**Abstract** In the methylotrophic yeast, *Hansenula polymorpha*, peroxisomes are formed during growth on methanol as sole carbon and energy source and contain the key enzymes for its metabolism, one of the major enzymes being alcohol oxidase (AO). Upon a shift of these cells to glucose-containing medium, peroxisomes become redundant for growth and are rapidly degraded via a highly selective process designated macroexophagy. *H. polymorpha* pdd mutants are disturbed in macroexophagy and hence retain high levels of peroxisomal AO activity upon induction of this process. To enable efficient isolation of *PDD* genes via functional complementation, we make use of the fact that AO can convert allyl alcohol into the highly toxic compound acrolein. When allyl alcohol is added to cells under conditions that induce macroexophagy, *pdd* mutants die, whereas complemented *pdd* mutants and wild-type cells survive. Besides isolating bona fide *PDD* genes, we occasionally obtained *pdd* transformants that retained high levels of AO activity although their allyl alcohol sensitive phenotype was suppressed. These invariably contained extra copies of a gene cluster encoding homologues of *Saccharomyces carlsbergensis* old yellow enzyme. Our data suggest that the proteins encoded by these genes detoxify acrolein by converting it into less harmful components.

**Keywords** Detoxification · *HYE* gene cluster · Methylotrophic yeast · NADPH dehydrogenase · Selective peroxisome degradation

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**Introduction**

When *Hansenula polymorpha* cells are grown in media containing methanol as sole carbon and energy source, peroxisomes are formed that contain the key enzymes involved in methanol metabolism, alcohol oxidase (AO), dihydroxyacetone synthase and catalase (van der Klei et al. 1991; Veenhuis and Harder 1991). However, when methanol-grown cells are shifted to conditions of excess glucose, peroxisomes are rapidly degraded by a highly selective process designated macroexophagy (reviewed by Bellu and Kiel 2002; Abeliovitch and Klionsky 2001). Mutants defective in macroexophagy (*pdd* mutants) have been isolated by chemical mutagenesis (Titorenko et al. 1995). The most straightforward technique to isolate the genes affected in these mutants is by functional complementation of the Pdd phenotype. Usually, transformants are screened using a colorimetric plate assay based on visualisation of AO activity that remains 6 h after induction of macroexophagy. In this assay, 2,2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) is used as a substrate (Titorenko et al. 1995).

*pdd* mutant cells do not degrade peroxisomes and hence retain high AO activities while, in functionally complemented strains, most of the peroxisomal AO has been degraded in the vacuole in the 6-h time course. Therefore, complemented strains do not stain significantly in these plate assays, whereas *pdd* mutants produce a blue-green colour. This assay has allowed the isolation of many mutants with a Pdd phenotype. However, the assay is laborious, and does not allow positive selection of complemented *pdd* mutants.

This prompted us to find a method that allows positive selection of functionally complemented *pdd* mutants. The method is based on an earlier observation that wild-type *H. polymorpha* cells that contain high levels of AO activity cannot grow in the presence of allyl alcohol (AA; Sibyrny et al. 1989). AA is a substrate for AO, which catalyses its conversion into...
acrolein. This highly reactive substance is toxic for cells and therefore the cells die. We argued that this method could potentially discriminate between cells that are impaired in macropexophagy (and thus maintain high AO activity) and wild-type or complemented pdd cells that are susceptible to peroxisome degradation (and thus degrade AO protein). At present, this screen is used for the isolation of putative PDD genes.

However, in addition to isolating actual PDD genes, we consistently isolated plasmids that suppressed the growth defect of pdd cells on plates containing AA but did not complement the actual Pdd phenotype. These plasmids contained a gene cluster encoding three homologues of *Saccharomyces cerevisiae* old yellow enzyme (OYE), a putative NADPH dehydrogenase (Saito et al. 1991). The details of this work are the content of this paper.

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### Material and methods

#### Strains and media

All *H. polymorpha* strains used in this study are derivatives of *H. polymorpha* NCYC495 *leu1.1* (Gleeson and Sudbery 1988). *H. polymorpha* pdd mutants were generated either by chemical mutagenesis (Titorenko et al. 1995) or RALF mutagenesis (van H. *polymorpha* pdd mutations were grown on plates containing 0.67% yeast nitrogen base without amino acids (Difco) and 1.5% granulated agar supplemented with 1% glucose (YNM plates), 0.5% methanol (YNM plates) or 0.4% glucose, together with various concentrations of AA (AA selection plates).

*Escherichia coli* DH5α (Sambrook et al. 1989) was used for plasmid constructions and was cultured on LB medium supplemented with the appropriate antibiotics.

#### DNA procedures

Standard DNA techniques were carried out essentially according to Sambrook et al. (1989). Transformation of *H. polymorpha* was performed as described by Faber et al. (1994). DNA sequencing reactions were performed at BaseClear (Leiden, The Netherlands), using a LiCor automated DNA-sequencer and dye primer chemistry (LiCor, Lincoln, Neb.). For DNA and amino acid sequence analysis, the Clone Manager 5 program (Scientific and Educational Software, Durham, USA) was used. The TBLASTN algorithm (Altschul et al. 1997) was used to screen databases at the National Centre for Biotechnology Information (Bethesda, Md.). Protein sequences were aligned using the CLUSTAL X program (Thompson et al. 1997). The nucleotide sequence of the region comprising HYE1, HYE2 and HYE3 was deposited at GenBank and was assigned accession no. AF486188.

#### AA and AO plate assays

Growth on plates that contained AA was determined as follows. *H. polymorpha* transformants were replica-plated from YND plates and replica-plated to AA selection plates. Growth of colonies was scored 3 days after the transfer. The AO activity plate assay (Titorenko et al. 1995) was used to select functionally complemented *H. polymorpha* pdd transformants.

### Results

#### Positive selection of *H. polymorpha* PDD genes

When *H. polymorpha* cells, pregrown on methanol, are incubated in medium supplemented with AA, peroxisomal AO enzyme activity converts this compound into the highly reactive acrolein, which kills the cells (Izard and Libermann 1978). The use of AA to detect the presence of enzymatically active AO protein was described before, for the isolation of mutants disturbed in AO activity (Sibyrny et al. 1989) and peroxisome biogenesis (Johnson et al. 1999).

We used a variant of this assay to screen large numbers of transformants of *H. polymorpha* pdd mutants for functional complementation. When pdd transformants are replica-plated from methanol-containing (YNM) plates to fresh plates containing glucose and AA (AA selection plates), non-complemented colonies die because of the remaining AO enzyme activity. Wild-type *H. polymorpha* colonies and complemented pdd mutants may survive since, in these strains, the majority of AO enzyme is degraded, along with the peroxisomes. This is illustrated in Fig. 1. Wild-type colonies containing vector pYT3 and Δpdd7 colonies (Komduur et al. 2002) containing a plasmid with the complementing PDD7 gene (pYT3-PDD7), show good growth on AA selection plates. In contrast, Δpdd7 colonies containing the vector pYT3 fail to grow at the same conditions.

We successfully used this method to isolate *H. polymorpha* PDD genes. One example is the PDD4 gene that we isolated by functional complementation of the pdd4-43 mutant (Titorenko et al. 1995). The pdd4-43 mutant
was transformed using a H. polymorpha gene library in vector pYT3 (Tan et al. 1995). Some 5,000 leucine-protoprotrophic transformants were tested on AA selection plates containing 0.08 mM AA, and three colonies appeared to grow after 3 days of incubation at 37 °C. Subsequently, these transformants were analysed using the colorimetric AO plate assay to screen for colonies restored in peroxisome degradation (Titorenko et al. 1995). Indeed, one of the transformants showed true complementation of the Pdd phenotype of the pdd4-43 mutant. The other transformants did not display typical macropexophagy characteristics, as exemplified by the retained high levels of AO activity (data not shown). Currently, we are analysing the role of the PDD4 gene in selective peroxisome degradation in detail. This indicates that the AA selection method in principle provides an easy screening method to isolate PDD genes by functional complementation.

Overexpression of genes encoding homologues of OYE allows growth of H. polymorpha pdd mutants on AA plates

As described above, we observed that the AA selection method also resulted in the isolation of plasmids that suppressed the growth defect of pdd mutants on AA plates, whilst not complementing their Pdd phenotype. We observed this phenomenon infrequently in multiple independent transformation experiments using various pdd strains (data not shown). Analysis of this type of plasmid isolated from mutants pdd9-F52 and pdd11-M indicated that they contained either identical or overlapping genomic H. polymorpha inserts. To understand more as to why these plasmids suppress the growth defect of pdd cells on AA selection plates, we decided to analyse the gene(s) causing this phenotype. Two plasmids containing overlapping fragments were selected (Fig. 2). Subcloning revealed that plasmid pHYE-2.5, containing an approximately 2.5-kb BamHI genomic fragment, allowed slow growth of pdd9-F52 colonies on plates containing 0.1 mM AA, as compared with transformants that contained the original plasmid, pHYE-9 (Fig. 2). This suggested that not the entire DNA region responsible for the suppression phenotype was present on pHYE-2.5. Initial sequencing of this fragment demonstrated that it contained part of a gene cluster of highly similar genes (Fig. 2). Subsequently, we isolated the entire gene cluster that was located on an approximately 4.7-kb genomic region. Partial sequencing upstream and downstream from this region revealed no additional genes belonging to the same family (data not shown). Analysis of the sequence showed that the genomic region contained three highly similar genes (Fig. 2). They encoded proteins with high similarity to OYE, a putative NADPH dehydrogenase from brewer’s yeast (S. carlsbergensis; Saito et al. 1991). An alignment of S. carlsbergensis OYE and the H. polymorpha homologues is presented in Fig. 3; and similarities between the H. polymorpha gene products and with a series of relevant homologues are shown in Table 1. Consistent with the nomenclature used in Kluyveromyces lactis (KYE1; Miranda et al. 1995), we designated the H. polymorpha genes HYE1, HYE2 and HYE3 (Fig. 2; see GenBank accession number AF486188).

Analysis of the primary sequences of HYE1, HYE2 and HYE3 showed that, in these proteins, most residues comprising the co-factor flavin mononucleotide (FMN)-binding site as determined for S. carlsbergensis OYE are conserved (Thr38, Gly73, Gln115, Arg244, Gly325, Asn326, Phe327, Gly346, Gly348, Arg349 in OYE; Karplus et al. 1995). Of the putative active site residues (His192, Asn195, Tyr197, Tyr376 of OYE; Fig. 3; see Karplus et al. 1995), only the two tyrosine residues are conserved in all HYE enzymes. In contrast, in HYE2 and HYE3, Asn195 is not conserved, but is replaced by histidine; and both His192 and Asn195 are converted into non-conserved residues in HYE1 (Fig. 3). The effect of these changes on the activity of the HYE enzymes is unknown.

Overproduction of OYE in both wild-type H. polymorpha and pdd mutants allows growth at enhanced concentrations of AA

To investigate whether (over-) expression of the plasmid-based H. polymorpha HYE gene cluster resulted in a general increase in the ability of H. polymorpha colonies to grow on AA selection plates, we transformed H. polymorpha wild-type, pdd2-1, pdd5-J40 and Δpdd7 cells with plasmids containing the entire HYE gene cluster (pHYE20; see Fig. 2), using the empty vector pYT3 as control. After selection of transformants on YND plates, colonies were replica-plated to YNM plates, grown for 2 days at 37 °C and finally replica-plated to AA selection plates containing 0.08 mM and 0.16 mM AA. Growth was scored 3 days after replica-plating. The results (Table 2) indicate that the plasmid containing the entire HYE gene cluster allowed both
Fig. 3. Sequence alignment of the deduced primary sequences of *H. polymorpha* HYE1, HYE2 and HYE3 with that of *S. carlsbergensis* old yellow enzyme (OYE; PIR-A39495), using the one-letter code. Gaps were introduced to maximise the similarity. Identical and conserved residues are shaded. Active site residues in OYE are indicated by an arrow.

<table>
<thead>
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<th>HYE1</th>
<th>HYE2</th>
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Table 1. Sequence identities between *Hansenula polymorpha* HYE1, HYE2 and HYE3 and their counterparts in other organisms. Protein sequences of *H. polymorpha* HYE1, HYE2 and HYE3 were aligned with putative orthologues of other eukaryotes: *Saccharomyces carlsbergensis* old yellow enzyme (OYE; PIR-A39495), *S. cerevisiae* OYE2 (PIR-A46009) and OYE3 (PIR-A55569), *Kluyveromyces lactis* KYE1 (PIR-S55844), *Candida albicans* oestrogen-binding protein (EBP; PIR-A36990), *Arabidopsis thaliana* 12-oxophytodienoate reductase (OPDA; EMBL-CAA71627) and *Pseudomonas putida* morphinone reductase (MorB; PIR-S64687). Sequence identities (%) between the proteins are indicated.
Table 2. Effect of the expression of the plasmid-based HYE gene cluster in H. polymorpha on growth on allyl alcohol (AA) selection plates. Colonies were grown on methanol-containing media and, after 2 days, shifted to AA selection plates. Growth was scored after 3 days of incubation at 37 °C. + Growth, – no significant growth. Overexpression of the HYE gene cluster allows growth on enhanced concentrations of AA in both pdd mutants and wild-type H. polymorpha

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<th>Strain</th>
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<td>Wild type (pHYE-20)</td>
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<td>Wild type (pYT3)</td>
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<tr>
<td>Δpdd7 (pHYE-20)</td>
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<tr>
<td>pdd2-1 (pHYE-20)</td>
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<tr>
<td>pdd2-1 (pYT3)</td>
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<tr>
<td>pdd5-J40 (pHYE-20)</td>
<td>+</td>
</tr>
<tr>
<td>pdd5-J40 (pYT3)</td>
<td>–</td>
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</table>

mutant and wild-type H. polymorpha colonies to grow on plates containing 0.16 mM AA, a concentration at which wild-type controls containing pYT3 are unable to grow. This indicates that the HYE gene cluster promotes growth of H. polymorpha colonies in the presence of AA.

Discussion

Positive selection of PDD genes by the AA screening method

Here, we describe a positive selection method for the isolation of genes that are involved in selective peroxisome degradation (macropexophagy) in the methylo-trophic yeast H. polymorpha. The method is based on the ability of H. polymorpha cells not affected in macropexophagy to grow on enhanced concentrations of AA after induction of peroxisome degradation. These conditions are lethal for pdd cells, due to the formation of acrolein from AA by the high AO activity that remains in these cells. The AA assay has been used successfully in our group to isolate several PDD genes via functional complementation.

We observed that, in addition to PDD genes, the described method also selects for genes that suppress the growth defect of pdd mutants on AA selection plates, but do not complement the Pdd phenotype of these mutants. We have identified these genes as homologues of OYE which, in H. polymorpha, is encoded by at least three genes present in a gene cluster, which we designated HYE1, HYE2 and HYE3.

The presence of the HYE gene cluster as a set of genes suppressing the growth defect of pdd mutants on AA selection plates may interfere with the isolation of actual PDD genes. This potential drawback is overcome by using the AA assay as an initial screen, followed by an analysis of the few colonies that survive the AA selection method by a subsequent AO activity plate assay. Therefore, suppression by the HYE gene cluster may not represent a major disadvantage of our selection method.

It must be noted that, besides screening for complementation pdd mutants, the described method also allows isolation of novel pdd mutants. Using the recently described RALF gene-tagging technique (van Dijk et al. 2001), we obtained approximately 1,500 transformants and identified 50 colonies that were unable to grow on plates containing 0.08 mM AA after induction of macropexophagy. At least seven of these showed a strongly reduced ability to degrade peroxisomes, as judged from reduced AO degradation patterns (our unpublished data). Thus, the AA method is a powerful tool that can be used to isolate PDD genes by functional complementation and also to identify novel H. polymorpha pdd mutants.

Overproduction of OYE probably suppresses the growth defect on AA by detoxification of acrolein.

OYE was first isolated almost 70 years ago from brewer’s bottom yeast (S. carlsbergensis; Warburg and Christian 1933). It was the first enzyme found to require a cofactor (FMN) for its function (reviewed by Karplus et al. 1995; Schöpfer and Massey 1991). OYE has been described to exhibit NADPH oxidoreductase activity using oxygen as terminal electron acceptor. OYE can also reduce olefinic bonds of α, β-unsaturated ketones and aldehydes via its co-factor FMN (Vaz et al. 1995). Although the physiological function of OYE in yeast is still unknown; functions have been attributed to OYE homologues in other organisms. For instance, in Pseudomonas putida, the homologous enzyme was shown to be an active morphinone reductase, an enzyme involved in the degradation of morphine (French and Bruce 1995). Furthermore, the Arabidopsis thaliana enzyme was shown to contain 12-oxophytodienate (OPDA) reductase activity, which is involved in signalling in plant-defensive reactions (Schaller and Weiler 1997). In the latter study, it was also demonstrated that yeast OYE could reduce OPDA. Our data indicate that over-expression of the HYE gene cluster in H. polymorpha from a plasmid confers enhanced resistance towards higher concentrations of AA. In H. polymorpha, AA is converted by AO into the toxic compound acrolein, which, like morphinone and OPDA, is an unsaturated aldehyde/ketone. We hypothesise that, in H. polymorpha, the HYE gene cluster encodes enzymes that can also reduce α, β-unsaturated aldehydes and therefore may detoxify acrolein by converting it into propionaldehyde, which is less harmful to cells (Slott and Hales 1985). In line with this, the ability of wild-type H. polymorpha cells to grow on plates containing 0.08 mM AA may reflect the net result of a balance between the endogenous detoxifying activity of the HYE gene products and the residual AO activity after induction of macropexophagy. In this manner, the acrolein that is formed under these conditions is effectively...
detoxified by HYE activities. In contrast, pdd mutants retain high AO activity, which promotes acrolein formation to toxic levels. However, artificially enhanced HYE protein levels allow both pdd mutants and wild-type *H. polymorpha* to grow on higher concentrations of AA.

In conclusion, our data suggest that the HYE proteins in *H. polymorpha* and possibly OYE-like proteins in general, have a function in the detoxification of dangerous \( \alpha, \beta \)-unsaturated ketones and aldehydes. In fact, other NADPH dehydrogenases (e.g. thioredoxin reductase, glutathione reductase) are known to have roles in the detoxification of reactive oxygen species (Costa and Moradas-Ferreira 2001; Moradas-Ferreira et al. 1996).

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