

The role of PDR13 in tolerance to high copper stress in budding yeast

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Abstract *PDR13* in *Saccharomyces cerevisiae* contributes to drug resistance via sequential activation of *PDR1* and *PDR5*. In this study, we found that a *PDR13* deletion mutant was hypersensitive to Cu^{2+} compared to the wild-type counterpart. The Cu^{2+} tolerance mechanism mediated by Pdr13 does not seem to involve Pdr1 or Pdr5, since mutants harboring a deletion of either the *PDR1* or *PDR5* gene did not show elevated Cu^{2+} sensitivity. Instead, we found that the *PDR13* null mutant could not express *CUP1* or *CRS5* metallothionein at wild-type levels when subjected to high Cu^{2+} stress. These results suggest that Pdr13 contributes to high Cu^{2+} tolerance of *S. cerevisiae*, at least in part, via a mechanism involving metallothionein expression. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: PDR13; High Cu stress; Budding yeast; Metallothionein

1. Introduction

Copper (Cu) is an essential element required by all living organisms. However, high Cu levels are toxic, mainly because Cu catalyzes the synthesis of reactive oxygen species that cause cellular damage [1,2]. It is important that organisms elaborate appropriate mechanisms for Cu uptake [3–5] and detoxification [6,7] to ensure that sufficient Cu is present in the cell to drive essential biochemical processes while preventing its accumulation to toxic levels. To tolerate high Cu levels, yeast cells express metallothioneins (MTs), Cup1 and Crs5 [8–12]. These MTs sequester Cu ions and thus protect yeast cells from high Cu toxicity [8,13].

From our screening of heavy metal-sensitive mutants of *Saccharomyces cerevisiae*, we found that the yeast cell line harboring a *PDR13* (pleiotropic drug resistance) deletion was more sensitive to Cu^{2+} compared to wild-type cells. *PDR13* encodes an Hsp70 homolog that has a role in drug resistance. Pdr13 elevates the function of Pdr1 [14], a transcription factor that up-regulates the expression of *PDR5* and *YOR1*, genes encoding ATP-binding cassette transporters (ABC transporter) involved in drug efflux [15–18]. Pdr13 may have other functions as well, since *PDR13* deletion mutants show elevated expression of several stress-responsive genes, including *CUP1*, *CTT1*, *HSP12* [14] and have increased

sensitivity to high-osmolarity stress [19]. The possible roles of Pdr13 in stress responses have not been intensively investigated. In this study we show that Pdr13 has a role in resistance to high Cu^{2+} stress, and that it functions not via activation of a drug resistance pathway involving Pdr1 or Pdr5, but rather via expression of the MT genes.

2. Materials and methods

2.1. Yeast growth conditions and metal treatment

Yeast cells were grown in either YPD medium (1% yeast extract, 2% Bacto Peptone, 2% dextrose) or in synthetic complete (SC) medium for selection of strains transformed with plasmids. Liquid cultures were seeded to an optical density of 0.5 and grown to exponential phase at 30°C and 200 rpm. Harvested yeast cells were grown on 1/2 YPD or 1/2 SC agar medium (1× YPD or SC broth diluted with an equal volume of 3% microagar) containing the indicated concentrations of CuCl_2 or CdCl_2 .

2.2. Construction of plasmids and yeast strains

The genotypes of the yeast strains used in this study were SEY6210 (*MATα leu2-3,112ura3-52 lys2-801 trp1-Δ901 his3-Δ200 suc2-Δ9 Mel⁻*) and Y800 (*MATα leu2-Δ98 CRY1 ade2-101 his3-Δ200 ura3-52 CAN1 lys2-801 cyh2^R TRP1*). *PDR1*, *PDR5*, *PDR13* null mutants and their isogenic wild-type strain (SEY6210) were kindly provided by Dr. W.S. Moye-Rowley. Yeast cells were transformed using the lithium-acetate method [20]. A 3.2-kb fragment containing the *PDR13* open reading frame was amplified by PCR using gene specific primers PDR13-F (5'-CCGTCGACTATGACTT CCAAAATTGATAAAT-TACAAAG-3') and PDR13-R (5'-CGAATCGAAATGTTGTTC-CATTTGTCTTC-3'). The amplified DNA was cloned into the YC-plac33 (single-copy) and YEplac195 (multi-copy) vectors [21], which were named YCplac-*PDR13* and YEplac-*PDR13*, respectively. YC-plac33 and YEplac195 vectors were kindly provided by Dr. S.H. Leem. For overexpression of *CUP1* gene in *PDR13* null mutant (*Δpdr13*), *CUP1* gene was PCR-amplified using an N-terminus primer 5'-CATCACATAAAATGTTTCAGC-3' and a C-terminus primer 5'-CCGGTACCGACGTTCTCATAATAC-3', cloned into the pYES2 vector (Invitrogen), and then transformed into *Δpdr13* yeast cells. For expression of *CUP1* gene, the cells were grown on 1/2 SC agar medium in which galactose substituted for dextrose.

2.3. Chromosomal deletion of the *PDR13* gene

The chromosomal *PDR13* gene was replaced with *URA3*. The *PDR13*-containing pGEM T-easy vector was cleaved by *Hind*III, which resulted in *PDR13*-flanking sequences from 220 to 1330, and ligated with *URA3* derived from pRS316 [22]. The PCR-amplified *PDR13-URA3* construct was transformed into wild-type cells (Y800) by the lithium-acetate method. *URA3* transformants were selected, and correct integration was confirmed by PCR.

2.4. Measurements of cellular Cu levels

Yeast strains were cultured on YPD agar plates for 1 day at 30°C, transferred to metal-containing 1/2 YPD agar plates, cultured for 1 day at 30°C, and then harvested. They were washed twice in ice-cold 1 mM citrate solution for 30 min each. Cells were briefly centrifuged, collected, and digested with 65% HNO_3 at 200°C for 6 h. Digested samples were diluted with 0.5 N HNO_3 and analyzed using an atomic absorption spectrometer (AAS; SpectrAA-800, Varian).

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Abbreviations: MTs, metallothioneins; PDR, pleiotropic drug resistance; ABC transporter, ATP-binding cassette transporter

2.5. RNA extraction and Northern blot analysis

Total RNA was prepared from exponentially growing cells under conditions indicated in the figure legends. 30 µg of total RNA was separated in a formaldehyde-containing agarose gel and then transferred onto nylon membranes. After UV crosslinking, hybridization was carried out in 6×SSPE, 0.5% SDS, 1% non-fat milk and 50% formamide at 42°C for 16 h with ³²P-labeled gene probes. The hybridized membrane was exposed to a phosphorimager screen (Fuji film) or X-ray film (Kodak). The mRNA expression levels were analyzed by the Mac-BAS image-reader program.

3. Results

3.1. Involvement of *Pdr13* in tolerance to high copper and cadmium levels

The *PDR13* null mutant ($\Delta pdr13$), which grew to similar cell numbers as its isogenic wild-type after three days of growth in control YPD medium (Fig. 1A), showed a more sensitive phenotype than the wild-type strain when grown in 1/2 YPD agar plates containing 4.5 mM or higher concentrations of Cu²⁺ (Fig. 1B). The null mutant was also more sensitive to Cd²⁺ (50 µM) than the wild-type strain (Fig. 1C). However, the mutant did not show elevated sensitivity to Pb²⁺, Ni²⁺ or Zn²⁺ (data not shown). The Cu²⁺-sensitive phenotype of the mutant was consistently strong, and thus we decided to concentrate our studies on the mechanism of Cu²⁺ sensitivity in this mutant.

To confirm that the Cu²⁺-sensitive phenotype of $\Delta pdr13$ was indeed due to the deletion of *PDR13*, we transformed the $\Delta pdr13$ strain with low copy- and high copy-number plasmids carrying the *PDR13* gene. In both cases, $\Delta pdr13$ yeast cells transformed with *PDR13*-containing plasmids recovered

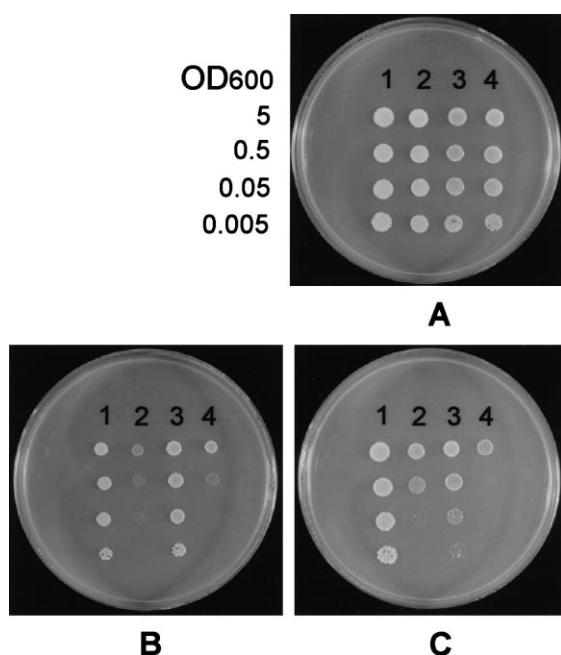


Fig. 1. The $\Delta pdr13$ strain is more sensitive to Cu²⁺ and Cd²⁺ than wild-type cells. Yeast cells were grown in YPD medium to an OD₆₀₀ = 2. Cells were then diluted, spotted onto the indicated metal-containing 1/2 YPD plate, and grown for 3 days. Two strains of yeast were used to test Cu²⁺ sensitivity. Lanes 1 (SEY6210) and 3 (Y800) are wild-type cells, and 2 and 4 are $\Delta pdr13$ strains of SEY6210 and Y800 cells, respectively. (A) Control medium, (B) 4.5 mM Cu²⁺-containing medium, (C) 50 µM Cd²⁺-containing medium.

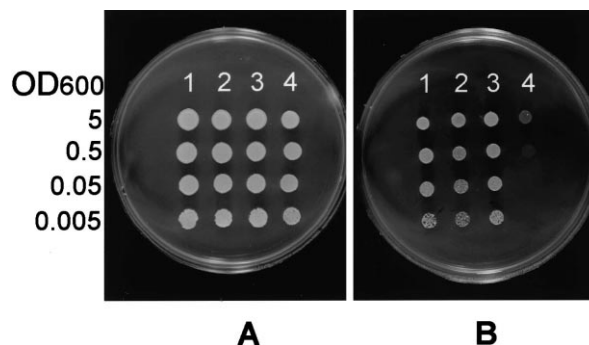


Fig. 2. The Cu²⁺-sensitive phenotype of the $\Delta pdr13$ strain is not caused by defects in the drug resistance pathway mediated by Pdr1. The Cu²⁺ sensitivity was tested as described in Fig. 1. Lane 1: WT, lanes 2, 3, 4: *PDR1*, *PDR5*, *PDR13* null mutants, respectively. (A) Control medium, (B) 4.5 mM Cu²⁺-containing medium.

the phenotype of the wild-type strain, while the same strain of cells transformed with empty vectors failed to do so (data not shown). However, there was no difference in Cu²⁺ resistance between low- and high-copy number *PDR13*-plasmid transformants (data not shown).

3.2. Cu²⁺ and Cd²⁺ elevate *PDR13* mRNA levels

We tested whether Cu²⁺ and Cd²⁺ could induce the expression of the *PDR13* gene in wild-type *S. cerevisiae*. The level of *PDR13* transcript was increased by 2.6 fold by Cu²⁺ and 1.9 fold by Cd²⁺ treatments at concentrations that inhibit growth by 30–40% in YPD-liquid medium (data not shown).

3.3. Cu²⁺ detoxification by *PDR13* is not via the drug resistance pathway

Pdr13 has been reported to elevate the function of Pdr1, a protein that regulates the expression of ABC transporter-encoding genes (*PDR5* and *YOR1*), which are required for normal cycloheximide and oligomycin tolerance [14,17,18]. To examine whether Pdr13 also requires Pdr1 and Pdr5 for high Cu²⁺ tolerance, we tested the phenotypes of the *PDR1* and *PDR5* null mutants in media containing 4.5 mM Cu²⁺. The *PDR1* and *PDR5* null mutants were no more sensitive to high Cu²⁺ concentrations than the wild-type (Fig. 2). These results suggest that the *PDR1* and *PDR5* multi-drug resistance genes are not involved in the Cu²⁺-detoxifying mechanism mediated by Pdr13.

3.4. Cellular Cu content of $\Delta pdr13$ is lower than that of wild-type cells

When Cu²⁺ is accumulated in excess, it is toxic due to its propensity to participate in Fenton-like reactions that lead to the generation of highly reactive hydroxyl radicals that cause cellular damage. We measured Cu levels in wild-type cells and the $\Delta pdr13$ strain to see whether the mutant had higher levels of cellular Cu leading to its more sensitive phenotype.

In contrast to our prediction, a much lower level of cellular Cu was detected in the $\Delta pdr13$ strain than in wild-type cells when exposed to YPD-agar media containing 5 mM Cu²⁺ (Fig. 3). At lower concentrations of Cu²⁺, no consistent difference in cellular Cu²⁺ levels between the two lines was evident. Similarly, the Cu²⁺-sensitive phenotype only appeared when Cu²⁺ concentrations above 4.5 mM were added to the YPD-agar medium, at which point the $\Delta pdr13$ strain became more

sensitive to Cu^{2+} than its wild-type (Fig. 1, our unpublished data).

3.5. Changes in the expression of Cu metallothioneins

Since MTs are important for high Cu^{2+} tolerance, we examined whether deletion of the *PDR13* gene affects MT gene expression in yeast. Cup1 and Crs5 are well-known copper MTs, which bind to cellular free Cu ions and detoxify their toxic effects [11]. When we treated the wild-type and $\Delta pdr13$ strains with 4.5 mM Cu^{2+} , the difference between the 2 strains in the levels of *CUP1* expression increased with time (Fig. 4). After a 15 h treatment with 4.5 mM Cu^{2+} , *CUP1* gene expression in $\Delta pdr13$ cells was much lower than that in wild-type cells. The levels of expression of the *CRS5* gene were also lower in the mutant than in wild-type yeast cells, although its overall expression levels were much lower than those of *CUP1* (data not shown).

3.6. Overexpression of *CUP1* metallothioneins in $\Delta pdr13$ cells

If the reduced expression of Cu metallothionein caused the Cu^{2+} sensitivity of $\Delta pdr13$ cells, the Cu^{2+} -sensitive phenotype may be rescued by overexpression of *CUP1*, a major Cu metallothionein gene. Indeed, $\Delta pdr13$ cells transformed with a multi-copy vector containing *CUP1* grew better than the same line of cells transformed with empty vector on 1 mM Cu^{2+} -containing SC-ura agar medium (data not shown). However, $\Delta pdr13$ cells overexpressing *CUP1* still could not grow as well as the wild-type cells or $\Delta pdr13$ cells complemented with *PDR13* gene (data not shown).

4. Discussion

In this paper, we showed that Pdr13 contributes to detoxification of excess Cu^{2+} in yeast. The $\Delta pdr13$ strain was sensitive to Cu^{2+} and Cd^{2+} (Fig. 1), and complementation of the mutant cells with *PDR13* recovered its Cu^{2+} tolerance to levels comparable to those of wild-type cells. We also observed that Pdr13 is up-regulated by Cu^{2+} and Cd^{2+} (data not shown), and is important for elevated MT gene expression under high Cu^{2+} conditions (Fig. 4), through which it confers tolerance to excess Cu^{2+} .

Previously it has been reported that Pdr13 activates Pdr1 at a post-translational step, resulting in increased expression of some PDR genes, including *PDR5* and *YOR1* [14,23]. When

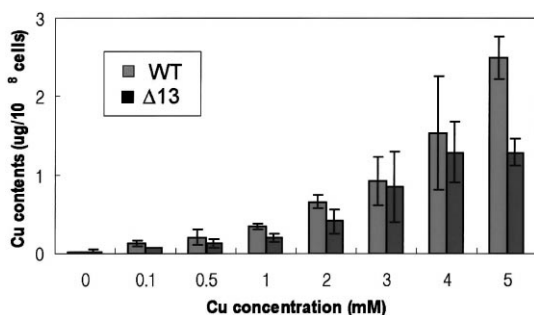


Fig. 3. Cellular Cu levels in the $\Delta pdr13$ mutant and its isogenic wild-type strain. The wild-type (SEY6210) and $\Delta pdr13$ strains were grown on a 1/2 YPD agar plate at various concentrations of Cu^{2+} at 30°C for 24 h. Harvested cells were washed, digested with 65% HNO_3 at 200°C for 6 h, and analyzed for their Cu contents as described in Materials and Methods. Data are the mean values from three different experiments.

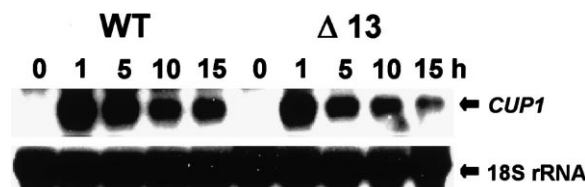


Fig. 4. Expression of *CUP1* in the $\Delta pdr13$ strain was lower than that in wild-type cells. Exponential-phase cultures of wild-type and $\Delta pdr13$ cells were plated on 4.5 mM Cu^{2+} -containing 1/2 YPD plates and incubated at 30°C for the times indicated. Total RNA was isolated as described in the Materials and Methods. Membranes were hybridized with a *CUP1* specific probe labeled with [α -³²P]-dCTP and exposed to X-ray film. 18S rRNA was used as a loading control.

the *PDR1* or *PDR5* gene was deleted, yeast cells became sensitive to cycloheximide and oligomycin [14,24]. However, $\Delta pdr1$ and $\Delta pdr5$ strains did not show a Cu^{2+} -sensitive phenotype (Fig. 2). Therefore, we conclude that the Cu^{2+} resistance mechanism mediated by Pdr13 is different from multidrug resistance pathway mediated by Pdr1 or Pdr5.

To understand the Cu^{2+} -sensitive phenotype of $\Delta pdr13$, we measured the Cu content in wild-type and mutant cells. When grown in medium containing 5 mM Cu^{2+} , the Cu content in $\Delta pdr13$ was about 51% of that in wild-type cells (Fig. 3). This result indicates that tolerance to excess Cu^{2+} mediated by Pdr13 is not caused by the activation of a Cu^{2+} -extruding transporter protein present in the plasma membrane. There are two possible ways to explain why low Cu-containing mutant cells showed higher sensitivity to Cu^{2+} than their wild-type counterparts. First, sequestration of Cu^{2+} into vacuoles is defective in the mutant. Vacuolar function and integrity have been shown to be important for normal Cu^{2+} homeostasis [25], but the mechanism of sequestration of Cu^{2+} into vacuoles is not well understood. Second, mechanisms of Cu^{2+} chelation are defective in the mutant. Cu MTs, Cup1 and Crs5 are well known for their Cu^{2+} chelating activity in budding yeasts. Therefore, we tested whether Cu MTs gene expression was altered in the mutant. Indeed, when cells were grown in 1/2 YPD medium containing 4.5 mM Cu^{2+} , expression of both *CUP1* and *CRS5* MTs were lower in the mutant compared to the control (Fig. 4, our unpublished data). Interestingly, when cells were grown in minimal medium without additional Cu^{2+} , the *PDR13* deletion mutant expressed higher levels of *CUP1* compared to the wild-type control ([14], our unpublished data). Therefore, deletion of the *PDR13* gene adversely affects normal regulation of the MT gene, resulting in aberrant MT expression levels under normal and high Cu^{2+} conditions.

To test whether the reduced expression of Cu MTs contributed to the Cu^{2+} -sensitive phenotype of $\Delta pdr13$, we overexpressed *CUP1* gene in $\Delta pdr13$ cells. *CUP1* gene expression partially rescued the Cu^{2+} -sensitive phenotype of $\Delta pdr13$ (data not shown). Therefore, modulation of Cu MT gene expression is one way in which Pdr13 may induce high Cu^{2+} tolerance. Many other factors may also be involved, including the previously-documented stress-responsive genes [14,19]. In short, our data strongly support a role for Pdr13 in the regulation of MT gene expression, which constitutes a mechanism of tolerance to excess Cu^{2+} in *S. cerevisiae*.

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