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SHORT COMMUNICATION

Heterologous expression of *Saccharomyces cerevisiae MPR1* gene confers tolerance to ethanol and L-azetidine-2-carboxylic acid in *Hansenula polymorpha*

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Abstract *Hansenula polymorpha* is a naturally xylose-fermenting yeast; however, both its ethanol yield from xylose and ethanol resistance have to be improved before this organism can be used for industrial high-temperature simultaneous saccharification and fermentation of lignocellulosic materials. In the current research, we checked if the expression of the *Saccharomyces cerevisiae MPR1* gene encoding *N*-acetyltransferase can increase the ethanol tolerance of *H. polymorpha*. The *S. cerevisiae MPR1* gene was cloned in the *H. polymorpha* expression vector under the control of the *H. polymorpha* strong constitutive promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*). *H. polymorpha* recombinant strains harboring 1–3 copies of the *S. cerevisiae MPR1* gene showed enhanced tolerance to L-azetidine-2-carboxylic acid and ethanol. The obtained results suggest that the expression of the *S. cerevisiae MPR1* gene in *H. polymorpha* can be a useful approach in the construction of *H. polymorpha* strains with improved ethanol resistance.

Keywords Fuel ethanol · Ethanol resistance · *MPR1* · AZC · *Hansenula polymorpha*

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Introduction

Biofuel production, including that of fuel ethanol, is an example of a high-priority technology [10]. Microbial fermentation of renewable resources (plant biomass, mostly consisting of lignocellulose) is one of the ways to produce ethanol [21], as lignocellulose is a ubiquitous and cheap feedstock. Several nonconventional yeast species have some advantages over the traditional ethanol producer, *Saccharomyces cerevisiae*, for biofuel ethanol production from lignocellulose. Whereas the fermentation of glucose by *S. cerevisiae* is very efficient, wild-type strains are not capable of fermenting and utilizing xylose (the main pentose sugar of lignocellulose) [2, 11].

The nonconventional methylotrophic yeast *Hansenula polymorpha* is one of the most thermotolerant yeasts identified to date, and is able to grow at a maximal temperature of 50°C [3]. This yeast utilizes xylose and ferments both glucose and xylose at high temperature [7, 12, 13, 26]. Xylose fermentation at 50°C is important for the development of high-temperature SSF (simultaneous saccharification and fermentation) technology for fuel ethanol production, as the optimum conditions required by many fungal cellulases are in the range of 50–60°C at a pH of 5.0 [35]. This makes *H. polymorpha* a suitable organism for the development of a biofuel ethanol process that uses lignocellulosic feedstocks. The commercial application of *H. polymorpha* for bioethanol production will depend on further improvement of the xylose fermentation performance of this yeast.

Tolerance to ethanol is of great importance in industries in which ethanol is the primary fermentation product. Aside from its negative effects on cell growth and viability [23], the accumulation of ethanol during fermentation inhibits further ethanol production by yeast cells. In spite of numerous studies, the molecular mechanisms underlying ethanol

tolerance have not yet been fully elucidated [14]. Several factors were shown to contribute to the ethanol tolerance of yeast cells. Generally, these involve the composition of plasma membrane lipids [4, 6, 15, 17], the activity of plasma membrane ATPase [1, 25] or superoxide dismutase [5], the accumulation of trehalose [19, 20, 29] and proline [31], and upregulation of the expression of heat-shock proteins [23, 28]. In addition to the above, the vacuole protein sorting and peroxisome protein import machinery and especially the *FPS1* gene encoding plasma membrane aquaglyceroporin have been shown to play a role in yeast ethanol tolerance [32].

Recently, the *S. cerevisiae MPR1* gene coding for *N*-acetyltransferase was reported to confer ethanol tolerance in this yeast species [8]. In this study, we investigated the effect of heterologous *S. cerevisiae MPR1* gene expression on ethanol tolerance in *H. polymorpha*. Recombinant strains of *H. polymorpha* harboring the *S. cerevisiae MPR1* gene proved to be more resistant to both ethanol and L-azetidine-2-carboxylic acid than the wild-type strain is. This is a promising finding that will aid in the further development of *H. polymorpha* for biofuel ethanol production.

Materials and methods

Strains and growth conditions

The following yeast strains were used: *S. cerevisiae* Σ1278b (α wild-type *MPR1 MPR2 AZC*^{R}), kindly provided by Prof. Penninckx M. J. (Laboratoire de Microbiologie de l'Université Libre de Bruxelles c/o Institut de Recherché du CERIA, Brussels, Belgium) and *H. polymorpha* 3Leu+ [13]. Yeast strains were maintained on YPS medium (0.5% yeast extract, 1% peptone and 2% sucrose) at 30°C in the case of *S. cerevisiae* or 37°C in the case of *H. polymorpha*. Yeast strains were also grown on YNB medium with 2% sucrose containing L-azetidine-2-carboxylic acid, AZC (Sigma–Aldrich Co., St. Louis, MO, USA) or 7% ethanol. *H. polymorpha* transformants were selected on YPS medium supplemented with zeocin at 140 mg L^{-1}.

The *Escherichia coli* strain DH5α [Φ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (r_K^-, m_K^+), *supE*44, *relA*1, *deoR*, Δ (*lacZYA-argF*) U169] was used in experiments that required a bacterial host. The bacterial strain was grown at 37°C in LB medium as described by Sambrook et al. [27]. The recombinant *E. coli* strains were grown on a medium containing 100 mg L^{-1} of ampicillin. 2% agar was added to solidify the media.

Construction of the *MPR1* expression plasmid

The genomic DNA of the Σ1278b strain of *S. cerevisiae* served as a template for isolating the ORF of the *MPR1* gene with the primers IS239 5′-CACAAGCTTATGG ATGCGGAATCCATCGAATG-3′ and IS240 5′-CCCAA GCTTTTATTCCATGGAGAGGAATTCGG-3′. The PCR fragment of 690 bp was treated with restriction endonuclease *Hin*dIII and cloned into the expression vector for *H. polymorpha* p70 (Fig. 1) under the control of the strong constitutive promoter *GAPDH*. The resulting recombinant plasmid was designated p70 + MPR1Sc (Fig. 1).

Molecular biology techniques

The following published methods were used for DNA manipulation [27] and yeast transformation [9]. Plasmid

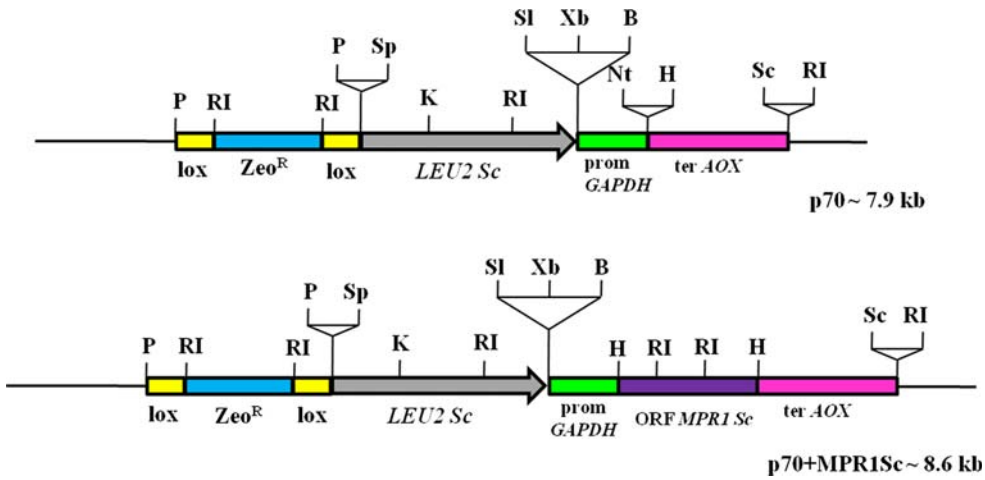

Fig. 1 Linear schemes for the plasmids p70 and p70 + MPR1Sc. The *S. cerevisiae* ORF of *MPR1* is shown as a *violet box*, the promoter of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) of *H. polymorpha* as a *green box*, the terminator of alcohol oxidase (*AOX*) of *H. polymorpha* as a *pink box*, the *LEU2* gene of *S. cerevisiae* as a *gray arrow*, the zeocin resistance gene Zeo^R as a *blue box*, and *lox*P sequences as *yellow boxes*. The pUC18 part: *thin line*. Restriction sites: P, *Pst*I; RI, *Eco*RI; Sp, *Sph*I; K, *Kpn*I; SI, *Sal*I; B, *Bam*HI; H, *Hind*III; Sc, *Sac*I

DNA isolations from *E. coli* were carried out using NucleoSpin® Plasmid QuickPure (Macherey–Nagel, Düren, Germany). *Taq* DNA polymerase and Vent$_R$® DNA polymerase (both New England Biolabs, Frankfurt am Main, Germany) were used for analytical and preparative PCR, respectively. T4 DNA ligase, T4 DNA polymerase and restriction enzymes were purchased from Fermentas (Vilnius, Lithuania). Preparations of genomic DNA from yeast species were carried out using the DNeasy® tissue kit (Qiagen, Hilden, Germany). Southern blotting analysis was performed using the Amersham ECL direct nucleic acid labeling and detection system (GE Healthcare, Freiburg, Germany).

Reverse transcription (RT)-PCR analysis

Yeast strains were grown in YPS medium to the mid-exponential phase (OD_{600} of 0.5). Total RNA was extracted from the yeast cells using the Trizol method (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. RNA was quantified by UV spectrophotometry and diluted in RNase-free water. Single-stranded cDNA was synthesized using MuLV reverse transcriptase (First Strand cDNA Synthesis Kit, Fermentas, Vilnius, Lithuania). Quantitative RT–PCR analysis was carried out using gene-specific primer pairs and cDNA as a template: IS239 and IS240 for ORF of *S. cerevisiae MPR1* gene; IS381 5′-GAGGTTACACGTTCTCCACCACTGC-3′ and IS382 5′-GTGATCACCTGTCCATCAGGCAACTC-3′ for *H. polymorpha* ORF of *ACT1* gene (orf262, Hp_contig01).

Acetyltransferase activity assay

Cell-free extracts of yeast strains were prepared as described earlier [13]. Acetyltransferase activity was measured in cell-free extracts with 1 mM AZC as a substrate as described by Nomura et al. [22]. Enzyme assay was performed at 37°C by monitoring the increase in 5-thio-2-nitrobenzoic acid (TNB) produced from the reaction of acetyl-CoA with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). The enzyme reaction rate was calculated using an extinction coefficient for TNB of 15,570 M^{-1} cm^{-1}. One unit is defined as the amount of enzyme catalyzing the formation of 1 μmol TNB/min at 37°C. Protein concentration was determined using the Lowry method, with bovine serum albumin used as a standard [18].

Results

Construction of the recombinant *H. polymorpha* strains expressing the *S. cerevisiae MPR1* gene

The plasmid p70 + MPR1Sc was linearized with *Sac*I and used for transformation of the *H. polymorpha* strain 3Leu+. Yeast transformants were selected on YPS medium with zeocin. The stability of the transformants was checked by alternating cultivation on selective medium (YPS with zeocin) and nonselective medium (YPS), as described previously [12]. The transformants that did not lose the plasmid after being grown in nonselective medium for 60 generations were assumed to be stable. The presence of desirable recombinant constructs (promoter *GAPDH* fused to *MPR1* ORF and terminator AOX) in the genome of stable integrants was checked by PCR (data not shown). Stable integrants of *H. polymorpha* carrying the plasmid were designated MPR1-2, MPR1-4 and MPR1-5. The expression of heterologous *MPR1* gene in these transformants was analyzed by RT–PCR (Fig. 2). All MPR1 transformants were shown to have mRNA of *MPR1* expressed from *H. polymorpha GAPDH* promoter in the cells cultured in YPS medium (Fig. 2).

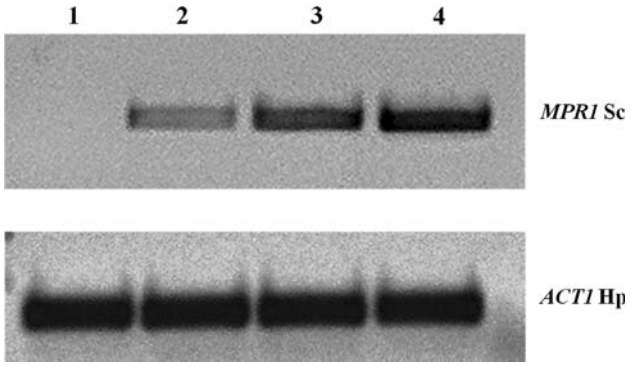

Fig. 2 RT–PCR analysis of *MPR1* expression in *H. polymorpha* transfromants. The RT–PCR reaction was performed on the cDNA of the transformants: *1*, 3Leu+; *2*, MPR1-2; *3*, MPR1-4; *4*, MPR1-5. Primers used in the reaction were designed for *S. cerevisiae MPR1* ORF (*MPR1* Sc) and for *H. polymorpha* actin (*ACT1* Hp), serving as a control

Resistance to AZC and ethanol

The growth phenotypes of the obtained recombinant strains were examined on media supplemented with either AZC or ethanol (Fig. 3). The transformants expressing *MPR1* of *S. cerevisiae* were more tolerant of both AZC and ethanol as compared to the recipient strain 3Leu+. Among the transformants tested, the stress resistance varied in a manner consistent with the incorporation of different copy numbers of p70 + MPR1Sc in the genome. Judging from the growth on the solid media, the resistance to AZC/ethanol of the MPR1-5 transformant was approximately 10-fold higher (Fig. 3).

Genomic DNA of the *H. polymorpha* transformants MPR1-2, MPR1-4, MPR1-5 and the recipient strain 3Leu+ was isolated and treated with restriction endonuclease *Bam*HI, which does not cut within the coding region

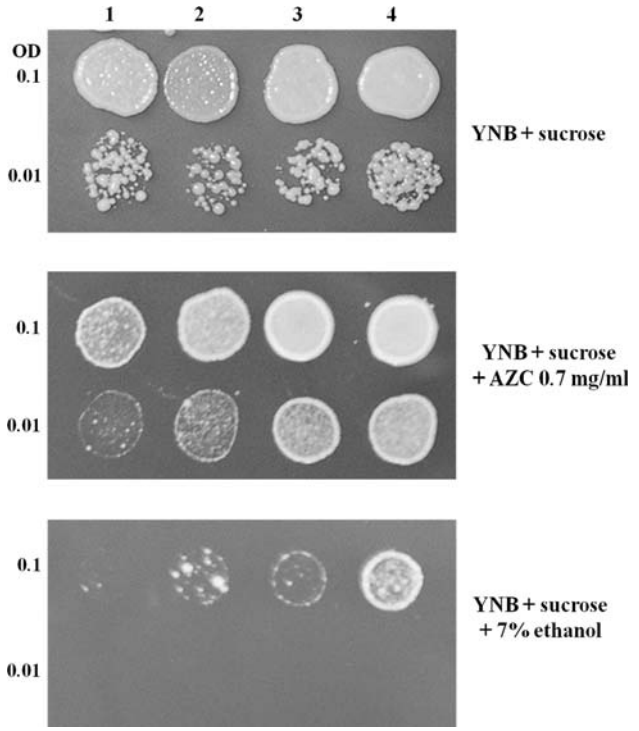

Fig. 3 Growth phenotypes of *H. polymorpha* transformants carrying the *MPR1* expression plasmid on the minimal media supplemented with AZC and 7% ethanol. Yeast strains were grown in the liquid YPS medium overnight; serial dilutions with the same OD were spotted on the agar media and incubated at 37°C for 3 days. Strains: *1*, 3Leu+; *2*, MPR1-2; *3*, MPR1-4; *4*, MPR1-5

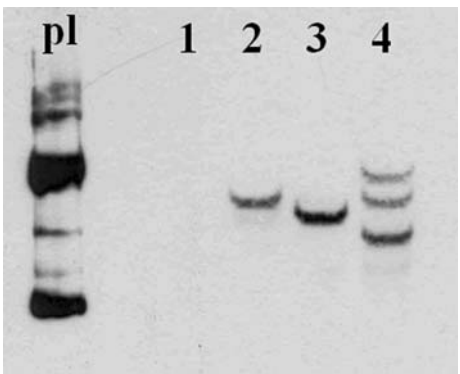

Fig. 4 Southern blot analysis showing the *S. cerevisiae MPR1* gene copy number in the genomes of the *H. polymorpha* transformants. The genomic DNA of the *H. polymorpha* strains was digested with *Bam*HI. *S. cerevisiae MPR1* was used as a probe. Strains: *1*, 3Leu+; *2*, MPR1-2; *3*, MPR1-4; *4*, MPR1-5. *pl*, plasmid p70 + MPR1Sc loaded

of the *MPR1* gene and is unique on the p70 + MPR1Sc vector. Southern blot analysis with *S. cerevisiae MPR1* used as a probe (Fig. 4) revealed that MPR1-2 and MPR1-4 have one copy of the plasmid while MPR1-5 carries three plasmid copies. The hybridization signals for the MPR1 transformants differed in size, indicating that the plasmid p70 + MPR1Sc was integrated at different genomic locuses (Fig. 4).

Table 1 Acetyl transferase activities of *H. polymorpha* strains

Strain	Acetyl transferase activity (U)	
	YNB + sucrose	YNB + sucrose + 7% ethanol
3Leu+	14.2 ± 0.4	2.3 ± 0.5
MPR1-2	53.2 ± 6.3	20.9 ± 5.1
MPR1-4	94.14 ± 2.9	8.9 ± 0.8
MPR1-5	201.7 ± 3.5	57.9 ± 4.1

For enzyme assay measurements, the yeast strains were grown in liquid YNB media with/without ethanol. The data shown are the means of three independent experiments

The acetyltransferase activities of the *H. polymorpha* transformants showed a correlation with *MPR1* copy number in the genome and with resistance to AZC and ethanol. It was found that a MPR1-5 transformant carrying three plasmid copies had the highest acetyltransferase activity and AZC/ethanol resistance on the minimal medium and on the minimal medium supplemented with 7% ethanol (Table 1; Fig. 3). The acetyltransferase activities measured in the transformants MPR1-2, MPR1-4 and MPR1-5 were correspondingly 3.8-, 6.6- and 14-fold higher than that of the recipient strain 3Leu+ on the YNB with sucrose medium (Table 1). On the minimal media supplemented with 7% ethanol, the acetyltransferase activities measured in the transformants MPR1-2, MPR1-4 and MPR1-5 were correspondingly 9.1-, 3.9- and 25.2-fold higher than that of the control strain (Table 1). The acetyltransferase activities of the transformants MPR1-2 and MPR1-4 carrying one plasmid copy in the genome (Fig. 3) appear to differ in media with or without ethanol (Table 1), which can be explained by the influence of the plasmid integration position in the genomes of these transformants [33].

Discussion

MPR1 was first identified as a gene coding for novel acetyltransferase in the *S. cerevisiae* Σ1278b strain that detoxifies the proline analog AZC [30]. AZC is incorporated into protein competitively with proline and causes reduced thermal stability due to misfolding. It was suggested that Mpr1 detoxifies AZC by acetylating this compound, with the resulting *N*-acetyl-AZC not being incorporated into proteins. The Mpr1 was also reported to protect *S. cerevisiae* cells from oxidative damage, including H_2O_2, heat-shock and ethanol stress, by reducing intracellular ROS levels [8]. The *MPR1* gene was shown to be widely distributed among various yeast and fungi, but enzymatic

analysis of these *MPR1* homologs has been limited to *S. cerevisiae*-complex species and some other genera [16, 22, 34].

Using the genome database of the sequenced *H. polymorpha* CBS4732 strain [24], a BLAST search was performed against the *S. cerevisiae MPR1* gene amino acid sequence, and this did not reveal any proteins with substantial homology. We have shown that *H. polymorpha* CBS4732 and 3Leu+ (NCYC495 *leu1-1* strain derivative) differ in their resistance to AZC. While 3Leu+ grows at AZC concentrations of up to 1 mg/mL, CBS4732 does not even grow on AZC media at the smallest concentration tested (0.3 mg/mL; data not shown). The mechanism of AZC detoxification in the NCYC495 strain is not known, and may possibly involve a native acetyltransferase gene.

In this study, we decided to check if heterologous *S. cerevisiae MPR1* can increase *H. polymorpha* ethanol tolerance. A 3Leu+ strain was chosen for *MPR1* expression, as its parental strain NCYC495 *leu1-1* is a better ethanol producer from xylose than CBS4732 is. To do this, the *S. cerevisiae MPR1* gene was amplified from the Σ1278b strain and cloned into the *H. polymorpha* expression vector under the control of the strong *GAPDH* promoter and transformed into the *H. polymorpha* 3Leu+ strain. It was shown that mRNA of the *S. cerevisiae MPR1* gene is efficiently transcribed in *H. polymorpha* from the *GAPDH* promoter. The constructed recombinant strains carrying *MPR1* expression cassettes were tested for sensitivity to AZC and ethanol. All *MPR1* transformants had improved resistance to AZC and ethanol. The resistance level correlated with *S. cerevisiae MPR1* copy number in the genomes of the transformants and acetyltransferase activity. The obtained data support the successful expression of the heterologous *MPR1* gene in *H. polymorpha*. The *S. cerevisiae MPR1* gene can be used for the construction of *H. polymorpha* strains with improved ethanol resistance and as a dominant selective marker for the genetic transformation of this yeast species.

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