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Characterization of the *Hansenula polymorpha* *PUR7* gene and its use as selectable marker for targeted chromosomal integration

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Abstract

The *Hansenula polymorpha* genes encoding the putative functional homologs of the enzymes involved in the seventh and eighth step in purine biosynthesis, *HpPUR7* and *HpPUR8*, were cloned and sequenced. An overexpression vector designated pHIPA4 was constructed, which contains the *HpPUR7* gene as selectable marker and allows expression of genes of interest via the strong, inducible alcohol oxidase promoter. An *ade11* auxotrophic mutant that is affected in the activity of the *HpPUR7* gene product was used to construct strain NCYC495 *ade11.1 leu1.1 ura3*. This strain grew on methanol at wild-type rates (doubling time of approximately 4 h) and is suitable for independent introduction of four expression cassettes, each using one of the markers for selection, in addition to the zeocin resistance marker. It was subsequently used as a host for overproduction of two endogenous peroxisomal matrix proteins, amine oxidase and catalase. Efficient site-specific integration of pHIPA4 and overproduction of amine oxidase and catalase is demonstrated. The expression cassette appeared to be pre-eminently suited to mediate moderate protein production levels. The advantages of pHIPA4 and the new triple auxotrophic strain in relation to the use of *H. polymorpha* as a versatile cell factory or as a model organism for fundamental studies on the principles of peroxisome homeostasis is discussed. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Adenine; Auxotrophic marker; Cell factory; Genetic tool; Methylotrophic yeast

1. Introduction

Methylotrophic yeast species such as *Pichia pastoris* and *Hansenula polymorpha* are attractive hosts for the production of various proteins of scientific or commercial interest (for review, see [1]). The ability of these organisms to grow to high cell densities on relatively inexpensive carbon sources as well as the availability of strong, tightly regulated promoters renders them favorable cell factories. Over the past years, advanced molecular-genetic tools have become available for *H. polymorpha*, including an efficient electrotransformation procedure [2], efficient methods for homologous integration into genomic sequences [3,4], as well as a method for the rapid selection of integrants with various copy numbers [5].

Several selectable markers have been used to enable

selection of *H. polymorpha* transformants. Auxotrophic mutants that are available for this purpose include *leu1.1*, *ura3*, and *trp3*. Vectors carrying either homologous (*LEU1*, *URA3*, and *TRP3*) or heterologous (*LEU2* and *URA3* from *Saccharomyces cerevisiae*, *LEU2* from *Candida albicans*) marker genes were constructed that functionally complement these mutations [6,7]. Hollenberg and co-workers used the *S. cerevisiae* *URA3* marker that poorly complements the *H. polymorpha* *ura3* mutation to obtain high copy number integration of expression cassettes in *H. polymorpha* [8,9]. However, the availability of additional selectable markers is crucial to further increase the potentials of the organism.

The present paper describes the cloning and sequencing of the *H. polymorpha* *PUR7* gene, encoding phosphoribosyl-aminoimidazole-succinocarboxamide synthetase (SAICAR synthetase; EC 6.3.2.6), and the putative *HpPUR8* gene for adenylosuccinate lyase (EC 4.3.2.2). These enzymes perform the seventh and eighth step in the purine biosynthetic pathway, respectively (for review, see [10]).

The *H. polymorpha* *ade11.1* (*pur7*) mutant, which is un-

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able to grow in the absence of adenine, accumulates the typical red intracellular pigment, presumed to be 5-aminoimidazole-4-carboxylic acid ribonucleotide, when grown on media containing limiting amounts of adenine [11,12]. The red color of this mutant provides an easy additional criterion for distinguishing transformants from non-transformed cells and thus the mutant is a useful host strain for genetic manipulation. We describe the construction of a vector carrying the *HpPUR7* gene as a selectable marker that cannot replicate in *H. polymorpha*, and the successful application of this integrative vector for overexpression of two homologous genes, encoding *H. polymorpha* amine oxidase (AMO) and catalase (CAT).

2. Materials and methods

2.1. Strains, media and growth conditions

For cloning purposes, *Escherichia coli* DH5 α was used and grown as described [13]. *H. polymorpha* strain NCYC495 and auxotrophic derivatives [14] were grown at 37°C in YPD medium containing 1% yeast extract, 1% peptone, and 1% glucose, or in synthetic medium [15]. Glucose or methanol was added as a carbon source to a final concentration of 0.5%. For selective growth, cells were plated on YND medium containing 0.67% yeast nitrogen base (Difco, Detroit, MI, USA) and 1% glucose, supplemented with 1.5% agar. When necessary, adenine, leucine, and uracil were added to a final concentration of 30 $\mu\text{g ml}^{-1}$ each.

For biochemical analyses, selected strains were pre-cultured at least three rounds in synthetic medium containing glucose and subsequently shifted to methanol-containing synthetic medium to induce expression of the gene under the control of the alcohol oxidase promoter. Crossing of auxotrophic mutants of *H. polymorpha* was performed using the procedure described by Gleeson and Sudbery [14].

2.2. Molecular biological techniques

Standard recombinant DNA procedures were carried out as described [13]. Restriction and DNA modifying enzymes were obtained from Roche Molecular Biochemicals (Almere, The Netherlands). PCR was performed with *Pwo* DNA polymerase as described by the supplier, using a Perkin-Elmer GeneAmp PCR 2400 thermocycler. Oligonucleotides were synthesized by Baseclear (Leiden, The Netherlands). Genetic manipulations of *H. polymorpha* were performed as detailed before [2,3]. Double-stranded DNA sequencing was carried out using both a LiCor automated DNA sequencer (LiCor, Lincoln, NE, USA) with dye-primer chemistry and an ABI 310 Genetic Analyzer (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands), using dye-terminator chemistry.

Alignments of amino acid sequences were produced us-

ing Clustal_X [16]. The TBLASTN algorithm [17] was used to search the DDBJ/EMBL/GenBank database (release of February, 2001) for protein sequences showing similarity to the putative *HpPUR7* and *HpPUR8* gene products. The sequence data for *HpPUR7* (909 bp) are available from EMBL/GenBank/DDBJ under accession number AY034035, those for *HpPUR8* (1485 bp) under accession number AY033990.

Chromosomal DNA was extracted from cells grown overnight on YPD, as described by Sherman et al. [18], but the procedure included a protein precipitation step using 1.5 M of NaCl prior to DNA precipitation. Southern blot analysis was performed using the ECL direct nucleic acid labeling and detection system, as described by the manufacturer (Amersham Pharmacia Biotech, Little Chalfont, UK).

2.3. Construction of plasmids

To facilitate cloning, an *Asp718* site was introduced upstream of the *HpPUR7* gene and its promoter by PCR, using primer 5'-CAAGGTACCAACACAGATCGCC-3' and the M13/pUC reverse sequencing primer, with as template pHS5-*ADE11*, which fully complements the *ade11* mutant. The resulting 2.1-kb PCR product was cloned as an *Asp718*-*Bam*HI fragment into pBluescript SK⁺ (Stratagene, San Diego, CA, USA), digested with *Asp718* and *Bam*HI, resulting in plasmid pHA1. Subsequently, a 46-bp fragment containing the *Bam*HI-*Sal*I fragment of the multiple cloning site of pBluescript SK⁺ was inserted into the 6.7-kb *Bam*HI-*Sal*I fragment of plasmid pHIPX4-

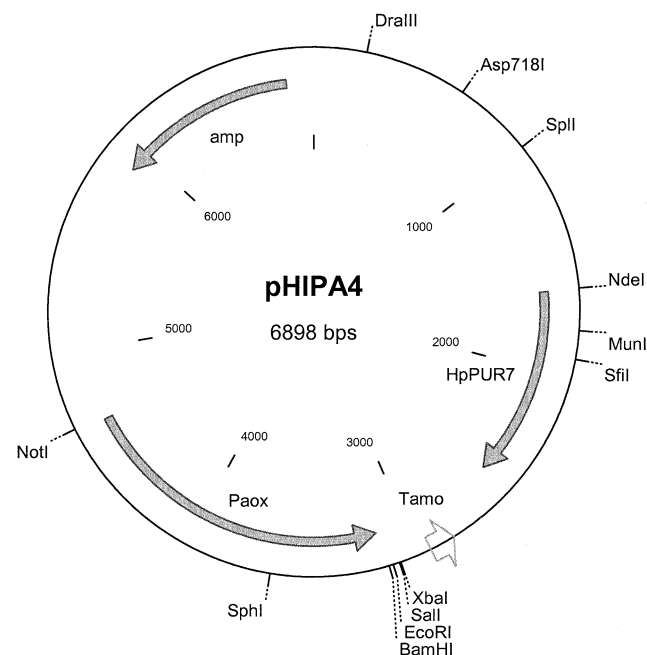


Fig. 1. Physical map of integrative plasmid pHIPA4. Indicated are: amp, ampicillin resistance gene; P_{aox}, *H. polymorpha* alcohol oxidase promoter; HpPUR7, *H. polymorpha* PUR7 gene; T_{amo}, *H. polymorpha* amine oxidase terminator.

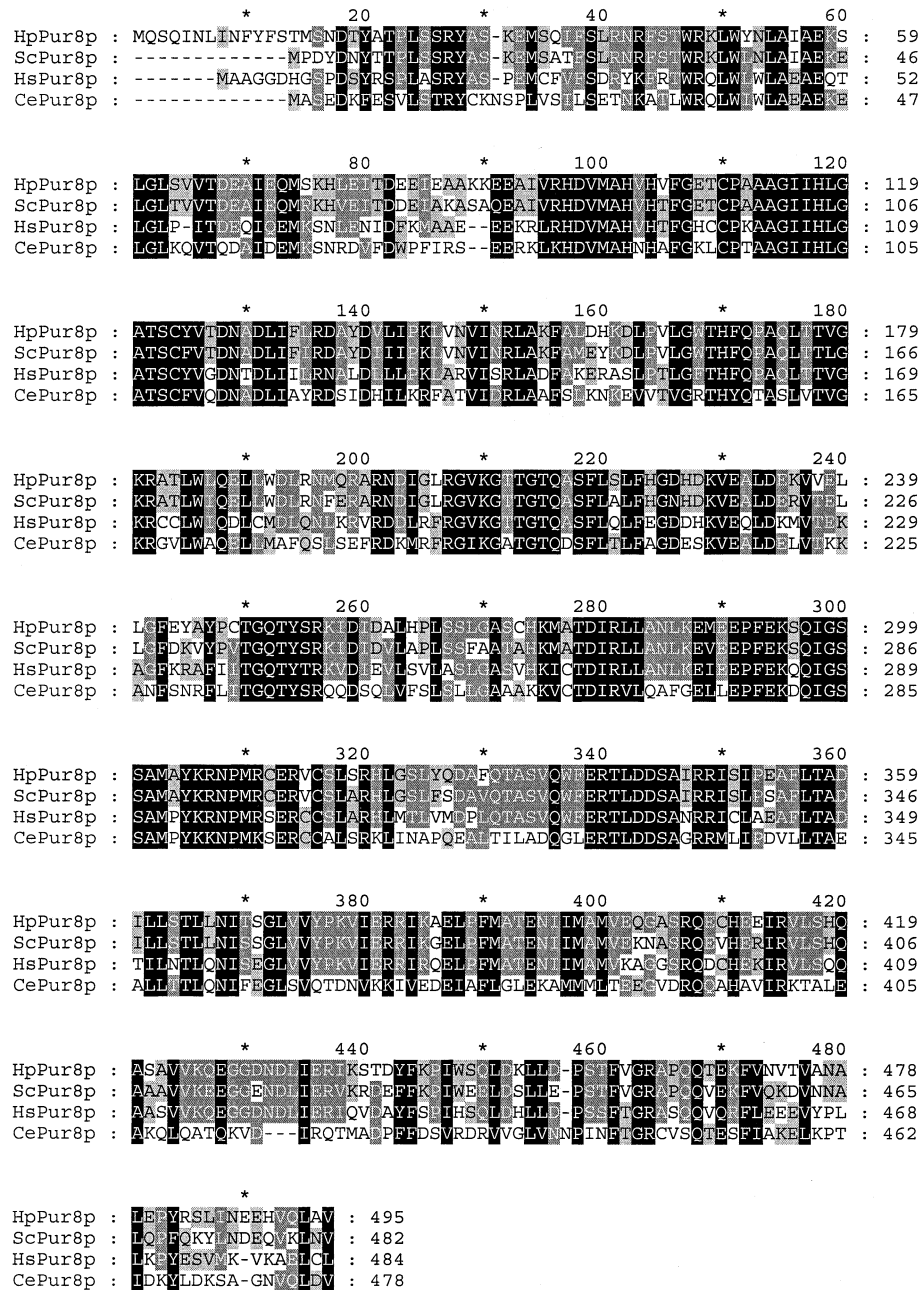


Fig. 2. Alignment of the amino acid sequences of putative Pur8 proteins of *H. polymorpha* (Hp), *S. cerevisiae* (Sc), *H. sapiens* (Hs), and *C. elegans* (Ce). The one-letter code is shown. Gaps are introduced to maximize the similarity. The shading of amino acids indicates degree of conservation: black box: conserved in four out of four sequences; dark gray: conserved in three out of four sequences; light gray: conserved in two out of four sequences. This figure was prepared using the Genedoc program (Nicholas and Nicholas, 1997, <http://www.psc.edu/biomed/genedoc/>).

PAS3 [19], resulting in pHIPX4-C. Finally, the 1.9-kb *NotI*–*BglIII* fragment of pHIPX4-C, containing the alcohol oxidase promoter (P_{AOX}), a short multiple cloning site, and the terminator region of the amine oxidase (*AMO*) gene (T_{AMO}), was cloned into pHA1 digested with *BamHI* and *NotI*. This resulted in plasmid pHIPA4 (see Fig. 1).

A plasmid for overexpression of the *H. polymorpha* *AMO* gene was constructed as follows: the *AMO* gene was cloned as a *NheI* (Klenow-treated)–*SphI* fragment of pGF71 [20] into pHIPA4 digested with *BamHI* (Klenow-treated) and *SphI*, thereby placing *AMO* under control of

P_{AOX} . This resulted in plasmid pHIPA4-AMO. An overexpression vector for the *H. polymorpha* catalase gene (*CAT*) was constructed by cloning a 1.5-kb PCR product obtained using primers 5'-AGA AAG CTT ATG TCA AAC CCC CCT G-3' and 5'-TCT GTC GAC GAT TAT ATT TTG GAT GGA G-3' as a blunt-*SalI* fragment into *BamHI* (Klenow fill-in)–*SalI*-digested pHIPA4. The resulting plasmid, containing the *CAT* gene placed under the transcriptional control of P_{AOX} , was designated pHIPA4-CAT.

To identify the DNA fragment complementing mutant

KL110 [21], a 2.2-kb *Xba*I fragment of the original 6.3-kb complementing fragment was cloned into the *Xba*I site of pHS5. This resulted in pHS5-PUR8, which failed to complement KL110, but was demonstrated by sequencing to contain the putative *HpPUR8* gene.

2.4. Biochemical procedures

Crude extracts were prepared as described [22]. Extracts prepared from an equivalent of 0.3 OD units of cells were subjected to SDS-PAGE and Western blotting. Western blots were decorated using polyclonal rabbit anti-AMO or anti-CAT antiserum. AMO and CAT activities were assayed as described [23,24].

2.5. Electron microscopy

Whole cells were prepared for electron microscopy and immunocytochemistry as described [25]. Immunolabeling was performed on ultrathin sections of uncryl-embedded cells, using specific antibodies against AMO and CAT and gold-conjugated goat anti-rabbit antibodies according to the instructions of the manufacturer (Amersham, Arlington Heights, IL, USA).

3. Results

3.1. Cloning and sequence analysis of the putative *H. polymorpha* *PUR7* and *PUR8* genes

In order to enhance the possibilities for genetic manipulation of *H. polymorpha*, we set out to isolate genes involved in purine biosynthesis to create *ade*⁻ auxotrophs. By coincidence, we discovered a putative *HpPUR8* gene that appeared to be present on the chromosomal insert of a clone from our genomic DNA library of *H. polymorpha* [26]. This particular plasmid functionally complemented the *ass3* mutant that was defective in the assembly of peroxisomal alcohol oxidase [21]. The putative *H. polymorpha* *PUR8* gene encoded a protein of 495 amino acids, with a calculated MW of 55931 Da. Its protein product, HpPur8p, showed high sequence similarity to Pur8 proteins of several eukaryotic organisms, including *S. cerevisiae* (Ade13p, 80% identity, 88% similarity), *Homo sapiens* (63% identity, 76% similarity) and *Caenorhabditis elegans* (42% identity, 60% similarity). A sequence alignment reveals a high degree of conservation throughout the entire sequence (Fig. 2). As expected, *H. polymorpha* Pur8p displays a fumarate lyase signature at position 298–307

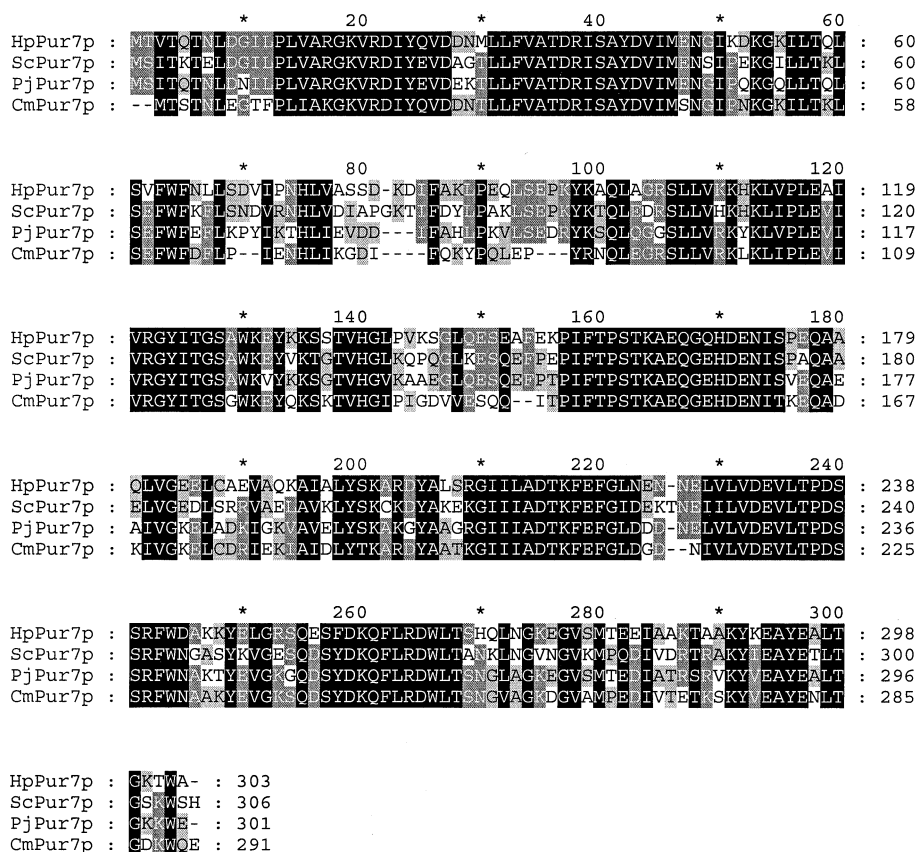


Fig. 3. Alignment of the amino acid sequences of Pur7 proteins of *H. polymorpha* (Hp), *S. cerevisiae* (Sc), *P. jadinii* (Pj), and *C. maltosa* (Cm). For details see legend to Fig. 2. In higher eukaryotes, including man, the corresponding *PUR7* gene encodes a multifunctional protein, the N-terminal half of which is homologous to SAICAR synthetases, the C-terminal half being the catalytic subunit of phosphoribosyl-aminoimidazole-carboxylase (AIR carboxylase, EC 4.1.1.21). Therefore, only the functional homologs of several yeast species are depicted in this figure.

(GSSAMAYKRN). Based on these similarities, we conclude that this protein probably catalyzes the eighth step in purine biosynthesis in *H. polymorpha*.

A screen of the available *H. polymorpha ade* mutants [14] did not result in identification of a mutant phenotype that was functionally complemented by *HpPUR8*. Therefore, we used the reciprocal approach to isolate the gene that functionally complemented selected *ade* mutants, using our genomic DNA library. Strains from two out of the 12 available complementation groups [14], namely *ade11* and *ade12*, displayed the typical red color also observed for *S. cerevisiae ade1* and *ade2* mutants upon growth of cells in adenine-limiting conditions. Of these, *H. polymorpha ade11.1*, which is presumed to accumulate 5-aminoimidazole-4-carboxylic acid ribonucleotide, was selected to isolate the corresponding gene by functional complementation. The original *ade11.1* mutant was backcrossed three times with strain *H. polymorpha* NCYC495 *leu1.1* prior to transformation with a *H. polymorpha* genomic library in pYT3, in order to eliminate possible secondary mutations accumulated during mutagenesis. After transformation and plating on YND containing adenine, leucine-prototrophic colonies were replica-plated on medium lacking adenine to select for adenine prototrophs. A plasmid (designated pYT3-ADE11) containing a 10-kb genomic DNA fragment was rescued from a complemented mutant strain. Initial DNA sequence analysis of the complementing part of the insert revealed the presence of an open reading frame (ORF), encoding a protein of 303 amino acids, with a calculated MW of 33 890 Da. A database search revealed high sequence similarity to several Pur7 proteins of other yeast species, including *Pichia jadinii* (70% identity, 82% similarity), *S. cerevisiae* (Ade1p, 67% identity, 80% similarity) and *Candida maltosa* (63% identity, 78% similarity). Sequence alignment of these proteins reveals a high degree of conservation throughout the entire sequence (Fig. 3). The *H. polymorpha* protein contains two SAICAR synthetase 1 signatures at positions 113–127 (LVPLEAIVRGYITGS) and 212–220 (LADTKFEFG).

3.2. Construction of strains overproducing AMO or CAT

The original *H. polymorpha ade11.1* grew very poorly on methanol (final OD₆₆₀ 1.0 versus 3.5 for wild-type after overnight culturing), a phenomenon that appeared to be due to a partial peroxisomal import defect of catalase protein (data not shown). Therefore, this mutant was backcrossed three times with NCYC495 *leu1.1* to obtain strain *H. polymorpha* NCYC495 *ade11.1 leu1.1*. This strain grew on methanol at wild-type rates and catalase was normally localized in peroxisomes of these cells. This indicates that the observed effects on growth and catalase localization are not linked to the *ade11.1* phenotype, but instead are second-site mutations. To allow full exploitation of the new auxotrophic marker, we crossed this strain with NCYC495 *ura3* to yield strain NCYC495 *ade11.1 leu1.1*

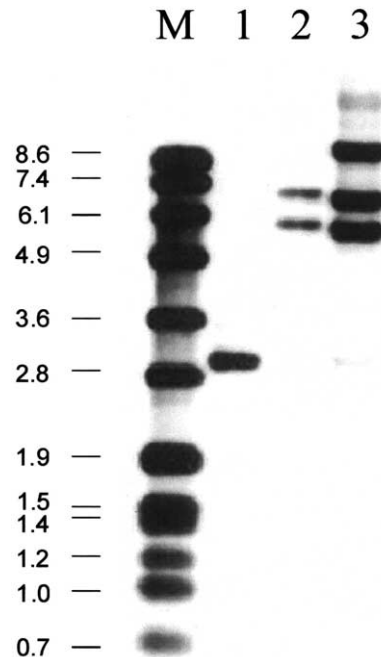


Fig. 4. Southern blot analysis of selected *H. polymorpha* pHIPA4-AMO transformants. Chromosomal DNA was digested using *EcoRI*, electrophoresed and blotted onto Hybond-N⁺ nylon transfer membrane (Amersham Pharmacia Biotech) by capillary blotting. Bacteriophage SPP1 DNA digested with *EcoRI* was used as molecular mass marker (fragment sizes indicated in kb). The blots were hybridized using the 1.5-kb *HindIII/NotI* fragment of pHIPX4 [33] (containing the *P_{AOX}* locus) mixed with a small amount of SPP1 DNA digested with *EcoRI* as a probe. M: molecular mass marker; lane 1: *H. polymorpha* NCYC495 *ade11.1 leu1.1 ura3*; lane 2: NCYC495 *ade11.1 leu1.1 ura3*::[*P_{AOX}*AMO]₁; lane 3: NCYC495 *ade11.1 leu1.1 ura3*::[*P_{AOX}*AMO]₂.

ura3. This strain is suitable for independent introduction of three expression cassettes, each using one of the auxotrophic markers for selection. We constructed vector pHIPA4 to mediate overexpression of genes under control of *P_{AOX}* while using the *ADE11* gene as selection marker in an *ade11.1* background. The application of pHIPA4 to mediate protein overproduction was analyzed using *H. polymorpha* AMO and CAT as model proteins.

For integration at the *AOX* locus, plasmids pHIPA4-AMO and pHIPA4-CAT were linearized by digestion with *SphI* and subsequently used to transform strain *H. polymorpha* NCYC495 *ade11.1 leu1.1 ura3*. The transformation procedure yielded approximately 1000 transformants per μ g of linearized DNA. After 3 days of growth on YND plates supplemented with leucine and uracil, white Ade⁺ colonies appeared on plates containing cells transformed with the linearized plasmids. To confirm proper integration of the constructs at the *P_{AOX}* locus, Southern blotting was performed using chromosomal DNA isolated from selected Ade⁺ transformants. Out of 20 pHIPA4-AMO transformants tested, 15 showed insertion of the construct at the *P_{AOX}* in a single copy (data not shown). Two transformants contained two copies of the construct. Multi-copy integrants were not observed. Three transformants still contained the wild-type fragment of

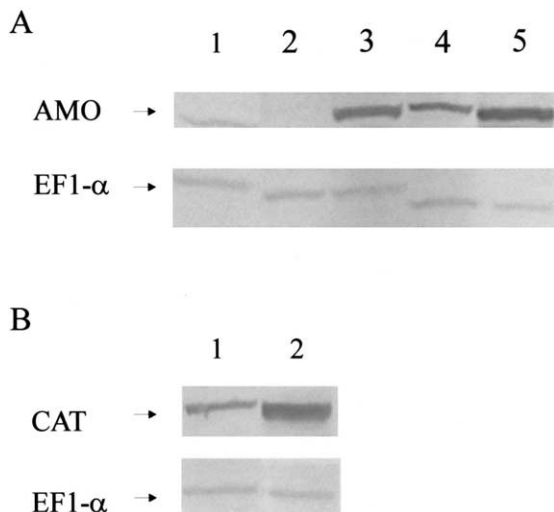


Fig. 5. Western blot analysis of strains overproducing AMO (A) or CAT (B). Per lane an amount of protein corresponding to 0.3 OD units of cells was loaded; as loading control the level of the constitutive elongation factor 1- α (EF1 α) was used. Blots were decorated with polyclonal α -AMO, α -CAT, or α -EF1 α antibodies. Detection was performed using secondary alkaline phosphatase-conjugated goat anti-rabbit antibodies and NBT-BCIP substrate. A: lane 1: wild-type *H. polymorpha* NCYC495 *ade11.1 leu1.1 ura3* cells grown on glucose/ammonium sulfate; lane 2: wild-type cells grown on methanol/ammonium sulfate; lane 3: wild-type cells grown on glucose/ethylamine; lane 4: NCYC495 *ade11.1 leu1.1 ura3::[P_{AOX}AMO]*₁ cells grown on methanol/ammonium sulfate; lane 5: NCYC495 *ade11.1 leu1.1 ura3::[P_{AOX}AMO]*₂ cells grown on methanol/ammonium sulfate. B: lane 1: wild-type *H. polymorpha* NCYC495 *ade11.1 leu1.1 ura3* cells grown on methanol/ammonium sulfate; lane 2: NCYC495 *ade11.1 leu1.1 ura3::[P_{AOX}CAT]*₁ cells grown on methanol/ammonium sulfate.

P_{AOX} suggesting that integration had occurred at a locus other than P_{AOX} . A characteristic Southern blot of two selected Ade⁺ transformants obtained using pHIPA4-AMO is shown in Fig. 4. One of these, designated NCYC495 *ade11.1 leu1.1 ura3::[P_{AOX}AMO]*₁, contains a single copy of the expression cassette, the second one, NCYC495 *ade11.1 leu1.1 ura3::[P_{AOX}AMO]*₂, contains two copies. Both strains were used for further analysis. Similar integration frequencies were obtained for pHI-

PA4-CAT-transformed cells (data not shown). This suggested that the construct containing the full-length promoter of the *ADE11* gene complemented the mutant efficiently. Double or multiple-copy integration events, which can be specifically selected for when a construct is used that complements the mutant phenotype poorly if present in a single copy [5], did not occur at high frequencies. Our results indicate that plasmid pHIPA4 is pre-eminently suited for the construction of mutants containing a single copy of the overexpression cassette.

The production levels of AMO and CAT protein in the overproducing strains are shown in Fig. 5. The data indicate that in the host strain AMO protein is solely synthesized in cells grown in the presence of the AMO-inducing substrate, ethylamine, but not in the presence of ammonium sulfate, independent of the carbon source used for growth (Fig. 5A). In the single-copy transformant AMO protein is detected upon growth of cells on methanol/ammonium sulfate at levels comparable to those observed in the amine-induced host strain. As expected, enhanced levels are observed in extracts prepared from the two-copy transformant (Fig. 5A). Comparable results were obtained for CAT protein, which is present in enhanced levels in extracts prepared from the single-copy transformant compared to the control host strain (Fig. 5B).

Immunocytochemistry revealed that the rate of AMO and CAT overproduction exceeded the maximum uptake capacity for these proteins of the peroxisomes present in the cells, in that the proteins were found in both the organelles and the cytosol (shown for CAT, Fig. 6A). Remarkably, CAT protein was also observed in mitochondria (Fig. 6B), presenting a phenotype that was observed before in some of the *ass* mutants [21].

4. Discussion

In this paper we report the isolation of the putative *H. polymorpha* *PUR7* and *PUR8* genes that are involved

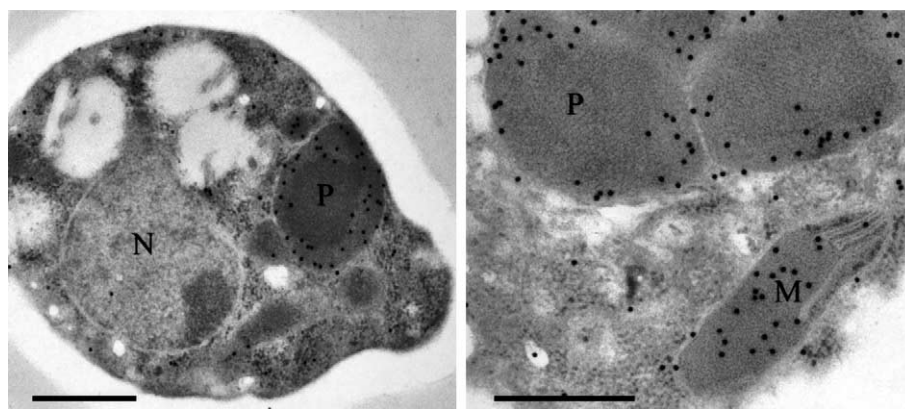


Fig. 6. Immunocytochemical experiments to demonstrate the location of CAT protein, produced in methanol-grown NCYC495 *ade11.1 leu1.1 ura3::[P_{AOX}CAT]*₁ cells. α -CAT-dependent labeling is observed on peroxisomes and cytosol (A) but also on mitochondria (B). P, peroxisome; N, nucleus; M, mitochondrion; V, vacuole. Bar represents 0.5 μ m.

in purine biosynthesis in this organism. An expression vector containing the putative *HpPUR7* gene, pHIPA4, was designed and successfully used for production of the homologous peroxisomal matrix proteins AMO and CAT. The *H. polymorpha PUR7* gene was cloned by functional complementation of the *ade11* mutant strain. The red colonies, which are formed by the *ade11* mutant when grown on rich solid media under adenine-limiting conditions, enable an easy distinction between complemented and non-complemented *ade11* colonies (see below). Previously, only three other selectable genetic markers were available in *H. polymorpha* (*leu1.1*, *trp3*, and *ura3*). Therefore, the additional possibilities provided by the *ade11* mutant will be of value for future applied and fundamental studies using this organism. For this reason we constructed the three-fold auxotrophic mutant, NCYC495 *ade11.1 leu1.1 ura3*, and the expression vector pHIPA4. The feasibility of this system is convincingly demonstrated by the successful overproduction of the homologous proteins AMO and CAT.

Several authors have reported on plasmid-based systems for overproduction of proteins in methylotrophic yeast species. These systems have been used successfully for production of both homologous and heterologous polypeptides. Furthermore, very strong and well-regulated promoters have been isolated (e.g. promoters of the alcohol oxidase, dihydroxyacetone synthase, and formaldehyde dehydrogenase genes; for review see [1]). The use of these systems has led to the successful overproduction of various proteins, e.g. *Aspergillus niger* glucose oxidase, *S. cerevisiae* invertase, *Aspergillus aculeatus* cellulase I, *H. sapiens* urokinase, and hepatitis B antiserum (reviewed in [1]).

The *H. polymorpha ade11.1 leu1.1 ura3* mutant, together with the newly developed expression vector pHIPA4, provides an additional tool to enhance the possibilities to introduce foreign or homologous genes into this organism. All three selectable markers have now been used successfully for genetic modification of *H. polymorpha*. In addition, the zeocin resistance marker has recently been used in *H. polymorpha* to construct an *AOX* disruption strain [27]. Use of this dominant marker creates the possibility of introducing a gene into a prototrophic strain. Thus, the tools are now available for independently introducing four expression cassettes into *H. polymorpha*, either by genomic integration or as replicating plasmids. Genomic integration generally generates stable mutants, as indicated by the fact that we routinely grow transformants on rich media. During these procedures we never observe any significant phenotypic reversion.

Analysis of transformants obtained using the expression vector pHIPA4 revealed that the integration cassette typically inserts in a single copy into the genome of the *ade11* mutant. This indicates that the adenine-auxotrophic phenotype is efficiently complemented upon insertion of a single copy of the homologous *HpPUR7* (*ADE11*) gene. The expression system described here is therefore particu-

larly suited for obtaining modest production levels of selected proteins. This was shown for the *H. polymorpha* AMO and CAT proteins. Especially in those cases where high-level overproduction of proteins might cause artifacts, the system for obtaining moderate expression levels described here possesses distinct advantages. However, it remains possible to utilize the *HpPUR7* gene for multi-copy integration, e.g. by reducing the complementation efficiency of the mutant phenotype [5]. This can be achieved by using plasmids with poor complementing activity, e.g. plasmids that contain fragments of the promoter of the complementing gene, or by using heterologous instead of homologous markers.

Using electron microscopy we examined the subcellular fate of the overproduced proteins in *H. polymorpha*. Two separate pathways for the import of peroxisomal matrix proteins have been identified. The PTS1 pathway is used for the import of most of the matrix proteins, e.g. AO and CAT [28,29]. The alternative PTS2 pathway is used for import of only a small number of peroxisomal matrix proteins, e.g. AMO. Efficient targeting of AMO to the peroxisome requires induction of the PTS2 pathway [30]. In the AMO-overproducing cells analyzed here, AMO is present both in peroxisomes and in cytosol. As shown before, in *H. polymorpha* the PTS2 import machinery is severely repressed in the presence of ammonium and has to be induced by amines [20]. Therefore, the observed dual location of AMO protein upon overproduction is in agreement with earlier data [20,30]. In the CAT-overproducing cells, a similar localization of the overproduced protein was observed. Although a large portion of CAT is found in the peroxisomal matrix, a significant amount is mislocalized to the cytosol and the mitochondria. This result shows that even though the PTS1 import route, which is used for CAT import into peroxisomes, is fully active under the conditions used, a large import defect is observed. Mislocalization of CAT to mitochondria was also observed in some of the AO assembly (*ass*) mutants isolated in our lab [21]. At this point, we can only speculate on the mechanisms underlying this phenomenon.

Recently, approximately 50% of the genome of *H. polymorpha* has been sequenced in an effort of Genoscope (National Centre for Sequencing, France; www.genoscope.cns.fr) [31]. During an initial search we identified several partial ORFs sharing homology with *PUR* genes from *S. cerevisiae*. In the course of this search we found the ORF of *ADE13* (*HpPUR8*). Furthermore, the coding sequences for (part of) the putative *ADE2*, *ADE3*, *ADE5/7*, *ADE6*, *ADE8*, *ADE12*, *ADE16*, and *ADE17* homologs of *H. polymorpha* were found, using the *S. cerevisiae* proteins as queries. We plan to combine this sequence information with the results presented above to set up a synthetic lethality screening system [32], which can be used to study the interaction of gene products, e.g. using plasmids containing genes involved in purine biosynthesis.

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