Research Paper

Mutations and environmental factors affecting regulation of riboflavin synthesis and iron assimilation also cause oxidative stress in the yeast *Pichia guilliermondii*

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Iron deficiency causes oversynthesis of riboflavin in several yeast species, known as flavinogenic yeasts. However, the mechanisms of such regulation are not known. We found that mutations causing riboflavin overproduction and iron hyperaccumulation (*rib80, rib81* and *hit1*), as well as cobalt excess or iron deficiency all provoke oxidative stress in the *Pichia guilliermondii* yeast. Iron content in the cells, production both of riboflavin and malondialdehyde by *P. guilliermondii* wild type and *hit1* mutant strains depend on a type of carbon source used in cultivation media. The data suggest that the regulation of riboflavin biosynthesis and iron assimilation in *P. guilliermondii* are linked with cellular oxidative state.

Keywords: Yeast / Regulation of riboflavin biosynthesis / Iron accumulation / Oxidative stress

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Introduction

The capacity both of iron and flavin coenzymes to facilitate electron transfer during oxidation-reduction reactions makes these compounds potentially toxic due to their ability to generate reactive oxygen species that damage cellular components (Crichton and Charloteaux-Wauters 1987, Meneghini 1997, Massey 2000). Therefore, both iron and riboflavin (RF) metabolism have to be regulated tightly.

In ascomycetous yeasts, the main features of iron uptake systems are similar. However, the set of genes expressed under iron deprivation may vary (Askwith *et al.* 1996, De Freitas *et al.* 2000, Lan *et al.* 2004). In contrast to *Saccharomyces cerevisiae*, *P. guilliermondii* exhibits a coordinated regulation of iron accumulation and RF biosynthesis. Mechanisms and factors of this coordinated regulation are still poorly understood (Shavlovsky *et al.* 1992, 1993, Fedorovich *et al.* 1999).

Although RF can be toxic when living cells are exposed to a light irradiation (Chelala and Margolin 1983), there are many species of microorganisms that are naturally capable of RF overproduction. Various strains of microorganisms have been engineered to produce significant amounts of RF (*e.g.* the bacterium *Bacillus subtilis* and the yeast *Candida famata)* (Stachmann *et al.* 2000). Some yeast species overproduce RF in response to iron deficiency. This group includes the industrially important species *Debaryomyces hansenii* (anamorph, *Candida famata*), *Pichia guilliermondii* (anamorph, *Candida guilliermondii*), *Schwanniomyces occidentalis* and the human conditional pathogen *Candida albicans* (Shavlovsky and Logvinenko 1988, Sibirny 1996, Knight *et al.*, 2002). Despite the fact that iron-dependent regulation of flavinogenesis was first described in 1945 (Tanner *et al.* 1945), neither the physiological role nor the mechanism of this phenomenon are known. Recently, it has been shown that iron represses transcription of RF structural genes *RIB1* and *RIB7*; while *rib81* and *red6* mutations alleviate this iron repression (Boretsky *et al.* 2005). Also it is known that cobalt ions (Co^{2+}) cause overproduction of RF in flavinogenic yeasts (Enary

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Table 1. Strains of *P. guilliermondii* used in this study.

Strain	Genotype	Phenotype	References
L2 (ATCC 201911)	*RIB80, RIB81, HIT!, RIB83, hisX, MAT*$^-$	wild type	Shavlovsky *et al*. (1992, 1993)
rib80 1026-7	*rib80, metX, MAT*$^+$	RF oversynthesis; hyper accumulation of iron	Shavlovsky *et al*. (1992)
rib81 131-6	*rib81, hisX, MAT*$^-$	RF oversynthesis; hyper accumulation of iron	Shavlovsky *et al*. (1993)
hit1 (ATCC 201910)	*hit1, hisX, MAT*$^-$	RF oversynthesis; hyper accumulation of iron	Fedorovych *et al*. (1999)
rib83-LV251	*rib83, hisX, MAT*$^+$	unable to RF oversynthesis; reduced iron transport	Shavlovsky *et al.* (1989), Stenchuk *et al.* (2001)

1955) but mechanism of that phenomenon has not yet been explained.

P. guilliermondii appears to be a most suitable organism for studying interrelationship between iron and flavin metabolisms. In contrast to other RF-producing yeast species, *P. guilliermondii* can be stimulated to mate and sporulate (Sibirny *et al.* 1996). Genome of this yeast is publicly available at http://www.broad.mit.edu (*Candida guilliermondii* Sequencing Project) or at http://www.ebi.ac.uk/ (*Pichia guilliermondii*). In both databases, genome of the same strain *Pichia guilliermondii* ATCC 6260 is presented.

During past years, this yeast was used for extensive research of regulation of flavins biosynthesis. A number of *P. guilliermondii* mutants defective in the regulation of RF biosynthesis and iron acquisition has been isolated (Shavlovsky *et al.* 1992, 1993, Sibirny 1996, Fedorovich *et al.* 1999, Boretsky *et al.* 2005). These mutants roughly could be divided in two groups. The first group includes *rib80, rib81, hit1, red1-red6* which exhibits constitutive RF over-production, possess both elevated ferrireductase activity and iron transport. Representative of the second group, *rib83* mutant strain, is unable to overproduce RF and exhibits a substantially reduced iron transport (Stenchuk *et al.* 2001).

Earlier we reported that *P. guilliermondii* cells treated with superoxide generating agent, methylviologen (MV), produced more RF and accumulated more iron in the cells (Protchenko *et al.* 2000]. We hypothesized that the upregulation of RF biosynthesis and iron accumulation could occur in response to the oxidative stress. In this paper, we demonstrate that mutations affecting the regulation of RF biosynthesis and iron acquisition cause oxidative stress in *P. guilliermondii*. In this yeast species, iron deficiency or cobalt upload elevated production of RF and malondialdehyde (MDA), well known marker of oxidative stress. Our data suggest that the regulation of RF biosynthesis and iron accumulation in *P. guilliermondii* is associated with cellular oxidative state.

Materials and methods

The *P. guilliermondii* strains used in this study are listed in Table 1.

The cells were grown on modified synthetic Burkholder medium containing per liter: 20 g saccharose or an appropriate quantity of other carbohydrate; 3 g $(NH_4)_2SO_4$; 0.5 g KH_2PO_4; 0.2 g $MgSO_4 \times 7\ H_2O$; 0.2 g $CaCl_2 \times 6\ H_2O$; 2 μg biotin, 0.06 mg H_3BO_3, 0.04 mg $CuSO_4 \times 5\ H_2O$, 0.05 mg $MnSO_4 \times 7\ H_2O$, 0.12 mg $(NH_4)_6Mo_7O_{24} \times 4\ H_2O$, 0.3 mg $ZnSO_4 \times 7\ H_2O$. 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 (Shavlovsky *et al*. 1989). Cultivation media were supplemented with appropriate amino acids (40 mg l^{-1}) whenever required. The cells were grown in Erlenmeyer flasks on a gyro-shaker (200 rpm) at 30 °C. To study an influence of carbon source or cobalt ions on RF biosynthesis and iron assimilation exponentially growing yeast cells were harvested and re-suspended (1 mg of dry weight cells per 1 ml) in fresh media supplemented with appropriate carbon source or 0.9 mM of cobalt chloride. Time of incubation is given in legend to an appropriate illustration.

RF was assayed fluorometrically using solution of synthetic RF as a standard. Cellular iron content was determined with 2,2′-dipiridyl as described earlier (Shavlovsky *et al*. 1992). The ferrireductase activity of washed cells was measured spectrophotometrically with ferric citrate (0.2 mM) as a substrate (Fedorovich *et al.* 1999). Protein concentration was determined by the Lowry method (Lowry *et al.* 1951]. Electron microscopy and estimation of average number of mitochondria per thin section of cells was performed as described earlier (Kulachkovsky *et al.* 1997).

MDA content was measured in exponentially growing yeast cultures by a modified method of Bozkaya *et al.* (2001). We found that *P. guilliermondii* cells ex-

creted a major part of thiobarbituric acid reactive species (TBA-RS), apparently MDA, into culture media. To check that 0.8 ml of exponentially growing culture (the cells together with the culture medium) was mixed with 0.8 ml 4% H_3PO_4 and 0.25 ml 0.8% TBA and incubated at 100 °C for 30 min. After extraction with equal volume of n-butanol, aliquots of the upper phase were applied on Silufol (Chemapol) plates and a thin-layer chromatography was carried out in a mixture of n-butanol: acetic acid: water (10:3:7 v/v/v) or using 3% NH_4Cl. In addition this compound was separated from other interfering unidentified compounds by C18 reverse-phase HPLC techniques with detection at 532 nm. It was found that the major coloured compound migrated identically with synthetic MDA-TBA complex in all systems. Finally, the absorption spectra of that compound and synthetic MDA-TBA complex were found to be identical. Absorption spectra of another unidentified complex and synthetic MDA-TBA adduct had different λ_{max}. The absorption of MDA-TBA complex at λ_{440} was very low: $A_{440}/A_{532} = 0.0125$, whereas the unidentified complex had substantial absorbance at λ_{532} giving ratio $A_{532}/A_{440} = 0.187$. The obtained data were used to design a formula for calculation of MDA production by yeast cells:

MDA production
$= (A_{532} - K \times A_{440}) \times d \times V \times 10^6/1.56 \times 10^5/N$

$K = 0.187$ – a coefficient for calculation of absorption of unidentified complex at λ_{532};
d – dilution; V – sample volume (L); N – mass of dry cells in a sample (mg);
1.56×10^5 cm^{-1} M^{-1} – molar absorption coefficient of MDA-TBA complex at 532 nm [19].

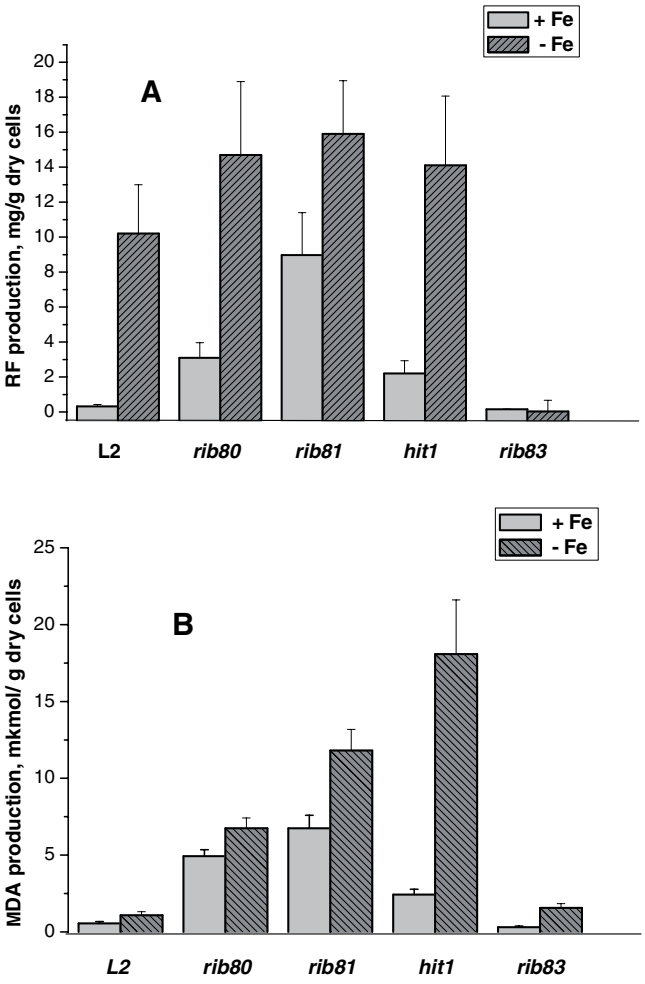

Figure 1. Riboflavin (A) and MDA (B) production by *P. guilliermondii* mutant and wild type strains grown in media containing 3.6 μM (+Fe) and 0.18 μM (–Fe) of iron. Cells were grown for 16 hours. Means ± SD from five experiments are shown.

Results

To adress whether regulation of vitamin B_2 is related to oxidative stress, we compared the production of RF and MDA (well known marker of oxidative stress) in wild-type and in mutant strains *rib80*, *rib81*, *hit1* and *rib83*. Mutants *rib80*, *rib81* and *hit1* produced more MDA and RF than the wild-type strain when grown in an iron supplemented medium (Fig. 1, Table 2). Omitting iron from the growth media caused additional increase in MDA production in all strains and especially in *hit1* mutant. The *rib83* strain was anable to oversynthesize RF in response to an iron deficiency but displayed an increase in MDA production.

The increased MDA production by mutant strains could indicate that selected mutations affect oxidative state of the cells. To check this hypothesis, we compared sensitivity of mutant and wild type strains to the factors causing oxidative stress, *i.e.* MV and cobalt ions (Fukushima *et al.* 2002). The wild-type and *rib*83 strains were resistant to both agents at concentrations of 2 mM. Mutant strains *rib80* and *hit1* grew poorly in media supplemented with MV or cobalt. Surprisingly mutant *rib81* exhibited different sensitivity to these agents. It was slightly inhibited by MV but resistant to Co ions (Fig. 2). The tolerance of the *P. guilliermondii* mutant *rib81* to Co ions does not contradict to the statement that mutation *rib81* leads to oxidative stress since it causes increased production of MDA (Fig. 1). We observed that in the presence of cobalt, all the tested strains (even *rib81* mutant, which was resistant to Co ions, as demonstrated in Fig. 2) displayed increased MDA production as well as ferrireductase activity of the

Table.2. Influence of Co ions on ferrireductase activity, iron content, RF and MDA production of *P. guilliermondii* mutant and wild type strains. Means ± SD from five experiments are given.

Strain	Conditions of growth	RF production, (mg/g dry cells)	Ferrireductase, μmol Fe × 10^3/(mg cells × min)	Iron content (μg/g dry cells)	MDA production (μmol/g dry cells)
L2	–	0.07 ± 0.04	1.8 ± 0.4	69 ± 9	0.34 ± 0.04
	+Co	0.53 ± 0.12	7.7 ± 0.8	113 ± 17	0.87 ± 0.12
rib80	–	1.36 ± 0.15	5.9 ± 0.7	182 ± 21	0.65 ± 0.23
	+Co	3.43 ± 0.36	9.0 ± 1.2	336 ± 29	3.28 ± 0.44
rib81	–	1.12 ± 0.07	3.9 ± 0.6	120 ± 13	1.94 ± 0.23
	+Co	2.46 ± 0.23	12.5 ± 1.6	281 ± 34	4.40 ± 0.51
hit1	–	0.51 ± 0.15	7.6 ± 0.9	204 ± 19	1.45 ± 0.15
	+Co	7.42 ± 0.98	13.4 ± 1.3	430 ± 47	2.60 ± 0.28
rib83	–	0.06 ± 0.02	2.3 ± 0.6	59 ± 5	1.06 ± 0.13
	+ Co	0.13 ± 0.03	5.9 ± 0.8	90 ± 8	1.90 ± 0.22

Exponentially growing cells were harvested, washed with water and inoculated (1 mg/ml of cell dry weight) into the fresh medium supplemented with cobalt chloride (0.9 mM). The same medium without cobalt was used as a control. For MDA and ferrireductase activity measurements the cells were incubated for 8 h. For RF and iron measurements the cells were incubated for 24 h.

cells (Table 2). Under these conditions, mutant strains *rib80, rib81, hit1* possessed 4–15 fold increase in RF production and 1.5–2 fold increase of cellular iron content. Thus, cobalt treatment exaggerated phenotypic characteristics of these mutants. At the same time, the changes observed in the case of the mutant *rib83*, similarly to the wild-type strain, were significantly lower. RF production by this mutant was very weak, as it was reported earlier (Shavlovsky *et al.* 1989).

Interestingly that cells of the wild type strain grown in cobalt supplemented medium exhibit increased number of mitochondria in addition to increased production of riboflavin and MDA (Fig. 3). Under these conditions, the average fractional volume of mitochondria increased two-fold. Similar morphological changes were reported for mutants *hit1*, *rib80* and *rib81* grown on iron supplemented medium (Fayura *et al.* 2007).

Thus, it could be suggested that the regulation of both RF and iron metabolism in *P. guilliermondii* is

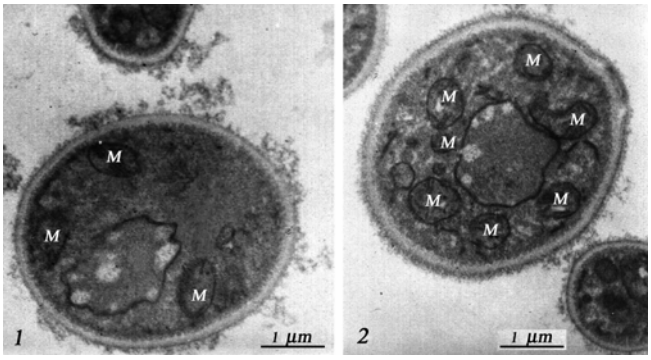

Figure 3. Electron micrographs showing sub-cellular morphology of *P. guilliermondii* wild-type strain grown in the medium supplemented with cobalt chloride (0.9 mM).
Labels are: M – mitochondria; Bar – 1.0 μm.

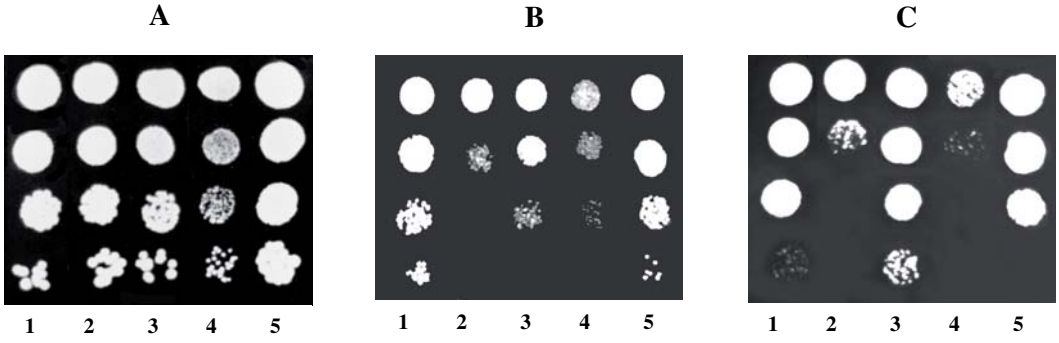

Figure 2. Inhibition of growth of *P. guilliermondii* mutants defective in regulation of RF biosynthesis by Co^{2+} and MV.
1 – L2 (wild type); 2 – *rib80*; 3 – *rib81;* 4 – *hit1*; 5 – *rib83*.
Cultures of the wild-type strain (L2) and the mutants *rib80*, *rib81*, *hit1* and *rib83* were grown aerobically in YPD medium for 24 h. Cells were harvested, washed with water and appropriate serial dilutions were made. 5 μl aliquots were plated onto plates with synthetic media without supplements (A), with 2 mM MV (B) or 2 mM Co^{2+} (C). Plates were incubated at 30 °C for 3 days.

Table 3. Iron accumulation, RF and MDA production by *hit1* mutant and wild type strain of the yeast *P. guilliermondii* grown in media supplemented with different carbon sources. Means ± SD from five experiments are shown.

Strain	Carbon source	RF production (mg/g dry cells)	Iron content (μg/g dry cells)	MDA production (μmol/mg dry cells)
L2	Glucose	0.08 ± 0.01	137.7 ± 17.4	0.69 ± 0.11
	Glycerol	0.09 ± 0.01	99.6 ± 10.7	0.39 ± 0.05
	Dulcitol	0.09 ± 0.01	67.4 ± 7.5	0.25 ± 0.03
hit1	Glucose	1.94 ± 0.26	235.3 ± 28.8	2.20 ± 0.38
	Glycerol	0.84 ± 0.09	98.1 ± 12.4	0.32 ± 0.05
	Dulcitol	0.63 ± 0.08	99.5 ± 11.9	0.41 ± 0.06

Exponentially growing cells were harvested, washed with water and inoculated into fresh medium supplemented with glucose, glycerol or dulcitol. For the MDA measurement, cells were incubated for 8 h. For RF and iron measurements, the same cells were incubated for 24 h.

linked to cellular oxidative state. One may suggest that the increased RF production is an element of antioxidant defense in *P. guilliermondii*. In this case, phenotype of the mutant strains checked would not be so pronounced under conditions at which cells exhibit higher oxidative-stress tolerance, for instance when glucose is substituted by alternative carbon source in the medium (Moradas-Ferreira and Costa 2000, Osorio *et al.* 2003). We observed that the substitution of glucose by glycerol or dulcitol decreased MDA and RF production by the *hit1* mutant significantly (Table 3). In addition, the iron content in the cells was also decreased in both *hit1* and the wild-type strain grown in glycerol or dulcitol-containing media. Thus, the substitution of glucose by these carbohydrates provokes the decrease in the intracellular iron content, which correlates with the drop in MDA and RF production by *P. guilliermondii.*

Discussion

We observed that mutations affecting regulation of RF biosynthesis and iron acquisition cause oxidative stress in *P. guillermondii*. Also, these mutations reduce the viability of the cells in media containing superoxide-generating agents, MV or cobalt ions. It could be suggested that oxidative stress in RF-overproducing mutants is caused by hyper-accumulation or missorting of iron in the cells. It is known that iron overload promotes oxidative stress due to the formation of free radicals in Fenton reactions (Crichton and Charloteaux-Wauters 1987 Meneghini 1997).

Cobalt also is thought to promote oxidative stress but mechanisms behind its action are not clear. In *S. cerevisiae,* cobalt stress selectively induces a number of genes of the so called "iron regulon", coding mainly for iron transport proteins, a response strikingly similar to iron starvation (Leonard *et al.* 1998, Stadler and Schweyen 2002). Perhaps similar mechanisms are involved in the regulation of iron homeostasis in *P. guilliermondii.* In support of this hypothesis, mutants *rib80, rib81, rib83* and *hit1* as well as the wild-type strain possessed significantly higher iron content in the cells when grown in the medium supplemented with cobalt. Under these conditions, all strains tested produced more MDA, possibly due to iron hyperaccumulation, and synthesized more RF (except of *rib83* mutant strain). Mutant strains *rib83* and *rib81* both were resistant to cobalt ions like the wild-type strain (Fig. 2). This observation does not contradict to the statement that mutation *rib81* leads to oxidative stress since it causes increased production of MDA (Fig. 1). Since both cobalt treatment and iron overload cause oxidative stress, it could be hypothesized that the tolerance of the *P. guilliermondii* strains to cobalt depends on their ability to accumulate iron in the cells. Strains that accumulate less iron are more resistant to cobalt ions (Table 2).

We found that the cobalt stress increased number of mitochondria in the cells of the wild type strain. Increased number of mitochondria accompanied with a slight decrease of respiratory activity of the cells was reported recently for *hit1* and *rib80* mutants of *P. guilliermondii* (Fayura *et al.* 2007]. Taken together, these data suggest that cobalt treatment and genetic defects affecting regulation of iron metabolism both caused oxidative stress in *P. guilliermondii.*

It is thought that yeast cells are more resistant to oxidative stress when grown on an alternative carbon source due to glucose-dependent repression of genes coding for enzymes involved in antioxidant defense (Moradas-Ferreira and Costa 2000). We also observed influence of carbon source on cellular oxidative state in *P. guilliermondii.* The *hit1* mutant and the wild-type strain produced less MDA when glucose was substituted for glycerol or dulcitol (Table 2). Under these condi-

tions both strains exhibited reduced iron content and RF production which is in accordance with previous study of *P. guilliermondii* mutant *rib80* (Shavlovsky *et al.* 1990).

Perhaps RF or its derivatives serve as an antioxidant in this yeast, like it was shown for other vitamins in eukaryotic cells (Schroeder and Johnson 1995, Chaudiere and Ferrari-Iliou 1999). In favour to this hypothesis, cells of mutant and wild type *P. guilliermondii* strains contained more RF upon cobalt overload. It should be noted that the major part of synthesized RF was excreted into a culture medium. Physiological role of RF production in response to cobalt overload has to be studied in more detail.

Surprisingly, we found that iron deficiency also led to oxidative stress in *P. guillermondii*. MDA production by all the strains tested was significantly enhanced in iron- deficient medium. One could suggest an inconsistency in the data since both iron deficiency and increased iron content in the cell (in the case of mutants) were accompanied by RF overproduction. It should be pointed out, however, that mutant strains *rib80, rib81* and *hit1* accumulated iron only under iron repletion. At the same time, similarly to the wild-type strain, the cellular iron content was significantly reduced when the mutants were grown in iron- deficient media (Fedorovich *et al.* 1997). Mutant strain cells could contain iron in an inaccessible form. There is an early observation indirectly supporting this view, as cells of *rib80, rib81* and *hit1* strains contain significantly more Fe (III) as compared to the wild-type strain (Fedorovich *et al.* 1997). It could be hypothesized that cells of mutant strains are under conditional iron starvation despite the apparent increase of its total cellular content. We could also assume that they might be defective in an unknown transcription factors and/or factors involved in intracellular iron sensing. In favour of this hypothesis, mutant cells (but not the wild-type cells) exhibited high activity of ferrireductase (Table 1) which is known to be transcriptionally activated in yeast under iron deficiency conditions (Dancis *et al.* 1992, Fedorovich *et al.* 1999, Rutherford *et al.* 2003). We can also suppose that iron-sulfur clusters rather than the free iron are involved in regulation of iron acquisition and RF biosynthesis in *P. guillermondii.* It is known that activation of the iron regulon by the yeast Aft1/Aft2 transcription factors depends on mitochondrial iron-sulfur protein biogenesis and not on cytosolic iron content (Rutherford *et al.* 2005). It is very likely that similar mechanisms function in *P. guillermondii.* Rapid degradation of iron-sulfur clusters under oxidative stress conditions (Hentze *et al.* 2004) could be implied for explanation of presented data on association oxidative stress response and coordinated regulation of RF biosynthesis and iron metabolism in *P. guilliermondii.*

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