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Regular paper

Characterization of thiamine uptake and utilization in *Candida* spp. subjected to oxidative stress*

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Candida **species are associated with an increasing number of life-threatening infections (candidiases), mainly due to the high resistance of these yeast-like fungi to antifungal drugs and oxidative stress. Recently, thiamine (vitamin B1) was found to alleviate stress responses in *Saccharomyces cerevisiae*; however, thiamine influence on defense systems in pathogenic fungi has never been investigated. The current work was aimed to elucidate the role of thiamine in stress reactions of *C. albicans, C. glabrata, C. tropicalis* and *C. dubliniensis*, subjected to hydrogen peroxide treatment. As compared to *S. cerevisiae*, *Candida* strains exposed to oxidative stress showed: (i) a much higher dependence on exogenous thiamine; (ii) an increased demand for thiamine diphosphate (TDP) and TDP-dependent enzyme, transketolase; (iii) no changes in gene expression of selected stress markers — superoxide dismutase and catalase — depending on thiamine availability in medium; (iv) a similar decrease of reactive oxygen species (ROS) generation in the presence of thiamine. Moreover, the addition of therapeutic doses of thiamine to yeast culture medium revealed differences in its accumulation between various *Candida* species. The current findings implicate that the protective action of thiamine observed in *S. cerevisiae* differs significantly form that in pathogenic *Candida* strains, both in terms of the cofactor functions of TDP and the effects on fungal defense systems.**

Key words: vitamin B1, thiamine diphosphate-dependent enzymes, antioxidants, reactive oxygen species, *Candida* spp.

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INTRODUCTION

Candida albicans is the most prevalent infectious fungus, present in numerous niches as a part of normal microbiota within a human host. In healthy individuals, its occurrence may be completely unnoticed; however, under conditions of severe defects in immune defenses, *C. albicans* can cause life-threatening systemic infections (Molero *et al.,*1998; Dantas *et al.,* 2015). In recent years, several non-albicans *Candida* species, e.g., *C. glabrata* and *C. tropicalis*, has been found to be associated with an increasing number of systemic candidiases, reaching a mortality rate of up to 40% (Pfaller & Diekema, 2007). *C. tropicalis* is closely related to *C. albicans* and both organisms represent the 'CTG clade' species that possess a unique CUG codon for serine (Fig. 1) (Dujon *et al.,* 2004). In contrast, *C. glabrata* is evolutionary more related to baker's yeast *Saccharomyces cerevisiae,* and thus, its adaptation as mammalian commensal developed independently from other *Candida* species (Roetzer *et al.,* 2011b). In 1995, a new *C. dubliniensis* strain was isolated from HIV-infected individuals (Sullivan *et al.,* 1995). Despite its extremely high similarity to *C. albicans*, allowing to differentiate the two species only with DNA fingerprinting methods, *C. dubliniensis* was identified in less than 3% of all candidiases and is associated mainly with benign superficial infections of vaginal and oral mucosa (Sullivan *et al.*, 1995; Pfaller & Diekema, 2007; Moran *et al.,* 2012).

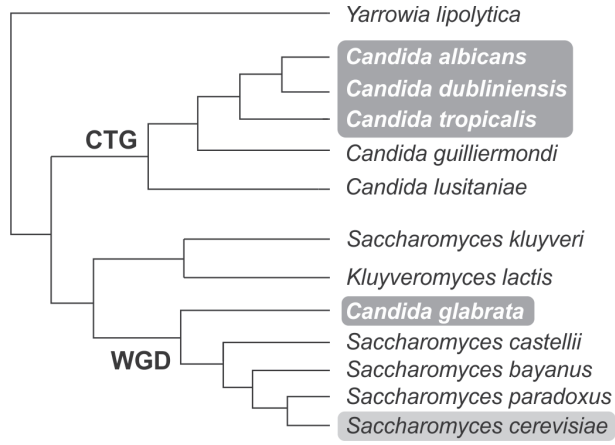

Figure 1. Phylogenetic tree of selected *Saccharomycotina* species.
CTG denotes the appearance of a new CUG codon for serine in selected *Candida* species, while WGD stands for the whole genome duplication that occurred in the lower branch of presented phylogenic tree. According to (Brown *et al.,* 2014), modified.

Integrated genomic and proteomic approaches are routinely applied to identify factors that contribute to *C. albicans* virulence and to investigate their occurrence in other *Candida* strains (Fernández-Arenas *et al.,* 2007; Selmecki *et al.,* 2010). In the light of the ongoing process of genome sequencing of the non-albicans species, the gene expression analyses using microarrays in selected strains at various stages of infection seems to be especially important (Fradin *et al.,* 2003).

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Abbreviations: CAT, catalase; KGDH, α-ketoglutarate dehydrogenase complex; ROS, reactive oxygen species; SOD, superoxide dismutase; TA, thiamine; TDP, thiamine diphosphate; THI80, thiamine pyrophosphokinase; TKL, transketolase.

The large differences in pathogenicity observed between various *Candida* species are shown to be strongly connected to the adaptations to stress conditions, and specific virulence factors utilized during the contact with host defense systems (d'Enfert, 2009). The latter include mainly adhesins, extracellular proteolytic enzymes and morphological changes (Naglik *et al.,* 2003; Sudbery *et al.,* 2004). The resistance of *Candida* species to oxidative stress is also of particular importance, as the main mechanism utilized by phagocytic cells — neutrophils and macrophages — to kill pathogens, involves the release of large amounts of reactive oxygen species (ROS) in the process known as oxidative burst (Babior *et al.,* 1973; Wojtaszek, 1997).

The antioxidative mechanisms that are exploited by *Candida* cells to deal with stress conditions, include both enzymatic action of catalase, superoxide dismutases and different peroxidases and non-enzymatic protective molecules, such as glutathione and trehalose (Alvarez-Peral *et al.,* 2002; Miramón *et al.,* 2012; Dantas *et al.,* 2015). Recently, thiamine (vitamin B1) has been found to ameliorate the effects of cellular stress in *S. cerevisiae*, through lowering the intracellular ROS level and reducing their deleterious effects on protein oxidation (Wolak *et al.,* 2014). It is already known that despite the role of thiamine diphosphate (TDP) in basic cellular metabolism (Sauberlich, 1967; Bettendorff *et al.,* 1996), thiamine can play other roles in stress responses in various organisms. In particular, thiamine can confer the resistance against oxidative agents in plants and bacteria (Jung & Kim, 2003; Tunc-Ozdemir *et al.,* 2009; Rapala-Kozik *et al.,* 2012) and, in forms of thiamine triphosphate and its adenylated derivatives, it can also serve as a signaling molecule under stress conditions (Lakaye *et al.,* 2004; Gigliobianco *et al.,* 2010). Although the mechanisms of the protective action of thiamine has not yet been recognized, the proposed thiamine oxidation upon the contact with free radicals can result in formation of thiamine thiols and tricyclic thiochrome derivatives (Lukienko *et al.,* 2000; Stepuro *et al.,* 2012).

A possible involvement of thiamine in stress responses of pathogenic *Candida* species has not been investigated previously. As the resistance of *Candida* cells to oxidants is much higher than that of *S. cerevisiae* (Alvarez-Peral *et al.,* 2002), it is particularly interesting if thiamine can affect the redox status in these fungal organisms, so specifically adapted to severe stress conditions. Thus, the main aim of this work was to analyze the oxidative stress response in several *Candida* species, in comparison to yeast *S. cerevisiae*, depending on the thiamine availability in culture medium.

MATERIALS AND METHODS

Materials. Culture media, YPD and Edinburgh Minimal Medium (EMM2), were obtained from Difco and US Biological respectively. Reagents for molecular biology experiments were obtained from Fermentas (GeneJet RNA Isolation Kit, dNTPs), Sigma (On-Column DNase, TRI Reagent), Promega (M-MLV Reverse Transcriptase) and KAPA (Universal SYBR Green Kit). All other reagents were purchased from Sigma.

Yeast strains and culturing. *Saccharomyces cerevisiae* BY4741 wild type strain was purchased from Euroscarf (Germany). *Candida albicans* ATCC 10231 strain was obtained from American Type Culture Collection (USA) and *Candida dubliniensis* NCYC 2670 was obtained from National Collection of Yeast Cultures (Great Britain). *Candida glabrata* and *Candida tropicalis* strains were kindly provided by Dr Trojanowska (Jagiellonian University in Krakow, Poland).

Yeasts were grown in standard YPD medium or in a defined EMM2 medium, supplemented with vitamin-free casein hydrolysate (20 mg/ml), amino acids (20 μg/ml tryptophan and 40 μg/ml methionine, leucine and histidine), uracil (120 μg/ml) and vitamins (0.4 μg/ml pyridoxine, niacin and pantothenic acid, 0.2 μg/ml riboflavin and 2 ng/ml biotin), at 30°C on orbital shaker (180 r.p.m.), until they reached an optimal growth phase (OD_{600} value of 0.4–0.5 for the gene expression analyses and of 0.8–1 for the other assays). Stress conditions were established by transferring cell pellets into fresh medium with hydrogen peroxide for 1 hour. Unless stated otherwise, *S. cerevisiae* cells were treated with 1 mM H_2O_2 and *Candida* cells with 5 mM H_2O_2.

Measurement of growth rates. Yeasts were grown overnight in minimal EMM2 medium with or without thiamine and then diluted to final OD_{600} value 0.2 in fresh medium with hydrogen peroxide. After 1 hour of stress treatment, yeast cells were transferred to medium without the stressor and the further growth was monitored; for this monitoring, small amounts of cultures were withdrawn every 1 hour and the optical density was measured at $\lambda = 600$ nm.

RNA isolation and quantitative PCR. Yeast cells were disrupted with glass beads (425–600 μm, Sigma) and TRI Reagent using FastPrep Instrument (6.0 m/s, 45 seconds). Due to the stronger cell wall, the disruption for *Candida* cells was performed in two 45-second cycles. Total RNA was isolated using GeneJet RNA Isolation Kit with DNase treatment and the quality of RNA was assessed by separation in agarose gel under denaturing conditions. First strand cDNA was synthesized using 2 μg of total RNA and dT18 primers with M-MLV Reverse Transcriptase, and subsequently diluted two fold with water. Real Time PCR was performed on Step One Instrument (Applied Biosystems) with SYBR Green for fluorescent labeling, in a final volume of 10 μl. The applied pairs of gene-specific primers (Genomed) are listed in Table 1. The reaction conditions were: 95°C for 10 min, followed by 40 cycles of 94°C for 15 s, 57°C for 15 s, and 72°C for 20 s. The *RDN18* and *ACT1* genes were used as references as they showed the most stable expression under stress conditions. Appropriate negative controls with RNA or water instead of cDNA were also used. Relative fold changes in expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Intracellular thiamine and TDP levels. Yeast cells were disrupted as for RNA isolation in the presence of 12% trichloroacetic acid (TCA) that was then removed with ether extraction. Samples were analyzed using reverse-phase high pressure liquid chromatography (RP-HPLC) on SUPELCOSIL™ LC-18 column (Sigma), with a post-column derivatization using 90 μM sodium hexacyanoferrate in 0.56 M NaOH. The fluorescence was monitored at 365 nm and 430 nm excitation and emission wavelengths (Rapala-Kozik *et al.,* 2008). For RP-HPLC separation, a gradient elution (0–98% B, 16 min) was used, (solvent A: 15 mM ammonium citrate (pH 4.2); solvent B: 0.1 M formic acid with 55 mM diethylamine).

Detection of reactive oxygen species. The ROS levels were determined with dihydroethidium (DHE) fluorescent dye (Fink *et al.,* 2004). Yeast cells were grown overnight in the presence of thiamine (1.4 μM) or its absence, then washed twice with phosphate-buffered saline

Table 1. List of primers used in this study.

Gene	Forward	Reverse	Strain
CTT1	GTCCATACTCCAAAGGTGATT	TACTTCGTCGTTGTCTTCATT	*S. cerevisiae*
KGD1	GGAAGCAACGCTCTGGTTTA	TCTTGGGTCTTCATTGGCTAGT	*S. cerevisiae*
RDN18	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT	*S. cerevisiae*
SOD2	TCACAAACCACTGTCTATTCTGG	GACTGCCAAACTGCTCGTC	*S. cerevisiae*
THI80	AGAACTAATCCATCCAAACG	TCAAGTCATGCAGCTTCC	*S. cerevisiae*
TKL1	AGCCCTTGACTTCCAACC	ATAGCGTGTTCTCTAATACCG	*S. cerevisiae*
ACT1	GATTTTGTCTGAACGTGGTAACAG	GAGTTGAAAGTGGTTTGGTCAATAC	*C. albicans*
CAT1	GATTCTCTACTGTTGGTGGTG	GTGAGTTTCTGGGTTTCTCTT	*C. albicans*
KGD1	TTAGAGTGTCGGGTCAAGAT	CTGGGGAGGTCAAGGAGTA	*C. albicans*
SOD2	CGTTGAAGCCAAATCTAAAG	GAGAGACAGGAGCCAAGTTT	*C. albicans*
THI80	ATCTCTCCACCTTCAGACTCAT	ACTATTTGCTCCACCATCG	*C. albicans*
TKL1	TCAAGAAAGACAACCCAGAC	GCAAGGAAACAACATTAGCC	*C. albicans*
ACT1	CCTCCAGAAAGAAAATACTCTG	TTGTGATGAACAATAGATGGAC	*C. dubliniensis*
CAT	GTTTTGGGATTACTTGACTAGC	TAAGAAGCTGGAGTACCTCTGT	*C. dubliniensis*
KGDH	GCTTACCAAGTTAGAGGTCATC	TACTCTTCTTACCACCTTGAGC	*C. dubliniensis*
SOD	GAGCTAAATACTCCGCTAGAAA	GGGTATAGACGTTGTCAGTTTT	*C. dubliniensis*
THI80	TAATTTCTCCACCTTCAGACTT	TGGTTTGTGATGTAGTTGTCTT	*C. dubliniensis*
TKL	AATGTCCCAGAAAGATACAGAG	CAAAGTTGGTTTATCGGTAGAT	*C. dubliniensis*
ACT1	GAGGTATTTTGACTTTGCGTTA	GTGTTCTTCTGGGGCGACT	*C. glabrata*
CTA1	GCGTAGAGTCGGTAAGATGGTC	GACAGGGATTTGGTGGAAGTTA	*C. glabrata*
KGDH	CGTAAACCAAACGAATCCATCT	TCTACCAAGGACAACAGGGTCT	*C. glabrata*
SOD	TAGAGTGGGACTTCGGTGCT	ATAGGTCTGGTGGTGCTTGG	*C. glabrata*
THI80	CTGTTGCCTGTTGGTCTTCC	GCTGCTCACTCGTCCAGATT	*C. glabrata*
TKL1	ATCGTCTTCCAAAGCATCTACG	CATACCGTGTTCTCTGATACCG	*C. glabrata*
ACT1	ATACTCTGTCTGGATCGGTGGT	TTTGTGGTGGACAATAGATGGA	*C. tropicalis*
CAT	GATTGATTCCTTGGCTCATTTC	AACACCATAAGCACCAGAACCT	*C. tropicalis*
KGDH	AAGAGATTTGGTTTGGAAGGTG	AGATGGATTCGTTTGGTTTACG	*C. tropicalis*
SOD	CAATGTTACCCAAGTCACCAAC	CTGAATCCGAACCAACCACTAT	*C. tropicalis*
THI80	ATCCTTGTTTTGCGATTCTGG	CACCATCTGTGTTGTCCATTCT	*C. tropicalis*
TKL	ATTTCCAACCACCATCTACTGG	AATAGCACCCATACCGTGTTCT	*C. tropicalis*

(PBS) and resuspended in PBS with 25 μg/ml DHE to final OD_{600} = 0.8 and incubated for 10 minutes at 30°C in the dark.

After washing twice with PBS, the fluorescence signal in the sample was detected in the presence of 0–25 mM H_2O_2, using a BioTek SYNERGY H1 microplate reader (λexc = 520 and λem = 610 nm).

Determination of protein concentration. Protein concentration was measured by the Lowry method (Lowry *et al.,* 1951).

Statistical analysis. All experiments were repeated at least 3 times to ensure proper analysis of statistical significance (*t*-test, $P<0.05$).

RESULTS

Candida spp. are present in numerous niches within the human host, that significantly differ in terms of thiamine availability. A particularly high amount of thiamine compounds was found in colon and skin, in comparison to a rather low concentration in vaginal mucosa (Gangolf *et al.,* 2010). Moreover, thiamine is known to be used in large doses for treatment of several pathological conditions, such as thiamine-responsive megaloblastic anemia (TRMA), Alzheimer disease or diabetes (Ozdemir *et al.,* 2002; Thornalley, 2005; Gibson & Blass, 2007), resulting in much higher local concentrations of thiamine than

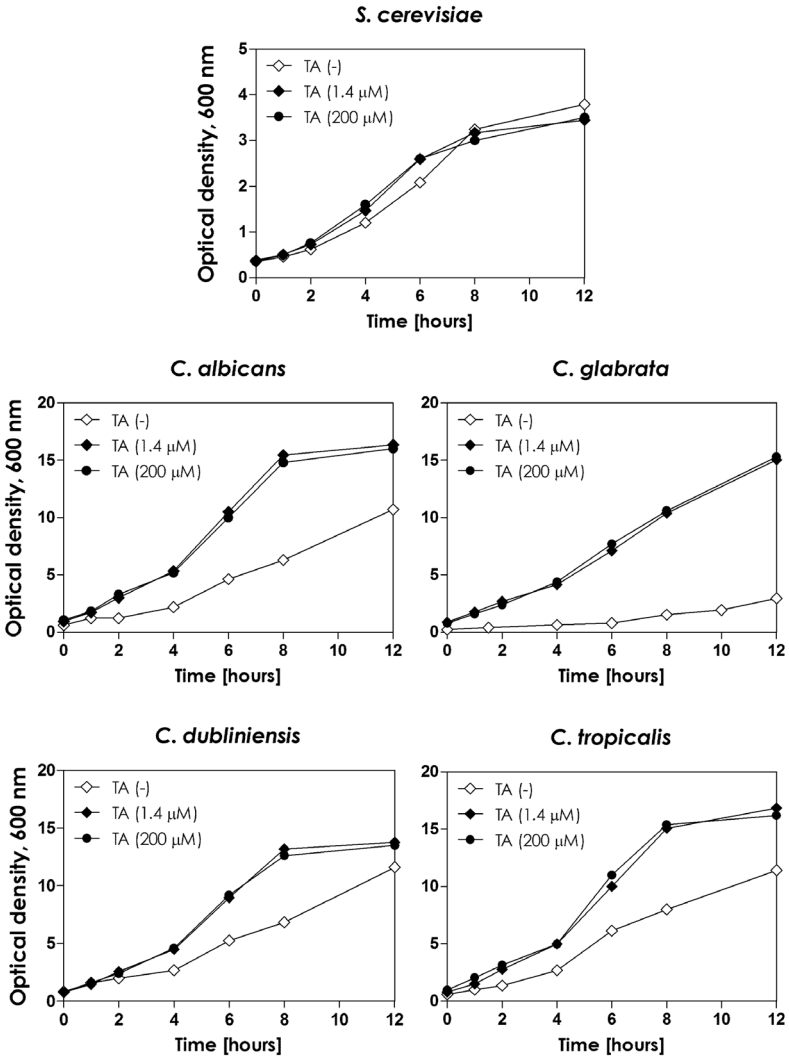

Figure 2. The growth of baker's yeast and selected *Candida* species, depending on thiamine availability in culture medium.
Yeasts were grown overnight in minimal EMM2 medium with or without thiamine and then were diluted to final OD_{600} value 0.2. The growth rate was then monitored at λ = 600 nm. Error bars represent the standard deviation.

under physiological state. In order to determine how different thiamine availability can affect *Candida* cells, three thiamine concentrations were selected, 0, 1.4 μM and 200 μM. The 1.4 μM concentration represents the level of thiamine in the YPD medium, that is used for optimal yeast propagation, whereas the 200 μM concentration corresponds to conditions during the medical treatments.

Effects of exogenous thiamine on the growth of selected *Candida* strains and the intracellular thiamine accumulation

A significant growth impairment of *Candida* cultures in the absence of thiamine was observed, especially in the case of *C. glabrata* (Fig. 2). The differences in growth rates between TA(–) and TA(+) conditions were much higher than in *S. cerevisiae* cultures, suggesting a lower rate of thiamine biosynthesis in *Candida* cells. The results correlated with intracellular thiamine accumulation, as no thiamine was detected in case of all selected *Candida* strains (Fig. 3). Probably, all thiamine synthesized under these conditions was already transformed to the active form (TDP), that could be detected at a similar level in *S. cerevisiae*. Only in the case of *C. glabrata,* no TDP was found in thiamine deficient medium, the finding that explains the impaired growth of this species under these conditions.

The addition of larger amounts of thiamine to culture medium did not affect the growth of *S. cerevisiae* or *Candida* spp., suggesting that the 1.4 μM concentration is high enough to ensure optimal growth of yeasts. However, the supplementation of medium with 200 μM thiamine revealed differences in intracellular thiamine accumulation between analyzed strains (Fig. 3). Under these conditions, both *S. cerevisiae* and *C. tropicalis* showed at least 8-times higher amount of thiamine than other species, suggesting that they possess mechanisms of a much tighter control of thiamine uptake. The differences in thiamine uptake regulation may be a part of the adaptation to the conditions within the host, and in this aspect *C. tropicalis* seems to have diverged from other *Candida* strains.

The analyses of gene expression of thiamine pyrophosphokinase (*THI80*), that converts thiamine into TDP, revealed its 6-fold upregulation in *C. tropicalis* when compared to other strains under TA(–) conditions (Fig. 4). The results may indicate a higher importance of the role of TDP-dependent enzymes in this

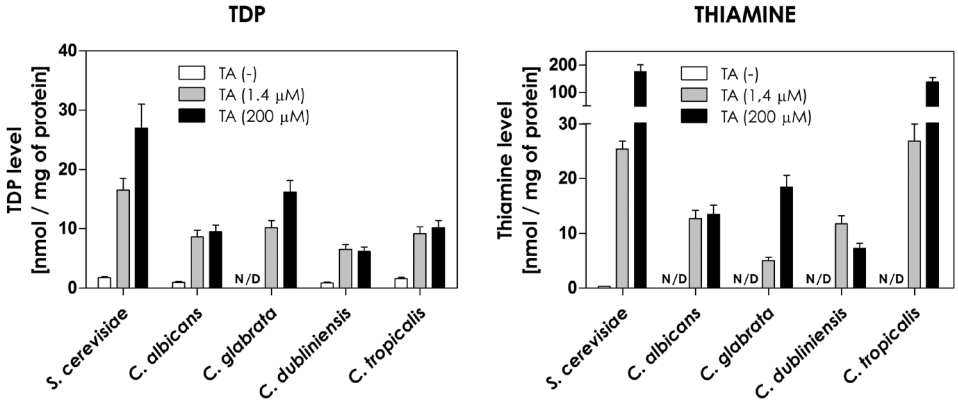

Figure 3. The intracellular accumulation of thiamine and TDP in *S. cerevisiae* and selected *Candida* species, depending on thiamine availability in culture medium.
Yeasts were grown overnight in minimal EMM2 medium with or without thiamine and then transferred to fresh medium for additional 1 hour. Thiamine compounds were extracted with 12% TCA and quantified using RP-HPLC method with post-column derivatization and fluorometric detection. N/D — thiamine or TDP not detected.

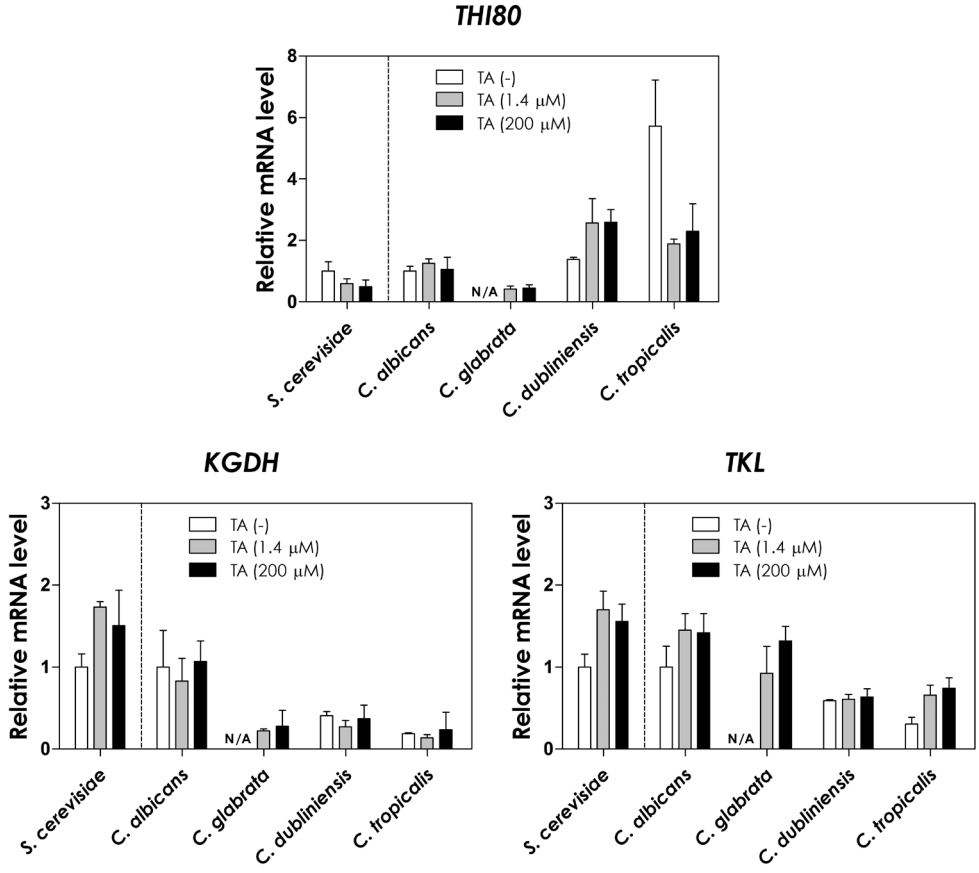

Figure 4. The expression of genes coding for thiamine pyrophosphokinase and major TDP-dependent enzymes — transketolase (TKL) and α-ketoglutarate dehydrogenase (KGDH) — in baker's yeast and selected *Candida* species, depending on thiamine availability in growth medium.
The gene expression analysis was performed using Real Time PCR with SYBR Green for fluorescent labeling and *ACT1/RDN18* genes as references. The results for all *Candida* strains were calculated versus *C. albicans* TA(–) samples (equal 1); a separate reference value was made for *S. cerevisiae* cells. Error bars represent the standard deviations calculated according to Livak & Schmittgen, 2001. N/A — sample not available due to very poor growth of *C. glabrata* culture in the absence of thiamine in medium.

Candida strain. Indeed, 2-fold higher expression of transketolase gene (*TKL*) was observed in *C. tropicalis* cells in the presence of thiamine in the medium. Intuitively, one could expect that the higher expression should be associated with thiamine deficiency; however, the same dependence was recently observed in *S. cerevisiae* (Wolak *et al.,* 2014) and in previous analyses of human cells and brain sections (Pekovich *et al.,* 1998; Shi *et al.,* 2008). The other *Candida* species did not reveal any significant differences in expression of *THI80* and main TDP-dependent enzymes, e.g., transketolase (*TKL*) and α-ketoglutarate dehydrogenase (*KGDH*).

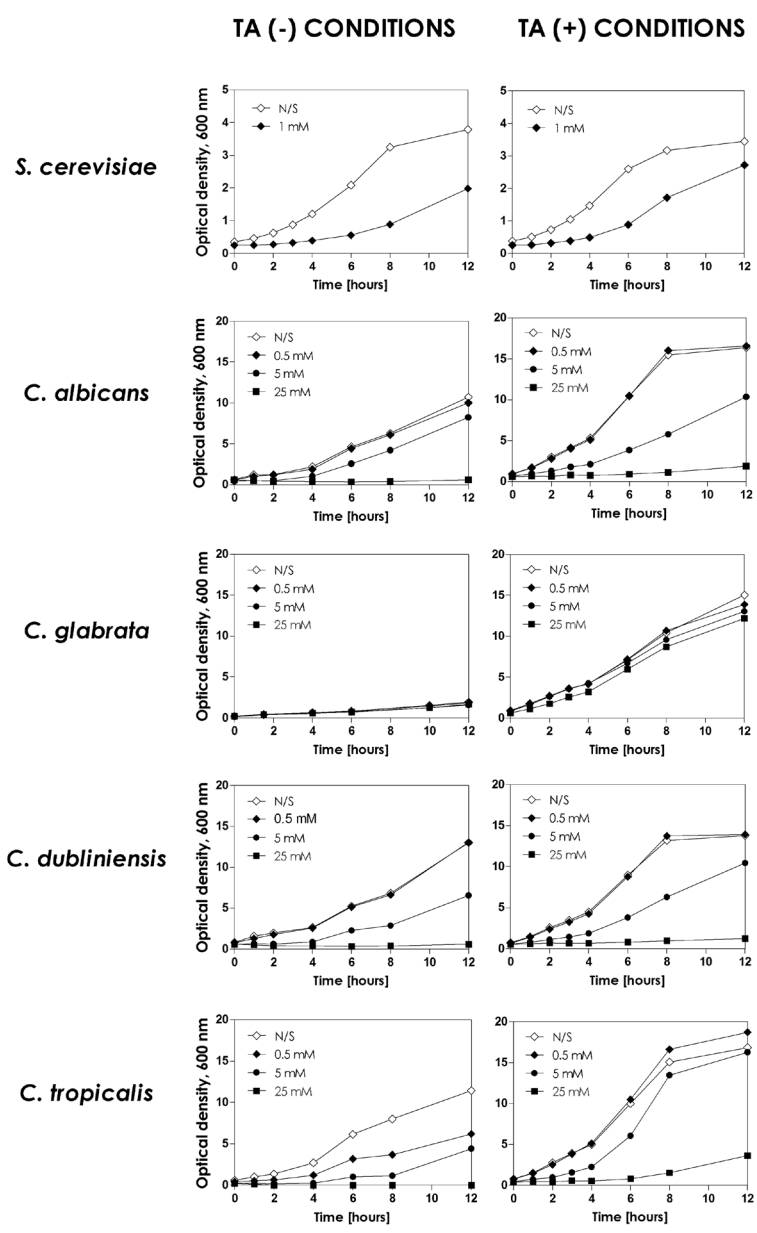

Figure 5. The growth of baker's yeast and selected *Candida* species under conditions of oxidative stress, depending on thiamine availability in culture medium.
Yeasts were grown overnight in minimal EMM2 medium with or without thiamine and then transferred to fresh medium with hydrogen peroxide. After 1 hour of treatment, the further growth in a new portion of medium without the stressor was monitored at λ = 600 nm. Error bars represent the standard deviation. N/S — non-stressed control.

An increased demand for thiamine and TDP in *Candida* cells under oxidative stress

Candida species are known to be much more resistant to oxidants than yeast *S. cerevisiae*, being able to survive short treatment of hydrogen peroxide at up to 50 mM concentrations (Alvarez-Peral *et al.,* 2002). In order to compare the stress responses of those organisms, their viability at different concentrations of hydrogen peroxide was tested (Fig. 5).

For further studies, 1 mM and 5 mM H_2O_2 were chosen for *S. cerevisiae* and *Candida* species, respectively, as both concentrations caused comparable survival of the cells.

The same analysis showed that under conditions of thiamine availability in the medium, *C. glabrata* cells showed the highest resistance to hydrogen peroxide, with almost no growth inhibition even when subjected to 25 mM stressor. Increased survival under stress and ability to growth at 25 mM H_2O_2 was observed also for *C. tropicalis*. Interestingly, both species presented also the largest growth defects under stress when relied completely on thiamine biosynthesis, showing the negative correlation between stress resistance and ability to synthesize thiamine.

The same species, *C. glabrata* and *C. tropicalis* accumulated more thiamine when subjected to stress treatments (Fig. 6). When thiamine was not present in the medium, thiamine again was not detected, whereas TDP level was unchanged, apart from *C. dubliniensis* (almost 2-fold less TDP under stress). The results were correlated with a very high expression of *THI80* gene in *C. dubliniensis* (more than 7-fold), that was probably aimed to compensate the small TDP level (Fig. 7). The high expression of thiamine pyrophosphokinase was also observed in *C. tropicalis* cells (more than 2-fold), possibly supporting the previous hypothesis on the increased importance of the TDP-dependent enzymes in this *Candida* strain under stress conditions.

An important difference between *S. cerevisiae* and *Candida* spp. was found in the transketolase gene expression. While in *S. cerevisiae* the *TKL1* gene is strongly repressed after hydrogen peroxide treatment as a part of the general metabolic shutdown (Gasch *et al.,* 2000; Ralser *et al.,* 2007), in all *Candida* cells transketolase was upregulated. Although it is known that in *S. cerevisiae* cells the activity of transketolase is largely controlled metabolically (Ralser *et al.,* 2009), its higher activity was already shown under oxidative stress (Kowalska *et al.,* 2012), as well as for *C. glabrata* and under heavy metal treatment for *C. albicans* (Yin *et al.,* 2009; Seneviratne *et al.,* 2010).

Changes in defense systems of *Candida* strains under oxidative stress, depending on thiamine availability

In order to determine the protective effect of thiamine on *Candida* cells, the expression of two main stress markers, superoxide dismutase (*SOD*) and catalase (*CAT*), depending on thiamine availability in medium was measured (Fig. 8). In *S. cerevisiae* cells, the addi-

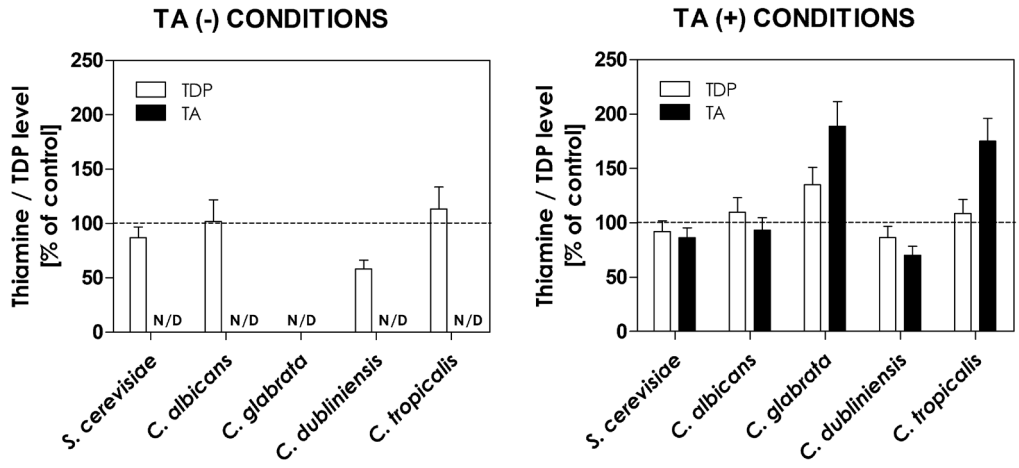

Figure 6. The intracellular accumulation of thiamine and TDP in *S. cerevisiae* and selected *Candida* species subjected to oxidative stress, depending on thiamine availability in culture medium.
Yeasts were grown overnight in minimal EMM2 medium with or without thiamine and then transferred to fresh medium with hydrogen peroxide for 1 hour. Thiamine compounds were extracted with 12% TCA and quantified using RP-HPLC method with post-column derivatization and fluorometric detection. N/D — thiamine or TDP not detected.

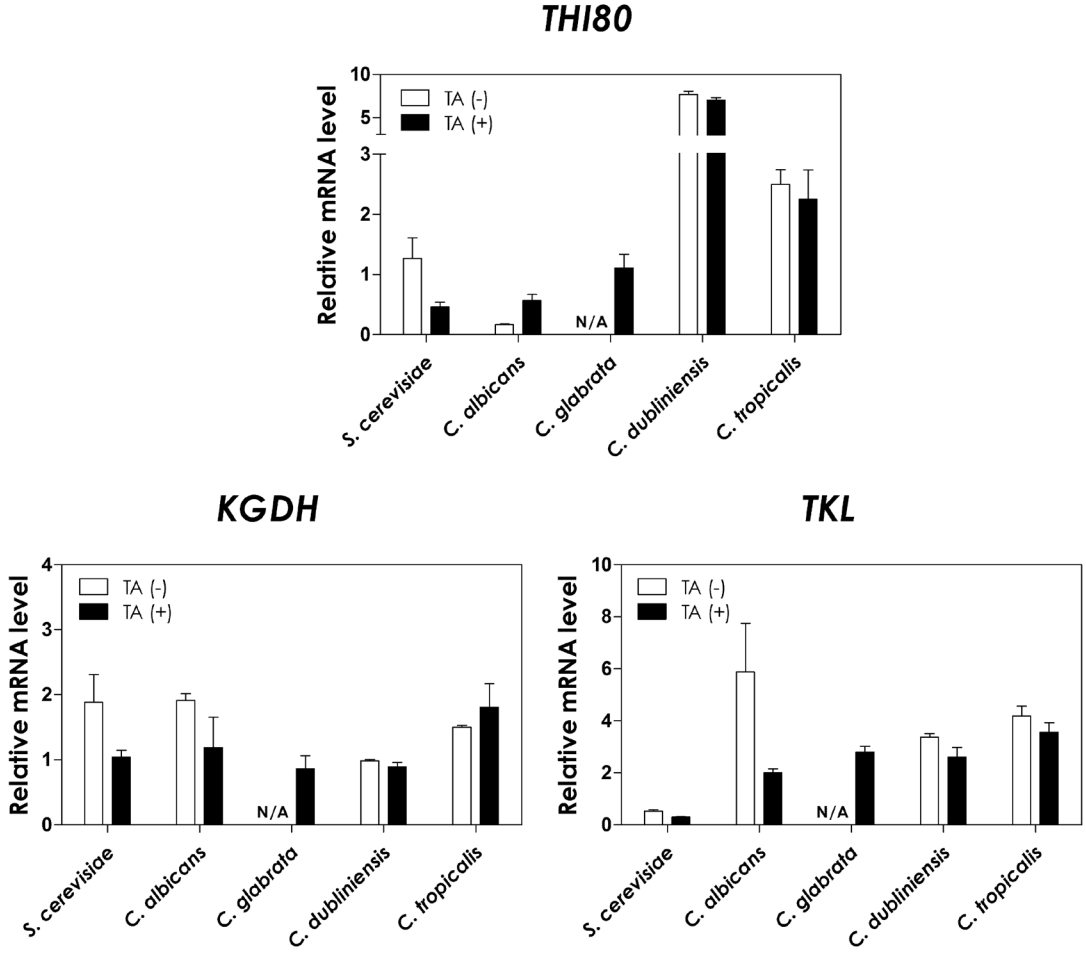

Figure 7. The expression of genes coding for thiamine pyrophosphokinase and major TDP-dependent enzymes — transketolase (*TKL*) and α-ketoglutarate dehydrogenase (*KGDH*) — in baker's yeast and selected *Candida* species under oxidative stress conditions, depending on thiamine availability in growth medium.
Yeasts were grown overnight in minimal EMM2 medium with or without thiamine and then transferred to fresh medium with hydrogen peroxide for 1 hour. The gene expression analyses were performed as described on Fig. 4. The results represent relative gene expression compared to the control conditions (equal 1) for each strain separately. Error bars represent the standard deviations calculated according to Livak & Schmittgen, 2001. N/A — sample not available due to very poor growth of *C. glabrata* culture in the absence of thiamine in medium.

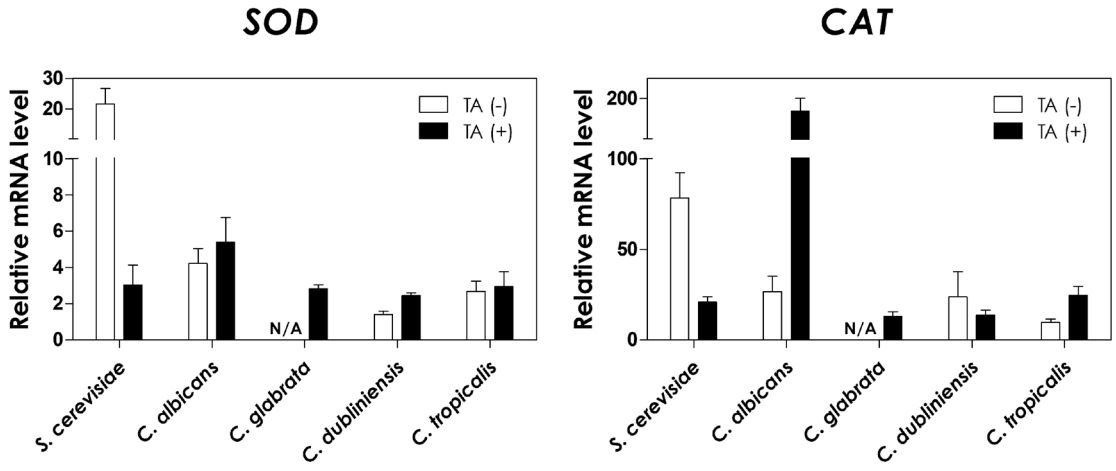

Figure 8. The expression of genes coding for major stress markers, superoxide dismutase (*SOD*) and catalase (*CAT*), in baker's yeast and selected *Candida* species under oxidative stress conditions, depending on thiamine availability in growth medium.
Yeasts were grown overnight in minimal EMM2 medium with or without thiamine and then transferred to fresh medium with hydrogen peroxide for 1 hour. The gene expression analyses were performed as described on Fig. 4. Error bars represent the standard deviations calculated according to Livak & Schmittgen, 2001. N/A — sample not available due to very poor growth of *C. glabrata* culture in the absence of thiamine in medium.

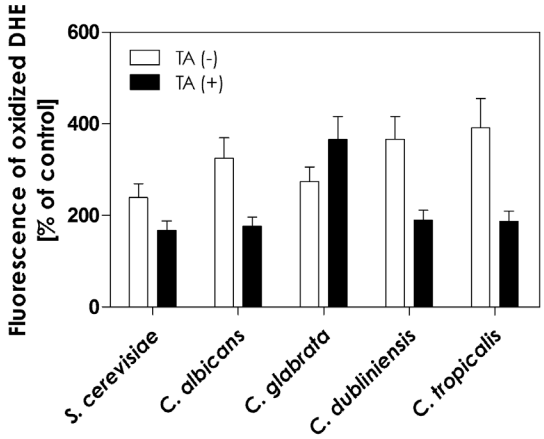

Figure 9. Reactive oxygen species (ROS) generation in *S. cerevisiae* and selected *Candida* strains, subjected to oxidative stress treatment, depending on thiamine availability in growth medium.
Yeasts were grown overnight in minimal EMM2 medium with or without thiamine. The ROS level was measured using 25 μg/ml dihydroethidium and the fluorescence signal was measured in the presence of hydrogen peroxide (λexc = 520 nm, λem = 610 nm).

tion of thiamine lowered the expression of both markers, suggesting its positive effect on the cellular redox state. This effect was not observed in any of the *Candida* strains tested, with even higher expression of *SOD* and *CAT* when thiamine was present in culture medium, especially in the case of catalase gene in *C. albicans* cells (up to 10-fold). The results may point at differential effects of thiamine on benign and pathogenic fungi under stress conditions. Most of *Candida* strains differ from *S. cerevisiae* with a number of isoforms of superoxide dismutase and catalase and their intracellular localization (Wysong *et al.,* 1998; Frohner *et al.,* 2009), a factor that could also affect the results.

However, most of *Candida* strains, similarly to *S. cerevisiae* cells, showed decreased ROS level when thiamine was present in culture medium (Fig. 9). Thus, thiamine can probably perform a protective action on *Candida* cells but the detailed mechanism may not be so straightforward as in *S. cerevisiae*. The only strain that revealed an opposite effect in ROS production was *C. glabrata* but this could be due to the very poor growth of this fungus under TA(–) conditions. Nevertheless, when thiamine was present in the medium, *C. glabrata* cells showed 2-fold higher level of ROS than other species. This effect may be associated with their highest resistance to oxidative stress and ability to survive for a long time inside phagosomes, where *C. glabrata* cells are exposed to large doses of reactive species (Roetzer *et al.,* 2010; Seider *et al.,* 2014).

DISCUSSION

Candida yeasts are the major causative agents of invasive fungal infections among hospitalized patients, with non-albicans strains representing a significant number of total isolates (Krcmery & Barnes, 2002; Zaoutis *et al.,* 2005). In many of them, the process of the adaptation to human host environment developed differently, resulting in several specific traits, associated mainly with the virulence level. One of them is the ability to survive under oxidative stress conditions, commonly encountered during the contact with host defense system or with other microorganisms inhabiting the same niches in human body (Dantas *et al.,* 2015). It is already known that thiamine can increase cell survival upon hydrogen peroxide treatment in baker's yeast *S. cerevisiae*, closely related to *C. glabrata,* mainly by reducing the intracellular ROS level and alleviating their negative effects on protein oxidation (Wolak *et al.,* 2014). No study, however, was conducted in this regard to pathogenic *Candida* species.

Thiamine is an essential compound for all living organisms but only plants, microorganisms and some fungi are capable of its biosynthesis (Begley *et al.,* 1999; Goyer, 2010). The synthesis of one thiamine molecule in yeast requires the equivalent of at least five adenosine-5-triphosphate molecules; thus, when thiamine can be taken up from the external environment, the biosynthesis process is almost completely blocked (Iwashima & Nose, 1976; Mojzita & Hohmann, 2006). The thiamine biosynthesis pathway is well recognized in *S. cerevisiae* (Nosaka, 2006; Kowalska & Kozik, 2008) but little is

known about this process in *Candida* species. Recently, some homologues of the thiamine biosynthetic enzymes of *S. cerevisiae* have been characterized in *C. albicans* and *C. glabrata* (Paul *et al.,* 2010; Lai *et al.,* 2012). However, our analyses of *Candida* growth under different thiamine concentration in the medium revealed the significant growth impairment when thiamine was not present, in comparison to *S. cerevisiae* cells, indicating that thiamine biosynthesis is much less effective in *Candida* species. The results correlated with the intracellular levels of thiamine, that in all strains were under detection limits. In *C. glabrata* cells, no TDP could also be detected, explaining almost complete growth inhibition of this strain under TA(–) conditions. The high dependence on the thiamine availability in host environment can be seen as a part of the adaptation to the commensal lifestyle of *Candida* species. Indeed, the loss of several genes involved in important cellular pathways, such as the metabolism of galactose (*GAL1/7/10*), phosphate (*PHO3/5/11/12*), nitrogen (*DAL1/2*) or sulphur (*SAM4*) was already observed in *C. glabrata*. Additionally, *C. glabrata* cells have lost the ability to synthesize some vitamins, such as niacin and pyridoxine (Kaur *et al.,* 2005). Although the thiamine biosynthesis rate seem to be very low, the main protein involved in this process, Thi6, has been recently characterized (Paul *et al.,* 2010), indicating that the thiamine biosynthetic ability may not be lost completely. The reduction in metabolic pathways was not observed in other *Candida* species, the feature that can be associated with a rather large evolutionary distance between *C. glabrata* and other *Candida* spp.. The differences, while comparing to *C. albicans*, may be also related to the ability to penetrate tissues by various *Candida* species (Fradin *et al.,* 2003). *C. albicans* is able to form hyphae and actively migrate between different niches in the host organism (d'Enfert, 2009), where they can experience various thiamine availability. *C. glabrata* cells do not change their morphological form and localize mainly in the mucosa (Roetzer *et al.,* 2011b), where main nutrients should be provided.

The addition of high doses of thiamine (200 μM) to culture medium did not affect the growth of neither *Candida* nor *Saccharomyces* strains, however, it revealed differences in thiamine accumulation. In comparison to *S. cerevisiae* and *C. tropicalis*, that accumulated larger amounts of thiamine, other species seemed to regulate the uptake process much more tightly. However, the specific carrier for thiamine has been identified only in *S. cerevisiae* (Enjo *et al.,* 1997; Singleton, 1997) and *C. glabrata* (*Candida Genome Database*, unpublished data). Both characterized proteins show very high similarity of amino acid sequences but our search for their homologues in other *Candida* strains was not successful.

The main part of our current work was devoted to the thiamine action in *Candida* cells under conditions of oxidative stress. The positive correlation between the resistance to hydrogen peroxide and to thiamine supplementation was observed. The low viability under TA(-) conditions is directly connected to previously described decreased ability to synthesize thiamine. Apart from *C. glabrata*, also *C. tropicalis* showed much more impaired growth under those conditions, suggesting that it can currently undergo the same process of metabolic reduction that was observed in *C. glabrata*. However, the identification of homologous proteins requires better characterization of genomes of many non-albicans *Candida* species.

The functions of thiamine are associated largely with the cofactor role of TDP in basic cellular metabolism. The higher mRNA level of thiamine pyrophosphokinase, enzyme that activate thiamine to TDP, suggests that the cofactor function may be more important in *Candida* than *Saccharomyces*. Indeed, the expression of transketolase under oxidative stress was also upregulated in all *Candida* species, in contrast to its downregulation in *Saccharomyces*. An increased activation of transketolase upon hydrogen peroxide treatment was previously shown in *C. glabrata* (Seneviratne *et al.,* 2010) and after cadmium treatment in *C. albicans* (Yin *et al.,* 2009). The difference between *Saccharomyces* and *Candida* in transketolase gene expression may be associated with the higher dependence of the latter species on the oxygen metabolism and, thus, higher generation of oxygen radicals (Vázquez- Torres & Balish, 1997). In *Saccharomyces*, the presence of even low amounts of ROS results in a temporary inhibition of Krebs cycle and redirection of metabolism to pentose phosphate pathway (Ralser *et al.,* 2007).

The analyses of gene expression for selected stress markers revealed, that their downregulation in the presence of thiamine in *S. cerevisiae* was not observed in *Candida* cells. This finding may be interpreted in terms of different isoenzymes, expressed between both genera. *C. albicans* is equipped with five superoxide dismutases genes, that allow to expose the encoded Sod proteins on the yeast cell surface (Martchenko *et al.,* 2004; Roetzer *et al.,* 2011b). *C. glabrata* has two *SOD* genes, similarly to *S. cerevisiae*; however, their expression is differently regulated rendering the ability to survive at severe oxidative stress. Adaptation to host environment resulted also in reduction of catalase genes. *C. albicans* and *C. glabrata* appear to have only one catalase but it combines the different transcriptional regulation and different intracellular localization (Roetzer *et al.,* 2010).

Despite the results obtained with stress markers, the protective effects of thiamine in *Candida* cells were confirmed by the observation of diminished generation of ROS in the presence of thiamine. An adverse response was observed only for *C. glabrata,* which was associated with poor growth of this yeast in the absence of thiamine and partially could result from their exceptional resistance to stress conditions (Roetzer *et al.,* 2011a). It should be mentioned that *Candida* species developed different strategies to survive on contact with phagocytic cells, neutrophils and macrophages. Some of them, like *C. albicans*, can escape from phagosomes, whereas *C. glabrata* can survive for longer time inside phagosomes (Ferrari *et al.,* 2011). This is mainly owing to ability of this species to actively suppress ROS production by neutrophils and relative resistance to starvation (Wellington *et al.,* 2009).

Thus, our current findings implicate that the protective action of thiamine, observed in *S. cerevisiae*, differ significantly in pathogenic *Candida* species, both in terms of thiamine cofactor functions and the thiamine effects on defense systems.

CONCLUSIONS

Despite evolutionary differences between *Candida* strains, their adaptation to human host resulted in developing similar traits, allowing them for an effective invasion under conditions of weakened immune defenses. One of them is an increased resistance to oxidative stress but the role of thiamine in this process is less clear than in the stress reaction of *S. cerevisiae*. The more detailed analyses of defense system in *Candida* would require the use of other isoforms of stress markers, which diverged slightly from those observed in *Saccharomyces* ge-

nus. Nevertheless, the reduced amount of oxygen species observed in the presence of thiamine confirmed at least its partial protective effect on *Candida* species.

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