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Transcriptional expression of selected genes associated with excretion of carboxylic acids from *aci* mutants of *Saccharomyces cerevisiae*

Transkrypcyjna ekspresja genów kodujących białka biorące udział w procesie wydzielania kwasów karboksylowych przez mutanty aci *Saccharomyces cerevisiae*

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: *Saccharomyces cerevisiae* is an excellent model organism for studies of transcriptional regulation of metabolic processes in other eukaryotic cells including human cells. Cellular acid-base balance can be disturbed in pathologic situations such as renal acidosis or cancer. The extracellular pH of malignant solid tumors is acidic in the range of 6.5-6.9. EG07 and EG37 *aci* mutants of *Saccharomyces cerevisiae* excessively excrete carboxylic acids to glucose-containing media or distilled water. The excreted acids are Krebs and/or glyoxylate cycle intermediates. The genes restoring the wild-type phenotype have function that does not easily explain the Aci^+ phenotype.

Material/Methods: In this study, using real-time PCR we measured relative mRNA expression, in the mutants compared to the wild-type strain, of selected genes associated with both carboxylic acid cycles and two cell transporters, *Pma1* and *Pdr12*, of organic acids.

Results: Unexpectedly, we found that the relative expression of the selected Krebs cycle and glyoxylate cycle genes did not change significantly. However, the expression of the two transporter genes was strongly elevated in EG37 and moderately increased in EG07.

Conclusion: These results indicate that the induction of the two cell transporter genes plays an important role in acid excretion by the aci mutants.

Keywords: *Saccharomyces cerevisiae* • ***aci*** **mutants** • **Krebs and glyoxylate cycle** • **acid transporters**

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Abbreviations: **Aci**$^{+}$ – mutant phenotype excreting acids; **aci** – mutated gene; **Cit1, Cit2** and **Cit3** – citrate synthase isozymes; ***ICL1*** – isocitrate lyase isozyme; MDH***1,*** MDH***2*** and MDH***3*** – malate dehydrogenase isozymes; ***PDR12*** – plasma membrane ABC transporter; ***PMA1*** – plasma membrane H+-ATPase; **R** – relative expression ratio: mutant/wild type; WAR1 – transcription factor of *PDR12*.

Introduction

The yeast *Saccharomyces cerevisiae* is an excellent model organism for studies of transcriptional regulation of metabolic processes, such as Krebs cycle, glyoxylate cycle or cellular transport of organic acids in eukaryotic cells [10,12,13,17,21]. For example, such model studies may be very helpful in finding molecular mechanisms that explain metabolic acidosis in renal cells [18] or how solid tumors generate acid and export it into the surrounding parenchyma [24]. The entire genome of *S. cerevisiae* was sequenced in 1996 [7]. However, despite a broad knowledge of many metabolic reactions in this organism, the transcriptional regulation of the metabolic pathways, including the ones named above, is complex and remains unclear [6]. Metabolic mutants like those defective in Krebs cycle genes [1,23] have been very useful in this kind of research. Gonchar et al. [8] using a mutagenic reagent ethyl methanesulfonate (EMS) generated a group of *S. cerevisiae* aci mutants that on complete Kok medium [11] containing glucose excrete acids. The excreted acids were later identified as intermediates of the Krebs cycle or the glyoxylate cycle [2,16]. Quick release of acid was also observed when the mutants were suspended in water [16]. The aci mutants were found to be the result of single gene mutations and distributed among 16 complementation groups [9]. Transformation of the mutants with a genomic DNA library on the multicopy shuttle plasmid pFL44L [3] restored the wild-type Aci^{-} [2]. Also, in our preliminary studies (unpublished results) a single-copy plasmid containing the *YLR376C* gene had this ability. Overall, three genes, *YJL185C, YLR376C* and *YJR129C*, restoring the Aci^{-} phenotype were identified. *YJL185C* encodes Pex3p interacting protein required for pexophagy (www.yeastgenome.org). *YLR376C* encodes a component of the Shu complex, which promotes error-free DNA repair (www.yeastgenome.org). *YJR129C* encodes a putative adenosylmethionine-dependent methyltransferase (www.yeastgenome.org) [2]. However, the functions of these proteins do not easily explain the Aci+ phenotype. Presumably, the discovered genes play unknown regulatory roles that indirectly influence the function of the Krebs and/or glyoxylate cycle. In such a case the excessive accumulation of the organic acids in the cytoplasm induces an acid stress response that leads to the removal of the acids from the cell. Another possibility is that the mutated genes regulate the expression or activity of an organic anion transporter such as *Pdr12* cooperating with a proton pump such as *Pma1* [21].

Our ultimate goal is the identification of the mutated genes, and genetic cascades with corollary metabolic pathways triggered by the mutations. However, before the attainment of this goal we decided to broaden biochemical characterization of the aci mutants. In this study, using a real-time PCR technique, we compared mRNA expression of several important metabolic genes in two selected aci mutants to that in the wild-type strain D273-10B/A1 of *S. cerevisiae*. The two selected aci mutants, EG07 and EG37, display the strongest aci character but differ in their growth ability on non-fermentable carbon sources [16]. The chosen metabolic genes encode enzymes of the Krebs and glyoxylate cycles, ABC anion transporter *Pdr12* [21], proton pump *Pma1* [25], and the transcription factor War1 that induces *Pdr12* activity [12].

Materials and methods

Yeast strains

Saccharomyces cerevisiae strains, wild type and mutants, used in this work are listed in Table 1.

Table 1. *Saccharomyces cerevisiae* strains

Strains	Markers	Origin
Wild-type D273-10B/A1	*MATa met6*	Dr. Grenson M., Universite Libre de Bruxelles, Belgium
Mutant EG07	*MATa met6*	Dr. Boniewska-Bernacka E. University of Opole, Poland
Mutant EG37	*MATa met6*	Dr. Boniewska-Bernacka E. University of Opole, Poland

Growth conditions

The yeast cells were maintained on YPD (yeast extract 1%, bacto-peptone 1%, dextrose 2%) agar slopes at 4°C, and cultivated 24 hours at 28°C in liquid YPD medium with shaking.

PolyA+ mRNA isolation

Total RNA was extracted from yeast cells using a Total RNA kit (A&A Biotechnology, Gdansk, Poland) according to the manufacturer's protocol. PolyA+ mRNA purification was performed using FastTrack® MAG mRNA Isolation Kits (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using SuperScriptTM III Platinum® RT--PCR System (Invitrogen) in a total volume of 20 µl, then obtained cDNA was used to analyze gene expression by real-time PCR.

Real-time PCR assay

The PCR primers (Table 2) were custom synthesized by the DNA Sequencing and Oligonucleotide Synthesis Laboratory at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland (www.oligo.pl). The synthetic oligonucleotides were purified by HPLC. The gene *specificity* of the primers was checked by searching the Saccharomyces Genome Database at www.yeastgenome.org with the Wu-BLAST2 program available on the same Internet site.

Table 2. Real-time PCR primers

Gene	Primers	Sequence 5′→ 3′	Amplicon size (bp)
MDH1	sense	GGGTTTACTCCAGAAGAGC	147
	antisense	TGCCAAATCGCGAACGATGC	
MDH2	sense	AAGAAAACCTGGCATGACTC	162
	antisense	ATGTTAGAAACCATCACTGG	
MDH3	sense	AGGCATTGGTAAGGATTTATC	143
	antisense	CTCTAGTTAAACCGGGCTTTC	
PDR12	sense	GTCGTTGAATCTGGTGAAATG	153
	antisense	AGACATCATTTCGCTTTGGTC	
PMA1	sense	TACAAACTGACCCATCTTACG	155
	antisense	AGCGGCTTCCATAACGAATTG	
CIT1	sense	ACTTCAACCGATCCTAATGC	162
	antisense	GGCAGAACCCACTAAATGTG	
CIT2	sense	GACCCAAATGCCGATTATGC	158
	antisense	GATAGTGCTGAGCCCACAAG	
CIT3	sense	CAGTCAATGTTTTGGCAAGG	177
	antisense	TTGCTGGAAAGTTGGCACAC	
ICL1	sense	GCATCAGGACAAGAACTAGC	170
	antisense	TGGGATGTTTCAGTCAATGG	
WAR1	sense	TGATGAGGAAACACAGAACG	148
	antisense	CCAAGTGTCAAGCTTCATCG	
ACT1	sense	TCTCCACCACTGCTGAAAGAG	149
	antisense	AGTGATGACTTGACCATCTGG	
PDA1	sense	TCGTGTTTTGCTGTGAGAAC	156
	antisense	TAGCAAACTTGGATGCTTGG	

Real-time PCR was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster *City*, CA, USA) in the presence of SYBR Green. Individual real-time PCR reactions were carried out in a 96-well plate containing 10 µl of 2x SYBR Green Master Mix, 10 µl of 0.25 µM primers (Table 2) and 5 µl of cDNA template. All reactions were performed using SYBR Green Two-Step qPCR-PCR Kit with ROX (Invitrogen). We used the *PDA1* gene as a primary endogenous control to standardize the amount of cDNA added to the reactions. The PCR reactions of target genes were run in triplicate or occasionally in duplicate. The duplicates were accepted only if the obtained Ct values were very similar (with a deviation less than 0.40 from the average, and only one duplicate had a Ct deviation of 0.72). The endogenous controls had 4 or 5 data points. The PCR amplification protocol was as follows: incubation of reaction mixture at 50°C for 2 minutes, the initial denaturation step at 95°C for 2 minutes, then 40 cycles of heating and cooling at 95°C for 15 seconds and 60°C for 1 minute, respectively. After the runs, melting curves were analyzed to check if dimer primers or unspecific products were formed.

The ΔΔCt [15,28] and Pfaffl methods [20,28] were used to calculate relative amounts (R or LogR values) of mutant's mRNA vs. wild strain's mRNA normalized to the endogenous reference (*PDA1*). Here the ΔΔCt values were calculated from: ΔΔCt = ΔCtwild - ΔCtmutant, where ΔCt = Ctsample - Ct*PDA1*. Both methods basically gave the same results except for *ICL1*, which displayed about 20% lower amplification efficiency than the other genes. To estimate experimental variability of the results, propagated standard deviations (SD) were calculated for the ΔΔCt values, then the range of the relative expression ratios (Rmin to Rmax) was calculated using the E-ΔΔCt-SD and E-ΔΔCt+SD formulas [29] adjusted for the amplification efficiency if needed [15,29]. The Ct, ΔCt and ΔΔCt values were normalized for a constant efficiency (E) of 1.83 that was obtained for *PDA1*. The measured efficiencies were in the range of 1.82–1.94, except for the *ICL*1 efficiency of 1.65. In addition, the standard statistical t-test was used for comparison of the means of the mutant's and wild strain's ΔCt values.

Results

Endogenous reference genes

To normalize the mRNA levels in different samples of the yeast strains we chose *PDA1* as a reference gene. *PDA1* is recommended here rather than *ACT1* because it has been shown that in the case of *S. cerevisiae*, mRNA of *PDA1* is expressed at a constant level over a longer period than that of *ACT1*, whose expression substantially drops when cultures of *S. cerevisiae* reach the stationary phase [27]. Anyway, we included *ACT1* because this reference gene is frequently reported in the literature. The expression level of *ACT1* vs. *PDA1* in our mutants was somewhat higher than that in the wild strain, probably due to faster growth of the latter (data not shown).

Expression of selected structural genes associated with Krebs and glyoxylate cycles

Since the aci mutants excrete acids that are intermediates of the Krebs and glyoxylate cycles [16] we first examined several genes, *CIT1, CIT2, CIT3, MDH1, MDH2, MDH3* and *ICL*, of enzymes involved in these two cycles. The *Cit* and Mdh isozymes are the first and last enzymes in the two cycles, respectively, and changes in their expression levels could result in accumulation of the acidic intermediates. These enzymes are often induced due to mutation of other related metabolic genes [4,14] or changes of cell environmental conditions [14,19]. *Cit1, Cit3* and Mdh*1* are the mitochondrial isozymes involved in the Krebs cycle. *Cit2,* Mdh*2*, and *Icl*1 are involved in the glyoxylate cycle, and Mdh*3* is the peroxisomal isozyme. Somewhat surprisingly, the expression levels of these genes in the mutants, except for *CIT3* in EG37 (3.7-fold increase), were not significantly different from those in the wild strain (Figures 1 and 2).

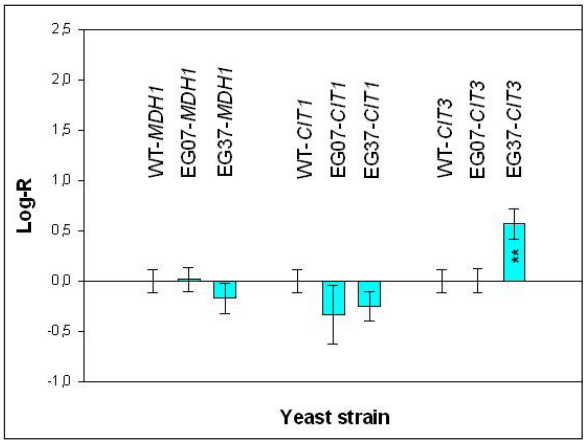

Fig. 1. Relative expression of Krebs cycle genes; R, mRNA expression ratio relative to WT strain; WT, wild-type; EG07 and EG37, mutants; *CIT1* and *CIT3*, genes coding citrate synthase isozymes 1 and 3; *MDH1*, gene coding malate dehydrogenase isozyme 1; Error bars represent propagated SD (see Materials and Methods); two asterisks denote t-test significance level at p<0.05 (see Materials and Methods).

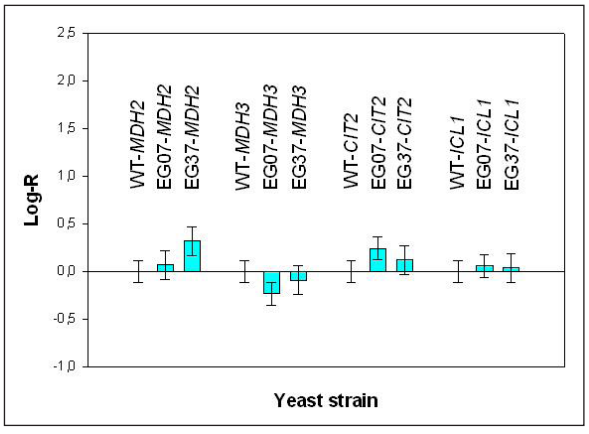

Fig. 2. Relative expression of glyoxylate cycle genes; *CIT2,* gene coding citrate synthase isozyme 2; *MDH2* and *MDH3,* genes coding malate dehydrogenase isozymes 2 and 3; *ICL1,* gene coding isocitrate lyase isozyme 1; other symbols are the same as described in Fig. 1.

Expression of transporter genes, *PDR12* and *PMA1*

PDR12 and *PMA1* were included in the study because Piper et al. [21] reported that *S. cerevisiae* cultures incubated at pH 4.5 displayed 10-fold elevated *PDR12* expression, when compared with pH 7.0 cultures. In the proposed mechanism of anion extrusion by the *Pdr12* transporter, the activity of *Pdr12* is postulated to be coupled to that of the *Pma1* proton pump [21]. Since our mutants overproduce carboxylic acids, it was probable that the expression level of the two major acid transporters would increase relative to the wild strain. Indeed, this supposition has been confirmed in our experiments. The relative expression values (R) were 2.4 and 24 (0.38 and 1.38 on log scale) for *PDR12* in EG07 and EG37, respectively. The corresponding R values for *PMA1* were 7.5 and 53 (0.88 and 1.72 on log scale), respectively (Figure 3). We also measured the expression level of *WAR1* (Figure 3) encoding a transcription factor that mediates *PDR12* induction [12]. The expression levels of *WAR1* in the mutants were not significantly different from those in the wild strain (R values were 0.46 and 1.1 for EG07 and EG37, respectively).

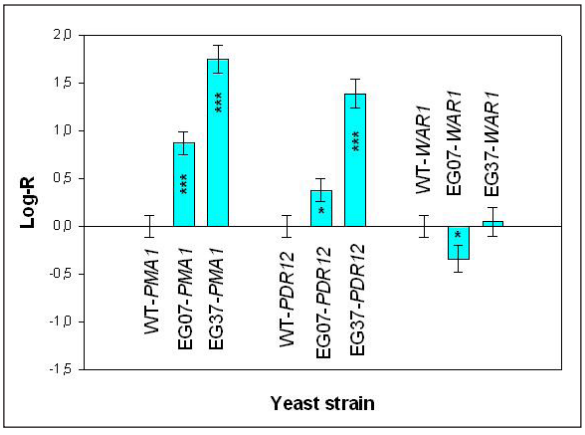

Fig. 3. Relative expression of cell transporter genes *PMA1, PDR12* and transcription factor gene *WAR1; PMA1*, gene coding *Pma1* proton pump; *PDR12*, gene coding *Pdr12* anion transporter; one and three asterisks denote t-test significance level at p<0.1 and p<0.01, respectively (see Materials and Methods); other symbols are the same as described in the previous figures.

Discussion

In our previous studies we isolated and biochemically characterized 16 *S. cerevisiae* mutants that excessively excreted acidic intermediates of the Krebs and/or glyoxylate cycle. Genetic analysis indicated single mutation of different genes [9,16]. Since the acid excretion occurs in the presence of glucose, one could assume that the glyoxylate cycle is turned off. However, this is not necessarily true for the mutants; therefore we decided to include this cycle in our studies. Our primary supposition was that the excretion of the organic acids was caused by a blockage of one of the cycles resulting in the accumulation of the acidic intermediates. However, there is no simple explanation for these findings because the genes restoring the wild-type phenotype Aci$^-$ do not encode the enzymes of the two cycles and have functions not directly related to the acid excretion (www.yeastgenome.org) [2].

In this work we decided to investigate the mRNA expression of selected genes associated with both cycles in two mutants displaying the strongest Aci+ phenotype. In addition, since the excretion of the acids involves membrane transporters, two of them, *Pdr12* and *Pma1*, extensively described in the literature, were included in the present studies. The two chosen aci mutants, EG07 and EG37, belong to different complementation groups [16] and therefore are assumed to have different mutated genes. Aside from the strong Aci+ phenotype, EG07 and EG37 are also interesting because they require very different growth conditions. EG37 easily utilizes non-fermentable substrates (except for ethanol) such as acetate but EG07 does not [16]. So, we expected to detect differences in regulation of both the cycles not only between each mutant and the wild strain but also between the mutants themselves (EG07 and EG37). The selected *CIT1*, *CIT2*, *CIT3*, *MDH1*, *MDH2*, *MDH3* and *ICL* genes encode important Krebs and glyoxylate cycle enzymes whose level can be expected to influence the kinetics of the two cycles. Since malate together with other carboxylic acid intermediates (*cit*rate, succinate, and fumarate) accumulated to high concentrations in the medium [16] we first focused our attention on *MDH* and *CIT*. Mdh is the last enzyme in the two cycles and it catalyzes the energetically unfavorable reaction. The product oxalacetate has to be removed efficiently and therefore Mdh acts in a complex with *Cit* preventing excessive accumulation of oxalacetate [5,26]. Moreover, literature data show that the expression of the genes selected here, including *ICL*1 associated with the glyoxylate cycle only, changes dramatically when glucose is replaced with non-fermentable substrates [19]. However, except for *CIT3* in EG37, our real-time PCR experiments did not reveal any significant expression changes of these genes between the studied strains (Figures 1 and 2). Only the R value for *CIT3* in EG37 was moderately elevated to 3.7 but we do not have a good explanation for this result. By contrast, we observed significant increases in the expression levels of the cell transporter genes *PDR12* and *PMA1* in the mutants relative to the wild strain (Figure 3). Especially large R values of 24 and 53, respectively, were obtained for EG37. EG07 displayed moderate R values of 2.4 and 7.5, respectively. The R value of 2.4 is low and its significance at $p < 0.1$ questionable, but *PDR12* expression in EG07 might be higher at the protein level. It is probable that EG07 and EG37 have different mutations. This supposition is supported by the fact that these two mutants belong to different complementation groups [9] and behave differently with respect to acetate as a source of carbon. So far we have been mostly focusing our attention on genetic regulation of the Krebs and glyoxylate cycles and more studies are needed in this area, but the present results point to a different and probably more promising avenue of research dealing more with the regulation of the transporters rather than the enzymes of both cycles. After obtaining the first results for *PDR12* and *PMA1* we added *WAR1* to our studies. *PMA1* expression is known to be regulated by transcription factors Rap1 and Gcr1 [22], but first we focused our attention

on *Pdr12*, which transports organic anions. *WAR1* is a gene encoding the transcription factor War1 of *PDR12* [12]. We hoped that the expression of *WAR1* at least in EG37 would be higher than that in the wild strain but only small differences were observed. The R value for EG07 was even below one (0.46) with the 90% confidence level (Figure 3). Interestingly, this result is consistent with the low expression of *PDR12* in EG07 but further studies are necessary to exclude experimental coincidence. Anyway, such a result is not very surprising because literature data [12] suggest that War1 requires phosphorylation for its activation, yet the kinase responsible for the phosphorylation is not known. At this moment, an unresolved question is whether the induction of *PDR12* and *PMA1* is caused by the accumulation of the acids in the mutant cells or by the mutation(s) of regulatory gene(s) associated with the transporters. This issue will be addressed in subsequent studies. In addition, we can conclude that one of the previously considered possibilities, that a physical leakage of the acids through the cell membrane is responsible for the Aci+ phenotype [16], should probably be excluded.

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