. Finally, the cells accumulate glycerol at high concentrations. In response to hypo-osmotic stress, yeast cells cease to accumulate glycerol and undergo activation of a mitogen-activated protein kinase cascade, which is regulated by Pkc1p (for review, see Ref. 31). We speculate that Aqy1p may mediate rapid movements of water preceding the osmotic responses.

It has recently been shown that the wild-type strain Σ1278b is capable of pseudohyphal growth under nitrogen starvation conditions, whereas laboratory strain S288C is unable to form pseudohyphae (32, 33). The authors demonstrated a point mutation in FLO8 from strain S288C leading to a block in growth of pseudohyphae. Because many wild-type yeast strains are filamentous, pseudohyphal formation may contribute to their ability to survive in their normal environments. Many laboratory strains are defective in pseudohyphal formation and carry the same FLO8 mutation, leading to the conclusion that these mutations were selected during laboratory cultivation. Thus, comparisons of data between laboratories using different putative wild-type strains must be undertaken with the utmost care.

Although our studies predict that Aqy1p will function to facilitate osmotically directed movements of water, firm identification of the physiological need for this process may not be achieved directly. The *E. coli* homolog AqpZ was also found to be nonessential for life; however, the null phenotype only became obvious under conditions sustaining maximum growth rates (7). The known sequence-related protein Fps1p was predicted by gene sequence comparisons to be a glycerol facilitator (11). Bacterial glycerol facilitators have been defined in oocytes for their ability to transport glycerol (34) and are related to aquaglyceroporins. Much effort was needed to demonstrate the function of Fps1p in releasing glycerol (an osmolyte) in response to hypo-osmotic stress (12). Nevertheless, Fps1p is not essential for life, and other functions are still being studied including possible roles in phospholipid biogenesis (13) or in maintenance of osmotic balance, which regulates cell fusion during mating (35).

Elucidation of the normal functions of Aqy1p will require additional studies. It is possible that Aqy1p may be involved in specific phases of the cell cycle. If control of the osmotic balance is important for mating, it may also be critical for processes like budding, pseudohyphal formation, or sporulation. It has been already shown that laboratory strains, industrial strains, and wild-type strains of yeast may have physiological differences in osmosensitivity, but the genetic explanation is not yet known (36). Our studies showing that wild-type and laboratory strains of *S. cerevisiae* contain distinct *AQY1* sequences, resulting in major functional differences that may provide an explanation for these differences.

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