Curr Microbiol
DOI 10.1007/s00284-012-0242-0

Oversynthesis of Riboflavin in the Yeast *Pichia guilliermondii* is Accompanied by Reduced Catalase and Superoxide Dismutases Activities

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Received: 2 March 2012 / Accepted: 14 September 2012

Abstract Iron deficiency causes oversynthesis of riboflavin in several yeast species, known as flavinogenic yeasts. Under iron deprivation conditions, *Pichia guilliermondii* cells increase production of riboflavin and malondialdehyde and the formation of protein carbonyl groups, which reflect increased intracellular content of reactive oxygen species. In this study, we found that *P. guilliermondii* iron deprived cells showed dramatically decreased catalase and superoxide dismutase activities. Previously reported mutations *rib*80, *rib*81, and *hit*1, which affect repression of riboflavin synthesis and iron accumulation by iron ions, caused similar drops in activities of the mentioned enzymes. These findings could explain the previously described development of oxidative stress in iron deprived or mutated *P. guilliermondii* cells that overproduce riboflavin. Similar decrease in superoxide dismutase activities was observed in iron deprived cells in the non-flavinogenic yeast *Saccharomyces cerevisiae*.

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Introduction

Pichia guilliermondii belongs to a group of so-called flavinogenic yeast species that overproduce riboflavin under iron limitation conditions. This group also includes *Candida albicans*, *Debaryomyces hansenii*, industrial riboflavin overproducer *Candida famata* and some other yeast species [1, 14]. The molecular mechanisms of such regulation and physiological advantages of this phenomenon are not known. It has to be mentioned that iron-dependent repression of riboflavin synthesis is also found in some species of bacteria and plants. It has been hypothesized that excreted riboflavin promotes iron acquisition in bacteria, yeasts, and plants that overproduce riboflavin under iron deficiency. The readers are referred to the recent review on riboflavin synthesis and its regulation for more details [1].

The *P. guilliermondii* mutants *rib*80, *rib*81, and *hit*1 that constitutively overproduce riboflavin under iron repletion condition mimic to some extent the wild-type strain under iron deficiency though it was unexpectedly shown to contain more iron in the cells as compared to the wild-type cells [10, 11]. Mutations *rib*80, *rib*81, and *hit*1 are recessive, monogenic and possess nuclear localization [11, 31, 32]. So, the strains bearing these mutations are useful models to study interrelationships between iron and riboflavin metabolisms despite the fact that none of the mutated genes has been identified so far.

It is known that both iron and riboflavin are able to facilitate transfer of electrons during oxidation–reduction reactions, which are essential for all living cells. Though, the same property makes these compounds potentially toxic because of their ability to generate reactive oxygen species that damage cellular components by oxidizing lipids, proteins, and nucleic acids [25]. Superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are known to be

Published online: 09 October 2012

potentially dangerous products of oxygen metabolism in aerobic organisms. To reduce the harmful effects of superoxide anion and hydrogen peroxide, cells produce detoxifying enzymes. Enzymatic mechanisms include superoxide dismutases (SODs) that convert superoxide anion to hydrogen peroxide and oxygen, whereas catalases convert hydrogen peroxide to water and oxygen [13, 36].

Superoxide dismutases (SOD) can be classified according to metal cofactor(s) utilized: the copper and zinc (Cu, Zn-SOD), manganese (Mn-SOD), iron (Fe-SOD), and nickel (Ni-SOD) containing enzymes [3, 13, 26, 28]. Typically, eukaryotes express cytosolic Cu,Zn-SOD and mitochondrial Mn-SOD. *Saccharomyces cerevisiae* possesses two forms: the cytosolic Cu,Zn-SOD and the mitochondrial Mn-SOD which have been shown to play an important role in protecting the cells against oxygen toxicity [26]. *Candida albicans* genome harbors six genes encoding SODs. Four of them, namely *SOD*1, *SOD*4, *SOD*5, and *SOD*6, encode Cu,Zn-dependent enzymes whereas *SOD*2 and *SOD*3 both encode Mn-dependent enzymes that are localized to mitochondria and cytoplasm, respectively [4, 15–17, 24, 29].

In *S. cerevisiae*, there are two forms of catalase, cytosolic Ctt1 and peroxisomal Cta1 encoded by genes YGR088W and YDR256C, respectively, both utilizing heme as a co-factor [34]. In contrast, there is only one gene encoding catalase in the genome of the methylotrophic yeast *Hansenula polymorpha* (http://genomeportal.jgi-psf.org/Hanpo2/Hanpo2.info.html). There is very little information available regarding *P. guilliermondii* genes encoding catalases and SODs. Genes encoding these enzymes are annotated in the database for *Candida guilliermondii* genome (anamorph of *P. guilliermondii*) (http://www.broad.mit.edu/annotation/genome/candida_guilliermondii/Info.html#t0); although the functions of these genes have not been studied yet.

We have demonstrated earlier, that mutations affecting the regulation of riboflavin biosynthesis and iron acquisition as well as iron deficiency itself cause oxidative stress in *P. guilliermondii* [2]. We hypothesized that the up-regulation of riboflavin biosynthesis and iron hyper-accumulation could occur in response to oxidative stress. Though the mechanisms connecting riboflavin oversynthesis, iron metabolism, and development of oxidative stress by *P. guilliermondii* were not discussed. Here, we present evidence that disturbing the iron metabolism dramatically decreases catalase and SOD activities, which in turn may be a primary reason for evolving the oxidative stress in *P. guilliermondii* under iron starvation.

Materials and Methods

The strains used in this study are listed in Table 1. The cells were grown on modified synthetic Burkholder medium as described [33]. Iron-supplemented media contained 3.6 μM of iron added as ammonium ferrous sulfate hexahydrate. Iron-deficient media contained approximately 0.18 μM of iron. Iron was removed from the medium with 8-hydroxyquinoline as described earlier [33]. Cobalt-supplemented media contained 0.9 mM of cobalt added as cobalt chloride. Cultivation media were supplemented with appropriate amino acids (40 mg/l) whenever required. The cells were grown in Erlenmeyer flasks on a gyro-shaker (200 rpm) at 30 °C.

Riboflavin was assayed fluorometrically (Turner Quantech FM 109510-33 fluorometer) using solution of synthetic riboflavin as a standard. Protein concentration was determined by the Lowry method [23].

Intracellular reactive oxygen species (ROS) was detected using the oxidant-sensitive probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) [8]. Measurements of intracellular oxidation were performed with 20 mg (dry weight) of mid-exponential phase grown cells. A fresh ethanol stock solution of DCFH-DA was added to the culture to a final concentration of 10 μM and cells were incubated for 30 min at 28 °C. After cells were cooled on ice, harvested by centrifugation and washed twice with distilled water. The samples were viewed with a fluorescent microscope (Axio Imager A1; Carl Zeiss MicroImaging, Jena, Germany) coupled to a monochrome digital camera (Axio Cam MRm; Carl Zeiss MicroImaging) and processed using the AxioVision 4.5 (Carl Zeiss MicroImaging) and Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA). The increase in DCF fluorescence represents

Table 1 Strains used in this study

Strain	Genotype	Phenotype	References
P. guilliermondii L2 (ATCC 201911)	*RIB80 RIB81 HIT1 hisX*, MAT^-	Wild type	[32]
P. guilliermondii rib80-1018-31	*rib80 RIB81 HIT1 metX*, MAT^+	Riboflavin oversynthesis; hyper-accumulation of iron	[33]
P. guilliermondii rib81-131-6	*RIB80 rib81 HIT1 hisX*, MAT^-	Riboflavin oversynthesis; hyper-accumulation of iron	[32]
P. guilliermondii hit1-1	*RIB80 RIB81 hit1 hisX*, MAT^-	Riboflavin oversynthesis; hyper-accumulation of iron	[11]
S. cerevisiae L 3262	MAT^+ *ura3-52 leu2-3, 112 his4-34*	Wild type	[20]

the generation of ROS. Cell extracts were prepared by vigorous vortexing of cells with an equal volume of glass beads (0.3–0.4 mm) and 0.15 ml of 10 mM sodium phosphate buffer, pH 7.8. After centrifugation (14,000×*g* for 20 min) supernatant was used for fluorescence measurement using Turner Quantech FM 109510-33 fluorometer with excitation at 490 nm and emission at 515 nm. The values were normalized by protein content in the mixture.

Protein oxidation was measured as total carbonyl groups content using 2,4-dinitrophenylhydrazine as described [7]. Carbonyl protein content was evaluated spectrophotometrically at 370 nm using a reported molar extinction coefficient [22] and normalized by protein content in the mixture.

To evaluate malondialdehyde (MDA), an end-product of fatty acid peroxidation 0.8 ml of exponentially growing culture (culture medium together with cells) were treated with thiobarbituric acid as described [2].

The MDA production was calculated as follows

$$\text{MDA production} = (A_{532} - K \times A_{440}) \times d \times V/1.56 \times 10^5 \times N$$

$K = 0.187$	coefficient for calculation of absorption of unidentified complex at λ_{532}
d	dilution
V	sample volume (L)
$1.56 \times 10^5\ \text{cm}^{-1}\ \text{M}^{-1}$	coefficient of molar absorption of MDA-TBA complex at 532 nm
N	g of dry cells in a probe

For assay of SOD activity the modified method [5] was used. After electrophoresis the gel was washed twice in 50 mM phosphate buffer (pH 7.8) for 20 min. Then it was soaked in 50 mM phosphate buffer pH 7.5, 0.064 mg/ml of riboflavin, 3.2 µl/ml *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, 0.3 mg/ml of 2,3,5-triphenyltetrazoliumchloride for 20 min. Area of SOD activity remained yellowish-clear after the gel was exposed to the UV light (using UV-transilluminator (SPECTROLINE), whereas the rest of the gel turned red due to reaction of 2,3,5-triphenyltetrazoliumchloride with superoxide anion that resulted in formation of colored formazan.

To find out which band corresponds to Cu,Zn-SOD 10 µg of total cellular proteins were applied on to the gel. Loading buffer was supplemented with 1 mg/ml of bovine serum albumin (BSA). After electrophoresis, the gel was washed as described above. Then, the stripes of the gel were soaked for 60 min in reaction buffer supplemented with 5 mM of potassium cyanide or 0.1 mM of diethyldithiocarbamate. The reaction buffer without additional supplements was used as a control. The subsequent processing of the gel strips was identical.

Results

Earlier it has been reported, that iron deficiency causes pleiotropic effect on yeast *P. guilliermondii* resulting in increased production of riboflavin and MDA [2]. Mutations *rib*80, *rib*81, *hit*1 causing iron hyper-accumulation and riboflavin overproduction [2, 10] as well as wild-type cells supplementation with cobalt exerted similar effects on *P. guilliermondii* (Table 2). Iron content in the mutant cells grown under iron deprivation decreased similarly to that in the wild-type cells. Increased production of MDA in the mutants tested could suggest that mutations *rib*80, *rib*81, and *hit*1 cause chronic oxidative stress to *P. guilliermondii* cells. Iron depletion evoked significant increase in MDA production, especially pronounced in mutant cells. To further study development of the oxidative stress caused by the mentioned mutations and iron deprivation, we measured both ROS level and content of protein carbonyl groups in mutant and wild-type strains grown in iron defined media. As expected, intracellular oxidation levels in the cells of *rib*80, *rib*81, *hit*1 mutants grown under iron repletion condition was several folds higher as compared to the wild-type cells (Fig. 1; Table 2). In the presence of cobalt ions, the highest intracellular oxidation levels were observed in mutant cells again. Observed increase of protein carbonyl groups further supports this suggestion (Table 2).

The search of the *P. guilliermondii* genome database (http://www.broad.mit.edu/annotation/genome/candida_guilliermondii/Info.html#t0) suggests that the presence of two putative catalases genes, PGUG_01850.1 and PGUG_05581.1 that are similar to peroxisomal and cytosolic catalases reported in *S. cerevisiae*, respectively [6, 34]. We hypothesise that decrease of iron availability would lower catalase activity since both enzymes require iron ions for their activity. To check that, we compared catalase activity in the *P. guilliermondii* cells grown in media supplemented with different concentration of iron. In addition, we tested cells grown in the same medium supplemented with 3.6 µM of iron and 0.9 mM of cobalt (added as $CoCl_2 \cdot 6H_2O$) since it was suggested that cobalt ions act as a competitor of ferrous ions [9]. As was expected, iron limitation led to significant decrease (up to 9 times) of catalase activity in the cells of the wild-type strain (Fig. 2). Cobalt supplementation caused similar but less pronounced effect. In addition, we found that catalase activity in the cells of riboflavin overproducing mutants grown in iron sufficient medium is also decreased (Fig. 3). Riboflavin production caused by iron deficiency, Co supplementation or mutations mentioned above was increased in these experiments (data not shown), as previously described [2, 11, 31, 32]. Notwithstanding that all *P. guilliermondii* riboflavin overproducing mutants were shown to contain several times more iron as compared to the wild-type strains [10].

Table 2 Cellular iron content, intracellular oxidation level, protein carbonyl groups content and MDA production by *P. guilliermondii* mutant and wild-type strains grown in iron defined media

Strains	Iron content in the medium	Iron content in the cells, μg/g dry cells	Intracellular oxidation level, relative fluorescence/mg of protein	Protein carbonyl groups, nmol/mg of protein	MDA production, μmol/g dry cells
L2	+Fe	99 ± 7	21 ± 1	2.2 ± 0.1	0.5 ± 0.1
	+Fe+Co	113 ± 8	171 ± 3	Nd	1.4 ± 0.3
	−Fe	48 ± 3	31 ± 2	3.0 ± 0.1	1.2 ± 0.2
*rib*80	+Fe	280 ± 10	81 ± 5	4.0 ± 0.2	2.8 ± 0.2
	+Fe+Co	336 ± 12	374 ± 15	Nd	7.0 ± 0.4
	−Fe	60 ± 3	Nd	Nd	6.6 ± 0.7
*rib*81	+Fe	119 ± 6	53 ± 3	2.1 ± 0.1	4.3 ± 0.4
	+Fe+Co	281 ± 9	603 ± 21	Nd	9.8 ± 0.5
	−Fe	51 ± 3	Nd	Nd	12.8 ± 1.4
*hit*1	+Fe	235 ± 9	78 ± 5	2.9 ± 0.10	2.0 ± 0.3
	+Fe+Co	430 ± 18	272 ± 14	Nd	3.6 ± 0.2
	−Fe	60 ± 4	Nd	Nd	18.3 ± 3.5

Nd not determined

For MDA measurement, cells were incubated for 8 h

−Fe medium supplemented with 0.18 μM of iron

+Fe medium supplemented with 3.6 μM of iron

+Co medium supplemented with 3.6 μM of iron and 0.9 mM of cobalt

Mean ± SD from at least three experiments are shown

SOD activity in different *P. guilliermondii* strains were also analyzed. Since this yeast species is closer to *C. albicans* than to *S. cerevisiae* (http://www.broadinstitute.org/annotation/genome/candida_group/MultiHome.html), multiple genes encoding SOD could be expected in its genome. Search for homologies to SODs reported in *C. albicans* revealed five *P. guilliermondii* genes putatively encoding SODs (Table 3). Due to multiplicity of SOD genes and isozymes, we applied a gel staining method to evaluate SOD activity in *P. guilliermondii* cells instead of measurement of the total activity in the cell-free extracts.

Cells of the wild-type strain (L2) grown to the late logarithmic phase exhibited two distinct bands of SOD activity. One of them (fast migrating) was partially inhibited by both 5 mM of potassium cyanide or 0.1 mM of diethyldithiocarbamate (Fig. 4) [3, 24]. Contrary, the activity in slowly migrating upper band was not inhibited by these agents (Fig. 4). Thus, the fast migrating band would represent Cu,Zn-SOD activity(-ies) while slow migrating band could be attributed to other SODs encoded by *P. guilliermondii* nuclear genes (Table 3).

The activity of slow migrating form of SOD is slightly visible in the younger cells (Fig. 5). Activity of Cu,Zn-SOD which is the major form in younger cells, apparently did not depend on the growth phase. Contrary, the activity of this form of SOD is dramatically decreased in cells of rib80-1018-31 mutant as compared to the wild-type cells. Cells of rib81-131-6 strain also possess decreased activity of this form as compared to the wild-type cells. Contrary to all other tested strains, the cells of *hit1-1* mutant possessed Cu,Zn-SOD activity only.

We expected that *P. guilliermondii* iron deprived cells possess increased activity of SODs since they are oxidatively stressed under such conditions. Unexpectedly, we revealed a completely opposite phenomenon. Iron deprived *P. guilliermondii* cells exhibited dramatic decrease in activity of SODs (Fig. 6). The band of Cu,Zn-SOD activity was slightly visible in the cells from earlier logarithmic phase. There were only traces of activity of this form and no activity of slow migrating SOD in the cells harvested at middle or late logarithmic phase as well as in the cells grown to earlier stationary phase. Contrary to that, two typical bands of SOD activity were found in the cells of the same strain grown in the medium supplemented with 3.6 μM of iron (iron sufficient conditions). Only the fast migrating band of SOD activity (apparently Cu,Zn-SOD) was found in the cells grown in the same medium supplemented with 0.9 mM cobalt and 3.6 μM iron. These results suggested that decrease in iron availability leads to dramatic reduction of SODs activity in *P. guilliermondii* cells.

Interestingly, the model organism non-flavinogenic yeast *S. cerevisiae* possessed very similar changes in SOD activity as both, iron deprived or cobalt loaded, cells exhibited decreased SOD activities (Fig. 7). Thus, decrease in SOD activities under iron starvation occurs in both flavinogenic (*P. guilliermondii*) and non-flavinogenic (*S. cerevisiae*) yeast species.

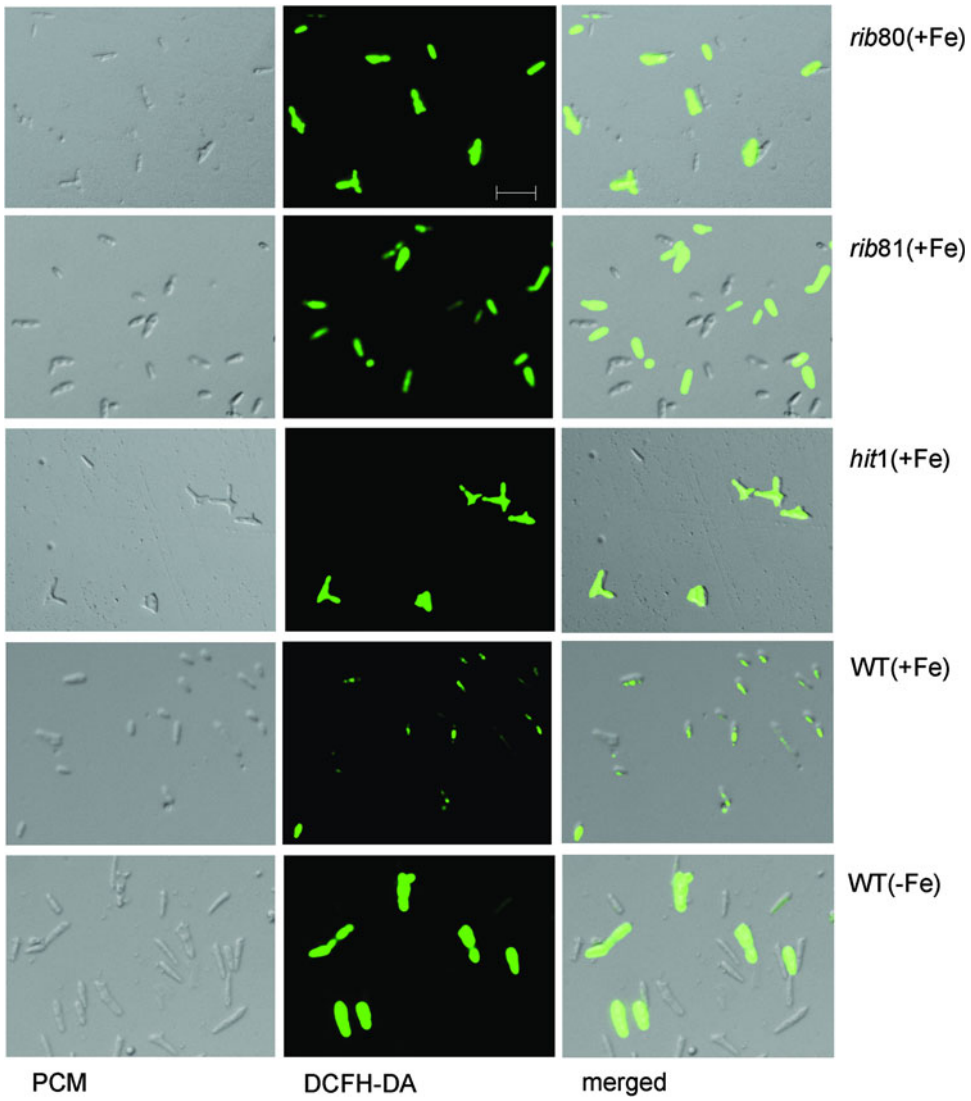

Fig. 1 Fluorescence microscopy analysis of ROS generation by *P. guilliermondii*. *Pichia guilliermondii* rib80-1018-31 (*rib*80 +Fe), rib81-131-6 (*rib*81 + Fe), and hit1-1 (*hit*1 + Fe) mutant were grown in the synthetic medium containing 3.6 μM of iron. Wild-type cells L2 were grown in the synthetic medium containing 3.6 μM (WT + Fe) and 0.18 μM (WT-Fe) of iron, respectively. *PCM* phase contrast microscopy, *DCFH-DA* DCFH-DA stained cells. Representative images are shown

Discussion

Earlier we observed that mutations *rib*80, *rib*81, *hit*1 affecting regulation of riboflavin biosynthesis and iron acquisition cause oxidative stress in *P. guilliermondii* [1, 27]. Data on the elevated ROS level and content of protein carbonyl groups in the cells of riboflavin overproducing mutants further confirmed this assumption. It should be stressed that the mutants exhibited chronic oxidative stress without pro-oxidants treatment. It could be suggested that oxidative stress in these mutants is caused by hyper-accumulation of iron that promotes formation of free radicals via Fenton reactions [10, 25]. To analyze this phenomenon, we evaluated both catalase and superoxide dismutase activities in the *P. guilliermondii* cells undergoing oxidative stress caused by mutations *rib*80, *rib*81, *hit*1 or iron deficiency. As was expected, we found substantial reduction of catalase activity in *P. guilliermondii* cells grown under iron-deficient conditions (as compared to iron-supplemented cells). Interestingly, mutations leading to riboflavin oversynthesis and iron hyper-accumulation also caused reduction of catalase activity in the cells though to the lesser extent. Apparently this hyper-accumulated iron remains in an unavailable form, which could not be utilized by the cells. Indeed, the mutant cells with iron hyper-accumulation behave like iron deprived wild-type cells as both are characterized by increased riboflavin production [1, 11, 31, 32].

To prove that defects in iron metabolism can cause decrease of catalase activity we used cobalt since this metal is thought to be a competitor of iron [9]. Earlier it was shown that in *S. cerevisiae* cobalt stress selectively induces a number of genes of a so called "iron regulon", coding mainly for iron transport proteins, a response strikingly similar to iron starvation. As result of cobalt-mediated activation of "iron regulon", this treatment led to twofold

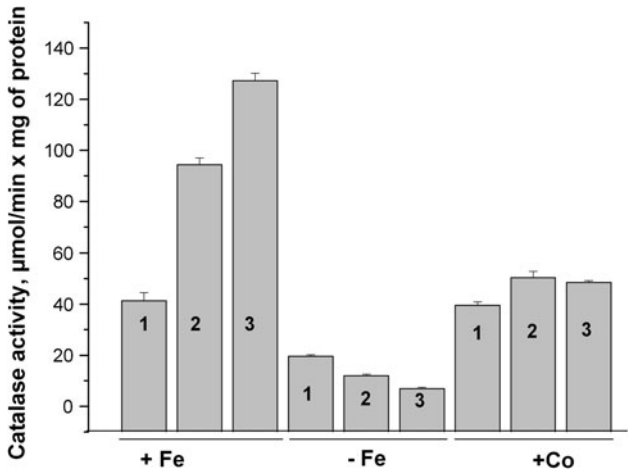

Fig. 2 Catalase activity in *P. guilliermondii* wild-type strain cells grown in iron defined and cobalt-supplemented media. *Pichia guilliermondii* L2 cells were grown in the synthetic medium containing 3.6 µM of iron (+Fe), 0.18 µM of iron (−Fe), and 3.6 µM of iron and 0.9 mM Co (+Fe+Co). Cells from earlier (*1*), late (*2*) logarithmic and stationary (*3*) growth phase were analyzed. Means and SD from triplicate experiments are shown. *Error bars* represent SD

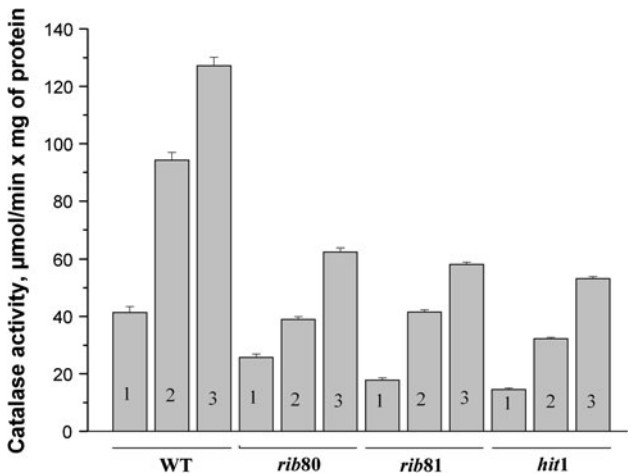

Fig. 3 Catalase activity in *P. guilliermondii* strains grown in iron sufficient medium. Cells of *P. guilliermondii* wild-type strain L2 (WT) and rib80-1018-31 (*rib*80), rib81-131-6 (*rib*81), and hit1-1 (*hit*1) riboflavin producing mutants were grown in the synthetic medium containing 3.6 µM of iron. Cells from earlier (*1*), late (*2*) logarithmic, and stationary (*3*) growth phase were analyzed in each case. Means and SD from triplicate experiments are shown. *Error bars* represent SD

increased iron content in *S. cerevisiae* cells [35]. Similarly to that, *P. guilliermondii* wild-type strains treated with cobalt possessed significantly higher riboflavin production and decreased catalase activity, thus behaving like the mutants or like iron deprived wild-type cells [2, 11, 31, 32]. So, availability of iron has significant impact on catalase activity in *P. guilliermondii*.

Table 3 *Pichia guilliermondii* proteins exhibiting significant similarity to *C. albicans* SODs

Putative *P. guilliermondii* SODs	Protein of *C. albicans*	Identical residues	Similar substitutions	% of similarity
PGUG_00172.1	SOD3 [21]	133/201	23/201	77
	SOD2 [29]	129/199	25/199	77
PGUG_00849	SOD4 [24]	80/170	33/170	66
	SOD5 [24]	75/162	31/162	64
	SOD6 [24]	63/161	27/161	55
	SOD1 [16]	38/124	22/124	48
PGUG_02053.1	SOD6 [24]	73/155	31/155	67
	SOD5 [24]	51/118	22/118	61
	SOD4 [24]	53/117	15/117	58
PGUG_02952.1	SOD2 [29]	147/207	27/207	84
	SOD3 [21]	131/199	28/199	79
PGUG_03463.1	SOD6 [24]	93/165	26/165	72
	SOD5 [24]	71/166	23/166	56
	SOD4 [24]	69/166	25/166	56

* Only proteins exhibiting more than 48 % of homology are shown

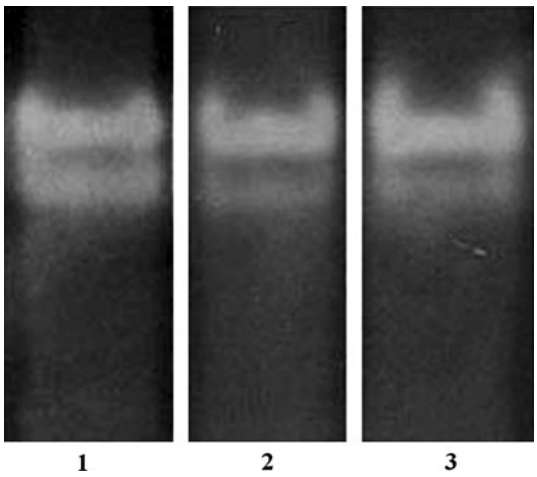

Fig. 4 Inhibition of SOD activity in *P. guilliermondii.* Cells of *P. guilliermondii* wild-type strain L2 were grown to middle logarithmic growth phase in the synthetic medium containing 3.6 µM of iron (iron repletion conditions). 10 µg of cell protein were applied on to the gel in each case. **1** reaction mixture without inhibitors added, **2** reaction mixture supplemented with 0.1 mM of diethyldithiocarbamate, **3** reaction mixture supplemented with 5 mM potassium cyanide

Unexpectedly, we observed that iron availability had much more pronounced effect on SOD activity. Traces of activity were found in iron deprived *P. guilliermondii* cells of wild-type strain, whereas cobalt loaded cells possessed only one band of activity (apparently belonging to Cu, Zn-SOD(s) (Fig. 6), as observed for hit1-1 mutant. The activity of this form of SOD is substantially decreased in cells of both rib80-1018-31 and rib81-131-6 mutants, as compared to the wild-type strain (Fig. 5). The situation is

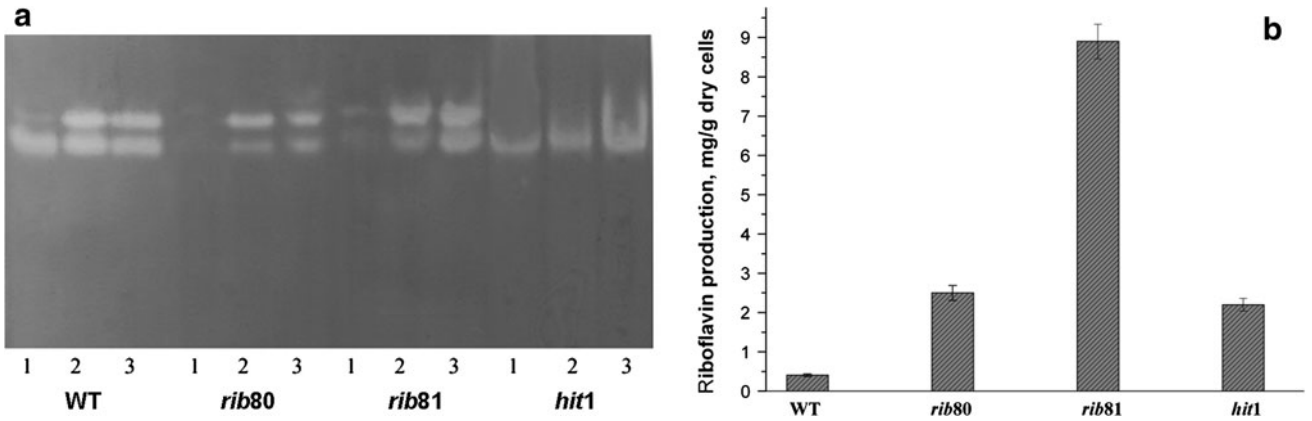

Fig. 5 Areas of SOD activity (**a**) and riboflavin production (**b**) by *P. guilliermondii* strains. Cells were grown in the synthetic medium containing 3.6 μM of iron (iron repletion conditions). 40 μg of cell protein were applied in each case. *WT* wild-type strain L2, *rib*80 strain rib80-1018-31, *rib*81 strain rib81-131-6, *hit*1 strain hit1-1. Cells from earlier (*1*), middle (*2*), and late (*3*) logarithmic growth phase were analyzed in each case. Results of the typical representative experiment are shown. For riboflavin measurement cells were grown to late logarithmic phase. Means and SD from triplicate experiments are shown. *Error bars* represent SD

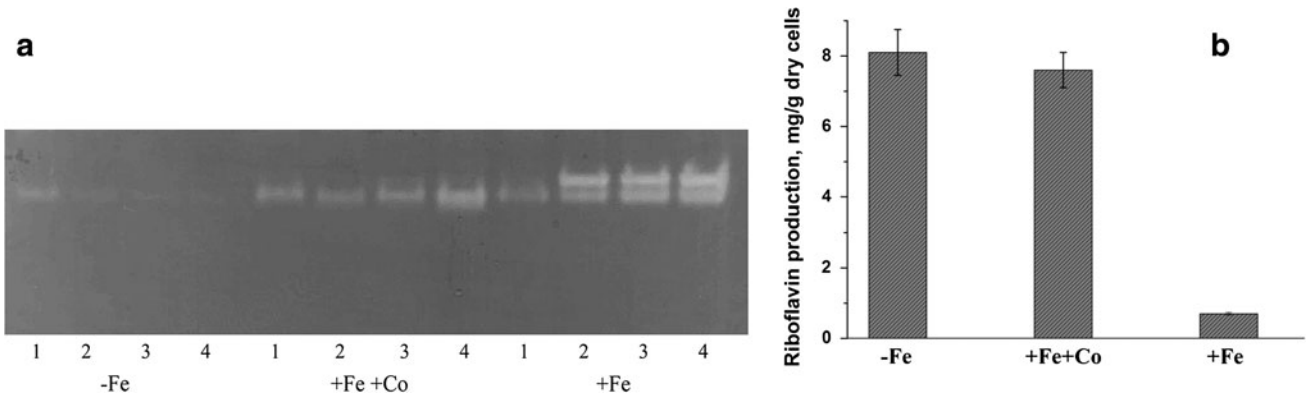

Fig. 6 Areas of SOD activity (**a**) and riboflavin production (**b**) by *P. guilliermondii* wild-type strain. Cells of *P.guilliermondii* strain L2 were grown in the synthetic medium containing 0.18 μM of iron (−Fe), 3.6 μM of iron, and 0.9 mM Co (+Fe+Co) and 3.6 μM of iron (+Fe). Cells from earlier (*1*), middle (*2*), late logarithmic (*3*) and stationary (*4*) growth phase were analyzed. 40 μg of cell protein were applied in each case. For riboflavin measurement cells were grown to late logarithmic phase. Means and SD from triplicate experiments are shown. *Error bars* represent SD

really complicated since data show multiplicity of SOD coding genes. There are at least five genes in *P. guilliermondii* genome potentially encoding SODs. It should be noted that two of the proteins listed in Table 3, namely PGUG_02952.1 and PGUG_00172.1 were annotated as putative Fe,Mn-containing SOD. Apparently these two enzymes but not others could be inactive in the case of decreased availability of iron. Also it should not be excluded that transcription of SOD genes in *P. guilliermondii* is repressed under iron deficiency through Hap43 transcriptional repressor like it was reported for other genes in *C. albicans* [14]. *Pichia guilliermondii* five putative SODs apparently are sorted to different cellular compartment (cytosol and mitochondria). To further investigate the role of the particular SOD genes in oxidative resistance (and subsequently in riboflavin biosynthesis regulation) we plan to construct strains containing double or triple deletions of the particular genes in the future. Such study should provide important insights into this phenomenon.

Involvement of iron-sulfur clusters in a link between Cu,Zn-SOD activity and iron metabolism was suggested earlier in *S. cerevisiae* [12, 18, 19]. It was supposed that increased iron demand in the *sod1* mutant may be a reflection of the cell efforts to reconstitute iron-sulfur cluster-containing enzymes that are continuously inactivated in conditions of superoxide excess [12]. It is important to mention here that iron deficiency strongly diminished SOD activity in *S. cerevisiae* (Fig. 7); so the observed phenomenon is similar between flavinogenic (*P. guilliermondii*) and non-flavinogenic (*S. cerevisiae*) yeasts. Iron-sulfur clusters are universally involved in regulation of iron metabolism in yeast cells [30]. It can be supposed that in *P. guilliermondii*

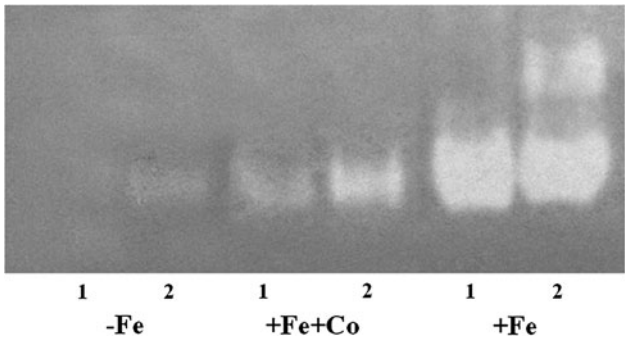

Fig. 7 Detection of SOD activity in *S. cerevisiae*. *Saccharomyces cerevisiae* L3262 cells were grown in the synthetic medium containing 0.18 μM of iron (−Fe), 3.6 μM of iron, and 0.45 mM Co (+Fe+Co) and 3.6 μM of iron (+Fe). Cells from logarithmic (*1*) and stationary (*2*) growth phase were analyzed. 40 μg of cell protein were applied in each case

iron-sulfur clusters are involved in regulation of riboflavin biosynthesis as well. This assumption could explain riboflavin overproduction by oxidatively stressed *P. guilliermondii* cells. Although these hypotheses need to be tested in greater details, the observed significant decrease, both catalase and SODs activities can be the main reasons of oxidative stress evolving in iron deprived *P. guilliermondii* wild-type cells as well as in cells of the riboflavin overproducing mutants mentioned above.

References

1. Abbas CA, Sibirny AA (2011) Genetic control of biosynthesis and transport of riboflavin and flavin nucleotides and construction of robust biotechnological producers. Microbiol Mol Biol Rev 75:321–360
2. Boretsky Y, Protchenko O, Prokopiv T, Mukalov I, Fedorovych D, Sibirny A (2007) Mutations and environmental factors affecting regulation of riboflavin synthesis and iron assimilation also cause oxidative stress in the yeast *Pichia guilliermondii*. J Basic Microbiol 47:371–377
3. Bridges SM, Salin ML (1981) Distribution of iron-containing superoxide dismutase in vascular plants. Plant Physiol 68:275–278
4. Chauhan N, Latge JP, Calderone R (2006) Signalling and oxidant adaptation in *Candida albicans* and *Aspergillus fumigatus*. Nat Rev Microbiol 4:435–444
5. Cizewski-Culotta V, Klomp LWJ, Strain J et al (1997) The copper chaperone for superoxide dismutase. J Biol Chem 272: 23469–23472
6. Cohen G, Fessl F, Traczyk A, Rytka J, Ruis H (1985) Isolation of the catalase A gene of *Saccharomyces cerevisiae* by complementation of the *ctal* mutation. Mol Gen Genet 200:74–79
7. Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R (2003) Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta 329:23–38
8. Davidson JF, Whyte B, Bissinger PH, Schiestl RH (1996) Oxidative stress is involved in heat-induced cell death in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 93:5116–5121
9. Enary TM (1955) Effect of cobalt and iron on riboflavin by *Candida guilliermondii*. Acta Chem Scand 9:19–23
10. Fedorovich DV, Kityk IV, Dzhala VI, Protchenko OV, Shavlovskii GM (1997) Accumulation and redox transformations of iron in the yeast *Pichia guilliermondii* and its flavinogenic mutants. Mikrobiologiya 66:48–51 (in Russian)
11. Fedorovich D, Protchenko O, Lesuisse E (1999) Iron uptake by the yeast *Pichia guilliermondii*. Flavinogenesis and reductive iron assimilation are co-regulated processes. Biometals 12:295–300
12. De Freitas JM, Liba A, Meneghini R, Valentine JS, Gralla EB (2000) Yeast lacking Cu–Zn superoxide dismutase show altered iron homeostasis. Role of oxidative stress in iron metabolism. J Biol Chem 275:11645–11649
13. Fridovich I (1998) Oxygen toxicity: a radical explanation. J Exp Biol 201:1203–1209
14. Hsu PC, Yang CY, Lan CY (2011) *Candida albicans* Hap43 is a repressor induced under low-iron conditions and is essential for iron-responsive transcriptional regulation and virulence. Eukaryot Cell 10:207–225
15. Hwang CS, Baek YU, Yim HS, Kang SO (2003) Protective roles of mitochondrial manganese-containing superoxide dismutase against various stresses in *Candida albicans*. Yeast 20:929–941
16. Hwang CS, Rhie GE, Kim ST et al (1999) Copper- and zinc-containing superoxide dismutase and its gene from *Candida albicans*. Biochim Biophys Acta 1427:245–255
17. Hwang CS, Rhie GE, Oh JH et al (2002) Copper- and zinc-containing superoxide dismutase is required for the protection from oxidative stresses and full virulence in *Candida albicans*. Microbiology 148:3705–3713
18. Irazusta V, Moreno-Cermeno A, Cabiscol E, Ros J, Tamarit J (2008) Major targets of iron-induced protein oxidative damage in frataxin-deficient yeasts are magnesium-binding proteins. Free Radic Biol Med 44:1712–1723
19. Jensen LT, Cizewski-Culotta V (2000) Role of *Saccharomyces cerevisiae ISA1* and *ISA2* in iron homeostasis. Mol Cell Biol 20:3918–3927
20. Kang HA, Jung MS, Hong WK et al (1998) Expression and secretion of human serum albumin in the yeast *Saccharomyces cerevisiae*. J Microbiol Biotechnol 8:42–48
21. Lamarre C, Le May JD, Deslauriers N, Bourbonnais Y (2001) *Candida albicans* expresses an unusual cytoplasmic manganese-containing superoxide dismutase (SOD3 gene product) up on the entry and during the stationary phase. J Biol Chem 276:43784–43791
22. Lenz AG, Costabel U, Shaltiel S, Levine RL (1989) Determination of carbonyl groups in oxidatively modified proteins by reduction with tritiated sodium borohydride. Anal Biochem 177:419–425
23. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagents. J Biol Chem 193: 265–275
24. Martchenko M, Alarco AM, Harcus D, Whiteway M (2004) Superoxide dismutases in *Candida albicans*: transcriptional regulation and functional characterization of the hyphal-induced *SOD5* gene. Mol Biol Cell 15:456–467
25. Meneghini R (1997) Iron homeostasis, oxidative stress, and DNA damage. Free Radic Biol Med 23:783–792
26. O'Brien KM, Dirmeier R, Engle M, Poyton RO (2004) Mitochondrial protein oxidation in yeast mutants lacking manganese-(MnSOD) or copper- and zinc-containing superoxide dismutase (CuZnSOD): evidence that MnSOD and CuZnSOD have both unique and overlapping functions in protecting mitochondrial proteins from oxidative damage. J Biol Chem 279:51817–51827
27. Protchenko OV, Boretsky YR, Romanyuk TM, Fedorovych DV (2000) Oversynthesis of riboflavin by yeast *Pichia guilliermondii* in response to oxidative stress. Ukr Biokhim J 72:19–23
28. Raychaudhuri S, Reddy MM, Rajkumar NR, Rajasekharan R (2003) Cytosolic iron superoxide dismutase is a part of the

triacylglycerol biosynthetic complex in oleaginous yeast. Biochem J 372:587–594
29. Rhie GE, Hwang CS, Brady MJ et al (1999) Manganese-containing superoxide dismutase and its gene from *Candida albicans*. Biochim Biophys Acta 1426:409–419
30. Rutherford JC, Ojeda L, Balk J et al (2005) Activation of the iron regulon by the yeast Aft1/Aft2 transcription factors depends on mitochondrial but not cytosolic iron-sulfur protein biogenesis. J Biol Chem 280:10135–10140
31. Shavlovsky GM, Fedorovich DV, Babyak LY (1993) The effect of *rib81* mutation on riboflavin biosynthesis and iron transport in yeast *Pichia guilliermonlii* yeast. Mikrobiologiya 62:897–903 (in Russian)
32. Shavlovsky GM, Fedorovich DV, Kutsiaba VI, Babyak LY, Stenchuk MM (1992) Participation of *RIB80* gene in regulation of riboflavin biosynthesis and iron transport in yeast *Pichia guilliermonlii*. Genetika 28:25–32 (in Russian)
33. Shavlovsky GM, Koltun LV, Kshanovskaya BV, Logvinenko EM, Stenchuk NN (1989) Regulation of biosynthesis of riboflavin by elements of the positive control in *Pichia guilliermonlii* yeast. Genetika 25:250–258 (in Russian)
34. Spevak W, Fessl F, Rytka J, Ruis H et al (1983) Isolation of the catalase T structural gene of *Saccharomyces cerevisiae* by functional complementation. Mol Cell Biol 3:1545–1551
35. Stadler JA, Schweyen RJ (2002) The yeast iron regulon is induced upon cobalt stress and crucial for cobalt tolerance. J Biol Chem 277:39649–39654
36. Temple MD, Perrone GG, Dawes IW (2005) Complex cellular responses to reactive oxygen species. Trends Cell Biol 15: 319–326