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A screen of yeast respiratory mutants for sensitivity against the mycotoxin citrinin identifies the vacuolar ATPase as an essential factor for the toxicity mechanism

Received: 18 November / 27 December 1999

Abstract In countries with a hot climate the mycotoxin citrinin represents a serious problem in fungal food poisioning. In humans the renal system is affected the most and the mitochondrial respiratory chain was identified as a possible sensitive target for this toxin. In addition, citrinin has an antifungal activity that also inhibits the growth of the yeast *Saccharomyces cerevisiae*. So far the precise mode of action and the subcellular targets for citrinin have not been identified. Therefore, we decided to use the model organism yeast for a genetic approach to identify genes that play a role in the sensitivity against this mycotoxin. A large collection of conditional respiratory deficient yeast mutants was screened for sensitivity against citrinin. One special *pet-ts* mutant was identified that exhibited a higher sensitivity against citrinin. The genetic system of yeast allowed the isolation of the respective wild-type gene. This yeast gene encodes the Vph2p subunit that is essential for the correct assembly of the vacuolar ATPase. Isolation of the mutated gene and gene-disruption experiments of *VPH2* and the partially overlapping small *YKL118W* gene verified this finding. The wild-type *VPH2* gene restores all defects of the mutants. In contrast to this, *YKL118W* gave no complementation and the null mutant showed no phenotype. Thereby the yeast vacuolar ATPase was found to be important for the toxic effect of citrinin in yeast cells. The consequences of this finding for the molecular mechanism of citrinin action and its relation to the mitochondrial respiratory chain are discussed.

Key words Citrinin · *Pet* mutants · Mitochondrial biogenesis · Vacuolar ATPase · *YKL118W* disruption · *Saccharomyces cerevisiae*

Introduction

Citrinin is a secondary toxic benzopyran metabolite (Hashimoto and Morita 1957) produced and secreted by some special *Aspergillus* and *Penicillium* species (Haraguchi et al. 1987, 1989). This toxin represents a severe problem especially in countries with a hot climate as under these conditions it is a major source of food poisoning after fungal contaminations (Braunberg 1994; El-Kady et al. 1995; Sinha and Prasad 1996; Prasad 1997). In animals and humans the toxin accumulates in the kidneys and can cause severe renal failure (Berndt 1983, 1998; Braunberg et al. 1992; Chagas et al. 1995a; Bondy and Armstrong 1998; Galtier 1998). Physiological investigations identified different adverse effects on kidney, liver and the gastro-intestinal tract (Siraj et al. 1981; Glahn et al. 1988; Krejci et al. 1996). Renal mitochondria and components of the respiratory chain were identified as possible subcellular targets (Yoneyama and Sharma 1987; Chagas et al. 1995a; Bondy and Armstrong 1998; Galtier 1998). But, so far, the precise mode of action and the subcellular target proteins have not been determined.

Citrinin also exhibits an antifungal activity against other microorganisms (Haraguchi et al. 1989) and initial experiments have shown that growth of the yeast *Saccharomyces cerevisiae* is affected by this toxin (Schappert and Khachatourians 1984; Haraguchi et al. 1987). In addition, differences in sensitivity against citrinin were found for yeast cells that have an intact or defective mitochondrial respiratory chain (Haraguchi et al. 1989).

This finding prompted us to use the yeast *S. cerevisiae* as a model organism (Grivell 1995) for the molecular identification of genes involved in the sensitivity against citrinin. The high amount of citrinin necessary for
sensitivity screens and the problems in the synthesis and isolation of this substance make it difficult, or even impossible, to use citrinin in a large genetic screen for spontaneous yeast mutants with higher resistance against this mycotoxin. Therefore, we decided to use a large collection of conditional respiratory deficient yeast mutants (pet-ts mutants) for the selection of yeast cells with higher sensitivity for this mycotoxin. This pet mutant collection represents 216 different complementation groups (Michaelis et al. 1982) and therefore allows a very efficient screening (Bröhl et al. 1994; Esser et al. 1996; Lisowsky and Michaelis 1988; Ziaja et al. 1993).

In the present study we report the first nuclear yeast mutant that has a higher sensitivity against citrinin, and the isolation and characterisation of the yeast gene that is responsible for this phenotype. With this approach it was found that an intact vacuolar ATPase is not only essential for regulation of the internal pH, cell growth and mitochondrial respiration but is also specifically necessary for resistance against the mycotoxin citrinin. This allows new insights into the toxicity mechanism of citrinin inside the cell.

**Materials and methods**

**Strains**

The *S. cerevisiae* strains employed were: JRY (1nMATa, his4-519, Δleu2, ura3-52), Jasper Nine, unpublished; Sc176 (1nMATa, ade1) and a collection of pet-ts mutants generated in this strain (Michaelis et al. 1982); W303 (MATα, ade2-1/ade2-1, his3-11/his3-11, leu2-3/leu2-3, 112 trp1-1/112 trp1-1, ura3-1/ura3-1, can1-100/can1-100) Thomas and Rothstein (1989); and BMA64-1B (α ade2-1, his3-11, Δleu2-3, 112 trp1α2, ura3-1, can1-100). Strains used and generated in this study included: pet3327a (MATα, ade1, pet3327aα); HAI-5D (MATα his4-519, Δleu2-3, 112 trp1α2, ura3-1, pet3327aα) from a cross of the mutant pet3327a with JRY; and HA2-2B (MATα α, ade2-1, his3-11, Δleu2-3, 112 trp1α2, ura3-1, pet3327aα) from a cross of HAI-5D with BMA64-1B. Disruption mutants for pet2H were generated in a diploid W303, and haploid disrupted cells were isolated after tetrad dissection: VPH2/vph2::KAN (MATα, α, ade2-1/ade2-1, his3-11/his3-11, Δleu2-3/Δleu2-3, 112 trp1-1/112 trp1-1, ura3-1/ura3-1, can1-100/can1-100, VPH2/vph2::KAN); and vph2::KAN (MATα ade2-1, his3-11, Δleu2-3, 112 trp1-1, ura3-1, can1-100, vph2::KAN).

Yeast strains were grown at 23 °C or 36 °C in glucose or glycerol complete medium [2% glucose (or glycerol)] with 1% peptone, 1% yeast extract and appropriate amino acids, adenine or uracil, or in minimal medium (2% glucose, 0.67% yeast nitrogen base, phosphate buffer pH 6.2 and appropriate amino acids, adenine or uracil).

*Escherichia coli* strain DH5-α (Hanahan 1983) was used for cloning experiments and amplification of plasmid DNA.

**Plasmids**

Subcloning of DNA fragments was done in the pUC19 vector (Yanisch-Perron et al. 1985). The yeast genomic library was cloned in the low copy number vector YCp50 (Rose et al. 1987). For complementation studies in yeast the high copy number vector YEp352 (Hill et al. 1986) and the low copy number vector YCp50 (Rose et al. 1987) were used. Cloning for expression studies in yeast were done in the YEp352 plasmid carrying the ADH promoter as a 408-bp HindIII-SalI fragment, as described earlier (Becher et al. 1999).

**PCR amplification**

PCR fragments were amplified according to standard protocols (Innis et al. 1990) using the Taq polymerase kit (TaKaRa) and the following primers:

P1: (1.ATG of VPH2 with SalI)

P2: (stop codon of VPH2 with BamH1)

P3: (1.ATG of YKL118W with SalI)

P4: (stop codon of YKL118W with BamH1)

**Gene disruption experiments**

The VPH2 gene, together with YKL118W, were inactivated according to the one-step gene disruption procedure (Rothstein 1983). For this purpose appropriate PCR fragments of the pet2H cassette (Wach et al. 1994) with 60 bp of the 3’ flanking region from VPH2 and the 3’ end of YKL118W were synthesized and transformed into the diploid yeast strain W303. Correct insertion was checked by hybridisation with specific gene probes.

**Standard techniques**

Plasmid DNA was isolated from *E. coli* by alkaline lysis (Birnboim and Doly 1979). Purification, restriction-enzyme digestion, ligation and analysis of yeast genomic DNA on agarose gels were performed as described (Sambrook et al. 1989). Nucleotide sequences were determined by the biochemical method of Sanger et al. (1977) using *T7* polymerase, *x*[^13]S]dATP and the appropriate primers. Intact yeast cells were transformed after lithium-acetate treatment according to the procedure of Gietz et al. (1995).

**Results**

Sensitivity test of yeast strains against citrinin

First, the general sensitivity of different laboratory yeast strains against citrinin was tested. Wild-type yeast strains, with reference to the pet-ts collection, and yeast strains with selection markers for high efficiency of transformation were incubated on glycerol complete medium with different citrinin concentrations. The respective data are summarized in Table 1. Normal respiratory competent wild-type strains JRY, W303 or Sc167 exhibited normal growth up to a concentration of 300 μg/ml of citrinin in the culture medium. The high concentrations of citrinin that had no effect on the wild-type strains under these conditions made it impractical,