Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1623 (2003) 1–5
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Oxidative stresses elevate the expression of cytochrome *c* peroxidase in *Saccharomyces cerevisiae*

Minsuk Kwon, Seonha Chong, Sanghwa Han, Kyunghoon Kim*

Division of Biological Sciences, Kangwon National University, Hyoja 2-dong, Chunchon 200-701, South Korea

Received 19 February 2003; received in revised form 7 July 2003; accepted 21 July 2003

Abstract

Cytochrome *c* peroxidase (C*c*P) uses hydrogen peroxide as an electron acceptor to oxidize cytochrome *c* (C*c*) in the mitochondrial intermembrane space. A null allele of yeast *CCP1* gene encoding C*c*P was created by one-step gene disruption method in a diploid yeast strain. Haploid yeast cells with the disrupted *CCP1* gene were viable and able to grow in a medium containing lactic acid or glycerol as an energy source, indicating that C*c*P is not essential for both cell viability and respiration. However, *CCP1*-disrupted cells were more sensitive to H_2O_2 than wild-type cells. We also constructed a *CCP1–lacZ* fused gene and integrated this gene into yeast chromosomal DNA to monitor the expression of *CCP1* gene. We found that expression of *CCP1* gene increases under respiratory culture conditions and by treatments with H_2O_2. These results hint that the biological function of C*c*P is to reduce H_2O_2 generated during aerobic respiratory process. Moreover, expression of *CCP1* gene increased by treatments with peroxynitrite, indicating that C*c*P may act as a peroxynitrite scavenger.

Keywords: Cytochrome *c* peroxidase; Hydrogen peroxide; *Saccharomyces cerevisiae*; Peroxynitrite; Reactive oxygen species

1. Introduction

Metabolically active cells generate reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (H.O), singlet oxygen ($^1O_2$), and organic peroxides [1]. ROS react with various cellular components such as DNA, proteins, and lipids inducing oxidative modifications and fragmentation [2–4]. To fight against the ROS, cells have developed antioxidant enzymes including superoxide dismutase, catalase, and several peroxidases [5].

Mitochondrial electron transport chain is a major source of ROS in eukaryotes. About 1–4% of oxygen molecules in the mitochondria are incompletely reduced to ROS. According to in vitro studies, the complex III of the respiratory chain is responsible for more than 80% of ROS produced in yeast [6]. In budding yeast, cytochrome *c* peroxidase (C*c*P) uses hydrogen peroxide as an electron acceptor to oxidize cytochrome *c* (C*c*) in the mitochondrial intermembrane space [7]. Consequently, electrons to be transferred to oxygen are consumed by C*c*P and, as a result, water is produced from hydrogen peroxide. Thus, C*c*P might be used to detoxify hydrogen peroxide generated during the process of mitochondrial respiration. Although biochemical studies on structure and function of the C*c*P have actively been carried on, the biological function of the C*c*P is still unclear. In this study, we found that the C*c*P is not essential for both cell viability and respiration. We also show that expression of the C*c*P increases under respiratory culture conditions and by treatments with H_2O_2 or peroxynitrite, suggesting that the biological function of C*c*P is to remove reactive H_2O_2 or peroxynitrite.

2. Materials and methods

2.1. Strains and culture conditions

The *Saccharomyces cerevisiae* strains used in this study were described in Table 1. The standard yeast methods and growth media were as described by Sherman et al. [8]. The yeast cells were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose) or YEPG (1% yeast extract, 2% peptone, 3% glycerol as non-fermentable carbon source) at 30 °C. For anaerobic growth, the culture medium was boiled and degassed before inoculation. Then, mineral oil was

* Corresponding author. Tel.: +82-33-250-8527; fax: +82-33-251-3990.
E-mail address: kkim@kangwon.ac.kr (K. Kim).

0304-4165/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0304-4165(03)00151-X

Table 1
Yeast strains, plasmids, and primers used in this study

	Description	Source
JM749	*MATa/MATα his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 lys2-801/lys2-801 trp1Δ101/trp1Δ101 ura3-52/ura3-52*	
KM1	*MATa his3Δ200 lys2-801 trp1Δ101 ura3-52 ccp1::LEU2*	this study
KM2	*MATα his3Δ200 lys2-801 trp1Δ101 ura3-52 ccp1::LEU2*	this study
KM3	*MATa his3Δ200 lys2-801 trp1Δ101 ura3-52 CCP1*	this study
KM4	*MATα his3Δ200 lys2-801 trp1Δ101 ura3-52 CCP1*	this study
KM5	*MATa/MATα his3Δ200/his3Δ200 lys2-801/lys2-801 trp1Δ101/trp1Δ101 ura3-52/ura3-52 ccp1::LEU2/ccp1::LEU2*	this study
YA5	*MATα spo11 ura3 his1 leu2 lys7 met3 trp5*	
YA6	*MATa spo11 ura3 his1 leu2 lys7 met3 trp5*	
pRS305	*LEU2,* YIp	[17]
pRS306	*URA3,* YIp	[17]
YEp357	*URA3,* YIp	[18]
pJH4	pRS305-*Δccp1* (3′Δ and 5′Δ)	this study
pJH5	YEp357-*ccp1-lacZ*	this study
T3	5′-ATTAACCCTCACTAAAGGGA-3′	
T7	5′-TAATACGACTCACTATAGGG-3′	
pRS5′	5′-GCTTCCGGCTCCTATGTTGTGTG-3′	this study
YEp3′	5′-CTTCGCTATTACGCCAGCTGGCG-3′	this study
CCP-NF	5′-GTAGTCATGACTACACCGCTCGTTCATGTC-3′	this study
CCP-NR	5′-AGTACTCGAGTCTTAAACCTTGTTCCTCTAAAGTC-3′	this study
CpF5′R	5′-CCCGAATTCGATCTCGCCAATGGAAATTCC-3′	this study
CpR3′H	5′-CCCAAGCTTGGAGAACAGGTAGAGAGACCT-3′	this study

overlaid onto the surface of the medium and the culture was placed in a candle jar at 30 °C. Exponentially grown cells were harvested at the OD_{600} of 0.5–1.0. Stationary-phase cells were grown for 2 days. The *Escherichia coli* strain JM109 [9] was used for cloning, maintenance, and propagation of recombinant plasmids. *E. coli* cells were grown in LB media (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5). When needed, ampicillin (50 mg/l) was added to the LB.

2.2. Manipulation of plasmid DNA

Plasmid DNA was prepared from overnight culture of *E. coli* by the boiling method of Holmes and Quigley [10] or by the large-scale alkaline extraction method of Marko et al. [11]. Genomic DNA was prepared from yeast cells as described by Hoffman and Winston [12]. Enzyme reactions for DNA manipulation and agarose gel electrophoreses of DNA were performed according to Sambrook et al. [13]. Specific DNA segments were amplified by polymerase chain reactions, and the amplified products were analyzed by agarose gel electrophoresis.

2.3. Transformation

Yeast transformation was carried out using the lithium chloride method of Ito et al. [14], and *E. coli* transformation was performed as Cohen et al. [15].

2.4. Disruption of CCP1 gene

The yeast *CCP1* gene encoding C*c*P was disrupted by the one-step gene disruption technique [16]. We have first constructed a recombinant plasmid pJH4 containing an internal 400-bp segment of the yeast *CCP1* gene by subcloning the *Sal*I–*Hin*dIII fragment located from +595 to +995 of the *CCP1* ORF into a yeast integrative vector pRS305 carrying a *LEU2* selectable marker [17]. Then, a diploid yeast strain JM749 (Table 1) was transformed to Leu^+ with the pJH4 linearized at the unique *Nco*I recognition site present on +772 of the *CCP1* ORF. One of the transformants was designated JM749 (*CCP1*/*ccp1*::*LEU2*) and the correct integration of the pJH4 into the chromosomal *CCP1* locus was confirmed by amplifying an 886-bp DNA from the genomic DNA of the transformant with the T7 primer and the CCP-NF primer (Table 1) ranging from +205 to +225 of the *CCP1*. The integration was further confirmed by amplifying a 552-bp DNA with the T3 primer and the CCP-NR primer (Table 1) complementary to a region from +1065 to +1086 of the *CCP1*.

2.5. Construction of ccp1–lacZ fusion

An upstream region of *CCP1* gene containing 303 nucleotides of 5′ noncoding region and 69 nucleotides of the amino terminal coding sequence of *CCP1* (−303 to +69) was PCR-amplified by using a paired set of primers CpF5′R and CpR3′H (Table 1) and fused to the promoterless *lacZ* gene of YEp357 [18] in frame resulting in the construction of a recombinant plasmid designated pJH5. Subsequently, The *Nde*I–*Eco*RI fragment containing the *URA3* marker gene of the yeast integrative plasmid pRS306 was replaced with the *Nde*I–*Eco*RI fragment containing the *URA3* marker [17] and the *ccp1–lacZ* reporter gene of pJH5, and the resulting integrative plasmid was designated pJH7.

2.6. β-Galactosidase assay

Logarithmically grown yeast cells in 5-ml liquid medium were harvested by centrifugation, washed once with 1-ml cold water and the pellet was resuspended in 500-μl Z buffer (40 mM NaH_2PO_4, 60 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM mercaptoethanol, pH 7.0) containing 0.4-g acid-washed glass beads with the diameter of 0.43–0.60 mm. Cells were disrupted for 30 s using a vortex mixer four times, with chilling on ice for 1 min after every agitation. The mixture was centrifuged at 15,000 × *g* at 4 °C for 10 min, and the supernatant was used for the β-galactosidase assay [19] and the determination of protein concentration [20]. Enzyme activities were measured for two independent cultures and averaged. Specific activities of β-galactosidase were expressed as Miller units (1000 × A_{420}/min/mg protein).

3. Results and discussion

3.1. CcP is not essential for viability and respiration

Cells of the diploid yeast strain JM749 (*CCP1*/*ccp1*::*LEU2*) were induced to sporulate by cultivating the cells in 1% potassium acetate for 3 days. The induced spores were separated individually with glass beads of 0.1 mm in diameter and their mating types were determined by crossing them with tester strains YA5 and YA6 (Table 1), respectively. Out of 138 haploid clones, 35 were a Leu^+ (*ccp1*::*LEU2*), 48 were α Leu^- (*CCP1*), 29 were a Leu^+ (*ccp1*::*LEU2*), and 26 were α Leu^- (*CCP1*). Because *CCP1* and *ccp1*::*LEU2* genotypes were segregated at about 1:1 ratio, C*c*P does not appear essential for sporulation and cell viability. This result is consistent with that of Winzeler et al. [21]. Representative haploid clones were then designated KM1 for a *ccp1*::*LEU2*, KM2 for α *ccp1*::*LEU2*, KM3 for a *CCP1*, and KM4 for α *CCP1*. All of the four strains grew well in YEPG medium which cannot support the growth of respiration-deficient cells, indicating that C*c*P is not essential for respiration, either. Thus, the ROS produced during aerobic respiratory growth of C*c*P-negative cells seem to be sufficiently detoxified by complementary defense mechanisms such as catalases, superoxide dismutases, and other peroxidases than C*c*P [22–24]. C*c*P, however, is likely to play an important role in scavenging an excess amount of H_2O_2. Growth of the C*c*P-deficient KM1 and KM2 was noticeably retarded in the presence of 2 mM H_2O_2, while the C*c*P-positive KM3 and KM4 grew normally in the presence of 2 mM H_2O_2 (Table 2). Furthermore, when both 2 mM H_2O_2 and 2 mM aminotriazole (3-amino-1,2,4-triazole), which is a catalase inhibitor [25], were treated, the C*c*P-

Table 2
Effects of hydrogen peroxide and 3-aminotriazole on the growth of yeast cells[a]

Strains	0 mM H_2O_2		2 mM H_2O_2		2 mM H_2O_2 + 2 mM 3-AT	
	1st	2nd	1st	2nd	1st	2nd
KM1	0.76	0.86	0.16	0.23	0.12	0.18
KM2	0.82	0.78	0.15	0.16	0.13	0.09
KM3	0.75	1.07	0.65	1.02	0.59	0.80
KM4	0.80	0.94	0.68	0.85	0.66	0.74

[a] Yeast cells treated with hydrogen peroxide and 3-aminotriazole were grown in YEPG overnight, and optical densities of the cells were measured at 600 nm. Results of two independent tests were shown.

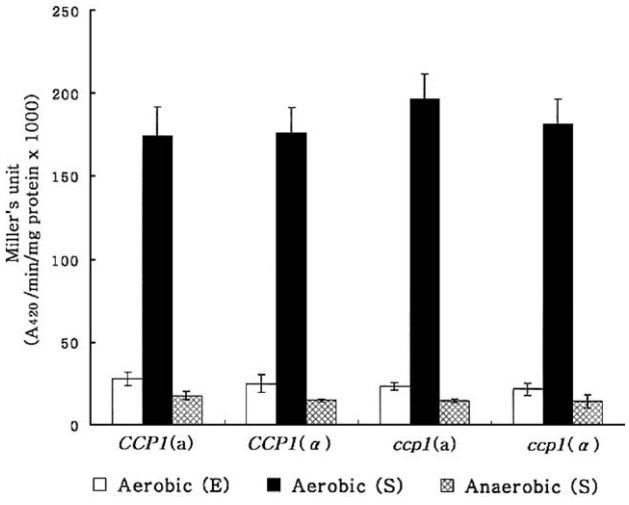

Fig. 1. β-Galactosidase activities expressed from *ccp1*–*lacZ* gene of yeast cells. Cells of KM1 to KM4 were grown with YEP + glucose media to exponential phase or stationary phase. Open, closed, and hatched bars represent the β-galactosidase activities of the strains grown to exponential phase aerobically, stationary phase aerobically, and stationary phase anaerobically, respectively.

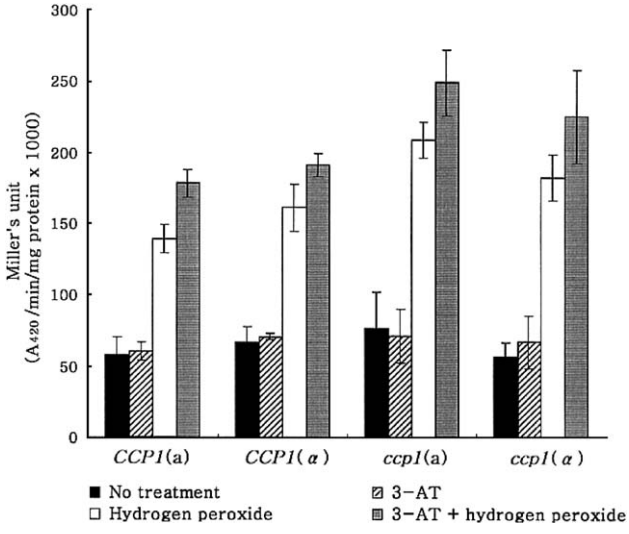

Fig. 2. β-Galactosidase activities expressed from *ccp1*–*lacZ* gene of yeast cells. Cells of KM1 to KM4 were grown with YEP + glucose media to exponential phase. Closed, slashed, open, and gray bars represent the β-galactosidase activities of the strains treated for 2 h with no chemical, 2 mM 3-aminotriazole, 2 mM hydrogen peroxide, and 2 mM 3-aminotriazole plus 2 mM hydrogen peroxide, respectively.

positive KM3 and KM4 grew slightly slower than the KM3 and KM4 cells with no treatment, but the C*c*P-negative KM1 and KM2 cells grew even slower than the KM1 and KM2 cells treated with only 2 mM H_2O_2 (Table 2).

3.2. The expression level of CCP1 is increased in aerobic condition

In order to investigate the quantitative response of *CCP1* gene under various growth conditions, we first constructed a recombinant plasmid pJH7 containing a *ccp1–lacZ* reporter gene as described in Section 2.5. To maintain the constant copy number of the *ccp1–lacZ* fusion, we integrated the pJH7 at the chromosomal *ura3* loci of KM1 to KM4 by transforming the yeast cells to Ura^+ with the pJH7 linearized at the unique *Stu*I recognition site within the *URA3* marker gene. Correct integrations of the fusion into the chromosomal *ura3* were confirmed by amplifying a 657-bp DNA with the pRS5′ primer and the YEp3′ primer (Table 1). The Ura^+ transformants of KM1 to KM4 with the integrated *ccp1–lacZ* at the *ura3* loci were grown in YEPD liquid media aerobically or anaerobically by using a candle jar, and β-galactosidase activities of the cultures were measured (Fig. 1). Cells grown aerobically to the stationary phase expressed the *ccp1–lacZ* about 8- to 10-fold higher than those grown anaerobically. The levels of the *ccp1–lacZ* expression from cells grown aerobically to an exponential phase, however, were much lower than those from stationary phase cells grown aerobically, although they were still higher than those grown anaerobically (Fig. 1). These results imply that the ROS produced during aerobic growth increase the expression of the *ccp1–lacZ* and the biological role of C*c*P is the scavenger of hydrogen peroxide produced during respiration. Consistently with our results, Izawa et al. [26] have previously shown that ROS-scavenging enzymes such as catalases and superoxide dismutase are detected to a higher extent from yeast cells of a stationary phase than those of an exponential phase.

3.3. H_2O_2 and peroxynitrite elevate the expression of the CCP1 gene

Exponentially grown yeast cells KM1 to KM4 with the integrated *ccp1–lacZ* were treated for 2 h with a sublethal concentration of 2 mM H_2O_2 and/or 2 mM aminotriazole, which is a catalase inhibitor [25], and β-galactosidase activities were measured from the cell extracts (Fig. 2). Expression levels of the *ccp1–lacZ* from cells treated with H_2O_2 were threefold higher than those without treatment (Fig. 2). While expression levels of the *ccp1–lacZ* from the yeast cells treated with aminotriazole were not much different from those of control cells, the *ccp1–lacZ* levels from the cells treated with H_2O_2 plus aminotriazole were slightly higher than those from the H_2O_2-treated cells (Fig. 2). These data indicate that the expression of the *CCP1* is increased by H_2O_2 or H_2O_2 plus 3-aminotriazole, which is consistent with the suggested idea that the main role of C*c*P is to remove H_2O_2 produced during respiration. In order to see if C*c*P can remove ROS other than H_2O_2, we treated the yeast cells with peroxynitrite, which is produced by the rapid reaction of superoxide anion and nitric oxide under certain conditions including phagocytosis. When the exponential-phase cells of KM1 to KM4 with the integrated *ccp1–lacZ* were treated for 2 h with 1 mM peroxynitrite, β-galactosidase activities from the cell extracts were about twofold higher than those from control cells (Fig. 3). This result suggests that C*c*P might also act as a scavenger of peroxynitrite, helping yeast cells survive phagocytic attacks.

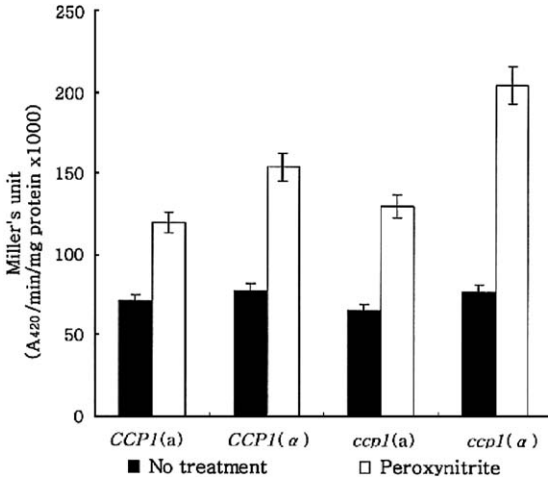

Fig. 3. β-Galactosidase activities expressed from *ccp1–lacZ* gene of yeast cells. Cells of KM1 to KM4 were grown with YEP + glucose media to exponential phase. Closed and open bars represent the β-galactosidase activities of the strains treated for 2 h with no chemical and 1 mM peroxynitrite, respectively.

Acknowledgements

This work was supported by a grant (KOSEF 981-0502-009-2) from the Korea Science and Engineering Foundation.

References

[1] B.N. Ames, M.K. Shigenaga, T.M. Hagen, Oxidants, antioxidants, and the degenerative diseases of aging, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 7915–7922.
[2] B.S. Berlett, E.R. Stadtman, Protein oxidation in aging, disease, and oxidative stress, J. Biol. Chem. 272 (1997) 20313–20316.
[3] G. Storz, M.F. Christman, H. Sies, B.N. Ames, Spontaneous mutagenesis and oxidative damage to DNA in *Salmonella typhimurium*, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 8917–8921.
[4] S.P. Wolff, A. Garner, R.T. Dean, Free radicals, lipids and protein degradation, Trends Biochem. Sci. 11 (1986) 27–31.
[5] P. Moradas-Ferreira, V. Costa, P. Piper, W. Mager, The molecular defences against reactive oxygen species in yeast, Mol. Microbiol. 19 (1996) 651–658.
[6] C. Richter, Do mitochondrial DNA fragments promote cancer and aging? FEBS Lett. 241 (1988) 1–5.

[7] C. Godon, G. Lagniel, J. Lee, J.M. Buhler, S. Kieffer, M. Perroti, H. Boucherie, M.B. Toledano, J. Labarre, The H_2O_2 stimulation in *Saccharomyces cerevisiae*, J. Biol. Chem. 273 (1998) 22480–22489.
[8] F. Sherman, G.R Fink, J.B. Hicks, Methods in Genetics, CSHL Press, Cold Spring Harbor, 1986.
[9] C. Yanisch-Perron, J. Vieira, J. Messing, Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors, Gene 33 (1985) 103–119.
[10] D.S. Holmes, M. Quigley, A rapid boiling method for the preparation of bacterial plasmids, Anal. Biochem. 114 (1981) 193–197.
[11] M.A. Marko, R. Chipperfield, H.C. Birnboim, A procedure for the large-scale isolation of highly purified plasmid DNA using alkaline extraction and binding to glass powder, Anal. Biochem. 121 (1982) 382–387.
[12] C. Hoffman, F. Winston, A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *E. coli*, Gene 57 (1987) 267–272.
[13] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: a Laboratory Manual, CSHL Press, Cold Spring Harbor, 1989.
[14] H. Ito, Y. Fukuda, K. Murata, K. Kimura, Transformation of intact yeast cells treated with alkali cations, J. Bacteriol. 153 (1983) 163–168.
[15] S.N. Cohen, A.C.Y. Chang, L. Hsu, Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA, Proc. Natl. Acad. Sci. U. S. A. 69 (1972) 2110–2114.
[16] R.J. Rothstein, One-step gene disruption in yeast, Methods Enzymol. 101 (1983) 202–211.
[17] R.S. Sikorski, P. Hieter, A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*, Genetics 122 (1989) 19–27.
[18] A.M. Myers, A. Tzagoloff, D.M. Kinney, C.J. Lusty, Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions, Gene 45 (1986) 299–310.
[19] M. Rose, D. Bostein, Construction and use of gene fusions to *lacZ* (beta-galactosidase) that are expressed in yeast, Methods Enzymol. 101 (1983) 167–180.
[20] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[21] E.A. Winzeler, D.D. Shoemaker, A. Astromoff, H. Liang, K. Anderson, B. Andre, R. Bangham, R. Benito, J.D. Boeke, H. Bussey, A.M. Chu, C. Connelly, K. Davis, F. Dietrich, S.W. Dow, M. El Bakkoury, F. Foury, S.H. Friend, E. Gentalen, G. Giaever, J.H. Hegemann, T. Jones, M. Laub, H. Liao, N. Liebundguth, D.J. Lockhart, A. Lucau-Danila, M. Lussier, N. M'Rabet, P. Menard, M. Mittmann, C. Pai, C. Rebischung, J.L. Revuelta, L. Riles, C.J. Roberts, P. Ross-MacDonald, B. Scherens, M. Snyder, S. Sookhai-Mahadeo, R.K. Storms, S. Véronneau, M. Voet, G. Volckaert, T.R. Ward, R. Wysocki, G.S. Yen, K. Yu, K. Zimmermann, P. Philippsen, M. Johnston, R.W. Davis, Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis, Science 285 (1999) 901–906.
[22] C.M. Grant, G. Perrone, I.W. Dawes, Glutathione and catalase provide overlapping defenses for protection against hydrogen peroxide in the yeast *Saccharomyces cerevisiae*, Biochem. Biophys. Res. Commun. 253 (1998) 893–898.
[23] Y. Inoue, T. Matsuda, K. Sugiyama, S. Izawa, A. Kimura, Genetic analysis of glutathione peroxidase in oxidative stress response of *Saccharomyces cerevisiae*, J. Biol. Chem. 274 (1999) 27002–27009.
[24] S. Izawa, Y. Inoue, A. Kimura, Oxidative stress response in yeast: effect of glutathione on adaptation to hydrogen peroxide stress in *Saccharomyces cerevisiae*, FEBS Lett. 368 (1995) 73–76.
[25] A.J. Kowaltowski, A.E. Vercesi, S.G. Rhee, L.E.S. Netto, Catalases and thioredoxin peroxidase protect *Saccharomyces cerevisiae* against Ca^{2+}-induced mitochondrial membrane permeabilization and cell death, FEBS Lett. 473 (2000) 177–182.
[26] S. Izawa, Y. Inoue, A. Kimura, Importance of catalase in the adaptive response to hydrogen peroxide: analysis of acatalasemic *Saccharomyces cerevisiae*, Biochem. J. 320 (1996) 61–67.