International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 12 (12), pp. 001-008, December, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

AQY1 gene from *Debaryomyces hansenii*

Juan Carlos González-Hernández

Facultad de Ciencias Médicas y Biológicas "Dr. Ignacio Chávez", Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México. E-mail: jcgh1974@yahoo.com. Tel: +52 433 312 05 10. Ext: 120. Fax: +52 433 312 00 14. Ext: 132.

Accepted 15 April, 2018

Aquaporins are members of the major intrinsic protein superfamily of integral membrane proteins that enable the transport of water, glycerol and other solutes across membranes in diverse organisms. In yeasts, the proposed physiological roles of aquaporins are related to their contribution to freeze tolerance, osmoregulation and water transport. This article reports a contribution to the freeze tolerance in the heterologous expression of the *Debaryomyces hansenii* aquaporin gene in *Saccharomyces cerevisiae*. In the present experiments, the transformant cells were similarly sensitive to osmotic stress conditions, since their growth capacity phenotype was equivalently abolished in the presence of 0.6 and 1.0 M NaCl. Northern analysis of the yeasts studied revealed a correlation between freeze resistance and the aquaporin gene *AQY1*.

Key words: *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, aquaporin, freeze tolerance.

INTRODUCTION

Aquaporin water channels have been identified in nearly all life forms. Although many aquaporin homologs transport only water (orthodox aquaporins), some transport water and glycerol (aquaglyceroporins). The aquaporin family of molecular water channels is widely expressed throughout the plant, yeast and animal kingdoms, many microorganisms have both. The baker's yeast *Saccharomyces cerevisiae* possesses two genes encoding putative aquaporins, *AQY1* and *AQY2*, that exhibit polymorphic variations at the gene level, leading to specific sequence differences at the protein level (Bonhivers, 1998; Laize, 2000). Aqy1p from strain Σ1278b functions as a water channel when expressed in *Xenopus oocytes* (Bonhivers, 1998; Laize, 1999). In addition, Σ1278b *aqy1*-null yeasts are less sensitive to repeated osmotic shocks than the parent strain (Bonhivers, 1998); however, a clear positive role for Aqy1p has not yet been identified. Aqy2p has been detected only during the exponential growth phase in rich medium containing glucose. Aqy2p expression is repressed by hyper-osmotic culture conditions, it is located in the endoplasmic reticulum (ER) as well as in the plasma membrane (Valérie, 2001). Thange et al. (2002) reported a correlation between freeze resistance and the aquaporin genes *AQY1* and *AQY2*; deletion of these genes in a laboratory strain rendered yeast cells more sensitive to freezing, whereas, overexpression of the respective genes, as well as heterologous expression of the human aquaporin gene *hAQP1*, improved freeze tolerance (Tanghe, 2002).

Thange et al. (2005), expressed the *S. cerevisiae* aquaporin-encoding gene *AQY2–1* in *Saccharomyces pombe* cells and found that the relatively low freeze tolerance of *S. pombe* could be significantly enhanced. These authors concluded that their results further support the correlation between aquaporin function and freeze tolerance in microorganisms (Tanghe, 2005; Kayingo et al., 2005). Thange et al. (2005) also found a clear correlation between expression of the *Candida albicans* aquaporin-encoding gene *AQY1* and freeze tolerance, reporting the first clear phenotype for a *C. albicans AQY1* deletion strain, confirming the correlation between aquaporin function and freeze tolerance in microorganisms.

Although biophysical features of membrane water permeability had been recognized, the molecular pathway of transmembrane water movement remained unknown until the discovery of aquaporins, a family of proteins that enables selective, osmotic flow of water across cell membranes (Chrispeels, 1994).

When yeasts are grown under high NaCl concentrations, a complex cellular response is elicited. The best studied systems involved in this response are those present in organisms like *S. cerevisiae*, which induce mechanisms for sodium exclusion from the cytoplasm and increase the production of compatible solutes. Although *S. cerevisiae* is considered as an important model to study the transcriptional transient response to saline shock, studies of the long-term evolutionary adaptation to the presence of salt in the environment which should operate in halotolerant-halophilic yeasts, could increase our understanding of this phenomenon. *Debaryomyces hansenii* is one of the most halotolerant-halophilic yeast species, able to grow in media containing a wide range of salt concentrations (Prista, 1997; González-Hernández, 2004). Like *S. cerevisiae*, when *D. hansenii* is grown in the presence of high salt concentration, it accumulates glycerol as the most important compatible solute (Gustafsson, 1976; Adler, 1980; González-Hernández, 2005). The objective of this work was to gain further insight on the characterization of *D. hansenii* aquaporin gene and its role in mechanisms underlying halotolerance and freezing.

MATERIALS AND METHODS

Yeasts strains and bacteria

The type strains *D. hansenii* PYC2968 (CBS767 and *S. cerevisiae* ANT29 (Mat α leu2::hisG trp1::hisG his3::hisG ura3-52) were used as genetic background strains (10560-6B); *S. cerevisiae* YSH1172 (Mat α leu2::hisG trp1::hisG his3::hisG ura3-52 *aqy1* ::kanMX4 *aqy2* ::HIS3) and *S. cerevisiae* ANT27, further indicated as overexpressing *AQY1* (10560-6B/pYX012 *KanMX AQY1-1)*, were used as recipient strains (Table 1). Competent cells of *Escherichia coli* XL1-Blue (Invitrogen) were also used for plasmid (Yep352), selection and propagation were performed and transformed according to Hanahan (1985).

Growth media

D. hansenii strain was routinely maintained in YPD medium (10 g yeast extract, 10 g peptone, 20 g glucose, and 20 g agar per liter). The transformant strains were routinely maintained on solid YNB-Ura (w/o amino acids) medium with 2% glucose, supplemented when required, with the adequate requirements for prototrophic growth (Pronk, 2002). *E. coli* XL1-Blue strain was routinely maintained in Luria-Bertani medium (LB) at 37°C, ampicillin (100 µg ml^{-1}) and 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal, 4 µg ml^{-1}) were used as supplements (Sambrook, 1989) when required.

Cloning strategy and DNA manipulations

Sequence data for *D. hansenii* were obtained from Génolevures Consortium website (Génolevures, 2001), by performing tblastn search with Aqy1p sequence from *S. cerevisiae* against the *D. hansenii* genomic sequence. Using the BLASTP 2.2.14 program (NCBI, Bethesda, MD, USA) (Altschul et al., 1997), ORF revealing homology to *S. cerevisiae* Aqy1p protein were identified. Based on the nucleotide sequences of this ORF together with the contiguous upstream and downstream regions, primers were designed in order to amplify a region that comprised of approximately +500 bp from the ATG start codon and +200 bp after the TAA stop codon of the gene. The specific primers used, designed and modified to incorporate a restriction site (underlined) were for *Eco*RI forward primer, (5´-GGAATTCCTATGAGAAACTAGCAACAA-3´); and for reverse primer, *Xba*I; (5´-GCTCTAGAGCCGATCCTATAAACTTTCAAG-3´) to amplify AQY1 ORF and the flanking regions (-500p, +200bp). PCR amplification was carried out in an Eppendorf thermocycler with DNA polymerase from BIOTOOLS, for 30 cycles, at 49°C (annealing temperature chosen according to the primer characteristics). Using this approach, one fragment of 1.6 kb was obtained, using *D. hansenii* CBS767 genomic DNA as template. The amplified products were digested with *Pst* 1, purified using the purification kit "GFX PCR DNA and Gel Band Purification" (GE Healthcare).

The fragment was cloned into the XL1-Blue *E. coli* strain (YEp352) (Table 1), characterized at the molecular level, and used to transform a wild type and deleting the *S. cerevisiae* strain for *AQY1* and *AQY2* genes by the lithium acetate method (Geitz and Schiestl, 1995). Current plasmid isolation was performed by alkaline extraction, as described in Birnboim and Doly (1979), modified as in Sambrook et al. (1989). For plasmid isolation from yeasts, the procedure described by Hoffman and Winston (1987) was followed. Agarose gel electrophoresis and restriction site mapping were performed according to standard methods (Sambrook, 1989). Yeast genomic DNA from *D. hansenii* was isolated (Cryer et al., 1975), after a previous treatment with lyticase. Yeast genomic pDNA was isolated by using QIAprep Spin Miniprep Kit Protocol according to manufacturer's directions. Constructs were named as: JCGHZERO for plasmids without *DhAQY1*; JCGHAQY1, for plasmids containing the *DhAQY1* gene (Table 1). Transformants were selected on minimal medium with methionine, leucine and tryptophan; 60 transformants (JCGHZERO), 167 transformants (JCGHAQY1), 58 transformants (JCGH*zero*), and 159 transformants (JCGH*aqy1aqy2*) were obtained. One representative clone from each transformant was used for heterologous expression studies.

Isolation of RNA and northern blot analysis

Total RNA was extracted from exponential phase yeast cells after 6 h of incubation under saline stress conditions. Cells were then collected by centrifugation, frozen using liquid nitrogen and kept at -80°C until RNA extraction was performed. Total RNA was extracted by the hot phenol extraction protocol (Schmitt et al., 1990), modified by Daniela Castro (personal communication) as described below. Frozen cells were re-suspended in 470 µl of 100 mM sodium acetate, pH 5.0, 5 mM $MgCl_2$ plus 1/10 volume of 10% SDS (w/v), 5 µl DEPC and 500 µl glass beads, and vortexed for 1 minute. After vortexing, cells were subjected to three hot phenol extractions (5 min at 65°C) with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1), pH 5.0, and one extraction with 1 volume of chloroform:isoamyl alcohol at room temperature. RNA precipitation was performed as described by Schmitt et al. (1990). Total RNA was fractionated through formaldehyde-agarose gels and

Table 1. List of bacteria, yeasts strains and plasmid used in the present work.

Plasmids and strains	Characteristics	Source
Bacteria		
E. coli XL1-Blue	(Invitrogen)	(Hanahan, 1985)
Strains		
D. hansenii (CBS767)	Type strain	Portuguese yeast culture collection
S. cerevisiae (10560-6B)	ANT29Mat α leu2::hisG trp1::hisG his3::hisG ura3-52	Dr. P. Van Dijck
S. cerevisiae (10560-6B)	YSH1172 Mat α leu2::hisG trp1::hisG his3::hisG ura3-52 *aqy1* ::kanMX4 *aqy2* ::HIS3	Dr. P. Van Dijck
S. cerevisiae (10560-6B)	ANT27 10560-6B/pYX012 *KanMX AQY1-1* (Indicated as overexpressing *AQY1* gene)	Dr. P. Van Dijck
JCGHZERO	ANT29 (YEp352 empty)	This work
JCGHAQY1	ANT29 (JCGHpAQY1)	This work
JCGH*zero*	YSH1172*aqy1aq2* (YEp352 empty)	This work
JCGH*aqy1aqy2*	YSH1172*aqy1aq2* (JCGHpAQY1)	This work
Plasmid		
YEp352	Yeast episomal vector, 2µm, URA3 yeast marker and	(Hill et al., 1986)
JCGHpAQY1	Amp^R YEp352 derivative containing *DhAQY1* gene	This work

transferred to N^+-Hybond membranes (GE Healthcare). Hybridization was performed with a digoxygenin (DIG)-labeled probe prepared from the *DhAQY1* gene labeled using the DIG system (Roche) by random priming, according to manufacturer's instructions. Hybridizations were performed in DIG Easy Hyb (Roche), at 50°C (14 h). Membranes were then washed under high-stringency conditions and exposed to X-ray films for a maximum 48 h.

Growth assays

Specific growth rates were determined in liquid YNB-ura medium (2% (w/v) of glucose or glycerol) starting from a 50 ml pre-inoculum of cells grown in the same medium. The pre-inoculum was grown at 28°C for 24 h in an orbital shaker at 180 rpm and used to inoculate 250 ml flasks containing 100 ml of liquid medium at 0.2 of the initial optical density. Growth was followed by measuring the absorbance at 640 nm, with a Shimadzu spectrophotometer, model UV-160A. The ability of transformant strains to grow in the presence of different NaCl and KCl concentrations (0.6, 1.5, and 3.0 M) at two different pH (5.6 and 7.5, adjusted with Trizma base), and two carbon source (2% (w/v) of glucose or glycerol) was assessed in solid YNB-ura media with KCl and NaCl to the desired final concentrations. Transformants were grown for 24 h in 25 ml of YNB-ura liquid medium (2% glucose) up to a final density of approximately 3×10^7 ml^{-1}. Plates were inoculated with serial 10-fold dilutions of these cultures and incubated at 28°C. Growth was recorded after 1 week or 2 weeks (in the case of the 3 M of NaCl or KCl media).

Determination of freeze resistance in liquid medium by using cells viability test

Cells were grown to stationary phase in YNB-ura medium. They were then diluted to about 1.1×10^7 ml^{-1} with YNB-ura (without glucose) and incubated for 30 min at 30°C. Thereafter, the cultures were diluted 1:25 with YNB-ura (with 2% glucose) and incubated in this medium at 30°C to initiate fermentation and induce the transition from stationary phase to log phase. Samples were taken at different time points, from 0 to 135 min, to determine freeze resistance. After a 1:25 dilution with water, 1-ml samples were frozen for 2 h or 1 week at -26°C. Nonfrozen controls were diluted and plated immediately on YNB-ura plates, and the number of colonies was counted after incubation at 30°C for 2 to 4 days. Frozen samples were thawed, diluted with H_2O and plated on YNB-ura medium for colony counting as appropriate. Heat resistance in liquid medium was assessed in the same way as freeze resistance, except that the cultures were heated to 38°C for 15 min instead of being frozen (Teunissen, 2002).

Reproducibility

All assays were repeated at least three times and the data reported are mean or representative values.

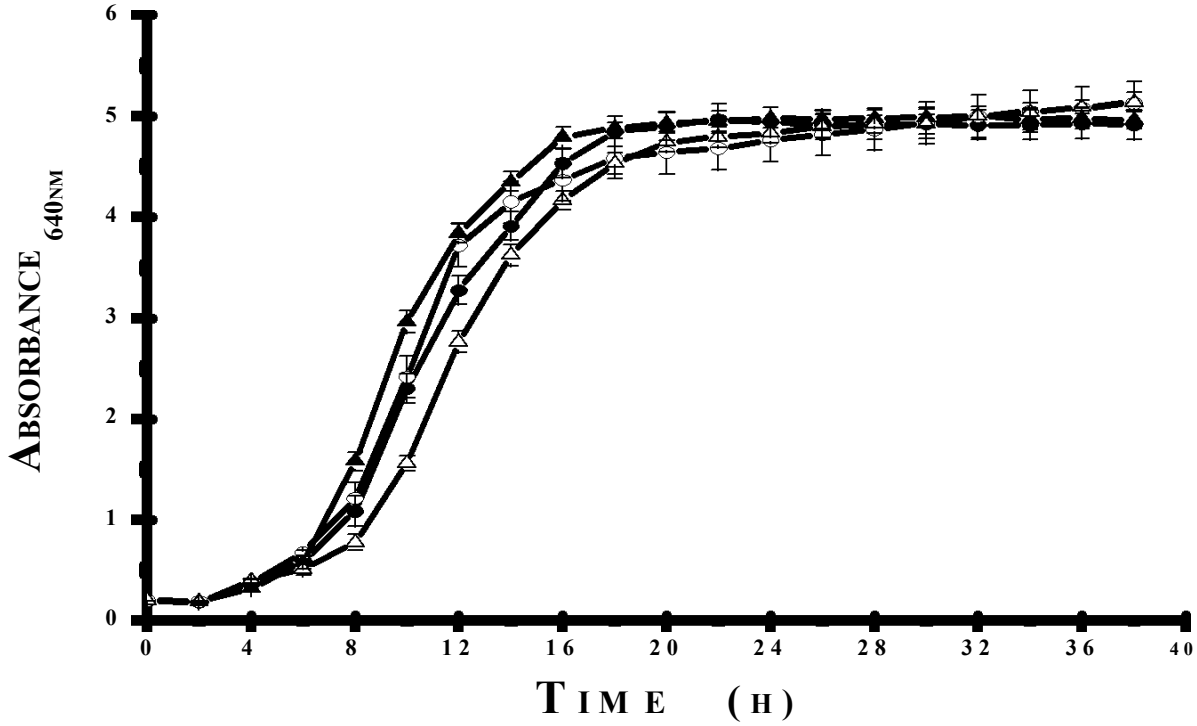

Figure 1. Growth of *S. cerevisiae* transformants cultivated in YNB-ura medium (2% (w/v) glucose) at pH 5.6 and 28°C. JCGHZERO (), JCGHAQY1 ( ), JCGH*zero* ( ), JCGH*aqy1aqy2* ( ). Data are representative of three experiments.

RESULTS

The release of the complete genome sequence of *D. hansenii* by the Génolevure consortium led me to look for *D. hansenii* sequences with homology to genes involved in glycerol and water transport in order to clone and characterize the putative transporter(s). By performing a tblastn search, Aqy1 protein sequence of *S. cerevisiae* against the translated sequence from the *D. hansenii* genome sequence database released by Génolevure was compared. An ORF (DEHA0F28787g) was found with 60% homology and 41% identity with Aqy1p from *S. cerevisiae*, located in chromosome F (anti-sense strand) and previously annotated as a gene of the water channel activity protein of 310 aa. The chromosomal regions corresponding to *DhAQY1* were amplified together with its own putative promoter and termination regions. The amplified fragments were cloned and used to transform a wild type and *S. cerevisiae* mutant (*aqy1aqy2*). Table 1 lists plasmids and strains used for cloning this gene.

To assess the possible contribution of the DhAqy1p protein under study, growth of the *S. cerevisiae* transformants was evaluated in YNB-ura liquid mineral medium without stress conditions with glucose as carbon source. Figure 1 illustrates the growth results obtained with the transformants. The heterologous expression of the *DhAQY1* gene did not affect or benefit the growth rate, the length of the lag phase, and the log phase (Figure 1) of any of the *S. cerevisiae* transformant yeasts when grown in liquid YNB-ura medium without salt. Growth of *D. hansenii* and *S. cerevisiae* transformant yeasts (Figure 2), containing YEp352 without or with the genomic DNA *AQY1* fragment from *D. hansenii* was evaluated also in solid YNB-ura medium (pH 5.6) and under saline stress growth conditions (Control = YNB-ura medium without salt, 0.6 or 1.5 M of NaCl, and KCl, respectivaly). The results obtained in this experiments show us that *D. hansenii* has the capacity to grow in all conditions evaluated, whereas, the JCGHZERO, JCGHAQY1 and JCGH*aqy1aqy2* did not grow in the presence of saline stress, that is, in the presence of NaCl; but, in the presence of KCl, a small similar effect but only in the presence of 1.5 M of this salt was observed. An important finding of this work is the capacity conferred by the *DhAQY1* gene to recover the phenotype of the wild type *S. cerevisiae* and the double null mutant of *AQY1* genes (JCGH*aqy1aqy2*).

Figure 3 describes the loss of stress resistance during the start of fermentation in liquid medium in transformant yeasts, which was evaluated at 2 h at -26°C, or at 1 week

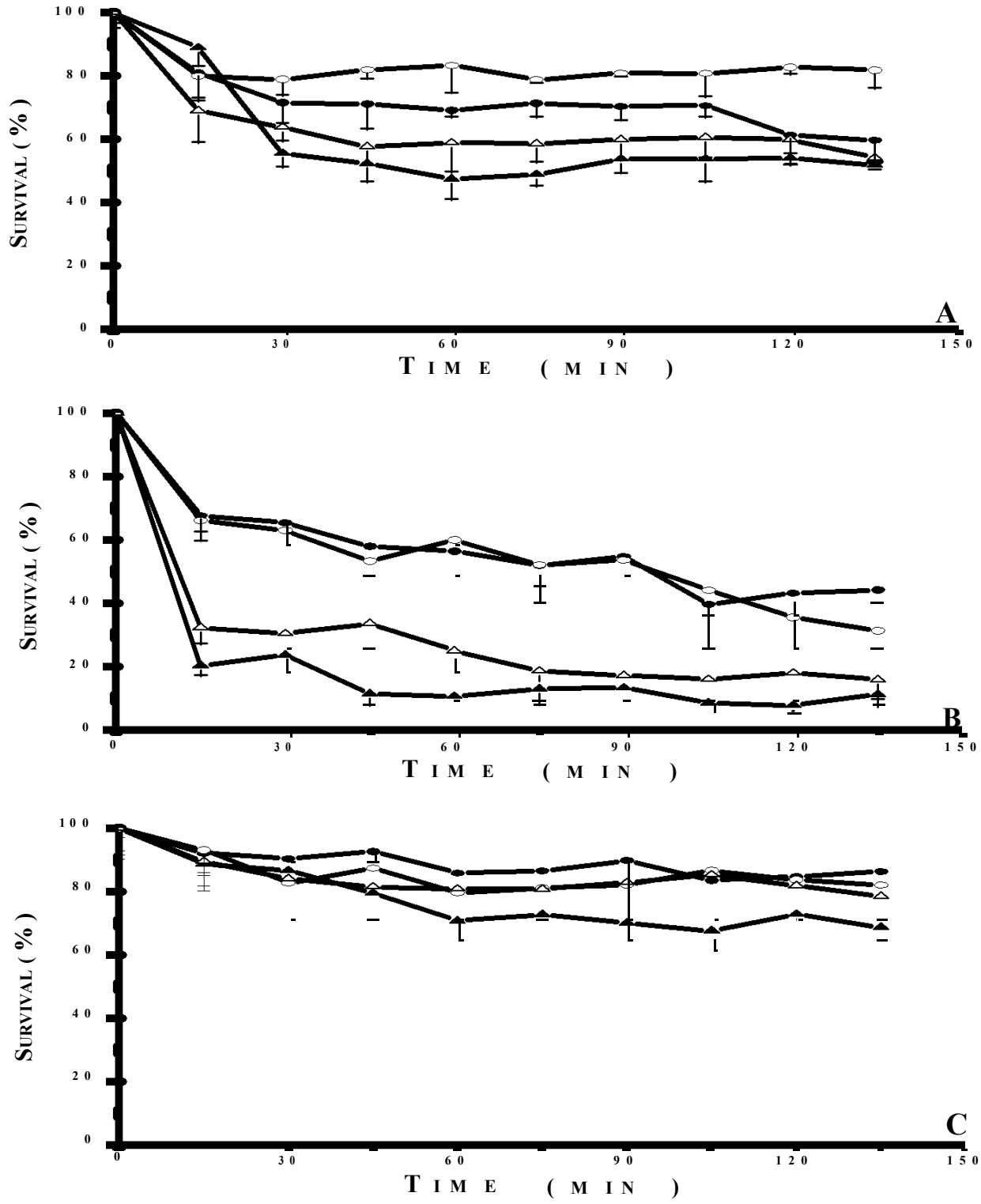

Figure 2. Growth of *D. hansenii* and *S. cerevisiae* transformants strains. Cells were grown for 24 h in 5 ml of YNB-ura liquid medium up to a final density of approximately 3×10^7 ml^{-1}. Plates were inoculated with serial 10-fold dilutions (the black arrow indicates the dilution direction) of these cultures in YNB-ura medium (pH 5.6) and different growth conditions (Control = YNB-ura medium without salt, 0.6 M or 1.5 M of NaCl [A] and KCl [B], respectively), and incubated at 28°C. *D. hansenii,* JCGHZERO (), JCGHAQY1 ( ), JCGH*zero* ( ) and JCGH*aqy1aqy* ( ). Growth was recorded after 1 week.

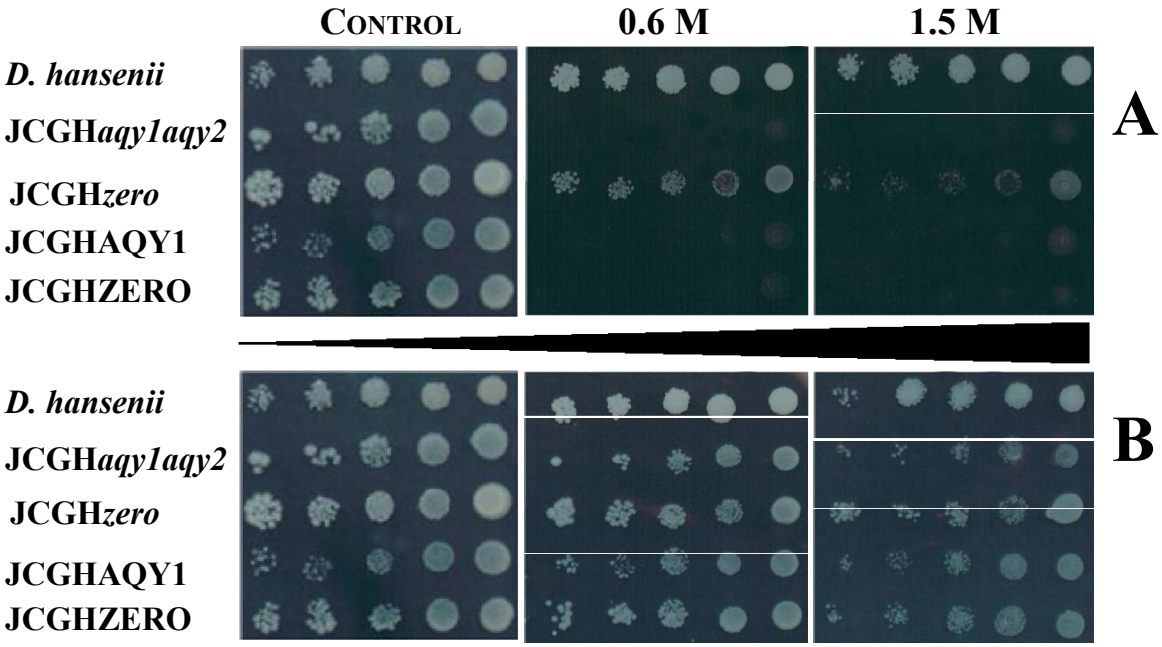

Figure 3. Loss of stress resistance during the start of fermentation in liquid medium in *S. cerevisiae* transformant strains, with the presence of the *DhAQY1* gene. JCGHZERO (), JCGHAQY1 ( ), JCGH*zero* ( ), JCGH*aqy1aqy2* ( ). Freeze stress 2 h at -26°C [A], 1 week at -26°C [B], cultures were heated to 38°C for 15 min instead of being frozen [C]. The samples were diluted with H_2O and plated on YNB-ura for colony counting, as appropriate.

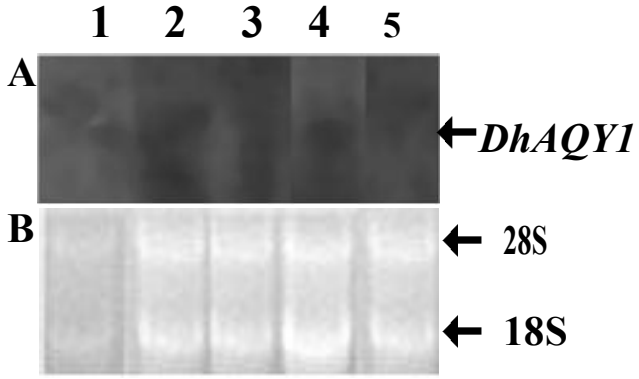

Figure 4. Northern blot analyses. Total RNA (20 µg) was extracted and loaded onto 1.0% agarose gel, transferred onto a membrane and hybridized with DIG-labeled *AQY1* Easy Hyb (Roche). Total RNA extracted from cells grown in YNB medium at pH 5.6, which were conserved in freeze stress conditions during 1 month at -26°C, ANT27 (line 1); *D. hansenii* (line 2); JCGHZERO (line 3); JCGHAQY1 (line 4); JCGH*aqy1aqy2* (line 5) (A). Ethidium bromide-stained (0.01 µg ml^{-1}) pattern (B). Arrowheads indicate the positions of ribosomal RNA.

at -26°C, cultures were heated to 38°C for 15 min instead of being frozen. A clear and little contribution on freeze resistance of the *DhAQY1* gene to the *S. cerevisiae* transformant yeasts was observed, when the cells were incubated 2 h (Figure 3A); during the first 30 minutes, survival of cells diminished 20 - 40%. The 1-week incubation under freezing conditions revealed only a small contribution of the *DhAQY1* gene to the JCGH*aqy1aqy2* transformant yeast (Figure 3B), and in this experiment the deleted transformant yeasts lost 60 - 80% survival. When the transformant yeast strains were heated, a similar survival rate was observed in all *S. cerevisiae* transformant yeasts. A clear diminution of survival in *D. hansenii* strain starting at 30 min of incubation was observed (data not shown).

The heterologous expression of the *DhAQY1* gene in *S. cerevisiae* under freeze treatment was evaluated by northern blot analyses. Figure 4 shows the Aqy1p expression when the cells were incubated 1 month. In this experiment, a control of the *S. cerevisiae* ANT27 was been included to further indicate overexpression of *AQY1* (10560-6B/pYX012 *KanMX AQY1-1)* in which, a small Aqy1p expression signal was observed.

DISCUSSION

Aquaporins belong to the major intrinsic protein (MIP) family of membrane proteins. Members of this family are channel proteins with six transmembrane domains. They

are involved in the transport of water and/or small neutral solutes such as glycerol (Park and Saier, 1996; Soveral et al., 2007). The starting point of this study was to search the recently available tool of the Genolevures consortium database, in which the genomes of various non-conventional yeasts have been sequenced. In the available genetic tools used, a sequence of *DhAQY1* gene with ample similarity with *S. cerevisiae* yeast was reported. This procedure enabled me to detect sequences and design primers to amplify and clone successfully the complete Aqy1 protein sequence, as well as the upstream and downstream regions eventually involved in their regulation.

It has been reported that when the *AQY1* gene is deleted from a wild-type yeast and cells are cultured *in vitro* with cycled hypo-osmolar or hyper-osmolar stresses, the *AQY1* null yeast shows significantly improved viability as compared with the parental wild-type strain. *S. 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 (Bonhivers et al., 1998). In these experiments, the JCGHZERO, JCGHAQY1 and JCGH*aqy1aqy2* transformant cells were similarly sensitive to osmotic stress under salt stress conditions, since their growth phenotype was equivalently abolished in presence of 0.6 and 1.0 M NaCl (Figure 2). Thus, the results obtained agree with the suggestion that increased survival under these conditions is significantly enhanced by the deletion of *AQY1*, and that the Aqy1p is not essential for life; as supported by the work of Bonhivers et al. (1998).

The present study demonstrates that the osmotic stress during growth was much better tolerated by *aqy1aqy2* mutant yeast than by the JCGHZERO, JCGHAQY1 and JCGH*aqy1aqy2* transformant cells, however, a role in osmoregulation seems likely. Current models have described how cells sense osmotic stress but 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 (Varela and Mager, 1996). In addition, the first study has been reported, in which the parameters of water transport in yeasts were assessed in intact cells of strains with different levels of *AQY1* expression (Soveral et al., 2007).

Little information is available about the precise mechanisms and determinants of freeze resistance in baker's yeast, *S. cerevisiae*. Genome-wide gene expression analysis and northern analysis of different freeze-resistant and freeze-sensitive strains have revealed a correlation between freeze resistance and the aquaporin genes *AQY1* and *AQY2*. Deletion of these genes in a laboratory strain rendered yeast cells more sensitive to freezing, whereas, overexpression of the respective genes, as well as heterologous expression of the human aquaporin gene *hAQP1*, improved freeze tolerance. These findings support a role for plasma membrane water transport activity in determinating freeze tolerance in yeast. This appears to be another clear physiological function identified for microbial aquaporins (Tanghe et al., 2002). Since aquaporin overexpression does not seem to affect the growth and fermentation characteristics of yeast, these results open new perspectives for the successful development of freeze resistant baker's yeast strains for use in frozen dough applications (Tanghe et al., 2002). Freeze tolerance has been reported in nonfermenting and fermenting cells. The *AQY1-1* gene is highly expressed in nonfermenting cells and poorly expressed in glucose medium, whereas, *AQY2-1* expression is very low in nonfermenting cells and increases after the addition of glucose. In accordance with the findings of expression studies of the two aquaporins using northern blot analysis (Tanghe et al., 2002), this could explain our weak signal in the *AQY1* expression, because cells were grown in glucose medium.

Higher levels of aquaporins in the plasma membrane would allow for faster water efflux, especially at freezing temperatures, at which water diffusion through the phosphorlipid layer of the membrane is much slower than at higher temperatures. Because reduction of ice crystal formation results in reduced destruction of cellular membranes and other components, it allows the cells to maintain higher activity and viability. This explanation agrees with previous observations on the protective effects of ethanol and methanol against freeze damage, which correlates with their stimulating effects on membrane permeability, presumably allowing faster water efflux during freezing (Lewis et al., 1994).

The aquaporin overexpression significantly improves the maintenance of viability of industrial yeast strains upon freezing and seems to have little effect on other yeast properties, it appears to be a promising tool for improvement of freeze tolerance in commercial baker's yeast strains (Tanghe et al., 2002).

The overexpression of the aquaporin-encoding genes *AQY1-1* and *AQY2-1* improves the freeze tolerance of industrial strain AT25, but only in small doughs under laboratory conditions and not in large doughs under industrial conditions (Tanghe et al., 2002). Freeze tolerance and expression of the aquaporin-encoding genes *AQY1* and *AQY2* have been correlated based on a

genome-wide gene expression analysis of the freeze-resistant industrial mutant strain AT25 (Randez-Gil et al., 1999).

ACKNOWLEDGMENT

This work was supported by grant SFRH/BPD/19913/2004 to Juan Carlos González-Hernández, granted by FCT, Portugal.

REFERENCES

Bonhivers M, Carbrey JM, Gould SJ, Agre P (1998). Aquaporins in *Saccharomyces*. Genetic and functional distinctions between laboratory and wild-type strains. J. Biol. Chem. 273: 27565-27572.

Laize V, Tacnet F, Ripoche P, Hohmann S (2000). Polymorphism of *Saccharomyces cerevisiae* aquaporins. Yeast 16: 897-903.

Laize V, Gobin R, Rousselet G, Badier C, Hohmann S, Ripoche P, Tacnet F (1999). Molecular and functional study of *AQY1* from Saccharomyces cerevisiae*:* Role of the C-terminal domain. Biochem. Biophys. Res. Commun. (257): 139-144.

Tanghe A, Van Dijck P, Dumortier F, Teunissen A, Hohmann S, Thevelein JM (2002). Aquaporin expression correlates with freeze tolerance in baker's yeast, and overexpression improves freeze tolerance in industrial strains. Appl. Environ. Microbiol. (68): 5981-5989.

Tanghe A, Kayingo G, Prior BA, Thevelein JM, Van Dijck P (2005). Heterologous aquaporin (AQY2-1) expression strongly enhances freeze tolerance of *Saccharomyces pombe*. J. Mol. Microbiol. Biotechnol. (9): 52-56.

Tanghe A, Carbrey JM, Agre P, Thevelein JM, Van Dijck P (2005). Aquaporin expression and freeze tolerance in *Candida albicans.* Appl. Environ. Microbiol. (71): 6434-6437.

Chrispeels MJ, Agre P (1994). Aquaporins: water channel proteins of plant and animal cells. Trends Biochem. Sci. (19): 421-425.

Prista C, Almagro A, Loureiro-Días MC, Ramos J (1997). Physiological basis for the high tolerance of Debaryomyces hansenii. Appl. Environ. Microbiol. (63): 4005-4009.

González-Hernández JC, Cárdenas-Monroy CA, Peña A (2004). Sodium and potassium transport in the halophilic yeast Debaryomyces hansenii. Yeast (21): 403-412.

González-Hernández JC, Jiménez-Estrada M, Peña A (2005). Comparative analysis of trehalose production by Debaryomyces hansenii and Saccharomyces cerevisiae under saline stress. Extremophiles 9: 7-16.

Adler L, Gustafsson L (1980). Polyhydric alcohol production and intracellular amino acid pool in relation to halotolerance of yeast Debaryomyces hansenii. Arch. Microbiol.(124): 123-130.

Gustafsson L, Norkrans B (1976). On the mechanism of salt tolerance. Production of glycerol and heat during growth of *Debaryomyces hansenii*. Arch. Microbiol. 110: 177-183.

Hanahan D (1985). Techniques for transformation of *Escherichia coli*. In: DNA Cloning: A Practical Approach (Glover, D.M., Ed.), IRLPress, Oxford pp. 109-135.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Birnboim HC, Doly J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523.

Hoffman CS, Winston F (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene 57: 267-272.

Cryer DR, Ecclesmall R, Marmur J (1975). Isolation of yeast DNA. In: Methods in Cell Biology (Prescott, D.M., Ed.), Academic Press, New York p. 12: 39.

Teunissen A, Dumortier F, Gorwa MF, Bauer J, Tanghe A, Loïez A, Smet P, Van Dijck P, Thevelein JM (2002). Isolation and characterization of a freeze-tolerant diploid derivative of an industrial baker's yeast strain and Its use in frozen doughs. Appl. Environ. Microbiol. (68): 4780-4787.

Schmitt ME, Brown TA, Trumpower BL (1990). A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae.* Nucl. Acids Res. 18: 3091-3092.

Park JH, Saier MH (1996). Phylogenetic characterization of the MIP family of transmembrane channel proteins. J. Membr. Biol. 153: 171-180.

Soveral G, Madeira A, Loureiro-Dias MC, Moura TF (2007). Water transport in intact yeast cells as assessed by fluorescence self-quenching. Appl. Environ. Microbiol. 73: 2341-2343.

Varela JCS, Mager WH (1996). Response of Saccharomyces cerevisiae to changes in external osmolarity. Microbiology 142: 721-731.

Lewis JG, Learmonth RP, Watson K (1994). Cryoprotection of yeast by alcohols during rapid freezing. Cryobiology 31: 193-198.

Randez-Gil F, Sanz P, Prieto JA (1999). Engineering baker's yeast: room for improvement. Trends Biotechnol. 17: 237-244.