Gentisate and 3-oxoadipate pathways in the yeast *Candida parapsilosis*: identification and functional analysis of the genes coding for 3-hydroxybenzoate 6-hydroxylase and 4-hydroxybenzoate 1-hydroxylase

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The pathogenic yeast *Candida parapsilosis* degrades various hydroxy derivatives of benzenes and benzoates by the gentisate and 3-oxoadipate pathways. We identified the genes *MNX1*, *MNX2*, *MNX3*, *GDX1*, *HDX1* and *FPH1* that code for enzymes involved in these pathways in the complete genome sequence of *C. parapsilosis*. Next, we demonstrated that *MNX1*, *MNX2*, *MNX3* and *GDX1* are inducible and transcriptionally controlled by hydroxyaromatic substrates present in cultivation media. Our results indicate that *MNX1* and *MNX2* code for flavoprotein monooxygenases catalysing the first steps in the 3-oxoadipate and gentisate pathways, respectively (i.e. 4-hydroxybenzoate 1-hydroxylase and 3-hydroxybenzoate 6-hydroxylase). Moreover, we found that the two pathways differ by their intracellular localization. The enzymes of the 3-oxoadipate pathway, Mnx1p and Mnx3p, localize predominantly in the cytosol. In contrast, intracellular localization of the components of the gentisate pathway, Mnx2p and Gdx1p, depends on the substrate in the cultivation medium. In cells growing on glucose these proteins localize in the cytosol, whereas in media containing hydroxyaromatic compounds they associate with mitochondria. Finally, we showed that the overexpression of *MNX1* or *MNX2* increases the tolerance of *C. parapsilosis* cells to the antifungal drug terbinafine.

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INTRODUCTION

Candida parapsilosis is a pathogenic yeast with increasing medical importance, which exhibits a number of peculiar features (Nosek *et al.*, 2009; Trofa *et al.*, 2008; van Asbeck *et al.*, 2009; Weems, 1992). This species is commonly isolated from environmental sources such as soil, plants and plant-associated insects (Carruba *et al.*, 1991; Suh *et al.*, 2008; Trindade *et al.*, 2002), suggesting that it participates in decomposition of decaying plant tissues including hemicellulose and lignin degradation products. This is in line with its ability to ferment D-xylose and to utilize diverse phenol derivatives as the sole carbon sources. Understanding the metabolism of phenolic compounds may be important for biotechnological applications and/or bioremediation. In *C. parapsilosis*, hydroxybenzenes and hydroxybenzoates are degraded via a hydroxyhydroquinone (HHQ, 1,2,4-trihydroxybenzene) variant of the 3-

oxoadipate pathway and a glutathione (GSH)-dependent variant of the gentisate pathway (Fig. 1). In the first pathway, 4-hydroxybenzoate 1-hydroxylase (Mnx1p) decarboxylates 4-hydroxybenzoate to hydroquinone (1,4-dihydroxybenzene), which is a substrate for hydroquinone hydroxylase (phenol 2-monooxygenase, Mnx3p). Both enzymes are NAD(P)H-dependent flavoprotein monooxygenases with broad substrate specificity. 4-Hydroxybenzoate 1-hydroxylase utilizes 4-hydroxybenzoate, resorcinol (2,4-dihydroxybenzoate) and protocatechuate (3,4-dihydroxybenzoate); and hydroquinone hydroxylase uses hydroquinone and resorcinol (1,3-dihydroxybenzene). These substrates are converted to HHQ, which is further metabolized via 2-maleylacetate and 3-oxoadipate to succinate and acetyl-CoA. In the second pathway, 3-hydroxybenzoate is hydroxylated by 3-hydroxybenzoate 6-monooxygenase (Mnx2p) to gentisate (2,5-dihydroxybenzoate), which is converted by gentisate 1,2-dioxygenase (Gdx1p) to 3-maleylpyruvate and subsequently metabolized via 3-fumarylpyruvate to fumarate and pyruvate (Cooper & Land, 1979; Middelhoven, 1993; Middelhoven *et al.*, 1992; van Berkel *et al.*, 1994).

Abbreviations: GSH, glutathione; HHQ, hydroxyhydroquinone.

Two supplementary figures and a supplementary table are available with the online version of this paper.

Fig. 1. Catabolic pathways involved in the degradation of hydroxybenzoates and hydroxybenzenes operating in *C. parapsilosis* cells. Identified genes coding for enzymes catalysing the corresponding reactions are indicated in bold; **MNX1** (CPAG01781), **MNX2** (CPAG03410), **MNX3** (CPAG00562), **GDX1** (CPAG03408), **HDX1** (CPAG05217), **FPH1** (CPAG03407). Note that Eppink *et al.* (1997, 2000) characterized the enzymatic activities of Mnx1p and Mnx3p. Abbreviations of the phenolic compounds are shown in parentheses.

In this study, we addressed two questions: (i) what is the genetic basis of utilization of hydroxybenzenes and hydroxybenzoates in *C. parapsilosis*; and (ii) what is the occurrence of the corresponding genes in related yeast species. We took advantage of the complete genome sequences of *C. parapsilosis* and closely related species

from the 'CTG' clade of hemiascomycetes (Butler *et al.*, 2009; Jeffries *et al.*, 2007; Jones *et al.*, 2004) and searched for candidate genes coding for the enzymes involved in both metabolic pathways mentioned above. We identified open reading frames (ORFs) coding for components of the 3-oxoadipate and gentisate pathways and we named

them *MNX1* (monooxygenase 1; systematic ORF name in the *C. parapsilosis* genome database CPAG01781), *MNX2* (monooxygenase 2; CPAG03410), *MNX3* (monooxygenase 3; CPAG00562), *GDX1* (gentisate dioxygenase 1; CPAG03408), *HDX1* (hydroxyquinol dioxygenase 1; CPAG05217) and *FPH1* (fumarylpyruvate hydrolase 1; CPAG03407). Next, we demonstrated that *MNX1*, *MNX2* and *MNX3* code for flavoprotein monooxygenases with the activity of 4-hydroxybenzoate 1-hydroxylase, 3-hydroxybenzoate 6-hydroxylase and hydroquinone hydroxylase, respectively. Comparative analysis revealed that most of the examined yeast species lack homologues of one or more genes present in *C. parapsilosis*, which is underlined by their inability to grow in media containing the corresponding hydroxybenzenes or hydroxybenzoates as the sole carbon source.

METHODS

Yeast strains and cultivation. *C. parapsilosis* strains CLIB214 (the wild-type strain; identical to CBS604^T), CDU1 (CLIB214 *ura3Δ::FRT/ura3Δ::FRT*; Ding & Butler, 2007), SR23 (=CBS7157; *ade⁻lys4⁻*) and SR23 met-1 (*ade⁻lys4⁻met2⁻*; Nosek *et al.*, 2002) were used for experiments described in this study. *C. albicans* CBS562^{NT}, *C. dubliniensis* CBS7987^T, *C. glabrata* CBS138^T, *C. metapsilosis* CBS10907^T (MCO448^T), *C. orthopsilosis* CBS10906^T (MCO457^T), *C. parapsilosis* CBS604^T, *C. tropicalis* CBS94^T, *Clavispora lusitanae* CBS6936^T, *Debaryomyces hansenii* CBS767^T, *Lodderomyces elongisporus* CBS2605^T, *Pichia guilliermondii* CBS2030^T, *Pichia stipitis* CBS5773^T and *Saccharomyces cerevisiae* L5366 (MATa/*ura3-52/ura3-52*; Gimeno & Fink, 1994) were used in assimilation tests of hydroxyaromatic compounds. Yeasts were cultivated in media listed in Table 1.

Plasmid constructs. The coding sequences of *C. parapsilosis* genes *MNX1*, *MNX2*, *MNX3* and *GDX1* were amplified with or without the termination codon from the genomic DNA of strain CBS604 by using

PCR with the primers listed in Table 2. The PCR products were cloned into pGEM-T Easy (Promega) or pDrive (Qiagen) vectors. The resulting plasmid constructs were then digested with the endonuclease *Xba*I and the DNA fragments coding for investigated proteins were inserted into the *Xba*I site of pBP7 and pBP8 (Kosa *et al.*, 2007). The gene promoters were amplified as PCR products derived 747–1051 bp upstream of the corresponding ORF, phosphorylated with T4 polynucleotide kinase and cloned into the pPK3 vector (Kosa *et al.*, 2007) linearized with the endonuclease *Sma*I. The inserts in all plasmid constructs were verified by DNA sequencing and comparison with the genome sequence of the *C. parapsilosis* reference strain CDC317 (<http://www.sanger.ac.uk/sequencing/Candida/parapsilosis/>). All plasmid constructs are shown in Table 3. The plasmid DNA was transformed into *C. parapsilosis* cells by lithium acetate/polyethylene glycol/carrier DNA protocol (Nosek *et al.*, 2002) or by electroporation (Zemanova *et al.*, 2004).

NADPH oxidation assays. Yeast cells grown in synthetic media (Table 1) were harvested at late exponential phase by centrifugation (10 min, 2500 g at 4 °C), washed with water and resuspended in 5 vols 50 mM Tris/HCl, 5 mM EDTA (pH 8.0). Samples were vortexed with glass beads (0.45–0.5 mm in diameter, 0.8 g ml⁻¹) four times for 1 min with 1 min intermissions on ice. After centrifugation (10 min, 1500 g at 4 °C) the supernatant was transferred to a new Eppendorf tube. Enzymatic activities of Mnx1p, Mnx2p and Mnx3p in cell lysates were measured essentially as described by Eppink *et al.* (1997). Briefly, 250–500 µg protein extract was added to 50 mM potassium phosphate buffer (pH 7.6) containing 0.2 mM NADPH, 10 µM FAD and 1 mM of appropriate substrate. Oxidation of NADPH at 340 nm and 25 °C was continuously monitored by using a Hitachi U-2001 spectrophotometer. The amount of oxidized NADPH min⁻¹ (mg protein)⁻¹ was calculated from the decrease in the absorbance at defined time intervals.

Oxygen consumption measurements. Yeast cells grown in synthetic media (Table 1) were harvested at late exponential phase by centrifugation (10 min, 2500 g at 4 °C), washed twice with water, then with 40 mM potassium phosphate buffer (pH 7.4) and

Table 1. Yeast cultivation media

Agar (2%) was added for solid media. YNB-AA-AS, yeast nitrogen base without amino acids and ammonium sulphate (Difco). Synthetic media (S) were supplemented with 20 µg adenine ml⁻¹ and 50 µg lysine ml⁻¹ for cultivation of strains SR23 and SR23 met-1. Hydroxyaromatic compounds were dissolved in DMSO as 0.5 or 1 M stocks. All percentage values are w/v.

Name	Composition
YPD	1 % yeast extract, 2 % peptone, 2 % glucose
SD	0.17 % YNB-AA-AS, 0.5 % (NH ₄) ₂ SO ₄ , 2 % glucose
SGal	0.17 % YNB-AA-AS, 0.5 % (NH ₄) ₂ SO ₄ , 2 % galactose
SGly	0.17 % YNB-AA-AS, 0.5 % (NH ₄) ₂ SO ₄ , 3 % glycerol
S2OH	0.17 % YNB-AA-AS, 0.5 % (NH ₄) ₂ SO ₄ , 10 mM salicylate
SAsp	0.17 % YNB-AA-AS, 0.5 % (NH ₄) ₂ SO ₄ , 10 mM aspirin
S3OH	0.17 % YNB-AA-AS, 0.5 % (NH ₄) ₂ SO ₄ , 10 mM 3-hydroxybenzoate
S4OH	0.17 % YNB-AA-AS, 0.5 % (NH ₄) ₂ SO ₄ , 10 mM 4-hydroxybenzoate
S2,4diOH	0.17 % YNB-AA-AS, 0.5 % (NH ₄) ₂ SO ₄ , 10 mM resorcinol
S3,4diOH	0.17 % YNB-AA-AS, 0.5 % (NH ₄) ₂ SO ₄ , 10 mM protocatechuate
S2,5diOH	0.17 % YNB-AA-AS, 0.5 % (NH ₄) ₂ SO ₄ , 10 mM gentisate
SCat	0.17 % YNB-AA-AS, 0.5 % (NH ₄) ₂ SO ₄ , 10 mM catechol
SRes	0.17 % YNB-AA-AS, 0.5 % (NH ₄) ₂ SO ₄ , 10 mM resorcinol
SHyd	0.17 % YNB-AA-AS, 0.5 % (NH ₄) ₂ SO ₄ , 10 mM hydroquinone

Table 2. Oligonucleotide primers

Name	Sequence (5'→3')
Mnx1up_XbaI	TCTAGAATGGCAGTTCAAGCACCAT
Mnx1dn_XbaI	TCTAGATTAGCCTGATGCACTTAATGGA
Mnx1dn_XbaI_w/o_Stop	TCTAGAGCCTGATGCACTTAATGGA
Mnx1upProm	TTGTTGGTATAAGCAAAGAGC
Mnx1dnProm	CATTTCTCTGGTGTGTACTTGT
Mnx2up_XbaI	TCTAGAATGAATCTGCTCTCATTT
Mnx2dn_XbaI	TCTAGACTATAACTTAGATTCAAAAGT
Mnx2dn_XbaI_w/o_Stop	TCTAGATAACTTAGATTCAAAAGTACTG
Mnx2upProm	TCATTGTGTTCTTGTGGTAA
Mnx2dnProm	CATGAACCTTATATATAGTTTCATG
Mnx3up_XbaI	TCTAGAATGCCCTCACAAACAAACA
Mnx3dn_XbaI	TCTAGATTATACTGCTAATCTTGGT
Mnx3dn_XbaI_w/o_Stop	TCTAGATACTGCTAATCTTGGTTGAAG
Mnx3upProm	TTATGGTTTGTAGCCCTGTTG
Mnx3dnProm	CATGTTGTTTGTGAAGTTTGGT
Gdx1up_XbaI	TCTAGAATGTCTCCAGTCGAAGCATC
Gdx1dn_XbaI	TCTAGATTAAATTTGCAGCAGTTTGTG
Gdx1dn_XbaI_w/o_Stop	TCTAGAATTTGCAGCAGTTTGTGGAT
Gdx1upProm	CGTAGCTTAAAGCATCTTCTTC
Gdx1dnProm	CATTCTAAATGATTCTTGAGTGA

resuspended in the same buffer. Oxygen consumption was measured at 30 °C in a thermostatically controlled chamber equipped with a Clark electrode. Oxygen depletion by endogenous respiration and after addition of 10 mM phenolic substrates was recorded and analysed by using an Oxygen meter 782 with data analysis module (Strathkelvin Instruments).

Fluorescence microscopy. Intracellular localization of the proteins Mnx1, Mnx2, Mnx3 and Gdx1 was observed in *C. parapsilosis* CDU1 cells transformed with pBP8-*MNX1*, pBP8-*MNX2*, pBP8-*MNX3* and pBP8-*GDX1* plasmids expressing the proteins fused with the enhanced version 3 of green fluorescent protein (yEGFP3). To visualize the fusion proteins, the transformants were cultivated overnight at 28 °C in synthetic media containing various carbon sources and observed by using a BX50 microscope equipped with the

appropriate filter set and digital camera DP70 (Olympus). Mitochondria were counterstained with 25 nM MitoTracker Red CMXRos (Molecular Probes) for 15–60 min.

Bioinformatic analyses. The gene sequences of *C. parapsilosis*, their systematic names and homologues were identified using the BLAST tools at the *C. parapsilosis* and *C. dubliniensis* sequencing sites (<http://www.sanger.ac.uk/sequencing/Candida/parapsilosis/>; <http://www.sanger.ac.uk/sequencing/Candida/dubliniensis/>), *Candida* comparative site (http://www.broadinstitute.org/annotation/genome/candida_group/MultiHome.html), *P. stipitis* v2.0 database (<http://genome.jgi-psf.org/Picst3/Picst3.home.html>) and the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). Syntenic context was examined using the *Candida* gene order browser (<http://cgob.ucd.ie/>; Fitzpatrick *et al.*, 2010).

Table 3. Plasmid constructs

C. parapsilosis CDU1 was the host strain for all pBP7 and pBP8 constructs, and *C. parapsilosis* SR23 met-1 was the host cell for all pPK3 constructs.

Name	Expressed gene
pBP7- <i>MNX1</i>	<i>C. parapsilosis</i> <i>MNX1</i> driven by the <i>GAL1</i> promoter
pBP7- <i>MNX2</i>	<i>C. parapsilosis</i> <i>MNX2</i> driven by the <i>GAL1</i> promoter
pBP7- <i>MNX3</i>	<i>C. parapsilosis</i> <i>MNX3</i> driven by the <i>GAL1</i> promoter
pBP8- <i>MNX1</i>	<i>C. parapsilosis</i> <i>MNX1</i> fused with the yEGFP3 ORF
pBP8- <i>MNX2</i>	<i>C. parapsilosis</i> <i>MNX2</i> fused with the yEGFP3 ORF
pBP8- <i>MNX3</i>	<i>C. parapsilosis</i> <i>MNX3</i> fused with the yEGFP3 ORF
pBP8- <i>GDX1</i>	<i>C. parapsilosis</i> <i>GDX1</i> fused with the yEGFP3 ORF
pPK3-P _{<i>MNX1</i>}	<i>K. lactis</i> <i>LAC4</i> driven by the <i>MNX1</i> promoter
pPK3-P _{<i>MNX2</i>}	<i>K. lactis</i> <i>LAC4</i> driven by the <i>MNX2</i> promoter
pPK3-P _{<i>MNX3</i>}	<i>K. lactis</i> <i>LAC4</i> driven by the <i>MNX3</i> promoter
pPK3-P _{<i>GDX1</i>}	<i>K. lactis</i> <i>LAC4</i> driven by the <i>GDX1</i> promoter

RESULTS AND DISCUSSION

Utilization of hydroxybenzoates and hydroxybenzenes by *C. parapsilosis* and related yeast species

C. parapsilosis does not utilize phenol, catechol (1,2-dihydroxybenzene), salicylate (2-hydroxybenzoate) and 2,3-dihydroxybenzoate. However, it grows on several hydroxy derivatives of benzene and benzoate such as 3-hydroxybenzoate, 4-hydroxybenzoate, gentisate, hydroquinone, protocatechuate, resorcinol and resorcinol (Middelhoven, 1993; Middelhoven *et al.*, 1992). Catabolism of these compounds proceeds either via a HHQ variant of the 3-oxoadipate pathway or a GSH variant of the gentisate pathway (Fig. 1). In contrast with the *C. parapsilosis* type strain CBS604 (=CLIB214), we found that two laboratory strains, SR23 (=CBS7157) and SR23 met-1, do not grow on hydroquinone (data not shown). These strains were mutagenized and we assume that SR23, which is parental to SR23 met-1, may have also acquired a mutation in a component of the 3-oxoadipate pathway.

In order to identify whether both pathways are conserved in other yeasts, we examined the growth of selected species in synthetic media containing hydroxybenzenes or hydroxybenzoates as the sole carbon sources (Fig. 2a). Our results show that, in contrast with *C. parapsilosis*, the other yeasts examined either do not grow (i.e. *C. glabrata*, *C. lusitanae*, *D. hansenii*, *S. cerevisiae*) or utilize only a limited number of tested substrates (i.e. *C. albicans*, *C. dubliniensis*, *C. metapsilosis*, *C. orthopsilosis*, *C. tropicalis*, *L. elongisporus*, *P. guilliermondii*, *P. stipitis*), suggesting that the two pathways are compromised in these species. The 3-oxoadipate pathway appears to be conserved in *C. orthopsilosis* and *C. metapsilosis*, which were originally classified as *C. parapsilosis* groups II and III (Tavanti *et al.*, 2005), respectively. Both species utilize 4-hydroxybenzoate, resorcinol, protocatechuate, hydroquinone and resorcinol. Interestingly, these species do not grow on 3-hydroxybenzoate and gentisate, indicating that they lack a functional gentisate pathway. This pathway appears to be present only in *C. parapsilosis* and *P. stipitis*. Interestingly, none of the examined yeasts grew in media containing 10 mM salicylate as the sole carbon source. Since acetylsalicylate (aspirin) induces apoptosis-like cell death in *S. cerevisiae* (Balzan *et al.*, 2004) and exhibits antifungal properties in a number of yeast species (Leeuw *et al.*, 2007), we analysed whether the absence of growth of *C. parapsilosis* on salicylate is due to its inhibitory effect on the growth or inability of this yeast to utilize salicylate as the sole carbon source (Fig. 2b). We found that *C. parapsilosis*, as well as two other pathogenic species *C. albicans* and *C. glabrata*, are unable to grow in media with salicylate or acetylsalicylate (aspirin) even in combination with glucose or glycerol, suggesting that both hydroxybenzoates are cytotoxic. In addition, we observed that the treatment with salicylate or aspirin also reduces the viability of *C. parapsilosis* in non-growth conditions (data not shown). Moreover, we noticed

that the morphology of *C. parapsilosis* colonies developed from either single cells (dubbed 'clonal colonies') or cell suspensions ('macrocolonies') is a characteristic, substrate-dependent feature (Fig. 2c). This indicates that catabolic degradation of the hydroxyaromatic compounds investigated is associated with global cellular responses, reflected by changes in morphogenesis and/or cell differentiation.

Next, we examined the activities of enzymes catalysing the first steps of the gentisate and 3-oxoadipate pathways. 3-Hydroxybenzoate 6-hydroxylase, 4-hydroxybenzoate 1-hydroxylase and hydroquinone hydroxylase were analysed in protein extracts from *C. parapsilosis* cells cultivated in media containing glucose or phenolic substrate using the NADPH oxidation assay (Fig. 3a). We detected low enzymatic activities in extracts prepared from cells grown in SD medium. However, the activities were ~20- to >100-fold higher in extracts prepared from cells grown in media with hydroxybenzoates or hydroxybenzenes, indicating that the corresponding enzymes are induced on these substrates. In a complementary experiment, we analysed the enzymes mentioned above as well as gentisate dioxygenase, which does not use NAD(P)H as a cofactor. We inferred the enzymatic activities from oxygen consumption of *C. parapsilosis* cells grown in synthetic media differing by the carbon source (Fig. 3b). Using this assay, we detected a substrate-dependent increase in oxygen consumption in cells cultivated in media containing hydroxyaromatic compounds but not in cells grown on glucose or glycerol. For example, cells cultivated on 3-hydroxybenzoate exhibited increased oxygen consumption after addition of 3-hydroxybenzoate (6.6-fold), gentisate (3.6-fold) and protocatechuate (2.6-fold). Similar enzymatic activities were found in cells utilizing gentisate as the carbon source. In contrast, cells grown on 4-hydroxybenzoate (and similarly on hydroquinone) had increased activities with 4-hydroxybenzoate (4.3-fold), catechol (2-fold), resorcinol (3.2-fold), hydroquinone (4.5-fold), resorcinol (1.4-fold) and protocatechuate (4.3-fold). These results indicate that the two pathways are regulated independently and appear to be induced by the corresponding substrate (i.e. the gentisate pathway by 3-hydroxybenzoate and gentisate, and the 3-oxoadipate pathway by 4-hydroxybenzoate and hydroquinone).

C. parapsilosis genes encoding the enzymes of the gentisate and 3-oxoadipate pathways

To identify genes encoding the enzymes involved in metabolism of hydroxybenzenes and hydroxybenzoates, we performed BLASTP searches of the *C. parapsilosis* genome sequence using bacterial protein queries (e.g. NahG, NagIKL, HadC, TbuD and XlnDEF from *Ralstonia/Pseudomonas* species) and the hits were further analysed for the presence of conserved protein domains. These searches revealed two ORFs for homologues of salicylate monooxygenase, i.e. CPAG01781 (*MNX1*) and CPAG03410 (*MNX2*). Since *C. parapsilosis* does not grow on salicylate (see above), we speculated that these ORFs code for

Fig. 2. Utilization of hydroxybenzoates and hydroxybenzenes by *C. parapsilosis* CLIB214 and selected yeast species. (a) *C. parapsilosis* (CANPA), *C. orthopsilosis* (CANOR), *C. metapsilosis* (CANME), *L. elongisporus* (LODEL), *C. tropicalis* (CANTR), *C. dubliniensis* (CANDU), *C. albicans* (CANAL), *D. hansenii* (DEBHA), *P. guilliermondii* (PICGU), *P. stipitis* (PICST), *Clavispora lusitanae* (CLALU), *C. glabrata* (CANGL) and *S. cerevisiae* (SACCE) were grown for 7 days at 28 °C on the indicated media. Note that media were adjusted using NaOH to pH 6.0–6.5 and 0.01 % (w/v) bromothymol blue was used as pH indicator. Except *C. glabrata* and *S. cerevisiae*, the examined species belong to the 'CTG' clade of hemiascomycetes. The phylogenetic relationship among the examined species is indicated. *S. cerevisiae* strain L5366 has been transformed with pYES2/CT plasmid to complement the *ura3-52* mutation and ensure its prototrophy. (b) Aspirin, salicylate and catechol are toxic to *C. parapsilosis* cells. *C. albicans* (top row), *C. parapsilosis* (middle row) and *C. glabrata* (bottom row) were grown on the indicated media for 3 days at 28 °C. In (a) and (b), the yeast cultures were plated in tenfold serial dilutions. (c) Colony morphology of *C. parapsilosis* growing from a single cell (clonal colonies) or a suspension of $\sim 10^5$ cells (macrocolonies) grown for 21 days at 25 °C in synthetic media containing the indicated carbon sources. The solid media on plates shown in (b) and (c) did not contain bromothymol blue and their pH was not adjusted.

Fig. 3. Enzymatic activities inferred from NAD(P)H oxidation (a) and oxygen consumption (b) assays. *C. parapsilosis* CLIB214 cells were grown in the indicated synthetic media and processed for analyses of enzymatic activities (see Methods). (a) Flavoprotein monooxygenase activities ascertained by NADPH oxidation in protein extracts were measured after addition of 0.2% DMSO or 1 mM phenolic substrates, i.e. 3-hydroxybenzoate (3OH), 4-hydroxybenzoate (4OH), resorcinol (2,4diOH), protocatechuic acid (3,4diOH), resorcinol (Res) and hydroquinone (Hyd). (b) Oxygen consumption was analysed after addition of 2% DMSO or 10 mM phenolic substrates, i.e. 3-hydroxybenzoate, 4-hydroxybenzoate, salicylate (2OH), catechol (Cat), resorcinol (Res), hydroquinone, resorcinol, gentisate (2,5diOH) and protocatechuic acid. The assays were performed in three independent experiments with two parallel measurements in each case (bars, mean $\pm$ SEM). The significance of differences between the samples and the control (DMSO) was evaluated by Student's *t*-test (**P* < 0.05).

3-hydroxybenzoate 6-hydroxylase and/or 4-hydroxybenzoate 1-hydroxylase. Although the latter enzyme has been characterized previously (Eppink *et al.*, 1997), both protein products deduced from these ORFs have similar molecular masses of about 50 kDa [52.6 kDa (Mnx1p) versus 49.9 kDa (Mnx2p)] and the sequence analysis alone did not allow identification of the corresponding enzyme unequivocally. Next, we identified CPAG00562 (*MNX3*), coding for a homologue of hydroquinone hydroxylase. Our prediction has been confirmed by the presence of peptide sequences determined from the purified protein (Eppink *et al.*, 2000). Moreover, we found candidate ORFs encoding additional components of the investigated pathways such as CPAG03408 (*GDX1*) and CPAG05217 (*HDX1*). In the case of fumarylpyruvate hydrolase, we found two candidates, CPAG03407 and CPAG04256. Based on the sequence

comparison of deduced protein products we propose that CPAG03407 and CPAG04256 code for fumarylpyruvate hydrolase (*FPH1*) and fumarylacetoacetate hydrolase (*FAA1/FAH1*), respectively (Supplementary Table S1 and Supplementary Fig. S1, available with the online version of this paper). Our search for genes encoding homologues of maleylacetate reductase (*MAR*) and maleylpyruvate isomerase (*MPI*) involved in the 3-oxoadipate and gentisate pathway, respectively, revealed multiple candidate ORFs. However, we did not investigate which of them are involved in the analysed pathways.

The ORFs CPAG03410 (*MNX2*) and CPAG03408 (*GDX1*) appear to be species-specific, as they do not have counterparts in the *C. albicans* genome (Logue *et al.*, 2005). Since our results show that *C. albicans* lacks the gentisate

pathway (Fig. 2a), we assume that *MNX2* codes for 3-hydroxybenzoate 6-hydroxylase. *MNX2* is located within the gene cluster present in the subtelomeric region of the 957 321 bp chromosomal contig corresponding to chromosome V (Supplementary Fig. S2). The cluster consists of ORFs coding for presumed components of the gentisate pathway (*MNX2*, *GDX1*, *FHP1*) and three additional ORFs potentially related to gentisate metabolism, i.e. CPAG03409, CPAR873 and CPAG03406, coding for MFS (major facilitator superfamily) transporter, Gfa1 (glutathione-dependent formaldehyde-activating enzyme) and zinc-finger transcription factor, respectively. Using the *Candida* gene order browser (Fitzpatrick *et al.*, 2010) we found that the cluster CPAG03410–CPAG03406 is partially conserved in *P. stipitis* (PICST55211–PICST30014–PICST41230–PICST65252), which utilizes substrates metabolized in the gentisate pathway (Fig. 2a). This strengthens the idea that monooxygenase encoded by *MNX2* is involved in this pathway. We found that *D. hansenii* also contains two ORFs, DEHA0C01045g and DEHA0C01067g, from this cluster that are orthologues of CPAG03408 (*GDX1*) and CPAG03407 (*FHP1*), respectively. However, the deduced protein product of DEHA0C01045g appears to be truncated (Supplementary Fig. S1d), which explains why this yeast does not grow on gentisate (Fig. 2a).

Our search for homologues of *MNX1*, *MNX2*, *MNX3*, *GDX1*, *HDX1* and *FHP1* revealed their presence in the genome sequences of yeasts belonging to the ‘CTG clade’, although most species do not contain the complete set (Supplementary Table S1). This is in line with their inability to utilize corresponding substrates (Fig. 2a). In contrast, the species outside the ‘CTG clade’ such as *C. glabrata*, *Kluyveromyces lactis*, *S. cerevisiae*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica* lack orthologues of all these genes and hence the corresponding enzymatic pathways.

***MNX1* and *MNX2* code for 4-hydroxybenzoate 1-hydroxylase and 3-hydroxybenzoate 6-hydroxylase, respectively**

Biochemical activities of the protein products encoded by *MNX1* and *MNX2* were identified as follows. Since we observed that chromosomal copies of the examined genes are not induced in the wild-type cells cultivated in SGal medium (data not shown), we cloned corresponding ORFs into the expression vector pBP7 under the *GAL1* promoter. Next, we used the NADPH oxidation assay to detect flavoprotein monooxygenase activities in protein extracts prepared from *C. parapsilosis* transformants cultivated overnight in SGal medium (Fig. 4). The extracts from cells transformed with the pBP7 vector and the pBP7-*MNX3* construct were used as the negative and positive control, respectively. This experiment showed that cells transformed with pBP7-*MNX1* exhibited increased enzymatic activities when 4-hydroxybenzoate and protocatechuate were used as substrates in the assay. In contrast, we observed the highest activity with 3-hydroxybenzoate in the extracts prepared from the pBP7-*MNX2* transformants. In the case of cells expressing hydroquinone hydroxylase (pBP7-*MNX3*) we detected increased activities using resorcinol, hydroquinone and catechol. In all three cases, enzymatic activities detected using other substrates were substantially lower or undetectable. Our results indicate that *MNX1* and *MNX2* code for 4-hydroxybenzoate 1-hydroxylase and 3-hydroxybenzoate 6-hydroxylase, respectively. Importantly, the results obtained with *MNX1* and *MNX3* are consistent with the substrate specificity of the purified enzymes reported by Eppink *et al.* (1997, 2000).

Hydroxyaromatic compounds induce promoters of *MNX1*, *MNX2*, *MNX3* and *GDX1*

Enzymatic activities involved in the gentisate and 3-oxoadipate pathways appear to be regulated independently

Fig. 4. Flavoprotein monooxygenase activities in *C. parapsilosis* transformants expressing *MNX1*, *MNX2* and *MNX3*. *C. parapsilosis* CDU1 cells transformed with pBP7-*MNX1*, pBP7-*MNX2* and pBP7-*MNX3* expressing the examined genes under the control of the *GAL1* promoter were grown overnight at 28 °C in SGal medium, then lysed and assayed for NADPH oxidation activity (see Methods) in the presence of 1 mM phenolic substrates, i.e. salicylate (2OH), 3-hydroxybenzoate (3OH), 4-hydroxybenzoate (4OH), resorcinol (2,4diOH), protocatechuate (3,4diOH), catechol (Cat), hydroquinone (Hyd) and resorcinol (Res). Transformants with the vector pBP7 were used as the control. The experiment was repeated three times and the data were processed in a similar manner to that described in Fig. 3.

and are induced by corresponding hydroxyaromatic substrates (Fig. 3). Therefore, we investigated the gene expression using promoter fusions with β -galactosidase encoded by *K. lactis* gene *LAC4*. We constructed plasmids derived from the pPK3 vector containing yeast β -galactosidase driven by promoters of the examined genes. Subsequently, we analysed β -galactosidase activity in *C. parapsilosis* SR23 met-1 transformants cultivated in synthetic media containing various substrates as the sole carbon sources. Our results show that both pathways are controlled at the level of transcription. The levels of β -galactosidase in cells cultivated in media with glucose and galactose are relatively low. However, we observed increased activities on hydroxyaromatic substrates (Table 4). For example, we detected very strong induction of β -galactosidase in transformants containing pPK3- P_{MNX1} or pPK3- P_{MNX3} . The activity expressed from the *MNX1* promoter was ~2300 times higher in cells grown on 4-hydroxybenzoate than on glucose. Importantly, the promoters of gene pairs *MNX1*-*MNX3* and *MNX2*-*GDX1* appear to be co-regulated depending on the substrate. While the former pair of promoters was highly induced on 4-hydroxybenzoate, protocatechuate and resorcinol, the induction of the latter pair was observed on 3-hydroxybenzoate and gentisate. This is in line with our conclusion that *MNX1* and *MNX2* are involved in the 3-oxoadipate and gentisate pathways, respectively.

Enzymes of the gentisate and 3-oxoadipate pathways differ by intracellular localization

We expressed recombinant proteins Mnx1p, Mnx2p, Mnx3p and Gdx1p tagged at their C-termini with yEGFP3 to identify their intracellular localization. First, we prepared a set of plasmid constructs derived from the pBP8 vector. These constructs contain corresponding ORFs lacking the terminal codon fused in-frame with the sequence coding for yEGFP3. Next, we transformed the plasmids into *C. parapsilosis* CDU1 cells and cultivated the transformants in synthetic media. Using fluorescence microscopy we observed the fusion proteins Mnx1-yEGFP3 and Mnx3-yEGFP3 in the cytosol. In contrast, we found that localization of Mnx2-yEGFP3 and Gdx1-yEGFP3 depends on the corresponding substrate present in the medium. In SD medium, we detected both proteins in the cytosol. However, in media containing 3-hydroxybenzoate or gentisate as the sole carbon source, we detected the fusion proteins as discrete spots or foci. Subsequent staining with MitoTracker Red revealed that they co-localize with mitochondria (Fig. 5). This indicates that enzymes of the gentisate pathway are associated with mitochondria in a substrate-dependent manner, although the molecular mechanism controlling intracellular distribution of these proteins remains unknown. Nevertheless, we assume that the gentisate pathway components associate with mitochondria in the wild-type cells, as the expression of corresponding enzymes is induced only in media with hydroxyaromatic substrates. Since Mnx2p and Gdx1p do not have a recognizable mitochondrial import sequence, the

Table 4. Promoter activities of *MNX1*, *MNX2*, *MNX3* and *GDX1* genes were assayed in whole-cell lysates of *C. parapsilosis* SR23 met-1 transformed with corresponding plasmid constructs

The cultures were grown to OD₆₀₀ 1 at 28 °C in the indicated medium supplemented with adenine and lysine. Transformants containing the pPK3 vector lacking a promoter were used as a negative control. β -Galactosidase activity was determined essentially as described by Rose & Botstein (1983). Each value represents the mean $\pm$ SEM from three independent experiments. Note that strain SR23 met-1 does not grow in SHyd medium so we did not test the effect of hydroquinone on the genes expression.

Growth medium	Plasmid construct	Specific β -galactosidase activity (Miller units)
SD	pPK3- P_{MNX1}	1.45 $\pm$ 0.15
	pPK3- P_{MNX2}	1.66 $\pm$ 0.04
	pPK3- P_{MNX3}	13.20 $\pm$ 0.49
	pPK3- P_{GDX1}	1.47 $\pm$ 0.28
SGal	pPK3- P_{MNX1}	0.75 $\pm$ 0.36
	pPK3- P_{MNX2}	1.09 $\pm$ 0.01
	pPK3- P_{MNX3}	17.24 $\pm$ 1.10
	pPK3- P_{GDX1}	1.76 $\pm$ 0.53
S3OH	pPK3- P_{MNX1}	3.33 $\pm$ 0.50
	pPK3- P_{MNX2}	14.78 $\pm$ 0.32
	pPK3- P_{MNX3}	17.32 $\pm$ 0.22
	pPK3- P_{GDX1}	423.13 $\pm$ 37.96
S4OH	pPK3- P_{MNX1}	3412.38 $\pm$ 129.81
	pPK3- P_{MNX2}	3.87 $\pm$ 0.42
	pPK3- P_{MNX3}	5154.72 $\pm$ 196.34
	pPK3- P_{GDX1}	13.23 $\pm$ 0.82
S2,5diOH	pPK3- P_{MNX1}	3.89 $\pm$ 0.37
	pPK3- P_{MNX2}	5.11 $\pm$ 0.54
	pPK3- P_{MNX3}	13.19 $\pm$ 0.37
	pPK3- P_{GDX1}	698.81 $\pm$ 77.99
S3,4diOH	pPK3- P_{MNX1}	1943.89 $\pm$ 122.88
	pPK3- P_{MNX2}	0.62 $\pm$ 0.27
	pPK3- P_{MNX3}	2146.17 $\pm$ 200.03
	pPK3- P_{GDX1}	7.11 $\pm$ 0.78
SRes	pPK3- P_{MNX1}	998.37 $\pm$ 119.07
	pPK3- P_{MNX2}	1.12 $\pm$ 0.06
	pPK3- P_{MNX3}	3083.31 $\pm$ 436.53
	pPK3- P_{GDX1}	5.61 $\pm$ 0.25

question remains whether they are imported into mitochondria or attached to the mitochondrial outer membrane. The mitochondrial localization of the gentisate pathway is also supported by the putative N-terminal mitochondrial import signal found in Fph1p, which acts downstream of Gdx1p in the pathway. Taken together, we conclude that the 3-oxoadipate pathway operates in the cytosol and the gentisate pathway in, or in close proximity to, the mitochondria.

MNX1 and *MNX2* increase the tolerance of *C. parapsilosis* to terbinafine

C. parapsilosis isolates are highly sensitive to the antifungal drug terbinafine (lamisil), an inhibitor of squalene

Fig. 5. Intracellular localization of MnX1p, MnX2p, MnX3p and Gdx1p. *C. parapsilosis* CDU1 transformed with pBP8-MNX1, pBP8-MNX2, pBP8-MNX3 and pBP8-GDX1 expressing the examined proteins tagged with yEGFP3 were grown at 28 °C for 48 h in synthetic media containing the indicated carbon sources and examined by fluorescence microscopy (Olympus BX50). Transformants with the vector pBP8 were used as a control. Mitochondria were stained with MitoTracker Red CMXRos.

epoxidase involved in ergosterol biosynthesis (Ryder *et al.*, 1998). Understanding the possible causes of the drug resistance is therefore important for clinical microbiology. It has been reported that salicylate monooxygenase encoded by the *salA* gene mediates the resistance of *Aspergillus nidulans* to terbinafine (Graminha *et al.*, 2004). These authors proposed that the naphthalene nucleus of terbinafine is degraded to salicylate or a similar product. In bacteria, naphthalene can be metabolized via salicylate and gentisate (Grund *et al.*, 1992). Therefore, we examined

whether *MNX1* and *MNX2* contribute to the survival of *C. parapsilosis* treated with terbinafine. We cultivated *C. parapsilosis* CDU1 cells transformed with pBP7, pBP7-MNX1 and pBP7-MNX2 in SGal media containing increasing amounts of terbinafine for 48 h and observed that cells expressing *MNX1* and *MNX2* under the *GAL1* promoter exhibit increased tolerance to terbinafine (Fig. 6). This suggests that degradation of terbinafine in *C. parapsilosis* proceeds via both 3-oxoadipate and gentisate pathways. We did not observe MnX1p- and/or MnX2p-

Fig. 6. *MNX1* and *MNX2* increase tolerance of *C. parapsilosis* cells to terbinafine. *C. parapsilosis* CDU1 transformed with pBP7, pBP7-MNX1 and pBP7-MNX2 were grown in liquid SGal medium containing terbinafine (0, 1.0, 2.5, 5.0, 7.5 and 10 µg ml⁻¹) for 48 h at 28 °C. The growth yield was determined by measurement of OD₆₀₀. The experiment was performed twice (bars, mean ± SEM). The statistical analysis was performed using the two-factor analysis of variance (ANOVA) test (**P*<0.05).

mediated degradation of salicylate (Fig. 4). This corresponds to the inability of *C. parapsilosis* to use salicylate and catechol as the growth substrates. However, we demonstrate that catechol is utilized as a substrate in the assays shown in Figs 3(b) and 4, presumably due to the activity of hydroquinone hydroxylase, which has low affinity ($K_m=500\text{ }\mu\text{M}$) for this substrate (Eppink *et al.*, 2000). Although in the presence of glucose *C. parapsilosis* wild-type cells do not express *MNX1* and *MNX2* and are susceptible to terbinafine, our experiment demonstrates that the activation of these genes may generate cells tolerant to higher doses of this drug.

Conclusions

C. parapsilosis metabolizes hydroxybenzenes and hydroxybenzoates using inducible gentisate and 3-oxoadipate pathways. We propose that catabolic degradation of these compounds may have a particular role in the physiology of *C. parapsilosis* and could provide a growth advantage in a specific ecological niche. Both pathways can contribute to the survival of yeast cells in an environment enriched in phenolic derivatives (e.g. lignin degradation products) and can potentially mediate the pathogen's tolerance to antifungal treatment with terbinafine. In contrast with *C. parapsilosis*, the range of hydroxyaromatic compounds utilized as the sole carbon sources differs in closely related species. Comparative analysis of the complete genome sequences revealed that most yeast species lack homologues coding for enzymes involved in these pathways. This evokes questions concerning evolutionary and ecological constraints leading to diversity in the catabolism of phenolic compounds observed in the 'CTG' clade species. Further studies should address how these pathways and corresponding genes evolved in hemiascomycetes and whether they were present in their common ancestor and subsequently lost in most lineages or, alternatively, entered the 'CTG' clade relatively recently by horizontal gene transfer.

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REFERENCES

Balzan, R., Sapienza, K., Galea, D. R., Vassallo, N., Frey, H. & Bannister, W. H. (2004). Aspirin commits yeast cells to apoptosis depending on carbon source. *Microbiology* **150**, 109–115.

Butler, G., Rasmussen, M. D., Lin, M. F., Santos, M. A., Sakthikumar, S., Munro, C. A., Rheinbay, E., Grabherr, M., Forche, A. & other authors

(2009). Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* **459**, 657–662.

Carruba, G., Pontieri, E., De Bernardis, F., Martino, P. & Cassone, A. (1991). DNA fingerprinting and electrophoretic karyotype of environmental and clinical isolates of *Candida parapsilosis*. *J Clin Microbiol* **29**, 916–922.

Cooper, B. H. & Land, G. A. (1979). Assimilation of protocatechuic acid and *p*-hydroxybenzoic acid as an aid to laboratory identification of *Candida parapsilosis* and other medically important yeasts. *J Clin Microbiol* **10**, 343–345.

Ding, C. & Butler, G. (2007). Development of a gene knockout system in *Candida parapsilosis* reveals a conserved role for *BCR1* in biofilm formation. *Eukaryot Cell* **6**, 1310–1319.

Eppink, M. H., Boeren, S. A., Vervoort, J. & van Berkel, W. J. (1997). Purification and properties of 4-hydroxybenzoate 1-hydroxylase (decarboxylating), a novel flavin adenine dinucleotide-dependent monooxygenase from *Candida parapsilosis* CBS604. *J Bacteriol* **179**, 6680–6687.

Eppink, M. H., Cammaert, E., Van Wassenaar, D., Middelhoven, W. J. & van Berkel, W. J. (2000). Purification and properties of hydroquinone hydroxylase, a FAD-dependent monooxygenase involved in the catabolism of 4-hydroxybenzoate in *Candida parapsilosis* CBS604. *Eur J Biochem* **267**, 6832–6840.

Fitzpatrick, D. A., O'Gaora, P., Byrne, K. P. & Butler, G. (2010). Analysis of gene evolution and metabolic pathways using the *Candida* Gene Order Browser. *BMC Genomics* **11**, 290.

Gimeno, C. J. & Fink, G. R. (1994). Induction of pseudohyphal growth by overexpression of *PHD1*, a *Saccharomyces cerevisiae* gene related to transcriptional regulators of fungal development. *Mol Cell Biol* **14**, 2100–2112.

Graminha, M. A., Rocha, E. M., Prade, R. A. & Martinez-Rossi, N. M. (2004). Terbinafine resistance mediated by salicylate 1-monooxygenase in *Aspergillus nidulans*. *Antimicrob Agents Chemother* **48**, 3530–3535.

Grund, E., Denecke, B. & Eichenlaub, R. (1992). Naphthalene degradation via salicylate and gentisate by *Rhodococcus* sp. strain B4. *Appl Environ Microbiol* **58**, 1874–1877.

Jeffries, T. W., Grigoriev, I. V., Grimwood, J., Laplaza, J. M., Aerts, A., Salamov, A., Schmutz, J., Lindquist, E., Dehal, P. & other authors (2007). Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. *Nat Biotechnol* **25**, 319–326.

Jones, T., Federspiel, N. A., Chibana, H., Dungan, J., Kalman, S., Magee, B. B., Newport, G., Thorstenson, Y. R., Agabian, N. & other authors (2004). The diploid genome sequence of *Candida albicans*. *Proc Natl Acad Sci U S A* **101**, 7329–7334.

Kosa, P., Gavenciakova, B. & Nosek, J. (2007). Development of a set of plasmid vectors for genetic manipulations of the pathogenic yeast *Candida parapsilosis*. *Gene* **396**, 338–345.

Leeuw, N. J., Swart, C. W., Ncango, D. M., Pohl, C. H., Sebolai, O. M., Strauss, C. J., Botes, P. J., van Wyk, P. W., Nigam, S. & Kock, J. L. (2007). Acetylsalicylic acid as antifungal in *Eremothecium* and other yeasts. *Antonie van Leeuwenhoek* **91**, 393–405.

Logue, M. E., Wong, S., Wolfe, K. H. & Butler, G. (2005). A genome sequence survey shows that the pathogenic yeast *Candida parapsilosis* has a defective *MTLa1* allele at its mating type locus. *Eukaryot Cell* **4**, 1009–1017.

Middelhoven, W. J. (1993). Catabolism of benzene compounds by ascomycetous and basidiomycetous yeasts and yeastlike fungi. A literature review and an experimental approach. *Antonie van Leeuwenhoek* **63**, 125–144.

Middelhoven, W. J., Coenen, A., Kraakman, B. & Sollewijn Gelpke, M. D. (1992). Degradation of some phenols and hydroxybenzoates by

the imperfect ascomycetous yeasts *Candida parapsilosis* and *Arxula adeninivorans*: evidence for an operative gentisate pathway. *Antonie van Leeuwenhoek* **62**, 181–187.

Nosek, J., Adamikova, L., Zemanova, J., Tomaska, L., Zufferey, R. & Mamoun, C. B. (2002). Genetic manipulation of the pathogenic yeast *Candida parapsilosis*. *Curr Genet* **42**, 27–35.

Nosek, J., Holesova, Z., Kosa, P., Gacser, A. & Tomaska, L. (2009). Biology and genetics of the pathogenic yeast *Candida parapsilosis*. *Curr Genet* **55**, 497–509.

Rose, M. & Botstein, D. (1983). Construction and use of gene fusions to *lacZ* (beta-galactosidase) that are expressed in yeast. *Methods Enzymol* **101**, 167–180.

Ryder, N. S., Wagner, S. & Leitner, I. (1998). *In vitro* activities of terbinafine against cutaneous isolates of *Candida albicans* and other pathogenic yeasts. *Antimicrob Agents Chemother* **42**, 1057–1061.

Suh, S. O., Nguyen, N. H. & Blackwell, M. (2008). Yeasts isolated from plant-associated beetles and other insects: seven novel *Candida* species near *Candida albicans*. *FEMS Yeast Res* **8**, 88–102.

Tavanti, A., Davidson, A. D., Gow, N. A., Maiden, M. C. & Odds, F. C. (2005). *Candida orthopsilosis* and *Candida metapsilosis* spp. nov. to replace *Candida parapsilosis* groups II and III. *J Clin Microbiol* **43**, 284–292.

Trindade, R. C., Resende, M. A., Silva, C. M. & Rosa, C. A. (2002). Yeasts associated with fresh and frozen pulps of Brazilian tropical fruits. *Syst Appl Microbiol* **25**, 294–300.

Trofa, D., Gácsér, A. & Nosanchuk, J. D. (2008). *Candida parapsilosis*, an emerging fungal pathogen. *Clin Microbiol Rev* **21**, 606–625.

van Asbeck, E. C., Clemons, K. V. & Stevens, D. A. (2009). *Candida parapsilosis*: a review of its epidemiology, pathogenesis, clinical aspects, typing and antimicrobial susceptibility. *Crit Rev Microbiol* **35**, 283–309.

van Berkel, W. J., Eppink, M. H., Middelhoven, W. J., Vervoort, J. & Rietjens, I. M. (1994). Catabolism of 4-hydroxybenzoate in *Candida parapsilosis* proceeds through initial oxidative decarboxylation by a FAD-dependent 4-hydroxybenzoate 1-hydroxylase. *FEMS Microbiol Lett* **121**, 207–215.

Weems, J. J., Jr (1992). *Candida parapsilosis*: epidemiology, pathogenicity, clinical manifestations, and antimicrobial susceptibility. *Clin Infect Dis* **14**, 756–766.

Zemanova, J., Nosek, J. & Tomaska, L. (2004). High-efficiency transformation of the pathogenic yeast *Candida parapsilosis*. *Curr Genet* **45**, 183–186.

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