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Iron uptake by the yeast *Pichia guilliermondii*. Flavinogenesis and reductive iron assimilation are co-regulated processes

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Abstract

Pichia guilliermondii cells overproduce riboflavin (vitamin B2) in responce to iron deprivation. The increase in ferrireductase activity in iron-starved *P. guilliermondii* cells correlated with the increase in flavin excretion. As in *Saccharomyces cerevisiae*, a typical b-type cytochrome spectrum was associated with the plasma membrane fraction *of P. guillermondii* and the cell ferrireductase activity was strongly inhibited by diphenylene-iodonium, an inhibitor of flavoproteins, in both yeasts. Mutants of *P. guilliermondii* with increased ferrireductase activity were selected for further investigation of the relationship between iron reduction/uptake and flavin production. The obtained mutation has been called *hit* (high iron transport). A *hit* mutant with a single recessive mutation showed the following phenotype: high ferrireductase activity, increased rate of iron uptake and elevated flavinogenic activity. Cu(II) (50 μm) strongly inhibited the growth of the *hit* mutant compared to the wild-type. The mutant cells grown in copper-supplemented medium (5–25 μm) showed an increase of the ferrireductase activity (up to 2–3 fold). The copper content of the mutant cells grown under these conditions was also higher (1.5–2 fold) than that of the wild-type. The role of the *HIT* gene of *P. guillermondii* in the regulation of iron, copper and flavin metabolisms is discussed.

Abbreviations: TTC, triphenyltetrazolium chloride; BPS, bathophenanthrolin disulfonic acid.

Introduction

Reductive iron uptake is a two-step process by which extracellular ferric chelates are reduced at the cell surface before the iron enters the cell. The reduction step is catalysed by a plasma membrane-bound electron transport system, which is induced under iron deprivation (Lesuisse *et al.* 1987; Dancis *et al.* 1990). This reductive system of iron assimilation has been well characterized in *Saccharomyces cerevisae* (review: Askwith & Kaplan 1998), and we have previously shown that several other fungi, in addition to *S. cerevisiae*, have inducible plama membrane-bound ferrireductase activity (Lesuisse *et al.* 1995). One of them, *P. guilliermondii*, a yeast of industrial interest, takes up iron by a reductive system very similar to that of *S. cerevisiae* (Fedorovich *et al.* 1995), as does *Candida albicans* (Morrissey *et al.* 1996).

The molecular basis of reductive iron uptake in *S. cerevisiae* has been well studied. This yeast has two genes encoding structural components of the plasma membrane reductive system, *FRE1* (Dancis *et al.* 1990, 1992) and *FRE2* (Georgatsou & Alexandraki 1994), and their transcription is regulated by iron and copper. The high-affinity transport of iron into the cell is independent of the reduction step, and involves the formation of a plasma membrane complex between a mulicopper oxidase (Fet3p) (Askwith *et al.* 1994)

and a permease (Ftr1p) (Stearman *et al.* 1996). Thus, the copper which coregulates the expression of *FRE* genes, is also needed for the high affinity iron uptake by the cells. Two other genes are important for the regulation of reductive iron uptake, these are *MAC1*, whose product probably interacts with copper and regulates the expression of *FRE* genes (Jungmann *et al.* 1993), and *AFT1*, whose product interacts with iron and regulates the transcription of most of the genes involved in reductive iron uptake (Yamaguchi-Iwai *et al.* 1995, 1996).

It has been known for some time that there is a link between iron metabolism and flavinogenesis in the eukaryotic cell (Demain 1972). However, the physiological implications of this link have never been determined. *P. guilliermondii* is a flavin overproducer, which makes it a good tool for studying the interactions between iron metabolism and flavinogenesis. The products of at least two genes, *RIB80* and *RIB81*, regulate both iron uptake and flavinogenesis in this yeast (Shavlovsky *et al.* 1992, 1993). The mutants *rib80* and *rib81* have increased flavin production and increased ferrireductase activity (Fedorovich *et al.* 1992). This report shows that a third gene, *HIT*, is also involved in the regulation of iron/copper uptake and of flavinogenesis in *P. guilliermondii.*

Materials and methods

Yeast strains and growth conditions

The *P. guilliermondii* strains used were: ATCC9058 (wild-type), L2 (*hisX*, MAT−), L4 (*cysX*, MAT+), RG104 (*rib1*, *hisX*, MAT−), *RIB80*-1026-7 (*RIB80*, *metX*, MAT+), *RIB81*-131-6 (*RIB81*, *hisX*, MAT+). The *S. cerevisiae* strain used as a reference was S150-2B (*ura3*, *his4*, *leu2*, *trp1*, MATα). Unless specified, the yeasts were grown on complete YPG medium, or on synthetic medium containing (per liter): 20 g sucrose, 3 g $(NH_4)2SO_4$, 0.5 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.2 g $CaCl_2{\cdot}6H_2O$, 1.5 mg $FeSO_4$, 2 mg biotin, 0.06 mg H_3BO_3, 0.04 mg $CuSO_4{\cdot}5H_2O$, 0.05 mg $MnSO_4{\cdot}7H_2O$, 0.12 mg $(NH_4)6Mo_7O_{24}{\cdot}4H_2O$, 0.3 mg $ZnSO_4{\cdot}7H_2O$. The cells were grown in Erlenmeyer flasks on a gyro-shaker (200 rpm) at 30 °C.

Ferrireductase activity and iron/copper uptake

The ferrireductase activity of washed resting cells was measured spectrophotometrically as previously described (Lesuisse *et al.* 1987; Fedorovich *et al.* 1992) with different ferric chelates (0.2 mM) as substrate, in 50 mM phosphate buffer (pH 5.5) containing 2% glucose. The cells were incubated at 30 °C for 15 min with agitation (200 rpm) before the iron and the iron-trapping reagent (2,2′-bipyridyl or bathophenantroline disulfonate) were added. Iron uptake was measured in 50 mM citrate buffer (pH 6.5) containing 5% glucose, using ^{55}Fe as iron source. The cells were harvested in exponential growth phase, washed with distilled water and resuspended (1 mg wet weight/ml) in the citrate-glucose buffer. After 3 min preincubation at 30 °C, iron was added as 2 μM Fe(III)-citrate (1:20) and the incubation was continued for 15 min. The reaction was stopped by adding an excess (1 mM) of cold ferric citrate and the cells were then washed on a filter with 10 ml ice-cold synthetic medium. The radioactivity on the filters was counted by liquid scintillation.

Quantitative estimation of the total iron and copper contents of the cells was carried out by Roentgen fluorescence method (Iida & Gohshi 1991). Riboflavin was assayed fluorometrically (fluorometer EF-3M).

Mutant isolation and genetic analysis

Mutagenesis was performed by irradiating the cells with UV light to obtain a 10% survival. The cells were plated onto synthetic agar medium containing 40 mg/ml triphenyltetrazolium chloride to get about 100–200 colonies/plate. The colonies with high reductase activity turned red after 2–3 days.

Hybridization, sporulation and random spore analysis were done as previously described (Sibirny *et al.* 1977). Haploid strains of opposite mating types and complementary auxotophies were crossed on acetate medium (1% Na acetate, 0.5% KCl) and replica-plated onto minimum synthetic medium to select the prototrophic diploid strains. Sporulation was done on acetate medium. The spores were selected by selective killing of the vegetative cells with nystatine or 20% ethanol.

Isolation of plasma membranes

Cell fractionation and plasma membrane purification were done as described by Dufour *et al.* (1988) using disruption of cells with glass beads followed by differential centrifugation and acid precipitation (acetic acid) of mitochondrial membranes. Aliquots of plasma membranes were suspended at about 5 mg/ml in 10 mM Tris acetate buffer (pH 7.5) and frozen. Low temperature spectra (−191 °C) of plasma membrane

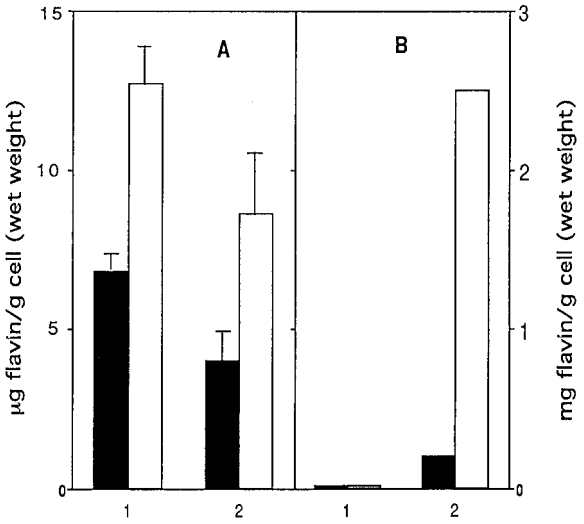

Figure 1. Total flavin associated with the cells (A) or excreted in the medium (B) by *S. cerevisiae* (1) or *P. guilliermondii* (2). The cells were grown to stationary phase in complete medium with no addition (■, iron-sufficient conditions) or in complete medium added with 0.2 mM BPS (□, iron-deficient conditions).

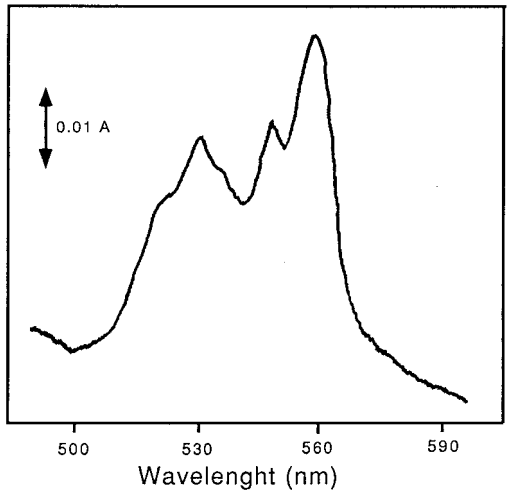

Figure 2. Low-temperature absorbance spectrum of purified plasma membranes from *P. guilliermondii*. The cells (wild-type strain) were grown to late exponential growth phase in complete (YPG) medium.

suspensions were recorded with an optical path length of 1 mm with one sheet of wet filter paper in the reference path. Spectra were corrected for the baseline shift.

Results and discussion

The cell iron status affected the flavin contents of both *S. cerevisiae* and *P. guilliermondii* cells, but only

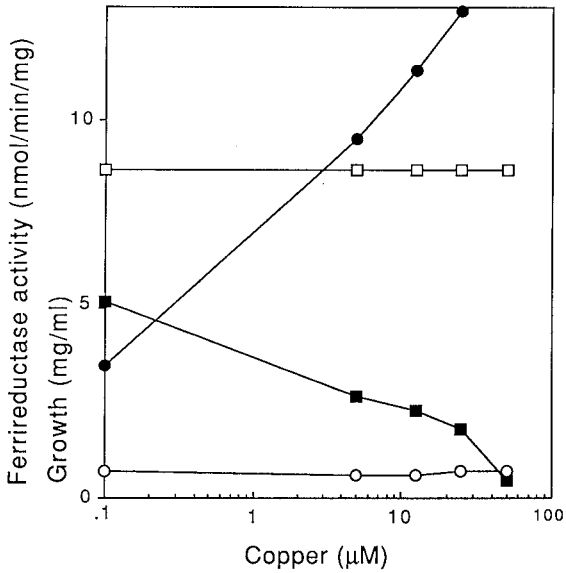

Figure 3. Ferrireductase activity (circles) and growth (squares) of *P. guilliermondii* cells as a function of the concentration of copper in the growth medium. Cells of the wild-type strain (open symbols) and of the *hit* mutant strain (closed symbols) were grown on minimum medium containing different concentration of copper. The cell ferrireductase activity and the growth yield were measured respectively in exponential growth phase and in stationary phase.

the *P. guilliermondii* cells excreted large amounts of flavins into the extracellular medium in response to iron deprivation (Figure 1). The increase in ferrireductase activity in iron-starved *P. guilliermondii* correlated well with the increase in flavin excretion (data not shown). As in *S. cerevisiae* (Lesuisse *et al.* 1996), a typical b-type cytochrome spectrum was associated with the plasma membrane fraction of *P. guillermondii* (Figure 2), and the cell ferrireductase activity was strongly inhibited by diphenylene-iodonium, an inhibitor of flavoproteins, in both yeasts (not shown). Thus, both reductase systems could include two redox centers, involving flavin(s) and heme(s). The need for increased flavin production during iron starvation could be partly due to the flavinic nature of one of the redox centers involved in ferrireductase activity. In this context, heme-deficient *S. cerevisiae* cells are deficient for ferrireductase activity (Lesuisse & Labbe 1989) while heme-deficient *P. guilliermondii* cells have no flavin overproduction when they are starved of iron (Schavlovsky & Laska 1973). However, the amount of flavin produced by iron-starved *P. guillermondii* clearly exceeds any flavin requirement for some redox centers involved in iron metabolism, and it is not clear why massive amounts of flavins are excreted

Table 1. Phenotype of *hit* mutant and wild-type strains

	Wild-type	Mutant
Reduction of TTC (mg/g cell)	1.1	42.4
Ferrireductase activity (nmol/mg cell/min)	0.42	4.35
Iron uptake rate (increase factor)	1	67
Riboflavin excretion (mg/g cell)	0.2	2.2

Table 2. Random spore analysis of hybrids of hit mutant and wild-type strains of *P. guillermondii.* Segregants of group 1 formed red colonies and segregants of group 2 white colonies on TTC-containing medium

	Ferrireductase activity (nmol/mg/min)	Iron uptake rate (increase factor)	Riboflavin excretion (mg/g)
Parental strains			
Wild-type	0.3	1	0.2
hit mutant	5.7	76	2.4
Hybrids	0.4	1.2	0.4
Segregants			
Group 1 (305 clones)	2.7–4	51–79	1.4–2.2
Group 2 (310 clones)	0.4–0.65	1.4–3.3	0.2–0.3

into the extracellular medium under such conditions. The extracellular concentration of riboflavin does not influence the rate of iron uptake (from ferric citrate) by resting cells of *P. guilliermondii* (not shown). We further investigated the relationship between iron reduction/uptake and flavin production, mutants of *P. guilliermondii* with increased ferrireductase activity were selected, and their ability to produce flavins was examined. Some of the mutants obtained had high ferrireductase activity so that various electron acceptors (ferricyanide, various ferric chelates, triphenyltetrazolium, FMN, riboflavin, etc.) were efficiently reduced by the cells as in *S. cerevisiae* (Lesuisse & Labbe 1994). They also had high rates of iron transport when the iron was presented in either the ferric or the ferrous forms. Thus, the increased rate of iron uptake of the mutants was not due to the increased ferrireductase activity. Lastly, they had increased flavinogenic activity. The phenotypic differences between the mutants and wild-type strains are summarized in Table 1. We determined whether the mutant phenotype resulted from the mutation of one or several genes by backcrossing the original isolates with the parental strains. All the heterozygous diploid strains were recessive for all the phenotypes. These diploid strains were then analysed for random spore production on sporulation medium and the phenotypes of the segregants were determined. An example of the results obtained is given in Table 2. Crossing the mutant strains with the wild-type yielded, after sporulation, a segregation ratio of 1/1 for the three phenotypic characteristics of the mutant. Thus, mutants were affected in a single gene, that we called *HIT* (high iron transport). Previous studies (Shavlovsky *et al.* 1985; Babyak *et al.* 1993) have shown that cells mutated in the *RIB80* or *RIB81* genes, two genes involved in the regulation of flavin production, have increased ferrireductase and flavinogenic activities. We checked to see if the *HIT* gene was different from the *RIB80* or the *RIB81* genes by crossing *HIT* mutant strains with *RIB80* and *RIB81* mutants. The ferrireductase activity of various hybrid strains is shown in Table 3. The data presented clearly show that *HIT* is a third gene involved in the regulation of flavinogenesis and reductive iron uptake. *HIT* mutants, and not *RIB80* or *RIB81* mutants, showed an unusual sensitivity to copper. 50 μM of that metal strongly inhibited cell growth (Figure 3). Curiously, a non-lethal concentration of copper in the medium (5–25 μM) resulted in an increase in the ferrireductase activity (Figure 3) of *HIT* mutant cells. The copper content of

Table 3. Ferrireductase activity of various *P. guillermondii* strains

Strain and Genotype	Ferrireductase activity (nmol/mg cell/min)
Wild-type, *RIB80 RIB81 HIT*	0.4
hit mutant, *RIB80 RIB81 hit*	8.2
rib80 mutant, *rib80 RIB81 HIT*	4.6
rib81 mutant, *RIB80 rib81 HIT*	5.4
hybrid *hit* mutant X *rib80* mutant	0.8
hybrid *hit* mutant X rib81 mutant	0.9

the mutant cells was also higher (1.5–2 fold) than that of the wild-type (data not shown). Thus, the *HIT* gene is involved in the regulation of iron, copper and flavin metabolisms. The increased sensitivity to copper of the cells and their increased ferrireductase activity are phenotypical characteristics that are also found in the $MAC1^{up}$ mutants of *S. cerevisiae* (Jungmann *et al.* 1993). In this yeast, the *MAC1* gene encodes a transcriptional activator acting on genes involved in iron and copper metabolisms (*FRE1*, *CTR1*, *CTR3*). The activation domain of Mac1p is repressed by copper (Graden & Winge 1997). In *P. guilliermondii*, Shavlovski & Logvinenko (1982) have proposed that riboflavin biosynthesis is transcriptionally controlled *via* a complex involving Fe(II) and the products of the *RIB80* and *RIB81* genes. The same complex could also regulate genes involved in reductive iron uptake. This would explain why both iron deficiency and mutations in the *RIB80*/*RIB81* genes lead to flavin overproduction and increased ferrireductase activity. We now know that the situation is still more complex, since a third gene, *HIT*, is involved. The product of this gene could interact with copper, as does Mac1p in *S. cerevisiae.* Interactions between the metabolisms of iron and copper have been well characterized in *S. cerevisiae*. A common mechanism for the control of iron/copper acquisition and riboflavin biosynthesis could be essential for the regulation of electron transport systems in the yeast cells. *P. guillermondii* could be a good model to study these mechanisms, as shown by the present work.

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